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Aims and Scope

The Clinical and Molecular Hepatology, is an international, peer-reviewed, open- access journal published quarterly in English. ***The Clinical and Molecular Hepatology*** aims to share advanced and latest knowledge, trend, and understanding of hepatobiliary diseases, to provide a wide open academic forum for active debate and discussion among clinical doctors, translational researchers, and basic scientists, and to improve public health through a multidisciplinary approach, especially in resource-limited Asia-Pacific area with high prevalence of B viral infection and hepatocellular carcinoma. In addition, ***the Clinical and Molecular Hepatology*** gives priority to epidemiological studies of hepatobiliary diseases in East Asia, North Asia, Southeast Asia, Central Asia, South Asia, Southwest Asia, Pacific, Africa, Central Europe, Eastern Europe, Central America, and South America.

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Editorials

Reviews

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Editorial

Congratulatory remarks

Si Hyun Bae

The Catholic University of Korea, College of Medicine, Seoul, Korea
President, the Korean Association for the Study of the Liver

Congratulations on the successful publication of this special review series on non-alcoholic fatty liver disease (NAFLD). I would like to express my sincere gratitude to the renowned hepatologists who willingly agreed to participate as authors, and to the Editor-in-Chief, Professor Seung Up Kim, and the members of the editorial board for their dedication and hard work.

Clinical and Molecular Hepatology (CMH) is the official journal of the Korean Association for the Study of the Liver (KASL), and it aims to share the latest knowledge through the publication of distinguished research in the field of hepatology. *CMH* started as '*The Korean Journal of Hepatology*' in 1995, and changed its name to *CMH* from 2012. *CMH* has continuously developed through the submission of outstanding papers by numerous domestic and foreign researchers, and has been listed in the Science Citation Index Expanded since November 2019. Today, *CMH* continues to develop rapidly as one of Asia's leading hepatology journals.

I believe the commendable attempt to publish this special review series on NAFLD reflects the constant effort made by the editorial board members, which has led to such developments of *CMH* . In the future, I look forward to publishing various special review series on diverse liver diseases, which will cover the most up-to-date as well as controversial topics. *CMH* and the KASL will continuously pursue novel changes and strive for further development in research. I hope that the publication of this special review series on NAFLD will serve as an opportunity for *CMH* to advance one more step. Furthermore, I hope this review will also provide a forum for researchers to share their achievements and innovative ideas through active intellectual collaboration, and ultimately contribute to improving patient care and research in the field of hepatology.

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Editorial

Preface

Yoon Jun Kim

Seoul National University College of Medicine and Liver Research Institute, Seoul, Korea
Editor-in-Chief Emeritus, Clinical and Molecular Hepatology, Seoul, Korea
President of the Korean NAFLD Study Group

This special review series on non-alcoholic fatty liver disease (NAFLD) deals with the most recent and controversial issues related to NAFLD, which continues to increase worldwide and has emerged as a disease with high social burden. In addition, hepatocellular carcinoma associated with NAFLD has also increased, and the importance of NAFLD in future liver disease research is expected to increase gradually. Unfortunately, an effective treatment for NAFLD has not yet been developed; but as continuous clinical research is being conducted on candidate substances, we expect the development of an effective medication in the near future. A review series that will serve as the guideline for fatty liver research is absolutely necessary at this critical time.

The current issue consists of 26 reviews that contain a wide range of contents, such as updates on the latest knowledge and future research prospects for NAFLD, which would greatly help the readers to broaden their views on NAFLD and provide novel ideas for future studies. We tried to cover all aspects of NAFLD, including the definition, nomenclature, epidemiology, causes and comorbidities, screening, risk factors, non-invasive markers, high-risk population, surveillance, prevention and treatment, liver transplantation, and pathology. Of course, NAFLD-related research is developing rapidly, so there are many areas that we have not covered in this issue, and new research and concepts will be introduced soon. However, it would still be meaningful to compile the research results that are available so far.

We hope that this special review series on NAFLD will serve as an opportunity for all researchers and clinicians to resolve their queries and find the optimal answers related to NAFLD based on current literature. Above all, we would like to thank Professor Si Hyun Bae, the President of the Korean Association for the Study of the Liver, and the members of the Korean NAFLD Study Group for their financial and clerical support, as well as manuscript writing and research support. We also express our sincere gratitude to the current Editor-in-Chief of *Clinical and Molecular Hepatology*, Professor Seung Up Kim, who organized and published this special review series on NAFLD, along with the editorial board members and the distinguished scholars who contributed to the special review series.

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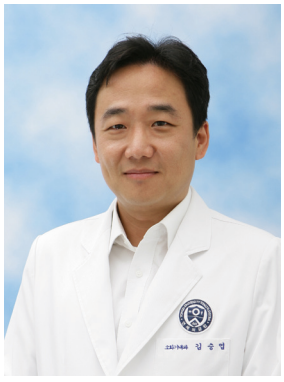
Editorial

Preface

Seung Up Kim

Yonsei University College of Medicine, Seoul, Korea
Editor-in-Chief, Clinical and Molecular Hepatology, Seoul, Korea



First of all, I would like to thank all the editorial board members, distinguished foreign and domestic authors, and reviewers for their efforts in getting the non-alcoholic fatty liver disease (NAFLD) review series published. This NAFLD review series is on a continuum with "KASL Clinical Practice Guideline: Management of Nonalcoholic Fatty Liver Disease" published in *Clinical and Molecular Hepatology* in 2021. The contents that could not be described in detail due to the limited space in this KASL guideline were divided into more detailed categorization, so that we can further provide useful information to our readers. This NAFLD review is composed of 26 topics, covering everything about NAFLD from its epidemiology to treatment. Recently, many researchers have been working to find drugs that help treat NAFLD, and I believe that the now is the best time to publish this NAFLD review series, as we are expecting new effective drugs to be released soon.

I would also like to thank Professor Si Hyun Bae, the President of the Korean Association for the Study of the Liver, as well as Professor Yoon Jun Kim, the President of the Korean NAFLD Study Group, for their support for the completion of the NAFLD review series. Thank you very much.

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Editorial

Special thanks

Seung Up Kim

Editor-in-Chief, Clinical and Molecular Hepatology, Seoul, Korea

We sincerely express our gratitude to all editors and members of KASL, CMH, and Korean NAFLD study group listed below who devoted their efforts for the Special Edition of Nonalcoholic Fatty Liver Disease review series 2023.

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Review

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Non-alcoholic fatty liver disease: Definition and subtypes

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Non-alcoholic fatty liver disease (NAFLD) is one of the most common liver diseases worldwide, with a global prevalence of approximately 30%. However, the prevalence of NAFLD has been variously reported depending on the comorbidities. The rising prevalence of obesity in both the adult and pediatric populations is projected to consequently continue increasing NAFLD prevalence. It is a major cause of chronic liver disease worldwide, including cirrhosis and hepatocellular carcinoma (HCC). NAFLD has a variety of clinical phenotypes and heterogeneity due to the complexity of pathogenesis and clinical conditions of its occurrence, resulting in various clinical prognoses. In this article, we briefly described the basic definition of NAFLD and classified the subtypes based on current knowledge in this field. **(Clin Mol Hepatol 2023;29(Suppl):S5-S16)**

Keywords: Non-alcoholic fatty liver disease; Steatohepatitis; Fibrosis

INTRODUCTION

The term non-alcoholic fatty liver disease (NAFLD) was first introduced by Schaffner in 1986.[1] It is characterized by excessive hepatic fat accumulation, associated with insulin resistance and defined as the histological presence of steatosis in >5% hepatocytes. As non-invasive measurement, proton magnetic resonance spectroscopy or quantitative fat/water selective magnetic resonance imaging (MRI) can be used to measure steatosis by determining the proton density fat fraction (rough estimation of the fat volume fraction in the liver; steatosis >5.6%).[2-4] A diagnosis of NAFLD is made after excluding other obvious factors that influence the liver profile or could induce steatosis, such as significant alcohol intake, viral hepatitis, and medications that cause fatty changes. NAFLD is an integrated term for heterogeneous pathological states; therefore, the therapeutic approach should be chosen considering each cause and subtype. In recent years, there have been several attempts to refine NAFLD stages and phenotypes.

The diagnosis of NAFLD is based on radiological or histopathological findings that demonstrate fatty changes in the liver. Biopsy is the gold standard for confirming fatty changes, but there are limitations of sampling error, intra-observers' discrepancy, and invasiveness. Non-invasive modalities, such as computed tomography (CT), ultrasonography (US), and MRI are used to detect fatty changes in the liver. Therefore, the incidence and prevalence of NAFLD have been re-

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ported differently depending on the diagnostic tool.

The annual incidence (diagnosis made using abdominal US) in the general population was approximately 48.2 cases/1,000 persons (range, 13.4–77.7).[5-7] Using another diagnostic method, the hepatic steatosis index, the annual incidence rate was 21.1 cases/1,000 persons per year[8]. In a meta-analysis, the annual incidence rate in Korea was 45.1 cases/1,000 persons.[9,10] The prevalence of NAFLD varied from 21–44%.[11-13] In a meta-analysis conducted in Korea, the prevalence rate of NAFLD was reported as 12.6–51.0%[9,14,15] according to diagnostic modality. However, the data of incidence and prevalence, according to various classification and subtypes of NAFLD, were insufficient until now.

TRADITIONAL DEFINITION AND CLASSIFICATIONS

NAFLD is a generic term that encompasses the spectrum of non-alcoholic fatty liver (NAFL), non-alcoholic steatohepatitis (NASH), and NASH-related cirrhosis. NASH is the inflammatory subtype of NAFLD, and it is characterized by steatosis, evidence of hepatocyte injury (ballooning), and inflammation with or without fibrosis. NASH-cirrhosis is the presence of cirrhosis with current or previous histological evidence of steatosis or steatohepatitis.[4]

The 2018 American Association for the Study of Liver Diseases (AASLD) NAFLD guidelines recommend that the classification of biopsy specimens should include a distinction between NAFL (steatosis), NAFL with inflammation, and NASH (steatosis with lobular and portal inflammation and hepatocellular ballooning). A comment on severity (mild, moderate, or severe) might be useful.[2] Specific scoring systems, such as NAFLD activity score (NAS) and/or steatosis, activity, and fibrosis score, and the presence of fibrosis might be used in description.[2,16] In 2005, the NASH Clinical Research Network (CRN) published the NAS to provide a standard measure for assessing histological changes in NAFLD during clinical trials.[16] This score can be used for assessing the full spectrum of NAFLD, including simple steatosis. The score is calculated as the unweighted sum of the scores for steatosis (0–3), lobular inflammation (0–3), and hepatocellular ballooning (0–2), and it ranges from 0 to 8. The main purpose of the NAS is to evaluate histological changes over time rather than to serve as diagnostic criteria for NASH.

However, some studies have used the threshold values of NAS, specifically NAS ≥5, as a surrogate for the histological diagnosis of NASH because NAS ≥5 has been reported to correlate with a diagnosis of NASH, and biopsies with scores ≤2 were diagnosed as 'not NASH'.[16] Brunt et al.[17] reviewed biopsies obtained from 976 adults in NASH CRN studies and reported that only 75% of the biopsies with definite NASH had NAS ≥5, whereas 28% of the borderline NASH and 7% of the 'not NASH' biopsies had NAS ≥5. In addition, 3% of the patients with NAS ≥5 were 'not NASH', and 29% of the patients with NAS ≤4 were diagnosed as NASH.[17] Therefore, caution is needed in the clinical application of NAS, and it should not be confused with diagnostic or classification criteria.

Non-alcoholic fatty liver (simple steatosis)

Hepatocellular steatosis is the hallmark of NAFL, and presence of more than 5% is required for diagnosis.[18-20] It is classified into two types: macrovesicular and microvesicular steatosis. Steatosis in NAFLD is usually macrovesicular; however, microvesicular steatosis may also be present in approximately 10% of patients with NAFLD.[21,22]

Many previous studies have suggested that NAFL is a benign disease. Through the several studies performing paired or repeat liver biopsy, NAFL showed significantly superior overall prognosis, including progression to cirrhosis rather than NASH.[23,24] However, the concept that NAFL is a benign disease was challenged with the accumulation of evidence; it is now regarded as a progressive disease. Recent data suggest that nearly 25% of the patients with NAFL may develop fibrosis.[25] In another study that included patients with NAFLD who underwent serial biopsy (25 with simple steatosis and 45 with NASH), 64% of the 25 patients with steatosis showed

Abbreviations:

AASLD, American Association for the Study of Liver Disease; BMI, body mass index; CRN, Clinical Research Network; EASL, European Association for the Study of Liver; *HSD17B13*, hydroxysteroid 17β-dehydrogenase 13; MAFLD, metabolic (dysfunction)-associated fatty liver disease; MHO, metabolically healthy obesity; NAFL, non-alcoholic fatty liver; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NAS, NAFLD activity score; *PNPLA3*, patatin-like phospholipase domain-containing protein 3; *TM6SF2*, transmembrane 6 superfamily member 2

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rapid progression to NASH after 3.7 years.[26] The increasing severity of steatosis has been reported to be positively associated with lobular inflammation, zone 3 fibrosis, and definite steatohepatitis.[27] In a meta-analysis comparing NAFL and NASH, the percentage of patients who progressed by one or more stage of liver fibrosis was similar (39.1% and 34.5%, respectively).[28] Overall, roughly 30–40% of patients with NAFL show fibrosis progression in studies with sequential biopsies. Therefore, follow-up can be considered even in patients with simple NAFL without evidence of inflammation.

The European Association for the Study of the Liver (EASL) Clinical Practice Guidelines recommend that patients with NAFL without metabolic risk factors should be monitored at 2–3-year intervals considering the low risk of progression.[29] The clinical factors associated with progression to NASH include hypertension, diabetes or insulin resistance, and low aspartate aminotransferase/alanine aminotransferase (AST/ALT) ratio at the time of liver biopsy.[26] Rapid progression was also often observed with concomitant hepatic injury related to alcohol, toxin exposure, nutrients, drugs, chronic hepatitis C, or autoimmune liver disease.[30] In contrast, there has been no consensus on surveillance strategy for NAFL with risk factors.

Non-alcoholic steatohepatitis without fibrosis

NASH was first described in 1980 and represents a state of chronic liver inflammation.[31] NASH is currently defined as very heterogeneous, especially according to the presence or absence of fibrosis. A diagnosis of NASH requires a biopsy with histological findings demonstrating hepatocellular ballooning degeneration and hepatic lobular inflammation with hepatic steatosis.[2,3] However, histological confirmation is not frequent; thus, the accurate estimation of the prevalence of NASH in the general population is limited. The prevalence of NASH has been known to be approximately 1.4–15.0% in the general population, and 20% of the patients with NAFLD histologically show NASH in biopsy specimens.[10,32,33] The incidence of NASH doubled between 1990 and 2017, and its age-standardized incidence rate has increased by 1.35% per year, from 3.31 to 4.81 per 1,000,000 persons.[34] Current guidelines from the AASLD recommend biopsy for patients with NAFLD who are at increased risk of steatohepatitis and/or advanced fibrosis and for those in whom the coexisting liver disease cannot be ruled out.[2] High-risk factors for progression to NASH include coexisting metabolic diseases (hypertension, diabetes mellitus, or obesity), elevated levels of aminotransferases, older age (>60 years), and Hispanic ethnicity.[30] Non-invasive scoring systems and methods for the prediction of fibrosis include NAS, Fibrosis-4 index, AST-to-platelet ratio index (APRI), and enhanced liver fibrosis (ELF) panel and Vibration Controlled Transient Elastography and magnetic resonance elastography (MRE).[4]

Brunt et al.[35] classified the inflammatory grades of NASH as grade 1 (mild), grade 2 (moderate), and grade 3 (severe). The NASH CRN later subclassified grade 1 according to the degree and location of fibrosis (Table 1). Intralobular inflammation is also present in NASH and usually consists of a mixed inflammatory cell infiltrate.[36] In NAFLD/NASH, portal inflammation is usually absent or mild and mainly involves lymphocytic infiltration. When portal inflammation is disproportionately severe, the possibility of concurrence with other liver diseases (such as hepatitis C and autoimmune hepatitis) should be considered. Hepatocellular ballooning is characterized by swollen hepatocytes with rarefied cytoplasm, reflecting hepatocellular injury. Hepatocellular ballooning is believed to result from the alteration of the intermediate filament cytoskeleton. In a meta-analysis of 10 longitudinal histological studies, older age and parenchymal or portal inflammation on initial biopsy were independent predictors of progression to advanced fibrosis in NASH.[37]

Until these days, there are insufficient data about the relationship between the degree of inflammation and prognosis. Therefore, the clinical importance between simple NAFL and NASH (without fibrosis) has not yet been fully investigated. A recent study showed that the presence of biopsy-proven NASH was not related to liver-specific morbidity or overall mortality.[38] More prospective studies on the prognosis of NASH without fibrosis are needed.

Non-alcoholic steatohepatitis with fibrosis

The characteristic pattern of fibrosis in NASH is perisinusoidal/pericellular fibrosis, which typically begins in zone 3. Fibrosis in NAFLD typically involves an active necroinflammatory reaction. As NASH progresses, portal/periportal and bridging fibrosis and liver cirrhosis may develop. Those with histologic evidence of NASH with pronounced fibrosis have a higher risk of adverse hepatic outcomes (hepatic decompensation, HCC, and liver-related mortality), and this risk increas-

Table 1. Grading and staging system for non-alcoholic steatohepatitis

Grading		
Grade 1 (mild)	Steatosis	Up to 66%
	Ballooning	Occasional in zone 3
	Inflammation	Intralobular inflammation: scattered polymorphs±lymphocytes
	Portal inflammation	Portal inflammation: no or mild
Grade 2 (moderate)	Steatosis	Any degree
	Ballooning	Obvious, predominantly zone 3
	Inflammation	Polymorphs and chronic inflammation noted
	Portal inflammation	Mild to moderate
Grade 3 (severe)	Steatosis	Panacinar
	Ballooning	Ballooning and disarray obvious, predominantly in zone 3
	Inflammation	Scattered polymorphs±mild chronic inflammation
	Portal inflammation	Mild or moderate
Staging		
Stage 1	Zone 3 perisinusoidal/pericellular fibrosis, focal or extensive	
Stage 2	Zone 3 perisinusoidal/pericellular fibrosis+focal or extensive periportal fibrosis	
Stage 3	Zone 3 perisinusoidal/pericellular fibrosis+portal fibrosis+bridging fibrosis	
Stage 4	Cirrhosis	

es exponentially as fibrosis advances to cirrhosis. In addition, many observational studies have shown that biopsy-confirmed liver fibrosis is a major predictor of not only liver-related but also overall mortality in patients with NAFLD.[39]

A recently published systematic analysis including 4,428 patients with biopsy-confirmed NAFLD, of which 2,875 patients (65%) had a histologically proven NASH, revealed that the unadjusted risk increased with increasing stage of fibrosis relative to no fibrosis stage (stage 0): a relative risk for all-cause mortality 3.42 (95% confidence interval [CI], 2.63–4.46) and a relative risk for liver-related events, 12.78 (95% CI, 6.85–23.85).[40] Sanyal et al.[41] from the NASH CRN also reported a prospective study on the outcomes of NAFLD, including the entire spectrum of NAFLD. In this study, all-cause mortality increased with increasing fibrosis stages, with 0.32 deaths per 100 person-years for stage F0 to F2, 0.89 deaths per 100 person-years for stage F3, and 1.76 deaths per 100 person-years for stage F4. The incidence of other complications of cirrhosis also increased as the fibrosis grade increased.[41,42] Therefore, many clinical trials on NASH treatment aim to reduce fibrosis.

NASH-related cirrhosis

In advanced fibrosis or cirrhosis, steatosis and necroinflammatory reactions may disappear; this condition is known as burn-out NASH.[43,44] Patients with this presentation could be diagnosed with cryptogenic cirrhosis, of which the leading cause is believed to be NAFLD/NASH.[45,46] The prevalence of NASH-related cirrhosis was 0.178% in a study including 417,524 American adults performed between 2009 and 2012, which showed a 2.0–2.5-fold increase from the values obtained between 1999 and 2002.[47] Recently, rapid progression to NASH-cirrhosis was reported in patients with advanced fibrosis. In these studies, approximately 20% of the patients with NASH and advanced fibrosis (F3) may develop cirrhosis within 2 years.[48,49] Prospective studies for the natural courses for NASH-cirrhosis need to be accumulated.

NASH-related cirrhosis is most commonly macronodular or mixed,[50] and often, specific histological features related NASH or even steatosis were missed out in advanced cirrhosis.[44] Most patients with cryptogenic cirrhosis in the United States have been diagnosed with 'burnt-out' NASH.[51-54] This concept was indirectly supported by the fact that patients with cryptogenic cirrhosis who undergo liver transplantation had higher rates of obesity and other metabolic risk factors

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and a higher risk of developing recurrence of NASH and metabolic conditions after transplantation.[52,53] A study that compared 103 and 144 patients with cryptogenic cirrhosis and biopsy-proven NASH, respectively, reported that cryptogenic cirrhosis was demographically similar to NASH-related cirrhosis.[55]

The diagnosis of NASH cirrhosis is based on: (1) having risk factors for progression to cirrhosis, (2) excluding the other causes of cirrhosis, and (3) having cirrhosis complications. The majority of patients with NASH-cirrhosis are women, older than 50 years, and with obesity and/or diabetes mellitus and dyslipidemia as comorbidities. Patients with NASH-advanced fibrosis (F3-4) showed an overall 10-year survival of 81.5% during the follow-up period. NASH-cirrhosis had lower rates of liver-related complications and HCC than cirrhosis related with hepatitis C infection.[56] In a recent study, all-cause mortality rate in NASH-cirrhosis is 1.76 deaths per 100 person-years. Patients with NASH-cirrhosis also had a higher risk of diabetes and chronic renal disease.[41] In a retrospective study that included the United Network for Organ Sharing Data, the authors reported that the number of NASH-related transplant cases increased.[57] With the increasing prevalence of risk factors, the number of NASH-cirrhosis patients would consistently increase.

VARIANTS IN CLASSIFICATION OF NON-ALCOHOLIC FATTY LIVER DISEASE

Lean non-alcoholic fatty liver disease

Risk factors for NAFLD include insulin resistance and metabolic syndrome i.e., three or more of the following: obesity, diabetes mellitus, hypertension, low high-density lipoprotein levels, and high triglyceride levels.[2] Among these, obesity is the most common risk factor. However, people with normal body weight (body mass index [BMI; kg/m^2] <23 kg/m^2 for Asians and <25 kg/m^2 for Westerners) or non-obese weight (BMI <25 kg/m^2 for Asians and <30 kg/m^2 for Westerners) can also be diagnosed with NAFLD, referred to as lean or non-obese NAFLD. The lean NAFLD is more prevalent in Asia.[4,58] Data on the prevalence of lean NAFLD in the general population varies from 7.8–74.0% across studies.[58-61] This variation is mainly because of the variation in the BMI cut-off used to define lean individuals. In one Asian study that included 307 biopsy cases, 23.5% were diagnosed as lean NAFLD.[62]

Compared to healthy people, patients with lean NAFLD had higher metabolic syndrome occurrence, diastolic blood pressure, hemoglobin A1c, and insulin resistance.[63,64] Additionally, biochemical and hematologic markers, such as serum ALT, AST, Gamma glutamyl peptidase (γ-GT), and total bilirubin levels, were higher in patients with lean NAFLD than in healthy participants.[60,61,63] Although the prevalence of metabolic syndrome in lean NAFLD was lower than in obese NAFLD, the impact of lean NAFLD was a stronger risk factor for higher rates of all-cause mortality, cirrhosis, and HCC than obese NAFLD.[63] Zou et al.[65], reported that patients with lean NAFLD showed advanced fibrosis stage, higher incidence of metabolic comorbidities, and higher all-cause mortality than obese NAFLD. Additionally, Hagström et al.[66] reported that patients with lean NAFLD had a higher risk for cirrhosis, HCC than obese NAFLD. These results suggest the important role of metabolic disorders in this population.

The etiology of lean NAFLD is assumed to be based on central obesity and visceral fat.[67] Therefore, the BMI-driven approach for NAFLD may need to be reappraised. BMI does not entirely explain the association between visceral fat and NAFLD. Moreover, the relationship between lean NAFLD and metabolic syndrome is still not fully understood, and more long-term studies are required.

Metabolically healthy non-alcoholic fatty liver disease

Obese patients present with significant variations in metabolic abnormalities, such as hyperglycemia, hypertension, and dyslipidemia. Recently, these patients have been classified into different subphenotypes depending on their metabolic health status. Metabolically healthy obesity (MHO) is a concept derived from clinical observations that some obese people do not present with common metabolic abnormalites[68]; the implications of this for the development of NAFLD across its subphenotypes remain vague.

In a study that included 4,432 MHO people, 2,145 patients (48.4%) were presented NAFLD simultaneously.[67] On the contrary, in 225 patients with NAFLD, 14 (6.2%) were metabolically healthy.[61] MHO was considered as a risk factor of NAFLD development. Chang et al.[5] reported that the metabolically healthy obesity was an independent risk factor for NAFLD development with hazard ratio as 2.15–3.55 than lean pa-

tients. Metabolic healthy people with NAFLD had a favorable biochemical profile i.e., lower γ-GT, fasting glucose, and triglycerides levels and higher high-density lipoprotein cholesterol levels than metabolic unhealthy people. However, they had been diagnosed with NAFLD at a younger age, similar to metabolically unhealthy people.[69]

Despite the consensus that obesity is a prerequisite for MHO, more than 30 different definitions of metabolic health are used in clinical studies.[70] According to the previous studies, MHO is still considered as preliminary status toward metabolic syndrome and NAFLD; therefore, surveillance strategy of these groups has not been established. A consensus on the concept of MHO and metabolic health is required, and in NAFLD, a cohort study that includes a large number of patients is need to be accumulated.

Metabolic (dysfunction)-associated fatty liver disease

As mentioned earlier, the definition of NAFLD must exclude other causes that can result in inflammation and fatty changes. The significant amount of alcohol intake that differentiates NAFLD from alcoholic fatty liver disease ranges from 10 to 40 g (pure alcohol) a day, and this range varies between studies. The EASL guideline defined the amount of significant alcohol consumption as ≥210 g in men and ≥140 g in women weekly.[3] These criteria were also applied in the Korean Association for the Study of Liver NAFLD guidelines.[4] In the AASLD guidelines, the standard alcohol drink was defined as 14 g of pure alcohol, and significant alcohol consumption was defined as more than 21 standard drinks in men and 14 in women per week.[2]

Recently, it has been suggested that the term NAFLD does not reflect the heterogeneous pathogenesis or various courses of fatty liver disease. Furthermore, the overestimation of the exclusion of alcohol has induced debate about the threshold of 'significant' alcohol consumption which is required for the diagnosis of NAFLD. In 2019, a consensus by 32 experts suggested an alternative terminology, metabolic (dysfunction)-associated fatty liver disease (MAFLD), to more accurately reflect the pathogenesis of this disease.[71] The diagnosis of MAFLD is based on the evidence of fat accumulation in the liver in the presence of one of the following three criteria: overweight/obesity, type 2 diabetes mellitus, and evidence of metabolic dysregulation.

Prevalence of MAFLD was estimated to be approximately 50.7% in general population, and it varied substantially across countries and regions, from 22.3% to 81.5%.[72,73] According to a recently published study, the prevalence of MAFLD in Korea was reported to be 33.9%.[74] Patients with MAFLD were significantly older and had higher BMI and prevalence of metabolic comorbidities (diabetes and hypertension) than those with NAFLD.[73,75] In a study that included 756 Japanese patients with fatty liver, the MAFLD definition better identified a group with fatty liver and significant fibrosis, which were evaluated using non-invasive tests.[76]

The term MAFLD implies that fatty change is a risk factor in patients with other causes of chronic liver disease, including viral hepatitis B and C, autoimmune diseases, or alcohol intake above the threshold levels. Whether MAFLD can replace NAFLD is still under debate in several studies.[73,77] Further research and comparative analyses of the risk associated with fatty changes are needed to validate this term.

Genetic variants

Genetic factors play a major role in NAFLD development. Many studies have explored the genetic drivers of NAFLD beyond metabolic syndrome and insulin resistance. Typically, patatin-like phospholipase domain-containing protein 3 *(PNPLA3*) and transmembrane 6 superfamily member 2 (*TM6SF2*) nucleotide polymorphisms affect the development and progression of the disease.[78] Furthermore, homozygous carriers of p.148M mutations show a 12-fold increased risk of developing HCC, suggesting the potential for monogenic inheritance.[79-81] The mutation occurs with the greatest frequency in Hispanics, followed by non-Hispanic whites, and the least in African Americans.[81]

The rs738409[G] allele of *PNPLA3* has been consistently shown to be associated with higher liver fat content and necroinflammatory scores and a substantially increased risk of developing fibrosis.[82] The *PNPLA3* rs738409[G] allele is more common in Asians with lean NAFLD without metabolic syndrome, which could account for the observation that Asian and Caucasian populations have a similar prevalence of NAFLD.[33] In another study, patients with cryptogenic cirrhosis had a similar prevalence of *PNPLA3* rs738409 genotypes as those with NASH.[55] These associations were independent of the presence of type 2 diabetes mellitus and obesity.[83,84] However, high *PNPLA3* allele expression was related to other

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factors, such as lifestyle, viral infection, and alcohol consumption.[82]

Another genetic variant that is associated with NASH is the rs58542926 allele of *TM6SF2*. The *TM6SF2* E16K variant is associated with an increased risk of progressive NASH,[85] although a recent study has reported that the variant may reduce the risk of cardiovascular disease.[85] In a more comprehensive discussion on NAFLD genetics, including *TM6SF2* and *MBOAT7* gene variants, genetic risk factors for liver fibrosis were identified.[86]

Another example is the enzyme hydroxysteroid 17β-dehydrogenase 13 (*HSD17B13*), a member of a large family of enzymes primarily involved in sex hormone metabolism, which is a novel liver-specific lipid droplet-associated protein in mice and humans with NAFLD. Hepatic overexpression of *HSD17B13* promotes lipid accumulation in the liver, suggesting the pathogenic role of *HSD17B13* in NAFLD.[87] A recent study showed that a loss-of-function variant of *HSD17B13* was associated with a reduced risk of chronic liver disease and progression from steatosis to steatohepatitis, highlighting it as a potential therapeutic target.[88]

Many other genes involved in carbohydrate and lipid me-

Table 2. The definition and subtypes of non-alcoholic fatty liver disease

Classification	Definition	Prevalence	Clinical implications
Traditional classification			
NAFL	5% of steatosis in hepatocytes Without any cause of fatty change	5–30% of general populations	30–40% of patients with NAFL seem to experience progression of fibrosis
NASH	NAFLD+hepatocyte ballooning degeneration and hepatic lobular inflammation	2–30% of NAFLD 3–6% of the general population	Fibrosis is a major prognostic predictor of liver-related and overall mortality
NASH-Cirrhosis	NAFLD+necroinflammatory reactions may disappear, and cirrhosis without other specific causes may be present.	20% of patients with NASH 0.18% of the general population	Cryptogenic cirrhosis is presumed to be an advanced form of NASH
Variants of NAFLD			
Lean NAFLD	NAFLD in people with normal body weight (BMI <23 for Asians or <25 for Westerners)	23.5% of the general population More prevalent in Asia	Compared with non-lean NAFLD, lean NAFLD had a stronger correlation with metabolic deterioration The risk of fibrosis is increased
Metabolically healthy NAFLD	Steatosis above 5% Does not meet any metabolic syndrome criteria	6.2% of NAFLD	Diagnosed with NAFLD at a younger age The disease progression from metabolically healthy to unhealthy is higher in obesity group than normal weight group
MAFLD	Steatosis above 5% The presence of one of the following three criteria: overweight/obesity, type 2 diabetes mellitus, and evidence of metabolic dysregulation	50.7% of the general population; varies across countries and regions	Paradigm shift from NAFLD to MAFLD
Genetics			
PNPLA3			Common in Asians with lean NAFLD Associated with cryptogenic cirrhosis
TM6SF2			Increased risk for progressive NASH
HSD17B13			Loss-of-function variant was associated with progression of NAFLD

NAFL, non-alcoholic fatty liver; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; BMI, body mass index; MAFLD, metabolic (dysfunction)-associated fatty liver disease; PNPLA3, patatin-like phospholipase domain-containing protein 3; HSD17B13, hydroxysteroid 17β-dehydrogenase 13; TM6SF2, transmembrane 6 superfamily member 2.

tabolism, insulin signaling pathways, inflammatory pathways, oxidative stress, and fibrogenesis have been shown to play a role in the development and progression of NAFLD/NASH. Some of these include *GCKR*, *APOB*, *LPIN1*, *UCP2*, and *IFLN4*.[89-91]

Although these genetic advancements have increased our understanding of the pathogenesis of NAFLD, routine testing for these genetic variants is currently not advocated. The relationship between genetic diversity and NAFLD progression requires further investigation.

We show several subtypes and definitions for NAFLD (Table 2).

CONCLUSION

NAFLD affects a heterogeneous patient population. Although the primary driver in many patients is metabolic syndrome, a complex and dynamic heterogeneous interaction of different factors are involved. Therefore, the response to therapy differs among patients depending on sex, the presence of genetic variants, coexistence of different comorbidities, and various amounts of alcohol consumption. In this review, we addressed this heterogeneity and subtypes of NAFLD by analyzing published data on the differential contributions of known factors to the pathogenesis and clinical expression of NAFLD. We need to consider this heterogeneity and the dominant drivers of this disease in patients according to subtypes and make predictions to provide precision-targeted therapy for NAFLD.

Authors' contribution

All authors contributed to the study conception and design, material preparation, data collection. The first draft of the manuscript was written by Seul Ki Han and Moon Young Kim. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest

The authors have no conflicts to disclose.

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Review

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MAFLD: How is it different from NAFLD?

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"Metabolic dysfunction-associated fatty liver disease (MAFLD)" is the term suggested in 2020 to refer to fatty liver disease related to systemic metabolic dysregulation. The name change from nonalcoholic fatty liver disease (NAFLD) to MAFLD comes with a simple set of criteria to enable easy diagnosis at the bedside for the general medical community, including primary care physicians. Since the introduction of the term, there have been key areas in which the superiority of MAFLD over the traditional NAFLD terminology has been demonstrated, including for the risk of liver and extrahepatic mortality, disease associations, and for identifying high-risk individuals. Additionally, MAFLD has been adopted by a number of leading pan-national and national societies due to its concise diagnostic criterion, removal of the requirement to exclude concomitant liver diseases, and reduction in the stigma associated with this condition. The current article explores the differences between MAFLD and NAFLD diagnosis, areas of benefit, some potential limitations, and how the MAFLD terminology has opened up new fields of research. **(Clin Mol Hepatol 2023;29(Suppl):S17-S31)**

Keywords: MAFLD; NAFLD; MAFLD vs. NAFLD

INTRODUCTION

Excess fat deposition within the liver has been recognized for centuries. In a landmark paper published by Ludwig et al.[1] (1980), the term "non-alcoholic steatohepatitis (NASH)" was first used to describe the liver histology associated with excess liver fat in the absence of significant alcohol consumption. The term "non-alcoholic" used by the researchers was derived from similarities in the histopathological findings of these patients compared to those with alcohol-related liver disease, due to the lack of knowledge about its pathophysiological basis at that time.[1]

Ever since the introduction of the term nonalcoholic fatty liver disease (NAFLD) into the medical compendium, there has been discussions around changing the name to better reflect the disease process and extending the terminology beyond the superficial histopathological similarities to alcohol-related liver disease.[2,3] In early 2020, an international panel of experts led a consensus-driven process to develop a more appropriate term for the disease. Utilizing a 2-stage Delphi consensus, the term that was proposed was "metabolic dysfunction-associated fatty liver disease," or

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"MAFLD".[4]

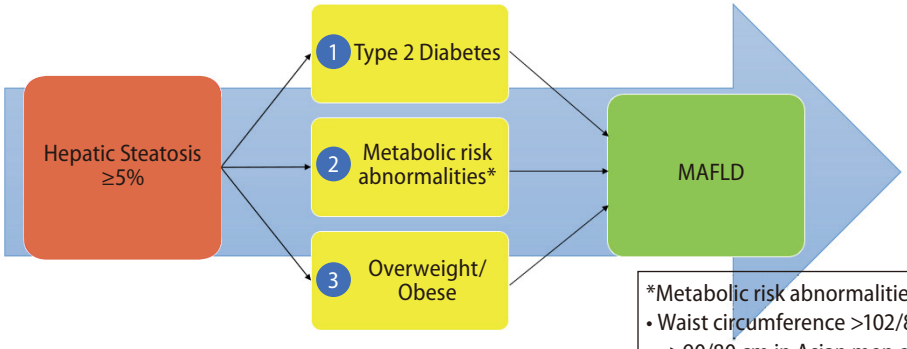
In addition to the name change, the consensus proposed a set of simple positive criteria to diagnose and evaluate individuals for the disease.[4] The diagnostic criterion highlighted the contribution that systemic metabolic dysregulation plays in driving the liver disease (Fig. 1). These contributory factors have since been identified as core research in the field of "NAFLD" and its extra-hepatic associations.[5]

Since the introduction of MAFLD in 2020 as an alternative term with its own set of diagnostic criteria, there have been more than 800 unique articles referencing the new diagnosis. There has also been controversy with some societies supporting its usage and introducing it as a formal change in terminology and diagnosis in their guidelines.[6-10] This article expands on the differences between MAFLD and NAFLD and the potential benefits and detriments of this change.

MAFLD VS. NAFLD – THE DIFFERENCES

NAFLD vs. MAFLD diagnosis – Criterion changes

The NAFLD diagnosis, as published in guidelines, requires hepatic steatosis of ≥5% without concurrent liver disease, including "significant" alcohol usage (Fig. 2).[11] The criterion for MAFLD utilizes the same standard for hepatic steatosis, but identifies metabolic dysregulatory factors as a prerequisite for the diagnosis to be entertained (Fig. 1).[4] The metabolic risk drivers, according to the MAFLD criteria, are type 2 diabetes mellitus and overweight/obesity by ethnic-specific body mass index (BMI) classifications. Both of these risk factors are classically involved in liver fat deposition, and have been noted to be associated with an increase in disease progression and of hepatic and extra-hepatic complications. The third dysregulatory pathway is less commonly recognized but is part of the operational definition of metabolic syndrome. For the diagnosis of MAFLD in healthy weight



*Metabolic risk abnormalities - 2 out of 7
- Waist circumference >102/88 in Caucasian men and women, (or >90/80 cm in Asian men or women)
- Blood pressure >130/85 mmHg or specific drug treatment
- Plasma triglycerides >150 mg/dL (>1.70 mmol/L) or specific drug treatment
- Plasma HDL-cholesterol <40 mg/dL (<1.0 mmol/L) for men and <50 mg/dL (<1.3 mmol/L) for women or specific drug treatment
- Prediabetes (i.e. fasting glucose levels 100-125 mg/dL (5.6- 6.9 mmol/L) or 2-hour post-load glucose levels 140-199 mg/dL (7.8-11.0 mmol/L) or HbA1c of 5.7-6.4% (39-47 mmol/mol)
- Homeostasis model assessment of insulin resistance score >2.5
- Plasma high-sensitivity C-reactive protein level >2 mg/L

Figure 1. Diagnostic criterion for MAFLD. MAFLD, metabolic dysfunction-associated fatty liver disease; HDL, high-density lipoprotein.

Abbreviations:

MAFLD, metabolic dysfunction-associated fatty liver disease; NAFLD, nonalcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; ALRD, alcohol-related liver disease; BMI, body mass index; NHANES, National Health and Nutrition Examination Surveys; FIB-4, fibrosis-4; FLD, fatty liver disease; CKD, chronic kidney disease; FEV1, forced expiratory volume in 1 second

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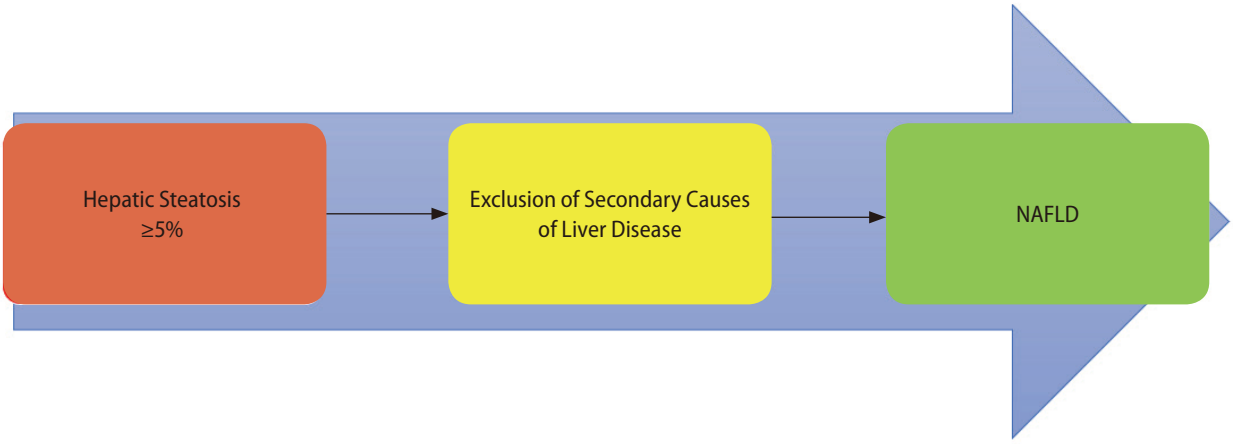

Figure 2. Diagnosis of NAFLD. NAFLD, nonalcoholic fatty liver disease.

people, an individual needs to have two of the seven risk factors to make a diagnosis. The risk factors include waist circumference, blood pressure, plasma triglycerides, plasma high-density lipoprotein-cholesterol, prediabetes, homeostasis model assessment of insulin resistance score, and plasma high sensitivity C-reactive protein. The combination of hepatic steatosis with one of these three metabolic risk stratifications results in the diagnosis of MAFLD.[4]

The most significant difference between NAFLD and the diagnosis of MAFLD, however, is not the formal recognition of metabolic dysregulatory pathways in the development of the disease, but rather the removal of exclusion of concurrent liver disease to entertain the diagnosis.[4,12] Multiple studies have shown the synergistic effects of comorbid liver disease, including viral hepatitis, and concurrent alcohol usage; however, the exclusion of these in the diagnosis of NAFLD underpins a cognitive dissonance between these disease processes, attempting to exclude their contribution to individualized patient outcomes.[13,14] In short, MAFLD tells us what the disease is and not what it is not, and MAFLD is unrelated to the presence or absence of other causes of liver disease. This simple change has allowed clinicians to identify and treat all the liver diseases that might exist in a given patient in a holistic manner. The latter is important, given that in many countries and regions, overweight or obesity impacts over 60% of the adult population.

Positive diagnostic criterion versus negative diagnosis criterion

The switch to a set of positive diagnostic criteria results in the ability to detect all underlying liver diseases, particularly in patients without apparently clear metabolic features. A recent study by Alexander et al.[15] (2018) utilized multiple European primary care databases to determine the prevalence of NAFLD in general practice among 17.7 million patients. It found that the pooled prevalence of NAFLD was significantly lower than expected, with only 1.9% given this diagnosis in 2015 compared to prior observational studies estimating a prevalence of 20–30% in the European population. Although the prevalence has doubled since 2007 in these general practice databases, their recognition has not increased to meet the conservative estimates of this disorder.

In the age of improved investigations, there has been a significant move in most specialties to create positive diagnostic criterion for diseases. The benefits include decreased time to diagnosis and initiation of treatment, as well as consistent diagnosis facilitating both collaboration and research into the underlying disorders.[4,16] While this has occurred in a number of specialties, the prevalence of these disorders has been rare, and has not garnered the level of controversy that the proposed terminology of MAFLD has within the liver community. A recent example is the change from primary biliary cirrhosis to primary biliary cholangitis, which better reflects the pathophysiological manifestations of the disease process, as this condition has rarely resulted in cirrhosis.

For a disorder with a significant prevalence in the community, the NAFLD diagnostic criteria did not lead to increased understanding in the healthcare community and the wider general public. Fatty liver deposition is one of the most prevalent conditions affecting up to 40% of the general population; however, the recognition of the condition and its associated complications is poor.[17] One of the major controversies regarding the change in terminology to MAFLD is the suggestion that a name change will impair the current work to improve public awareness of NAFLD. However, the public awareness of NAFLD as a condition of concern is surprisingly low (around 4%), despite being included in the medical compendium for over 40 years.[17] There have been some suggestions that the change in name from NAFLD to MAFLD will increase public awareness of this condition.[16,18,19]

Contributory factors to development of fatty liver disease

MAFLD diagnosis has been crucial in identifying higher-risk patients who would benefit from targeted management. Several studies have highlighted that a MAFLD diagnosis better correlates with higher liver fibrosis stage and non-invasive markers of fatty infiltration.[20-23] This recognition that metabolic dysregulatory pathways contribute to more significant liver disease highlights the important difference of the MAFLD diagnostic criteria over the NAFLD exclusionary criteria to assess individuals suffering from the disease.

NAFLD – A nebulous diagnosis

Despite the histopathological premise for the term NAFLD and advances in understanding the pathophysiological basis of the disease with many patient and healthcare suggestions for a name change, no new terminology has been developed and NAFLD has subsisted in the literature for decades.[24] Moreover, utilization of the diagnosis of NAFLD in healthcare outside of the gastroenterology specialty has been sparse. In a survey conducted by non-gastroenterology specialists in Australia, 56% of the respondents believed that NAFLD was related to alcohol intake.[25] This suggests that, despite non-alcoholic being the defining feature of the term "NAFLD" documented clearly within the name, the term is nebulous even among hospital specialists and not reflective of the practice need.

Another key characteristic of NAFLD is the exclusion of harmful alcohol intake in individuals with the disease. There are a number of reasons why harmful alcohol intake should not be used as an exclusionary tool in fatty liver disease. The first is that alcohol intake is a self-reported measure by a patient and has a variable designation of volume in different societal settings. Due to the stigma associated with alcohol consumption and its effects on the liver, under-reporting by patients has been identified.[26] A recent study performed by Staufer et al.[27] in 2022 also has also called into question the utilization of NAFLD after examining ethyl glucuronide in hair samples collected to assess alcohol consumption. In this prospective study, 114 patients were diagnosed with NAFLD after exclusion of other chronic liver diseases and alcohol consumption by patient recall. Harmful alcohol consumption was designated as >20 g of EtOH/day for women and >30 g of EtOH/day for men. The study found that 29% of the patients diagnosed with NAFLD had high to moderate risk of alcohol-related liver damage with repeated moderate to excessive alcohol consumption after being confronted with hair analysis, showing elevated levels of ethyl glucuronide. In a study directly assessing NAFLD diagnosis, almost 30% of the patients had elevated alcohol levels, which contradicted the basis for the diagnosis of NAFLD.[27]

Confounding the picture even further is a recent paper by Meijnikman et al.[28] (2022) regarding the role of the gut microbiome in generating endogenous ethanol. In that study assessing obese NAFLD and NASH patients, portal vein and peripheral blood were taken to assess ethanol. It showed that microbiome-related ethanol production occurs in all populations, but was significantly higher in NASH and NAFLD when compared to patients without hepatic steatosis. This microbiome-induced ethanol production did not produce high peripheral concentrations of alcohol due to the livers' ability to process large quantities of ethanol. The main point of this study was that, even though exogenous ethanol has been accounted for in the diagnostic terminology, there is a possibility that endogenous ethanol production by the microbiome could be contributory to its development.[28] Due to the histopathological similarities between alcohol-related liver disease and NAFLD, it is possible that the mechanism of injury is similar, but from different sources.

Secondly, there is heterogeneous reporting requirements across geographic regions governing the volume of alcohol considered to be harmful. Examples of this include the

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American Associated for the Study of Liver Disease and the Asian Pacific Association for the Study of the Liver guidelines, which define heavy or at-risk drinking as more than 14 drinks per week for men or more than seven drinks per week for women.[11] In the European Associated for the Study of the Liver guidelines, the diagnosis of NAFLD requires the exclusion of daily alcohol consumption of >30 g for men and >20 g for women.[29] Thirdly, even light or moderate alcohol consumption in the setting of NAFLD, which does not meet the exclusionary criteria set above, can cause significant worsening of fibrosis when compared to no consumption.[30] This has been shown in studies where even mild alcohol usage worsened fibrosis and may synergistically cause cirrhosis in patients diagnosed with NAFLD.

Due to the lack of histological characteristic features distinguishing alcohol-related fatty infiltration from non-alcohol-related fatty liver infiltration, the utilization of "non-alcoholic" via comprehensive alcohol assessment as a patient-reported measure with the associated stigmatization calls into question its ongoing use. This is particularly important, as international guidelines have recommended that "non-harmful" alcohol consumption has been shown to worsen fibrosis in patients with fatty liver disease. Additionally, evidence pointing towards increased endogenous ethanol production by the microbiome in fatty liver disease could be contributory to the underlying pathogenesis.

MAFLD VS. NAFLD – THE OVERALL BENEFITS

Identification of at-risk individuals

The utilization of previously collected databases to assess the applicability of MAFLD has been undertaken by several authors. The first of these studies performed by Lin *et al*. used the National Health and Nutrition Examination Surveys (NHANES) from 1988–1994, which examined 13,083 patients with complete ultrasonography and laboratory data.[31] Patients who met the MAFLD diagnostic criteria had statistically significant increases in metabolic comorbidities, liver enzymes, and non-invasive liver fibrosis scores compared to the NAFLD group.

A review performed by Kang et al.[32] in 2021 on behalf of the Korean NAFLD study group examined the publications that compared MAFLD to NAFLD, with a particular focus on the combined associations of risks in retrospective studies. It showed that MAFLD had statistically significant increases in alanine transferase (23.96±22.22 vs. 22.31±21.34, P≤0.001), NAFLD fibrosis score (–2.05±1.51 vs. –2.18±1.52, P≤0.001), and fibrosis-4 (FIB-4) scores (1.06±1.35 vs. 1.01±0.84, P≤0.001) compared to NAFLD. This indicates that MAFLD more specifically selects patients with worse liver function and non-invasive scores. These differences were even more striking in the comparison of MAFLD to non-metabolic risk (MR) NAFLD (or NAFLD patients without the necessary metabolic risk factors to meet the criteria for MAFLD). Utilizing MAFLD diagnostic criteria compared to non-MR NAFLD, the increases became more marked in alanine transferase (23.96±22.22 vs. 16.81±17.84, P≤0.001), NAFLD fibrosis score (–2.05±1.51 vs. –3.00±1.32, P≤0.001), and FIB-4 scores (1.06±1.35 vs. 0.87±1.05, P≤0.001). This highlights the utility of the MAFLD criteria over the traditional NAFLD diagnostic criteria in assessing patients for worsening liver disease. These analyses of large patient cohorts have also correctly identified most patients who have higher related risks for comorbidities and increased mortality. For example, the diagnosis of MAFLD has been shown to be superior in identifying patients who are most at risk for clinical disease progression compared to NAFLD.[21,33]

Public awareness

There is limited historical evidence for the recognition of NAFLD and its contributory factors in the literature. Evidence that is available suggests that NAFLD recognition and diagnosis in primary care settings that manage the majority of patients are poorly understood and applied.[15,25,31,34,35] The simple criteria for MAFLD have been purported to increase the recognition and understanding outside of gastroenterology and hepatology specialists, and it will also enable primary care practitioners and others to initiate early management.[16,18,19,36] This has not been studied in the literature to date, but would be significant to public health as early interventions, similar to cardiovascular disease and diabetes mellitus, are more likely to be efficacious in preventing adverse outcomes.

From an individual patient perspective, the utilization of the term NAFLD has led to many patients trivializing their condition. Several studies have reported that up to 95% of

patients with suspected NAFLD are unaware of having liver disease, and that >75% do not feel they are at risk of developing NAFLD.[34,35,37] This minimization of potential harms does a disservice to the prevalence and potential severity of the disease, creating a lack of engagement among patient populations who suffer from NAFLD. Evidence suggests that trivialization mainly arises through an inappropriate name of the condition, or when disease perceptions or diagnoses are confusing to people. Expert opinion governing this area of terminology believe that the negative prefix "non-" carries a perception that the disease is unimportant.[16]

Stigma associated with NAFLD diagnosis

One of the particularly onerous societal burdens of NAFLD is the utilization of alcohol in its name. Alcohol usage carries with it a significant stigma, and that stigma has overlapped into the diagnosis of NAFLD.[16] This is particularly damaging in discussing the disease with pediatric patients and practicing Muslim patients, where stigma may prohibit practitioners from discussing the disease with the patients. Recent correspondence regarding the change in terminologies' impact on the Arab world, with the largest practicing Muslim population, has highlighted the benefit of changing the name to MAFLD.[10,38]

Stigmatization of healthcare conditions carries a significant burden. Stigma has negative effects on self-esteem and can lead to decreased self-management of the condition, decreased quality of life, and increased inability to cope with a disease.[16] Stigma can also induce fear in patients, which can lead to adverse health behaviours, including denial of diagnosis, treatment avoidance, lack of compliance with treatment and healthcare advice, and ultimately, termination of treatment.[16] Therefore, stigma should be avoided with any diagnosis label to increase the patients' motivation to manage their condition and to seek ongoing treatment.

Increase in prevalence of MAFLD compared with NAFLD

One of the benefits of utilizing MAFLD compared to NAFLD is the increase in the identification of individuals with high-risk features for progressive liver disease. In a study by Ayada et al.[39] (2021), 17 studies containing both a diagnosis of NAFLD and MAFLD comprising 9,808,677 individuals were reviewed. This study showed that the prevalence of MAFLD was 33.0% (95% CI 29.7–36.5), with a NAFLD prevalence of 29.1% (95% CI 27.1–31.1). The surprising detail of this study was that of all the fatty liver identified in the combined studies, 15.1% were identified with MAFLD-only diagnosis (95% CI 11.5–19.5). Several of the studies showed that large increases in the patients diagnosed were undertaken in Asian populations. This indicates that the new diagnostic criteria is better suited to identify patients over the traditional NAFLD diagnosis label.[39] Whilst this has been replicated in other reports, there are geographic variations to this increase in the identification of significant fatty liver disease.

Non-MR NAFLD

One of the potential detractions from utilizing MAFLD exists in the patients who fulfill the criteria for NAFLD without metabolic risk factors or other identifiable aetiologies of liver disease. When examining retrospective data comparing the two diagnoses, the majority of patients fulfill both the MAFLD and NAFLD criteria. However, there is a small proportion of patients who make up the non-MR NAFLD group across these studies, ranging from 0.6–16.1%, with most consistently estimating this group to make up around 5% of the fatty liver disease population.[20,22,32,33,40-43] While most risks were associated with MAFLD diagnosis, some studies did show that non-MR NAFLD patients had increased risks of cardiovascular disease during follow-up, though the majority of studies showed no increase in liver-related risk compared to the control populations.[39] The presence of severe hepatic steatosis has been shown to have implications on metabolic complications, including metabolic syndrome, and thus, these patients should be monitored for the development of complications, especially since metabolic risk factors, including weight and dysglycemia, can increase over time.

MAFLD CLINICAL DIFFERENCES

Mortality

Whilst a number of articles have been published on MAFLD vs. NAFLD, there have been numerous negative articles suggesting that MAFLD does not contribute to mortality

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Table 1. Mortality difference between MAFLD and NAFLD

Study	Patients (n)	Outcomes
Huang et al.[45] (2021) NHANES III	Total/MAFLD/NAFLD=4,437/3,909/3,779 Both MAFLD and NAFLD=3,251 MAFLD only=658 NAFLD only=528	1. MAFLD increased overall mortality compared to NAFLD (HR 2.07 vs. 1.47) – difference was non-significant after adjusting for metabolic parameters. 2. NAFLD-only had reduced total mortality (HR 0.46, 95% CI 0.24–0.89). .
Muthiah et al.[46] (2022) NHANES III – Type 2 diabetes patients	Total/MAFLD/NAFLD=4,982/4,982/excluded Both MAFLD and NAFLD=2,950 MAFLD only=2,032 NAFLD only= Excluded	MAFLD-only had increased overall all-cause mortality compared to MAFLD+NAFLD (HR 1.27, 95% CI 1.11–1.48).
Younossi et al.[14] (2022) NHANES III+NHANES 2017–2018 participants	Total/MAFLD/NAFLD=2,617/2,332/2,122 Both MAFLD and NAFLD=1,915 MAFLD only=418 NAFLD only=207 Fatty liver disease not defined by MAFLD or NAFLD=78	1. Study excluded all patients with viral hepatitis. 2. MAFLD-only had significantly increased all-cause mortality when compared to MAFLD+NAFLD (HR 2.28, 95% CI 1.84–2.82, *P*<0.001 vs. 1.89, 95% CI 1.68–2.11, *P*<0.001). 3. NAFLD-only had a protective effect on all-cause mortality (HR 0.57, 95% CI 0.34–0.96, P=0.0335). 4. When adjusted for age, sex, race, education, income, marital status, smoking status, healthy eating index, BMI, physical activity, and alcohol-related liver disease, only MAFLD+NAFLD had increased all-cause mortality (HR 1.15, 95% CI 1.04–1.28, *P*=0.0091).
Nguyen et al.[47] (2021) NHANES III	Total/MAFLD/NAFLD=2,997/2,742/2,494 Both MAFLD and NAFLD=2,240 MAFLD only=503 NAFLD only=254	1. On unadjusted modelling MAFLD-only had higher increased overall mortality compared to NAFLD+MAFLD (HR 4.6, 95% CI 2.6–7.9, *P*<0.001 vs. 3.2, 95% CI 2.0–5.2, *P*<0.001). On adjusted modelling, MAFLD-only was associated with increased mortality (HR 2.4, 95% CI 1.2–4.6, *P*=0.01). 2. On unadjusted modelling, MAFLD-only had higher increased cancer related mortality vs. NAFLD+MAFLD (HR 3.9, 95% CI 1.7–8.9, *P*=0.002 vs 2.7, 95% CI 1.2–6.0, *P*=0.02). On adjusted modelling, neither MAFLD only nor NAFLD+MAFLD had statistically significant association with cancer-related mortality. 3. On unadjusted modelling, MAFLD-only had higher increased other cause mortality vs. NAFLD+MAFLD (HR 4.0, 95% CI 2.1–7.6, *P*=<0.001 vs. 2.8, 95% CI 1.5–5.2, *P*=0.002). On adjusted modelling, neither MAFLD-only nor NAFLD+MAFLD had statistically significant associations with other cause mortality.
Semmler et al.[48] (2021) SAKKOPI database	Total/MAFLD/NAFLD=4,718/2,189/2,262 Both MAFLD and NAFLD MAFLD only NAFLD only=73	1. Increased mortality was observed in lean MAFLD compared to lean NAFLD and no hepatic steatosis (8.6% vs. 2.7% vs. 5.6%). 2. Increased mortality was observed in overweight MAFLD compared to overweight without hepatic steatosis (6.8% vs. 3.7%). 3. Increased mortality was observed in obese MAFLD compared to obese without hepatic steatosis (7.1% vs. 6.5%). 4. On adjusted modelling with age and components of metabolic syndrome, MAFLD was not associated with increased mortality (HR 1.115, 95% CI 0.822–1.512, *P*=0.484).

NHANES, National Health and Nutrition Examination Surveys; MAFLD, metabolic dysfunction-associated fatty liver disease; NAFLD, nonalcoholic fatty liver disease; HR, hazard ratio; 95% CI, 95% confidence interval; BMI, body mass index; SAKKOPI, Austrian Screening Cohort for Colorectal Cancer.

(Table 1).[14,44-48] The main point suggested by these articles is that the metabolic dysregulatory features are the cause for mortality, and not the underlying MAFLD diagnosis. This has been shown using adjusted modelling considering type 2 diabetes mellitus and BMI, which were treated as confounders of the demonstrated association that MAFLD displays with mortality. However, there are two major concerns that these articles fail to acknowledge. First, as the diagnosis of MAFLD relies on metabolic dysregulation, type 2 diabetes mellitus and BMI cannot be treated as confounders—they are an integral part of the diagnosis. Put simply, without metabolic dysregulatory changes, there is no MAFLD; therefore, their inclusion as confounders in these adjustment models revokes the diagnosis of MAFLD. These adjustment models only assess fatty liver without a metabolic component, which is hepatic steatosis.

The second point that adjustment modelling indicates is that MAFLD without metabolic derangements does not have any association with mortality. While this has been highlighted to show that the MAFLD diagnosis is "wrong" and is then discussed at length, the opposite has been unwittingly demonstrated. What each of these articles has failed to recognize is that adjustment models show that the utilization of metabolic dysregulatory factors is the key cause of increased mortality in fatty liver deposition. Without further metabolic dysregulation, fatty liver *per se* poses no threat of increased mortality. As metabolic dysregulation is required for the diagnosis of MAFLD, in sum, these articles show that the consensus group was correct in selecting these factors to underpin the major causative pathways that lead to increased mortality.

A study by Moon et al.[44] (2022) assessed individuals from two community-based cohorts, between the ages of 40 and 70 years, and prospectively followed them for a median of 15.7 years. Using the diagnostic criterion for MAFLD and NAFLD and adjusting for confounders, they showed that MAFLD independently predicted the overall mortality with a hazard ratio (HR) 1.33 (95% CI 1.05–1.69), while NAFLD was not associated with the overall mortality with a HR of 1.20 (95% CI 0.94–1.53). MAFLD also predicted cardiovascular disease after adjustment for age, sex, and BMI, but lost its significance when adjusted for other metabolic dysfunction risk factors, most notably type 2 diabetes mellitus. The latter is not surprising, as discussed earlier, and since these risk factors are more proximal to adverse organ-specific outcomes (e.g., hypertension or atherogenic dyslipidemia for cardiovascular disease).

Metabolic risk factors

In utilization of the MAFLD criteria, there is an understanding of the individual phenotypic profiles of the patient that has contributed to the development of fatty liver infiltration.[4] These risks not only provide clues to the causation of fatty liver, but also on the possible treatment and management options. This is important when we address each of the individual phenotypes separately, but also when we note the synergistic effects that each pathway provides for the overall patient outcomes. In contrast, with the diagnosis of NAFLD, a one-size-fits-all approach governs the phenotypic presentation and management.

An example is the risk of type 2 diabetes mellitus in overweight or obese patients. It has been shown that being overweight or obese significantly increases the risks for developing type 2 diabetes mellitus.[49] The underlying mechanisms have not been fully established; however, weight loss in these individuals can ameliorate or even normalize the risk of type 2 diabetes mellitus. Targeted weight loss should be the first step in reducing the risk of developing type 2 diabetes mellitus in overweight or obese patients by decreasing peripheral and hepatic insulin resistance;[49] whereas, in patients with type 2 diabetes mellitus, the first step in management is the normalization of blood sugars. Whilst this can be targeted by weight loss, the specific goal is the normalization of blood sugars. This highlights the contributory metabolic risk factors in the development of further metabolic co-morbidities. Each needs a tailored response to address the underlying needs, despite the interrelated effects of each.

Of note, there have been recent studies demonstrating that a MAFLD diagnosis is associated, on multivariate analysis, with an increased risk of type 2 diabetes mellitus in patients whose metabolic phenotype at diagnosis does not include type 2 diabetes mellitus, compared to a NAFLD diagnosis.[50] This is significant as it demonstrates the early diagnosis of MAFLD over that of NAFLD, which can help target individuals at risk of developing other significant complications, such as type 2 diabetes mellitus. It, therefore, allows clinicians to appropriately target patient populations to modify their metabolic risk profile to prevent complications.[50]

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Utilizing the definition of MAFLD also highlights the importance of holistic patient management.[4] Currently, the mainstay of initial management of all metabolic disorders is dietary change and exercise. Targeting them holistically, rather than in an organ-specific manner, can lead to widespread improvements in outcomes, particularly with regard to cardiovascular health and cancer, which are the greatest causes of adverse outcomes in fatty liver disease.[5] This is also particularly important for clinical research which focuses on metabolic dysregulation to improve both liver and systemic outcomes.

Metabolic complications

Outside of the traditional metabolic dysregulatory environments that are included in the diagnostic algorithm for MAFLD, there have been studies that showed an association between MAFLD and other disease processes.[51,52]

This is to be expected when placing MAFLD in alignment with other metabolic dysregulation-associated disorders, such as cardiovascular disease, rather than the stand-alone disease entity of NAFLD (Table 2). While cardiovascular disease is the major mortality burden in fatty liver disease, other disorders associated with MAFLD include peripheral vascular disease, chronic kidney disease, and some cancers, especially of the gastrointestinal tract.[5]

There have been studies assessing the cardiovascular risk association of MAFLD vs. NAFLD. A study by Lee et al.[53] (2021) evaluated incident cardiovascular disease risk from a nationwide health screening database involving 9,584,399 participants followed for a median of 10.1 years. Patients were placed in fatty liver disease (FLD), NAFLD-only, MAFLD-only, or both FLD groups. Cardiovascular risk was elevated in all fatty liver disease; however, NAFLD-only group had significantly decreased hazard ratio (HR 1.09, 95% CI 1.03–1.15) compared to MAFLD-only (HR 1.43, 95% CI 1.41–1.43) and both FLD groups (HR 1.56, 95% CI 1.54–1.58).

Recent studies assessing NAFLD vs. MAFLD have identified that asymptomatic atherosclerotic cardiovascular disease has an independent association on multivariable logistic regression models with MAFLD, but not with NAFLD diagnosis.[54] This is significant due to the burden of cardiovascular disease in patients suffering from fatty liver infiltration. Therefore, MAFLD diagnosis assists in identifying patients who should undergo cardiovascular assessment and intervention over the traditional NAFLD diagnosis.

Non-metabolic complications

Other associations made with NAFLD have been assessed against the MAFLD criteria to assess the strength of the associations with the change in terminology. A study by Sun et al.[49] (2021) utilized the NHANES database to assess the correlation of MAFLD with chronic kidney disease (CKD) and abnormal albuminuria. In that study, MAFLD patients had a lower estimated glomerular filtration rate (74.96±18.21 vs. 76.46±18.24 mL/min/1.73m^2, P<0.001) and a greater prevalence of CKD (29.60% vs. 26.56%, P<0.005) compared to those with NAFLD.

Studies addressing the association between MAFLD and other conditions are currently underway. While several conditions, such as breast lesions, have shown that MAFLD is related with these conditions, similar to NAFLD, no direct comparison has been published. It would be interesting to note the strength of association of the conditions that were previously noted to be associated with NAFLD, as well as the impact of the MAFLD criteria on them.

Somewhat surprisingly, MAFLD has shown associations with lung conditions over a NAFLD diagnosis, with poorer lung function and higher rates of mortality associated with COVID19 infection. A study performed by Miao et al.[56] (2022) compared the association of lung function parameters in patients diagnosed with MAFLD vs. NAFLD. After adjusting for age, sex, adiposity measures, smoking status, and alcohol intake, MAFLD subjects had significantly lower predicted forced vital capacity (88.27±17.60% vs. 90.82±16.85%, P<0.005) and lower 1 second forced expiratory volume (FEV_1) (79.89±17.34 vs. 83.02±16.66%, P<0.005) when compared to those diagnosed with NAFLD. While the results suggest that MAFLD has a greater role in identifying patients with reduced lung function, it is likely related to MAFLD selecting patients with higher non-invasive liver fibrosis scores. Every 1-point increase in FIB-4 resulted in a decrease in FVC by 0.507 (95% CI −0.840 to −0.173, P=0.003) and a decrease in FEV_1 by 0.439 (95% CI −0.739 to −0.140, P=0.004).

Dual etiology liver disease and synergistic effects

The additive basis of MAFLD with other liver diseases is a

Table 2. Cardiovascular mortality difference between MAFLD and NAFLD

Study	Patients (n)	Outcomes
Muthiah et al.[46] (2022) NHANES III – Type 2 diabetes patients	Total/MAFLD/NAFLD=4,982/4,982/excluded Both MAFLD and NAFLD=2,950 MAFLD only=2,032 NAFLD only=Excluded	1. MAFLD-only had increased cardiovascular mortality compared to MAFLD+NAFLD (HR 1.26, 95% CI 1.05–1.52).
Niriella et al.[51] 2021 Community-based cohort study with 7-year follow-up	Total/MAFLD/NAFLD=2,985/990/940 Both MAFLD and NAFLD=902 MAFLD only=88 NAFLD only=38	1. MAFLD had increased overall cardiovascular non-fatal and fatal events when compared to NAFLD (RR 4.2, 95% CI 1.5–11.5 vs. RR 3.7, 95% CI 1.3–10.3, $P<0.006$). 2. MAFLD-only had significantly higher rates of cardiovascular non-fatal and fatal events when compared to NAFLD only (RR 7.2, 95% CI 2.4–21.5 vs. RR 1.9, 95% CI 0.25–14.8).
Nguyen et al.[47] (2021) NHANES III	Total/MAFLD/NAFLD=2,997/2,742/2,494 Both MAFLD and NAFLD=2,240 MAFLD only=503 NAFLD only=254	1. On unadjusted modelling, MAFLD-only had higher increased cardiovascular disease mortality vs. NAFLD+MAFLD (HR 9.4, 95% CI 2.6–34.6, $P=0.001$ vs. HR 7.0, 95% CI 2.1–23.1, $P=0.002$). On adjusted modelling, neither MAFLD-only or NAFLD+MAFLD had statistically significant associations with cardiovascular mortality, but MAFLD-only had a trend towards significance (HR 6.7, 95% CI 0.9–47.1, $P=0.06$).
Lee et al.[53] (2021) Nationwide Korean health screening database	Total/MAFLD/NAFLD=3,628,540/2,680,217/3,573,644 Both MAFLD and NAFLD=2,625,321 MAFLD only=948,323 NAFLD only=54,896	1. NAFLD+MAFLD had higher increased cardiovascular events when compared to MAFLD-only and NAFLD-only (HR 1.56, 95%CI 1.54–1.58 vs. HR 1.43, 95% CI 1.41–1.45 vs. HR 1.09, 95% CI 1.03–1.15).
Guerreiro et al.[52] (2021) Database of Brazillian patients undergoing liver biopsy at university hospital	Total/MAFLD/NAFLD=171/154/109 Both MAFLD and NAFLD MAFLD only NAFLD only	1. Non-significant higher prevalence of high-risk cardiovascular scores was observed in MAFLD group compared to NAFLD group (36.4% vs. 25.7%, $P=0.209$).

MAFLD, metabolic dysfunction-associated fatty liver disease; NAFLD, nonalcoholic fatty liver disease; NHANES, National Health and Nutrition Examination Surveys; HR, hazard ratio; 95% CI, 95% confidence interval; RR, risk ratio.

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main advantage over the traditional NAFLD definition. Since NAFLD excludes concomitant liver diseases, such as hepatitis B or C, there was no ability for the patients to have dual etiologies for their liver disease. Substantive literature has shown that individuals who have underlying liver diseases from hepatitis B and hepatitis C, with a diagnosis of MAFLD, have significantly increased complications, both intra- and extra-hepatic.[13] The additional diagnosis of MAFLD coupled with hepatitis B, for example, increases the rates of complications and mortality.[57]

In a recent study by Zheng et al.[58], among 780 patients with liver biopsies, 773 were given a diagnosis of MAFLD. Of the patients with MAFLD, 66 also had excess alcohol consumption. On subgroup analysis assessing MAFLD patients with significant alcohol consumption, the patients had high gamma-glutamyl transferase levels and exhibited more hepatic steatosis when compared to patients with MAFLD without co-existing liver disease. This outcome could not be evaluated in previous studies with NAFLD due to the requirement to exclude co-existing liver disease.

Future treatment pathways – Exclusionary diagnosis of NAFLD limits treatment options for patients

Due to the restrictive nature of NAFLD not allowing concurrent liver disease as a requirement for diagnosis, treatment strategies have focused on single liver disease entities.[11] With the more finessed MAFLD diagnosis, the co-existence of separate entities of liver disease can be entertained.[4] This allows clinicians to manage one or more conditions simultaneously, rather than treating a "dominant" liver disease.

While there is currently no approved medical treatment for MAFLD, there are a number of phase III trials underway that are showing promising preliminary results.[59] One of the major benefits of a MAFLD diagnosis, which has been overlooked in the debate over terminology, is the potential inability to provide treatment for fatty liver infiltration in individuals with concurrent liver pathologies.[4,7] This underscores the most serious implication of the NAFLD terminology in excluding significant proportions of the population who would benefit from future treatments.

MAFLD RESEARCH

Exploring phenotypic conditions

The inclusion for NAFLD clinical studies has been based on a hepatic phenotype in the absence of significant alcohol intake and all concurrent steatosis-associated liver pathologies. The move forward with MAFLD proposes that the basis for intervention should focus on the pathogenic drivers. This change will move research on fatty liver from a "one-size-fits-all" situation to a more nuanced treatment of its pathophysiological determinants.[36]

Previous correspondence has suggested that the name change to MAFLD may hinder the interpretation of studies that are currently ongoing.[60] The major concern is regarding the utilization of "resolution of NASH with no worsening of liver fibrosis," which is a key histological endpoint for conditional drug approval.[61] Negating this argument, the MAFLD criteria do not propose any change in pathological criteria for a diagnosis of metabolic steatohepatitis.

Positive diagnostic criteria – Less confounding bias in patient selection for research

When selecting patients for fatty liver disease trials, there is confounding bias associated with the NAFLD terminology.[62] Whilst the exclusionary criteria of alcohol and other contributory liver diseases are standard, there is no mechanism to explore the pathogenic aspects of the underlying liver fat infiltration. We have already discussed concerns with alcohol usage in patients with NAFLD with significant underestimation likely in clinical practice. With the utilization of MAFLD and the strict criteria for assessing metabolic co-factors, however, clinical trials inclusion will identify a more homogenous group of patients.

While the controversy regarding NAFLD vs. MAFLD is ongoing, the debate is also polarizing. Although MAFLD will not capture every single patient, it does capture those who require early intervention and are at increased risk of disease progression. Therefore, in our perspective, it would on balance be more beneficial to further develop the MAFLD concept for improved patient care and clinical research (Table 3).

Table 3. Open research questions

Synergistic liver disease	What is the synergistic relationship between MAFLD and other liver diseases?
	What is the clinical relevance of MAFLD in chronic hepatitis B not on treatment vs. on treatment?
	What is the natural history of MAFLD post-treatment for chronic hepatitis C with direct-acting antivirals?
Phenotypes	Which MAFLD phenotype is most associated with HCC?
	Which phenotype of MAFLD-HCC has higher risks of progression?
	Which MAFLD phenotype is predominant with other liver diseases?
	Which MAFLD phenotype responds best to weight loss interventions?
	Which MAFLD phenotype is associated with the different pathophysiological pathways?
	Which MAFLD phenotype is most associated with hepatic inflammation?
	Do multiple MAFLD phenotypes convey higher risks of liver related complications and mortality?
Treatments	Do previously trialled treatments have different responses based on MAFLD phenotype (i.e., were some null trials hampered by patient selection in NAFLD, and could prove beneficial based on subgroup analysis of MAFLD)?
	Which phenotype of MAFLD-HCC responds best to which treatment (i.e., surgical resection vs. locoregional vs. systemic therapy)?
	Which treatments work best for MAFLD in the context of other concomitant liver diseases?
	What is the impact of reversal of MAFLD on its long-term natural history, including cardiovascular complications and liver-related complications?

MAFLD, metabolic dysfunction-associated fatty liver disease; NAFLD, nonalcoholic fatty liver disease; HCC, hepatocellular carcinoma.

CONCLUSION

There are significant clinical, research, and patient benefits to the utilization of MAFLD over the NAFLD terminology. MAFLD establishes a clear diagnosis due to a set of positive diagnostic criteria that allows clinicians to better tailor practice to target individuals at high risk of developing complications or other metabolic co-morbidities. Therefore, we contend that the term "MAFLD" is a step in the right direction to decrease the stigma associated with a NAFLD diagnosis, to increase public awareness and to improve clinical care.

Authors' contribution

All authors were responsible for drafting and critical revision of the manuscript.

Conflicts of Interest

The authors have no conflicts to disclose.

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Review

Global incidence and prevalence of nonalcoholic fatty liver disease

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Nonalcoholic fatty liver disease (NAFLD) is a leading cause of liver disease worldwide. The estimated global incidence of NAFLD is 47 cases per 1,000 population and is higher among males than females. The estimated global prevalence of NAFLD among adults is 32% and is higher among males (40%) compared to females (26%). The global prevalence of NAFLD has increased over time, from 26% in studies from 2005 or earlier to 38% in studies from 2016 or beyond. The prevalence of NAFLD varies substantially by world region, contributed by differing rates of obesity, and genetic and socioeconomic factors. The prevalence of NAFLD exceeds 40% in the Americas and South-East Asia. The prevalence of NAFLD is projected to increase significantly in multiple world regions by 2030 if current trends are left unchecked. In this review, we discuss trends in the global incidence and prevalence of NAFLD and discuss future projections. **(Clin Mol Hepatol 2023;29(Suppl):S32-S42)**

Keywords: Nonalcoholic fatty liver disease; Incidence; Prevalence; Epidemiology

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a leading cause of chronic liver disease globally.[1] It encompasses a spectrum ranging from simple hepatic steatosis to nonalcoholic steatohepatitis (NASH), which can progress to liver fibrosis and cirrhosis.[2] The global prevalence of NAFLD has been increasing over time, with a recent meta-analysis estimating that 32% of the adult population is afflicted by NAFLD.[3] This has occurred in tandem with the global obesity and diabetes epidemics.[4,5]

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NASH is now the fastest-rising cause of hepatocellular carcinoma worldwide[6,7] and is also the fastest-rising indication for liver transplantation in the United States.[8]

INCIDENCE OF NAFLD

A recent meta-analysis by Riazi et al.[3] estimated the incidence of NAFLD at 46.9 cases per 1,000 person-years. The incidence of NAFLD was higher in males (70.8 cases per 1,000 person-years) vs. females (26.9 cases per 1,000 person-years, $P<0.0001$). However, all included studies were conducted in Asia, hence it is unclear whether these data are generalizable to other parts of the world. A previous meta-analysis published in 2016 had estimated the NAFLD incidence at 52.34 per 1,000 person-years in Asia and 28.01 per 1,000 person-years in Israel.[9] Another meta-analysis focused on NAFLD in Asia reported an incidence of 50.9 per 1,000 person-years, with the highest incidence of 63 per 1,000 person-years in mainland China and the lowest incidence of 29 per 1,000 person-years in Japan (Fig. 1).[10] The NAFLD incidence in South Korea was around 45 cases per 1,000 person-years.[10,11] Taken together, the estimates for NAFLD incidence in Asia remain consistent across several meta-analyses (Table 1).

PREVALENCE OF NAFLD

Riazi et al.[3] pooled data from 72 studies (1,030,160 individuals) and estimated that the global prevalence of NAFLD in adults was 32% (Table 2). The prevalence was higher in males than females (40% vs. 26%, $P<0.0001$). The prevalence of NAFLD increased from 26% in studies from 2005 or earlier to 38% in studies from 2016 or beyond. However, data from this study by Riazi et al.[3] requires cautious interpretation, as data were available from only 17 countries, hence it is unclear if the estimates from this study are a true reflection of 'global' prevalence. The relative lack of studies emphasizes the need to improve data collection from regions such as Africa, Oceania, and South America, where data was lacking. Le et al.[12]

Figure 1. Estimated incidence of nonalcoholic fatty liver disease. Data for China, Hong Kong, Japan, and South Korea was obtained from Li et al.[10] and Riazi et al.[3]. Data for Israel was obtained from Riazi et al.[3] and Younossi et al.[9]. NAFLD, nonalcoholic fatty liver disease.

Abbreviations:

NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; HCC, hepatocellular carcinoma; BMI, body mass index; NHANES, National Health and Nutrition Examination Surveys; PNPLA3, patatin-like phospholipase domain-containing protein 3; FLI, fatty liver index

Table 1. Selected meta-analyses providing data for the incidence of nonalcoholic fatty liver disease

Study	No. of studies	No. of individuals	Study years	Regions/Countries included	Main findings	Comments
Riazi et al.[3] (2022)	16	381,765	1994–2018	Asia (China, Japan, South Korea, Hong Kong, Israel)	Incidence 46.9 cases per 1,000 person-years; the incidence in men (70.8 cases per 1,000 person-years) was higher than in women (29.6 cases per 1,000 person-years)	The majority of included studies were from Asia hence data may not be generalizable Substantial heterogeneity
Li et al.[10] (2019)	18	416,988	2002–2017	Asia (China, Japan, South Korea, Hong Kong)	Incidence 50.9 cases per 1,000 person-years; incidence highest in China (63 per 1,000 person-years), lowest in Japan (29 per 1,000 person-years)	All studies were from Asia hence data may not be generalizable
Younossi et al.[9] (2016)	5	4,895	1997–2013	Asia (China, Japan, Israel)	Incidence 52.34 cases per 1,000 person-years (China and Japan), 28.01 cases per 1,000 person-years (Israel)	A limited number of included studies All studies were from 3 Asian countries

also pooled data from 245 studies (2,699,627 individuals) and estimated the global prevalence of NAFLD at 29.8%, which is consistent with Riazi's findings. Likewise, in this study, there was limited or no data from Africa, Oceania, and North and South America.

Asia

The prevalence of NAFLD varies substantially by region (Fig. 2). The overall prevalence of NAFLD in Asia is approximately 30%. A meta-analysis by Le et al.[12] conducted a literature search in 2019 (182 studies with 2,385,999 individuals) and estimated NAFLD prevalence in Asia at 30.5%. A recent meta-analysis by Riazi et al.[3] which included 63 studies (1,000,681 individuals) found that NAFLD prevalence in Asia was 31.6%. This is consistent with a previous meta-analysis by Li et al.[10] which reported NAFLD prevalence in Asia to be 29.62%.

The prevalence of NAFLD within Asia is highly variable as it encompasses countries with a wide spectrum of ethnicities and socioeconomic factors. Among Asian subregions, Southeast Asia had the highest NAFLD prevalence of 42%. Among Asian countries with more than 3 studies available, Li et al.[10] determined that the highest pooled NAFLD prevalence was in Iran (38.07%), and the lowest pooled NAFLD prevalence was in Japan (22.28%). Riazi et al.[3] determined similar results and found that Iran had the highest NAFLD prevalence (40.8%), followed by Taiwan (36.1%), South Korea (34.6%), and China (32.5%). On the other hand, Japan had a strikingly low NAFLD prevalence of 22.3%, which may be related to a low prevalence of obesity.

In China, a meta-analysis by Wu et al.[13] estimated a NAFLD prevalence of 29.88%, and another study by Zhou et al.[14] estimated that NAFLD prevalence was 29.2%. NAFLD prevalence in South Korea is also approximately 30%—a meta-analysis by Im et al.[15] reported a NAFLD prevalence of 30.3%, and Li et al.[10] reported a similar prevalence of 32.9%.[11] A large cross-sectional study of 571,872 Korean males in their early 20s found that even among young adult males, NAFLD prevalence was 13.47%, with an increase from 10.66% in 2015 to 16.44% in 2021. There was a higher prevalence of metabolic risk factors such as hypertension, hypercholesterolemia, and hyperglycemia during the same period.[16] Another study utilizing data from Korea National Health and Nutrition Examination Survey found that NAFLD prevalence increased from 18.6% in 1998–2001 to 21.5% in 2016–2017, and there was a

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Table 2. Selected meta-analyses providing data for the prevalence of non-alcoholic fatty liver disease

Study	No. of studies	No. of individuals	Study years	Regions/Countries included	Main findings	Comments
Riazi et al.[3] (2022)	72	1,030,160	1994–2019	Asia (63 studies), Europe (7 studies), North America (USA only), Africa (Egypt only)	The estimated global prevalence was 32.4% Prevalence was higher in men (39.7%) than in women (25.6%) Prevalence increased over time, from 25.5% ≤2005 to 37.8% ≥2016	Limited data from North America and Africa; no data from South America
Le et al.[12] (2021)	245	5,399,254	1991–2018	Asia (182 studies), Europe (11 studies), North America (3 studies), South America (4 studies), Africa (2 studies)	Estimated global prevalence 29.8% Prevalence highest in South America (35.7%) and North America (35.3%) Prevalence increased from 21.9% in 1991 to 37.3% in 2019 (yearly increase 0.7%)	Limited data from North America, South America, and Africa
Younossi et al.[9] (2016)	86	8,515,431	1989–2015	Asia (20 studies), Middle East (3 studies), Europe (21 studies), North America (35 studies), South America (3 studies), Africa (2 studies), Oceania (1 study)	Estimated global prevalence 25.2% Prevalence highest in South America (30.5%) and the Middle East (31.8%); lowest in Africa (13.5%)	Limited data from South America and Africa Included case series and case-control studies
Rojas et al.[43] (2022)	19	5,625		South America only (Brazil, Mexico, Chile, Argentina, Peru)	Estimated overall prevalence 59%; prevalence in general and 'captive' population 24%	High heterogeneity A large proportion (2,948) were patients visiting healthcare facilities and hence susceptible to selection bias Data applicable only to South America
Cholongitas et al.[31] (2021)	17	85,203	2005–2018	Europe	Estimated overall prevalence 26.9% Prevalence in Mediterranean countries 23.9%, non-Mediterranean countries 28.5% Prevalence higher in men (32.8%) than women (19.6%)	Studies that used elevated aminotransferases alone as a method for diagnosis of NAFLD were included Data are applicable only to Europe

Table 2. Continued

Study	No. of studies	No. of individuals	Study years	Regions/Countries included	Main findings	Comments
Li et al.[10] (2019)	237	13,044,518	1994–2017	Asia	Estimated overall prevalence 29.6% Prevalence increased over time – 25.3% (1995–2005), 28.5% (2006–2011), 33.9% (2012–2017)	Data are applicable only to Asia

NAFLD, nonalcoholic fatty liver disease.

higher prevalence of obesity and diabetes over the same period.[17] These suggest that the increasing NAFLD prevalence may be driven by an increase in metabolic risk factors. Ito et al.[18] reported a comparatively lower NAFLD prevalence of 25.5% in Japan, in line with the findings by Li and Riazi. This could be attributed to a lower prevalence of obesity and diabetes in Japan compared to other countries,[19,20] and may be related to a diet that is traditionally lower in fat and red meat.[21]

In South Asia, India had a NAFLD prevalence of 25.7–32.74%, Bangladesh had a NAFLD prevalence of 26.2–33.86%, and Sri Lanka had a NAFLD prevalence of 24.74%.[3,10] In Southeast Asia, Li et al.[10] reported that NAFLD prevalence was 38.5% in Malaysia, 40.43% in Singapore, and 51.04% in Indonesia. Data from Central Asia is lacking, but the Global Burden of Disease Study (GBD) 2019 reported NAFLD prevalence in Central Asia increased from 12.4% in 1990 to 19.7% in 2019, although these estimates require cautious interpretation as the Global Burden of Disease Study relied on complex modeling and past trends when data was limited.[22]

A distinct feature of the NAFLD epidemic in Asia is the high prevalence of lean NAFLD (body mass index [BMI] <23) and non-obese NAFLD (BMI <25).[23] Up to 19% of non-obese Asians have NAFLD,[24] which may be contributed to a higher percentage of visceral adiposity in Asians compared to other ethnicities.[25] Visceral adiposity plays an important role in atherogenic dyslipidemia and insulin resistance. It is a major risk factor for type 2 diabetes and has been implicated in the development and progression of NAFLD.[26] Asians also tend to develop diabetes at a younger age and lower BMI level, resulting in a longer duration of disease and increased likelihood of complications.[27-29] Worryingly, emerging data suggest that individuals with lean NAFLD may be at a higher risk of progressive liver disease, but this hypothesis requires validation.[30]

Europe

Meta-analyses by Le and Riazi had similar estimates of the prevalence of NAFLD in Europe at 30.9% (11 studies with 15,062 individuals)[12] and 32.6% (7 studies with 14,111 individuals),[3] respectively. Another meta-analysis by Cholongitas et al.[31] pooled data from 17 studies (85,203 individuals) and estimated NAFLD prevalence in Europe to be 26.9%. Cholongitas also found that NAFLD prevalence in Mediterranean coun-

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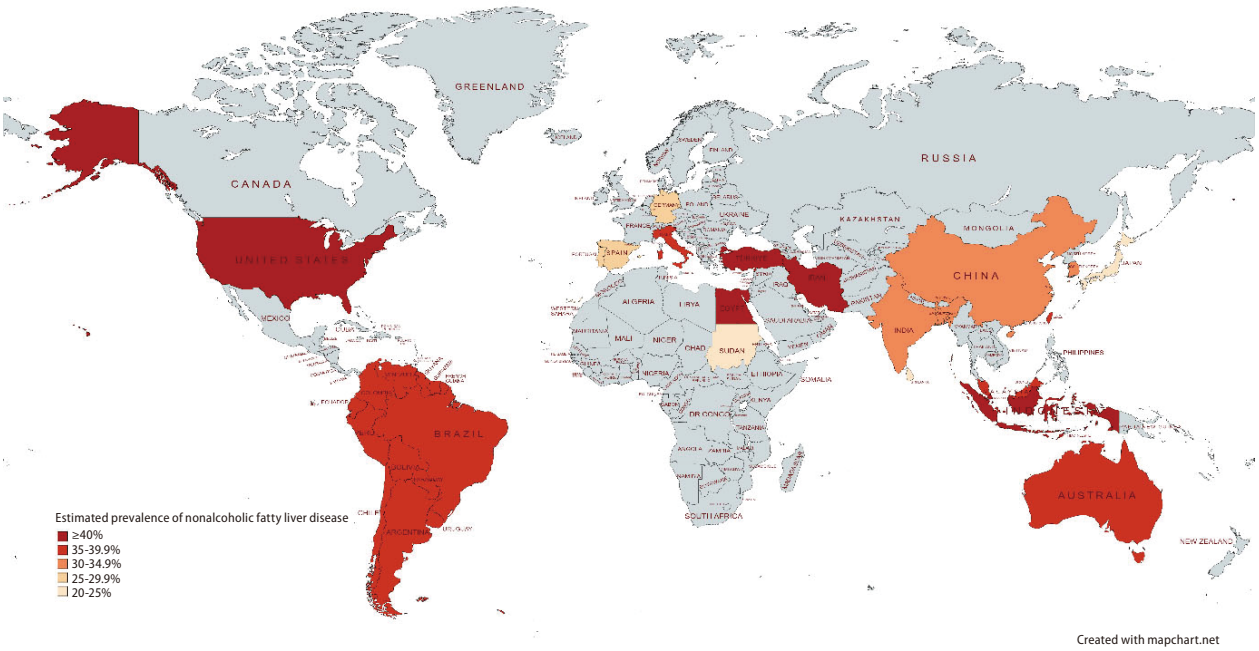

Figure 2. Estimated prevalence of nonalcoholic fatty liver disease (NAFLD). Data for Iran, China, Taiwan, South Korea, Europe (Turkey, Italy, Germany, Portugal, Spain), North America (USA), and Egypt was obtained from Riazi et al.[3]. Data for South America was obtained from Le et al.[12]. Data for South Asia (India, Bangladesh, Sri Lanka) and Southeast Asia (Indonesia, Malaysia, Singapore) was obtained from Li et al.[10]. Data for Japan was obtained from Li et al.[10] and Riazi et al.[3]. Data for Sudan was obtained from Younossi et al.[9]. Data for Australia was obtained from population-based studies by Farrell et al.[55] and Roberts et al.[54].

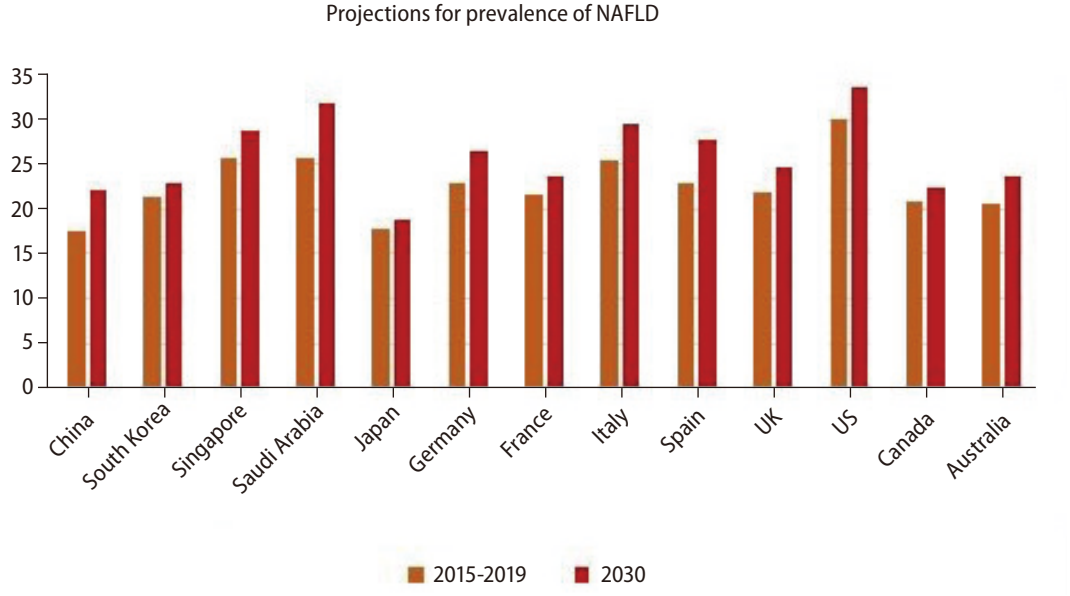

Figure 3. Estimated projections for the prevalence of nonalcoholic fatty liver disease (NAFLD). Data was obtained from Estes et al.[58,59,61]. Data for Saudi Arabia was obtained from Alswat et al.[60], data for Canada was obtained from Swain et al.[62], and data for Australia was obtained from Adams et al.[63].

tries at 23.9% compared to non-Mediterranean countries at 28.5%, although the difference was not statistically significant.

Within Europe, Turkey had the highest NAFLD prevalence at 48.4%, followed by Italy at 38.2%. Germany, Portugal, and Spain had NAFLD prevalence between 25–27%.[3] A cross-sectional study utilizing data from a large population-based cohort in France found that NAFLD prevalence in France was 18.2%.[32] A study involving individuals from population-based studies in Russia reported that NAFLD prevalence was 40% in the Ural Eye and Medical Study (UEMS) (5,852 individuals), and 69.8% in the Ural Very Old Study (UVOS) (1,130 individuals).[33] However, it should be noted that in the UVOS, individuals were older with minimum age of 85 years, and methods for diagnosis of NAFLD differed between studies as well.

North America

Based on subgroup data from 4 studies (18,356 individuals), Le et al.[12] estimated that the prevalence of NAFLD in North America was 35.3%. More recently, NAFLD prevalence was reported at 47.8% in the meta-analysis by Riazi which included 2 large studies with 15,178 individuals from the USA.[3] This is driven by a high prevalence of obesity in the USA. In North America, Hispanics have the highest NAFLD prevalence, followed by non-Hispanic Whites and non-Hispanic blacks.[34-39] Based on data from National Health and Nutrition Examination Surveys (NHANES) 2017–2018, NAFLD prevalence was estimated at 63.7% in Hispanics, 56.8% in non-Hispanic whites, and 46.2% in non-Hispanic blacks.[37] This could be attributed to genetic factors like the patatin-like phospholipase domain-containing protein 3 (*PNPLA3*) mutation, which is associated with elevated risk for hepatic steatosis and NASH, occurring more frequently in Hispanics.[40,41] This could also be related to metabolic factors like the higher prevalence of central adiposity and insulin resistance in Hispanics compared to non-Hispanic whites.[34,42] Lower serum triglyceride levels in African-Americans may also contribute to reduced NAFLD prevalence.[38]

South America

A meta-analysis comprising 19 studies (5,626 individuals) by Rojas et al. estimated the prevalence of NAFLD in South America at up to 59%.[43] Notably, the majority of the studies included in this meta-analysis were hospital-based studies and included patients with risk factors for NAFLD, hence the results may not have been fully representative of the general population. Le et al.[12] pooled data from 3 studies (5,716 individuals) and determined that South America had the greatest estimated NAFLD prevalence among the continents at 35.7%. This may be due to a combination of genetic susceptibility and a greater prevalence of metabolic risk factors.[44] There is a high prevalence of PNPLA3 genetic polymorphism in the general population, especially among individuals with Native American ancestry.[45-47] Furthermore, obesity is extremely common in the region—a cross-sectional study across 4 geographical regions found that central obesity was highest in South America.[48] Type 2 diabetes has also been rising in prevalence in South America.[49] Data from the meta-analysis by Le showed that compared to other regions, NAFLD individuals in South America had a higher likelihood of having diabetes and higher mean cholesterol levels.[12] In addition, physical activity is often inadequate—Latin America was ranked as the top region for physical inactivity, with a third of the population experiencing a lack of physical activity.[50]

Africa

There is a paucity of data from Africa on the epidemiology of NAFLD. A meta-analysis estimated the prevalence of NAFLD in Africa at 13.5%, ranging from 9% in Nigeria to 20% in Sudan.[51] More recently, NAFLD prevalence was reported at 28.2% in the meta-analysis by Le, and 56.8% in the meta-analysis by Riazi.[3,12] Of note, the meta-analysis by Riazi only included 1 study from Egypt. The wide variation in estimates of NAFLD prevalence is likely related to a lack of reliable data from Africa.[52,53]

Oceania

Likewise, there is scarce data from Oceania on the incidence and prevalence of NAFLD. Population-based studies using fatty liver index have demonstrated NAFLD prevalence of 35.7–38% in Australia.[54,55] There are no population-based studies on NAFLD prevalence using imaging modalities such as ultrasound.[56]

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PROJECTIONS IN THE PREVALENCE OF NAFLD

Based on mathematical modeling studies, the burden of NAFLD and NASH will continue to increase over the next 10 years worldwide (Fig. 3). The global prevalence of NAFLD is forecasted to reach 55.4% by 2040.[57] It was estimated by Estes et al.[58] that China would have the greatest overall and relative increase in NAFLD prevalence, with the estimated number of individuals afflicted by NAFLD increasing from 243.67 million in 2016 to 314.58 million in 2030. Comparatively, Japan was forecasted to have the lowest increment in NAFLD population from 22.67 million in 2016 to 22.74 million in 2030, with an estimated prevalence of 18.8% in 2030.[58] A similar modeling study including 4 other Asian countries predicted that Singapore would have the highest relative increase of 20% in NAFLD cases, from 1.49 million in 2019 to 1.8 million in 2030, with an expected prevalence of 28.7% in 2030.[59] South Korea was predicted to have the lowest relative increment of 6% from 10.95 million in 2019 to 11.64 million in 2030, with an expected prevalence of 22.8% in 2030.[59] These models were based on data on obesity prevalence and were predicated on the assumption that changes in NAFLD prevalence would occur in concordance with changes in obesity prevalence. In the Middle East, it was projected that in Saudi Arabia, NAFLD cases would increase from 8.45 million in 2017 to 12.53 million in 2030, with an expected prevalence of 31.7% by 2030; in the United Arab Emirates (UAE), NAFLD cases were projected to increase from 0.255 million in 2017 to 0.372 million in 2030, with an expected prevalence of 30.2% by 2030.[60]

In Europe, a modeling study found that between 2016 to 2030, the number of NAFLD cases could potentially increase from 13.98 million to 16.05 million in France; 18.45 million to 20.95 million in Germany; 15.22 million to 17.42 million in Italy; 10.53 million to 12.65 million in Spain; and 14.08 million to 16.92 million in the United Kingdom (UK). By 2030, the estimated prevalence of NAFLD was forecasted to be highest in Italy (29.5%), followed by Spain (27.6%), Germany (26.4%), the UK (24.7%), and France (23.6%).[58]

This trend of increasing NAFLD prevalence has also been predicted to occur in North America and Australia. A modeling study based on data from the US predicted that the number of individuals with NAFLD would increase by 21% from 83.1 million in 2015 to 100.9 million in 2030, reaching an expected prevalence of 33.5% in 2030.[61] A separate modeling study from Canada projected that NAFLD individuals would rise by 20% from an estimated 7.76 million in 2019 to 9.31 million in 2030.[62] A similar study from Australia estimated that NAFLD cases would increase by 25% from 5.55 million in 2019 to 7.02 million in 2030, and NAFLD prevalence was expected to rise from 22% to 23.6% in 2030.[63] Taken together, these data suggest that the prevalence and burden of NAFLD is likely to increase across multiple world regions if current trends are left unchecked. This serves as a call to action for greater political will and resources directed toward combating metabolic risk factors for NAFLD, at a regional and global level.[64,65]

CONCLUSION

In summary, the global burden of NAFLD is substantial and is projected to increase. It is important to maintain and increase data collection from all world regions to improve the understanding of the burden of disease associated with NAFLD and NASH worldwide. Improving our understanding of the burden of NAFLD can facilitate the development of healthcare policies and strategies to slow this epidemic.

Authors' contribution

All authors have made substantial contributions to all of the following: (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, (3) final approval of the version to be submitted. No writing assistance was obtained in the preparation of the manuscript. The manuscript, including related data, figures and tables has not been previously published and that the manuscript is not under consideration elsewhere.

All authors approve the final version of the manuscript, including the authorship list and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflicts of Interest

The authors have no conflicts to disclose.

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Review

Causes and risk profiles of mortality among individuals with nonalcoholic fatty liver disease

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Nonalcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease in the United States and worldwide. Though nonalcoholic fatty liver *per se* may not be independently associated with an increased risk for all-cause mortality, it is associated with a number of harmful metabolic risk factors, such as type 2 diabetes mellitus, hyperlipidemia, obesity, a sedentary lifestyle, and an unhealthy diet. The fibrosis stage is a predictor of all-cause mortality in NAFLD. Mortality in individuals with NAFLD has been steadily increasing, and the most common cause-specific mortality for NAFLD is cardiovascular disease, followed by extra-hepatic cancer, liver-related mortality, and diabetes. High-risk profiles for mortality in NAFLD include *PNPLA3 I148M* polymorphism, low thyroid function and hypothyroidism, and sarcopenia. Achieving weight loss through adherence to a high-quality diet and sufficient physical activity is the most important predictor of improvement in NAFLD severity and the benefit of survival. Given the increasing health burden of NAFLD, future studies with more long-term mortality data may demonstrate an independent association between NAFLD and mortality. **(Clin Mol Hepatol 2023;29(Suppl):S43-S57)**

Keywords: Non-alcoholic fatty liver disease and fatty liver; Death; Risk factor; NASH; Outcome

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is defined as hepatic steatosis in the absence of significant alcohol consumption or other alternative explanation for hepatic fat deposition, such as underlying other chronic liver diseases.[1,2] It is closely associated with type 2 diabetes mellitus, hyperlipidemia, obesity, gallstone disease, a sedentary lifestyle, and an unhealthy diet.[3-5] NAFLD is the most common cause of chronic liver disease in the United States, where prevalence passed over 30% in 2017–2018.[6] Prevalence of NAFLD is similarly high in other parts of the world, particularly the Middle East and South America.[1] While the prevalence of chronic viral hepatitis has decreased over the past decade, the prevalence of NAFLD has steadily increased over the same period, coinciding with increasing rates of obesity and type 2 diabetes.[7,8] The US national prevalence of NAFLD-related advanced fibrosis increased from 2.6% in 2005–2008 and 4.4% in 2009–2012 to 5.0% in 2013–2016.[7] Age-standardized mortality in individuals with NAFLD has also been steadily increasing over the past decade at an annual rate of 7.8%.[9] Though projected to further increase by 44% between now and 2030,[10]

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mortality for NAFLD still remains lower than those seen in chronic hepatitis C virus (HCV) infection or alcohol-related liver disease (ALD).[9] The most common cause-specific mortality in individuals with NAFLD is cardiovascular disease, followed by mortality due to extra-hepatic cancer, liver-related mortality (including hepatocellular carcinoma, HCC), and diabetes.[11] When controlling for comorbid conditions such as diabetes, hypertension, smoking status, hyperlipidemia, and obesity, NAFLD *per se* is not associated with increased all-cause or cause-specific mortality, likely because a large proportion of this mortality is due to cardiovascular deaths driven by comorbid metabolic abnormalities.[12,13] In contrast, metabolic dysfunction-associated fatty liver disease, which requires the presence of metabolic risk factors in the setting of hepatic steatosis, is associated with increased all-cause and cardiovascular mortality.[13,14] In this review, we focus on the causes and risk profiles of mortality among individuals with NAFLD (Fig. 1).

EPIDEMIOLOGY OF MORTALITY IN NAFLD

All-cause mortality in NAFLD

We summarized essential studies regarding all-cause mortality in individuals with NAFLD in Table 1. The first US community-based retrospective cohort study (n=435) of its kind showed there was a significantly lower survival for populations with NAFLD defined by ultrasonography or histology compared to the age- and sex-matched general population during 7.6 years of follow-up (77% vs. 87%, respectively, $P<0.005$).[15] Several subsequent studies revealed similar results with a significant increase in all cause-mortality with ranges of the hazard ratio (HR) of 1.004–1.038 and standardized mortality ratio of 1.34–2.6.[16] Although earlier studies showed that NAFLD was associated with a higher risk of all-cause mortality compared to the general population of the same age and sex, it is unclear whether NAFLD-related liver disease is an independent risk factor, or if it is associated with the underlying metabolic abnormalities responsible for the increased risk of all-cause and cause-specific mortalities.[17] A US population-based study determined that NAFLD *per se* did not increase mortality risk after adjusting for multiple

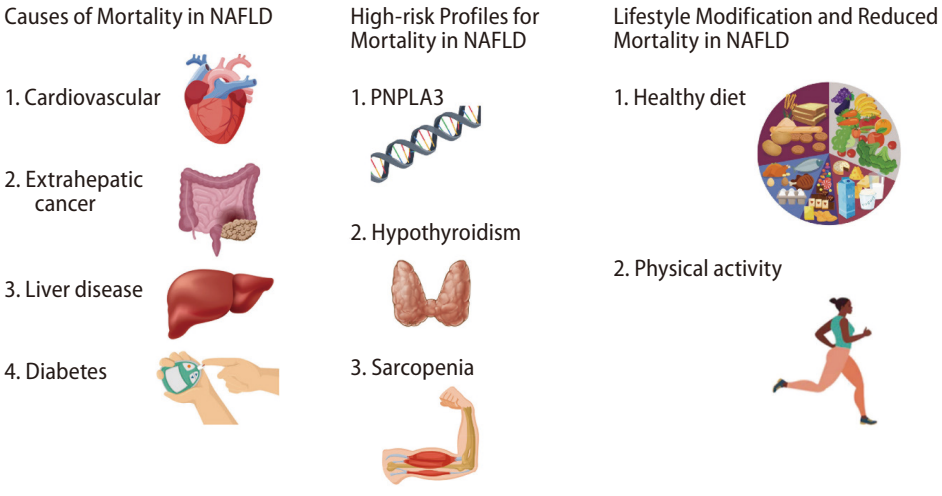

Figure 1. Causes and risk profiles of mortality among individuals with nonalcoholic fatty liver disease. NAFLD, nonalcoholic fatty liver disease; PNPLA3, patatin-like phospholipase domain-containing 3.

Abbreviations:

ALD, alcohol-related liver disease; CI, confidence interval; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HR, hazard ratio; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; RR, relative risk; PA, physical activity; PNPLA3, patatin-like phospholipase domain-containing 3; TSH, thyroid-stimulating hormone

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Table 1. Essential studies evaluating all-cause mortality in individuals with NAFLD

Study	Country	Total population (number of NAFLD)	Diagnostic method	Average follow-up (years)	Outcomes	Confounder adjustment
Dam-Larsen et al.[19] (2004)	Denmark	215	Fatty liver: liver biopsy	NAFLD: 16.7 Alcoholic fatty liver: 9.2	Overall estimated survival in NAFLD was not different from general Danish population	None
Adams et al.[15] (2005)	USA	420 NAFLD	NAFLD: ultrasonography, computed tomography, magnetic resonance imaging, liver biopsy, or cryptogenic cirrhosis + metabolic syndrome	7.6	Overall survival in NAFLD was lower than the expected survival for the general population (HR, 1.34; 95% CI, 1.003–1.76)	Age and sex
Kim et al.[12] (2013)	USA	11,154 (NAFLD: 34%)	NAFLD: ultrasonography Fibrosis: non-invasive panels	14.5	NAFLD had no association with all-cause mortality (HR, 0.89; 95% CI, 0.78–1.02). Advanced fibrosis had a 69% increase in all-cause mortality (HR, 1.69; 95% CI, 1.09–2.63)	Age, sex, race or ethnicity, education, income, diabetes, hypertension, history of cardiovascular disease, lipid-lowering medication, smoking status, waist circumference, alcohol consumption, caffeine consumption, total cholesterol, high-density lipoprotein cholesterol, transferrin saturation, and C-reactive protein
Estes et al.[10] (2018)	USA	N/A	N/A	N/A	Total annual deaths in NAFLD patients were projected to reach 1.83 million in 2030, a 44% increase from a baseline of 1.27 million in 2015	N/A
Kim et al.[9] (2018)	USA	25,379,768 (NAFLD: 30,091)	NAFLD: ICD-10 codes	10	Between 2007 and 2016, there was a linear increase in age-standardized all-cause mortality for NAFLD (APC, 7.8; 95% CI, 6.3–9.4). NAFLD-related mortality increased continuously in Hispanics and non-Hispanic whites from 2007 to 2016, while mortality remained stable in non-Hispanic black	Age

Table 1. Continued

Study	Country	Total population (number of NAFLD)	Diagnostic method	Average follow-up (years)	Outcomes	Confounder adjustment
Taylor et al.[21] (2020)	Multinational	4,428 NAFLD	NAFLD: liver biopsy Fibrosis: liver biopsy	6.2	Biopsy-confirmed fibrosis was associated with increased all-cause mortality in NAFLD, which increased incrementally with increasing fibrosis stage. Stage 1: HR 1.12 (95% CI, 0.91–1.38) Stage 2: HR 1.50 (95% CI, 1.20–1.86) Stage 3: HR 2.13 (95% CI, 1.70–2.67) Stage 4: HR 3.42 (95% CI, 2.63–4.46)	Variable
Alvarez et al.[22] (2020)	USA	12,253 NAFLD	NAFLD: ultrasonography	23.3	The population attributable fraction for overall mortality associated with NAFLD was 7.5% (95% CI, 2.1–79.6)	Age, sex, race/ethnicity, years of education, physical activity score, cigarette smoking, moderate alcohol consumption, body mass index
Kim et al.[13] (2021)	USA	7,761 (NAFLD: 29.5% MAFLD: 25.9%)	NAFLD: ultrasonography MAFLD: criteria proposed by international panel	23	MAFLD(–)/NAFLD(+) had no association with all-cause mortality (HR, 0.94; 95% CI, 0.60–1.46). MAFLD(+)/NAFLD(–) (HR, 1.66; 95% CI, 1.19–2.32) and MAFLD(+)/NAFLD(+) (HR, 1.13; 95% CI, 1.00–1.26) were both associated with an increase in all-cause mortality	Age, sex, race/ethnicity, education, marital status, smoking status, alanine aminotransferase, sedentary lifestyle, body mass index, diabetes, hypertension, fasting triglycerides, high-density lipoprotein cholesterol, waist circumference, and C-reactive protein
Simon et al.[20] (2021)	Sweden	10,568 NAFLD	NAFLD: liver biopsy Fibrosis: liver biopsy	14.2	NAFLD at all histological stages was associated with increased all-cause mortality when compared to the general population (HR, 1.93; 95% CI, 1.64–1.79). Overall mortality increased with the worsening stage of fibrosis. Simple steatosis: HR 1.71 (95% CI, 1.64–1.79) NASH without fibrosis: HR 2.14 (95% CI, 1.93–2.38) Non-cirrhotic fibrosis: HR 2.44 (95% CI, 2.22–2.69) Cirrhosis: HR 3.79 (95% CI, 3.34–4.30) *P* trend: <0.01	Age at the index date, sex, county, calendar year, education level, cardiovascular disease, and the metabolic syndrome, defined as a composite categorical variable (ranging from 0 to 4) with 1 point given for each of the following conditions (i.e., diabetes, obesity, hypertension and/or dyslipidemia)

NAFLD, nonalcoholic fatty liver disease; HR, hazard ratio; CI, confidence interval; APC, annual percentage change; MAFLD, metabolic (dysfunction)-associated fatty liver disease; NASH, nonalcoholic steatohepatitis; ICD-10, International Classification of Diseases 10th revision; N/A, not applicable.

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clinical and metabolic confounders beyond age and sex.[12,13] Consistent with these results, several studies have reported no significant difference in all-cause mortality in individuals with NAFLD.[16,18,19] Stratification by fibrosis using non-invasive panels was associated with a higher risk of all-cause mortality.[12] A Swedish nationwide, matched cohort study with 10,568 biopsy-confirmed NAFLD reported that significant excess mortality risk was noted in nonalcoholic steatohepatitis (NASH) without fibrosis (adjusted HR, 1.14; 95% confidence interval [CI], 1.03–1.26), non-cirrhotic fibrosis (adjusted HR, 1.26; 95% CI, 1.15–1.38) and cirrhosis (adjusted HR, 1.95; 95% CI, 1.75–2.18) compared with nonalcoholic fatty liver (simple steatosis).[20] Dose-response association along with the severity of NAFLD was observed (*P* for trend <0.01).[20] A recent meta-analysis showed that compared with no fibrosis (stage 0), the unadjusted risk increased with increasing stage of fibrosis (stage 0 vs. 4) with all-cause mortality relative risk (RR) of 3.42 (95% CI, 2.63–4.46) irrespective of the presence or absence of NASH.[21] The stage of fibrosis and rate of fibrosis development associated with mortality in NAFLD may be utilized as a predictor to differentiate between low-risk NAFLD and those that will progress to fibrosis or cirrhosis, which result in all-cause mortality. Therefore, better phenotyping of NAFLD may be needed to determine the relationship of NAFLD with all-cause mortality.

The recent trends in NAFLD-related all-cause mortality showed an initial linear increase, which then accelerated in recent years in the US.[9,11] Although the International Classification of Diseases 10th revision (ICD-10) code for NAFLD underestimated the true prevalence of NAFLD, the mortality due to NAFLD increased from an annual rate of 6.1% (95% CI, 4.5–7.8%) in 2007–2013 to 11.3% (95% CI, 6.3–16.6%).[9] Compared with other racial/ethnic subgroups, non-Hispanic whites had higher mortality due to NAFLD.[9] NAFLD-related mortality increased continuously in Hispanics and non-Hispanic whites from 2007 to 2016, while mortality remained stable in non-Hispanic blacks.[9] A recent study showed that the attributable risk of NAFLD for all-cause mortality is 7.5% (95% CI, 3.0–12.0%), although the attributable risk of diabetes was 38.0% (95% CI, 13.1–63.0%).[22] NAFLD-related mortality is expected to increase by 44% to 1.83 million annual deaths by 2030 in the US.[10]

Cause-specific mortality in NAFLD

The leading cause of death in individuals with NAFLD is cardiovascular disease (summarized in Table 2), followed by extra-hepatic cancer and then liver-related mortality (summarized in Table 3).[12,15]

Cardiovascular mortality

NAFLD has been associated with an increased risk for the development of cardiovascular disease compared to those without NAFLD. A recent meta-analysis reported that NAFLD was associated with a moderately increased risk of fatal or non-fatal cardiovascular disease events (pooled HR, 1.45; 95% CI, 1.31–1.61).[23] This risk markedly increased across the severity of NAFLD, especially the fibrosis stage (pooled HR, 2.50; 95% CI, 1.68–3.72).[23] This effect is even more substantial with more advanced liver disease, especially with higher fibrosis stage, suggesting that the severity of NAFLD may independently predict risk for incident cardiovascular disease. Even relative to other causes of liver disease, such as viral hepatitis or ALD, the underlying cause of death in individuals with NAFLD is more likely to be cardiovascular disease. Though the independent association between NAFLD and increased cardiovascular mortality may be inconclusive, the underlying cause of death in individuals with NAFLD was more likely to be cardiovascular disease compared with other chronic liver diseases.[9] According to a study from the US national mortality data, the proportion of deaths due to cardiovascular disease in individuals with NAFLD was 16.2%, notably higher than that seen for those with HCV infection (10.3%), hepatitis B virus infection (7.2%), and ALD (5.0%).[7] This is likely due to the fact that many of the comorbid metabolic abnormalities associated with NAFLD confer an increased risk of cardiovascular mortality. In particular, the accumulation of ectopic fat and resulting pro-inflammatory milieu work synergistically with associated dyslipidemia to accelerate the process of atherosclerosis. Among individuals with NAFLD, a high probability of advanced fibrosis by non-invasive markers was significantly associated with an increased risk of cardiovascular mortality (HR: 3.46, 95% CI: 1.91–6.25 for NAFLD fibrosis score; HR: 2.68, 95% CI: 1.44–4.99 for fibrosis-4 [FIB-4]; HR: 2.53, 95% CI: 1.33–4.83 for aspartate aminotransferase to platelet ratio index).[12] A multinational study with 458 biopsy-proven NAFLD with bridging fibrosis (n=159) or compensated cirrhosis (n=222) showed

Table 2. Essential studies evaluating cardiovascular mortality in individuals with NAFLD

Study	Country	Total population (number of NAFLD)	Diagnostic method	Average follow-up (years)	Outcomes	Confounder adjustment
Adams et al.[15] (2005)	USA	420 NAFLD	NAFLD: ultrasonography, computed tomography, magnetic resonance imaging, liver biopsy, or cryptogenic cirrhosis + metabolic syndrome	7.6	Cardiovascular disease was identified as the cause of death in 28% of participants.	Age and sex
Kim et al.[12] (2013)	USA	11,154 (NAFLD: 34%)	NAFLD: ultrasonography Fibrosis: non-invasive panels	14.5	Increased mortality in individuals with NAFLD and hepatic fibrosis was driven mostly by cardiovascular death. NFS: HR 3.56 (95% CI, 1.91–6.25) APRI: HR 2.53 (95% CI, 1.33–4.83) FIB-4: HR 2.68 (95% CI, 1.44–4.99)	Age, sex, race or ethnicity, education, income, diabetes, hypertension, history of cardiovascular disease, lipid-lowering medication, smoking status, waist circumference, alcohol consumption, caffeine consumption, total cholesterol, high-density lipoprotein cholesterol, transferrin saturation, and C-reactive protein
Vilar-Gomez et al.[24] (2018)	Multinational	458 NAFLD (Bridging fibrosis: 35%, Compensated cirrhosis: 65%)	NAFLD, fibrosis, or cirrhosis: liver biopsy	5.5	Cardiovascular deaths made up a higher proportion of overall mortality in patients with NAFLD and bridging fibrosis (5%) than in cirrhosis (1–2%). Annualized incidence of major vascular events in the entire cohort was 0.9 (95% CI, 0.5–1.8).	Center, race/ethnicity, age, sex, calendar year of patients' recruitment, baseline body mass index, hypertension, history of previous vascular events or malignant neoplasm, anti-diabetic, antihypertensive, and hypolipidemic drugs, aspirin, current smoking and diagnosis of type 2 diabetes as time-varying covariates.
Kim et al.[9] (2018)	USA	25,379,768 (NAFLD: 30,091)	NAFLD: ICD-10 codes	10	Cardiovascular disease made up a higher proportion of overall mortality in individuals with NAFLD than those with other chronic liver diseases. NAFLD-related cardiovascular mortality steadily decreased over the period.	Age

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Table 2. Continued

Study	Country	Total population (number of NAFLD)	Diagnostic method	Average follow-up (years)	Outcomes	Confounder adjustment
Kim et al.[11] (2019)	USA	27,903,198 (NAFLD: 33,945)	NAFLD: ICD-10 codes	11	The cause of death in NAFLD was more likely to be cardiovascular disease (approximately 20%), which increased at a gradual rate (APC, 2.0%; 95% CI, 0.6–3.4), whereas liver-related mortality increased rapidly (APC, 12.6%; 95% CI, 11.7–13.5).	Age
Mantovani et al.[23] (2021)	Multinational	5,802,226	NAFLD: liver biopsy, imaging techniques, or ICD-10 codes in the absence of significant alcohol consumption	6.5	Incidence of fatal or non-fatal cardiovascular events was higher in individuals with NALFD (HR: 1.45; 95% CI, 1.31–1.61). Incidence increased with increasing severity of fibrosis (pooled random-effects HR, 2.50; 95% CI, 1.68–3.72).	Age, sex, adiposity measures, diabetes, and other common cardiometabolic risk factors

NAFLD, nonalcoholic fatty liver disease; NFS, NAFLD fibrosis score; HR, hazard ratio; CI, confidence interval; APRI, aspartate aminotransferase to platelet ratio index; APC, annual percentage change; ICD-10, International Classification of Diseases 10th revision; FIB-4, fibrosis-4.

that NAFLD with bridging fibrosis had extra-hepatic cancers and cardiovascular events predominantly, while NASH cirrhosis had liver-related events predominantly.[24] Although all-cause mortality was significantly lower in NAFLD with bridging fibrosis, 50% of deaths were directly attributed to extra-hepatic cancers or cardiovascular events. In contrast, patients with compensated cirrhosis were at significantly lower risk for non-liver-related deaths (12%).[24] Therefore, it is essential to identify advanced fibrosis at increased risk of cardiovascular mortality among individuals with NAFLD.

Extra-hepatic cancer-related mortality

A Korean cohort study reported the association between NAFLD and incident cancer. During the follow-up of the median of 7.5 years, the cancer incidence rate in NAFLD was higher than that of non-NAFLD (HR, 1.32; 95% CI, 1.17–1.49).[25] NAFLD was strongly associated with two extra-hepatic cancers: colorectal cancer in men (HR, 2.01; 95% CI, 1.10–3.68) and breast cancer in women (HR, 1.92; 95% CI, 1.15–3.20).[25] A high probability of advanced fibrosis was associated with developing all cancers and HCC.[25] A US cohort study with age and sex-matched individuals with and without NAFLD reported that NAFLD was associated with a 90% increased risk of cancer.[26] The incidence of uterine, stomach, pancreas, and colon cancer was higher in those with NAFLD than those without.[26] Other cancers that have been demonstrated to have a higher incidence in those with NAFLD include male genital, female breast, and skin cancer in any gender.[27] Interestingly, NAFLD carries an independent association with an increased risk for cancer, while obesity alone does not.[26] NAFLD is associated with an increased risk for cancer-related mortality even outside the liver, and mortality due to extra-hepatic cancer is rising faster than any other cause of death in individuals with NAFLD at an annual percent change of 15.1% (95% CI, 13.0–17.2%).[11] A recent meta-analysis reported that NAFLD was significantly associated with a 1.5–2 fold higher risk of incident gastrointestinal cancers (esophagus, stomach, colorectal, or pancreas) independent of age, sex, obesity, diabetes, smoking, or other potential confounders.[28] In addition, NAFLD was associated with a nearly 1.2–1.5-fold higher risk of incident lung, breast, urinary tract, or gynecological cancers.[28] Extra-hepatic cancer and cardiovascular mortality rates in NAFLD-related cirrhosis were more pronounced than in NAFLD without cirrhosis.[11] Though the mechanism of hepatic fibrosis facilitating carcinogenesis in

Table 3. Essential studies evaluating liver-related mortality in individuals with NAFLD

Study	Country	Total population (number of NAFLD)	Diagnostic method	Average follow-up (years)	Outcomes	Confounder adjustment
Younossi et al.[37] (2015)	USA	19,916 (NAFLD: 1,944)	NAFLD: ICD-9 codes	10	14.1% of HCC cases were related to NAFLD. The proportion of HCC related to NAFLD had a 9% average annual increase between 2004–2009. NAFLD-related HCC was associated with increased risk of 1-year overall mortality (OR, 1.21; 95% CI, 1.01–1.45)	Age, gender, cancer stage, residence region, education, median household income, modified Charlson comorbidity index, and date of diagnosis
Dulai et al.[32] (2017)	Multinational	1,395 NAFLD	NAFLD: liver biopsy	11.7	Individuals with NAFLD and stage 2 fibrosis or higher had increased risk for liver-related mortality when compared to individuals with NAFLD and stage 0 fibrosis (MRR, 9.57; 95% CI, 0.17-11.95) Liver-related mortality rates increased exponentially with increasing stage of fibrosis. Liver-related deaths made up 59% of all-cause mortality in individuals with stage 4 fibrosis.	None
Kim et al.[33] (2019)	USA	25,379,768 (NAFLD: 12,099)	NAFLD: ICD-10 codes	10	Age-standardized cirrhosis-related mortality rates in individuals with NAFLD increased linearly from 2007 and 2016 with an average annual percent change of 15.4% (95% CI, 14.1–16.7). Age-standardized HCC-related mortality rates in individuals with NAFLD increased linearly from 2007 and 2016 with an average annual percent change of 19.1% (95% CI, 14.0–24.5).	Age
Taylor et al.[21] (2020)	Multinational	4,428 NAFLD	NAFLD: liver biopsy Fibrosis: liver biopsy	6.2	Biopsy-confirmed fibrosis was associated with increased liver-related mortality in NAFLD. This increased incrementally with increasing fibrosis stage, reaching significance at stage 3 fibrosis. Stage 1: HR 1.05 (95% CI, 0.35–3.16) Stage 2: HR 2.53 (95% CI, 0.88–7.27) Stage 3: HR 6.65 (95% CI, 1.99–22.25) Stage 4: HR 11.13 (95% CI, 4.15–29.84)	Variable

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Table 3. Continued

Study	Country	Total population (number of NAFLD)	Diagnostic method	Average follow-up (years)	Outcomes	Confounder adjustment
Kim et al.[41] (2020)	USA	25,907,886 (NAFLD: 15,812)	NAFLD: ICD-10 codes	10	Age-standardized cirrhosis-related mortality rate in individuals with NAFLD increased linearly with an average annual percent change of 16.2% (95% CI, 15.4–17.0) between 2009–2018. Age-standardized HCC-related mortality rate in individuals with NAFLD increased linearly with an average annual percent change of 21.1% (95% CI, 16.9–25.4) between 2009–2018.	Age

NAFLD, nonalcoholic fatty liver disease; HCC, hepatocellular carcinoma; OR, odds ratio; CI, confidence interval; MRR, mortality rate ratio; HR, hazard ratio; ICD-10, International Classification of Diseases 10th revision.

the liver is well-described, how NAFLD and metabolic syndrome are associated with the development of extra-hepatic cancer is less well-understood. It is theorized that hepatic fat deposition results in the release of pro-inflammatory cytokines, leading to extra-hepatic tissue damage, remodeling, and immune cell dysfunction.[29] This theory partly explains why obesity in the absence of hepatic steatosis is not associated with an increased risk of cancer. However, future mechanistic studies are warranted.

Liver-related mortality

Individuals with NAFLD are at risk for progression to liver fibrosis and cirrhosis. This is especially true of the inflammatory subtype of NASH, which carries a 20% lifetime risk of progression to cirrhosis.[30] Prevalence of NAFLD-associated advanced fibrosis in the US has increased markedly in recent years, doubling from 3% in 2005–2006 to 6% in 2013–2016.[7] Increased age, insulin resistance, and genetic polymorphisms may be associated with an increased risk for the development of fibrosis in individuals with NAFLD.[31] Liver fibrosis is one of the most important predictors of mortality in NAFLD, and liver-related mortality increases exponentially with the increasing fibrosis stage.[32] A recent meta-analysis showed that individuals with NAFLD and fibrosis were at an increased unadjusted RR of liver-related mortality and all-event liver morbidity compared with those with NAFLD and no fibrosis, and this risk was incremental according to the fibrosis stage.[21] Liver-related mortality included deaths due to compensated cirrhosis, complications of decompensated cirrhosis (ascites or bleeding esophageal varices, hepatic encephalopathy), acute on chronic liver failure, and/or HCC. A recent US national study showed that liver-related mortality among individuals with NAFLD was responsible for 58.9% of deaths in 2017, although liver-related mortality among those with NAFLD was lower than among those with other chronic liver diseases.[11] NAFLD-related liver mortality markedly increased in recent years with an annual percentage change of 4.9% (95% CI, 4.2–5.5%) during the recent decade.[9]

In terms of cirrhosis-related mortality, there was an initial increase in cirrhosis due to HCV infection at a rate of 2.9% per year (95% CI, 2.3–3.5%) in 2007–2014, followed by a decrease in 2014–2016 at an annual rate of 6.5% (95% CI, –10.3% to –2.6%) after the introduction of direct-acting antiviral agents.[33] In contrast, mortality due to NASH cirrhosis increased with an average annual rate of 15.4% (95% CI, 14.1–

16.7%) during the recent decade.[33]

NAFLD is the fastest-growing cause of HCC in the world.[34] HCC risk associated with diabetes seemed to be highest in NAFLD, followed by ALD.[35] Based on dynamic modeling after accounting for current trends in diabetes and obesity, the annual incidence of NAFLD-associated HCC is projected to increase by 137%, from 5,160 cases in 2015 to 12,240 cases in 2030.[10] A meta-analysis showed that the annual incidence of HCC was 0.44 per 1,000 person-years in those with NAFLD, and even higher in those with biopsy-proven NASH (5.29 per 1,000 person-years).[36] In addition, HCC is an increasingly-recognized contributor to mortality in individuals with NAFLD, as metabolic syndrome and NAFLD cause almost 10% of cases of HCC in the world and 14.1% of cases of HCC in the US.[37] HCC usually arises in the background of cirrhosis, thought to be related to increased cell turnover from chronic inflammation leading to the formation of driver gene mutations. However, NAFLD and NASH are among the most common causes of HCC in the absence of cirrhosis.[38] HCC is the fourth leading cause of cancer-related mortalities globally, accounting for 810,000 mortalities in 2015.[39] Globally, deaths from HCC increased by 60% from 1990 to 2013,[40] and HCC remained the second leading cause of years of life lost due to cancer from 2005 to 2015.[39] In addition, HCC is a growing burden in individuals with NAFLD. A recent study based on the US National Vital Statistics System demonstrated an increase in the annual rate of HCV infection-related HCC mortality of 5.4% (95% CI, 3.6–7.4%) was noted from 2009 to 2014, followed by a decrease from 2014 to 2018 at a rate of 3.5% per year (95% CI, –5.9% to –1.1%) after the introduction of potent direct-acting antiviral agents.[41] In contrast, age-standardized mortality for HCC from NAFLD demonstrated a linear increase with an annual percentage change of 21.1% (95% CI, 16.9–25.4%) from 2009 to 2018.[41]

Diabetes-related mortality

Type 2 diabetes is common among individuals with NAFLD and NASH, with a global estimated prevalence of 22.5% (95% CI, 17.9–27.9%) and 43.6% (30.3–58.0%), respectively,[36] compared to a contemporary US national prevalence of 14.3% (95% CI, 12.9–15.8%).[42] This strong association reflects the overlapping pathogenesis of metabolic dysregulation shared between the two conditions. However, the relationship between type 2 diabetes and NAFLD is complex and may be bidirectional.[43] The global prevalence of NAFLD and NASH among individuals with type 2 diabetes was 55.5% (95% CI, 47.3–63.7%) and 37.3% (95% CI, 24.7–50.0%).[44] A recent US population-based study showed that the prevalence of NAFLD by transient elastography was high in individuals with prediabetes (38.5–52.9%) and diabetes (70.7–82.1%).[45] Significant fibrosis and cirrhosis were observed in about one-fourth of individuals with NAFLD and diabetes and one-sixth with NAFLD and prediabetes.[45] In the US general population, age-standardized mortality due to diabetes declined from 112.2 per 100,000 individuals in 2007 to 104.3 in 2017, with the decline of annual percentage change of –1.4% (95% CI, –1.9% to –1.0%) in 2007–2014 and stabilization of annual rate of 1.1% (95% CI, –0.6% to 2.8%) in 2014–2017.[46] When looking specifically at individuals with NAFLD and diabetes, however, mortality in individuals with NAFLD increased at an annual rate of 11.6% (95% CI, 9.5–13.8%) during the same period.[47] Therefore, clinicians bear in mind the harmful impact of NAFLD among individuals with diabetes and vice versa.

HIGH-RISK PROFILES FOR MORTALITY IN NAFLD

As commented above, it is essential to identify and phenotype high-risk profiles at increased risk of all-cause mortality among individuals with NAFLD.

Genetic polymorphism

Outcomes of individuals with NAFLD are impacted by several associated factors, including genetic mutations such as polymorphisms in the patatin-like phospholipase domain-containing 3 (PNPLA3) gene. PNPLA3 encodes an enzyme involved in the hydrolysis of triglycerides, and mutations affecting its function have been associated with increased risk for the development of NAFLD, NASH, advanced fibrosis, and HCC.[48] *PNPLA3 I148M* polymorphism is more common among Hispanics, contributing to a higher incidence of advanced fibrosis and poorer outcomes from NAFLD compared with any other race/ethnicity.[49] *PNPLA3 I148M* polymorphism is associated with an earlier age of NAFLD, observation most pronounced in Hispanic Americans.[50] Earlier studies on the association between *PNPLA3 I148M* polymorphism and all-cause mortality have reported inconsistent results.[51,52] A US population-based study determined that individuals with NAFLD

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who are homozygous for the *PNPLA3 I148M* mutation are at increased risk of all-cause mortality as well as liver-related mortality when compared to those with NAFLD and wildtype *PNPLA3* genotype during a follow up of 20 years.[53,54] Risk for cardiovascular mortality does not appear to be increased in individuals with *PNPLA3 I148M polymorphism*. A Chinese study with a mean age of 64 years showed that being homozygous for the *PNPLA3 I148M* mutation was independently associated with increased liver-related mortality (HR, 3.34; 95% CI, 1.01–11.17) but not associated with all-cause and cardiovascular mortality during 5.3 years follow-up.[55] Further studies are warranted to confirm these associations.

Low thyroid function and hypothyroidism

Low thyroid function, defined as higher levels of thyroid-stimulating hormone (TSH) level within the normal reference range of thyroid hormone ("low-normal" thyroid function and subclinical hypothyroidism), may cause adverse health effects similar to overt hypothyroidism. Hypothyroidism and low thyroid function are closely associated with increased risk for NAFLD, and a more advanced spectrum of NAFLD, including NASH, and significant fibrosis independent of clinical and metabolic risk factors.[56-58] A recent longitudinal study showed that increasing TSH levels during a median follow-up of 4 years were associated with incident NAFLD independent of other metabolic factors.[59] An US population-based study determined a strong association between NAFLD with increasing plasma TSH levels and all-cause mortality, mainly from cardiovascular mortality.[60] During the median follow-up of 23 years, low thyroid function was independently associated with an increased risk for all-cause mortality in individuals with NAFLD (HR, 1.24; 95% CI, 1.02–1.50), while this association was absent in those without NAFLD.[60] "Low-normal" thyroid function and subclinical hypothyroidism were significantly associated with an increase in the risk for all-cause mortality among individuals with NAFLD of 18% and 38%, respectively.[60] Low thyroid function was associated with cardiovascular mortality in individuals with NAFLD (HR, 1.62; 95% CI, 1.11–2.34). "Low-normal" thyroid function and subclinical hypothyroidism were significantly associated with a increase in the risk for cardiovascular mortality among individuals with NAFLD of 50% and 94%, respectively.[60]

Sarcopenia

NAFLD and sarcopenia, which share various pathophysiologic mechanisms, have become increasingly prevalent conditions, resulting in a significant health burden.[61] Previous Asian studies determined sarcopenia is independently associated with NAFLD[62,63] and NAFLD-associated fibrosis.[64] A US population-based study showed an independent association between sarcopenia and NAFLD across various ethnicities.[65] During a median follow-up of 23 years, individuals with both NAFLD and sarcopenia had an increased risk for all-cause mortality (HR, 1.28; 95% CI, 1.06–1.55) compared with those without NAFLD and sarcopenia.[66] Sarcopenia was associated with an increased risk for all-cause mortality only in individuals with NAFLD after adjusting for advanced fibrosis, whereas this association was absent in those without NAFLD.[66] Other research is consistent with this finding.[67,68] Both NAFLD and sarcopenia confer increased risk for adverse outcomes mediated by a combination of additive and synergic risk factors for all-cause and cardiovascular-related mortality.[61]

LIFESTYLE MODIFICATION IN NAFLD AND MORTALITY

Lifestyle modification is the staple of the management of NAFLD of any severity. Guidelines from both the American Gastroenterological Association and American Association for the Study of Liver Diseases on the management of NAFLD recommend lifestyle modification with a combination of physical activity (PA) and dietary modifications to achieve a weight loss of ≥5% of total body weight for NAFLD reduction, ≥7% for NASH resolution, and ≥10% for fibrosis regression/stability.[69,70] Though achieving weight loss is the most important predictor of improvement in NASH or fibrosis, adherence to a high-quality diet and sufficient PA have each been associated with improvement in NAFLD, even in the absence of weight loss.[71,72] Dietary modification includes restriction of caloric intake by 500–1,000 kcal as well as prioritization of foods low in carbohydrates and saturated fats, such as in the Mediterranean diet.[69,70] Higher diet quality was associated with significantly lower odds of NAFLD and a lower risk for all-cause mortality.[3] Clinicians focusing on primary prevention with high diet quality may be the ideal way to help curb the rising prevalence of NAFLD.

Practice guidelines recommend that individuals with NAFLD should achieve more than 150 minutes/week of moderate-intensity or more than 75 minutes/week of vigorous-intensity PA,[70] which mirrors guideline recommendations for PA in the general population for the primary prevention of cardiovascular disease.[73] Although the prevalence of meeting the PA guidelines for leisure time increased in individuals without NAFLD from 2007 through 2016, the trends in meeting PA guidelines for any type of PA remained stable among those with NAFLD, with downtrends in transportation-related PA in the US.[74] Increasing PA beyond the amount recommended by PA guidelines may have an additional benefit to the management for NAFLD.[74,75] While 150–299 minutes/week of PA was associated with 40% lower odds of NAFLD, that risk reduction was 49% in those who achieved ≥300 minutes/week.[75] PA ≥300 minutes/week was also associated with 59% lower odds of fibrosis and 63% lower odds of cirrhosis.[75] Similar to diet quality, the level of PA has also been demonstrated to influence mortality. A recent US population cohort study with an average follow-up of 10.6 years showed that increasing duration of objectively-measured PA was associated with a reduced risk of all-cause mortality (*P* for trend <0.001) among individuals with NAFLD.[76] Furthermore, longer total PA was associated with a lower risk for cardiovascular mortality in individuals with NAFLD (*P* for trend=0.007).[76] In summary, increasing PA has beneficial survival impacts on all-cause and cardiovascular mortality in individuals with NAFLD. Increasing PA in individuals with NAFLD should be recommended for its benefits on survival.

CONCLUSION

NAFLD is a highly prevalent and growing problem in the United States and worldwide. The overall incidence of the disease, as well as associated mortality rates, are continually increasing. While NAFLD *per se* may do not independently increase the risk for all-cause mortality, more severe NAFLD is associated with the underlying metabolic complications responsible for the increased risk of all-cause and cause-specific mortalities. The most common causes of death in individuals with NAFLD are cardiovascular disease, extra-hepatic cancer, liver disease (including decompensated cirrhosis and HCC), and diabetes. Mortality in NAFLD is further influenced by mutations in the PNPLA3 gene, low thyroid function, and sarcopenia. Weight loss through diet and PA is the recommended approach for NAFLD. Both diet and exercise have each been demonstrated to have significant effects on mortality, including all-cause and cardiovascular mortality. As the health burden of NAFLD increases, future studies may demonstrate an association between NAFLD and mortality, especially as more long-term mortality data is available that captures the downstream cardiovascular consequences of long-standing NAFLD and fibrosis.

Authors' contribution

Dr. Peter Konyn was involved in the study concept and design, acquisition of data, interpretation of data, and drafting of the manuscript. Dr. Aijaz Ahmed and Dr. Donghee Kim were involved in the study concept and design, interpretation of data, drafting of the manuscript, critical revision of the manuscript, and study supervision.

Conflicts of Interest

The authors have no conflicts to disclose.

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Review

Comparison between obese and non-obese nonalcoholic fatty liver disease

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Nonalcoholic fatty liver disease (NAFLD) encompasses a spectrum of liver conditions that are characterized by excess accumulation of fat in the liver, and is diagnosed after exclusion of significant alcohol intake and other causes of chronic liver disease. In the majority of cases, NAFLD is associated with overnutrition and obesity, although it may be also found in lean or non-obese individuals. It has been estimated that 19.2% of NAFLD patients are lean and 40.8% are non-obese. The proportion of patients with more severe liver disease and the incidence of all-cause mortality, liver-related mortality, and cardiovascular mortality among non-obese and obese NAFLD patients varies across studies and may be confounded by selection bias, underestimation of alcohol intake, and unaccounted weight changes over time. Genetic factors may have a greater effect towards the development of NAFLD in lean or non-obese individuals, but the effect may be less pronounced in the presence of strong environmental factors, such as poor dietary choices and a sedentary lifestyle, as body mass index increases in the obese state. Overall, non-invasive tests, such as the Fibrosis-4 index, NAFLD fibrosis score, and liver stiffness measurement, perform better in lean or non-obese patients compared to obese patients. Lifestyle intervention works in non-obese patients, and less amount of weight loss may be required to achieve similar results compared to obese patients. Pharmacological therapy in non-obese NAFLD patients may require special consideration and a different approach compared to obese patients. **(Clin Mol Hepatol 2023;29(Suppl):S58-S67)**

Keywords: Nonalcoholic fatty liver disease; Non-obese; Lean

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) encompasses a spectrum of liver conditions that are characterized by excess accumulation of fat in the liver. The diagnosis is made following the exclusion of significant alcohol intake and other causes of chronic liver disease.[1,2] In the majority of cases, NAFLD is associated with overnutrition and obesity, although it may be also found in non-obese patients. The condition is closely associated with metabolic syndrome, which is a constellation of risk factors for cardiovascular disease.[3] The prevalence of NAFLD has been increasing, and it is recognized as the most common cause of chronic liver disease worldwide.[4,5] In 2020, an international panel of experts proposed a new term, "metabolic dysfunction-associated fatty liver disease (MAFLD)," which is diagnosed in persons with fatty liver in the presence of overweight or obesity, type 2 diabetes mellitus, or at least two metabolic risk abnormalities.[6] The present

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review primarily focuses on the comparison between obese and non-obese NAFLD, for which there is a richer body of literature, given that the term NAFLD has been in existence for a much longer period of time. However, the literature on MAFLD is rapidly expanding, and a similar review on MAFLD in due time would be of great interest. In general, a body mass index (BMI) cut-off of 25 and 30 kg/m^2 is used for the definition of obesity for Asian and Caucasian populations, respectively. In studies using the term "lean NAFLD," the non-lean patients included those who were overweight, defined by a BMI of ≥23 and ≥25 kg/m^2 for Asian and Caucasian populations, respectively.

EPIDEMIOLOGY AND NATURAL HISTORY OF NON-OBESE NAFLD

Initial recognition and increasing interest

One of the earliest reports on non-obese NAFLD came from India. In a study on 1,911 subjects from the rural administrative unit of West Bengal that was published in 2013, Das and colleagues found the prevalence of NAFLD to be 8.7%. While this was relatively low compared to studies from other parts of India, the prevalence was considerably high, given that the majority of study subjects were young, physically active, less affluent, and non-obese. The term "third-world NAFLD" was used to describe this phenotype, where instead of overt obesity, subtle measures of increased adiposity predisposed to NAFLD.[7] The interest in non-obese NAFLD sky-rocketed after an abstract was presented at the Digestive Disease Week in the following year. In a study on 1,090 biopsy-proven NAFLD patients who were followed for 133 months, Dela Cruz and colleagues found that lean NAFLD patients had a significantly shorter survival compared to non-lean NAFLD patients.[8] Subsequently, a population-based study on 911 patients using proton-magnetic resonance spectroscopy and transient elastography in Hong Kong found non-obese patients to have less severe liver disease based on significantly lower serum cytokeratin-18 level and liver stiffness measurement.[9] Furthermore, in another study on 307 biopsy-proven NAFLD patients, non-obese NAFLD patients had significantly lower serum cytokeratin-18 level, liver stiffness measurement, and histological fibrosis stage. During follow-up, six patients died, two developed hepatocellular carcinoma, and one had liver failure, all of whom in the obese patients.[10]

Possible reasons for disparities in data

Several other longitudinal studies have shown conflicting results (Table 1).[11-15] A study in Sweden found that patients with lean NAFLD were paradoxically more likely to develop develop severe liver disease, despite having less severe liver disease at baseline, compared to non-lean patients.[11] Further studies are warranted to understand the reasons behind these inconsistent findings. One possible explanation is that the lean NAFLD patients in the study had more severe liver disease than expected compared to the general population, which could be expected given that the patients were seen in a secondary or tertiary care setting and underwent liver biopsy. This was evident from the high proportion of lean patients with nonalcoholic steatohepatitis (NASH) and advanced liver fibrosis at 50% and 9.8%, respectively. Furthermore, important confounding factors, such as changes in alcohol intake and body weight over time, were not taken into account. Alcohol intake is an important confounding factor and may not be adequately captured due to under-reporting. In a study on 184 patients, repeated moderate to excessive alcohol intake was detected in 28.6% of patients with presumed NAFLD, and patients with repeated moderate to excessive alcohol intake had significantly lower BMI.[16] This may partly contribute to the high proportion of lean or non-obese NAFLD patients with more severe liver disease. Assessment of alcohol intake by ethylglucuronide in hair had an area under curve of 0.93 for the detection of repeated moderate to excessive alcohol consumption,[16] which may be useful to more accurately classify patients with fatty liver as NAFLD or not.

Epidemiology and clinical characteristics

A systematic review and meta-analysis estimated the prev-

Abbreviations:

NAFLD, nonalcoholic fatty liver disease; MAFLD, metabolic dysfunction-associated fatty liver disease; BMI, body mass index; NASH, nonalcoholic steatohepatitis; PNPLA3, patatin-like phospholipase domain-containing-3; TM6SF2, transmembrane 6 superfamily member 2; E167K, glutamate by lysine at position 167

Table 1. Summary of longitudinal studies on lean or non-obese NAFLD patients with or without baseline liver biopsy

Study	Population	Proportion of patients with lean and/or non-obese NAFLD	Main findings
Leung et al. (2017)[10]	342 biopsy-proven NAFLD patients in Hong Kong	23.5% were non-obese.	Non-obese NAFLD patients had lower NAFLD activity scores, histological fibrosis stage, serum cytokeratin-18 levels, and liver stiffness measurement by transient elastography. During a median follow-up of 49 months, six patients died, two developed hepatocellular carcinoma, and one had liver failure, all of whom were in the obese group.
Hagström et al. (2018)[11]	646 biopsy-proven NAFLD patients in Sweden	19% were lean.	Lean patients had less severe liver disease. NASH: 50% among lean vs. 64.6% among overweight patients and 79.8% among obese patients, $P<0.001$. Advanced fibrosis: 9.8% vs. 10.8% and 15.9%. During a mean follow-up of 19.9 years, compared to patients who were overweight, patients with lean NAFLD had no increased risk for overall mortality (hazard ratio 1.06, $P=0.73$) but had an increased risk for developing more severe liver disease (hazard ratio 2.69, $P=0.007$).
Chang et al. (2019)[12]	437,828 Korean adults	Prevalence of NAFLD was 20.9%. Among individuals with NAFLD, 61.7% were obese.	Compared with individuals without fatty liver, the liver-related mortality was higher among non-obese NAFLD individuals (hazard ratio 2.12, 95% CI 1.12–4.02) than among obese NAFLD individuals (hazard ratio 0.54, 95% CI 0.25–1.14). The liver-related mortality increased with increasing Fibrosis-4 index category, especially in non-obese NAFLD patients.
Golabi et al. (2019)[13]	5,375 lean participants from the third National Health and Nutrition Survey (NHANES) in the United States	Prevalence of NAFLD was 10.8%.	The presence of NAFLD in lean individuals was independently associated with increased all-cause and cardiovascular mortality.
Zou et al. (2020)[14]	21,827 participants from the 1999–2016 NHANES in the Unites States	Prevalence of NAFLD was 32.3%. Among individuals with NAFLD, 29.7% were non-obese and 13.6% were lean.	Greater proportion of non-obese NAFLD individuals had elevated Fibrosis-4 index (41.4%) compared to obese NAFLD individuals (29.9%) and non-NAFLD individuals (27.1%) ($P<0.001$). Non-obese NAFLD individuals had higher 15-year cumulative all-cause mortality (51.7%) compared to obese NAFLD individuals (27.2%) and non-NAFLD individuals (20.7%) ($P<0.001$).
Younes et al. (2022)[15]	1,352 biopsy-proven NAFLD patients in Italy, United Kingdom, Spain, and Australia	14.4% were lean.	Lean patients had less severe liver disease. NASH: 54.1% among lean vs. 71.2% among non-lean patients, $P<0.001$. Advanced fibrosis: 10.1% vs. 25.2%, $P<0.001$. During a median follow-up of 94 months, 4.7% of lean patients had liver-related events compared to 7.7% among non-lean patients, $P=0.37$. Overall survival was not significantly different when comparing lean to overweight and obese patients ($P=0.069$), but was significantly better when comparing non-obese to obese patients ($P=0.021$).

NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; CI, confidence interval.

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alence of lean NAFLD and non-obese NAFLD in the general population to be 5.1% (95% confidence interval [CI] 3.7–7.0%) and 12.1% (95% CI 9.3–15.6%), respectively. Among NAFLD patients, an estimated 19.2% (95% CI 15.9–23.0%) were lean and 40.8% (95% CI 36.6–45.1%) were non-obese. Among patients with non-obese or lean NAFLD, 39.0% (95% CI 24.1–56.3%) had NASH, 29.2% (95% CI 21.9–37.9%) had significant fibrosis, and 3.2% (95% CI 1.5–5.7%) had cirrhosis. The corresponding rates among obese NAFLD were 52.9% (95% CI 38.3–67.0%), 38.3% (95% CI 30.6–46.6%), and 2.0% (95% CI 0.4–5.7%). In the largest multicenter biopsy-proven NAFLD registry in Asia to date consisting of 1,812 patients, 21.6% of patients were non-obese. The proportion of patients with NASH and advanced liver fibrosis among non-obese NAFLD patients were 50.5% and 14%, respectively, while the corresponding rates among obese NAFLD patients were 56.5% and 18.7%, respectively.[17]

Natural history and prognosis

The incidence rates of all-cause mortality, liver-related mortality, and cardiovascular-related mortality among patients with lean or non-obese NAFLD were found to be 12.1 (95% CI 0.5–38.8), 4.1 (95% CI 1.9–7.1), and 4.0 (95% CI 0.1–14.9) per 1,000 person-years, respectively. The corresponding rates among obese NAFLD patients were 7.5 (95% CI 0–33.6), 2.4 (95% CI 1.0–4.4), and 2.4 (95% 0–13.3) per 1,000 person-years, respectively (Fig. 1).[18] Although it appeared that lean or non-obese NAFLD patients have higher all-cause mortality, liver-related mortality, and cardiovascular mortality, the results should be interpreted with caution due to the small number of related studies. The authors have also cautioned that further research is needed before any conclusions are made on this due to the scarcity of data for obese and non-obese populations.[18] The results on all-cause mortality, liver-

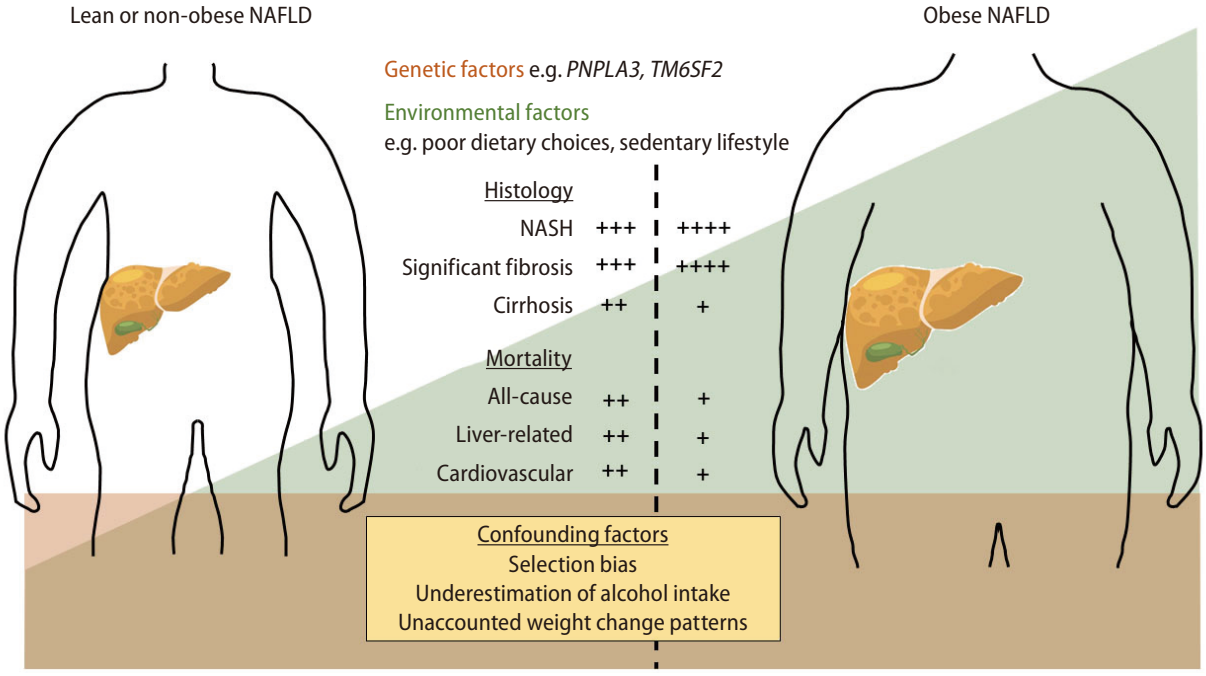

Figure 1. The effect of genetic, environmental, and confounding factors in the severity of liver disease and outcomes of lean or non-obese patients compared with obese NAFLD patients. Genetic factors may have a more pronounced effect towards the development of NAFLD in lean or non-obese individuals, but the effect may appear less pronounced in the presence of strong environmental factors, such as poor dietary choices and a sedentary lifestyle, in the obese state. Selection bias, underestimation of alcohol intake, and unaccounted weight loss over time from poorly controlled diabetes mellitus and/or loss of muscle mass from advanced liver disease are important confounding factors for varying severity of liver disease and outcomes in lean or non-obese NAFLD patients compared to obese NAFLD patients, although genetic factors may play a role. NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PNPLA3, patatin-like phospholipase domain-containing-3; TM6SF2, transmembrane 6 superfamily member 2. +: Relative frequency of the corresponding variable when comparing between lean or non-obese NAFLD and obese NAFLD.

related mortality, and cardiovascular mortality were based on only three studies. Furthermore, only one study provided all-cause mortality, cardiovascular mortality, and liver-related mortality for lean and non-lean NAFLD patients;[11] another study provided all-cause mortality and cardiovascular mortality for obese and non-obese NAFLD patients;[12] a third study provided all-cause mortality and cardiovascular mortality only for non-obese NAFLD patients.[13]

PATHOPHYSIOLOGY OF NON-OBESE NAFLD

The role of obesity and lipotoxicity in the development of NAFLD and NASH has been well described.[19] Briefly, obesity and insulin resistance lead to excess free fatty acids and increased *de novo* lipogenesis in the liver. Free fatty acids are either stored as triglyceride, exported from the liver, or undergo oxidation. The excess in free fatty acids causes oxidative stress, liver cell injury and death, inflammation, and eventually fibrosis. On the other hand, the pathophysiology of lean or non-obese NAFLD is not completely understood. Despite having a normal or lower BMI, lean or non-obese NAFLD patients have excess visceral adiposity. Lean or non-obese NAFLD patients share common altered metabolic and cardiovascular profile as their non-lean or obese counterparts, although the alterations are generally less severe.[20] While it is reasonable to think that lean or non-obese NAFLD is the early phase of NAFLD or the less severe end of the NAFLD spectrum, evidence suggests that there may be more to it.

Ethnic differences in body fat distribution and genetic factors

It is well-known that different ethnic groups have different tendency to accumulate visceral and liver fat and to develop metabolic syndrome. Ethnic difference in the prevalence of hepatic steatosis was first pointed out in the landmark paper by Browning and colleagues in 2004, where Hispanics were found to have the highest prevalence of hepatic steatosis, while the prevalence was significantly lower among Blacks despite an equally high prevalence of obesity and insulin resistance.[21] In a subsequent multi-ethnic cohort study on 1,794 subjects of African, European, Japanese, Latino, or Native Hawaiian ancestry in the United States, the mean visceral and liver fat were greatest among the Japanese Americans, which jointly accounted for a statistically significant fraction of the difference in metabolic syndrome prevalence compared to other ethnic groups independently of total fat mass.[22] Studies on multi-ethnic Malaysians have also consistently found the prevalence of NAFLD to be higher among the Indians and Malays compared to the Chinese,[23,24] with the ethnic predilection seen as early as young adulthood.[25] Consistent with this is the greater prevalence of metabolic syndrome among the Indians and Malays compared with the Chinese.[26] The difference in tendency for visceral adiposity, NAFLD, and metabolic syndrome between the different ethnic groups may be explained by genetic differences. A single nucleotide polymorphism in the patatin-like phospholipase domain-containing-3 (*PNPLA3*) gene, the rs738409 C>G variant, which results in substitution of isoleucine by methionine at position 148 (I148M), was found to be associated with increased liver fat in a genome-wide association study, and the risk allele was found to be the highest among Hispanics and the lowest among Blacks,[27] providing an explanation to the initial observation by Browning and colleagues. Genetic polymorphisms in the *PNPLA3* gene have subsequently been recognized as a major genetic determinant of NAFLD and its severity.[28] The *PNPLA3* protein has lipase activity in hepatocytes and I148M leads to loss of function that promotes accumulation of triglycerides in liver cells.[29] Interestingly, a population-based study in Hong Kong found that the *PNPLA3* gene polymorphism had a greater effect on liver fat in lean individuals compared to overweight and obese individuals. Furthermore, lean individuals were significantly more likely to carry the risk allele compared with overweight and obese individuals.[30] Therefore, genetic factors may have a greater effect towards the development of NAFLD in lean or non-obese individuals, but the effect may be less pronounced in the presence of strong environmental factors, such as poor dietary choices and a sedentary lifestyle, with increasing BMI and in the obese state (Fig. 1). The findings were somewhat different in a study in the Western population, which found that the effect of the risk allele was amplified by increasing adiposity.[31] The inconsistent findings may be due to other genetic determinants at play, environmental factors such as diet, or differences in the metabolic profile of the study populations. A difference in the effect of genetic polymorphisms in the *PNPLA3* gene on NAFLD has been observed among different ethnic groups, with the effect lowest among the Chi-

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nese compared to the Indians and Malays.[32] In a subsequent study, the *HSD17B13* rs72613567 and rs6834314 variants were found to be associated with a lower risk of NASH and adverse liver-related outcomes among the Chinese but not the Indians and Malays, supporting the role of polygenic determinants in the disease phenotype.[33] The transmembrane 6 superfamily member 2 (*TM6SF2*) encodes a membrane protein required for normal very low density lipoprotein secretion. The rs58542926 C>T variant, which results in substitution of glutamate by lysine at position 167 (E167K), was found to be associated with higher circulating levels of serum alanine aminotransferase, a marker of liver injury, but lower level of serum low density lipoprotein cholesterol and triglycerides.[34] In a retrospective cohort study on 669 consecutive patients with biopsy-proven NAFLD in Italy, a significantly greater proportion of patients with lean NAFLD had E167K compared to their non-lean counterparts. In the same study, I148M was the only independent factor found to be associated with NASH and significant fibrosis among lean patients.[35] Additionally, lean NAFLD may be also driven by other rare genetic disorder, such as familial hypobetalipoproteinemia and cholesteryl ester storage disease.[36,37]

More severe liver disease in some non-obese NAFLD patients

Even among lean or non-obese NAFLD patients, varying proportions of more severe liver disease have been observed. As elucidated earlier, this may be due to the under-reporting of alcohol intake, particularly in populations with high alcohol consumption, as well as genetic factors. For example, in a study on an outpatient population in the United States, ethic differences in the prevalence of cryptogenic cirrhosis mirrored the prevalence of hepatic steatosis and the frequency of I148M among the different ethnic groups.[27] Another point for consideration is the loss of weight from poorly controlled diabetes mellitus and the loss of muscle mass associated with more advanced chronic liver disease in patients with long-standing history of obesity, NAFLD, and diabetes mellitus. The inclusion of these patients as lean or non-obese NAFLD will paradoxically enrich the population with patients who are worse metabolically and have more severe liver disease with resultant poorer outcomes. The gut microbiome may play a role in the pathogenesis of NAFLD,[38] but this remains unclear and deserves further studies, especially in non-obese NAFLD.

NON-INVASIVE TESTS IN NON-OBESE NAFLD

It is well-recognized that the fibrosis stage is the single most important predictor for overall and liver-related mortality in patients with NAFLD.[39] The same has been observed for the subpopulation of lean or non-obese NAFLD patients.[11] Due to the high prevalence of NAFLD in the general population and only a small yet significant proportion of patients having advanced liver fibrosis,[40] a simple assessment and referral pathway is needed to identify the patients who are more likely to have more severe liver disease for specialist care and to limit unnecessary referrals.[41] Although liver biopsy is considered the reference standard for fibrosis assessment and required for the diagnosis of NASH, it is not routinely performed as it is invasive and associated with a small risk of serious complications. Since the initial description and following refinement and validation, sequential testing with simple fibrosis score followed by liver stiffness measurement has become the backbone for fibrosis assessment in patients with NAFLD.[41-44] In a multicenter study in France, Malaysia, and Hong Kong, all non-invasive tests that were tested, including the Fibrosis-4 index, NAFLD fibrosis score, and liver stiffness measurement, were performed equally well in non-obese compared with obese patients, and the same cut-offs can be used with similar or higher sensitivities and specificities. Furthermore, the negative predictive value of every non-invasive test was found to be higher due to the lower prevalence of advanced fibrosis among non-obese compared to obese patients.[45] A subsequent individual patient data meta-analysis evaluating non-invasive tests against liver histology using data from 5,705 patients (15.2% of patients had a BMI of <25 kg/m^2) found that non-invasive tests, namely the Fibrosis-4 index, NAFLD fibrosis score, and liver stiffness measurement, performed better in patients with lower BMI.[46] The area under the curve of some of the most commonly used non-invasive tests among non-obese patients compared to obese patients are summarized in Table 2.

Table 2. The area under the curve for some of the most commonly used non-invasive tests for NAFLD according to BMI category based on a multicenter study and an individual patient data meta-analysis

Study	BMI	Non-invasive test		
		Fibrosis-4 index	NAFLD fibrosis score	Liver stiffness measurement
Fu et al. (2020)[45]	<25 kg/m^2	0.86 (0.75–0.98)	0.85 (0.73–0.96)	0.93 (0.87–0.98)
	≥25 kg/m^2	0.73 (0.69–0.77)	0.69 (0.64–0.73)	0.83 (0.80–0.87)
Mózes et al. (2022)[46]	<25 kg/m^2	0.81 (0.78–0.84)	0.76 (0.71–0.81)	0.91 (0.89–0.94)
	25–29.9 kg/m^2	0.77 (0.75–0.80)	0.74 (0.71–0.77)	0.87 (0.85–0.89)
	≥30 kg/m^2	0.74 (0.72–0.76)	0.69 (0.66–0.72)	0.81 (0.79–0.83)

Values are presented as the area under the curve (95% confidence interval).
NAFLD, nonalcoholic fatty liver disease; BMI, body mass index.

LIFESTYLE INTERVENTION AND PHARMACOLOGICAL TREATMENT IN NON-OBESE NAFLD

Lifestyle intervention is the cornerstone for the management of NAFLD. A landmark study on comprehensive lifestyle programs for patients with biopsy-proven NASH has shown that weight loss of ≥10% can result in NASH resolution and fibrosis improvement in 90% and 45%, respectively.[47] In a randomized controlled trial of a 12-month lifestyle intervention program, a significantly greater proportion of patients in the intervention group achieved remission of NAFLD based on proton-magnetic resonance spectroscopy compared with the control group (64% vs. 20%, P<0.001) with 97% of patients with ≥10% weight loss achieving remission of NAFLD.[48] More importantly, a secondary analysis found similar beneficial effect of lifestyle intervention program regardless of the baseline BMI. The proportion of patients achieving remission of NALFD was 67% in the intervention group and 18% in the control group among non-obese patients. The corresponding proportions among obese patients were 61% and 21%, respectively. Furthermore, 50% of non-obese patients achieved remission of NAFLD with 3–5% weight loss, while the same could be also achieved with 7–10% weight loss among obese patients.[49]

To date, there is no pharmacological therapy approved for NAFLD. However, multiple drugs targeting obesity and the metabolic syndrome have shown promising results. In a multicenter, randomized, double-blind, placebo-controlled trial on biopsy-proven NASH patients, liraglutide 1.8 mg daily for 48 weeks resulted in significantly greater resolution of definite NASH compared to placebo.[50] In another study, semaglutide at increasing dosages resulted in significantly greater NASH resolution without worsening fibrosis compared with placebo, but there was no significant difference in fibrosis improvement.[51] However, these studies enrolled only overweight patients with BMI ≥25 kg/m^2.[50,51] Whether glucagon-like peptide-1 receptor agonists will be beneficial over standard of care and have acceptable profiles of side effect in lean NAFLD patients is not clear. Another concern related to marked weight loss, although desirable for the underlying NAFLD, is whether it comes with an associated loss of muscle mass. Sarcopenia is a common and important complication of chronic liver disease, including NAFLD, and has been associated with poorer outcomes.[52] However, post-hoc analysis of the STEP 1 trial, which was a trial evaluating semaglutide 2.4 mg once-weekly for adult patients with BMI ≥27 kg/m^2 with ≥1 weight-related comorbidity or BMI ≥30 kg/m^2, without diabetes mellitus, found semaglutide to be associated with reduced total fat mass and regional visceral fat mass, and an increased proportion of lean body mass. Although the total lean body mass decreased from baseline (–9.7%), the proportion relative to total body mass increased by 3.0% with improvement in lean body mass to fat mass ratio.[53] Another study found that semaglutide resulted in significant declines in fat mass index and visceral adipose tissue, but not skeletal mass index, fat free mass index, and muscle strength.[54] However, further studies are needed on the use of these emerging novel therapies in lean or non-obese NAFLD patients.[55]

CONCLUSION

Lean or non-obese NAFLD is a common entity and may be more than just the early phase or the less severe end of the NAFLD spectrum. While confounding factors, such as alcohol intake and weight loss following disease progression, could

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explain more severe liver disease and a worse outcome in some patients with lean or non-obese NAFLD, genetic factors are increasingly recognized to play an important role. Further studies to understand these genetic determinants in lean or non-obese NAFLD patients may open the door to better diagnostics and therapeutics that may have the potential to be expanded to obese NAFLD patients. Overall, non-invasive tests perform better in lean or non-obese NAFLD patients than in their obese counterparts. Lifestyle intervention works for lean or non-obese NAFLD patients, and less amount of weight loss may be required to achieve similar results compared to obese NAFLD patients. The role of emerging therapeutics in lean or non-obese NAFLD patients is unclear, and further studies are warranted.

Conflicts of Interest

Wah-Kheong Chan has served as a consultant or advisory board member for Roche, Abbvie, Boehringer Ingelheim and Novo Nordisk; and a speaker for Viatris and Hisky Medical.

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Review

Interaction between sarcopenia and nonalcoholic fatty liver disease

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Sarcopenia and nonalcoholic fatty liver disease (NAFLD) are common health problems related to aging. Despite the differences in their diagnostic methods, several cross-sectional and longitudinal studies have revealed the close link between sarcopenia and NAFLD. Sarcopenia and NAFLD are linked by several shared pathogenetic mechanisms, including insulin resistance, hormonal imbalance, systemic inflammation, myostatin and adiponectin dysregulation, nutritional deficiencies, and physical inactivity, thus implicating a bidirectional relationship between sarcopenia and NAFLD. However, there is not sufficient data to support a direct causal relationship between sarcopenia and NAFLD. Moreover, it is currently difficult to conclude whether sarcopenia is a risk factor for nonalcoholic steatohepatitis (NASH) or is a consequence of NASH. Therefore, this review intends to touch on the shared common mechanisms and the bidirectional relationship between sarcopenia and NAFLD. **(Clin Mol Hepatol 2023;29(Suppl):S68-S78)**

Keywords: Sarcopenia; NAFLD

INTRODUCTION

The global epidemic of obesity and metabolic syndrome in an aging population has led to growing health problems including nonalcoholic fatty liver disease (NAFLD) and sarcopenia. Sarcopenia is defined as the progressive and generalized loss of skeletal muscle mass, strength, and/or function with a risk of adverse outcomes such as physical disability, hospitalization, and mortality.[1,2] Despite the differences in their diagnostic methods, several studies have revealed the close link between sarcopenia and NAFLD.[3-16] This review focuses on the shared mechanisms and a bidirectional relationship between sarcopenia and NAFLD.

OPERATIONAL DEFINITION OF SARCOPENIA

Sarcopenia, previously considered an aging-related syndrome, is now recognized as a progressive disease associated with type 2 diabetes mellitus (T2DM), metabolic syndrome, liver disease, and cardiovascular disease.[17-20] It is primarily associated with aging and secondarily with diseases mediated by systemic inflammation and insulin resistance (IR).[21] In 2018, the European Working Group on Sarcopenia in Older People defined sarcopenia by low levels across three parameters: muscle strength, muscle quantity/quality, and physical performance. The presence of low muscle strength is the primary parameter to suspect sarcopenia, while the presence of

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low muscle mass (quantity) and quality are confirmatory. The coexistence of these factors represents severe sarcopenia.[2] Therefore, all these parameters enable improved understanding and awareness of sarcopenia.

SHARED MECHANISMS OF SARCOPENIA AND NAFLD

Sarcopenia and NAFLD share common underlying mechanisms, including IR, hormonal imbalance, systemic inflammation, myostatin and adiponectin dysregulation, nutritional deficiencies, and physical inactivity (Fig. 1).[22]

Insulin resistance

IR is the main pathologic mechanism causing both sarcopenia and NAFLD. IR results from the loss of skeletal muscle mass. It causes increased lipolysis with the consequent release of free fatty acids (FFA) from adipose tissue. IR also inhibits growth hormone (GH)/insulin growth factor-1 (IGF-1) axis that normally plays a protective role in muscle regeneration and age-related muscle loss.[17,23,24] It causes compensatory hyperinsulinemia, which leads to promotion of gluconeogenesis, upregulation of sterol regulatory element binding protein 1c, inhibition of β-oxidation, increased FFA delivery, and altered triglyceride (TG) transport. These events leads to accumulation of TGs in skeletal muscle and the liver, often referred to as ectopic fat.[25,26]

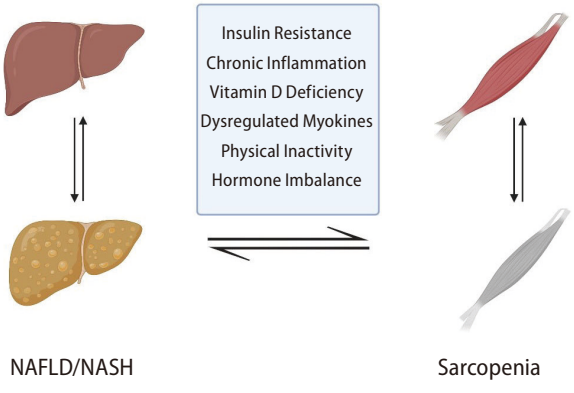

Figure 1. Bidirectional relationship between sarcopenia and NAFLD. NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis.

Impaired suppression of gluconeogenesis promotes proteolysis and reduces protein synthesis,[7] which results in age-related muscle depletion and sarcopenia.[27-29] Insulin activates the mammalian target of rapamycin (mTOR), 4E-binding protein 1, and ribosomal S6 kinase 1. These are involved in protein synthesis, maintenance of muscle mass, and skeletal muscle anabolism.[30] Skeletal muscle IR leads to increased muscle degradation with decreased mitochondrial content, function, and oxidative capacity.[31] A study demonstrated that T2DM was independently associated with sarcopenia, leading to metabolic disorders and physical disability in older adults with T2DM.[32] Furthermore, sarcopenia aggravates IR, since skeletal muscle is a primary insulin-responsive organ.[33] Likewise, myosteatosis, defined as fatty infiltration of muscle, is associated with reduced muscle function, IR, and a high risk of mortality in cirrhotic patients.[34,35] Both sarcopenia and obesity simultaneously induce more severe IR and glycemic dysregulation.[33]

Chronic inflammation

Inflammation and oxidative stress have been linked to the pathogenesis of NAFLD. Intramuscular lipid accumulation induces the secretion of proinflammatory cytokines from adipose tissue and generates oxygen-free radicals in the liver by inhibiting mitochondrial function for β-oxidation, leading to lipid peroxidation. Cytokines, such as interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and transforming growth factor-β (TGF-β) induce chronic low-grade inflammation.[36,37] Compared to healthy subjects, patients with isolated steatosis and steatohepatitis had increased TNF-α levels.[38] TNF-α causes lipid accumulation in the liver through activation of *de novo* lipogenesis (DNL).[39] It also stimulates nuclear factor κB, the main transcriptional factor for proinflammatory cytokines that contribute to the development of NAFLD and muscle catabolism.[36,39,40] Catabolic inflammation further worsens

Abbreviations:

CRP, C-reactive protein; FFA, free fatty acid; GH, growth hormone; IGF-1, insulin growth factor-1; IL-6, interleukin-6; IR, insulin resistance; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; TG, triglyceride; T2DM, type 2 diabetes mellitus; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; VDR, vitamin D receptor

sarcopenia among older patients because of the release of numerous inflammatory mediators from immune cells and adipocytes that contribute to the development of IR.[41] Patients with sarcopenia demonstrate chronic inflammation, increased levels of C-reactive protein (CRP) and proinflammatory cytokines, and decreased levels of anti-inflammatory cytokines.[3] IL-6 and CRP levels are also positively associated with total body fat mass and inversely associated with appendicular lean body mass.[4,42]

Vitamin D

Vitamin D is involved in the modulation of IR, NAFLD, metabolic syndrome, and sarcopenia.[43] It plays an essential role in myogenesis, myoblast proliferation and differentiation, production and growth of skeletal muscle cells, and skeletal muscle inflammation.[44-47] It exerts its effects through the nuclear vitamin D receptor (VDR), which is expressed in the liver and skeletal muscle.[48,49] Downregulation of VDR expression by vitamin D deficiency and aging may lead to sarcopenia.[36] Studies shows that subjects with sarcopenia have significantly lower vitamin D levels.[6,50] Decreased levels of vitamin D are associated with decreased muscle strength, poor muscle function, and an increased risk of sarcopenia among older adults.[51] However, vitamin D supplementation increases VDR expression in skeletal muscle, preventing the development of sarcopenia.[52]

The relationship between vitamin D and NAFLD has been already acknowledged. A meta-analysis including 17 cross-sectional and case-control studies showed that patients with NAFLD had decreased levels of serum vitamin D.[43] Hypovitaminosis D was strongly associated with the presence of NAFLD independent of metabolic syndrome, T2DM, and IR.[50]

Furthermore, vitamin D downregulates the expression of SREBP-1c, acetyl-coenzyme A carboxylase, and fatty acid synthase that modulate DNL, while peroxisome proliferator-activated receptor α and carnitine palmitoyltransferase-1 that mediate hepatic fatty acid oxidation are upregulated by vitamin D.[53] An animal study demonstrated that vitamin D deficiency worsened NAFLD by activating the inflammation-mediated pathway[43]. Vitamin D deficiency also causes IR via upregulation of hepatic IR, inflammatory, and oxidative stress genes.[54,55] Moreover, VDR-knockout mice spontaneously developed hepatic steatosis.[55] Most studies, to date, have shown that vitamin D plays a pivotal role in the development of sarcopenia and NAFLD. On the contrary, other studies demonstrated no significant relationship between vitamin D level and NAFLD/sarcopenia.[56,57]

Myokines

Skeletal muscle is an endocrine organ that releases myokines[58,59] after muscle contraction or strength training.[60] Myokines are involved in the autocrine regulation of muscle metabolism and the paracrine/endocrine regulation of other tissues and organs including the liver, adipose tissue, and brain.[61-63]

Myostatin, a member of the TGF-β family, is predominantly expressed in skeletal muscles.[64,65] It is an inhibitor of muscle mass and a key regulator of adipogenesis.[65-68] It mediates Smad 2/3 activation, inhibiting myogenesis and protein synthesis by suppressing the Akt-mediated mTOR signaling pathway.[69] This causes muscle atrophy. Muscle proteolysis is stimulated through FoxO-dependent activation of the ubiquitin-proteasome pathway and autophagy.[69] Myostatin also increases adipose tissue mass and inhibits adiponectin secretion.[22,70,71] Animal studies have demonstrated that blockage of myostatin significantly increases muscle mass, improves insulin sensitivity, and protects against liver steatosis.[72,73] Animal models have demonstrated increased expression of activin type IIB, a myostatin receptor expressed in stellate cells, in liver fibrosis.[74,75] Stellate cell cultures exposed to myostatin increase the expression of profibrotic proteins.[76] Therefore, myostatin, IR, and liver fibrogenesis are interconnected.

Irisin, an exercise-induced myokine, is inversely associated with the degree of fatty liver in obese patients and is a potential cause of sarcopenia and NAFLD. It increases energy expenditure through peroxisome proliferator- activated receptor α-dependent downstream signaling and improves insulin sensitivity and hepatic steatosis by upregulating fibroblast growth factor-21; these effects were independent of reduction in body weight and adiposity in a mouse model.[77,78] It increases glucose uptake by enhancing glucose transporter type 4 translocation and β-oxidation of FFA through AMP-activated protein kinase activation in muscle cells.[79] Irisin expression in muscle and serum irisin level are reduced in obese subjects.[80]

IL-6 has a dual metabolic effect. Muscle contrations stimulate acute IL-6 release from muscles,[81,82] with the levels increasing as the duration and intensity of muscle contraction

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increase.[83,84] IL-6 improves hepatic glucogenesis, lipolysis in adipose tissue, pancreatic β-cell viability, and insulin secretion.[81,85,86] It also enhances glucose uptake and fatty acid oxidation through adenosine monophosphate-activated protein kinase (AMPK) and phosphoinositide 3-kinase signaling processes.[87,88] However, IL-6 acts as a pro-inflmmatory cytokine in chronic inflammatory states such as obesity, infection, and cancer.[89] A study have demonstrated that increased IL-6 levels are associated with NASH, hepatic fibrosis, and IR.[90]

Physical inactivity

The lack of physical activity causes loss of muscle mass and reduces energy consumption, resulting in obesity and hepatic steatosis.[91] Both sarcopenia and NAFLD are worsened by chronic inflammation, oxidative stress, and IR.[92] During exercise, production of pro-inflammatory cytokines is decreased while anti-inflammatory cytokine production, muscle protein synthesis, regeneration, and glucose uptake are increased. Physical activity mitigates the risk of sarcopenia progression.[93] Exercise can improve metabolic health status even without significant weight loss.[94]

Other mechanisms

Adiponectin, a hormone secreted from adipose tissue, mediates glucose and lipid metabolism in insulin-sensitive tissues such as liver and muscle. In the liver, adiponectin promotes glucose use and enhances fatty acid oxidation by improvement of insulin action via activation of AMPK.[95,96] In addition, adiponectin has an anti-inflammatory effect by neutralizing TNF-α, and improves hepatic steatosis and inflammation.[97]

Anabolic hormones, such as GH and IGF-1, decline with aging process, which affects the progressive loss of muscle mass.[98] Fat accumulation and aging impair the GH/IGF-1 signaling pathway, leading to deterioration of muscle mass synthesis.[99,100] In an experiental mouse model of NAFLD, NAFLD was associated with decreased muscle mass and strength, and reduced IGF-1 level, implicating that IGF-1 reduction might play a role in the development of NAFLD-related sarcopenia.[101]

BIDIRECTIONAL RELATIONSHIP BETWEEN SARCOPENIA AND NAFLD

Numerous studies have reported a relationship between NAFLD and sarcopenia (Tables 1, 2). Sarcopenia is a risk factor for the presence and severity of NAFLD (Table 1).[7,22,102,103] The prevalence of sarcopenia is significantly increased in NAFLD and NASH compared to that in non-NAFLD (17.9% and 35.0% vs. 8.7%, respectively).[3] NAFLD patients with sarcopenia had a 2-fold higher risk of developing NASH and significant fibrosis independent of obesity and IR.[3] However, most studies were cross-sectional in design and the causal relationship between sarcopenia and NAFLD remains unclear. A recent study demonstrated that NAFLD was developed in 14.8% of its participants during a 7-year follow-up, with an increased incidence in participants with the lowest tertile of skeletal muscle mass at baseline. Baseline skeletal muscle mass was also positively associated with the resolution of existing NAFLD, regardless of metabolic risk factors.[10] Sarcopenia was associated with poor clinical outcomes, including severe hepatic fibrosis and increased mortality, in NAFLD patients.[104-106] Hence, low skeletal muscle mass may cause the development of NAFLD. In a multicenter prospective study, hepatic steatosis at baseline was significantly associated with the risk of sarcopenia in older adults. Lower muscle mass and strength were more common in NAFLD patients.[16] In another study, the loss of skeletal muscle mass was faster in subjects with NAFLD compared to those without NAFLD. When stratified by fibrosis severity, skeletal muscle mass loss was faster in NAFLD subjects with an intermediate-to-high probability of advanced fibrosis than in those without (Table 2).[107]

Muscle quality also plays a critical role in the development of NASH. Myosteatosis determines muscle strength and function, and metabolic and liver-related clinical outcomes.[108-110] It is a prognosticator for NASH development.[108,111,112] Studies have suggested that myosteatosis is a clinically useful surrogate marker for NASH[108] by demonstrating that severe myosteatosis, but not sarcopenia, predicts NASH development and fibrosis progression.[111] The prevalence of myosteatosis is increased in obese subjects with NASH; hence, myosteatosis could reflect the histological features of NASH.[110] Muscle alterations are linked with fibrosis severity in subjects with NAFLD.[3-5,9,22,113-117] These suggest that the role of sarcopenia in NASH development is unclear. Both sarcopenia and myosteatosis have been linked to advanced fibrosis and cirrho-

Table 1. Studies of sarcopenia as a risk factor for NAFLD

Study (yr)	Study design	Study size	Study population	Sarcopenia assessment	NAFLD assessment	Study conclusion
Hong et al. (2014)[4]	Cross-sectional	526	Korean	DXA	CT	5-fold increased risk of NAFLD
Lee et al. (2015)[6]	Cross-sectional	15,132	Korean	DXA	Noninvasive models	2.3- to 3.3-fold increased risk of NAFLD in patients with sarcopenia
Lee et al. (2016)[7]	Cross-sectional	2,761	Korean	DXA	Noninvasive models	2-fold increased risk of fibrosis in patients with sarcopenia
Kim et al. (2016)[8]	Cross-sectional	3,739	Korean	DXA	Noninvasive models	Low SMI is associated with NAFLD according to age group and menopause status
Koo et al. (2017)[3]	Cross-sectional	309	Korean	BIA	Liver biopsy	Increased prevalence of sarcopenia with NAFLD severity 2.5-fold increased risk of NASH and significant fibrosis in patients with sarcopenia
Petta et al. (2017)[9]	Cross-sectional	225	Italian	BIA	Liver biopsy	2-fold increased risk of fibrosis in NAFLD in patients with sarcopenia
Zhai et al. (2018)[12]	Cross-sectional	494	Chinese	DXA	US	NAFLD is not independently associated with sarcopenia.
Kim et al. (2018)[10]	Longitudinal	10,534	Korean	BIA	Noninvasive models	Increased incidence of NAFLD in patients with sarcopenia Increased resolution of baseline NAFLD with higher muscle mass
Wijarnpreecha et al. (2019)[13]	Cross-sectional	11,325	American	BIA	US	2.3-fold increased risk of NAFLD in patients with sarcopenia 1.8-fold increased advanced fibrosis in patients with sarcopenia
Hsieh et al. (2021)[104]	Cross-sectional	521	Korean	CT	Liver biopsy	Increased risk of significant fibrosis in NAFLD
Hsieh et al. (2022)[111]	Longitudinal	338	Korean	CT	Liver biopsy	Severe myosteatosis is significantly associated with early NASH and fibrosis progression in early stage NAFLD

NAFLD, nonalcoholic fatty liver disease; DXA, dual energy X-ray absorptiometry; CT, computed tomography; SMI, skeletal muscle index; BIA, Bioelectric impedance analysis; NASH, nonalcoholic steatohepatitis; US, ultrasonography.

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Table 2. Studies of NAFLD as a risk factor for sarcopenia

Study (yr)	Study design	Study size	Study population	Sarcopenia assessment	NAFLD assessment	Study conclusion
Issa et al. (2014)[5]	Cross-sectional	75	American	CT	Liver biopsy	Increased risk of sarcopenia in NASH and NASH cirrhosis
Sinn et al. (2022)[107]	Longitudinal	52,815	Korean	BIA	US	Faster loss of skeletal muscle mass in NAFLD Much faster loss of skeletal muscle mass in NAFLD according to fibrosis severity
Roh et al. (2022)[16]	Longitudinal	1,595	Korean	DXA	Noninvasive models	Increased risk of developing LMM (1.65-fold) and LMS (2.29-fold) in NAFLD

NAFLD, nonalcoholic fatty liver disease; CT, computed tomography; NASH, nonalcoholic steatohepatitis; BIA, Bioelectric impedance analysis; US, ultrasonography; DXA, dual energy X-ray absorptiometry; LMS, low muscle strength; LMM, low muscle mass.

sis.[22,34,118-121] However, the relatively low skeletal muscle mass observed in NAFLD patients may derive from increased body fat percentage.[15,110] Muscle wasting is often seen in patients with advanced fibrosis, implicating reverse causality between low skeletal muscle mass and NAFLD severity.[9,14] Patients with liver cirrhosis had concomitant sarcopenia (43%), sarcopenic obesity (low muscle mass with obesity) (26%), and myosteatosis (52%).[34] Hence, advanced fibrosis is more likely to cause sarcopenia rather than sarcopenia causing fibrosis progression.

CONCLUSIONS

It is currently difficult to conclude whether sarcopenia is a risk factor or a consequence of NASH. However, sarcopenia and NAFLD are linked by several shared pathogenetic mechanisms, implicating a bidirectional relationship between sarcopenia and NAFLD. Therefore, further studies are needed to investigate the effects of low muscle function and performance on NAFLD progression. In addition, prospective standardized trials with accurate diagnoses of sarcopenia and NAFLD are warranted to elucidate the cause-and-effect relationship between sarcopenia and NAFLD.

Authors' contribution

Sae Kyung Joo: drafting the manuscript; preparation of the figure and table. Won Kim: design of the work; supervision of the article; obtaining funding.

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Conflicts of Interest

The authors have no conflicts to disclose.

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Review

Risk factors in nonalcoholic fatty liver disease

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Nonalcoholic fatty liver disease (NAFLD) is the most common liver disease, with a global prevalence estimated at approximately 25%. NAFLD is also the leading cause of liver cirrhosis, hepatocellular carcinoma, and death. Additionally, the risk of cardiovascular disease increases with greater NAFLD severity. The liver- and cardiovascular disease-related mortality incident rate ratios among the NAFLD population were 0.77 and 4.79 per 1,000 person-years, respectively. We intend to discuss the risk factors associated with NAFLD in terms of development and progression. Obesity or higher body mass index is closely associated with NAFLD in a dose-dependent manner, but growing evidence suggests that central obesity plays a more important role in the development of NAFLD. Saturated fat and fructose have been reported to be closely related to NAFLD. Fructose intake promotes lipogenesis and impairs mitochondria fat oxidation. The presence of type 2 diabetes is the most powerful predictive risk factor for hepatic fibrosis in patients with NAFLD. Single nucleotide polymorphism is not only associated with the prevalence of NAFLD but also associated with increased liver disease mortality. Obstructive sleep apnea, intestinal dysbiosis, and sarcopenia are associated with the development of NAFLD. **(Clin Mol Hepatol 2023;29(Suppl):S79-S85)**

Keywords: Nonalcoholic fatty liver disease; Obesity; Diabetes mellitus, type 2; Sarcopenia

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is the most common liver disease, with a global prevalence of approximately 25%.[1,2] NAFLD is an umbrella terminology incorporating a spectrum of liver diseases ranging from simple steatosis (nonalcoholic fatty liver), steatohepatitis (nonalcoholic steatohepatitis, NASH), and cirrhosis.[3] NAFLD is also the leading cause of liver cirrhosis, hepatocellular carcinoma and death.[1] A study has forecasted that the burden of NAFLD is bound to rise through 2015–2030 with elevated prevalence and mortality.[4] For example, prevalence of NAFLD was approximately 25.8% in all ages in 2015 and would reach 28.4% in 2030, respectively. Moreover, the mortality of the NAFLD population is expected to increase by 23% by 2030, accounting for 13% of all deaths.[5] Patients with NAFLD have a higher risk of liver-related mortality, but cardiovascular disease is the leading cause of death with a 1.5-fold increase.[6,7] Additionally, the risk of cardiovascular disease increases with greater NAFLD severity (odds ratio [OR] 2.58).[8] The liver- and cardiovascular

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disease-related mortality incident rate ratios among the NAFLD population were 0.77 and 4.79 per 1,000 person-years, respectively.[9] Another notable cause of death in patients with NAFLD is neoplasms.[9-11] The overall cancer incidence is 1.3 times higher in patients with NAFLD than in controls (hazard ratio: 1.32, $P<0.001$).[11] Hepatocellular carcinoma and other gastrointestinal cancers, such as colorectal or stomach cancer, and breast cancer in women are the most prevalent neoplasms associated with the NAFLD population.[9,11,12] We intend to discuss the risk factors associated with NAFLD in terms of development and progression.

OBESITY AND CENTRAL OBESITY

Obesity or higher body mass index is closely associated with NAFLD in a dose-dependent manner, with approximately 20% increase in the risk of developing NAFLD for every unit increase in body mass index.[13] Furthermore, childhood obesity is also associated with fatty liver and a higher mortality overall.[14] Children with NAFLD show a 5.88-fold higher rate of all-cause mortality, including causes, such as cancer (hazard ratio 1.67 vs. 0.07/1,000 person-years), cardiometabolic disease (hazard ratio 1.12 vs. 0.14/1,000 person-years), and liver disease (hazard ratio 0.93 vs. 0.04/1,000 person-years) than the control group.[14] A retrospective cohort study has contributed to the association of central obesity and NASH and advanced fibrosis among lean patients with NAFLD.[15] In addition, both lean (OR 5.8; $P=0.004$) and overweight or obese (OR 4.2; $P=0.0001$) patients with NAFLD with central obesity (>102 cm for men, >88 cm for women) were closely associated with significant hepatic fibrosis.[15] Metaregression analysis of this cohort (n=11,400) found that waist circumference affects altered metabolic syndrome-related factors and fasting plasma glucose levels (slope: 1.55, $P=0.14$). Most studies focus on the relationship between obesity and NAFLD risk, as measured by body mass index. However, growing evidence suggests that central obesity, defined as waist circumference or waist-to-hip ratio, plays a more important role in NAFLD development.[16]

DIET

The total caloric intake is significantly higher among patients with NAFLD, but there is no significant difference in the pattern of consumption of macronutrients (e.g., proteins, fat, and carbohydrates) or micronutrients (e.g., vitamins, iron, or zinc) between the control and the NAFLD groups.[17] However, several food components, such as saturated fat and fructose, have been reported to be closely related to NAFLD development.[18] Fructose intake promotes lipogenesis and impairs mitochondrial fat oxidation, leading to increased uric acid production and depletion of adenosine triphosphate in the mitochondria, which triggers a series of reactions, such as oxidative stress.[19,20] Moreover, fructose metabolism may also affect intestinal permeability and dysbiosis, leading to the pathogenesis of NAFLD.[21] However, using Rotterdam cohort, Alferink et al.[22] could not confirm the association between NAFLD and monosaccharides and disaccharides.

TYPE 2 DIABETES MELLITUS (T2DM)

The estimated global prevalence of NAFLD, NASH and advanced hepatic fibrosis among patients with T2DM is 55.48%, 37.33%, and 17.02%, respectively (Table 1).[23] The prediabetes/diabetes status among patients with NAFLD is related to an increment in risk of severe hepatic steatosis (OR 2.00, $P<0.005$), severe lobular inflammation (OR 2.25, $P<0.005$), hepatic ballooning (OR 1.54, $P=0.069$), and significant fibrosis (OR 1.30, $P=0.45$).[24] The proportion of definite NASH is higher in patients with prediabetes/diabetes status than those with normal glucose tolerance (48.4% vs. 29.9%; $P<0.001$).[24,25] The proportion of patients with both the significant and advanced fibrosis in the T2DM group was 17.9%, whereas in the nondiabetic control group, it was 4.9% and 1.8%, respectively.[26] The findings strongly suggest that T2DM alone was an independent risk factor for hepatic fibrosis.[15] Moreover, presence of T2DM is the most powerful predictive risk factor for hepatic fibrosis even in lean patients with NAFLD.[26]

Abbreviations:

IR, insulin resistance; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; OR, odds ratio; OSA, obstructive sleep apnea; PNPLA3, patatin-like phospholipase domain-containing 3; T2DM, type 2 diabetes mellitus

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Table 1. Prevalence of NAFLD among patients with type 2 diabetes compared to the control group

Studies	Prevalence of NAFLD, NASH, and fibrosis among T2DM		
	NAFLD	NASH	Advanced fibrosis
Younossi et al.[23] (2019)*	55.48%	37.33%	17.02%
	Analyzed 80 studies, 49,419 patients	Analyzed 10 studies, 892 patients	Analyzed 7 studies, 439 patients
Le et al.[27] (2019)	72%	2.82% (2003–2006), 5.20% (2011–2014)	0.30% (2003–2006), 0.34% (2011–2014)
	Total 3,691 patients	"NAFLD-associated advanced fibrosis", APRI score >1	"NASH-cirrhosis", APRI score >2
Kwok et al.[28] (2016)	72.8%	-	17.1%
	1,799 patients with CAP measurement	-	1,770 patients with LSM measurement

NAFLD, nonalcoholic fatty liver disease; CAP, controlled attenuation parameter; LSM, liver stiffness measurement; T2DM, type 2 diabetes mellitus; APRI, aspartate aminotransferase/platelet ratio index.
*Majority of NAFLD diagnosed by radiologic imaging techniques like ultrasound and proton magnetic resonance spectroscopy, whereas nonalcoholic steatohepatitis (NASH) and advanced fibrosis were diagnosed using liver biopsy.

GENETIC POLYMORPHISMS

The pathogenesis of NAFLD or NASH is complex and involves multiple-hit pathogenic factors, such as adiposity, lipotoxicity, insulin resistance or genetic variations, acting in concert.[29] Single nucleotide polymorphism is one of the essential factors to note. Moreover, ethnic diversity and genetic predisposition suggest that single nucleotide polymorphism in NAFLD plays an important role in its pathogenesis.[30] Recent genome sequencing advancements have helped determine the association between specific genetic variations and NAFLD development. The most prominent variants are patatin-like phospholipase domain-containing 3 (PNPLA3) and the transmembrane 6 superfamily member 2.[30] More recently, novel variants like 17-beta hydroxysteroid dehydrogenase 13, glucokinase regulator, or protein phosphatase 1 regulatory subunit 3B have been investigated as well.[30,31] The 17-beta hydroxysteroid dehydrogenase 13 variation is notable as its wild-type plays a protective role against liver inflammation.[30] The rs738409 C>G single nucleotide polymorphism encoding I149M variant of PNPLA3 and the rs58542926 C>T encoding E167K variant of transmembrane 6 superfamily member 2 are the most studied genetic predispositions associated with NAFLD. Three genotypes included in PNPLA3 variants are CC, GC, and GG. The proportion of each genotype differs in patients with and without NAFLD. The proportion of CC genotype, the wild-type, is the highest in those without NAFLD (30.8% vs. 60.2%), whereas GC and GG genotypes, the variants, are more common among patients with NAFLD (43.0% vs. 35.6% and 26.2% vs. 4.2%, respectively).[31] Single nucleotide polymorphisms are closely associated with NAFLD pathogenesis in lean people. A recent study found a higher frequency of the non CC allele of PNPLA3 in lean patients with NAFLD than in overweight and obese patients.[32] In addition, a greater proportion of lean patients are associated with the transmembrane 6 superfamily member 2 gene single nucleotide polymorphism variation.[15] A more important point was that PNPLA3 I148M was associated with increased liver disease mortality.[33]

OBSTRUCTIVE SLEEP APNEA (OSA)

Obesity causes OSA and NAFLD. In addition, OSA can independently affect the development and progression of NAFLD.[34] As a result of meta-analysis of 18 cross-sectional studies, the pooling OR of OSA for the presence of NAFLD was 2.01 to 2.99.[35] The development of NAFLD in patients with OSA is strongly associated with chronic intermittent hypoxia. Cyclic hypoxia and reoxygenation can induce fatty liver directly via hypoxia-inducing factor-1, and promote tissue inflammatory responses through the accumulation of free radicals and NF-kB.[36] OSA also activates the sympathetic nervous system and induces systemic inflammatory responses and vascular endothelial dysfunction. Activating the sympathetic nervous system increases platelet activity and aggre-

gation, leading to insulin resistance, dyslipidemia, and metabolic syndrome.[36]

MICROBIOME

Gut-liver axis refers to the bidirectional relationship between the microbiome in the gut and the liver, communicating via dietary, genetic, and environmental signals.[37] Disturbance of the liver-gut axis is associated with the NAFLD pathogenesis through gut barrier disruption, bacterial translocation, and subsequent hepatic inflammation response.[38] Although the underlying mechanism or direct causality of NAFLD due to an altered gut microbiome remains unclear, various theories are being explored. For example, Martinez-Gurin et al.[39] showed that NAFLD did not occur due to decreased lipid metabolism and intestinal absorption even in a high-fat diet in germ-free mouse conditions. Resistance of NAFLD in germ-free mice is explained by the inhibition of lipid metabolism via disrupted enteroendocrine signaling (e.g., CCK) and fatty acid transportation (e.g., Cd36 and Dgat1). It was confirmed that absorption of intestinal fat was increased when a high-fat diet was administered after changing the germ-free mouse to general breeding conditions. These data showed how fat absorption changes according to the intestinal microflora's condition.

SARCOPENIA

Sarcopenia is defined as a progressive loss of muscle mass and its strength, more prevalent in patients with chronic medical conditions, such as chronic obstructive pulmonary disease, chronic kidney disease, or NAFLD, than in the healthy population.[40-43] Sarcopenia and NAFLD are associated in a bidirectional manner,[44] independent of insulin resistance (IR) or obesity[41] because they share common pathophysiological mechanisms.[40] It is also suggested that sarcopenia is associated with worse clinical outcomes in general.[43,45] Skeletal muscle plays a central role in glucose metabolism as one of the largest organs in our body to utilize glucose. Loss of muscle mass due to aging,[45] nutrient deficiency, or lack of physical activity leads to weaker muscle strength and dysregulated metabolic function. Skeletal muscle is one of the most significant insulin-stimulated sites in the body, which is generally considered the main culprit of IR.[46] A vicious cycle of local myosteatosis and muscle IR plays a major role in creating systemic inflammation and IR. This vicious loop, called the "metabaging cycle", comprises lipid metabolism dysfunction, lipotoxicity, IR, local inflammation, and lipolysis. Proinflammatory factors involved in the cycle, such as interleukin-6, and tumor necrosis factor-alpha, further induce secretion of cytokines positively, gradually spreading local inflammation into a systemic issue.[47] IR and chronic inflammatory status are common comorbidities among patients with NAFLD, including dysregulation of lipid metabolism.[48,49] Hong et al.[40] suggested that NAFLD and sarcopenia are negatively correlated with homeostasis model assessment of IR and high-sensitivity C-reactive protein. In addition, Koo et al.[42] showed that the prevalence of sarcopenia in patients with NAFLD was higher than in the control group (17.9% vs. 8.7%, $P<0.001$). The risk of NASH and significant fibrosis with sarcopenia is 2.30 and 2.05 times higher than the control group, respectively. The prevalence of significant fibrosis (≥F2) is higher in patients with sarcopenia than those without (OR 2.01, 45.7% vs. 24.7%; $P<0.001$).[42] Moreover, there was a higher prevalence of Child-Pugh class C cirrhosis than those with class B or A in patients with sarcopenia (46.7% vs. 37.9% vs. 23.3%, respectively; $P=0.007$).[50] It is also associated with a higher prevalence of cirrhosis-related complications (81.82% vs. 62.24%, $P<0.001$).[45] The overall survival rate seems significantly lower (relative risk 2.64) than cirrhosis without sarcopenia. It suggests the association of cirrhotic complications, such as ascites (relative risk of 1.82), spontaneous bacterial peritonitis (relative risk of 3.33), hepatic encephalopathy (relative risk of 1.96), and upper gastrointestinal varices (relative risk of 2.13).[45] Five-year survival probabilities of patients with cirrhosis and sarcopenia was shorter than those without (46.6% vs. 74.2%, $P<0.001$).[50]

Authors' contribution

Drafting the article, Eunji Ko; Critical revision of the article, Eileen L. Yoon and Dae Won Jun.

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Conflicts of Interest

The authors have no conflicts to disclose.

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Review

Nonalcoholic fatty liver disease and non-liver comorbidities

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Non-alcoholic fatty liver disease (NAFLD) is a chronic liver disease characterized by excess fat accumulation in the liver. It is closely associated with metabolic syndrome, and patients with NAFLD often have comorbidities such as obesity, type 2 diabetes mellitus, and dyslipidemia. In addition to liver-related complications, NAFLD has been associated with a range of non-liver comorbidities, including cardiovascular disease, chronic kidney disease, and sleep apnea. Cardiovascular disease is the most common cause of mortality in patients with NAFLD, and patients with NAFLD have a higher risk of developing cardiovascular disease than the general population. Chronic kidney disease is also more common in patients with NAFLD, and the severity of NAFLD is associated with a higher risk of developing chronic kidney disease. Sleep apnea, a disorder characterized by breathing interruptions during sleep, is also more common in patients with NAFLD and is associated with the severity of NAFLD. The presence of non-liver comorbidities in patients with NAFLD has important implications for the management of this disease. Treatment of comorbidities such as obesity, type 2 diabetes mellitus, and dyslipidemia may improve liver-related outcomes in patients with NAFLD. Moreover, treatment of non-liver comorbidities may also improve overall health outcomes in patients with NAFLD. Therefore, clinicians should be aware of the potential for non-liver comorbidities in patients with NAFLD and should consider the management of these comorbidities as part of the overall management of this disease. **(Clin Mol Hepatol 2023;29(Suppl):S86-S102)**

Keywords: Nonalcoholic fatty liver disease; Comorbidity

INTRODUCTION

As the incidence and prevalence of nonalcoholic fatty liver disease (NAFLD) continues to increase worldwide, the association of NAFLD with other comorbid conditions is an area of increasing interest and research.[1-9] In several studies, NAFLD has been found to be an independent risk factor for adverse outcomes, including mortality,[10] even after controlling for other known risk factors. However, the relationship of NAFLD to other comorbid conditions is still under investigation, especially when trying to understand whether these conditions coexist or if one causes the other. Furthermore, the presence of fibrosis complicates this relationship as when fibrosis is present, it becomes the number one predictor of mortality.[11-19] Nonetheless, having an understanding of what comorbidities are often associated with NAFLD is important so that proper treatment can be forthcoming. Therefore, the following will provide a brief review of these conditions (Fig. 1) and the current evidence regarding each association.

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CARDIOVASCULAR DISEASE

Ischemic heart disease

The most common cause of death in patients with NAFLD is the spectrum of cardiovascular disease (CVD) comprising coronary artery disease, angina, and ischemic stroke. The incidence of CVD in NAFLD has been estimated to be as high as 100.6 per 1,000 person-years.[20] Though it appears clear that the two conditions are associated, the proof for NAFLD being an independent cause of CVD has not been borne out by the evidence.[21] The absence of a causative link, however, may be due to a lack of data in stratifying CVD in relation to the level of fibrosis. NAFLD does appear to increase the overall risk of CVD, but it is not yet clear if it increases mortality caused by CVD. A meta-analysis of 16 studies showed that NAFLD significantly increased the risk of non-fatal cardiovascular events with an odds ratio (OR) of 2.52 when compared to patients without NAFLD, but no significant relationship was found between NAFLD and the risk of fatal cardiovascular outcomes. However, if severe NAFLD was assessed, as defined by fatty liver on imaging with either increased gamma-glutamyltransferase (GGT) or elevated NAFLD fibrosis score or positron emission tomography showing increased fluorodeoxyglucose (FDG) uptake or worsening fibrosis on pathology, then there was a higher risk of CVD mortality with an OR of 3.28 when compared to patients without NAFLD.[22]

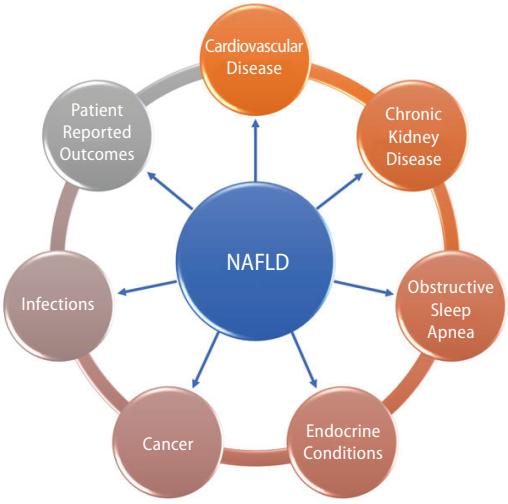

Figure 1. The Multisystem Impact of nonalcoholic fatty liver disease (NAFLD).

Pathophysiologically, the metabolic syndrome inflicts widespread end-organ damage which manifests as CVD and NAFLD. The mechanism is thought to be related to the accumulation of visceral and ectopic fat leading to the production and release of fat-derived toxic metabolites. These metabolites trigger systemic and local inflammation ultimately resulting in the progression of both NAFLD and CVD.[23]

As the mechanisms are similar, the treatment guidelines are shared among the diseases. The American Heart Association has released the "Life's Simple 7" guidelines with a stated goal of reducing deaths from CVD and stroke by 20%. A recent study conducted among patients with NAFLD using Life's Simple 7 guidelines did find that if all NAFLD subjects achieved an ideal rating on all 7 of the health metrics, 66% of all-cause deaths and 83% of cardiovascular (CV) deaths were preventable. In fact, among NAFLD subjects, lack of glycemic control (adjusted population attributable fraction [PAF] =28.3% all-cause; 38.1% CV) and hypertension (adjusted PAF of 23% all-cause; 52.8% CV) were the largest mortality contributors while obtaining ideal physical activity level provided an adjusted PAF=13.9% all-cause and 13.8% CV mortality.[24]

A Mediterranean style diet has also been proposed as an intervention that may help decrease the incidence of both NAFLD and CVD.[25]

Abbreviations:

NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; CVD, cardiovascular disease; HFpEF, heart failure with preserved ejection fraction; HFrEF, heart failure with reduced ejection fraction; AVS, aortic valve sclerosis; MAC, mitral annular calcification; OR, odds ratio; CIMT, carotid intima-media thickness; AST, aspartate transaminase; ALT, alanine transaminase; GGT, gamma-glutamyltransferase; HMG-CoA reductase, hydroxy-methyl-glutaryl-coenzyme A reductase; PCSK 9 and 7, proprotein convertase subtilisin/kexin type 9 and 7; CKD, chronic kidney disease; OSA, obstructive sleep apnea; BMI, body mass index; PSG, polysomnography; AHI, apnea-hypopnea index; CIH, chronic intermittent hypoxia; GH, growth hormone; BMI, body mass index; M/F, males/females; US, ultrasound; MRI, magnetic resonance imaging; CT, computerized tomography; DM, diabetes mellitus; PCOS, polycystic ovarian syndrome; OCP, oral contraceptive pills; LDL, low-density lipoprotein; GLP-1, glucagon-like peptide-1; TH, thyroid hormones; TSH, thyroid stimulating hormone; rhGH, recombinant human growth hormone; ICD-9, International Classification of Diseases, ninth revision; *H. pylori, Helicobacter pylori*; LFS, liver fibrosis score; CagA, cytotoxin associated gene A; VacA vacuolating cytotoxin A; CAP, community-acquired pneumonia; VAP, ventilator-associated pneumonia; CT, computerized tomography; ICU, intensive care unit; C. difficile and CD, *Clostridium difficile*; rCDI, recurrent *C. difficile* infection; GDH, glumatate dehydrogenase; PCR, polymerase chain reaction; ACE2, angiotensin-converting enzyme 2; COVID-19, coronavirus disease 2019; SARS-COV-2, severe acute respiratory syndrome coronavirus 2; PROs, patient-reported outcomes

However, just recently, the American Health Association updated their Life Simple 7 guidelines to include sleep as a new metric (Life's Simple 8 guidelines)[26] as well as updating their diet recommendations to include more food groups such as what is found in a Mediterranean style diet and to use non-high density lipoprotein (HDL) cholesterol measurement for lipid quantification. In this light, a recently published study looked at both sleep and fatigue and their impact on NAFLD mortality. Investigators reported that adults with NAFLD and fatigue experienced 2.3-fold higher mortality than adults with NAFLD but without fatigue. In addition, depression, sleep disturbance and CVD were all major predictors of fatigue, while not having a sleep disturbance had an inverse relationship with mortality.[27] As such, the association between NAFLD and CVD is complex which requires a systematic treatment approach as outlined in several recent guidelines.[28-35]

Congestive heart failure

As the end-stage phenotype of multiple cardiac conditions, congestive heart failure (CHF) is a widespread threat. Associations have been drawn between the presence of NAFLD and CHF. The risk of incident heart failure in patients with NAFLD is higher than patients without NAFLD with an estimated hazard ratio of 1.75, according to a study from Sweden looking at 10,422 patients over a median follow-up period of 13.6 years.[36] Increased epicardial fat in patients diagnosed with fatty liver leads to abnormal energy metabolism, especially in the left ventricle, despite seemingly normal systolic and diastolic function as measured by echocardiography.[37] Positive correlations are seen between hepatic and myocardial triglyceride content as measured by magnetic resonance. Rijzewijk et al.[38] showed that greater amounts of myocardial fat deposition contribute to left ventricular (LV) diastolic dysfunction, predisposing to heart failure with preserved ejection fraction.[38]

In the other well-known phenotype of CHF, heart failure with reduced ejection fraction, NAFLD appears to be an independent risk factor. Even after accounting for obesity, insulin resistance and a suboptimal diet, the presence of NAFLD remains an independent factor contributing to a lower ejection fraction.[39] Using ultrasound and echocardiography, Trovato et al.[39] performed multiple linear regression to compare the presence of fatty liver with ejection fraction and found a statistically significant negative correlation. This deeply concerning finding brings into perspective the much greater risk that these patients with NAFLD face. Further complicating the picture is the population that is not obese yet has underlying fatty liver. High clinical suspicion would be required at the frontlines to find these "lean NAFLD" patients and ensure adequate cardiovascular risk stratification in this population.

The prevalence of NAFLD is 36% in patients with, heart failure with reduced ejection fraction (HFrEF), significantly higher than in the general population. The combination of NAFLD and HFrEF may be particularly troublesome, as these patients are on average younger and have a higher body-mass index, larger LV mass, and greater fibrosis in the LV myocardium.[40] The changes are not all morphologic, as these patients with NAFLD in addition to HFrEF have higher rates of in-hospital and post-discharge all-cause mortality. Advanced fibrosis due to NAFLD is a specific cause of even greater all-cause mortality.[41]

Valvular heart disease

Studies have examined the presence of increased cardiac valvular calcification with NAFLD prevalence. Coexisting sclerosis of the aortic valve (AVS) along with calcification of the mitral annulus (MAC) appears to have the strongest correlation, while patients without any valvular calcification are the least likely to have NAFLD. Isolated AVS and isolated MAC have an intermediate probability.[42] These associations are present independent of diabetes, kidney disease, medications and even echocardiographic values. Along with valvular calcification, suboptimal glycemic control and advancing kidney disease were the other independent predictors of valvular calcification, implying common causative pathways.[43]

Treatment of valvular heart disease may also become more complicated. Anticoagulants like warfarin are frequently indicated in these patients to prevent thromboembolic events. Patients with NAFLD along with valvular disease are seen to require higher doses of warfarin and even then, they are less likely to stay in the therapeutic range as compared to patients without NAFLD.[44]

Ischemic stroke

NAFLD appears to increase the frequency of ischemic stroke though the evidence for it being a potential causative

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factor has been conflicting. Some earlier smaller studies had not shown a clear association between the two entities.[45,46] However, according to a study from Sweden by Simon et al.[36], patients with NAFLD have a significantly increased risk of incident stroke when compared to patients without NAFLD (hazard ratio of 1.58). A large meta-analysis published in 2022 looking at 64 studies from 1998 to 2016 showed that the more advanced the NAFLD, the higher the risk of ischemic stroke. Mild NAFLD had an OR of 1.47 when compared to ischemic stroke, while moderate NAFLD had an OR of 1.67 and severe NAFLD had an OR of 1.79. Mild, moderate, and severe NAFLD were assessed with the degree of hepatic echogenicity on ultrasound. The authors felt that the data was conclusive enough to suggest the use of carotid intima-media thickness (CIMT), assessed by duplex ultrasonography, as a screening tool for NAFLD.[47] While CIMT is not yet being used by clinicians to check for NAFLD, the relationship does bring into perspective the close ties shared by these conditions.

A smaller study from Korea suggests that the assessment of steatosis alone may not adequately predict risk. It concludes that fibrosis specifically, and not necessarily the degree of steatosis, is what increases the risk of ischemic stroke.[48] The association does not seem to differ based on ethnicity or the type of ischemic stroke,[49] though hemorrhagic stroke does not seem to have any relationship with the presence of NAFLD.[47]

It does appear that patients who present with an ischemic stroke are more likely to have underlying NAFLD. A recent study from Japan reports that the frequency of NAFLD is nearly 40% in patients with stroke but only 26.4% in the general Japanese population.[50] From a clinical standpoint, patients who have been diagnosed with a new ischemic stroke should have closer follow-up regarding the status of their liver function, as this follow-up is not routinely done at present.

Specific phenotypes of ischemic stroke caused by NAFLD have been considered. Large artery atherosclerosis and small vessel occlusions are most commonly seen in stroke patients with NAFLD, whereas a cardioembolic etiology is less commonly found.[51] Brainstem infarctions may also be more common in this patient population and have a higher risk of progression even after adjusting for comorbidities.[52]

Atrial fibrillation

An arrhythmia with already high prevalence in the general population, a diagnosis of NAFLD appears to push it higher. A prospective study from Finland on the Observational Pharmaco-Epidemiology Research & Analysis (OPERA) cohort looked at nearly 1,000 patients and established an independent association between the two conditions even after adjusting for age, sex and the presence of diabetes. The increase in risk was found to be nearly two-fold.[53] Though not directly assessing NAFLD, the Framingham study of 3,700 patients found that higher liver enzymes (aspartate transaminase and alanine transaminase) did correlate with an increased risk of incident atrial fibrillation.[54] At least 4 studies from 2014 to 2017 did suggest that elevated GGT levels were also independently associated with the development of atrial fibrillation. A review on the topic looking at 14 studies and 3 meta-analyses found one study that did not show an association between NAFLD and atrial fibrillation, while all the others suggested that NAFLD is associated with an increased risk of developing atrial fibrillation.[55]

Ventricular arrhythmias

Other more immediately dangerous arrhythmias are also being linked to the presence of NAFLD. An excessively prolonged corrected QT (QTc) interval on electrocardiography can often degenerate into ventricular tachyarrhythmias and has been associated with sudden cardiac death.[56] Interestingly, the degree of NAFLD has been found to increase the QTc interval on patient EKGs. A large study of over 30,000 patients from Taiwan found that mild NAFLD increased QTc intervals by 2.55 ms and severe NAFLD increased it by 12.13 ms.[57] Smaller studies have confirmed this association in other parts of the world.[58,59] Clearly, patients with NAFLD would benefit from having a lower threshold for undergoing rhythm monitoring if symptomatic though the evidence does not yet support screening for arrhythmias in NAFLD.

Impact of dyslipidemia treatment

Contrary to what one may hope, treatment of dyslipidemia has not been found to improve NAFLD, though newer targets in the pipeline may be able to alter disease progression. In a study of 2,566 patients, traditional antidyslipidemic treat-

ment, including hydroxy-methyl-glutaryl-coenzyme A reductase (HMG-CoA) reductase inhibitors, did not improve mortality or major adverse cardiovascular events in NAFLD.[60] It is possible that traditional therapies do not account for the specific phenotype of dyslipidemia that exists in NAFLD. A higher plasma apolipoprotein B to apolipoprotein A1 ratio has been found in patients with NAFLD even after taking obesity into account. Patients with NAFLD also have smaller low-density lipoprotein (LDL) particle size.

It is thought that these patients may require different treatment targets, which are currently being researched. Specifically, increasing hepatic fat metabolism is the proposed mechanism of action of resmetirom (MGL-3196), a selective thyroid hormone receptor agonist. This oral medication has shown increased reduction of hepatic fat as measured by magnetic resonance imaging (MRI) in phase 2 clinical trials though a clear mortality benefit is not yet evident.[61] Phase 3 trials confirm that resmetirom is as safe and as well tolerated as placebo while significantly improving liver transaminases and fibrosis biomarkers in addition to proton-density fat fraction on MRI.[62]

Though PCSK9 inhibitors such as alirocumab and evolocumab are coming into more widespread use, these medications have not yet been shown to improve NAFLD. However, studies have shown that specific gene variants of PCSK7 have been associated with higher levels of inflammation in the liver along with higher transaminases.[63] Specifically targeting PCSK7 may be able to target NAFLD and its downstream deleterious effects.[64]

CHRONIC KIDNEY DISEASE

Much research has been done into the risk of incident chronic kidney disease and nonalcoholic fatty liver disease. A 2018 meta-analysis including a total of 96,595 patients concluded that NAFLD did increase the risk of incident chronic kidney disease with a hazard ratio of 1.37. Multiple confounding factors including age, sex, body mass index, serum lipids, hypertension, tobacco use, baseline kidney function, and diabetes were assessed and the association persisted. Statistical analysis confirmed that the risk of developing chronic kidney disease increased as NAFLD advanced.[65] Cross-sectional analysis showed a patient with liver fibrosis has a 2.5 times greater likelihood of having CKD and is twice as likely to have albu-

Table 1. Studies Assessing the Relationship between NAFLD and OSA

Author	Year	Sample size	NAFLD diagnosis	OSA diagnosis	Significant findings
Tanne et al.	2005	163	Liver biopsy and liver enzymes	PSG	AHI predicted liver histology independent of age and BMI
Turkay et al.	2012	112	Ultrasound and liver enzymes	PSG	Increased prevalence and severity of steatosis in OSA AHI independently predicted presence of NAFLD
Minville et al.	2014	226	Biochemical markers	PSG	Steatosis increased with the severity of CIH
Qi et al.	2015	175	Ultrasound	PSG	Steatosis increased with the severity of CIH
Lin et al.	2015	85	Ultrasound	PSG	Steatosis increased with the severity of CIH
Angrawal et al.	2015		Liver enzymes and elastography	PSG	Fibrosis increased with the severity of CIH and higher AHI
Petta et al.	2018	126	Liver biopsy	PSG and questionnaires	Fibrosis increased with the severity of CIH
Trzepizur et al.	2018	1,285	Liver enzymes and biochemical markers	PSG or home sleep study	Steatosis increased with the severity of OSA Severe OSA associated with 2.5 folds higher risk of liver fibrosis

NAFLD, nonalcoholic fatty liver disease; OSA, obstructive sleep apnea; BMI, body mass index; PSG, polysomnography; AHI, apnea-hypopnea index; CIH, chronic intermittent hypoxia.

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minuria then a patient without NAFLD.[66] It is hoped that medications that would target inflammation and fibrosis in nonalcoholic steatohepatitis (NASH) and chronic kidney disease may delay the disease progression of both these conditions.

OBSTRUCTIVE SLEEP APNEA

Most studies have concluded that a greater degree of hepatic steatosis increases the severity of chronic intermittent hypoxia on polysomnography (Table 1).

More concerningly, obstructive sleep apnea may contribute to the development of insulin resistance and it may trigger the development of nonalcoholic fatty liver disease.[67] Highlighting the need for multimodal therapy in NAFLD, chronic positive airway pressure treatment decreases the concentrations of liver enzymes, specifically alanine transaminase and aspartate transaminase.[68]

ENDOCRINE CONDITIONS

Diabetes mellitus

The interest surrounding NAFLD and its predisposition to diabetes has been extensive. Patients with NAFLD generally have hepatic insulin resistance, which then increases the likelihood of developing diabetes mellitus. On a molecular level, it is thought that insulin resistance causes mitochondrial dysfunction which disrupts fatty acid beta oxidation and leads to lipid deposition in the liver.[69] Addressing NAFLD early on would decrease incident diabetes mellitus and its myriad associated complications. Measures aimed at weight loss, limiting saturated fats in the diet, and becoming physically active all increase insulin sensitivity and decrease hepatic steatosis.[70] Medications used for diabetes mellitus like pioglitazone are among the first line agents in the medical management of NASH.[71]

Polycystic ovarian syndrome

The hallmark feature of polycystic ovarian syndrome (PCOS), androgen excess, has been related to insulin resistance. It is well recognized that higher rates of diabetes, central obesity, and dyslipidemia are observed in patients with PCOS.[72] NAFLD has also been shown to affect 34 to 70% of women with PCOS, when NAFLD affects only 14 to 34% of women in the general population.[73]

Hyperandrogenism may independently increase the risk of NAFLD. A case-control study compared 275 non-obese women with PCOS to 892 non-obese women without PCOS. The PCOS cohort was found to have a NAFLD prevalence of 5.8%, while only 2.8% of women without PCOS had NAFLD. The study found that increased levels of free testosterone correlated to a higher risk for NAFLD even after adjusting for age, body-mass index, insulin resistance, and lipid profile.[74]

Specific treatment of PCOS has not been shown to improve NAFLD. A common treatment for PCOS, oral contraceptives, have not had a clear benefit in NAFLD. A cross-sectional study looking at NHANES data did find lower rates of NAFLD in women currently on oral contraceptives when compared to women who had used them in the past or had never used them.[75] A biopsy-based study, however, showed increased lobular inflammation, a histologic feature of NASH, in patients taking oral contraceptives.[76]

Weight loss, on the other hand, appears to be a more surefire way to ameliorate both conditions. Liraglutide 1.8 mg daily led to decreased rates of NAFLD along with downtrends in hepatic fat fraction and visceral adipose tissue.[77] The increased availability of glucagon-like peptide-1 receptor agonists worldwide remains a goal of clinicians invested in public health.

Hypothyroidism

The exact pathophysiological mechanisms for the development of NAFLD in the presence of hypothyroidism are yet to be elucidated. However, the most accepted mechanism of action is that hepatic steatosis results from decreased serum levels of thyroid hormone (TH). The decrease in TH stimulates lipolysis from fat stores in white adipose tissue and from dietary fat sources (high-fat diets) to generate free fatty acids that enter the hepatic cells via protein transporters causing an induction of *de novo* lipogenesis (DNL). In addition, TH indirectly controls the transcriptional regulation of hepatic DNL by regulating the expression and activities of other transcription factors such as sterol associated with NAFLD through increased levels of thyroid stimulating hormone (TSH) whereby high levels of TSH stimulate lipogenesis in the liver causing

Table 2. Studies Assessing the Relationship between NAFLD and Growth Hormone Deficiency

Author	Year	Study design	Sample size	NAFLD diagnosis	Significant findings
Adams et al.	2004	Retrospective; single center	21	10 based on liver biopsy and 11 based on imaging	NAFLD developed rapidly (on average 6.4 years) after the diagnosis of pituitary/hypothalamic dysfunction, and liver disease was severe; 60% of those biopsied had cirrhosis, and 14.3% (three) of the 21 received liver transplants or died.
Fukuda et al.	2008	Retrospective: single center	42	Ultrasound and elevated transaminases	Rate of NAFLD increased progressively after stopping GH therapy. The prevalence of NAFLD at 10 and 20 years after the cessation of GH was 22%/10% (M/F) and 33%/25% (M/F).
Hong et al.	2011	Cross-sectional; single center	34 males with 40 controls	Ultrasound	The degree of fatty liver on abdominal ultrasonography correlated with the degree of GH deficiency even after adjusting for BMI.
Nishizawa et al.	2012	Retrospective; single center	66 patients with 83 controls	Ultrasound; 16 had liver biopsy	GH replacement therapy significantly improved liver enzymes, histology, and levels of fibrotic markers in patients with NASH.
Gardner et al.	2012	Cross-sectional; single center	28 patients with 24 controls	Magnetic resonance spectroscopy	NAFLD was equally prevalent in patients with GH deficiency and matched controls. GH replacement significantly decreased abdominal subcutaneous and visceral fat though it did not reduce liver fat.
Meienberg et al.	2016	Cross-sectional	22 patients with 44 controls	Proton magnetic resonance spectroscopy	Liver fat content and the prevalence of NAFLD were similar in patients with GH deficiency and matched controls. GH-deficient patients had greater total and visceral fat mass. GH replacement therapy did not decrease hepatic fat fractions.
Kang et al.	2021	Cross-sectional	76 patients with 74 controls	Transient elastography and MRI	71% of patients with hypopituitarism had NAFLD, compared with 31% of controls.

NAFLD, nonalcoholic fatty liver disease; GH, growth hormone; BMI, body mass index; M/F, males/females; MRI, magnetic resonance imaging; NASH, nonalcoholic steatohepatitis.

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hepatosteatosis.[78] Disturbingly, hypothyroidism has been found to be more common in those with NASH and NAFLD related hepatocellular carcinoma.[79-82] Currently, there are no additional treatments recommended for this condition.[83]

Growth hormone deficiency

Ever since Takano et al published their case report of a 17 years old boy who presented with panhypopituitarism and fatty liver in 1997,[84] the therapeutic use of growth hormone (GH) in NAFLD has been explored by researchers around the world. In the case report, the patient was treated with GH and their fatty liver subsequently improved, as measured by ultrasound echogenicity and liver size. Numerous studies over the years looking at the relationship between NAFLD and GH deficiency are summarized in Table 2.

In patients with proven GH deficiency, replacing GH does decrease body fat content while increasing lean muscle mass.[85] Efforts at using GH in patients without GH deficiency with the aim of treating NAFLD have had mixed results so far. In a small pilot study, treatment with recombinant human growth hormone did not decrease liver fat content as assessed by magnetic resonance spectroscopy (MRS), though a lower body mass index was achieved.[86]

NON-LIVER CANCER

Colon cancer

The links being found between NAFLD and cancer are alarming. Allen et al.[87] longitudinally followed a population of 4,722 patients with NAFLD and compared them to 14,441 controls and found that NAFLD doubled the risk of developing cancer while obesity alone did not (incidence rate ratio [IRR]=2.0, 95% confidence interval [CI] 1.5–2.9 vs. IRR=1.0, 95% CI 0.8–1.4). This data raises the concern that NAFLD may play a role in mediating cancer development. One theory proposes that visceral adipose tissue produces adipocytokines that lead to tumor proliferation. Gastrointestinal cancers appear to have the strongest correlation with NAFLD. Colon cancer specifically had an IRR of 1.8 in the study by Allen et al.[87]. A large meta-analysis of 15 studies confirms a similar degree of association, with a pooled OR of 1.7 when looking at NAFLD and the risk of colorectal cancer.[88]

Table 3. Studies Assessing the Relationship between NAFLD and Breast Cancer

Author	Year	Study design	Sample size	NAFLD diagnosis	Cancer diagnosis	Significant findings
Nseir et al.	2017	Cohort	73 patients	CT imaging	Pathology	Odds ratio of 2.82 (breast cancer in NAFLD vs. in controls)
Kim et al.	2018	Longitudinal cohort	25,947 patients	Ultrasound	Pathology and radiology	Incidence rate ratio of 1.77 (breast cancer in NAFLD vs. in controls)
Allen et al.[87]	2019	Longitudinal cohort	4,722 patients with 14,441 controls	ICD-9	ICD-9	Incidence rate ratio of 1.6 (breast cancer in NAFLD vs. in obese controls)
Kwak et al.	2019	Case-control	270 patients with 270 controls	Ultrasound	Mammography and pathology	Odds ratio of 1.63 (breast cancer in nonobese NAFLD vs. in nonobese controls); no association between NAFLD and breast cancer was detected in the obese group

NAFLD, nonalcoholic fatty liver disease; CT, computerized tomography; ICD-9, International Classification of Diseases, ninth revision.

NAFLD appears to not only increase the risk of colon cancer, but precancerous lesions, as well. Adenomatous polyps, polyps with villous morphology, and lesions with high-grade dysplasia are all more common in patients with NAFLD.[89] The need for strict adherence to the recommended guidelines for colon cancer screening in patients with NAFLD are evident, though increased or earlier screening has not yet been suggested.

Gastric cancer

Another gastrointestinal cancer with links to NAFLD is stomach cancer. Data from six studies assessing the risk of incident stomach cancer in NAFLD showed a pooled random effects hazard ratio of 1.81.[90] It is likely that similar pathways of tumorigenesis play a role in the development of these gastrointestinal cancers.

Breast cancer

The presence of NAFLD may also be associated with extragastrointestinal cancers. A pooled OR of 1.69 was found when assessing the risk of breast cancer in patients with NAFLD.[88] Some of the relevant studies are noted in Table 3.

Uterine cancer

Gynecologic cancers appear to be more prevalent in patients with NAFLD. In a pooled analysis of 85,827 patients, of which 23% had NAFLD, patients with NAFLD had an approximately 60% greater risk of developing uterine cancer than the general population.[90]

INFECTIONS

Helicobacter pylori

Gastrointestinal-specific infections have been associated with NAFLD. *Helicobacter pylori* increases the generation of inflammatory markers like interleukin-1β and tumor necrosis factor-α, levels of which are increased in patients testing positive for *H. pylori*. These markers may increase hepatic inflammation and predispose patients to developing NAFLD. Indeed, studies have shown a 36% greater risk of NAFLD in

Table 4. Studies Assessing the Relationship between NAFLD and *H. pylori*

Authors	Year	Study design	Sample size	NAFLD diagnosis	*H. pylori* diagnosis	Significant findings
Abdel-Razik et al.[92]	2018	Cohort; multicenter	369 adults without NAFLD	Ultrasound	Fecal antigen	-The presence of *H. pylori* was an independent risk variable for the presence of NAFLD -After therapy of H. pylori infection, there was a significant reduction in NAFLD-LFS
Kang et al.	2018	Retrospective; NHANES data	5,404 patients	Ultrasound	*H. pylori* serology	-*H. pylori* infection was significantly associated with NAFLD in non-Hispanic black patients -CagA negative *H. pylori* was associated with an increased risk for NAFLD in the non-Hispanic white and non-Hispanic black populations
Alvarez et al.	2020	Cross-sectional; community	424 patients	Fatty Liver Index and Hepatic Steatosis Index	*H. pylori* multiplex serology	-No significant associations between *H. pylori*, *H. bilis*, or *H. hepaticus* and NAFLD or other metabolic or liver conditions -Seropositivity for *H. pylori* antigens, CagA and VacA, and *H. hepaticus* antigen HH0713 were each associated with a 2 to 3-fold increased prevalence of NAFLD

NAFLD, nonalcoholic fatty liver disease; *H. pylori*, *Helicobacter pylori*; LFS, liver fibrosis score, CagA, cytotoxin associated gene A; VacA, vacuolating cytotoxin A; *H. bilis*, *Helicobacter bilis*; *H. hepaticus*, *Helicobacter hepaticus*; NHANES, National Health and Nutrition Examination Survey.

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Table 5. Studies Assessing the Relationship between NAFLD and *Clostridium difficile*

Authors	Year	Study design	Sample size	NAFLD diagnosis	*Clostridium difficile* diagnosis	Significant findings
Papić et al.[113]	2020	Retrospective cohort; single-center	314 patients	Ultrasound	Screening GDH test confirmed with toxin A/B PCR	-OR 3.27 (P=0.04) - association of NAFLD with C difficile infection. Appears to be independent of other components of metabolic syndrome, such as obesity and diabetes mellitus
Nseir et al.	2020	Retrospective cross-sectional; single-center	115 patients	Ultrasound or abdominal CT	Positive stool test for CD Toxin A/B by enzyme immunoassay or PCR for CD	-OR 1.51 (P=0.05) - association of NAFLD with C difficile infection. -Majority of patients with NAFLD (63%) had a fibrosis score of more than two points. Consequently, it seems that NAFLD patients with liver fibrosis are at more risk for acquiring CDI
Šamadan et al.	2021	Retrospective cohort; single-center	329 patients	Ultrasound	Screening GDH test confirmed with toxin A/B PCR	-OR 1.81 (P=0.005) - association of NAFLD with recurrent C difficile infection. -DM and obesity were not associated with rCDI in this study -Statin use was associated with lower rCDI in patients both with and without NAFLD

NAFLD, nonalcoholic fatty liver disease; C. difficile and CD, *Clostridium difficile*; rCDI, recurrent C difficile infection; GDH, glumatate dehydrogenase; CT, computerized tomography; OR, odds ratio; PCR, polymerase chain reaction.

patients diagnosed with *H. pylori* infection[91] though the data does not universally affirm the risk (Table 4).

Data has been encouraging that treating *H. pylori* infection in patients with NAFLD does appear to improve fibrosis scores.[92] Clinicians should have a lower threshold for diagnosing and curing patients of *H. pylori* in cases of NAFLD.

Clostridium difficile

Altered gut microbiome in patients with NAFLD is also being explored. Patients with NASH are found to have increased amounts of Bacteroides and decreased amounts of *Prevotella* in their gastrointestinal flora, while *Ruminococcus* was associated with increased liver fibrosis.[93] It follows that patients with NAFLD have a higher risk of infection with *Clostridium difficile*, even after adjusting for the presence of diabetes and obesity (Table 5).

COVID-19

During the global pandemic of our time, front-line clinicians early on saw the increased mortality rates among patients with obesity. NAFLD by itself appears to increase the risk further, even after adjusting for the presence of obesity, especially in severe COVID-19 disease.[94] NAFLD also increases the duration of viral shedding,[95] a finding with public health implications. The liver is thought to be especially prone to this virus as SARS-COV-2 enters cells through the angiotensin-converting enzyme 2 (ACE2). These enzymes are abundant in both the liver and in the biliary epithelium.[96] Fighting NAFLD on all fronts may very well decrease the rapid spread of any future viral respiratory-borne infections.

Bacterial pneumonia

The increased inflammation associated with NAFLD may increase the susceptibility to certain infections. Bacterial pneumonia has been examined and some associations have been found (Table 6).

Table 6. Studies Assessing the Relationship between NAFLD and Bacterial Pneumonia

Authors	Year	Study design	Sample size	NAFLD diagnosis	Significant findings
Nseir et al.[71]	2017	Retrospective case-control	141 patients with 141 controls	Ultrasound or abdominal CT	NAFLD (odds ratio 2.5, P=0.023) was associated with CAP. 40.4% of the study group showed evidence of NAFLD compared to 27.6% in controls
Bailey et al.[72]	2017	Retrospective	147 trauma ICU patients with VAP and 130 trauma ICU patients without VAP	Abdominal CT	The presence of NAFLD on admission CT was significantly higher in ICU patients with post-traumatic VAP compared to the baseline trauma population
Nseir et al.[73]	2019	Retrospective cohort	561 patients	Ultrasound	NAFLD was independently associated with increased 30-day all-cause mortality in patients with CAP, especially in patients with advanced liver fibrosis

NAFLD, nonalcoholic fatty liver disease; CAP, community-acquired pneumonia; VAP, ventilator-associated pneumonia; CT, computerized tomography; ICU, intensive care unit.

OVERALL PATIENT REPORTED OUTCOMES (PROs)

The presence of NAFLD or NASH is associated with decreased PROs which is more evident in those with NASH and advanced fibrosis. Among the studies completed on health-related quality of life, results have consistently shown that patients with NAFLD and NASH report low physical functioning scores, fatigue and higher rates of depression and anxiety than the general population which can result in decreased productivity at work if employed (presenteeism) and/or performing their activities of daily living.[97-100] On the other hand, treatment of NAFLD or NASH that causes a regression in the disease state patients may show an improvement in their PROs.

Health care utilization for both inpatient and outpatient care is increased for those with NAFLD especially when the comorbidities of CVD, hypertension, and obesity were present for inpatients and CVD, diabetes mellitus, hypertension were present as outpatients. However, the presence of cirrhosis increased costs significantly among inpatients and outpatients. In addition, NAFLD has a significant economic impact on countries, as well.[101-113]

CONCLUSION

NAFLD is a complex metabolically based liver disease that is associated with a number of comorbidities. Through an increased awareness of the extrahepatic complications of NAFLD, clinicians can embark on a multi-pronged approach to tackle this insidious, mostly asymptomatic condition.[27-34] As more research is completed on finding patients with NAFLD who are at the highest risk for adverse outcomes, further study is required to determine the preventative screening guidelines to be implemented due to their demonstrably greater risk in several conditions. Due to its multifaceted nature, effective treatments of NAFLD may be generated in other fields not directly related to hepatology, and these developments will be followed with interest by hepatologists worldwide.

Authors' contribution

RM: study design, data collection, data synthesis and interpretation, and drafting of the manuscript. MHN: study con-

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cept, study supervision, data interpretation, and revision of the manuscript. All authors approved the final draft of the manuscript as well as the authorship list.

Conflicts of Interest

Mindie H. Nguyen: Research support: Pfizer, Enanta, Gilead, Glycotest, Vir, B.K. Kee Foundation, National Cancer Institute. Advisory board/consulting: Janssen, Spring Bank, Gilead, Novartis, Bayer, Eisai, Eli Lilly, Exact Sciences, Laboratory of Advanced Medicine, Helio Health, Intercept. Other authors have no disclosures.

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Review

Screening strategy for non-alcoholic fatty liver disease

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Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease, affecting approximately 25% of the general population worldwide, and is forecasted to increase global health burden in the 21st century. With the advancement of non-invasive tests for assessing and monitoring of steatosis and fibrosis, NAFLD screening is now feasible, and is increasingly highlighted in international guidelines related to hepatology, endocrinology, and pediatrics. Identifying high-risk populations (e.g., diabetes mellitus, obesity, metabolic syndrome) based on risk factors and metabolic characteristics for non-invasive screening is crucial and may aid in designing screening strategies to be more precise and effective. Many screening modalities are currently available, from serum-based methods to ultrasonography, transient elastography, and magnetic resonance imaging, although the diagnostic performance, cost, and accessibility of different methods may impact the actual implementation. A two-step assessment with serum-based fibrosis-4 index followed by imaging test vibration-controlled transient elastography can be an option to stratify the risk of liver-related complications in NAFLD. There is a need for fibrosis surveillance, as well as investigating the cost-effectiveness of different screening algorithms and engaging primary care for first-stage triage screening. **(Clin Mol Hepatol 2023;29(Suppl):S103-S122)**

Keywords: NAFLD; Metabolic diseases; Diabetes mellitus; Fatty liver; Fibrosis

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease that places an increasing burden on global health in the 21st century, and is known to affect approximately 25% of the general population worldwide.[1] NAFLD includes two pathologically distinct conditions: non-alcoholic fatty liver and non-alcoholic steatohepatitis (NASH); the latter covers a wide spectrum of disease severity, including inflammation, hepatocyte injury (hepatocellular ballooning), and fibrosis at different stages.[2,3] Without appropriate management, it can progress to cirrhosis and liver-related complications, including hepatocellular carcinoma (HCC) and liver failure.[4] Compared to the general population, individuals with NAFLD have an increased risk of overall mortality, with common causes of death, besides liver-related ones, being cardiovascular disease and malignancy.[5-8] A modelling study forecasted the total NAFLD population of eight major countries to increase by 18.3% from 2016 onward, reaching a prevalence of 28.4% by 2030.[9] Most individuals with NAFLD

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remain undiagnosed and, worryingly, the prevalence of advanced fibrosis and cirrhosis is projected to double by 2030.[9] Despite the high population prevalence of NAFLD, recognition and management of the condition varies, with improvements still required in investigations at the primary care level and in the staging of fibrosis.[10]

The need for NAFLD screening in the community has been questioned given the high associated direct and indirect costs, the low predictive value of non-invasive tests, the risks of liver biopsy, and the lack of effective treatment for NAFLD.[11] However, the progressive form of NAFLD (i.e., NASH), particularly when associated with advanced fibrosis, should be identified in patients at risk (age >50 years, type 2 diabetes mellitus or metabolic syndrome),[12] due to its prognostic implications. Although familial clustering occurs, based on current evidence, family screening is not generally advisable.[12] There is also a lack of validated cost-utility studies on the effectiveness of screening.

Currently, there is no consensus on the recommended population requiring screening for NAFLD. The American Association for the Study of Liver Diseases (AASLD) recommends against routine screening in any population, regardless of body mass index (BMI),[13] but also endorses "vigilance" in patients with type 2 diabetes mellitus (T2DM). The guidelines issued by the European Association for the Study of Liver (EASL), European Association for the Study of Diabetes (EASD), and European Association for the Study of Obesity (EASO) recommend screening in individuals with obesity or metabolic syndrome;[12] the recommendations from the Asian Pacific Association for the Study of the Liver (APASL)[14] and the Korean Association for the Study of the Liver (KASL)[15] are similar. There are also variations in the recommendations from British,[16] diabetic and pediatric professional associations (Table 1).[17-20]

In this review, we aimed to highlight the high-risk populations in which NAFLD screening may prove beneficial, summarize recent non-invasive tests for the screening for NAFLD, and discuss the importance of fibrosis surveillance.

SCREENING FOR NAFLD IN HIGH-RISK POPULATIONS: A PROMISING STRATEGY TO MITIGATE THE FUTURE BURDEN OF LIVER DISEASE

Screening should ideally be performed via an organized program that has the capacity to identify target populations, and perform thorough evaluation, monitoring, and treatment.[21] Screening should preferably be the main purpose of the program; if risk factors of NAFLD require management, patients should be referred to appropriate healthcare providers (Table 2).

DIABETES MELLITUS

NAFLD is found in 50–60% of T2DM patients and up to 45% of type 1 diabetes mellitus (T1DM) patients,[22] which raises an important question: Should we screen for NAFLD in the diabetic population?

Disease progression is more aggressive in T2DM patients with underlying hepatic necroinflammation and fibrosis. Mechanistically, lipotoxicity-induced mitochondrial dysfunction and activation of inflammatory pathways, rather than steatosis, cause progressive liver damage.[23] Among patients with T2DM, NASH is a leading cause of end-stage liver disease and a risk factor for cardiovascular disease.[24] Similar to diabetic retinopathy and nephropathy, NASH is increasingly being recognized as a complication of T2DM,[25] which may imply the condition should be considered for incorporation into diabetic complication screening programs. Since T2DM patients are at high risk of developing NASH, concomitant NAFLD can be present even when liver transaminases are

Abbreviations:

NAFLD, non-alcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; HCC, hepatocellular carcinoma; AASLD, American Association for the Study of Liver Diseases; BMI, body mass index; EASL, European Association for the Study of Liver; EASD, European Association for the Study of Diabetes; EASO, European Association for the Study of Obesity; APASL, Asian Pacific Association for the Study of the Liver; KASL, Korean Association for the Study of the Liver; T2DM, type 2 diabetes mellitus; T1DM, type 1 diabetes mellitus; ^{1}H-MRS, proton-magnetic resonance spectroscopy; MRI-PDFF, magnetic resonance imaging-estimated proton density fat fraction; MRE, magnetic resonance elastography; NFS, NAFLD fibrosis score; MAFLD, metabolic dysfunction-associated fatty liver disease; NASPGHAN, North American Society of Pediatric Gastroenterology, Hepatology and Nutrition; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ELF, enhanced liver fibrosis; NAS, NAFLD activity score; SHG/TPEF, second harmonic generation/two-photon excitation fluorescence; VCTE, vibration-controlled transient elastography; CAP, controlled attenuation parameter; SWE, shear wave elastography; pSWE, point shear wave elastography; PLIN2, perilipin-2; RAB14, ras-related protein 14; TSP2, thrombospondin-2; LCN2, lipocalin-2; EIT, electrical impedance tomography; FLI, fatty liver index

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normal.[26]

Several studies have reported the results of screening for liver fibrosis in the general population or individuals with T2DM using non-invasive methods (mainly by transient elastography). A population-based study from Hong Kong[27] investigated liver fat and fibrosis using proton-magnetic resonance spectroscopy (^{1}H-MRS) and transient elastography in 922 healthy individuals recruited by random selection. The prevalence of NAFLD (defined by an intrahepatic triglyceride content >5%) was 27.3%, and the prevalence of advanced fibrosis (liver stiffness >9.6 kPa) was 3.7%. In another study involving 1,918 T2DM patients,[28] the prevalence of increased liver stiffness (>9.6 kPa, suggestive of stage ≥F3) was 18%. Among approximately one-third of patients who underwent a liver biopsy, 56% had steatohepatitis, 21% had advanced fibrosis, and 29% had cirrhosis. A prospective study demonstrated the feasibility of using two accurate, precise, and validated non-invasive image-based biomarkers: magnetic resonance imaging-estimated proton density fat fraction (MRI-PDFF) to quantify liver fat, and magnetic resonance elastography (MRE) to detect advanced fibrosis in T2DM patients in a primary care setting,[29] with a 65% prevalence of NAFLD and a 7.1% prevalence of advanced fibrosis found in the study population.

Altogether, these results confirmed the increased prevalence of advanced fibrosis among individuals with T2DM,

Table 1. Current guidance on screening for NAFLD

Professional organizations	Year	Guidance statements
European Association for the Study of Liver (EASL), European Association for the Study of Diabetes (EASD), and European Association for the Study of Obesity (EASO)[12]	2016	Screening for NAFLD in people with obesity, metabolic syndrome, and in particular, T2DM
American Association for the Study of Liver Diseases (AASLD)[13]	2018	1. Routine screening for NAFLD in high-risk populations (obesity, T2DM) is not advised due to uncertainties in diagnostic testing, long-term management, and cost-effectiveness 2. Endorses "vigilance" in patients with T2DM 3. Systematic screening of family members for NAFLD is not currently recommended
Asian Pacific Association for the Study of the Liver (APASL)[14]	2020	Screening in those with T2DM or metabolic syndrome, or those who are overweight/obese according to ethnic-specific cut-offs
The American Academy of Pediatrics[17-19]	2007; 2014; 2017	1. Currently, the best screening test for NAFLD in children is ALT; however, it has substantial limitations. 2. Screening should be considered for obese youth with additional risk factors (central adiposity, insulin resistance, pre-diabetes or diabetes, dyslipidemia, sleep apnea, or family history of NAFLD/NASH) 3. Follow-up screening for NAFLD is recommended. When the initial screening test is normal, consider repeating ALT every 2–3 years if risk factors remain unchanged
The American Diabetes Association (ADA)[20]	2019	Patients with type 2 diabetes or prediabetes and elevated liver enzymes (ALT) or fatty liver on ultrasound should be evaluated for the presence of non-alcoholic steatohepatitis and liver fibrosis
Korean Association for the Study of the Liver (KASL)[15]	2021	1. Subjects who have persistent liver enzyme elevation, metabolic syndrome, or diabetes should be screened for NAFLD 2. Abdominal ultrasonography is the primary screening modality
British Association for the Study of the Liver (BASL) and British Society of Gastroenterology (BSG) NAFLD Special Interest Group[16]	2022	1. Services should have an agreed local clinical pathway for the investigation of suspected liver disease 2. Consider the possibility of liver fibrosis due to NAFLD in people with T2DM or metabolic syndrome

NAFLD, non-alcoholic fatty liver disease; T2DM, type 2 diabetes mellitus; ALT, alanine aminotransferase; NASH, nonalcoholic steatohepatitis.

Table 2. Differences among international guidelines in screening recommendations for NAFLD in high-risk populations

Populations	Supporting screening	Guidelines	Against screening	Guidelines
Age >50 years	√	2016 EASL-EASD-EASO		
Obesity	√√	2016 EASL-EASD-EASO 2019 APASL	X	2018 AASLD
Type 2 diabetes	√√√	2016 EASL-EASD-EASO 2019 APASL 2021 KASL	X	2018 AASLD
Metabolic syndrome	√√√	2016 EASL-EASD-EASO 2019 APASL 2021 KASL		
Persistently abnormal liver enzymes	√√√	2016 EASL-EASD-EASO 2019 APASL 2021 KASL		
Obese youth with additional risk factors*	√	The American Academy of ediatrics		
First-degree relatives of NAFLD			XX	2016 EASL-EASD-EASO 2018 AASLD
Genetic variants			XX	2016 EASL-EASD-EASO 2018 AASLD

NAFLD, non-alcoholic fatty liver disease; EASL, European Association for the Study of Liver; EASD, European Association for the Study of Diabetes; EASO, European Association for the Study of Obesity; APASL, Asian Pacific Association for the Study of the Liver; KASL, Korean Association for the Study of the Liver; AASLD, American Association for the Study of Liver Diseases; NASH, nonalcoholic steatohepatitis.
√ indicated the number of guidelines that support screening for NAFLD in this population. X indicated the number of guidelines against screening for NAFLD in this population. The number of markers indicate the strength of recommendation.
*Such as central adiposity, insulin resistance, pre-diabetes or diabetes, dyslipidemia, sleep apnea, and family history of NAFLD/NASH.

thereby justifying the potential benefits of screening for NAFLD among T2DM patients, although the use of magnetic resonance (MR)-based technologies would raise issues related to cost and accessibility.

OBESITY AND THE ENTITY OF LEAN NAFLD

It has been well-documented that obesity is associated with an increased risk of NAFLD. Increased BMI and waist circumference, a measure of visceral adiposity, are positively related to the presence of NAFLD[30] and predict advanced disease, particularly in the elderly.[31] Common obesity comorbidities, such as sleep apnea,[32] also contribute to the disease burden of NAFLD. The majority (>95%) of patients with morbid obesity undergoing bariatric surgery would have underlying NAFLD,[33,34] of which the prevalence of advanced fibrosis is estimated at 10%.[35] Since obesity can limit successful liver stiffness measurements, the XL probe (lower ultrasound frequency of 2.5 MHz; can reach deeper liver tissue 35–75 mm from the skin surface) has been shown to be effective in liver stiffness measurement in obese patients with increased success rates of measurements, compared to the standard M probe.[36,37]

In addition, patients with BMI <25 kg/m^2 but with visceral fat accumulation or dysfunctional adipose tissue can exhibit NAFLD with or without elevation in liver aminotransferases;[38,39] these individuals are usually described as "lean NAFLD." The populations of lean NAFLD vary worldwide, comprising 17.3% of the NAFLD cohort in the United States,[40] but with higher proportions of 50% and 75% in Japan[41] and India, respectively.[42] However, the concept of lean NAFLD is somewhat misleading and simplistic, as it draws a line at 25 kg/m^2 (or 23 kg/m^2 for Asian people). The definition of "lean" is based on BMI, but it does not consider how the weight is distributed in the body (fat vs. muscle, intra-abdominal fat vs. subcutaneous fat). Thus, lean NAFLD refers to the presence of NAFLD in lean people who often have some abdominal fat accumulation or other subtle metabolic abnormalities.[43] Caucasian lean subjects with NAFLD represent a wide spectrum

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of NAFLD, which can develop into advanced liver disease, metabolic comorbidities, cardiovascular disease, as well as liver-related mortality.[43] These findings illustrate the oversimplified concept of lean NAFLD.

The indications for screening of NAFLD in lean individuals are not well-defined; NAFLD may be easily missed since such patients do not fit the classic phenotype of obesity.[44] The fibrosis-4 (FIB-4) index and NAFLD fibrosis score (NFS), while well-validated, are generally more useful in excluding fibrosis than identifying it. A recent study found NFS and FIB-4 to be less accurate in discriminating the severity of disease in lean NAFLD patients.[45] Meanwhile, both non-obese and lean groups had substantial long-term liver and non-liver comorbidities. A retrospective study from 1999–2016 indicated that non-obese NAFLD individuals had higher 15-year cumulative all-cause mortality (51.7%) compared to obese NAFLD (27.2%) and non-NAFLD (20.7%) individuals in the United States.[46] These findings suggest that obesity should not be the sole criterion for NAFLD screening.[47]

METABOLIC SYNDROME

A third condition in which screening may be considered is metabolic syndrome, which comprises multiple metabolic and cardiovascular risk factors, primarily increased waist circumference, and a mixed combination of dyslipidemia, hypertension, and diabetes/prediabetes.[48] NAFLD parallels the prevalence of metabolic syndrome and its components, which also increases the risk of advanced disease. The link between metabolic syndrome and NAFLD is complex and bidirectional. Evidence indicated that NAFLD diagnosed via ultrasonography was associated with an increased risk of incident metabolic syndrome with a pooled relative risk of 3.22,[49] suggesting that a vicious cycle of worsening disease states is likely to exist.

A cohort study over a 6-year follow-up period has observed 3,913 new cases of NAFLD in 15,791 Han Chinese individuals, and the risk of incident NAFLD was markedly higher in those with metabolic syndrome.[50] The hazard ratios for incident NAFLD increased when three features of metabolic syndrome were present as compared to individuals who exhibited no metabolic syndrome components. Advanced fibrosis was observed in 10.4% of health checkup examinees by FIB-4 index and shear wave elastography in health checkup examinations.[51] Furthermore, metabolic syndrome with mild-to-moderate alcohol consumption was associated with advanced fibrosis.[51]

The EASL-EASD-EASO Clinical Practice Guidelines 2016 indicated that all individuals with steatosis should be screened for features of metabolic syndrome, independent of liver enzymes.[12] For patients with newly-presenting metabolic syndrome, screening for NAFLD by liver enzymes and/or ultrasound should be routine.[12] Since all components of metabolic syndrome correlate with liver fat level, regardless of BMI, the presence of metabolic syndrome in any particular patient should prompt an assessment of the risk of NAFLD, and vice versa, the presence of NAFLD should prompt an examination of all components of metabolic syndrome. A thorough evaluation of each element of the metabolic syndrome is required as part of the metabolic workup.

METABOLIC DYSFUNCTION-ASSOCIATED FATTY LIVER DISEASE IN CONCOMITANT LIVER DISEASE

The diagnosis of NAFLD conventionally requires the exclusion of other chronic liver diseases, including excess alcohol use and viral hepatitis.[13] Steatosis of metabolic origin can occur in chronic hepatitis B, chronic hepatitis C, and alcoholic liver disease. In fact, the distinction between "alcoholic" and "non-alcoholic" may not be clear-cut, with overlap and heterogeneity between the two conditions. One example would be a high-alcohol-producing bacteria-*Klebsiella pneumoniae,* which resides in the gut microbiota of >60% Chinese NAFLD patients, and produces high levels of ethanol which accelerates the development of steatosis regardless of alcoholic intake.[52]

In order to establish defined "positive" clinical criteria, an international panel of experts have detailed the rationale for an update of the nomenclature describing the liver disease associated with metabolic dysfunction, known as metabolic dysfunction-associated fatty liver disease (MAFLD).[53] According to the recent international consensus statement, the diagnosis of MAFLD is based on the detection of liver steatosis combined with the coexistence of at least one of three positive criteria, which include overweight or obesity, T2DM, or clinical evidence of metabolic dysfunction, such as an increased waist circumference and an abnormal lipid or glyce-

mic profile.[54] The diagnosis can be established irrespective of any presence of concomitant chronic liver disease. Concomitant MAFLD has been shown to be associated with adverse outcomes in both chronic hepatitis B virus (HBV) infection[55] and alcoholic liver disease.[56] Concomitant presence of diabetes, obesity, and metabolic screening should prompt screening, although it remains uncertain if screening may be beneficial for additional sub-groups.

AGE, SEX, AND ETHNICITY

An important risk factor for NAFLD development is increasing age, demonstrated by a NAFLD prevalence of over 50% in elderly Taiwanese (mean age: 70.3 years),[57] as well as over 60% of middle-aged (age >45 years) Southeast Asians.[58] Another important factor is sex, with NAFLD more common in men than in women, although NAFLD risk increases in women after menopause, suggesting that estrogen has a protective role.[59] Moreover, the impact of ethnicity cannot be ignored. As evidenced by a population–based cohort in the United States, NAFLD prevalence differs significantly between ethnicities, being more common in non-Hispanic whites (28.4%) compared to Asian Americans (18.3%).[60] Consistently, in another population study of 4,538 people, NAFLD prevalence was the lowest in non-Hispanic Blacks (18.0%) and Asians (18.1%), and the highest amongst Mexican Americans (48.4%). Within the NAFLD group, advanced fibrosis was the highest in non-Hispanic Blacks (28.5%) and the lowest amongst non-Hispanic Asians (2.7%).[61]

NAFLD is underdiagnosed in children due to a lack of recognition, screening, or appreciation of associated complications by healthcare providers. One study showed that less than one-third of children with obesity were screened for NAFLD through laboratory testing at clinic visits.[62] Children may not be recognized as being obese at clinic visits, and age-appropriate norms for BMI may go unacknowledged. Similar to adults, children with features of metabolic syndrome, such as obesity, hypertension, insulin resistance, and dyslipidemia, are at higher risk for NAFLD.[63] NAFLD may also be incidentally discovered in children while undergoing imaging. The 2017 North American Society of Pediatric Gastroenterology, Hepatology and Nutrition (NASPGHAN) guideline[16] recommends that screening for NAFLD should be considered for all obese youths starting at the age of 9–11 years with additional risk factors (central adiposity, insulin resistance, pre-diabetes or diabetes, dyslipidemia, sleep apnea, or family history of NAFLD/NASH) by alanine aminotransferase (ALT) levels, but recommends against using routine ultrasonography owing to low sensitivity. However, the 2018 AASLD guidance[13] has no recommendation regarding screening in children who are overweight and obese, due to a paucity of evidence.

GENETIC SUSCEPTIBILITY

Knowledge of the genetic component of NAFLD has grown exponentially, in part owing to genome-wide association studies and the advent of high-throughput omics technologies. Currently, at least five variants in different genes have been robustly associated with NAFLD,[64] such as patatin-like phospholipase domain-containing protein 3 (*PNPLA3*), transmembrane 6 superfamily member 2 (*TM6SF2*), membrane bound O-acyltransferase domain-containing 7 (*MBOAT7*), glucokinase regulator (*GCKR*), and Hydroxysteroid 17-Beta Dehydrogenase 13 (*HSD17B13*). Carriers of the PNPLA3 I148M[65-67] and the TM6SF2 E167K variants[68,69] have a higher liver fat content and increased risk of NASH. Nevertheless, the incorporation of NAFLD genetic markers into routine clinical testing for the dynamic assessment of disease status and response to therapy has been protracted. While PNPLA3 I148M is the best-characterized genetic variant associated with NAFLD, its contribution to NAFLD heritability remains modest.[70,71] Accordingly, the EASL-EASD-EASO Clinical Practice Guidelines 2016[12] do not recommend the testing of these genetic variants in routine clinical practice, although genotyping may be considered in selected patients and clinical studies.

FIRST-DEGREE FAMILY RELATIVES

The risk of undiagnosed liver disease in first-degree relatives of NAFLD patients has been of concern, particularly in those who have more advanced fibrosis. By using magnetic resonance elastography to quantify hepatic fibrosis in siblings, parents, and offspring of patients with NAFLD-cirrhosis,[72] first-degree relatives of patients with NAFLD-cirrhosis have a 12 times higher risk of advanced fibrosis than healthy

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controls, even after adjustment for age, sex, ethnicity, BMI, and diabetes status, signifying that screening for advanced fibrosis in first-degree relatives of patients with NAFLD-cirrhosis can be beneficial. With that being said, both the 2016 EASL-EASD-EASO[12] and 2018 AASLD guidelines[13] stated that, until further evidence emerges, systematic screening of family members for NAFLD is not advisable currently.

SCREENING IN THE PRIMARY CARE SETTING

Primary care would be taking up the main bulk of identifying patients with diabetes, dyslipidemia, hypertension, and components of metabolic syndrome; and are the optimal providers to identify patients with NAFLD, make appropriate referrals to specialists, and arrange appropriate surveillance. Once patients develop advanced fibrosis, the risk of liver-related mortality is exponentially increased.[73] Therefore, the challenge for primary care providers is the early identification of high-risk patients for specialist referral.

A prospective cohort study was designed to assess 1,118 patients with incidental abnormal liver function tests in the primary care setting and found the incidence rate of NAFLD to be 26.4%.[74] However, the number of primary care patients with abnormal liver enzymes may underestimate the true underlying prevalence, given the poor association between liver enzyme derangement and the presence of NAFLD. In terms of identifying patients with advanced fibrosis using the Enhanced Liver Fibrosis (ELF) test, with the low population prevalence of advanced fibrosis in the primary care setting, the positive predictive value of non-invasive testing was similarly low.[75] The use of non-invasive blood tests (a two-step algorithm combining FIB-4 score and ELF) for liver fibrosis improves the detection of advanced fibrosis and cirrhosis while reducing unnecessary referrals in patients with NAFLD.[76] With that being said, in order to implement primary care as a first-stage triage screening, primary care physicians need to be aware of the asymptomatic presentation of most NAFLD patients and understand the differences between NAFLD and NASH.[77]

MODALITIES OF SCREENING

Liver biopsy is essential for the diagnosis of NASH, and is the only procedure that reliably differentiates NAFL from NASH.[78] A histologically-based scoring system, NAFLD activity score (NAS),[79,80] was developed and validated to fulfill the diagnostic criteria for NASH and include the full spectrum of NAFLD. Recent accurate quantitative assessments of liver fibrosis based on liver biopsy, such as second harmonic generation/two-photon excitation fluorescence (SHG/TPEF) microscopy imaging,[81] can improve the efficacy endpoint for fibrosis in NASH clinical trials and give a more precise method for NASH staging. According to the 2018 AASLD guideline,[13] liver biopsy should be considered in patients with NAFLD who are at increased risk of steatohepatitis and advanced fibrosis. However, the risks of percutaneous liver biopsy, including bleeding, organ perforation, sepsis, and death, are also critical.[82]

With the vast majority of NAFLD patients being stable and asymptomatic, performing liver biopsies on all patients is unfeasible and unethical for disease screening, diagnosis, or progression assessment. Non-invasive diagnostic methods using plasma samples, ultrasonography, liver elastography (including both transient and magnetic resonance) have been developed with good diagnostic performance for liver steatosis and fibrosis.[83,84] These methods have been widely used for early steatosis detection, disease severity assessment, identification of patients needing a liver biopsy for confirmatory diagnosis (e.g., after discrepant results) and for the assessment of fibrosis progression. While avoiding the risks associated with a liver biopsy, these non-invasive tools, with the possible exception of transient elastography, are also hampered by several limitations, including suboptimal sensitivity to evaluate the complete spectrum of NAFLD histological lesions and the lack of validity to be used for routine diagnosis (Table 3).

Several scoring systems have been established for further elucidation of the presence of NAFLD.[85-93] The FIB-4 index (calculated by four clinical variables: age, aspartate aminotransferase [AST], ALT, and platelet count)[94] and NFS (age, BMI, impaired fasting glucose and/or diabetes, AST, ALT, platelet count, and albumin)[95-97] have been recommended by the EASL-EASD-EASO guidelines[12] as part of the diagnostic algorithm for ruling out advanced fibrosis. Importantly, the NFS has been shown to predict liver decompensation and mortality in patients with NAFLD.[95]

Conventional ultrasonography is the most common method for the qualitative assessment of hepatic steatosis due to

Table 3. Current non-invasive methods for NAFLD screening

Diagnostic panel	Cost	Features	Detection abilities		
			Steatosis	Advanced fibrosis	Cirrhosis
Serological markers					
Fatty liver index[85]	$	Common parameters involved (BMI, WC, triglycerides, and GGT) Cannot distinguish between steatosis grades	√	X	X
Hepatic steatosis index[86]	$	Common parameters involved (AST: ALT ratio, BMI, female sex, and DM) Inadequate distinction of the severity of steatosis	√	X	X
SteatoTest[87,88]	$$	Involves biomarkers that are not routinely done (α2M, haptoglobin, ApoA-1, total bilirubin, GGT, fasting glucose, triglycerides, cholesterol, and ALT, adjusted for patient's age, sex, weight, and height)	√	X	X
FIB-4[94]	$	A formula comprising age, platelet, AST, and ALT One of the best non-invasive tests for diagnosing advanced fibrosis in NAFLD Rules out advanced fibrosis	X	√	√
NFS[95-97]	$	A formula comprising age, hyperglycemia, BMI, platelet count, albumin, and AST/ALT ratio Identifies advanced fibrosis well Needs independent adjustment of BMI across ethnic groups	X	√	√
BARD score[95]	$	A formula comprising BMI, AST/ALT ratio, and diabetes Does not predict fibrosis well in patients with mild NAFLD (specifically in patients with obesity or T2DM), which limits its clinical use	X	√	√
ELF[89-91]	$$	Consists of an algorithm of three fibrosis markers (HA, PIIINP, and TIMP-1) that are not routinely measured Rules out advanced fibrosis	X	√	√
FibroTest[87,92,93]	$$	Involves biomarkers that are not routinely done (α2M, haptoglobin, ApoA-1, total bilirubin, GGT) Affected by other causes of hyperbilirubinemia and elevated GGT	X	√	X
Imaging modalities					
Ultrasonography[99-101]	$	AUROC 0.97 good predictive tool for steatosis but does not provide information regarding fibrosis, unless cirrhosis is established	√	X	√
VCTE[105-107,111]	$	AUROC 0.84 for F2 fibrosis with the M probe AUROC 0.93 for F3 fibrosis with the M probe AUROC 0.95 for F4 fibrosis with the M probe AUROC 0.80–0.85 for F2 fibrosis with the XL probe AUROC 0.84–0.90 for F3 fibrosis with the XL probe AUROC 0.91–0.95 for F4 fibrosis with the XL probe Not accurate in patients with cholestasis, ascites, and congestive heart failure	√ √	√ √	√ √
MRI-PDFF[110-112]	$$$	Good specificity and sensitivity in detecting steatosis Less reliable for grading steatosis in patients with advanced fibrosis or cirrhosis Cannot be performed in patients with claustrophobia, and the measurements are affected by hepatic iron deposition Not widely available	√ √	X	X

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Table 3. Continued

Diagnostic panel	Cost	Features	Detection abilities		
			Steatosis	Advanced fibrosis	Cirrhosis
MRS[112]	$$$	Results of this tool might be affected by respiration movements, claustrophobia, and implanted devices Only available in specialized centers	√ √ √	X	X
MRE[110,113-116]	$$$	AUROC 0.86–0.89 for F2 fibrosis AUROC 0.89–0.96 for F3 fibrosis AUROC 0.88–0.97 for F4 fibrosis Accessibility is limited by requirement of specific scanner hardware	X	√ √ √	√ √ √
SWE[88,117,118]	$	No well-established cutoffs for NAFLD Results may differ from liver biopsy; accurate if >30% of hepatocytes are steatotic Reduced sampling errors	X	√ √	√ √

NAFLD, non-alcoholic fatty liver disease; BMI, body mass index; WC, waist circumference; GGT, gamma–glutamyltransferase; DM, diabetes mellitus; ALT, alanine aminotransferase; AST, aspartate aminotransferase; FIB-4, fibrosis-4; NFS, NAFLD fibrosis score; T2DM, type 2 diabetes mellitus; ELF, enhanced liver fibrosis; AUROC, area under the receiver operating characteristic curve; VCTE, vibration-controlled transient elastography; MRI-PDFF, magnetic resonance imaging-estimated proton density fat fraction; MRS, magnetic resonance spectroscopy; MRE, magnetic resonance elastography; SWE, shear wave elastography; α2M, α2-macroglobulin; ApoA-1, Apolipoprotein AI; BARD, body mass index, AST/ALT ratio, and diabetes; HA, hyaluronic acid; PIIINP, type III procollagen peptide; TIMP-1, tissue inhibitor of metalloproteinases-1.
$ indicated the relative cost of using this method for NAFLD screening. $, relatively low; $$, relatively medium; $$$, relatively high. √ indicated the relative detection abilities of this method. $, relatively low; √, relatively medium; √, relatively high. X indicated that this screening method could not detect steatosis, advanced fibrosis, or cirrhosis.

its accessibility and low cost.[98] However, the ability to detect steatosis in patients with NASH is limited by the presence of advanced fibrosis.[99] Ultrasonography is useful at detecting moderate-to-severe steatosis with high diagnostic accuracy, with an area under the receiver operating characteristic curve (AUROC) of 0.93,[100] but is unable to discriminate between steatosis, fibrosis, inflammation, or NASH.[101] Furthermore, ultrasonography is also limited by both inter- and intra-observer reliability.[102]

Vibration-controlled transient elastography (VCTE) is the most validated and commonly used elastography method worldwide.[103] VCTE measures the tissue elasticity, which is directly related to liver stiffness, and in turn, is related to the degree of fibrosis.[104] Besides liver stiffness assessment, controlled attenuation parameter (CAP) is obtained by VCTE to quantify the liver fat.[105] A CAP value ≥248 dB/m is the commonly used cut-off to define hepatic steatosis.[106,107] Mild (equivalent to number of affected hepatocytes: 5–33%), moderate (34–66%), and severe (>66%) steatosis are defined as CAP 248–267 dB/m, CAP 268–279 dB/m, and CAP ≥280 dB/m, respectively.[106] According to recently published cut-offs in a large multicenter study[108] and a meta-analysis,[109] low risk of advanced fibrosis was defined as liver stiffness measurements <8.0 kPa, intermediate risk (8.0–12.0 kPa), and high risk >12.0 kPa.

MRI provides high specificity and sensitivity in detecting liver steatosis, especially MRI-PDFF. MRI-PDFF enables fat mapping of the entire liver, which is more accurate than CAP in detecting all grades of steatosis in NAFLD patients (AUROC 0.99).[110] MRI-PDFF is usually used as a research tool and is not easily accessible in clinical practice due to the logistical complexities, lengthy scan time, and lack of required expertise at the majority of medical imaging centers.[111] Additionally, H-magnetic resonance spectroscopy (H-MRS) is a well-established and validated method of non-invasive liver fat quantification by directly measuring chemical composition of tissue.[88] H-MRS is highly accurate for even minimal amounts of steatosis,[112] but its widespread application is also hampered by its cost and availability.

MRE enables non-invasive assessment of hepatic fibrosis, and is currently considered the most accurate non-invasive modality. MRE uses a modified phase-contrast method to

image the propagation of the shear wave in the liver parenchyma for quantitatively assessing tissue stiffness.[113,114] A meta-analysis found that MRE detected fibrosis in NAFLD with a high level of accuracy (AUROC 0.86–0.91) for all stages.[115] This technique is more accurate than VCTE in detecting F2 fibrosis (AUROC 0.86–0.89 vs. AUROC 0.84) and F4 fibrosis (AUROC 0.88–0.97 vs. AUROC 0.95).[110,116] However, its wider application is limited by cost, expertise, and availability. Currently, MRI-related techniques are unlikely to be applied as a first-line screening method in clinical practice.

Shear wave elastography (SWE) was developed based on the technological foundation of conventional ultrasonography. A potential advantage of SWE is the ability to perform measurements over a wider region of interest, thereby reducing sampling error.[117] Point shear wave elastography (pSWE) has similar advantages to VCTE in that the perfor-

Table 4. Potential future modalities for NAFLD screening

Developing modalities		Components	AUROC	Comments
Serum-based	Perilipin-2 (PLIN2)[119] mean fluorescence intensity	Combined with waist circumference, triglyceride, ALT and presence/ absence of diabetes as covariates as a biomarker for NASH	An accuracy of 93% in the discovery cohort and 92% in the validation cohort	Using flow cytometry to measure PLIN2 in peripheral blood monocytes Current form not feasible for screening
	Ras-related protein (RAB14)[119] mean fluorescence intensity	Combined with age, waist circumference, high-density lipoprotein cholesterol, plasma glucose, and ALT levels as covariates as a biomarker for NASH	99.3%, significantly higher than NFS (85.2%), FIB-4 (62.2%), APRI (61.8%)	Using flow cytometry to measure RAB14 in peripheral blood monocytes Current form not feasible for screening
	Thrombospondin-2 (TSP2)[120]	A novel fibrosis biomarker of NAFLD in T2DM	0.80, indicating fibrosis ≥F3 on VCTE, superior to both FIB-4 and NFS	Existing commercial enzyme-linked Immuno-sorbent Assay Cutoff: 3.6 ng/mL to identify ≥F3 fibrosis
	Lipocalin-2 (LCN2)[121]	A valuable NAFLD biomarker, especially for the transition from NAFL to NASH	AUC: 0.987 for NASH diagnosis, and AUC: 0.977 for steatosis	Unable to establish an optimal cut-off value for distinguishing NASH from NAFL Using a rapid, portable, point-of-care, and user-friendly point-of-care assay
Metabolomics	Amino acids[123,124]	The ratio of glutamate/ (serine+glycine)	F0–F2 vs. F3–F4, highest odds ratio (OR) for liver fibrosis (F3–4)	Using gas chromatography-mass spectrometry Current form not feasible for screening
	Bile acids[124,125]	7-ketodeoxycholic acid (7-Keto-DCA)	Advanced fibrosis (OR, 4.2), NASH (OR, 24.5), and hepatocellular ballooning (OR, 18.7)	Biomarkers for NAFLD progression Independent validation is required Using a stable isotope-dilution LC-MS/MS method Current form not feasible for screening
		7-ketolithocholic acid (7-Keto-LCA)	NASH (OR, 9.4) and ballooning (OR, 5.9)	
Stool-based	Fecal-microbiome derived metagenomic signature[126]	37 bacterial species are used to construct a Random Forest classifier model to detect advanced fibrosis in NAFLD	A robust diagnostic accuracy (AUC 0.936)	Need to utilize metagenomics sequencing Current form not feasible for screening

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Table 4. Continued

Developing modalities		Components	AUROC	Comments
Device-based	Multi-spectral EIT[122]	Using waist-over-height biometric as complementary information	Predict clinical-standard CAP in patients with or without NAFLD	Portable Self-administrable Potentially cost-effective and with a short acquisition time (3 minutes) Only with pilot results, need validation in large cohorts
	^{13}C-methacetin breath test[127,128]	Quantitative evaluation of the cytochrome P450-dependent liver function	A good tool for identifying patients with histologically proven NASH (AUROC: 0.824); Predicts F3 or F4 fibrosis (AUROC: 0.936 and 0.973)	Separate patients with normal/NAFL from patients with NASH Fail to detect early stages of fibrosis Mainly investigated in patients with chronic hepatitis C

NAFLD, non-alcoholic fatty liver disease; AUROC, area under the receiver operating characteristic curve; ALT, alanine aminotransferase; NASH, nonalcoholic steatohepatitis; NFS, NAFLD fibrosis score; FIB-4, fibrosis-4; AST, aspartate aminotransferase; APRI, AST to platelet ratio index; T2DM, type 2 diabetes mellitus; VCTE, vibration-controlled transient elastography; AUROC, the area under a receiver operating characteristic curve; AUC, area under the curve; EIT, electrical impedance tomography; CAP, controlled attenuation parameter; LC-MS/MS, liquid chromatography-mass spectrometry/mass spectrometry.

mance is better for severe fibrosis and cirrhosis than for the lower stages of fibrosis.[88,117] Unfortunately, pSWE does not allow for the assessment or quantification of steatosis. Values obtained with pSWE have a narrow range (0.5–4.4 m/s), which limits the definitions of cut-off values for discriminating different fibrosis stages, reducing its impact on management decisions.[118] There are no well-established cutoffs for pSWE in NAFLD patients.

In addition to the currently used screening modalities mentioned above, there are also various serum, metabolomic, stool, and device-based approaches (Table 4) that have potential for screening. Measuring the mean fluorescence intensity of perilipin-2 (PLIN2) or ras-related protein 14 (RAB14) in peripheral blood monocytes has been demonstrated to be an accurate liquid biopsy for NASH;[119] however, since it is detected by flow cytometry, its practicality for screening remains uncertain. Other promising markers, including serum thrombospondin-2 (TSP2)[120] and lipocalin-2 (LCN2),[121] lack validation and well-established cut-off values. Multi-spectral electrical impedance tomography (EIT)[122] is a self-administrative medical device for liver steatosis, but it is still in very early phases of development. Other methods with potential include metabolomic-based markers for fibrosis, ballooning and NASH,[123-125] fecal-based bacterial signatures,[126] and the ^{13}C-methacetin breath test.[127-129]

SURVEILLANCE AND FOLLOW-UP ARRANGEMENT

Most of the screening algorithms proposed to use these non-invasive assessments in a sequential algorithm.[130,131] A stepwise ultrasonography-FIB-4/NFS-VCTE strategy to screen for NAFLD is shown in Figure 1. First, ultrasonography is the preferred first-line diagnostic procedure for imaging of NAFLD. Fatty liver index (FLI), SteatoTest, and NAFLD liver fat score are acceptable alternatives for the diagnosis of steatosis if imaging tools are not available or feasible.[12] For fibrosis assessment, a non-invasive test with a single cut-off is performed in primary care or endocrinology units to exclude patients with a low risk of advanced fibrosis. FIB-4 or NFS are inexpensive, easy-to-perform tests for the exclusion of advanced fibrosis using a single cut-off (NFS <-1.455 and FIB-4 <1.30), and can be used as a first screening option for intermediate-to-high–risk patients. Both these tests may be influenced by age and should use a different cut-off for patients aged >65 years (NFS <0.12 and FIB-4 <2.0).

Once FIB-4 yields intermediate or high results, second-line VCTE can be used to improve the identification of advanced fibrosis, which has been shown to reduce the need for liver biopsy.[131,132] Patients can then undergo VCTE when advanced fibrosis cannot be excluded.[133] The cut-off for advanced fibro-

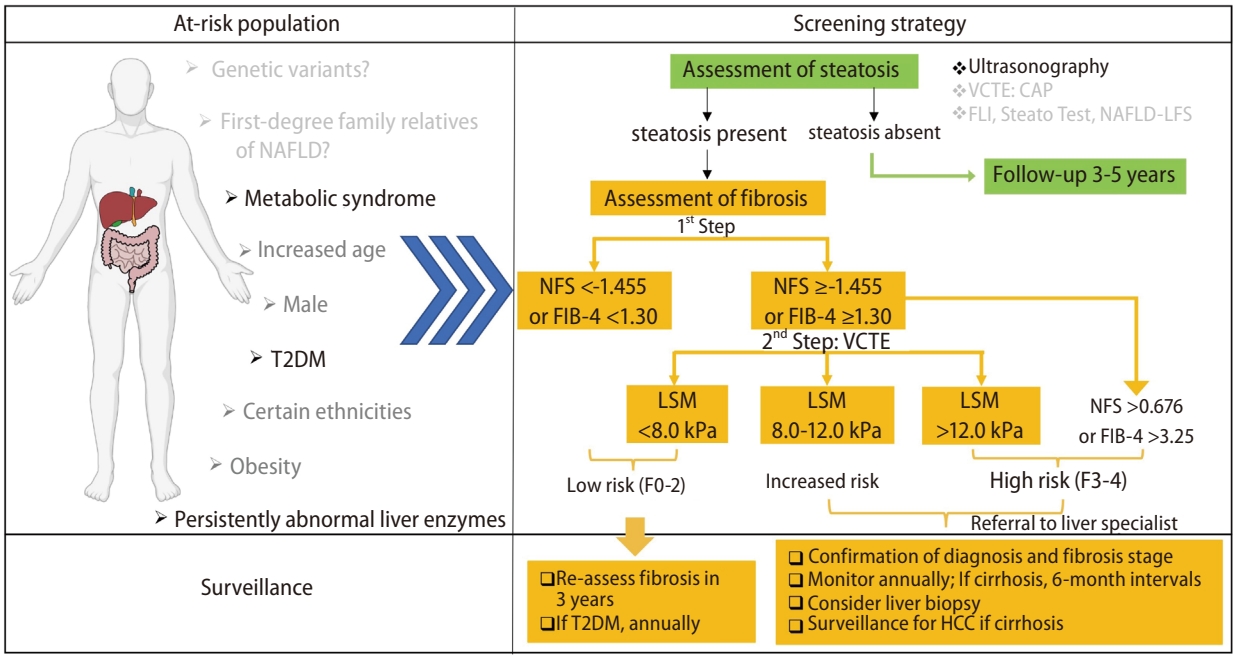

Figure 1. Diagnostic flow-chart to assess and monitor disease severity in the presence of suspected NAFLD. NFS threshold: -1.455 in patients aged <65 years, 0.12 in patients aged ≥65 years. FIB-4 threshold: 1.30 in patients aged <65 years, 2.0 in patients aged ≥65 years. CAP, controlled attenuation parameter; FIB-4, fibrosis-4 index; FLI, fatty liver index; HCC, hepatocellular carcinoma; NAFLD, nonalcoholic fatty liver disease; NFS, NAFLD fibrosis score; VCTE, vibration controlled transient elastography; T2DM, type 2 diabetes mellitus; LSM, liver stiffness measurement.

sis with VCTE is 8.0 kPa (M probe) or 6.2 kPa (XL probe) for the exclusion of advanced fibrosis. The XL probe is highly recommended in obese patients. Patients above the recommended thresholds should be referred to a hepatologist for subsequent management.

The optimal surveillance strategy for patients with NAFLD is undetermined. The variable risk of progression of both the hepatic disease and the underlying metabolic conditions, as well as the cost and workload for healthcare providers, need to be considered. According to the EASL-EASD-EASO algorithm,[12] monitoring should include routine biochemistry, assessment of comorbidities, and non-invasive monitoring of fibrosis. NAFLD patients without worsening of metabolic risk factors, should be monitored at 2- to 3-year intervals. Patients with NASH and/or fibrosis should be monitored annually, and those with NASH cirrhosis at 6-month intervals. If indicated on a case-by-case basis, liver biopsy could be repeated after 5 years.

COST-EFFECTIVENESS OF SCREENING

The question of whether NAFLD screening should be undertaken is deeply influenced by cost-effectiveness. High direct and indirect costs could be a barrier to screening. The AASLD guidelines do not recommend population screening for NAFLD.[13] Screening for liver fibrosis by VCTE at primary care centers is a highly cost-effective intervention and leads to earlier identification of patients in European and Asian populations, better than by standard of care alongside or using serum biomarkers.[134] Whether a two-step screening program using serum biomarkers followed by VCTE is more cost-effective and cost-saving in population screening should be tested in future studies. Moreover, the use of non-invasive liver fibrosis tests (FIB-4, ELF, or VCTE) in primary care increases early detection of advanced liver fibrosis, reduces unnecessary referral of patients with mild disease, and is cost-efficient.[135] Adopting a two-tier approach improves resource utilization.[135]

For high-risk populations, one study found screening for NASH in T2DM (age >50 years) by ultrasonography to lack

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cost-effectiveness; however, that may in part be related to the study's design, with the outcome measures of HCC and liver transplantation not being considered.[136] More recent data have supported the cost-effectiveness of screening. A comprehensive cost-utility analysis indicated that screening for NAFLD in patients with T2DM in the United States using an algorithm-based approach, starting with ultrasound and liver biochemistry and followed by VCTE for fibrosis to detect those most likely to have advanced fibrosis, was more cost-effective than the status quo of no screening.[137] Moreover, screening at a younger age will increase cost-effectiveness. However, comparisons of the cost-effectiveness of screening for NAFLD in general populations versus high-risk populations are still required.

FIB-4 followed by either VCTE, MRE, or liver biopsy can be cost-effective strategies for identifying cirrhosis in populations in whom the prevalence of cirrhosis varies between 0.27% and 4%.[138] Based on the U.S. health system, the combination of FIB-4 and VCTE, was the most cost-effective and the least costly, followed by the combination of FIB-4 and MRE. FIB-4 and VCTE remained the most cost-effective strategy if the aim were to avoid liver biopsy. Again, these findings require validation in other healthcare jurisdictions.

CONCLUSIONS

To this end, identifying high-risk populations based on the risk factors and metabolic characteristics for non-invasive screening is crucial. Screening all populations is generally not advisable and is not cost-effective.[136] Despite variations in international guidelines regarding how and who to screen, patients with T2DM, metabolic syndrome or persistently elevated liver enzymes may benefit the most from screening (Fig. 1). Screening for NAFLD in these high-risk patients, starting with ultrasound and liver biochemistry, and followed by non-invasive testing for fibrosis to detect advanced liver fibrosis, is more cost-effective than not screening this population.[137] The increasing availability of novel non-invasive tools, including transient elastography and MRI-based methods, will accurately quantify the severity of NAFLD and may help in screening and monitoring disease outcomes. The stepwise FIB-4/NFS-VCTE algorithm has been developed to rule out patients with a low risk of advanced fibrosis.

Regardless of screening strategies, patient participation will always be a key determinant of success. This is a social and behavioral challenge, as screening is a personal choice that is ideally based on informed decision-making. Increased patient participation[139] and physician awareness of the importance of screening will be crucial in reducing the morbidity and mortality related to NAFLD.

Authors' contribution

S Zhang: Conceptualization, Literature Review, Writing and Original Draft Preparation; LY Mak: Review, Critical Revision; MF Yuen: Review, Critical revision, final approval of published version; WK Seto: Review, Critical revision, final approval of published version.

Conflicts of Interest

MF Yuen is an advisory board member and/or received research funding from AbbVie, Arbutus Biopharma, Assembly Biosciences, Bristol Myer Squibb, Dicerna Pharmaceuticals, GlaxoSmithKline, Gilead Sciences, Janssen, Merck Sharp and Dohme, Clear B Therapeutics, Springbank Pharmaceuticals; and received research funding from Arrowhead Pharmaceuticals, Fujirebio Incorporation and Sysmex Corporation. WK Seto received speaker's fees from AstraZeneca and Mylan, is an advisory board member of CSL Behring, is an advisory board member and received speaker's fees from AbbVie, and is an advisory board member, received speaker's fees and researching funding from Gilead Sciences. No other authors have any conflict of interest to disclose.

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Review

Non-invasive imaging biomarkers for liver steatosis in non-alcoholic fatty liver disease: present and future

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Non-alcoholic fatty liver disease is currently the most common chronic liver disease, affecting up to 25% of the global population. Simple fatty liver, in which fat is deposited in the liver without fibrosis, has been regarded as a benign disease in the past, but it is now known to be prognostic. In the future, more emphasis should be placed on the quantification of liver fat. Traditionally, fatty liver has been assessed by histological evaluation, which requires an invasive examination; however, technological innovations have made it possible to evaluate fatty liver by non-invasive imaging methods, such as ultrasonography, computed tomography, and magnetic resonance imaging. In addition, quantitative as well as qualitative measurements for the detection of fatty liver have become available. In this review, we summarize the currently used qualitative evaluations of fatty liver and discuss quantitative evaluations that are expected to further develop in the future. **(Clin Mol Hepatol 2023;29(Suppl):S123-S135)**

Keywords: Non-alcoholic fatty liver disease; Liver steatosis; Biomarker; Ultrasonography

INTRODUCTION

Metabolic syndrome has been attracting attention owing to increasing obesity, diabetes, hypertension, and lipid metabolism abnormalities resulting from the westernization of diet. The prevalence of metabolic syndrome is estimated to be 25% worldwide,[1] with similarly high and increasing rates reported from Japan[2] and South Korea[3] in Asia. Fatty liver is known to be a frequent complication of metabolic syndrome. Fatty liver is collectively called non-alcoholic fatty liver disease) (NAFL[D]), in which patients drink no or little alcohol (less than 30 g/day ethanol equivalent in men and less than 20 g/day in) but have a fatty liver.

The term fatty liver was first described by Thomas Addison in the 1830s in Guy's Hospital Reports in the UK. In 1980, Ludwig proposed non-alcoholic steatohepatitis (NASH) as a condition in which a person does not drink alcohol but presents with a histology similar to an alcoholic.[4] In 1986, Schaffner first used the term NAFLD to describe the concept of fatty liver disease.[5] Subsequently, Matteoni et al.[6] published the di-

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agnostic criteria for NASH, based on the assumption that the findings correlating with prognosis among pathological findings of NAFLD are the characteristic findings of NASH.

NAFL often has a relatively benign course, but NASH comprises a group of advanced diseases that can lead to cirrhosis and hepatocarcinoma.[7] NASH accounts for approximately 10–20% of all NAFLD cases, and is pathologically distinguished by the presence of ballooning of hepatocytes and lobular inflammation as well as fat accumulation in more than 5% of the hepatocytes.[8] Moreover, NASH and NAFL are cross connectional conditions.

Although liver biopsy is considered the gold standard for the diagnosis of fatty liver, especially in NASH, it is not practical to perform liver biopsy in all patients due to its invasiveness, potential for sampling errors, and dependency on the pathologist.[9] As Kim[10] summarized, several studies have emerged showing the use of non-invasive biomarkers to reduce the invasiveness of liver biopsy. Recently, the diagnosis of NAFLD, especially liver steatosis, has been improved by magnetic resonance imaging-proton density fat fraction (MRI-PDFF)[11] and ultrasound-controlled attenuation parameter (CAP),[12] which are increasingly recognized as possible alternatives to liver biopsy.

The recommended treatment for NAFLD is weight loss and lifestyle and exercise modifications.[13] There is still no drug that fundamentally treats NAFLD. However, there are several reports of diabetes medications being effective.[14]

DEFINITION OF FATTY LIVER DISEASE AND ITS PROGNOSTIC FACTORS

Fatty liver disease is a general term for diseases that cause liver damage due to the deposition of triglycerides in hepatocytes. NAFLD is defined based on a pure ethanol equivalent intake of less than 20 g/day in women and less than 30 g/day in men. Pathologically, liver steatosis was conventionally defined as the presence of liver fat content in more than 30% of the hepatocytes; but currently, NAFLD is defined as liver fat content in more than 5% of the hepatocytes.[7,15-17]

Initially, the progression from NAFL to NASH was considered a prognostic factor of NAFLD.[18] However, it has been reported that liver fibrosis is the most important prognostic factor in NAFLD, independent of the degree of liver steatosis, intralobular inflammation, and ballooning degeneration of hepatocytes, which are the findings in NASH.[19-22] It was also found that liver fibrosis progresses both in NAFL and NASH, although at different rates.[23] Therefore, the importance of assessing the degree of fibrosis, rather than diagnosing NAFL or NASH or evaluating liver steatosis, for the diagnosis of NAFLD is now recognized.[23]

Since there were no comprehensive reports on the prognostic significance of NAFLD regarding the degree of liver steatosis and intralobular inflammation, simple fatty liver (NAFLD without fibrosis) development in the liver was regarded as a benign disease before 2021. Therefore, the progressive accumulation of steatosis in the liver was not recognized to have morbid implications. However, in 2021, a large Swedish cohort study showed that simple fatty liver disease, compared to the general population without fatty liver disease, was associated with a 1.9, 1.1, 7, 16.8, and 1.3 times higher risk of mortality from extrahepatic cancer, cardiovascular diseases, cirrhosis, hepatocellular carcinoma, and other causes, respectively,[24] which emphasizes the importance of appropriate evaluations of liver steatosis.

Qualitative evaluations of liver steatosis have been mainly performed by abdominal sonography, computed tomography (CT) scans, and magnetic resonance imaging (MRI), but with the advent of methods such as the CAP method by FibroScan® (Echosens, Paris, France) and MRI-PDFF, it is now possible to quantify liver steatosis.

The evolution of the disease concept and evaluation methods for NAFLD/NASH are summarized in Figure 1.

Abbreviations:

AASLD, American Association for the study of Liver; AI, artificial intelligence; AUROC, area under receiver operating characteristic curve; ATI, attenuation imaging; CAP, controlled attenuation parameter; CLD, chronic liver disease; CT, computed tomography; EASL, European Association for the study of the liver; KASL, Korean Associatoin for the Study of the liver, MAFLD, metabolic associated fatty liver disease; MRI, magnetic resonance imaging; MRI-PDFF, magnetic resonance imaging-proton density fat fraction; NAFL, non-alcoholic fatty liver; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; UGAP, ultrasound-guided attenuation parameter

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ABDOMEN ULTRASONOGRAPHY (QUALITATIVE ASSESSMENT)

Abdominal ultrasonography is simple and useful for the diagnosis of fatty liver. B-mode abdominal echo findings of fatty liver include bright liver,[25] hepatorenal echo contrast,[26] hepatosplenic echo contrast, vascular blurring and attenuation,[27] all of which are used in daily clinical practice.

B-mode findings have been reported to have good sensitivity and specificity when more than 30% of the hepatocytes have intrahepatic steatosis.[28-30] However, sensitivity and specificity are reduced when intrahepatic steatosis is less than 30%,[31,32] and no studies have found that B-mode findings can diagnose less than 5% liver steatosis.

Ultrasound is a popular and useful technique for detecting fatty liver. However, ultrasonography does not provide quantitative results, and it is unsuitable for determining increases or decreases in liver steatosis and the effectiveness of treatment. In addition, it cannot detect liver steatosis under 30%, its use varies largely among surgeons; and although it is useful in diagnosing fatty liver, false-positive or -negative cases may occur.[33] At the time when abdominal ultrasound was difficult to quantify fat, a scoring system was developed to predict whether a non-drinker had NAFLD, which had a high diagnostic performance with an area under receiver operating characteristic curve (AUROC) of 0.98 based on histological evaluation.[34]

ABDOMEN ULTRASONOGRAPHY (QUANTITATIVE ASSESSMENT)

The amplitude of ultrasound is attenuated exponentially as it propagates through the body. This attenuation can be broadly classified into scattering and absorption, but most of the transmitted waves on the beam are due to absorption. The attenuation constant, which represents the magnitude of attenuation, can be expressed as $\alpha=a\text{-}f^{n}$ (dB/cm) as a function of frequency f in case of living tissue (the value of n is almost always 1 in soft tissue). Instead of α, attenuation can be expressed as a proportionality constant a (dB/MHz/cm). This value varies depending on the tissue and lesion type. The fact that fatty liver exhibits more attenuation than normal liver has enabled the application of quantitative ultrasonography for liver steatosis.

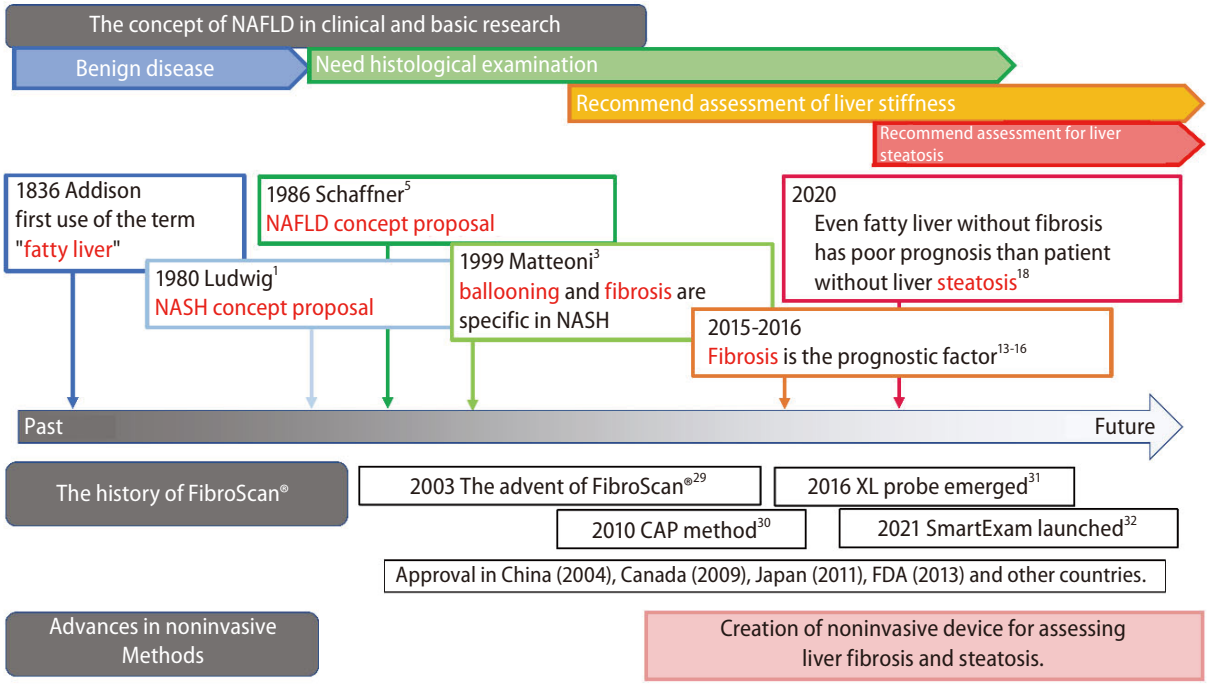

Figure 1. Landmark studies and advances of non-invasive methods in the assessment of NAFLD. NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; CAP, controlled attenuation parameter; FDA, food and drug administration.

CONTROLLED ATTENUATION PARAMETER (CAP)

FibroScan® (EchoSens, Paris, France), the pioneering instrument in vibration controlled transient elastography, was introduced in 2003.[35] Initially, it could only measure liver stiffness, but in 2010, CAP was introduced to measure the degree of fat attenuation.[36] This was the first time that a device was able to quantify liver steatosis. Although the CAP method was considered non-invasive, rapid, inexpensive, and reproducible, it was less suitable for obese patients, in whom acquiring ultrasound signals was difficult with the available M probe. However, with the introduction of the XL probe for obese patients,[37] shear waves are now able to penetrate deeper and generate signals in obese patients as well. The XL probe was also equipped with CAP, making it more useful for measurements in obese patients.[38]

In 2021, EchoSens launched the new computation method SmartExam allowing for deeper measurements and an increased number of CAP measurements, which is expected to further improve the accuracy of CAP measurements in obese patients.[39] Owing to its recency, there are few reports on this method, but further studies are in progress. Recently, we presented the first clinical report on the SmartExam-equipped FibroScan.[40] In our study, we compared the SmartExam-equipped FibroScan and the conventional FibroScan with the results obtained with magnetic resonance imaging (MRE)/MRI-PDFF, and reported that both are capable of comparable evaluation. We also concluded that the SmartExam-equipped FibroScan significantly reduced CAP variability, but tended to take slightly longer to obtain measurements compared to the conventional FibroScan. One limitation of this paper was the small number of obese patients, and further studies in a population with a large number of obese patients was recommended.

A meta-analysis of the diagnostic performance of CAP based on histological evaluation by liver biopsy in NAFLD showed high AUROCs of 0.924, 0,784, and 0.778 for S ≥1, 2, and 3, respectively.[41] The usefulness of CAP is emphasized in various NAFLD guidelines, including the American Association for the study of Liver (AASLD),[8] European Association for the study of the liver (EASL),[42] the Korean Association for the Study of the liver (KASL),[43] and Japanese guidelines.[16,17]

The advantage of CAP is that fatty liver quantification can be performed easily, quickly, and inexpensively with high diagnostic performance. However, the disadvantage is that the measurement results are affected by the distance to the liver surface making it necessary to change the probe to M or XL depending on advanced obesity and body size.[38] Different probes have different transmission frequencies; thus, resulting values cannot be simply compared. In addition, CAP measurements cannot be performed in cases of ascites or effusion, but some newer techniques have overcome such drawbacks.

Furthermore, it has been reported that liver stiffness measurements using FibroScan® are useful in assessing liver fibrosis in long-term follow-up.[44,45] However, it has not been reported whether the measurement of liver steatosis is also useful in long-term follow-up, and we hope that such studies are conducted in the future.

OTHER UPCOMING ULTRASOUND-BASED QUANTITATIVE EVALUATION METHODS

Since the advent of CAP, devices that measure attenuation coefficients simultaneously with B-mode images on conventional abdominal ultrasound systems have been developed and put into practical use, including UGAP (GE Healthcare, Wauwatosa, WI, USA), ATI (Canon Medical Systems, Tochigi, Japan), Attenuation Imaging (Fujifilm Healthcare, Tokyo, Japan),[46] ultrasound-derived fat fraction (UDFF) (Siemens Healthineers, Erlangen, Germany),[47-49] attenuation estimation algorithm (Hologic, Bedford, MA, USA), tissue-attenuation imaging (Samsung Medison, Seoul, Korea), and Philips attenuation (Philips Medical Systems, Amsterdam, The Netherlands).

ATTENUATION IMAGING (ATI)

ATI can also measure liver fat content without changing the probe. The principle of ATI is that it can avoid multiple reflections from a close range, which has been a disadvantage in diagnosis. It also eliminates the focal point dependence of the transmitted sound field characteristics, deep attenuation, and large vessels, which are dependent on the probe and affect the measured value, and it can automatically calculate and quantitatively evaluate the attenuation due to the properties of biological tissue in any part of the body. In addition,

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it is possible to automatically calculate and quantitatively evaluate the attenuation rate caused by the characteristics of the biological tissue in any part of the body. ATI has been reported to have as high diagnostic performance as MRI-PDFF in terms of liver fat quantification compared to MRI-PDFF.[50-61] It is reported that ATI has good correlation with CAP (r=0.65, *P*<0.0001) and the AUROC for detecting S >0 steatosis and S >1 steatosis was 0.91 and 0.88, respectively.[52] Tada et al.[50] also reported that ATI-induced attenuation coefficient values are not affected by liver stiffness.

As for ATI, it has only been studied on a small scale and is expected to be studied on a larger scale in the future. The advantage of ATI is that it has a high diagnostic performance and, unlike CAP, can be measured in the presence of ascites. It is also advantageous that the same machine can perform measurements while observing in B-mode. On the other hand, ATI is less commonly reported and less widely used than CAP.

ULTRASOUND-GUIDED ATTENUATION PARAMETER (UGAP)

UGAP is a fat quantification method based on measuring the attenuation coefficient (dB/cm/MHz) of the ultrasound signal in the common B mode. It was first reported in 2018 by Fujiwara et al.[62], and was shown to be comparable in terms of AUROC to CAP and MRI-PDFF, the latter being considered an alternative to liver biopsy for the evaluation of liver steatosis with comparable diagnostic performance, as shown in a multicenter study.[63] In this study, the AUROCs of UGAP for distinguishing steatosis grade ≥1 (MRI-PDFF ≥5.2%), ≥2 (MRI-PDFF ≥11.3%), and 3 (MRI-PDFF ≥17.1%) were 0.910 (95% confidence interval [95% CI], 0.891–0.928), 0.912 (95% CI, 0.894–0.929), and 0.894 (95% CI, 0.873–0.916), respectively, showing an excellent diagnostic accuracy for grading steatosis with reference to MRI-PDFF. The advantages and disadvantages of UGAP are similar to those of ATI. There have been a few reports, but further evaluations are expected.

Several new ultrasound techniques for measuring liver steatosis from various companies, including improved version of the attenuation coefficient (iATT) and UDFF, have been introduced, but they are still lacking evidence.

Table 1 summarizes the modalities and standard references for liver steatosis reported to date, and Table 2 summarizes the AUROCs of non-invasive imaging modalities.

STEATOSIS QUANTIFICATION AND QUALIFICATION USING CT

A comparison of CT values of the liver and spleen (liver/spleen ratio: L/S ratio)[64,65] is useful for the early detection of fatty liver. When the CT values of the liver are lower than those of the spleen due to increased fat accumulation in the liver, a fatty liver can be diagnosed. However, CT scans are costly and time-consuming; thus, a rapid and more readily available means of assessing NAFLD in routine clinical care is needed.[66] Unlike ultrasound and MRI, CT is now used less frequently due to exposure issues, its low quantitative nature, and its relatively poor performance in detecting mild steatosis and quantifying steatosis.[67-69]

Table 1. Standard reference and US techniques in the analysis of liver steatosis

US techniques	Company, Country	Liver biopsy	MRI-PDFF	CAP
Controlled attenuation parameter (CAP)	Echosens, Paris, France	○	○	-
Attenuation imaging (ATI)	Canon Medical Systems, Tochigi, Japan	○	○	○
Attenuation measurement (ATT)	Fujifilm Health Care, Tokyo, Japan	○	×	○
US-guided attenuation parameter (UGAP)	General Electric, Schenectady, NY, USA	○	○	○
US-derived fat fraction (UDFF)	Siemens Healthineers, Erlangen, Germany	×	○	×
Attenuation estimation	Hologic, Bedford, MA, USA	×	×	×
Tissue-attenuation imaging (TAI)	Samsung Medison, Seoul, Korea	×	×	×
Attenuation imaging	Philips Medical Systems, Amsterdam, the Netherlands	×	×	×

US, ultrasound; MRI-PDFF, magnetic resonance imaging-proton density fat fraction.

Table 2. AUCs of non-invasive imaging modalities

Study	Study population	Patient numbers	Imaging modality	Golden standard	AUROC		
					S≥1	S≥2	S=3
Nogami et al.[40]	CLD	167	CAP	MRI-PDFF	0.90 (0.83–0.94)	0.85 (0.78–0.90)	0.85 (0.89–0.90)
			CAPc		0.85 (0.77–0.90)	0.84 (0.76–0.89)	0.83 (0.75–0.89)
	NAFLD	97	CAP		0.83	0.77	0.78
			CAPc		0.84	0.77	0.78
Tada et al.[50]	nonBnonC	119	ATI	MRI-PDFF	0,81 (0.73–0.89)	0.87 (0.79–0.96)	0.94 (0.89–0.98)
Hsu et al.[51]	CLD	28	ATI	Liver biopsy	0.97 (0.83–1.00)	0.99 (0.86–1.00)	0.97 (0.82–1.00)
Ferraiolo et al.[52]	Consecutive adult subjects potentially at risk of steatosis and healthy controls	129	ATI	MRI-PDFF	0.91 (0.84–0.95)	0.95 (0.89–0.98)	
			CAP	MRI-PDFF	0.85 (0.77–0.91)	0.88 (0.81–0.93)	
Jeon et al.[53]	CLD	87	ATI	MRI-PDFF	0.76 (0.66–0.85)	0.88 (0.79–0.94)	
Bae et al.[54]	CLD	108	ATI	Liver biopsy	0.843 (0.761–0.906)	0.876 (0.799–0.931)	0.886 (0.811–0.949)
Dioguardi Burgio et al.[55]	CLD	101	ATI	Liver biopsy	0.805 (0.811–0.88)	0.892 (0.81–0.94)	
Lee et al.[56]	NAFLD suspected	108	ATI	Liver biopsy	0.93 (0.86–0.97)	0.88 (0.80–0.93)	0.83 (0.73–0.89)
Sugimoto et al.[57]	NAFLD suspected	120	ATI	Liver biopsy	0.88 (0.80–0.97)	0.86 (0.79–0.93)	0.79 (0.68–0.89)
Ferraioli et al.[58]	Patients with steatosis	72	ATI-Pen	MRI-PDFF	0.90 (0.81–0.96)		
			ATI-Gen		0.92 (0.82–0.96)		
			CAP		0.85 (0.74–0.92)		

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Table 2. Continued

Study	Study population	Patient numbers	Imaging modality	Golden standard	AUROC		
					S≥1	S≥2	S=3
Tada et al.[59]	Patients with steatosis	148	ATI	Liver biopsy	0.85 (0.72–0.88)	0.91 (0.84–0.97)	0.91 (0.82–0.99)
	Obese	41	ATI		0.72 (0.54–0.90)	0.72 (0.55–0.90)	0.78 (0.55–1.00)
	NAFLD	38	ATI		0.77 (0.61–0.94)	0.88 (0.77–0.99)	0.86 (0.69–1.00)
Kwon et al.[60]	Liver disease	100	ATI	MRI-PDFF	0.914 (0.858–0.969)	0.935 (0.886–0.985)	
Fujiwara et al.[62]	CLD	163	UGAP	Liver biopsy	0.900 (0.834–0.967)	0.953 (0.894–0.993)	0.959 (0.920–0.999)
			CAP		0.829 (0.743–0.914)	0.841 (0.728–0.953)	0.817 (0.703–0.932)
Imajo et al.[63]	CLD	1,010	UGAP	MRI-PDFF	0.910 (0.891–0.928)	0.912 (0.894–0.929)	0.894 (0.873–0.916)

Values are presented in 95% confidence interval.

AUROC, area under receiver operating characteristic curve; CLD, chronic liver disease; NAFLD, non-alcoholic fatty liver disease; nonBnonC, non hepatitis B non hepatitis C; CAP, controlled attenuation parameter; CAPc, continuous Controlled Attenuation Parameter; ATI, attenuation imaging; ATI-Pen, attenuation imaging–penetration; UGAP, ultrasound-guided attenuation parameter; MRI-PDFF, magnetic resonance imaging-proton density fat fraction.

Dual energy CT is a quantitative imaging method that uses two different X-ray tube voltages to estimate the composition of an imaging target using a material decomposition method that utilizes material-specific X-ray absorption characteristics.

Since the 1990s, reports on liver fat evaluation using dual energy CT have been published.[70,71] Using MRI-PDFF >6% as a reference diagnosis of fatty obesity, the diagnostic performance of fatty liver using dual energy CT was reported with an AUROC of 0.834. Optimal thresholds were 54.8 hounsfield unit (HU) (right) and 52.5 HU (left), with sensitivities/specificities of 57%/93.9% (right) and 67.9%/90% (left). For the hepatosplenic weight loss difference, the AUROCs were 0.808 (right) and 0.767 (left), with optimal sensitivities/specificities of 93.3%/57.1% (right) and 78.6%/68% (left).[72]

It has been suggested that positron emission tomography-computed tomography may be used in the future. Liver steatosis in NAFLD patients is independently associated with elevated liver enzymes, increased visceral adipose tissue volume, and decreased myocardial fluorodeoxyglucose-positron emission (FDG) uptake, but not with hepatic FDG uptake.[73] These properties could allow the clinical use of positron emission tomography—computed tomography for liver fat mass quantification in the future.

STEATOSIS QUANTIFICATION USING MRI

MRI signals are obtained from protons belonging to water and fat molecules, making it a good method for quantifying fat in the liver.

Proton magnetic resonance spectroscopy has been shown to be a safe and non-invasive method of quantifying liver fat content that correlates well with liver biopsy,[74-78] and can detect fat depositions as little as 2%.[79] However, it has not been widely adopted in general clinical practice, partly, due to specific software requirements.[80]

Subsequently, MRI-PDFF was introduced, which is a technique that allows the assessment of the amount of fat in the entire liver or in arbitrary regions of interest, even in small amounts.[81,82] Recently, studies have used MRI-PDFF instead of liver biopsy as a reference standard.[50-60,63,78-83] It has been reported that MRI-PDFF measurements correlate strongly with histological liver fattening.[84,85] In a comparison of pathological findings, the AUROC had an extremely high diagnostic accuracy of 0.99 for predicting hepatic steatosis by MRI-PDFF, which was much higher than that of CAP (AUROC 0.85).[86]

The AASLD,[8] KASL,[43] and Japanese guidelines[16,17] also emphasize the usefulness of MRI-PDFF. In addition to quantifying liver steatosis in clinical practice, recent clinical trials on NAFLD have examined histological evaluation, MRI-PDFF, and CAP reduction rates to investigate whether liver steatosis improves before and after investigational drug treatment.[87] Ac-

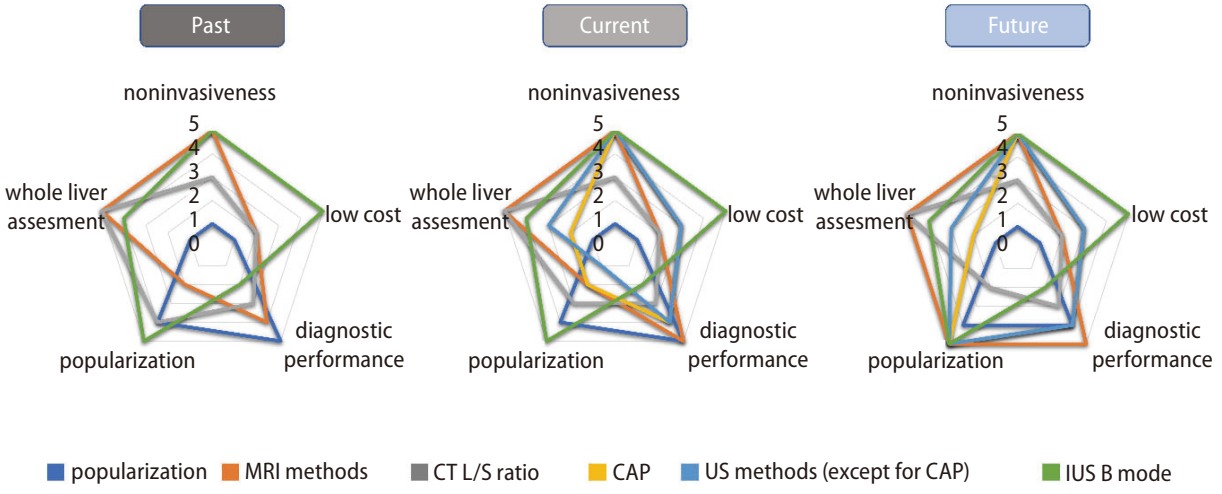

Figure 2. Characteristics of examinations to evaluate liver steatosis in the past, present, and future. MRI, magnetic resonance imaging; CT, computed tomography; CAP, controlled attenuation parameter; US, ultrasound; L/S ratio, liver-to-spleen ratio.

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cording to a recent review on quantitative liver steatosis assessment, MRI-PDFF should be used as a non-invasive reference standard in diagnostic studies.[46]

APPLICATION OF ARTIFICIAL INTELLIGENCE IN THE MEASUREMENT OF LIVER STEATOSIS

In recent years, artificial intelligence (AI) has been utilized in many fields. AI software tries to reproduce human logical thinking on a computer. With the development of deep learning technology, AI can autonomously learn and construct decision criteria from given data. The fields of pathology and imaging evaluation have a high affinity to AI which has enabled remarkable technological developments for clinical applications.

The advantages of AI are that it continuously provides stable results as it does not suffer from the exhaustion that occurs in humans, and that it prevents inter- and intra-observer variability. It has been reported that AI technology minimizes inter-observer variability in histological assessments.[88,89] Among other things, AI technology has the potential for the objective assessment of ballooning, which is a hallmark in the evaluation of NAFLD steatosis.[90]

Reports have also been published on AI-assisted ultrasound and MRI, which are expected to be useful in clinical practice. A meta-analysis on liver steatosis using AI technology was published by Decharatanachart et al.[91] They summarized 19 previous studies that assessed fibrosis and steatosis of the liver using AI-based ultrasound, elastography, CT, MRI, and clinical parameters. According to the pooled data, the sensitivity, specificity, positive predictive value, negative predictive value, and diagnostic odds ratio (DOR) for the diagnosis of liver steatosis were 0.97 (0.76–1.00), 0.91 (0.78–0.97), 0.95 (0.87–0.98), 0.93 (0.80–0.98), and 191.52 (38.82–944.81), respectively. AI technology is expected to be used in clinical practice in the future.

New concept, metabolic associated fatty liver disease (MAFLD)

It is known that fatty liver can occur whether one drinks alcohol or not; and since it is often complicated by lifestyle-related diseases, it has been proposed that fatty liver should be considered a MAFLD going forward, and not NAFLD.[92,93]

CONCLUSIONS

Fat content in NAFLD is nowadays evaluated quantitatively as well as qualitatively. Although histological evaluation remains the gold standard for liver steatosis measurement, it is likely to be replaced by MRI-PDFF in the future. Once additional evidence on the usefulness of fat determination by ultrasound using novel technology becomes available, liver fat content could potentially be measured easier than ever before in general clinical practice. Several methods have emerged to quantify liver steatosis, but each test has its own advantages and disadvantages in terms of diagnostic performance, cost, and invasiveness (Fig. 2).

Various liver steatosis measurement techniques are now available. However, the coherence between these techniques remains unclear. Further evidence and additional clinical studies are required.

Authors' contribution

AN wrote the manuscript and prepared the figures and tables. MY and AN revised the manuscript. All the authors read and approved the final version.

Conflicts of Interest

The authors have no conflicts to disclose.

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Review

Noninvasive imaging biomarkers for liver fibrosis in nonalcoholic fatty liver disease: current and future

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Nonalcoholic fatty liver disease (NAFLD) is increasingly prevalent worldwide and becoming a major cause of liver disease-related morbidity and mortality. The presence of liver fibrosis in patients with NAFLD is closely related to prognosis, including the development of hepatocellular carcinoma and other complications of cirrhosis. Therefore, assessment of the presence of significant or advanced liver fibrosis is crucial. Although liver biopsy has been considered the "gold standard" method for evaluating the degree of liver fibrosis, it is not suitable for extensive use in all patients with NAFLD owing to its invasiveness and high cost. Therefore, noninvasive biochemical and imaging biomarkers have been developed to overcome the limitations of liver biopsy. Imaging biomarkers for the stratification of liver fibrosis have been evaluated in patients with NAFLD using different imaging techniques, such as transient elastography, shear wave elastography, and magnetic resonance elastography. Furthermore, artificial intelligence and deep learning methods are increasingly being applied to improve the diagnostic accuracy of imaging techniques and overcome the pitfalls of existing imaging biomarkers. In this review, we describe the usefulness and future prospects of noninvasive imaging biomarkers that have been studied and used to evaluate the degree of liver fibrosis in patients with NAFLD. **(Clin Mol Hepatol 2023;29(Suppl):S136-S149)**

Keywords: Diagnostic imaging; Biomarkers; Liver fibrosis; Nonalcoholic fatty liver disease

INTRODUCTION

The prevalence of nonalcoholic fatty liver disease (NAFLD) is increasing worldwide, with approximately 25% of the global population being affected by this condition.[1] Accordingly, the burden on the global healthcare system posed by the treatment of NAFLD is increasing and becoming a serious public health problem.[2,3] NAFLD comprises a spectrum of liver disorders ranging from isolated steatosis to nonalcoholic steatohepatitis (NASH), which can lead to serious conditions such as cirrhosis, hepatocellular carcinoma (HCC), and liver-related death.[4,5] In particular, the progression of liver fibrosis in patients with NAFLD is considered one of the most important factors determining prognosis, with significant and ad-

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vanced liver fibrosis being an independent risk factor for both hepatic and extrahepatic complications and liver-related and overall mortality.[6,7] Therefore, accurate assessment of the degree of liver fibrosis in patients with NAFLD is the main issue to be addressed in modern medicine.

Although liver biopsy is the gold standard method for evaluating liver fibrosis in patients with NAFLD, its general clinical use is limited due to the high cost and potential complications.[8] Moreover, liver biopsy has a disadvantage in that it can sample only a limited portion (1/50,000) of the entire liver. Therefore, many noninvasive tests (NITs) have been developed to overcome the limitations of liver biopsy, and their use in clinical practice is gradually increasing.[9] Noninvasive imaging biomarkers can be broadly divided into ultrasound-based tests, such as vibration-controlled transient elastography (VCTE) and shear wave elastography (SWE) or acoustic radiation force impulse imaging (ARFI), and magnetic resonance imaging (MRI)-based tests, such as magnetic resonance elastography (MRE) (Fig. 1).[10] As each test has its strengths and limitations, understanding the characteristics of each test is essential to selecting the optimal modality for assessing the degree of liver fibrosis in patients with NAFLD.

As research on noninvasive imaging biomarkers continues, more efficient test equipment is expected to be developed and utilized in the future. In particular, methods that utilize artificial intelligence (AI), which have recently been in the spotlight, are expected to increase the accuracy and maximize the efficiency of existing inspection equipment.[11] Recent studies on the use of AI or deep learning methods in evaluating the degree of liver fibrosis showed promising results.[12,13]

This review describes the application and advantages of noninvasive imaging biomarkers that have been studied and used to evaluate liver fibrosis in patients with NAFLD, as well as the future prospects of such biomarkers.

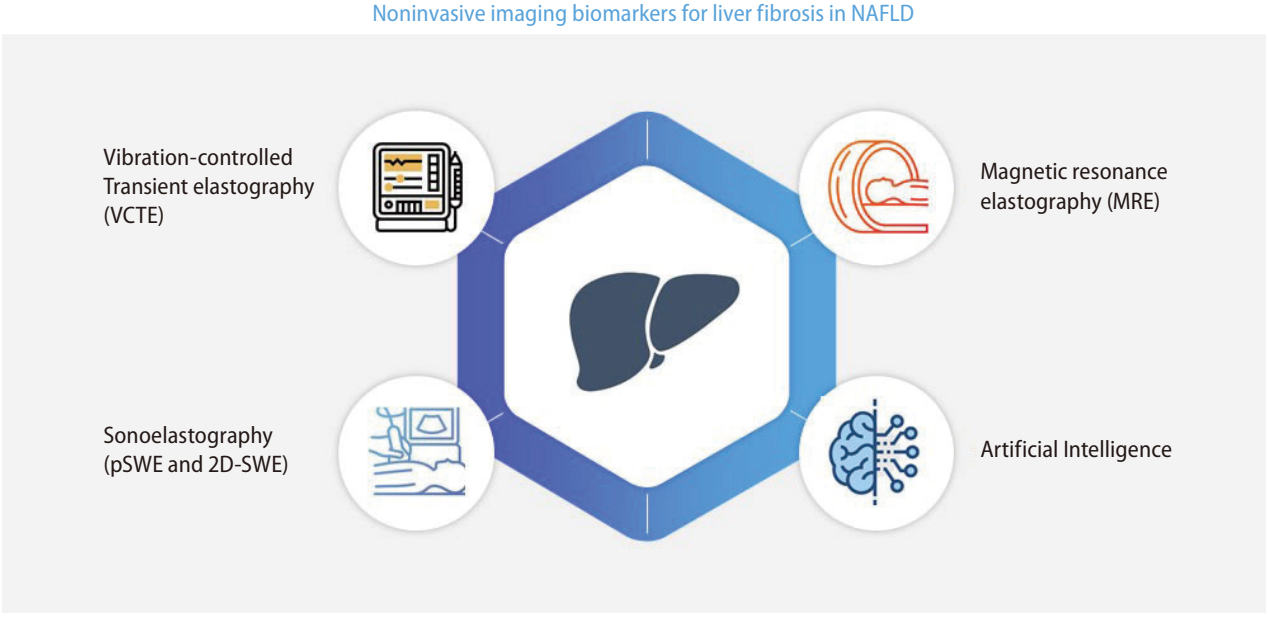

Figure 1. Currently used noninvasive imaging biomarkers in NAFLD. NAFLD, nonalcoholic fatty liver disease; SWE, shear wave elastography.

Abbreviations:

NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; HCC, hepatocellular carcinoma; NIT, noninvasive test; VCTE, vibration-controlled transient elastography; IQR, interquartile range; SWE, shear wave elastography; ARFI, acoustic radiation force impulse imaging; MRE, magnetic resonance elastography; AI, artificial intelligence; pSWE, point shear wave elastography; 2D-SWE, two-dimensional shear wave elastography; BMI, body mass index; LS, liver stiffness; kPa, kilopascals; AUROC, area under the receiver operating characteristic curve; ROI, region of interest; PPV, positive predictive value; HR, hazard ratio; FIB-4 index, fibrosis-4 index; CNN, convolutional neural networks; 3D, three-dimensional; MRI, magnetic resonance imaging

ELASTOGRAPHY

Elastography techniques are used to evaluate the stage of fibrosis by quantifying the shear wave velocity or tissue displacement generated by an ultrasonic or physical impulse, which represents liver stiffness (LS).[14] VCTE and MRE systems have mechanical drivers that generate shear waves and assess shear wave velocities using sonographic Doppler and magnetic resonance techniques, respectively.[15] High-frequency sonographic impulses generate shear waves in point SWE (pSWE), ARFI, and two-dimensional SWE (2D-SWE). Because different elastography techniques are based on different methods and use different frequencies, their values are not identical, and caution is required when interpreting the results. Therefore, the strengths and limitations of each modality must be considered (Table 1).

ULTRASOUND-BASED ELASTOGRAPHY

Vibration-controlled transient elastography

Technique

Transient elastography (FibroScan®; EchoSens, Paris, France) is an ultrasound-based elastography technique that is now a well-established noninvasive method for diagnosing and staging liver fibrosis in patients with NAFLD.[16] VCTE consists of a 3.5-MHz ultrasound transducer installed on the axis of a low-amplitude vibrator and utilizes monodimensional ultrasound to determine LS by measuring the velocity of low-frequency elastic shear waves propagating through the liver.[17] For a VCTE result to be reliable, a minimum of 10 valid measurements are required, and the ratio of the median valid LS measurement to the interquartile range (IQR) should be ≤0.3.[18]

Strengths and limitations

A transient elastography test can be completed in a relatively short time (generally within 5 minutes), and many studies have validated the reliability of this test in assessing liver fibrosis in patients with NAFLD.[19] Transient elastography also has excellent intraobserver and interobserver variability.[20] However, transient elastography has the following limitations: the optimal cutoff point is unclear; measurements may be impossible in patients with obesity; the scan results

Table 1. Strengths and limitations of noninvasive imaging tests for liver fibrosis in NAFLD

Methods	Validation	Reliability	Reliability criteria	Indeterminate cases	Failure rate	Factors related to failure	Invalid result rate	Confounders
TE	Fibrosis stage, liver-related outcomes	0.99	Yes	30–40%	3–14%	Obesity (less with XL probe), ascites	1–9%	Acute hepatitis, cholestasis, food ingestion, obesity, congestion
pSWE	Fibrosis stage	0.98	Yes	NA	0–1%	Obesity	16–24%	Acute hepatitis, food ingestion, obesity,
2D-SWE	Fibrosis stage	0.98–1.0	No	NA	1–13%	Obesity	0%	Acute hepatitis, food ingestion
MRE	Fibrosis stage, liver-related outcomes	0.99	Yes	NA	<5%	Massive ascites, poor contact between the passive driver and the abdominal wall, inconsistent breath holding and motion, claustrophobia, inability to fit in the MRI machine	Negligible	Iron overload, acute hepatitis, massive ascites

NAFLD, nonalcoholic fatty liver disease; TE, transient elastography; pSWE, point shear wave elastography; 2D-SWE, two-dimensional shear wave elastography; MRE, magnetic resonance elastography; NA, not available; MRI, magnetic resonance imaging.

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may be unreliable in the hands of inexperienced operators; and the diagnostic accuracy is limited in the early stages of fibrosis.[21]

Clinical applications

Detection and staging of liver fibrosis

Several recent studies have investigated the ideal cutoff value in VCTE to confirm significant liver fibrosis in patients with NAFLD.[22-27] In those studies, the average body mass index (BMI) of patients with NAFLD was 27.1–34.8 kg/m^2, and the BMI of patients in Asian studies was relatively lower than that in Western studies. The LS value measured by VCTE indicating the presence of significant liver fibrosis (F2) in patients with NAFLD ranged from 7.7 to 9.8 kilopascals (kPa), and the proportion of patients with significant liver fibrosis ranged from 30.9% to 70.8% of the study population. In addition, the LS value indicating the presence of advanced liver fibrosis or cirrhosis (F3 or higher) ranged from 7.3 to 12.5 kPa, which showed an acceptable area under the receiver operating characteristic curve (AUROC) values (0.80–0.92) (Table 2).

Prediction of liver-related outcomes

Recent studies have shown that baseline LS values measured by VCTE accurately predict the occurrence of liver decompensation, and higher baseline LS values can predict the development of liver-related events in patients with NAFLD.[28,29] In a multicenter cohort study that analyzed liver-related outcomes based on LS values measured by VCTE, baseline LS values were independently associated with the occurrence of hepatic decompensation (hazard ratio [HR]=1.03), HCC (HR=1.03), and liver-related death (HR=1.02).[29] In addition, an increase of >20% in the LS value during a mean follow-up period of 35 months was strongly associated with the risk of liver-related events and death, thus showing that LS values measured by VCTE are useful in predicting liver-related outcomes.[29] However, owing to the limitations inherent in retrospective studies, the study did not follow a standardized protocol for VCTE follow-up and could not accurately identify the use of alcohol and other drugs. Therefore, future prospective and validation studies are needed to clarify the association between LS values measured by VCTE and liver-related outcomes (Fig. 2).

Table 2. Performance of transient elastography in patients with NAFLD

Study	Number	Country	Mean BMI (kg/m^2)	Patients with advanced fibrosis (F≥2), n (%)	AUROC for F≥2 (95% CI)	Cut off for F≥2	Sensitivity/ specificity	AUROC for F≥3 (95% CI)	Cut off for F≥3	Sensitivity/ specificity
Cassinotto et al.[23] (2016)	291	France	32.1	206, 70.8%	0.82	9.8	0.60/0.90	0.86	12.5	0.57/0.90
Lee et al.[25] (2017)	94	Korea	27.1	46, 47.9%	0.76	7.4	0.62/0.92	0.87	8	0.82/0.85
Park et al.[24] (2017)	94	United States	30.4	29, 30.9%	0.86	6.9	0.79/0.85	0.80	7.3	0.78/0.78
Furlan et al.[26] (2020)	62	United States	34.8	44, 70.1%	0.77	8.8	0.51/0.94	0.86	10.5	0.50/0.92
Eddowes et al.[22] (2019)	450	United Kingdom	33.8	225, 60%	0.77	8.2	0.71/0.70	0.80	9.7	0.71/0.75
Imajo et al.[27] (2022)	201	Japan	27.1	-	0.89	8.4	0.86/0.74	0.92	9.7	0.84/0.85

NAFLD, nonalcoholic fatty liver disease; AUROC, area under the receiver operating characteristics curve; CI, confidence interval; BMI, body mass index.

Point shear wave elastography/acoustic radiation force impulse imaging

Technique

pSWE and ARFI are ultrasound-based elastography methods that enable the quantitative assessment of tissue stiffness. LS measurement with pSWE and ARFI is performed in the right lobe of the liver through the intercostal space. After selecting a region of interest (ROI), the shear wave velocity is measured within the defined region using ultrasound tracking beams laterally adjacent to a single push beam.[30] For the results of pSWE and ARFI to be reliable, the IQR/liver spasticity should be <30%.[31-33]

Strengths and limitations

Similar to VCTE, several meta-analysis studies have confirmed that pSWE and ARFI have good diagnostic accuracy for significant liver fibrosis, with a mean AUROC of 0.84–0.87, and excellent diagnostic accuracy for cirrhosis, with a mean AUROC of 0.91–0.94.[31,33] In addition, pSWE and ARFI have good intraobserver and interobserver agreement, with an intraclass correlation coefficient of between 0.84 and 0.87.[34,35] In addition, unlike VCTE, the accuracy of pSWE and ARFI is generally not limited by obesity or interfering structures such as blood vessels or the biliary tract, as the ROI can be manually positioned.[30] However, the disadvantages of pSWE and ARFI are that the size of the ROI is smaller than that in VCTE and the quality criteria are less evaluated.

Clinical applications

Detection and staging of liver fibrosis

Several studies have demonstrated the clinical application of pSWE and ARFI through noninvasive imaging biomarkers and the results showed that pSWE and ARFI are suitable diagnostic tools with higher diagnostic accuracy for advanced liver fibrosis (F3–4) than low-grade fibrosis (F1–2).[36,37] However, studies on pSWE and ARFI have been mainly monocentric retrospective studies; therefore, longitudinal validation in chronic liver diseases, especially NAFLD, is required to develop standardized quality criteria.

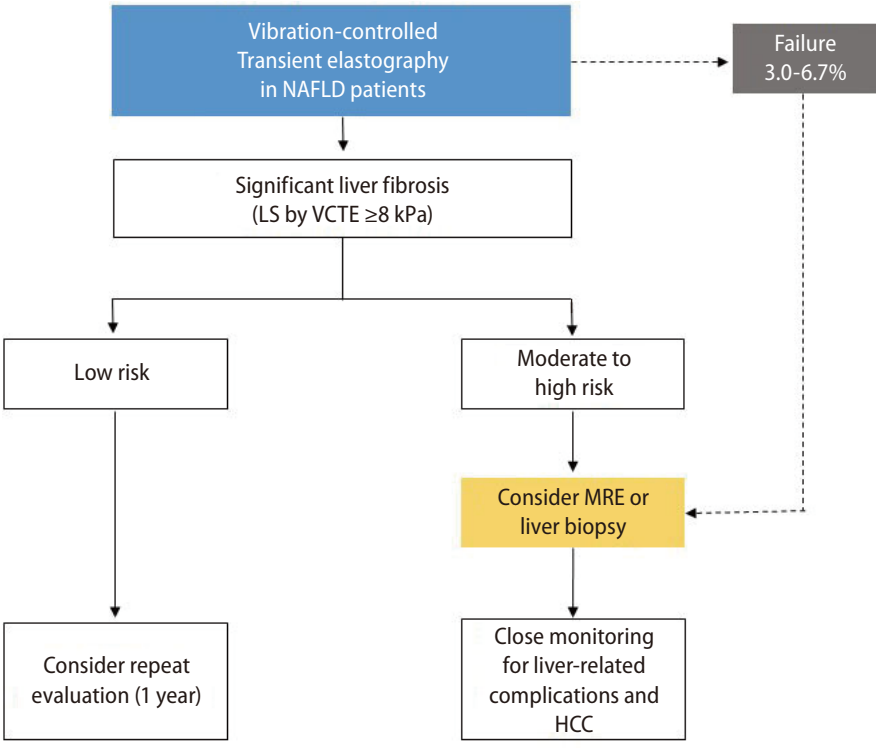

Figure 2. Algorithm for risk discrimination in patients with NAFLD using noninvasive imaging biomarkers. NAFLD, nonalcoholic fatty liver disease; LS, liver stiffness; VCTE, vibration-controlled transient elastography; MRE, magnetic resonance elastography; HCC, hepatocellular carcinoma.

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Two-dimensional shear wave elastography

Technique

Real-time 2D-SWE is performed rather similarly to pSWE and ARFI. It combines the initiation of a radiation force in tissues using focused ultrasonic beams and the acquisition of transiently propagating resultant shear waves in real-time with a high-frequency ultrasound imaging sequence.[38] In 2D-SWE, a two-dimensional parametric color map is generated by combining several shear waves over time with rapid ultrasound acquisition. Similar to pSWE and ARFI, 2D-SWE allows the operator to select the size and location of the ROI. When the operator "samples" a specific area within a color map, the shear-wave velocity is measured to obtain a quantitative measure of tissue elasticity using proprietary software (Aixplorer®; Supersonic Imaging, Aix en Provence, France).[39]

Strengths and limitations

The advantage of 2D-SWE is that it allows the operator to select the size and location of the ROI, thereby permitting the evaluation of the elasticity profile of a larger tissue section in a single acquisition.[40] In addition, 2D-SWE has the following advantages over pSWE and ARFI: qualitative (color-coded) and quantitative measurement, easier and more manageable measurement, and stability of the measured value.[41,42] However, 2D-SWE has some limitations, including the subjective nature of the color scale, potential bias when selecting the ROI, and a lack of meta-analysis confirming its clinical applications.

Clinical applications

Detection and staging of liver fibrosis

Several recent studies have confirmed that LS measured by 2D-SWE strongly correlates with the stage of liver fibrosis on liver biopsy in patients with NAFLD.[43] According to a meta-analysis conducted in Europe, 2D-SWE has good diagnostic performance for significant liver fibrosis (≥F2, AUROC=0.86) and excellent diagnostic performance for severe fibrosis (≥F3, AUROC=0.93) and cirrhosis (F4, AUROC=0.92). The optimal cutoff values for diagnosing significant liver fibrosis and cirrhosis were reported to be 7.1 and 13.0 kPa, respectively. In addition, the AUROC for the diagnosis of significant liver fibrosis (P=0.001) and cirrhosis (P=0.022) with 2D-SWE was higher than that with VCTE.[44] However, as studies on the clinical application of 2D-SWE and comparative studies with other noninvasive methods are lacking, follow-up studies are needed.

MAGNETIC RESONANCE IMAGING-BASED ELASTOGRAPHY

Technique

Liver MRE can be performed using existing magnetic resonance scanners. The setup includes an active pneumatic mechanical driver located outside the scanning room and a connected passive driver placed on the liver.[45] The active driver generates continuous acoustic vibrations that are transmitted to the passive driver and subsequently to the abdomen, including the liver. These waves produce microscopic shear displacement of tissues, which is visualized using MRE sequences as propagating shear waves.[46] Subsequently, a magnitude image revealing the anatomy of the upper abdomen and a phase-contrast image showing shear waves at the same level are reconstructed, and grayscale and colored stiffness maps, also known as elastograms, are produced.

Thereafter, readers draw the ROI within the confidence map of the liver, avoiding the liver edge, artifacts, fissures, fossa, and regions of wave interference.[45] The mean LS value is calculated using ROIs on four slices. The LS value measured by MRE is expressed in kPa, representing both the elasticity and viscosity of the tissue.

Strengths and limitations

MRE can examine the entire liver, and technical failure occurs in <5% of the examinations.[47-49] MRE measurements are highly reproducible, with robust intraobserver and interobserver agreements.[50-53] The LS value measured by MRE is not significantly affected by hepatic steatosis, and MRE can measure LS in patients with obesity.[54-57] In addition, hepatic inflammation does not affect the accuracy of MRE in patients with NAFLD.[55]

The most common cause of technical failure in MRI is iron overload.[55] Poor transmission of shear waves into the liver because of massive ascites increased subcutaneous fat thickness, and poor contact between the passive driver and the abdominal wall also led to a measurement failure. Inconsistent breath-holding and motion during the sequence are

common causes of technical failure in patients with massive ascites.[45] The heterogeneity of fibrosis progression in different liver lesions may lead to inaccurate LS measurements, particularly in small ROIs.[58] MRE cannot differentiate LS caused by congestion from that caused by increased vascular pressure; thus, the LS value measured by MRE should be carefully interpreted.[59] Differences in MRI specifications and vendors among institutions and studies are another concern in the interpretation of LS values measured by MRE. Finally, considering its cost and limited availability, MRE cannot be generally used in clinical practice at present.

Clinical applications

Detection and staging of liver fibrosis

Multiple studies have demonstrated that MRE has excellent accuracy in diagnosing and stratifying liver fibrosis in patients with NAFLD, predicting significant or advanced liver fibrosis and cirrhosis with consistent AUROC values of >0.90 (Table 3).[60-63] A recent meta-analysis showed the excellent accuracy of MRE, with an AUROC of 0.96 for advanced liver fibrosis and 0.92 for cirrhosis and LS cutoff values of 3.62–4.8 and 4.15–6.7 kPa, respectively.[58] A meta-analysis of nine studies that included 232 patients with NAFLD suggested reliable LS cutoff values of 2.88, 3.54, 3.77, and 4.09 kPa for detecting fibrosis stages 1, 2, 3, and 4, respectively.[55]

In a recent meta-analysis with individual data of 230 patients with biopsy-proven NAFLD, MRE outperformed VCTE in detecting all stages of fibrosis (AUROC for fibrosis stage ≥1, 0.87 vs. 0.82 [*P*=0.04]; stage ≥2, 0.92 vs. 0.87 [*P*=0.03]; stage ≥3, 0.93 vs. 0.84 [*P*=0.001]; and stage ≥4, 0.94 vs. 0.84 [*P*=0.005]).[64] Comparative studies between MRE and pSWE are limited; however, one study demonstrated that MRE was more accurate than pSWE in diagnosing any fibrosis stage in patients with NAFLD, especially in those with obesity.[56] A recent study demonstrated that MRE was more accurate than 2D-SWE in diagnosing stage ≥1 and ≥2 fibrosis but not stages ≥3 or 4 fibrosis.[27] Other MRI techniques, including diffusion-weighted imaging or contrast-enhanced MRI, were also reported to be less accurate than MRE in assessing liver fibrosis.[65,66] Consequently, the LS value measured by MRE can be considered the most accurate noninvasive imaging biomarker for detecting all stages of fibrosis (Table 4).

Recently, noninvasive LS-based models combining two different biomarkers have shown promising results in identify-

Table 3. Performance of magnetic resonance elastography in assessing liver fibrosis in patients with NAFLD

Study	Number	Country	Mean BMI (kg/m^2)	Patients with advanced fibrosis (F≥2) (%)	AUROC for F≥2 (95% CI)	Cut off for F≥2 (kPa)	Sensitivity/ specificity	AUROC for F≥3 (95% CI)	Cut off for F≥3 (kPa)	Sensitivity/ specificity
Imajo et al.[27] (2022)	201	Japan	27.1	-	0.927	3.19	0.90/0.81	0.929	3.90	0.83/0.92
Cui et al.[56] (2016)	125	United States	31.8	26.4	0.885	3.62	0.67/0.96	0.934	3.62	0.91/0.93
Loomba et al.[61] (2014)	117	United States	32.4	29.9	0.856	3.58	0.66/0.92	0.894	4.67	0.8/0.94
Costa-Silva et al.[62] (2018)	90	Brazil	32.2	24.4	0.932	4.14	0.92/0.97	0.928	4.39	0.91/0.97
Loomba et al.[63] (2016)	100	United States	32.1	27.0	0.878	3.65	-	0.921	3.80	0.87/0.94

NAFLD, nonalcoholic fatty liver disease; AUROC, area under the receiver operating characteristics curve; CI, confidence interval; BMI, body mass index.

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ing patients with significant liver fibrosis, with increased positive predictive value (PPV), thereby reducing screening failure rates in clinical trials and reducing unnecessary liver biopsies.[67-69] In previous studies, MEFIB (MRE plus fibrosis-4 [FIB-4]) had a significantly higher diagnostic accuracy than MRE alone and the FIB-4 index alone.[67] Notably, a recent study compared MEFIB, MAST (MRI–aspartate aminotransferase), and FAST (FibroScan–aspartate aminotransferase) in detecting stage ≥2 fibrosis among patients with NAFLD and demonstrated the superiority of MEFIB (PPV, 95%; negative predictive value, 90%) over MAST and FAST (both $P<0.001$).[69]

Prediction of liver-related outcomes

Multiple retrospective studies have suggested that MRE can play a role in predicting the long-term prognosis of patients with NAFLD.[70-72] A recent meta-analysis of six cohorts, including 1,707 patients with a median follow-up of 3 years, investigated the association between the LS value measured by MRE and liver-related outcomes.[67] The HR for liver-related outcomes in patients with an LS value of 5–8 kPa was 11.0 ($P<0.001$) and that in patients with an LS value of ≥8 kPa was 15.9 ($P<0.001$), compared with those with an LS value of <5 kPa. Furthermore, the MEFIB index was developed using the identified best cutoff values for LS and the FIB-4 index (defined as positive when the LS value measured by MRE was ≥3.3 kPa and the FIB-4 index was ≥1.6). A positive MEFIB index had a robust association with liver-related outcomes (HR=20.6; $P<0.001$), and a negative MEFIB had a high negative predictive value for liver-related outcomes (99.1% at 5 years).

However, few retrospective studies have described the association of MRE with the clinical outcomes of patients with NAFLD. Therefore, future multicenter prospective studies are required to clarify the association between LS measured by MRE and liver-related clinical outcomes.

Emerging magnetic resonance imaging-based techniques

Advances in MRE techniques, including automated liver elasticity calculations and improvements in shear-wave delivery, are promising to provide a faster and more reliable evaluation of the liver. Three-dimensional (3D)-MRE is a newly developed imaging technique that assesses shear-wave propagation in multiple planes to avoid mathematical assumptions.[63] For the 3D-MRE examination, a separate motion-sensitized, multislice, spin-echo echo-planar imaging sequence is performed to assess shear-wave displacements along the x-, y-, and z-directions.

Although 3D-MRE is more accurate than 2D-MRE in predicting advanced liver fibrosis in patients with NAFLD, further validation is required to prove the benefits of this technique.[63] Multiparametric MRI measures shear stiffness, loss

Table 4. Diagnostic accuracy of noninvasive imaging biomarkers for each stage of fibrosis in NAFLD

Method	TE	pSWE	2D-SWE	MRE
Stage ≥2 fibrosis				
AUROC	0.77	0.87	0.86	0.92
Sensitivity (%)	71.0	79.0	94.0	84.9
Specificity (%)	70.0	85.0	52.0	85.4
PPV (%)	78.0	91.0	65.1	79.8
NPV (%)	61.0	66.0	86.7	89.3
Stage ≥3 fibrosis				
AUROC	0.80	0.91	0.93	0.93
Sensitivity (%)	71.0	92.0	93.0	82.5
Specificity (%)	75.0	86.0	81.0	83.2
PPV (%)	63.0	82.0	77.0	61.8
NPV (%)	81.0	89.0	97.4	93.5

NAFLD, nonalcoholic fatty liver disease; TE, transient elastography; pSWE, point shear-wave elastography; 2D-SWE, two-dimensional shear-wave elastography; MRE, magnetic resonance elastography; AUROC, area under the receiver operating characteristic curve; PPV, positive predictive value; NPV, negative predictive value.

modulus, and MRI-derived fat fraction in a single scan. 3D-MRE incorporates a damping ratio at a lower frequency, which may further help in the detection of NASH and NASH-related fibrosis.[73]

ARTIFICIAL INTELLIGENCE

Recently, AI and deep learning methods have been incorporated into MRE and shown encouraging results. AI can make quantitative assessments objective, reproducible, and less ambiguous. Traditional (supervised) machine learning and deep learning algorithms use approaches that are dependent on predefined information or ROIs determined by experts.[11]

Deep learning does not rely on predefined features and does not always require a focus on ROIs. Convolutional neural networks (CNNs) are the most commonly applied deep learning methods in imaging analysis. In a retrospective study, LS measurements using an automated CNN-based method strongly agreed with manual ROI-based analysis across MRE systems (intraclass correlation coefficient, 0.98–0.99) and showed excellent discriminative performance for histology-determined stages of liver fibrosis (AUROC=0.89–0.93) in patients with NAFLD.[74] Considering the high incidence of NAFLD, CNN-based analysis may reduce reliance on expert image analysts.

Radiomic texture analysis is an evolving translational tool used to extract imaging information, which is prone to subjective and variable interpretation. A recent study applied texture analysis–derived parameters combined with machine learning to MRI-based techniques for the quantification of liver fibrosis.[12] Texture analysis and machine learning techniques were tested on T1- and T2-weighted MRI and MRE images of 62 participants with histologic evidence of chronic liver disease. The diagnostic accuracy for advanced liver fibrosis in T1-weighted MRI and MRE images was excellent (AUROC=0.82 vs. 0.92, P=0.41); however, T2-weighted MRI had a lower accuracy (AUROC=0.57).

Integrating AI into conventional noninvasive tools can provide an optimal balance between sensitivity and specificity in assessing liver fibrosis. Thus far, few studies have investigated the application of AI in the assessment of imaging biomarkers in NAFLD; however, studies evaluating liver fibrosis in patients with NAFLD are expected to gradually increase in the future.

ROLE OF NONINVASIVE TESTS IN DISEASE MONITORING

Repeated measurements using NITs can stratify the risk of liver-related events in patients with NAFLD. Currently, limited data are available on the impact of dynamic changes in LS values measured using NITs on the long-term outcomes of patients with NAFLD.

VCTE is useful for monitoring the severity of liver fibrosis not only in patients with NAFLD but also in patients with NASH-related cirrhosis, and LS can be a useful biomarker for predicting varices, HCC, and liver-related death.[75] According to a multinational study conducted in Europe in 790 patients with NAFLD-related compensated cirrhosis, the LS value measured by VCTE can effectively identify varices requiring treatment and reduce unnecessary endoscopies.[76] In addition, some studies have indicated that VCTE can be used to monitor fibrosis changes after treatment, although this should be confirmed by further studies using paired liver biopsies.[77,78]

In a prospective cohort study, 102 patients with biopsy-proven NAFLD underwent contemporaneous MRE and liver biopsy at baseline, followed by repeat paired liver biopsy and MRE assessment.[79] A 15% increase in the LS value measured by MRE was associated with histologic fibrosis progression and progression from early to advanced liver fibrosis. A retrospective study of 128 patients with NAFLD who underwent at least two serial MRE examinations showed a significantly higher risk of the development of cirrhosis and decompensation or death in patients with a ≥19% increase in LS value from baseline than in those without.[80]

Further studies are warranted to assess the implication of changes in LS measured using NITs over time on the risk of future liver-related events and mortality. Furthermore, although evidence is lacking and the optimal time interval remains to be determined, repeating NITs every 3 years in patients with early-stage NAFLD and every year in patients with advanced-stage disease seems reasonable.

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CONCLUSION

Currently, the main utility of noninvasive imaging biomarkers in NAFLD is discriminating patients with significant or advanced liver fibrosis from those with mild or no fibrosis for prognosis prediction and clinical decision-making. VCTE is the most widely validated test; pSWE and 2D-SWE have comparable performance to VCTE; and MRE is currently considered the most accurate noninvasive tool for the detection and staging of liver fibrosis. However, the clinical use of these tests is usually determined by the availability of the technology and the local expertise at each institution.

A major limitation of NITs is their suboptimal accuracy in diagnosing fibrosis in the early stages and in adequately discriminating between adjacent fibrosis stages. Differentiating other processes that cause increased LS values, such as inflammation, biliary obstruction, cholestasis, passive congestion, and increased portal venous pressure, from liver fibrosis is another challenge. Research on noninvasive imaging biomarkers in NAFLD, especially concerning their use in screening and risk prediction, will continue as the prevalence of the disease increases and as newer treatment methods emerge. Finally, noninvasive imaging biomarkers, liver biopsies, and clinical parameters must be used in combination for the accurate assessment of the fibrosis stage and risk stratification in patients with NAFLD.

Authors' contribution

Conception and design of the study: J.H. Yu, H.A. Lee, and S.U. Kim; Drafting or revision of the manuscript: J.H. Yu, H.A. Lee, and S.U. Kim; Approval of the final version of the manuscript: J.H. Yu, H.A. Lee, and S.U. Kim.

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Conflicts of Interest

Seung Up Kim served as an advisory committee member for Gilead Sciences, GSK, Bayer, and Eisai. He is a speaker for Gilead Sciences, GSK, Bayer, Eisai, Abbive, EchoSens, MSD, and Bristol-Myers Squibb. He also received a research grant from Abbive and Bristol-Myers Squibb. The other authors declare that they have no conflicts of interest.

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Review

Noninvasive serum biomarkers for liver steatosis in nonalcoholic fatty liver disease: Current and future developments

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Nonalcoholic fatty liver disease (NAFLD) affects approximately 30% of the population worldwide and includes nonalcoholic fatty liver, nonalcoholic steatohepatitis (NASH), and cirrhosis. Since NAFLD-associated diseases begin with steatosis, the early diagnosis of steatosis helps to prevent the progression of NASH and fibrosis. In addition, more convenient and easily diagnosable serum biomarkers are becoming crucial in disease diagnosis. In this report, we summarize the known serum biomarkers for liver steatosis and provide guidance for their application in clinical practice.
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Keywords: Biomarker; Liver steatosis; Nonalcoholic fatty liver disease

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is defined as the presence of >5% hepatic steatosis without evidence of liver injury.[1,2] However, the pathophysiology of NAFLD is complex and multifactorial. The most widely known mechanism is accumulated oxidative stress from insulin resistance, and others include an unhealthy diet, lifestyle, genetic factors, and the individual's microbiome. NAFLDs, regardless of their causative factors, are due to hepatic fat deposition, also known as steatosis; detecting hepatic steatosis is the first step in diagnosing NAFLD.

Liver biopsy, the gold standard for diagnosing NAFLD, is invasive, difficult to interpret, and expensive.[3] Moreover, only a limited range (1/50,000) of the entire liver can be assessed in this manner. Due to the limitations of liver biopsy, other noninvasive methods are being implemented. Ultrasonography is commonly used due to its low cost and wide availability. Recently, with the development of imaging technology, the diagnostic rate of fatty liver by ultrasound has increased to 83.4%.[4] Moreover, the accuracy of ultrasound has been improved by using the differences in the scatter and attenuation of ultrasound waves according to tissue type.[5] Further, the controlled attenuation parameter has the advantage of good feasibility for detecting steatosis and is widely used for steatosis evaluation. However, it cannot reliably differentiate between steatosis grades. Other techniques (such as computed tomography) carry risks associated with radiation exposure, and magnetic resonance imaging is not routinely used due to its cost.

Research into the noninvasive evaluation of hepatic steatosis is ongoing.[6] It is predicted that more than half of the pop-

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ulation will be diagnosed with fatty liver in the future, making it critical to find a simple and easy-to-use serum test.[7] Therefore, noninvasive tests have been developed to overcome these limitations, and their use is gradually increasing in clinical practice. This article aims to discuss the existing methods available for classifying steatosis using serum biomarkers.

NAFLD BIOMARKERS

Currently, the most commonly used serum markers are aminotransferase and γ-glutamyl transferase (GGT). Alanine aminotransferase (ALT) has long been used as a marker of liver fat accumulation; in 1986, Nanji et al. first reported the association between liver enzymes (i.e., the ALT-to-aspartate aminotransferase [AST] ratio) and fatty liver in obese patients.[8] As a marker of steatosis, the sensitivity and specificity of ALT are limited[9]; however, there is usually an absence of elevation in aminotransferase levels in steatosis-only conditions.[10,11] Moreover, patients with advanced liver disease show decreased aminotransferase levels.[12,13] GGT is often elevated in NAFLD patients and may be associated with advanced fibrosis and increased mortality rates.[14] However, GGT levels alone cannot identify the degree of steatosis.

The SteatoTest

The SteatoTest was developed using a combination of the six components of the FibroTest-ActiTest plus the body mass index (BMI), serum cholesterol, triglycerides (TG), and glucose after adjusting for age and sex.[15] It is known to have moderate accuracy in diagnosing liver steatosis (the area under the curve of the receiver operating characteristic [AUROC]: 0.79–0.80; sensitivity: 80–100%; specificity: 83–100%). The patients were classified according to hepatitis C treatment and alcoholic liver disease, and analyzed by dividing them into a training group and three validation groups. For the diagnosis of Grade 2–4 steatosis, the sensitivity values of the SteatoTest at the 0.30 cut-off value were 0.91, 0.98, 1.00, and 0.85, while the specificity data at the 0.70 cut-off were 0.89, 0.83, 0.92, and 1.00, respectively.

The SteatoTest has better predictive power than ALT and GGT serum markers: a meta-analysis has shown an AUROC of 0.80 for diagnosing steatosis >33%.[16] The disadvantage of this biomarker is that it is difficult to use in clinical practice and is expensive; it is also unable to discriminate between different levels of steatosis, and it cannot be used if the FibroTest-ActiTest is not available. (Table 1).

The fatty liver index (FLI)

The FLI utilizes four components: BMI, waist circumference, serum TG, and serum GGT. Based on abdominal ultrasonography studies, the FLI is moderately accurate (AUROC: 0.84; sensitivity: 87%; specificity: 64%).[17] An FLI <30 (negative likelihood ratio=0.2) rules out and an FLI ≥60 (positive likelihood ratio=4.3) confirms fatty liver. Another study has suggested that the FLI is associated with insulin resistance and all-cause, liver-related, and cancer mortality.[18]

The FLI uses information that can be easily obtained in clinical practice and is moderately accurate; however, ultrasonography, not liver biopsy, was used as a reference standard.

The hepatic steatosis index (HSI)

The HSI involves four components: the AST/ALT ratio, BMI, sex, and the presence of diabetes mellitus.[19] At values of <30.0 or >36.0, the HSI rules out NAFLD with a sensitivity of 93.1% or detects NAFLD with a specificity of 92.4%, respectively. The HSI was shown to have an AUROC of 0.81 in a large cohort study (n=10,724) of Korean patients. However, ultrasonography was used as a reference standard, and validation studies in other populations are required.

The nonalcoholic fatty liver disease liver fat score

The NAFLD liver fat score involves five components: the presence of metabolic syndrome or type 2 diabetes mellitus,

Abbreviations:

ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUC, area under the curve; AUROC, area under the curve of the receiver operating characteristic; BMI, body mass index; FLI, fatty liver index; GGT, γ-glutamyl transferase; HSI, hepatic steatosis index; LAP, lipid accumulation product; NAFL, nonalcoholic fatty liver; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; TG, triglyceride

Table 1. Serum biomarker testing for hepatic steatosis

Test	Parameter	Number of subject at the time of development	AUROC	Cut-off	Sensitivity	Specificity	Application	Reproducibility	Reference methods	Limitations
SteatoTest	ALT, A2M, ApoA1, haptoglobin, total bilirubin, GGT, total cholesterol, TG, glucose, age, gender and BMI	1,206	0.80	≥0.3 ≥0.7	90% 46%	54% 88%	SteatoTest <0.3 can exclude grade 2–4 steatosis; SteatoTest >0.72 is suggestive of grade 2–4 steatosis.	Reproducible	Liver Biopsy	Limited availability due to FibroTest-ActiTest, high cost
FLI	BMI, TG, WC, and GGT	496	0.84	<30 ≥60	87% 61%	64% 86%	A simple panel to detect fatty liver; FLI <30 rule out fatty liver, and >60 rule in fatty liver.	Reproducible	US	Suboptimal gold standard based on ultrasonography
HSI	AST/ALT, BMI, and diabetes	10,724	0.81	<30 >36	93% 46%	40% 92%	A simple panel to detect fatty liver; HSI <30 rule out fatty liver, and >36 rule in fatty liver.	Reproducible	US	Suboptimal gold standard based on ultrasonography
NAFLD liver fat score	MS, diabetes, insulin, AST/ALT	470	0.86	<-0.640 >-1.413	84% 95%	69% 56%	A simple tool to predict NAFLD	Reproducible	MRS	Limited availability due to insulin level is needed
NAFLD ridge score	ALT, HDL-C, TG, HbA1c, WBC, and hypertension presence	922	0.87	Dual cut-offs of 0.24 and 0.44	91%	90%	An accurate novel score with machine learning approach to predict NAFLD; NAFLD ridge scores <0.24 rule out NAFLD, and scores >0.44 rule in NAFLD.	Reproducible	MRS	No validation study
K-NAFLD score	Sex, WC, SBP, TG	3,634	0.929	0.884	-	-	An easy scoring system to identify NAFLD; K-NAFLD <-3.285 rule out NAFLD, and >0.884 rule in NAFLD.	Reproducible	NAFLD liver fat score	NAFLD is defined by NAFLD liver fat score. No validation study
NAFL screening score	Age, BMI, fasting plasma glucose, uric acid, TG, and AST to ALT ratio	46,493	0.825 (Male) 0.861 (Female)	33 29	80 89	66 69	A simple score to detect NAFL.	Reproducible	US	Suboptimal gold standard based on ultrasonography. No validation study

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Table 1. Continued

Test	Parameter	Number of subject at the time of development	AUROC	Cut-off	Sensitivity	Specificity	Application	Reproducibility	Reference methods	Limitations
NAFL risk score	BMI, TG multiplied by GGT, ratio of AST and ALT, LDL-C and HDL-C, uric acid	8,226	0.743 (Male) 0.820 (Female)	-	-	-	A simple score to predict 4-year risk of NAFL. Low-risk score group for male (0–6.5), for female (0–12.5). High-risk score group for male (7–18), for female (13–18).	Reproducible	US	Suboptimal gold standard based on ultrasonography. No validation study
LAP score	WC, TG and gender	588	0.79 0.68	>30 >40	93% 86%	34% 50%	Identify patients with hepatic steatosis clinically but could not predict liver fat content.	Reproducible	US	Suboptimal gold standard based on ultrasonography. No validation study
ION	Male: waist-to-hip ratio, TG, ALT and HOMA, Female: TG, ALT and HOMA	4,458	0.77	<11 ≥22	81% 60%	56% 82%	The ION model was superior compared with the FLI model. But, validation is needed.	Reproducible	US, liver biopsy	Suboptimal gold standard based on ultrasonography. No validation study

A2M, α2-macroglobulin; ALT, alanine aminotransferase; ApoA1, apolipoprotein A1; GGT, γ-glutamyltransferase; TG, triglyceride; BMI, body mass index; AUROC, area under the receiver-operating characteristics curve; AST, aspartate aminotransferase; FLI, fatty liver index; WC, waist circumference; US, ultrasonography; NAFLD, non-alcoholic fatty liver disease; HSI, hepatic steatosis index; MS, metabolic syndrome; MRS, magnetic resonance spectroscopy; HDL-C, high-density lipoprotein cholesterol; HbA1c, hemoglobin A1C; WBC, white blood cell; SBP, systolic blood pressure; NAFL, non-alcoholic fatty liver; LDL-C, low-density lipoprotein cholesterol; LAP, lipid accumulation product; ION, index of nonalcoholic steatohepatitis; HOMA, homeostatic model assessment for insulin resistance.

the fasting serum insulin, the serum AST, and the AST/ALT ratio.[20] A study based on magnetic resonance spectroscopy has shown high accuracy (AUROC, 0.86–0.87; sensitivity, 86%; specificity, 71% [cut-off point of -0.640]). NAFLD liver fat scoring was validated using magnetic resonance spectroscopy as a reference standard and showed relatively good diagnostic performance. The downside of this biomarker is that it requires fasting serum insulin test results, which are not yet standard.

The nonalcoholic fatty liver disease ridge score

The NAFLD ridge score is a machine learning-based method that utilizes seven components: serum ALT, high-density lipoprotein cholesterol, TG, hemoglobin A1c, leukocyte count, comorbidity data, and the presence of hypertension. NAFLD ridge scoring uses proton magnetic resonance spectroscopy as a reference standard. By using dual cut-offs of 0.24 and 0.44, the NAFLD ridge score achieved 92% (86–96%) sensitivity and 90% (86–93%) specificity. This method showed good accuracy levels (AUROC: 0.87; sensitivity: 92%; specificity: 90%) and excellent negative predictive values (96% to exclude NALFD).[21] The downside of this method is that there are no subsequent validation studies.

The K-nonalcoholic fatty liver disease score

This scoring system was created based on a sample of 3,634 patients and includes four components: sex, waist circumference, systolic blood pressure, and serum TG. A cut-off value for NAFLD was set at 0.884.[22] K-NAFLD scores <-3.285 and >0.884 were set as the cut-off values for no NAFLD and NAFLD. The K-NAFLD scoring method is based on data from a large cohort of patients, and it showed the most accurate (AUROC=0.929) predictive power compared to other biomarkers (FLI [AUROC=0.870]; LAP [AUROC=0.841]; and body mass index, age, alanine aminotransferase, and TG [BAAT] [AUROC=0.782]). However, the scoring system was created without using a liver biopsy or imaging study as a reference standard and therefore requires validation using other populations.

The nonalcoholic fatty liver screening score

The nonalcoholic fatty liver screening score (NSS) was based on a large cohort study of >40,000 people that utilized a total of six components: age, fasting plasma glucose, urinalysis, the ALT/AST ratio, BMI, and TG. A total score >29 correlates to a high risk for NAFL. For males, at the cut-off point of 33, the NSS had a sensitivity of 79.86% and a specificity of 66.13%. For females, at a value of 29, the sensitivity and specificity values of the NAFL screening score were 89.39% and 68.98%, respectively. This scoring system showed a higher accuracy than other NAFL models (male AUROC: 0.825 [0.806–0.843], and female AUROC: 0.861 [0.820–0.896], compared with the HSI: 0.791 [0.770–0.810] and the FLI: 0.805 [0.785–0.82]).[23]

The NAFL screening score was created based on a large cohort of patients and was particularly accurate for men, demonstrating higher AUROC values than other steatosis markers. However, ultrasonography was used as a reference standard, and validation studies have not yet been conducted.

The nonalcoholic fatty liver risk score

This scoring system was developed to predict the future four-year risk of NAFLD. The outcome is a score between 0 and 18 points that is based on five measurements: BMI, TG×GGT, ALT/AST ratio, low-density lipoprotein/high-density lipoprotein cholesterol ratio, and uric acid levels.

The advantage of this marker is that the NAFL risk score was relatively discriminative (AUROC=0.739 for males and 0.823 for females).[24] However, ultrasonography was used as a reference standard, and validation studies were not conducted.

The lipid accumulation product score

The LAP score uses three variables (waist circumference, TG, and sex) with moderate accuracy (AUROC: 0.79) to diagnose >30% of steatosis.[25] The degree of steatosis can be evaluated using this method, which has been validated as moderately accurate (AUROC: 0.79) in diagnosing >5% of steatosis.[26] Again, ultrasonography was used as a reference standard, and validation studies in other ethnic groups are warranted.

The index of nonalcoholic steatohepatitis (ION)

The ION Model was created using the data from 4,458

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NAFLD patients from the National Health and Nutrition Examination Survey III and 152 patients with biopsy-proven NAFLD.[27] This model uses different variables that are calculated using the sex/waist-to-hip ratio, TG, ALT, and the Homeostatic Model Assessment for Insulin Resistance (HOMA) in males and the TG, ALT, and HOMA in females. The ION had an AUROC of 0.77, a sensitivity of 81% for ruling out steatosis at a cut-off <11, and a specificity of 82% for ruling in steatosis at a cut-off >22. The ION model was superior in predicting NASH and mortality compared with the FLI model; however, ultrasonography was used as a reference standard.

DISCUSSION AND CONCLUSIONS

We investigated the biomarkers currently used in evaluating hepatic steatosis. Serum markers have several limitations in evaluating steatosis alone and are thus commonly combined with other markers including sex, age, BMI, and waist circumference.

Limitations exist when making direct comparisons between the methods mentioned above. First, the models were compared to different standards when assessing accuracy, such as liver biopsy, ultrasonography, and magnetic resonance spectroscopy. The FLI, NAFLD liver fat score, and HSI were obtained from the same cohort of patients and were hence directly comparable; however, the AUROC values between the methods were similar (0.83, 0.80, and 0.81, respectively). A previous study externally validated the involved hepatic steatosis formulas. In this study, the NAFLD liver fat score showed the best diagnostic performance and similar diagnostic agreement with ultrasonography.[28]

Novel serum markers to evaluate steatosis are being developed; however, a reliable method has not been widely validated, and further research is required. Since the long-term prognosis of NAFLD is more likely to be associated with fibrosis than steatosis, the focus on steatosis could be lessened. It is crucial to make an early diagnosis of steatosis to prevent the progression of NASH and fibrosis; this can be challenging since NAFLD and NASH are usually asymptomatic until patients reach the advanced stages. The high applicability, reproducibility, and widespread availability of serum biomarkers gives them an advantage over other methods. There is a demand for easy and precise methods, not only for diagnostic purposes but also to evaluate treatment outcomes. The lack of noninvasive methods to evaluate steatosis in both the clinical and research fields hinders the enrollment of new patient study objects. Thus, the development of a noninvasive steatosis marker is warranted for future pharmaceutical research and development.

While liver steatosis can be an effective measure of liver disease, this condition can also diminish during the progression of NAFLD to liver cirrhosis, which is known as the "burnout" effect. For patients with later-stage NAFLD, assessing the severity of NASH and fibrosis could be more critical than steatosis. Thus, it is crucial to identify high-risk groups that are likely to develop liver fibrosis to ensure that these groups are followed up regularly. Recognizing the limitations of serum markers is important; integrating imaging studies and patient information during the diagnosis process results in better outcomes. Furthermore, circulating biomarkers, such as microRNA and cell-free nuclear material DNA/RNA, and "omics" studies are yet to be developed for commercialization but may be critical in future clinical and research practices.[29]

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Review

Noninvasive serum biomarkers for liver fibrosis in NAFLD: current and future

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In the last 20 years, noninvasive serum biomarkers to identify liver fibrosis in patients with non-alcoholic fatty liver disease (NAFLD) have been developed, validated against liver biopsy (the gold standard for determining the presence of liver fibrosis) and made available for clinicians to use to identify ≥F3 liver fibrosis. The aim of this review is firstly to focus on the current use of widely available biomarkers and their performance for identifying ≥F3. Secondly, we discuss whether noninvasive biomarkers have a role in identifying F2, a stage of fibrosis that is now known to be a risk factor for cirrhosis and overall mortality. We also consider whether machine learning algorithms offer a better alternative for identifying individuals with ≥F2 fibrosis. Thirdly, we summarise the utility of noninvasive serum biomarkers for predicting liver related outcomes (e.g., ascites and hepatocellular carcinoma) and non-liver related outcomes (e.g., cardiovascular-related mortality and extra hepatic cancers). Finally, we examine whether serial measurement of biomarkers can be used to monitor liver disease, and whether the use of noninvasive biomarkers in drug trials for non-alcoholic steatohepatitis can accurately, compared to liver histology, monitor liver fibrosis progression/regression. We conclude by offering our perspective on the future of serum biomarkers for the detection and monitoring of liver fibrosis in NAFLD. **(Clin Mol Hepatol 2023;29(Suppl):S157-S170)**

Keywords: NAFLD; Liver fibrosis; Biomarker

INTRODUCTION

The global prevalence of nonalcoholic fatty liver disease (NAFLD) has been rising steadily since 2006[1] and NAFLD is estimated to affect a quarter of the world's adult population.[2] NAFLD represents a spectrum of liver fat-associated conditions that begins with liver fat accumulation and progresses to steatohepatitis, liver fibrosis and cirrhosis. Within that spectrum of liver disease, it is patients with F3[3] fibrosis and F4[3] cirrhosis who are at substantial risk of death from end-stage liver disease and liver cancer. However, the earlier stages of liver fibrosis lend themselves well to therapeutic interventions to either attenuate or ameliorate progression and potentially reverse liver damage.[4-7] Thus, managing patients with NAFLD necessitates identification of F1[3] and F2[3] stages and estimation of the risk of progression to a more advanced stage of fibrosis/cirrhosis. However, liver disease can be hard to identify before it has reached a very advanced stage be-

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cause it usually progresses without signs or symptoms.[8]

In the last 20 years significant advances have been made in the development of noninvasive serum biomarkers for the identification of liver fibrosis. In this brief review, we describe these biomarkers and discuss their current utility and their potential future use in clinical practice. We consider whether liver fibrosis biomarkers have a role in: a) identifying F2 (that might be amenable to treatment as a relatively early stage of fibrosis), b) predicting patient outcomes and c), whether biomarkers can be used to help track progression or amelioration of liver fibrosis.

INITIAL AND CURRENT USE OF NONINVASIVE SERUM BIOMARKERS FOR NAFLD

Liver fibrosis is one of the most relevant prognostic factors for important clinical outcomes in NAFLD,[9] yet liver fibrosis often remains undiagnosed until it has progressed to cirrhosis. With the global prevalence of NAFLD estimated to be between 31.6% and 40.8% of the population,[10] it is important to be able to detect liver fibrosis early in the disease process, so that effective interventions can be implemented before the disease becomes too advanced. The gold standard for identification and staging of liver fibrosis is liver biopsy, however, it is a diagnostic procedure that is time consuming, costly, invasive, subject to sampling error,[11] and not scalable considering the magnitude of the global health care burden imposed by NAFLD.

Noninvasive serum biomarkers for fibrosis were initially developed by and for secondary care physicians, to use as a diagnostic assessment tool to detect patients who have advanced liver fibrosis and/or cirrhosis, offering an alternative and potential replacement to liver biopsy. A number of noninvasive serum biomarkers have been developed over the last 20 years and we now have tests, that have been validated against liver biopsy, such as the enhanced liver fibrosis (ELF™) test,[12] fibrosis-4 (FIB-4) index,[13] NAFLD fibrosis score (NFS),[14] aspartate aminotransferase to platelet radio index (APRI)[15] and FibroTest®[16] (FibroSURE™ in the USA). These relatively common tests are widely available for use in both primary and secondary care and offer a variable degree of accuracy and reliability (Table 1).

Combining noninvasive serum biomarkers has been shown to further improve diagnostic performance compared with single biomarker performance alone.[17,18] Nevertheless, the current use of noninvasive serum biomarkers focuses on excluding disease, e.g., stratification of patients into those who have a high probability of ≥F3 fibrosis versus those who have a low probability of ≥F3 fibrosis. The utility of noninvasive serum biomarkers is therefore limited because even though they have been used to identify someone with a high probability of ≥F3 fibrosis, additional tests are required to confirm this. For example, in UK primary care, the biomarkers NFS, FIB-4 and ELF™ are recommended for use to identify patients with a high probability of ≥F3 fibrosis[19] but as the biomarker itself is not informative enough as a basis for intervention, the recommendation is to follow biomarker testing with vibration controlled transient elastography (VCTE),[20] to confirm the stage of fibrosis. In Korea, the recommendation is to assess for fibrosis using radiological examinations such as VCTE.[21] If this is not feasible then NFS or FIB-4 are the recommended tests.[21]

DO BIOMARKERS HAVE A ROLE IN IDENTIFYING F2 FIBROSIS?

We now know that F2 fibrosis has important consequences for patients.[22,23] F2 fibrosis is a risk factor for cirrhosis and overall mortality and F2 increases the risk of extra hepatic complications including cardio vascular disease.[22,23] Approximately 20% of patients diagnosed with low-levels of liver fibrosis (F1–F2) will progress to F3, or F4, within 5 years.[24] F2 is a stage of fibrosis that is easily managed in primary care and it is potentially treatable and maybe halted or reversed through lifestyle changes.[6,25,26] Alternatively, medications such as anti-fibrotic therapeutic drugs (currently in phase 3

Abbreviations:

APRI, aspartate transaminase to platelet ratio index; AUC, area under the curve; CI, confidence interval; CVD, cardio vascular disease; ELF™, enhanced liver fibrosis test; FDA, Food and Drug Administration; FIB-4, fibrosis-4 index; GLP-1, glucagon-like peptide-1; METAVIR, meta-analysis of histological data in viral hepatitis; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steohepatitis; NFS, NAFLD fibrosis score; NPV, negative predictive value; PPV, positive predictive value; PRO-C3, type III collagen marker of the N-terminal pro-peptide; VCTE, vibration controlled transient elastography

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trials[27]) or glucagon-like peptide-1 agonist medication[28] may have beneficial effects on the early stages of liver fibrosis. It is therefore important for clinicians to be able to identify F2 accurately, precisely, quickly and easily, which noninvasive serum biomarkers have the potential to do. However, there are difficulties in determining the optimum cut-off value to use to differentiate intermediate states of fibrosis from the more advanced stages.[29,30] To date no one biomarker is recommended for the detection of F2.[13,31]

Recent systematic reviews evaluating the five widely available noninvasive biomarkers concluded that APRI,[32] FIB-4,[32] FibroTest®[33] and NFS[32] showed a fair[34] performance for identifying ≥F2 fibrosis (Table 2). The performance of ELF™[35] however was evaluated as good,[34] although it should be noted that ELF™ may produce a high number of false positive tests (specificity=12%). In another systematic review, PRO-C3[36] (N-terminal type III collagen pro-peptide) a less widely available noninvasive blood biomarker, has been shown to match the performance of ELF™ and outperform APRI, FIB-4, FibroTest®, and NFS.[32] In this study PRO-C3 had a sensitivity and specific-

Table 1. Summary of the performance comparison of five widely available and frequently used noninvasive serum biomarkers for diagnosing ≥F3 liver fibrosis in NAFLD

Performance	Noninvasive blood biomarker				
	ELF™[35]	FIB-4[32]	NFS[32]	APRI[32]	FibroTest®[33]
AUC value	0.83	0.80	0.78	0.75	0.77
Sensitivity	0.42	0.32	0.43	0.33	0.72
Specificity	0.95	0.96	0.88	0.91	0.69
PPV	0.85	0.66	0.67	0.56	NR
NPV	0.71	0.85	0.89	0.79	NR
Notable differences					
Age included in algorithm		√	√	√	√
Score calculated from routine blood and anthropometric measurements*		√	√	√	
Additional costs beyond routine blood tests incurred	√				√
Utility for high prevalence setting only	√	√	√	√	√

NAFLD, non-alcoholic fatty liver disease; AUC, area under the curve; PPV, positive predictive value; NPV, negative predictive value; NR, not reported; ELF™, enhanced liver fibrosis; FIB-4, fibrosis-4; NFS, nonalcoholic fatty liver disease fibrosis score; APRI, aspartate transaminase to platelet ratio index. *Online calculators for FIB-4, NFS, and APRI are available:
FIB-4: e.g., https://gps.northcentrallondon.icb.nhs.uk/fib-4-calculator and https://www.hepatitisc.uw.edu/page/clinical-calculators/fib-4.
NFS: e.g., https://www.mdcalc.com/calc/3081/nafld-non-alcoholic-fatty-liver-disease-fibrosis-score and https://www.omnicalculator.com/health/nafld-fibrosis-score.
APRI: e.g., https://www.hepatitisc.uw.edu/page/clinical-calculators/apri and https://www.omnicalculator.com/health/apri.

Table 2. Comparison of the performance of ELF™, FIB-4, APRI, FibroTest®, and NFS for identifying ≥F2 fibrosis

Biomarkers	Cut-off values	AUC	Summary sensitivity (%)	Summary specificity (%)	Summary PPV (%)	Summary NPV (%)
APRI[32]	0.43–1.50	0.70	59.3 (33.3–71.1)	77.1 (66.2–90.6)	67.5 (61.1–74.3)	70.6 (57.6–87.5)
FIB-4[32]	0.37–3.25	0.75	64.4 (54.4–77.8)	70.0 (60.0–87.5)	73.3 (66.2–77.8)	60.6 (40.5–74.2)
FibroTest®[33]	0.30–0.75	0.77	56.0 (45.0–66.0)	77.0 (74.0–80.0)	NR	NR
NFS[32,*]	–1.1	0.72	66.5 (60.9–70.1)	82.5 (68.7–96.3)	81.7 (76.6–86.7)	73.6 (61.1–86.0)
ELF™[35]	7.7[†]	0.81	Sensitivity=0.96	Specificity=0.12	PPV=0.42	NPV=0.83

Values are presented as mean (range).
ELF™, enhanced liver fibrosis test; FIB-4, fibrosis-4; APRI, aspartate transaminase to platelet ratio index; NFS, nonalcoholic fatty liver disease fibrosis score; AUC, area under the curve; PPV, positive predictive value; NPV, negative predictive value; NR, not recorded.
*Two studies were used for to assess the performance of NFS for significant fibrosis. One cut point was reported.
[†]Manufacturers recommended cut-off value for moderate fibrosis.[50]

ity of 68% (95% confidence interval [CI], 0.50–0.82) and 79% (95% CI, 0.71–0.86) respectively, with an area under the curve (AUC) of 0.81 (95% CI, 0.77–0.84).[36] However, the availability of PRO-C3 is limited. Currently, the PRO-C3 assay is exclusively produced by a pharmaceutical company and at present is only used for research purposes and is not recommended for clinical use.[36]

Ideally, clinicians should be able to quickly and easily assess their patients for ≥F2 fibrosis without having to request additional costly blood tests that require specialist evaluation (e.g., ELF™ and FibroTest®). Sripongpun et al.[37] developed and validated a biomarker (Steatosis-Associated Fibrosis Estimator, SAFE) specifically to identify ≥F2 fibrosis. SAFE has seven variables (sex, body mass index [BMI], diabetes status, aspartate transaminase [AST], alanine transaminase [ALT], platelet and globulin).[37] SAFE is therefore similar to the NFS that includes age, BMI, platelet count, AST and ALT ratio.[14] SAFE was shown to outperform NFS,[37] suggesting that the coefficients applied to SAFE maybe a better fit for identifying ≥F2 fibrosis in modern NAFLD patients.[37]

The use of machine learning from serum biomarker data has been found to offer a good performance for identifying ≥F2 fibrosis, AUC 0.86.[38] A recently published study utilised routinely available data to develop and validate six algorithms (LiverAID XXS, XS, S, M, L, and 4XL) to identify ≥F2.[38] The diagnostic performance of all the LiverAID models for detecting ≥F2 outperformed FIB-4 and APRI, and in all cases was statistically significant (P≤0.001): the AUC of LiverAID-XXS=0.86, the AUC of LiverAID-XS=0.89, the AUC of LiverAID-S=0.91, the AUC of LiverAID-M=0.92, the AUC of LiverAID-L=0.92, the AUC of LiverAID-4XL=0.94, the AUC of FIB-4=0.70 and the AUC of APRI=0.74. This demonstrates how machine learning models can utilise data and very quickly learn to identify liver fibrosis. However, the performance of machine learning algorithms is dependent on the quantity and quality of the input data and using liver biopsy as the reference standard. To date, the data available from liver histology studies are not sufficient to develop and guide the algorithms and available datasets are currently far too small.[39] At present, the use of machine learning to identify fibrosis is still in its infancy. That said, machine learning is well positioned to deal with this type of dynamic data in the future (Fig. 1).[40]

CAN A SINGLE BIOMARKER TEST PREDICT PATIENT OUTCOMES?

Observational studies have shown biopsy-confirmed liver fibrosis is a prognostic factor for patients with NAFLD.[41,42] A single biomarker that can predict patient outcomes as well as, or better, than liver biopsy would be a useful tool for clinicians managing patients with liver disease. However, there is

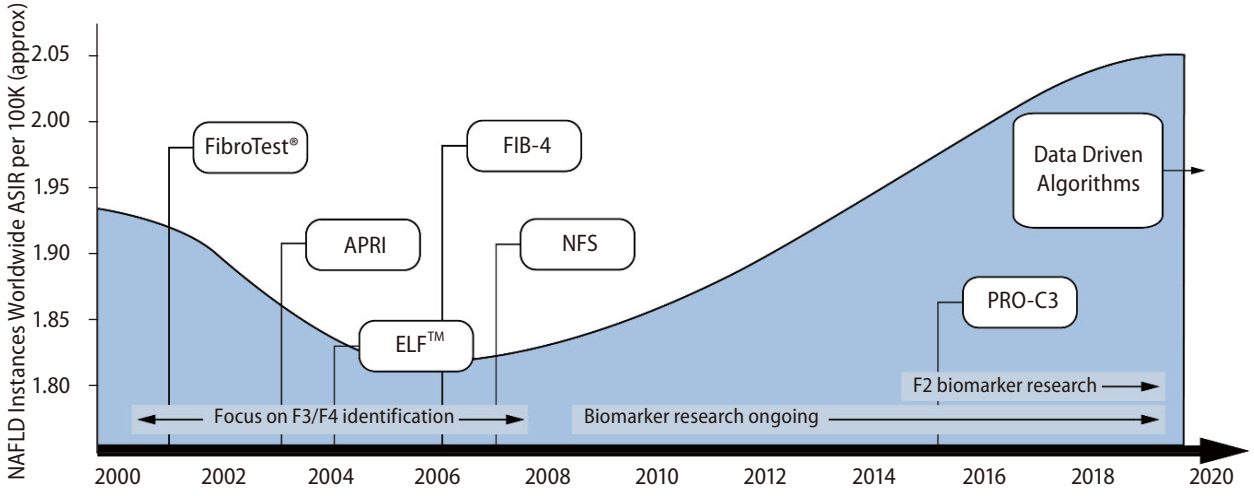

Figure 1. Timeline showing the global rise in NAFLD and the emergence of noninvasive biomarkers for fibrosis in NAFLD. NAFLD, non-alcoholic fatty liver disease; ASIR, age-standardised incidence rate per 100,000 persons; ELF™, enhanced liver fibrosis; FIB-4, fibrosis-4; NFS, nonalcoholic fatty liver disease fibrosis score; APRI, aspartate transaminase to platelet ratio index; PRO-C3, type III collagen marker of the N-terminal pro-peptide.

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conflicting evidence[43-45] and this may be in part due to the ethnicity of populations studied, the length of follow-up period, or inadequate sample sizes and the limited power of the studies to address these questions.[43-45]

A medium sized study (n=153) based in Israel,[43] with a follow-up period of 100 months, has shown that FIB-4 and NFS, but not APRI, when compared with liver biopsy, are good predictors of overall mortality. Higher FIB-4, NFS and APRI scores were also associated with hepatic and extra-hepatic malignancies.[43] A larger sized study (n=301) in Japan with a follow-up period of 84 months, has shown that FIB-4 and NFS are useful for predicting the occurrence of liver-related complications (e.g., varices, ascites or encephalopathy).[44] However, these scores were limited in their ability to predict extrahepatic malignancies.[44] A recent systematic review concluded that in secondary care, FIB-4, NFS and APRI show limited performance in predicting changes in fibrosis (as evaluated by biopsy).[45] However, these scores consistently predicted liver-related morbidity (e.g., ascites, esophageal varices or hepatocellular carcinoma), and also liver-related mortality.[45]

A more recent (2022) systematic review and meta-analysis has reaffirmed that NFS and FIB-4 are reliable and comparable to liver biopsy as prognostic markers of all-cause mortality in NAFLD patients. Additionally, NFS may be useful for predicting risk of cardiovascular death.[46] Further, a large retrospective study (n=5,123) in America[47] found that the risk of progression to cirrhosis and decompensation increased by FIB-4 strata at NAFLD diagnosis.[47] In Individuals with FIB-4 <1.3, the risk of NAFLD progression was higher than for those with 1.30-2.67 (hazard ratio [HR]=3.67; 95% CI=1.65–8.15; *P*=0.0014) and FIB-4 >2.67 (HR=56.26; 95% CI=25.77–122.83; *P*<0.001).[47] Also, the risk of death was higher in individuals with FIB-4 >2.67 (HR, 3.26; *P*<0.001).[47] In a different study, it has been shown that ELF™ predicts clinical outcomes more accurately than liver biopsy.[48] A one-point increase in ELF™ score was associated with a twofold increase in risk of liver-related clinical outcome (defined as liver-related death or episode of decompensated cirrhosis e.g., ascites or esophageal variceal hemorrhage).[48] Therefore, noninvasive serum biomarkers for liver fibrosis in NAFLD, e.g., NFS, FIB-4, and ELF™ may help predict non-liver-related outcomes e.g., cardiovascular-related mortality[46], and extra-hepatic cancers;[43,44] thus demonstrating their utility beyond simply diagnosing liver disease.

In the US, ELF™ has been granted marketing authorization by the American Food and Drug Administration (FDA) for use as a prognostic risk assessment tool for assessing the likelihood of fibrosis progression in patients with advanced fibrosis.[49] The guidance from the manufacturers of ELF™ is that in patients with F3 bridging fibrosis, an ELF™ score of ≥9.8 indicates an increased risk of progression to cirrhosis in 1–5 years.[50] The guidance also states that in patients with compensated cirrhosis, an ELF™ score of ≥9.8 indicates an increased risk of progression within 5 years to a liver-related event (e.g., development of hepatocellular carcinoma, liver failure or death).[50] The manufacturers of ELF™ do not, however, quantify how great the risk of progression is. In our opinion, a more accurate interpretation of their guidance should be that after a liver biopsy has diagnosed F3 bridging fibrosis, an ELF™ score of ≥9.8 indicates a risk of progression to cirrhosis in 1–5 years. In the UK, the ELF™ test is the recommended noninvasive blood biomarker test, to identify advanced fibrosis in patients diagnosed with NAFLD.[20] The guidelines are to repeat ELF™ every three years,[20] and not to use serial ELF™ measurements to monitor disease progression. Rather, the test should be used at any single moment in time to predict risk of prevalent ≥F3 liver fibrosis.

CAN SERIAL MEASUREMENT OF LIVER FIBROSIS BIOMARKERS HELP TRACK OR MONITOR DISEASE PROGRESSION?

As it is often uncertain how quickly liver disease will progress, a reliable noninvasive test to monitor progression over time is needed. Noninvasive serum biomarkers have the potential to monitor disease progression or amelioration over time. Having a baseline biomarker result that is repeated at regular intervals to monitor liver health would be useful for both patients and clinicians. However, repeating a biomarker and relying on the result to inform a prognosis requires the change in biomarker score to be independently validated against the change in liver biopsy, the gold standard for determining the presence and degree of liver fibrosis.

An alternative to using liver biopsy to validate biomarker score changes would be to examine retrospective biomarker scores over time in relation to liver disease progression, as was undertaken by Hagström et al.[51] These investigators used data from a retrospective population based cohort

(1986–1996) and showed that repeating FIB-4 within a 5-year period can, in comparison to a single measurement, help identify individuals who are at a higher risk of developing severe liver disease.[51] These authors noted that repeating FIB-4 is only recommended for individuals at a low risk of worsening fibrosis. The recommendation for a high risk patients was that these individuals should undergo additional diagnostic testing, e.g., VCTE, without repeat testing of FIB-4.[51] In another retrospective analysis, Balkhed et al.[52] examined data from a high prevalence of liver disease setting and showed the accuracy of FIB-4 (and APRI) is only weakly associated with disease progression. The authors concluded that the biomarkers have limited clinical utility in monitoring the course of NAFLD progression.[52]

Metabolomics analysis has been used as a promising method in NAFLD to investigate novel biomarkers involved in the pathogenesis of the disease.[53] In particular, serum lipocalin 2 has been identified as a key molecule participating in transport of fatty acids,[54] that may serve as a valuable NAFLD biomarker for monitoring the initiation and progression of fibrosis.[54]

Currently, there is still no licensed drug treatment for NAFLD. In the last decade, there have been many clinical trials testing new drugs for the treatment of liver disease in NAFLD. However, data obtained from these trials have shown suboptimal results, particularly for treatment of liver fibrosis.[55] In clinical trials for NAFLD treatment, liver biopsy is the reference standard used to assess liver fibrosis, which means that participants are required to have at least two (baseline and end of study) invasive procedures to assess the efficacy of a drug. In therapeutic drug trials for non-alcoholic steatohepatitis (NASH), noninvasive serum biomarkers are often (but not always) included to assess for changes in liver fibrosis. Therefore, when the liver biopsy findings in a drug trial show a change in the staging of fibrosis, the performance of biomarkers can be compared against the changes in liver histology.

We reviewed all 21 of the NASH drug trials from a recent systematic review and meta-analysis by Ampuero et al.[55] (Supplementary Table 1). Five[27,56-59] studies did not use any widely available noninvasive biomarker to assess changes in liver fibrosis, one[60] study stated that the data is not publicly available, and two[61,62] were conference reports/poster presentations. We tabulated the remaining 13 studies,[63-75] (Supplementary Table 2) and an abridged version shown as Table 3, to illustrate the biopsy-observed changes in liver fibrosis and the changes that occurred in serum biomarker scores (ELF™, NFS, APRI, FIB-4, FibroTest®, and PRO-C3) between baseline and follow-up assessment. It should be noted that the primary aim of the drug trials shown in the tables was to evaluate the efficacy of a therapeutic drug treatment for NASH, rather than to investigate the ability of noninvasive serum biomarkers to monitor change in histological measurement of fibrosis. As such, the value of the data reported and available from the published research papers is limited to address the question of whether biomarkers can be used to monitor changes in fibrosis attributed to a therapeutic intervention. For example, the biomarker scores at baseline and follow-up for ELF™, NFS, APRI, FIB-4, FibroTest®, and PRO-C3 in all the trials were all reported as an average score observed changes between baseline and follow up. Nine[63-71] of the studies included participants with F1 and F2 (and in some studies F0); yet the serum biomarkers used to assess fibrosis (ELF™, NFS, APRI, FIB-4, and FibroTest®) are currently only validated for ≥F3 fibrosis. The participant eligibility criteria for the remaining four[72-75] studies was F3 at baseline. Therefore a comparison of biomarker performance against changes in liver histology should be possible. However, only one of the studies (Harrison et al.[74], 2020) provided sufficient data to make this comparison. Therefore, the utility of noninvasive biomarkers to track changes in liver fibrosis needs further study in therapeutic trials targeting treatment of fibrosis.

CONCLUSION

The current use of widely available noninvasive serum biomarkers for fibrosis in NAFLD continues to be used to identify patients who have a high probability of ≥F3 fibrosis in settings where there is a high prevalence of more severe liver disease. It remains uncertain whether biomarkers have sufficient sensitivity and specificity to be able to monitor progression in fibrosis, or amelioration of fibrosis with therapeutic interventions. Although there is a recognized need to identify fibrosis earlier in the disease process, no single biomarker has been shown to be accurate or precise enough to identify patients with F2 liver fibrosis. Increased liver fibrosis biomarker scores are associated with liver-related morbidity and mortality and also associated with an increased risk of non-liver related patient outcomes. Currently, there is an insufficient evidence to demonstrate that a change in a biomarker

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Table 3. Comparison between change in noninvasive serum biomarkers and change in liver fibrosis assessed by liver histology, in therapeutic trials of nonalcoholic steatohepatitis (NASH)

Study	Study design, duration & numbers recruited	Relevant drug for NASH	Patient group	Fibrosis marker	Baseline	Follow-up	Change in mean	Change in serum biomarker score
Newsome et al.[63] (2021)	Phase 2, double-blind, randomised, placebo-controlled; 72 weeks; n=320	Semaglutide	0.4 mg	Mean fibrosis stage[a,*] (SD)	2.2 (0.6)	1.7 (0.4)	−0.5	
				Mean ELF™ score[d,f]	9.9±1.0	9.2[b]		−0.56[c]
				Mean VCTE reading, kPa[e]	11.5±87.1	7.68g		−3.82
			Placebo	Mean fibrosis stage[a,*] (SD)	2.2 (0.6)	2.0 (0.4)	−0.2	
				Mean ELF™ score[d,f]	9.6±0.9	9.77[b]		0.01[c]
				Mean VCTE reading, kPa[e]	8.7±90.0	10.84[g]		2.14[b]
Friedman et al.[64] (2018)	Phase 2b, double- blind, randomised, placebo-controlled; 52 weeks; n=288	Cenicriviroc	150 mg	Mean fibrosis stage[*] (SD)	2.1 (0.5)	1.9 (0.4)	−0.2	
				Median NFS score (min, max)	−0.942 (−4.55, 1.27)	−0.942 (−4.55, 1.27)		−0.942 (−4.55, 1.27)
				Median FIB-4 score (min, max)	1.239 (0.38, 4.20)	1.375 (0.42, 5.26)		0.080 (−1.81, 2.38)
				Median APRI score, (min, max)	0.470 (0.20, 3.12)	0.539 (0.15, 3.45)		0.024 (−1.30, 1.49)
				Median ELF™[§](min, max)	−0.892 (−2.70, 1.27)	−0.828 (−2.50, 1.08)		0.023 (−1.98, 1.65)
			Placebo	Mean fibrosis score[*] (SD)	2.0 (0.5)	2.1 (0.4)	0.1	
				Median NFS score (min, max)	−1.223 (−4.81, 2.46)	−1.190 (−4.27, 2.34)		0.102 (−1.74, 1.37)
				Median FIB-4 score (min, max)	1.303 (0.40, 4.14)	1.242 (0.36, 5.32)		0.006 (−1.18, 3.11)
				Median APRI score, (min, max)	0.568 (0.15, 2.26)	0.538 (0.13, 3.71)		−0.031 (−0.82, 3.46)
				Median ELF™[§](min, max)	−0.893 (−2.20, 1.62)	−1.003 (−2.53, 2.07)		−0.113 (−1.21, 1.60)
Francque et al.[65] (2021)	Phase 2b, double-blind, randomised, placebo-controlled; 24 weeks; n=247	Lanifibranor	1,200 mg	Mean fibrosis score (SD)[d,h,*]	2.1±0.8	NR	NR	
				Median ELF™ score[i] (IQR)	NR	NR		0.11 (−0.04 to 0.26)
				Median FIB-4 (IQR)	NR	NR		0.03 (−0.13 to 0.19)
				Median PRO-C3, µg/L (IQR)	NR	NR		−1.79 (−3.07 to −0.52)
				Mean VCTE reading, kPa (SD)	9.99 (5.46)	NR	−1.01 (3.88)	
			Placebo	Mean fibrosis score (SD)[d,h,*]	2.0±0.8	NR	NR	
				Median ELF™ score[i] (IQR)	NR	NR		−0.08 (−0.23 to 0.06)
				Median FIB-4 (IQR)	NR	NR		0.03 (−0.19 to 0.13)
				Median PRO-C3, µg/L (IQR)	NR	NR		−1.01 (−2.30 to 0.28)
				Mean VCTE reading, kPa (SD)	9.96 (4.89)	NR	−0.66 (3.04)	

Table 3. Continued

Study	Study design, duration & numbers recruited	Relevant drug for NASH	Patient group	Fibrosis marker	Baseline	Follow-up	Change in mean	Change in serum biomarker score
Harrison et al.[66] (2020)	Phase 2b, double-blind, randomised, placebo-controlled; 52 weeks; n=392	MSDC-0602K	250 mg	Mean fibrosis stage[a,*] (SD)	2.10 (0.53)	NR	−0.1	Reported as: the average effect of the combined highest doses relative to placebo on ELF™ FIB-4, FibroTest®, and CK-18 was a reduction of 0.21 (95% CI −0.39 to −0.03) SDs at 6 months and 0.17 (95% CI −0.37 to 0.02) SDs at 12 months.
				Mean APRI score (SD)	0.604 (0.4385)	NR		
				Mean ELF™ score (SD)	9.80 (1.052)	NR		
				Mean FIB-4 score (SD)	1.58 (0.909)	NR		
				Mean FibroTest® (SD)	0.33 (0.192)	NR		
			Placebo	Mean fibrosis stage[a,*] (SD)	2.2 (0.6)	NR	0.1	
				Mean APRI score (SD)	0.540 (0.2896)	NR		
				Mean ELF™ score (SD)	9.6 (0.850)	NR		
				Mean FIB-4 score (SD)	1.38 (0.688)	NR		
				Mean FibroTest® (SD)	0.31 (0.197)	NR		
Armstrong et al.[67] (2016)	Phase 2, double-blind, randomised, placebo-controlled; 48 weeks; n=52	Liraglutide	1.8 mg	Mean fibrosis stage[†] (SD)	2.3 (0.9)	NR	−0.2 (0.8)	
				Mean ELF™ score (SD)	9.3 (SD)	NR		−0.3 (0.8)
			Placebo	Mean fibrosis stage[†] (SD)	2.3 (1.3)	NR	0.2 (1.0)	
				Mean ELF™ score (SD)	9.4 (1.3)	NR		0.1 (0.8)
Chalasani et al.[72] (2020)	Phase 2b, double-blind, randomised, placebo-controlled; 52 weeks; n=162	Belapectin	8 mg/kg	Mean fibrosis stage[a,‡] (SD)	4.0[b]	3.75[a] (1.3)	−0.25[b]	
				Mean ELF™ score (SD)	10.64 (1.16)	NR		0.50 (0.78)
				Mean FibroTest® score (SD)	NR	NR		0.01 (0.02)
				Mean VCTE reading, kPa (SD)	29.3 (14.9)	NR		−2.34 (10.8)
			Placebo	Mean fibrosis stage[a,‡] (SD)	4.0[b]	3.7[a] (1.3)	−0.3[b]	
				Mean ELF™ score (SD)	10.81 (1.1)	NR		0.37 (0.63)
				Mean FibroTest® score (SD)	NR	NR		0.03 (0.02)
				Mean VCTE reading, kPa (SD)	29.9 (17.8)	NR	−0.47 (18.6)	
Harrison et al.[68] (2021)	Phase 2, double blind, randomised, placebo-controlled; 24 weeks; n=78	Aldafermin	1 mg	Mean fibrosis stage[a,*] (SD)	2.5a (0.7)	NR	NR[j]	
				Mean ELF™ score (SD)	9.8 (0.8)	NR		−0.2 (0.5)
				Mean PRO-C3 score, µg/L (SD)	17.5 (8.4)	NR		−5.4 (6.2)
			Placebo	Mean fibrosis stage[a,*] (SD)	2.4 (0.7)	NR	NR[j]	
				Mean ELF™ score (SD)	9.9 (1.0)	NR		0 (0.6)
				Mean PRO-C3 score, µg/L (SD)	17.1 (7.0)	NR		−1.2 (6.2)

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Table 3. Continued

Study	Study design, duration & numbers recruited	Relevant drug for NASH	Patient group	Fibrosis marker	Baseline	Follow-up	Change in mean	Change in serum biomarker score
Harrison et al.[69] (2021)	Phase 2a, double blind, randomised, placebo-controlled; 12 weeks; n=80	Efruxifermin	70 mg	Mean fibrosis stage[a,*] (SD)	2.0 (0.4)	NR	NR	
				Mean ELF™ score (SD)	9.5 (0.8)	NR		9.3[b,k]
				Mean PRO-C3 score, µg/L (SD)	17.2 (5.9)	NR		10.0[b,k]
			Placebo	Mean fibrosis stage[a,*] (SD)	2.0 (0.5)	NR	NR	
				Mean ELF™ score (SD)	9.5 (1.0)	NR		9.5[b,k]
				Mean PRO-C3 score, µg/L (SD)	16.1 (6.7)	NR		15.0[b,k]
Loomba et al.[73] (2021)	Phase 2b, double blind, randomised, placebo-controlled; 48 weeks; n=392	Cilofexor Firsocostat	Cilofexor 30 mg Firsocostat 20 mg	Biopsy confirmed F3/F4[*]	n=76 (98%)	NR	NR	
				Median ELF™ score (IQR)	10.0 (9.4, 10.7)	NR		−0.0 (−0.2, 0.20)
				Median VCTE reading, kPa (IQR)	15.7 (10.9, 22.2)	NR	−4.2 (−6.5, −1.9)	
Harrison et al.[70] (2019)	Phase 2, double blind, randomised, placebo-controlled; 36 weeks; n=125	Resmetriom	80 mg	Mean fibrosis stage[a,*] (SD)	1.6 (0.3)	NR	NR	
				Mean ELF™ score (SD)	9.2 (0.9)	NR		−0.38[m] (0.09)
				Mean PRO-C3 score, µg/L (SD)	17.8 (10.3)	NR		−2.2[p] (2.1); −6.5[q] (3.5)
			Placebo	Mean fibrosis stage[a,*] (SD)	1.6 (0.3)	NR	NR	
				Mean ELF™ score (SD)	9.2 (1.0)	NR		0.02[l] (0.12)
				Mean PRO-C3 score, µg/L (SD)	16.2 (59.0)	NR		7.4[n] (3.1); 14.9[o] (5.6)
Ratziu et al.[71] (2016)	Phase 2, double blind, randomised, placebo-controlled; 52 weeks; n=276	Elafibranor	120 mg	Mean fibrosis stage[*] (SD)	1.7 (0.9)	NR	NR	
				Mean NFS score (SD)	NR	NR		−0.25[b]
				Mean FibroTest[^] (SD)	NR	NR		−0.07[b]
			Placebo	Mean fibrosis stage[*] (SD)	1.5 (1.0)	NR	NR	
				Mean NFS score (SD)	NR	NR		−0.01[b]
				Mean FibroTest[^] (SD)	NR	NR		−0.01[b]
Harrison et al.[74] (2020)	Phase III (STELLAR-4), double blind, randomised, placebo-controlled; 48 weeks; n=877	Selonsertib	18 mg	Mean fibrosis stage[a,*] (SD)	4.0 (1.8)	3.7 (1.4)	−0.3[b]	
				Median ELF™ score (IQR)	10.61 (10.04 to 11.34)	10.73 (10.07 to 10.51)		0.10[b]
				Median FibroTest[^] (IQR)	0.58 (0.44 to 0.73)	0.58 (0.40 to 0.75)		NC
				Median APRI score (IQR)	0.8 (0.6 to 1.2)	0.8 (0.5 to 1.3)		NC
				Median FIB-4 score (IQR)	2.55 (1.76 to 3.62)	2.65 (1.74 to 3.76)		0.10[b]
				Median NFS score (IQR)	0.659 (−0.119 to 1.472)	0.816 (0.031 to 1.574)		0.157[b]
				Median VCTE reading, kPa (IQR)	21.10 (14.7 to 28.8)	19.4 (14.3 to 27.3)		−1.7[b]

Table 3. Continued

Study	Study design, duration & numbers recruited	Relevant drug for NASH	Patient group	Fibrosis marker	Baseline	Follow-up	Change in mean	Change in serum biomarker score
			Placebo	Mean fibrosis stage[a,*] (SD)	3.7 (1.4)	3.8 (1.5)		0.10[b]
				Median ELF™ score (IQR)	10.67(10.05 to 11.16)	10.66 (10.14 to 11.26)		−0.01[b]
				Median FibroTest® (IQR)	0.59 (0.40 to 0.77)	0.57 (0.39 to 0.73)		−0.02[b]
				Median APRI score (IQR)	0.8 (0.6 to 1.2)	0.7 (0.5 to 1.2)		−0.1[b]
				Median FIB-4 score (IQR)	2.50 (1.81 to 3.66)	2.50 (1.65 to 3.67)		NC
				Median NFS score (IQR)	0.682 (−0.304 to 1.450)	0.774 (−0.241 to 1.595)		0.092[b]
				Median VCTE reading, kPa (IQR)	20.00 (14.4 to 26.7)	19.30 (13.8 to 26.7)		0.70[b]
Loomba et al.[75] (2018)	Phase 2, double blind, randomised, *de facto* placebo-controlled; 24 weeks; n=72	Selonsertib±Simtuzumab	Selonsertib 18 mg ±Simtuzumab	Biopsy confirmed F3[*]	n=21 (66%)	Improvement n=13 (43%); Cirrhosis n=1 (3%)		
				Median ELF™ score (IQR)	NR	NR		0.02 (−0.34 to 0.52)
				Median FibroTest® (IQR)	NR	NR		−0.01 (−0.03 to 0.03)
				Median VCTE reading, kPa (IQR)	NR	NR		0.2 (−3.50 to 1.40)
			Simtuzumab	Biopsy confirmed F3[*]	n=6 (60%)	Improvement n=2 (20%); Cirrhosis n=2 (20%)		
				Median ELF™ score (IQR)	NR	NR		−0.13 (−0.35 to 0.05)
				Median FibroTest® (IQR)	NR	NR		0.01 (−0.04 to 0.05)
				Median VCTE reading, kPa (IQR)	NR	NR		−0.50 (−3.80 to 3.4)

NR, not reported; NC, no change; ELF™, enhanced liver fibrosis; FIB-4, fibrosis-4; NFS, NAFLD fibrosis score; APRI, aspartate transaminase to platelet ratio index; PRO-C3, Type III collagen marker of the N-terminal pro-peptide; SD, standard deviation; IQR, interquartile range; VCTE, vibration controlled transient elastography.

[a]Mean not provided, calculation made using data provided in the manuscript tables and supplementary information. [b]No standard deviation/IQR reported. [c]Change in biomarker score is the change reported in the research paper and not the exact difference between baseline and follow-up.[56] [d]Plus-minus values are means±SD. [e]Plus-minus values are geometric means±coefficient of variation. [f]An ELF™ score greater than 9.8 indicates a moderate risk of advanced fibrosis, and a score of greater than 11.3 denotes a high risk of advanced fibrosis. [g]No geometric means±coefficient of variation reported. [h]Fibrosis stage was classified according to the SAF-NASH CRN staging system. [i]An ELF™ score of less than 7.7 indicates none to mild fibrosis, and a score of 11.3 or greater indicates cirrhosis. [j]Improvement/no improvement or worsening reported, unable to calculate changes in fibrosis stage as data is not provided. [k]Estimated values only, exact values not recorded, data taken from manuscript[62] Figure 3, (F) and (G). [l]Mean difference reported for subjects with ELF™ ≥9.0 only (n=21) at week 12. [m]Mean difference reported for subjects with ELF™ ≥9.0 only (n=40) at week 12. [n]Mean difference reported for subjects with baseline ≥10.00 ng/mL (n=25). [o]Mean difference reported for subjects with baseline ≥17.50 ng/mL (n=12). [p]Mean difference reported for subjects with baseline ≥10.00 ng/mL (n=53). [q]Mean difference reported for subjects with baseline ≥17.50 ng/mL (n=29).

[*]Biopsy confirmed fibrosis stages using NASH CRN scoring system.

[†]Biopsy confirmed fibrosis stages using Kleiner scoring system.

[‡]Biopsy confirmed cirrhosis using Ishak scoring system.

[§]Data for baseline, follow up and change in ELF™ score taken from Supplementary Table 6.[57]

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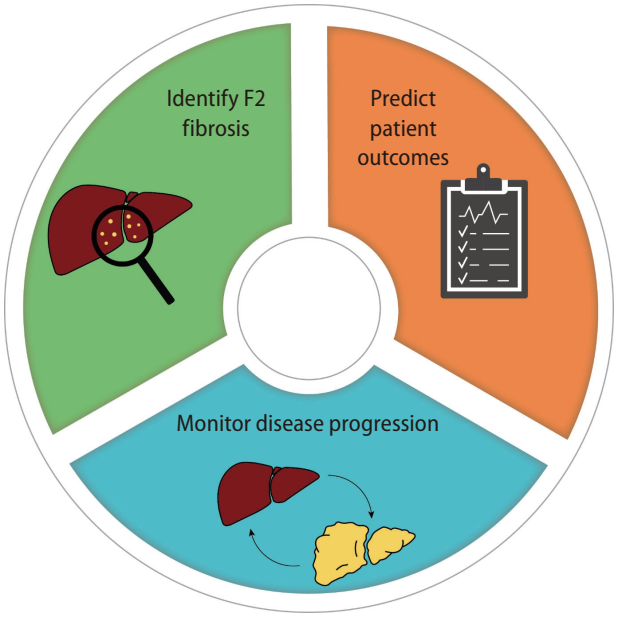

Figure 2. The future of noninvasive serum biomarkers for fibrosis in NAFLD. NAFLD, non-alcoholic fatty liver disease.

score allows prediction of a change in liver fibrosis. Finally, we consider that it is now crucial to develop biomarkers that accurately and precisely identify F2, and to continue to investigate whether biomarkers can be used for assessing and monitoring disease progression/regression with therapeutic interventions that include both drugs and lifestyle change (Fig. 2).

Authors' contribution

All authors (Tina Reinson, Ryan M. Buchanan, and Christopher D. Byrne) contributed to the review structure and concept; drafting of the manuscript and its critical revision; and approved the final version.

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Conflicts of Interest

The authors have no conflicts to disclose.

SUPPLEMENTARY MATERIAL

Supplementary material is available at Clinical and Molecular Hepatology website (http://www.e-cmh.org).

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Review

Non-invasive biomarkers for liver inflammation in non-alcoholic fatty liver disease: present and future

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Inflammation is the key driver of liver fibrosis progression in non-alcoholic fatty liver disease (NAFLD). Unfortunately, it is often challenging to assess inflammation in NAFLD due to its dynamic nature and poor correlation with liver biochemical markers. Liver histology keeps its role as the standard tool, yet it is well-known for substantial sampling, intraobserver, and interobserver variability. Serum proinflammatory cytokines and apoptotic markers, namely cytokeratin-18, are well-studied with reasonable accuracy, whereas serum metabolomics and lipidomics have been adopted in some commercially available diagnostic models. Ultrasound and computed tomography imaging techniques are attractive due to their wide availability; yet their accuracies may not be comparable with magnetic resonance imaging-based tools. Machine learning and deep learning models, be they supervised or unsupervised learning, are promising tools to identify various subtypes of NAFLD, including those with dominating liver inflammation, contributing to sustainable care pathways for NAFLD. **(Clin Mol Hepatol 2023;29(Suppl):S171-S183)**

Keywords: Cytokeratin-18; Deep learning; Fatty liver; Liver cancer; Machine learning

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) affects over 30% of the general adult population worldwide, and is emerging as an important cause of cirrhosis and hepatocellular carcinoma.[1] Its more active form, non-alcoholic steatohepatitis (NASH), is characterized by the presence of hepatic steatosis, inflammation (both lobular and portal), and hepatocyte ballooning. Assessment of inflammation is important. Although studies have consistently shown that the fibrosis stage[2] has a

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stronger correlation with adverse liver-related outcomes than features of NASH, inflammation is, after all, the driver of fibrosis progression.[3,4] Moreover, the United States Food and Drug Administration and the European Medicines Agency both accept NASH resolution with no worsening of fibrosis and/or fibrosis improvement with no worsening of NASH as key histological endpoints for conditional approval of new drugs for NASH.[5] Until the regulators accept the use of non-invasive surrogate biomarkers in place of liver biopsy, assessment of inflammation will remain crucial in the drug development process.

With that being said, the assessment of inflammation is difficult. Above all, there is substantial sampling, intraobserver, and interobserver variability in the histological assessment of inflammation and diagnosis of NASH.[6] When paired biopsies are performed to assess the treatment response, errors at each biopsy add up.[7] If the histological reference standard is unreliable, this would underestimate the performance of even an excellent biomarker. Moreover, compared with fibrosis, inflammation changes more rapidly. Therefore, the time interval between liver biopsy and non-invasive test assessment would have a greater impact on the evaluation of inflammation than fibrosis biomarkers. For the same reason, one may expect inflammatory markers to vary over time, and a single-point assessment may not mean much.

In this article, we review blood and imaging biomarkers of inflammation in NAFLD. We also highlight the emerging role of artificial intelligence and machine learning in diagnostics.

LIVER HISTOLOGY

Liver histology remains the standard to assess inflammation and diagnose NASH. Pathologists diagnose NASH based on a global picture that takes into account the degree and pattern of steatosis, inflammation, and hepatocyte ballooning and/or the presence of Mallory-Denk bodies.[8] In 2005, Kleiner and colleagues[9] from the NASH Clinical Research Network proposed the NAFLD activity score, which is the numerical sum of the steatosis grade (0–3), lobular inflammation (0–3), and ballooning (0–2). Later, it was apparent that it is inappropriate to use the score to diagnose NASH, mainly due to the heavy weighting assigned to steatosis.[10] Therefore, a patient can have severe steatosis but mild inflammation, resulting in a high NAFLD activity score but not meeting the pathological diagnosis of NASH. Currently, the NAFLD activity score is mainly used in early-phase clinical trials to evaluate treatment response.

In contrast, Bedossa and colleagues[11] proposed the Steatosis-Activity-Fibrosis score in 2012, thus separating the assessment of steatosis and inflammation. They also developed the Fatty Liver Inhibition of Progression algorithm, which essentially means that one can diagnose NASH when a patient scores 1 or more in steatosis, lobular inflammation, and ballooning.[12] The algorithm has demonstrated a higher degree of interobserver agreement.

One main limitation of the original scores is the relative underweighting of ballooning, which experts agree should be the defining feature of NASH. Besides, complete disappearance of ballooning is uncommon. This explains the very low percentage of patients with NASH resolution in clinical trials, rendering this histological endpoint often useless.[13] Recently, Pai and colleagues[14] proposed to expand the scale of ballooning scoring from 0–2 to 0–4 to increase granularity and reliability of the assessment of NASH.

Other than assessment variability, liver biopsy is also limited by its invasiveness nature, poor patient acceptance, cost, pain, and potential complications.[15] Therefore, it is important to develop non-invasive tests for routine clinical use.

SERUM MARKERS

Traditionally, alanine aminotransferase (ALT) and aspartate

Abbreviations:

ALT, alanine aminotransferase; CI, confidence interval; DM, diabetes mellitus; HCC, hepatocellular carcinoma; NAFLD, non-alcoholic fatty liver disease; CK-18, cytokeratin-18; CT, computed tomography; MRI, magnetic resonance imaging; NASH, non-alcoholic steatohepatitis; SAF, Steatosis-Activity-Fibrosis; FLIP, Fatty Liver Inhibition of Progression; AST, aspartate aminotransferase; HETE, hydroxyeicosatetraenoic acid; US, ultrasound; US-FLI, ultrasonographic fatty liver indicator; VCTE, vibration-controlled transient elastography; CAP, controlled attenuation parameter; LSM, liver stiffness measurement; NFS, NAFLD fibrosis score; FAST, FibroScan-AST; NECT, non-contrast-enhanced CT; DECT, dual-energy CT; pCT, perfusion CT; PCD-CT, photon-counting detector CT; LMS, LiverMultiScan; cT1, corrected T1; PDFF, proton density fat fraction; FIB-4, fibrosis-4; PPV, positive predictive value; NPV, negative predictive value; 3D, three-dimensional; CART, classification and regression trees; HbA1c, hemoglobin A1c; NAS, NAFLD activity score; CRN, Clinical Research Network; CNN, Convolutional Neural Network; GNN, Graph Neural Network; RNN, Recurrent Neural Network; EHRs, electronic health records; LSTM, long short-term memory

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aminotransferase (AST) have been used in routine clinical practice as biochemical markers of inflammatory damage in hepatocytes, or hepatitis in a simpler term. Unfortunately, a more active form of disease, such as NASH and advanced fibrosis, is often found in NAFLD patients exhibiting normal aminotransferase levels; such levels may even paradoxically decrease in patients with progressive fibrosis,[16] suggesting that ALT or AST levels are not reliable in establishing active inflammation in NAFLD. Combining routine clinical parameters is another popular approach; a handful of diagnostic panels were proposed and validated to identify liver inflammation in NASH (Table 1). Most of these models have the benefits of wide availability of parameters included and reasonably good diagnostic accuracy, but specific cut-offs need to be further optimized.[17]

Proinflammatory cytokines and apoptotic markers are possible diagnostic biomarkers for patients with NASH. The most evaluated NASH serum biomarker is cytokeratin-18 (CK-18), which is a well-recognized hepatocyte apoptosis product that accounts for about 5% of liver proteins.[18] Two antigens of CK-18, M30 and M65, are of the same protein yet distinctive mechanisms—M30 measures the caspase-cleaved CK-18 revealed during apoptosis, while M65 measures the full-length protein, including both caspase-cleaved and intact CK-18, which is released from cells undergoing necrosis.[18] In general, models with CK-18 perform better than those with solely routine laboratory parameters (Table 1).

Serum metabolomics[19] and lipidomics are also widely studied; pyroglutamic acid, phosphatidylcholine, sphingomyelin, fatty acids, hydroxyeicosatetraenoic acid, glycyrrhetinic acid, taurocholate, and various subtypes of triglycerides levels were incorporated in different models (Table 1).[20] Some diagnostic models have been commercially available (e.g., by OWL Metabolomics).[21]

While most of the biomarkers and models were derived and validated in a cross-sectional fashion, dedicated studies to evaluate the dynamic change, in particular, the reduction of score after treatment which correlates with inflammation improvement, are much warranted in the era of active development of novel therapeutics for NASH.

ULTRASOUND IMAGING (TABLE 2)

Transabdominal ultrasonography

Conventional B-model ultrasound (US) is the most widely used imaging technique for the non-invasive assessment of

Table 1. Diagnostic models for liver inflammation in non-alcoholic steatohepatitis (NASH) (adapted from Zeng et al.[17])

Models	Variables	AUROC	Cutoff	Sn	Sp
FLI	BMI, waist, TG, GGT	0.84	<30 and ≥60	87%	86%
HAIR score	HT, ALT, insulin, Glu	0.68	3	57%	77%
NASHTest-2	A2M, ApoA1, Hapt, TBil, GGT, TC, TG	0.59	0.5	83.3%	37.5%
MACK-3	CK-18 M30, AST, HOMA	0.81	≤0.167 and ≥0.551	84.2%	81.4%
G-NASH	CK-18 M30, GP73	0.85	NA	82.1%	80.5%
Nice model	CK-18, ALT, MS	0.88	0.14	84%	86%
FIC-22	CK-18 M30, FIB-4	0.82	1	89.1%	62.5%
NASH diagnostic™	CK-18 M30, adiponectin, resistin	0.91	0.2272	94.45%	70.21%
CHeK	CK-18 M30, GGT, age, HbA1c, adiponectin	0.73	NA	NA	NA
NASH score	PNPLA3, insulin, AST	0.77	-1.054	75%	74%
NASH PT score	PNPLA3, TM6SF2, diabetes, AST, HOMA-IR, hsCRP	0.86	-0.785	91%	58.1%
NIS4	miRNA-34a, A2M, YKL-40, HbA1c	0.80	<0.36 ≥0.63	80.8% 45.2%	65.2% 90.4%
GlycoNASHTest	Log (NGA2F/NA2)	0.74	NA	NA	NA

A2M, alpha-2 macroglobulin; ALT, alanine aminotransferase; ApoA1, apolipoprotein A1; AUC, area under the receiver operating characteristic curve; BMI: body mass index; CK-18, cytokeratin-18; FIB-4, fibrosis-4; GGT, γ-glutamyl transpeptidase; Glu, glucose; GP73, golgi protein 73; HT, hypertension; Hapt, haptoglobin; HbA1c, glycosylated hemoglobin; HOMA, homeostasis model assessment; hsCRP, high-sensitivity C-reactive protein; miRNA, MicroRNA; MS, metabolic syndrome; NA, not available; Sn, sensitivity; Sp, specificity; TBil, total bilirubin; TC, total cholesterol; TG, triglycerides.

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NAFLD. Focal steatosis tissue presents brighter than other parenchyma in ultrasound examination because of the increasing attenuation of US waves.[22] US is currently the first-line diagnostic approach for NAFLD suggested by clinical practice guidelines of the European Association for the Study of the Liver due to its low cost, wide availability, and repeatability.[23] In a meta-analysis with 2,815 patients performed on 34 studies, the overall sensitivity of US to detect moderate to severe fatty liver with liver biopsy as a reference standard was 84.8% (95% CI, 79.5–88.9%), specificity was 93.6% (95% CI, 87.2–97.0%) and the AUROC was 0.93 (0.91–0.95).[24] US has great diagnostic performance for NAFLD.

However, several studies found no correlation between the US characteristics and liver histologic features, including inflammation and ballooning.[25,26] Hamaguchi scoring system was developed based on US findings, including bright liver, and hepatorenal echo contrast (0–3), deep attenuation (0–2), and vessel blurring (0–1). The scoring system further improved the diagnostic performance of NAFLD in obese patients, with an area under the receiver operating characteristic curve (AUROC) of 0.98.[27] Ultrasonographic fatty liver indicator (US-FLI) is another scoring system ranging from 2–8 based on the intensity of liver or kidney contrast, attenuation of ultrasound beam, vessel blurring, and the visualization of gallbladder wall, diaphragm, and areas of focal sparing. The AUROC of US-FLI for predicting NASH was 0.80 (0.68–0.92), and US-FLI was correlated with lobular inflammation according to Kleiner's criteria.[28] Hamaguchi score and US-FLI score lack validation in large series of patients, and whether the dynamic change of scores correlates with inflammation progression or improvement needs to be validated in the future.

Vibration-controlled transient elastography

Vibration-controlled transient elastography (VCTE) technique measures the velocity of shear wave through the liver parenchyma, and the velocity is related to the degree of liver tissue stiffness. Controlled attenuation parameter (CAP) captures the attenuation in the amplitude of ultrasound waves to estimate the degree of hepatic steatosis, and it has been available for clinical practice since 2010. Fibroscan 502 Touch was the first VCTE device commercially available with CAP. An examination is considered valid in cases of ≥10 valid liver stiffness measurement (LSM) and CAP, and an interquartile range-to-median ratio of the measurements of ≤0.3 of LSM and CAP.[15,29] According to previous studies, Fibroscan has high accuracy, simplicity, and reproducibility to assess hepatic steatosis and fibrosis.[29] Series of studies have focused on the discriminative ability of CAP and LSM for NASH patients.[30,31] Lee et al.[30] conducted a prospective Korean study based on 183 patients with biopsy-proven NAFLD patients and showed that a cutoff value of 7 kPa for liver stiffness by VCTE can achieve an AUROC of 0.75 (95% confidence interval [CI] 0.68–0.82), a sensitivity of 73.4%, and a specificity of 78.7%. Based on VCTE, they developed a scoring system named "CLA score" using three independent predictors, including CAP value, liver stiffness by VCTE, and ALT level, to identify NASH patients. The CLA score had a significantly higher diagnostic performance than the NAFLD fibrosis score (NFS) (AUROC 0.81 vs. 0.62).[30] Recently, a randomized phase II drug trial showed that semaglutide in combination with cilofexor groups resulted in the reductions in liver stiffness by VCTE (-2.29 to -3.74 kPa), CAP (-52 to 80 db/m) in 24 weeks, with the improvement in Enhanced Liver Fibrosis score and other liver inflammation biomarkers.[32] The change of liver stiffness over time is also predictors of adverse clinical outcomes.[33]

Table 2. Diagnostic performance of ultrasound imaging for liver inflammation in non-alcoholic steatohepatitis (NASH)

Methods	Variables	Outcome	AUROC	Cutoff	Sn	Sp
US	NA	Severe NAFLD	0.93	NA	84.8%	93.6%
US-FLI	US findings	NASH	0.80	5	83.3%	62.9%
VCTE	NA	NASH	0.75	7	73.4%	78.7%
FAST score	Liver stiffness by VCTE, CAP and AST	Fibrotic NASH	0.74–0.95	≤0.35 and ≥0.67	64–100%	35–86%

AUROC, area under the receiver operating characteristic curve; NA, not available; Sn, sensitivity; Sp, specificity; US, Conventional B-model ultrasound; US-FLI, Ultrasonographic fatty liver indicator; VCTE, vibration-controlled transient elastography; CAP, controlled attenuation parameter; AST, aspartate aminotransferase; NAFLD, non-alcoholic fatty liver disease.

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FAST score

FibroScan-AST (FAST) score was a logistic regression-based scoring system for detecting fibrotic NASH, which includes liver stiffness by VCTE, CAP, and AST. The diagnostic performance of FAST score was validated in multiple large global cohorts. AUROCs ranged from 0.74 to 0.95, with sensitivity and specificity up to 1 and 0.86, and NPV ranged from 0.73 to 1. Compared to fibrosis-4 (FIB-4), NFS, and AST to platelet ratio index (APRI), the FAST score had a significantly higher diagnostic performance for fibrotic NASH.[34-36] FAST can be used as a non-invasive tool to screen fibrotic NASH to reduce the number of unnecessary liver biopsies. The relationship between dynamic changes of FAST score and liver inflammation should be explored in the future.

Computed tomography

Computed tomography (CT) uses computer processing of X-ray data of the body to produce images created from the detection of X-rays traversing tissues. Weakening of the X-ray as it passes through the body is a key parameter used to define the brightness of the tissue in the CT image. A healthy liver will appear brighter (i.e., parenchymal hyperdensity) than the spleen in a CT scan. As fat content in the liver increases, its corresponding image will become darker (i.e., parenchymal hypodensity).[37] CT liver images may be confounded by other factors such as concentration of iron, glycogen, and hematocrit. While CT is widely used to characterize focal liver lesions, in NAFLD patients, CT is more often studied to assess steatosis and fibrosis but not as much for inflammation.[38] Only one retrospective study of 88 NAFLD patients found that non-contrast-enhanced CT texture analysis with a 2-mm filter predicted NASH with accuracy above 90%; yet the accuracy dropped to 60% if a 4-mm filter was used.[39] Other emerging CT techniques, including dual-energy CT, post-processing software, perfusion CT, and photon-counting detector CT, are promising tools that are potentially more accurate to detect inflammation. Currently, CT is not the preferred primary modality to measure liver inflammation given its lack of sensitivity for steatohepatitis and the need for exposure of the subjects to radiation.

MAGNETIC RESONANCE IMAGING (TABLE 3)

LiverMultiScan

LiverMultiScan (LMS) is an emerging diagnostic tool using multiparametric magnetic resonance imaging (MRI) to quantify liver disease.[40] The technology is comprised of corrected T1 (cT1), T2, and liver fat assessment by advanced MRI. LMS measures the amount of iron in the liver to correct for its effect on T1-cT1, as excess iron in the liver reduces T1 relaxation time and leads to underestimation of liver disease. cT1 correlates with necroinflammation and fibrosis, and may serve as a non-invasive method in NASH. LMS had fewer technical failures, especially compared with ultrasound-based techniques which were less reliable in patients with a higher body mass index. The success rate exceeded 95% in previous clinical studies. One recent pooled study examined the utility of cT1 and proton density fat fraction (PDFF) for identifying NASH and fibrotic NASH.[41] The diagnostic accuracy (AUROC) of cT1 to identify patients with NASH was 0.78 (95% CI, 0.74–0.82), while that for MRI liver fat was 0.78 (95% CI, 0.73–0.82); and when combined cT1 with MRI liver fat, the diagnostic accuracy was 0.82 (95% CI, 0.78–0.85). The diagnostic accuracy of cT1 to identify patients with fibrotic NASH (AUROC [0.78; 95% CI, 0.74–0.82]) was superior to that of MRI liver fat (AUROC [0.69; 95% CI, 0.64–0.74]). There is one ongoing study

Table 3. Diagnostic performance of magnetic resonance imaging for non-alcoholic steatohepatitis

Models	Variables	Outcome	AUROC	Sn	Sp	PPV	NPV
LiverMultiScan	cT1, T2 and PDFF	Fibrotic NASH	0.69–0.79	0.39–0.86	0.56–0.90	0.45–0.60	0.78–0.91
MEFIB	MRE and FIB-4	Fibrotic NASH	0.84–0.90	0.85–0.94	0.94–0.98	0.91–0.95	0.85–0.92
MAST	MRE, PDFF and AST	Fibrotic NASH	0.86–0.93	0.89–0.94	0.89–0.90	0.50–0.55	0.91–0.98
3D MRE	-	NASH	0.73	0.67	0.80	0.73	0.74

AUROC, the area under the receiver operating characteristic curve; Sn, sensitivity; Sp, specificity; PPV, positive predictive value; NPV, negative predictive value; NASH, nonalcoholic steatohepatitis; MRE, MR elastography; FIB-4, fibrosis-4; AST, aspartate aminotransferase; PDFF, proton density fat fraction.

(NCT03743272) which aims to investigate the repeatability and reproducibility of LMS. Multiparametric MRI has been evaluated to be associated with liver-related clinical outcomes in a cohort of patients with chronic liver disease.[42] Longitudinal change of MRI-PDFF correlated well with the biopsy results, and there was one study evaluated that a 30% relative decline in MRI-PDFF predicted fibrosis regression in NAFLD patients.[43,44]

MEFIB

MEFIB index is a combination of MR elastography and FIB-4 used for the identification of fibrotic NASH.[45] In a validation cohort of the study by Jung et al.[45], the positive predictive value (PPV) exceeded 90% with an AUROC of 0.84 (95% CI, 0.78–0.89). MEFIB was evaluated to have a higher diagnostic accuracy than MAST and FAST score for significant fibrosis as well as fibrotic NASH.[46,47] The MEFIB index had a robust association with liver-related outcome with a hazard ratio of 20.6 (95% CI, 10.4–40.8), and the negative predictive value (NPV) for the outcome reached 99.1% at 5 years.[48] Future studies should explore if the dynamic change of MEFIB index is correlated with liver-related outcomes.

MAST

Given that MRI-PDFF has been shown to be more accurate than VCTE-based CAP in identifying all grades of steatosis in patients with NAFLD, and MR elastography is more accurate than VCTE in detecting liver fibrosis, Noureddin et al.[49] proposed the MAST score based on MRI-PDFF, MR elastography, and AST value. In their validation cohort, the MAST score demonstrated high performance and discrimination (AUROC 0.93, 95% CI 0.88–0.97), which was significantly better compared to the NAFLD fibrosis score, FIB-4 index, and FAST score. However, the MEFIB index showed a higher AUROC, and the PPV and NPV reached 95.3% and 90.1%, respectively, for ruling in and ruling out fibrotic NASH compared with MAST in a head-to-head comparison study.[47] There is still a lack of published studies on the prognostication as well as the dynamic change in fibrosis progression or regression by MAST score.

3D MR elastography

Recently, several studies by Allen et al.[50] from Mayo Clinic evaluated the role of three-dimensional (3D) MR elastography in identifying NASH in patients undergoing bariatric surgery. By combing the 3D MR elastography with MRI-PDFF, the AUROC was 0.73 for the diagnosis of NASH. Additionally, they demonstrated that the 3D MR elastography and MRI-PDFF could detect histologic changes in NASH resolution after bariatric surgery.[51] There are limited studies on the association between 3D MR elastography and liver-related outcomes.

MACHINE LEARNING MODELS

Over the past decade, the advancement of artificial intelligence has led to its numerous applications in hepatology. Artificial intelligence, machine learning, and deep learning can be considered three overlapping domains that use computer programs to mimic functions of human intelligence, including learning, problem solving, classification, and decision making.[52] Particularly, machine learning methods are usually applied for developing diagnostic or predictive models. Machine learning and deep learning algorithms can be supervised or unsupervised. Supervised learning methods occur when a label for the outcome is given in the training data. For example, if we aim to predict the presence of NASH among patients with biopsy-proven NAFLD, the information of whether the patients had NASH needs to be provided to the learning algorithms during training so that the model can distinguish patients with and without NASH based on that. As a result, the learning algorithm can identify combinations and interactions of factors that best separate the two groups of patients and yield an accurate prediction. In contrast, information on the presence and absence of NASH is not provided in unsupervised learning. The purpose of unsupervised learning is to identify several clusters of patients who are similar in terms of data distribution. In other words, patients within the same cluster have similar clinical characteristics, which may represent a certain disease phenotype or subtype.

Common supervised machine learning algorithms examined in identifying inflammation in NAFLD patients, including logistic regression with penalization, decision tree, random

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Table 4. Performance of machine learning or algorithm-based models in identifying inflammation in NAFLD

Study	Machine learning algorithms	Predicted variable	AUROC	Cutoff	Sn	Sp	PPV	NPV
Machine learning models								
Fialoke et al.[53]	DT with 3 temporal laboratory and 3 demographic variables	NASH vs. Healthy individuals	0.842*	0.5	74.5%	NA	78.6%	NA
	LR with 3 temporal laboratory and 3 demographic variables	NASH vs. Healthy individuals	0.835*	0.5	74.3%	NA	77.0%	NA
	RF with 3 temporal laboratory and 3 demographic variables	NASH vs. Healthy individuals	0.870*	0.5	76.8%	NA	80.4%	NA
	XGB with 3 temporal laboratory and 3 demographic variables	NASH vs. Healthy individuals	0.876*	0.5	77.4%	NA	80.8%	NA
Docherty et al.[54]	DT with 14 clinical and laboratory variables	NASH vs. NAFLD	0.72†	NA	78%	NA	76%	NA
	LR with 14 clinical and laboratory variables	NASH vs. NAFLD	0.77†	NA	79%	NA	79%	NA
	RF with 14 clinical and laboratory variables	NASH vs. NAFLD	0.82†	NA	82%	NA	80%	NA
	XGB with 14 clinical and laboratory variables	NASH vs. NAFLD	0.82†	NA	81%	NA	81%	NA
Canbay et al.[55]	LR with 5 clinical and laboratory variables	NASH vs. NAFLD among obese patients	0.70†	NA	NA	NA	NA	NA
Perakakis et al.[56]	SVM using 29 lipidomic features	NASH vs. Healthy individuals or NAFLD patients	0.96*	NA	92%	93%	NA	NA
	SVM using 20 lipidomic and hormonal features	NASH vs. Healthy individuals or NAFLD patients	0.96*	NA	91%	95%	NA	NA
	SVM using 20 lipidomic and glycomic features	NASH vs. Healthy individuals or NAFLD patients	0.96*	NA	89%	91%	NA	NA
Algorithm-based models								
Liu et al.[58]	qInflammation	Lobular inflammation‡ 0 vs. ≥1	0.838	1.251	83%	100%	100%	14%
	qInflammation	Lobular inflammation‡ ≤1 vs. ≥2	0.820	1.357	93%	58%	58%	93%
	qInflammation	Lobular inflammation‡ ≤2 vs. 3	0.831	1.503	100%	79%	12%	100%

NAFLD, non-alcoholic fatty liver disease; AUROC, are under the receiver operating characteristic curve; Sn, sensitivity; Sp, specificity; PPV, positive predictive value; NPV, negative predictive value; DT, decision tree; NASH, nonalcoholic steatohepatitis; NA, not available; LR, logistic regression; RF, random forest; SVM, support vector machine; XGB, XGBoost; CRN, Clinical Research Network.

*AUROC by internal validation with cross validation.

†AUROC in an independent validation cohort.

‡Lobular inflammation based on NASH CRN scoring system.

forest, support vector machine, and different boosting methods. Regarding the use of covariates, existing literature usually includes laboratory parameters or histological features from liver biopsy for the prediction. Fialoke and colleagues[53] utilized electronic health records from the Optum administrative claim dataset to develop machine learning models for identifying NASH patients from NAFLD patients or healthy patients without NAFLD. In this study, NAFLD and NASH were identified based on diagnosis codes. Supervised machine learning algorithms, including logistic regression, decision tree, random forest, and eXtreme Gradient Boosting (XGBoost), were examined. Temporal mean of laboratory parameters, including ALT, AST, and platelets, together with age, gender, race, and the presence of type 2 diabetes, were included as covariates. The four models yielded satisfactory classification performance with an AUROC of over 0.83 in internal validation (Table 4). This study demonstrated the possibility of using machine learning in identifying NASH in a large group of patients, while the good performance may be due to a more obvious separation between healthy individuals and NASH patients.

The NASHmap is another example of machine learning model for predicting NASH. Docherty and colleagues utilized a biopsy cohort to derive the machine learning models. Similarly, logistic regression, classification and regression trees (a.k.a. decision tree), random forest, and XGBoost were considered. Fourteen clinical and laboratory parameters were included in the models, which yielded AUROCs of around 0.7–0.8. Hemoglobin A1c (HbA1c) was found to be the most predictive covariate, followed by AST and ALT. The models were then externally validated in the Optum dataset and demonstrated comparable AUROC. Slightly reduced performance was observed in reduced models using five parameters, including HbA1c, AST, ALT, total protein, and triglycerides.[54] Moreover, Canbay et al.[55] developed a logistic regression model to distinguish NASH from NAFLD in obese patients, with an AUROC of 0.70 in an independent validation cohort. The logistic model included age, gamma-glutamyl transferase, CK-18 M30, adiponectin, and HbA_{1c}.[55] All of these laboratory-based machine learning models highlighted the importance of HbA1c, AST, and ALT in identifying NASH patients. On the other hand, there is emerging evidence of the difference in the characteristics of lipidomic, glycomic, and hormonal features in patients with NAFLD and NASH due to their strong relationship with metabolic syndrome. Perakakis and colleagues[56] incorporated these omics features into machine learning models including support vector machine, k-nearest neighbor classifier, and random forest. Using 29 features, the machine learning models achieved AUROCs of over 0.95 in selecting patients with NASH from patients with NAFLD or healthy individuals in internal validation (Table 4).[56]

Unsupervised learning can be useful to identify clinically relevant subtypes of NAFLD patients, including those with significant liver inflammation. Using a hierarchical clustering algorithm based on Manhattan distance of similarity, Vandromme and colleagues[57] identified five disease subtypes among NAFLD patients. Some of the subtypes showed evidence of liver inflammation, such as a high proportion of elevated ALT, as well as notable comorbidities, such as diabetes and hypertension.

The presence of lobular inflammation is one of the key histological characteristics of NAFLD activity score (NAS) besides the presence of hepatocyte ballooning and steatosis. Traditional scoring systems, such as the NAFLD activity score, only offer a non-linear and categorical assessment of the disease. Thus, machine learning has a role here to provide quantification of the assessment.[58] Liu and colleagues[58] developed an algorithm to analyze the liver biopsy and quantify different components of the NASH Clinical Research Network (CRN) scoring system. They used special microscopy and image analysis to visualize and quantify inflammation in liver biopsy.[58] The algorithms performed well in a three-center study to predict lobular inflammation and other components of the NASH CRN scoring system (Table 4).

DEEP LEARNING METHODS

Deep learning methods attempt to train deep neural networks for solving complex problems and show more promising prediction results compared to traditional methods based on handcrafted features. Recent deep learning techniques have led to wide applications in healthcare areas,[59] and they have been increasingly applied for the prediction and diagnosis of NASH. Popular deep learning approaches include the Convolutional Neural Network (CNN), Graph Neural Network (GNN), and Recurrent Neural Network (RNN). Besides developing sophisticated network architectures to improve prediction accuracy, other important questions in deep learning methods are also explored, such as model in-

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terpretability and annotation-efficient learning.

CNN is the most widely used technique of deep learning and has been proved effective in solving many medical problems. CNN achieves better performance when dealing with image-related tasks, such as analyzing CT, MRI, and pathology data. A typical model based on CNN contains a series of layers, including convolution layers, pooling layers, and fully connected layers. In convolution layers, each convolutional neuron only processes data within its receptive field, thus the architecture is ideal for large-scale data such as high-resolution images. NAS is important for diagnosing NASH, and liver biopsy is used for calculating NAS. CNN can be used for quantitative measurement of liver histology and disease monitoring in NASH, and CNN-based methods are proven accurate with strong correlations with expert pathologists and good risk stratification of patients with NASH.[60,61] CT is non-invasive and less expensive compared to liver biopsy, and recent works have proposed to combine the information from CT and pathology data for predicting NAS and fibrosis stage.[62] CNN is first used for feature extraction, and different fusion strategies are proposed to combine these two pieces of information for better prediction performance. Their results showed that combining data from different modalities is beneficial for improving the prediction performance of NAS. To conclude, existing studies have demonstrated that CNNs can automatically learn better features for NASH diagnosis compared to traditional approaches based on manually designed features.

GNN is a rapidly growing field of deep learning that is suitable for processing graph data which contains rich relation information among elements.[63] GNN is able to extract multi-scale localized spatial features by exchanging information between the nodes of graphs, and its key element is pairwise message passing. There is an increasing number of GNN applications, such as electrical health records modeling and synthesizing chemical compounds. GNN is also attracting more attention in pathology data analysis,[64] since it learns features that can well-represent the tissue spatial structure. A recent work proposed to study liver biopsy on two histological stains namely Trichrome (TC) and hematoxylin and eosin (H&E) with GNN.[65] The latent embeddings extracted from the graphs were concatenated to predict NAS, and their results showed superiority over competing methods. Graph representation is able to integrate the tissue features from the whole slide image, and deserves further study in the evaluation of tissue biopsies for NASH diagnosis.

RNN can process data with any length, and is a good choice for sequential data processing.[66,67] Electronic health records (EHRs) contain medical time series of laboratory tests, and RNN-based methods can analyze the conditions of patients using these records. Long short-term memory (LSTM) is a representative method of RNN, and its gating mechanism within each LSTM cell is effective to avoid the long-term dependency problem in standard RNNs. Deep learning approaches based on LSTM are utilized to identify patients at risk of developing NASH, and they have shown better performance compared to other competing methods, such as XGBoost.[68] Considering there is a large amount of EHRs available in hospitals, RNN-based methods can work as powerful tools to analyze these existing valuable data for NASH diagnosis.

Even though deep learning methods have achieved great success in solving many medical problems, applying them in clinical practice remains skeptical. However, deep learning methods are often described as "black boxes," and interpretability is especially important in the medical domain. Some recent works attempted to deal with the interpretability problem of deep learning methods. One promising solution is to incorporate domain knowledge into model design.[69] For example, clinically interpretable features (e.g., nuclei and fat droplets) can be incorporated into NAS prediction. Pathologists normally focus on the nuclei and fat droplet regions for evaluating a liver biopsy image and developing models to mimic the diagnosis process of pathologists is proven effective.[70] Moreover, the success of deep learning models depends on large-scale training data, while collecting such datasets is extremely difficult in the medical domain. Therefore, developing data-efficient deep learning models is important and requires further study for NASH diagnosis; and one possible solution is to fully utilize free-text reports stored in hospital archiving and communication systems.[71]

CONCLUSIONS AND PERSPECTIVES

This review summarizes the latest developments in histological and non-invasive assessments of inflammation in NAFLD. In routine clinical practice, non-invasive tests have already largely replaced liver biopsy in the evaluation of patients with NAFLD. However, liver biopsy remains valuable in cases of diagnostic uncertainty, such as uncertain etiology or

indeterminate or conflicting non-invasive test results. At present, liver biopsy is still required in late-phase clinical trials for NASH. The limitation of serial liver biopsies to determine NASH resolution has been well-documented. Artificial intelligence-aided assessment of key histological features, including ballooning and fibrosis, has made much progress and should be incorporated into future clinical trials, subject to agreement by the regulators. To the least, artificial intelligence has consistently demonstrated a much higher reproducibility than traditional pathological assessments. Eventually, the aim should be to use non-invasive tests in both clinical trials and routine clinical practice. With a disease that affects over 30% of the population, non-invasive tests are simply the only feasible option if we are to build robust and sustainable clinical care pathways and improve NAFLD management.

Authors' contribution

All authors were responsible for the writing plan, content, drafting and critical revision of the manuscript for important intellectual content.

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Conflicts of Interest

Terry Yip has served as a speaker and an advisory committee member for Gilead Sciences. Vincent Wong has served as an advisory committee member for AbbVie, Allergan, Echosens, Gilead Sciences, Janssen, Perspectum Diagnostics, Pfizer and Terns, and a speaker for Bristol-Myers Squibb, Echosens, Gilead Sciences and Merck. Grace Wong has served as an advisory committee member for Gilead Sciences and Janssen, as a speaker for Abbott, Abbvie, Bristol-Myers Squibb, Echosens, Furui, Gilead Sciences, Janssen and Roche, and received research grant from Gilead Sciences. The other authors declare that they have no competing interests.

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Review

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Genetics in non-alcoholic fatty liver disease: The role of risk alleles through the lens of immune response

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The knowledge on the genetic component of non-alcoholic fatty liver disease (NAFLD) has grown exponentially over the last 10 to 15 years. This review summarizes the current evidence and the latest developments in the genetics of NAFLD and non-alcoholic steatohepatitis (NASH) from the immune system's perspective. Activation of innate and or adaptive immune response is an essential driver of NAFLD disease severity and progression. Lipid and immune pathways are crucial in the pathophysiology of NAFLD and NASH. Here, we highlight novel applications of genomic techniques, including single-cell sequencing and the genetics of gene expression, to elucidate the potential involvement of NAFLD/NASH-risk alleles in modulating immune system cells. Together, our focus is to provide an overview of the potential involvement of the NAFLD/NASH-related risk variants in mediating the immune-driven liver disease severity and diverse systemic pleiotropic effects. **(Clin Mol Hepatol 2023;29(Suppl):S184-S195)**

Keywords: Nonalcoholic steatohepatitis; Genetics; PNPLA3; HSD17B13; Immune system

INTRODUCTION

The global trends in the prevalence and incidence of non-alcoholic fatty liver disease (NAFLD) represent a significant public health challenge. The disease prevalence has reached alarming figures not only in adults but also in the children's population.[1,2] Knowledge regarding the genetic component of NAFLD has grown exponentially over the last 10–15 years.[3-7] With this knowledge, it has become possible to translate information of risk alleles and its effects on the disease biology into clinical application.[6,8] Most importantly, knowledge on the genetic component of NAFLD may be lev-

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eraged to identify individuals at risk and/or to estimate the risk of severe histological outcomes, including non-alcoholic steatohepatitis (NASH)-fibrosis, cirrhosis, and hepatocellular carcinoma.[6,8]

While NAFLD is a disorder characterized by excess accumulation of fat in hepatocytes, in up to 40% of individuals with NAFLD, there are additional findings of portal and lobular inflammation and hepatocyte injury which characterize the severe histological forms of the disease.[3] Therefore, activation of the immune system is a key feature of the disease severity and progression.[3]

Furthermore, progressive clinical forms of NAFLD, including NASH-fibrosis, NASH-cirrhosis, and eventually hepatocellular carcinoma, are the main drivers of liver disease-associated mortality worldwide.[1,2]

Although remarkable progress has been made in understanding the disease biology, it remains unclear how to link NAFLD/NASH-associated variants with immune-specific cells mechanistically and how to explain the role of genetics in immune-driven disease progression.

In this review, we summarize the current evidence and the latest developments in the field of genetics of NAFLD and NASH—the disease' severe histological form—from the perspective of the role of risk alleles in modulating gene expression of cells of the immune system. Our focus is to provide an overview of the potential involvement of the NAFLD/NASH-related risk variants in mediating the immune-driven disease severity.

A SHORT OVERVIEW OF VARIANTS INFLUENCING THE RISK AND PROTECTION AGAINST NAFLD AND THE HISTOLOGICAL DISEASE SEVERITY

Genetic discoveries in the field of NAFLD have mainly been motorized by the use of genome-wide (GWAS),[9,10] exome-wide (EWAS),[11] and more recently, phenome-wide (PHEWAS) association studies using electronic health records,[12] as well as high-throughput sequencing technologies, which allow-refining and mapping of the discovered variants.[13]

Most relevant and replicated targets associated with the genetic component of NAFLD are illustrated in Figure 1, which depicts the primary protein function and subcellular localization. Notably, major candidate gene variants function in metabolic pathways.

Figure 2 summarizes the most replicated variants associated with NAFLD and NASH, including the global minor allele frequency, the variant's most severe consequence, the variant functionality, and the variant effect on the disease traits. It is interesting to point out that most of the variants associated with NAFLD and NASH are mapped to coding regions of the genome facilitating the variants' functional assessment.

The variants and single nucleotide polymorphisms (SNPs) identified in GWAS, EWAS, and PHEWAS, that were further replicated in extensive studies across the world as being associated with the NAFLD phenotype and the disease severity (NASH and NASH fibrosis), explain only approximately 30–50% of the estimated heritability of the disease. The effect of each SNP on NAFLD and disease-associated traits is relatively modest (Fig. 2).

However, the effect of rs738409 C/G variant located in *PNPLA3* (patatin-like phospholipase domain containing 3) on the risk of NAFLD and the disease progression is probably the strongest effect for a common variant modifying the genetic susceptibility of NAFLD and NASH (explaining ~5.3% of the total variance).[14] The evidence indicates that homozygous carriers of the G-risk allele of rs738409 present 3.24-fold greater risk of higher liver necroinflammatory scores and 3.2-fold greater risk of developing fibrosis when compared with homozygous CC.[14,15]

The rs58542926 C/T variant located in *TM6SF2* (Transmembrane 6 Superfamily Member 2) that was initially associated with liver fat accumulation and aminotransferase levels in a large GWAS study[11] and further replicated in subsequent candidate gene association studies[16,17] encodes for a protein involved in lipid metabolism. The rs58542926 is an important modifier of blood lipid traits in different populations. As a challenge in personalized medicine, the C-allele, which has an overall frequency as high as 93%, is associated with higher

Abbreviations:

GCKR, glucokinase regulator; GWAS, genome-wide association study; HSD17B13, hydroxysteroid 17-beta dehydrogenase 13; MBOAT7, membrane bound O-acyltransferase domain containing 7; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PNPLA3, patatin-like phospholipase domain containing 3; SNP, single nucleotide polymorphism; TM6SF2, transmembrane 6 superfamily member 2

blood lipids, whereas the T allele confers a moderate risk for NAFLD (carriers of the risk allele present approximately ~2.2% higher lipid fat content) but lower blood lipids.[18]

Likewise, the rs72613567 insertion/deletion variant in *HSD17B13* (hydroxysteroid 17-beta-dehydrogenase 13), the functional consequence of which is a splice donor variant of the *HSD17B13* [12], presents protective effect against NAFLD and severe histologic outcomes.[12,19,20]

The modest effects on NAFLD risk of the rs780094 in *GCKR* (glucokinase regulator)—odds ratio(OR) ~1.2[21] and rs641738 located in *TMC4* (transmembrane channel-like 4) exon 1 (p.Gly17Glu) and 500 bases downstream of the *MBOAT7* (*TMC4/MBOAT7*)—~OR 1.17,[22] are also highlighted in Figure 2.

In addition, the genetic architecture of NAFLD and NASH involves rare variants in other loci, for example, the recently discovered p.P426L loss-of-function variant (rs143545741 C>T) located in autophagy-related 7 (*ATG7*).[23] Furthermore, a rare nonsense mutation (rs149847328, p.Arg227Ter) in the glucokinase regulator (*GCKR*) has also been recently reported in an adult patient with NAFLD, morbid obesity, and type 2 diabetes. The p.Arg227Ter was associated with a rapidly progressive histological form of the disease.[24]

Besides, the genetic component of NAFLD and NASH involves mutations in genes of the oxidative phosphorylation (OXPHOS) chain of the mitochondrial DNA (mtDNA),[25,26] and variants in long noncoding RNAs (lncRNAs), which have a remarkable role in transcriptional and epigenetic regulation.[27,28] Moreover, we reported that deregulated expression of a particular lncRNA, metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*), stratifies patients into the histologic phenotypes associated with NAFLD severity.[28] *MALAT1* upregulation seems to be a common molecular mechanism in immune-mediated chronic inflammatory liver damage, which suggests that convergent pathophenotypes (inflammation and fibrosis) share similar molecular mediators leading to cancer.[28]

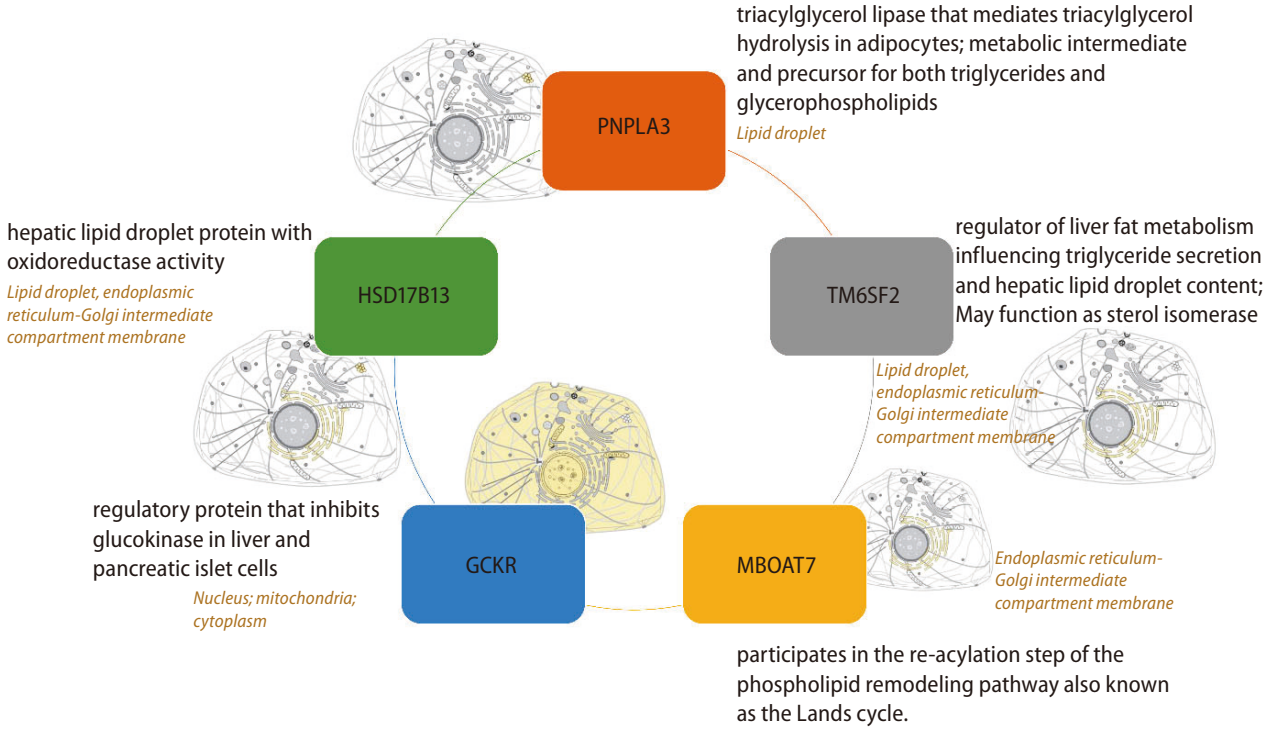

Figure 1. Most relevant and replicated targets associated with the genetic component of NAFLD. Figure depicts primary protein function and subcellular localization. Information was retrieved from UniProt, a comprehensive and freely accessible resource of protein sequence and functional information available at https://www.uniprot.org. NAFLD, non-alcoholic fatty liver disease; PNPLA3, patatin-like phospholipase domain containing 3; HSD17B13, hydroxysteroid 17-beta dehydrogenase 13; MBOAT7, membrane bound O-acyltransferase domain containing 7; GCKR, glucokinase regulator; TM6SF2, transmembrane 6 superfamily member 2.

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Genetic variants associated with NAFLD: effects and global MAF

Gene	Variant ID	Most severe consequence	Global MAF	Functionality	Effect
PNPLA3	**rs738409** SNP	missense variant	MAF: **0.26** (G) Highest population MAF: **0.48** (LA population)	Affect lipid trafficking in hepatocytes; I148M substitution renders PNPLA3 resistant to ubiquitylation.	**OR 3.24** ↑ risk of higher NAFLD/ **OR 3.44** ↑ risk NASH 5% variance
TM6SF2	**rs58542926** SNP	missense variant	MAF: **0.07** (T) Highest population MAF: **0.16** (East Asian)	Loss of function; liver allele-specific transcript abundance	**OR 2.2** ↑ risk NAFLD
HSD17B13	**rs72613567** INDEL	splice donor variant	MAF: **0.18** (A) Highest population MAF: **0.40** (East Asian)	Unstable and truncated protein with reduced enzymatic activity	**OR 0.80-0.67**↓ risk NASH
GCKR	**rs780094** SNP **rs1260326** SNP	Intron variant missense variant	MAF: **0.30** (T) Highest population MAF: **0.50** (East Asian) MAF: **0.29** (T) Highest population MAF: **0.50** (East Asian)	Unclear	**OR 1.2 -1.32** ↑ risk NAFLD
TMC4/ *MBOAT7*	**rs641738** SNP	TMC4: Missense Variant/ MBOAT7: 500B Downstream Variant	Highest population MAF: **0.49**	Changes in the hepatic phosphatidylinositol acyl-chain remodeling	**OR 1.17** ↑ risk NAFLD/1.22 ↑ risk fibrosis

http://www.ensembl.org/

Figure 2. Summary of variants influencing the risk and protection against NAFLD and the histological disease severity. The figure depicts the most replicated variants associated with NAFLD and NASH, including the global minor allele frequency, the most severe consequence of the variant, and the linked variant functionality. In addition, the figure highlights the main effect(s) on the risk and/or protection against NAFLD and NASH. Information was retrieved from Ensembl (available at https://www.ensembl.org/). NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PNPLA3, patatin-like phospholipase domain containing 3; HSD17B13, hydroxysteroid 17-beta dehydrogenase 13; MBOAT7, membrane bound O-acyltransferase domain containing 7; GCKR, glucokinase regulator; TM6SF2, transmembrane 6 superfamily member 2; TMC4, transmembrane channel-like 4; OR, odds ratio; MAF, minor allele frequency; SNP, single nucleotide polymorphism; LA, Latino population.

NOVEL ASPECTS OF GENETICS IN NAFLD: GENE VARIANTS AND INTERACTION EFFECTS

The nonsynonymous rs738409 variant in *PNPLA3* is regarded as the major genetic component of NAFLD and NASH.[9,14,15] The risk effect of this variant on developing fatty liver is the strongest ever reported for a common variant modifying the genetic susceptibility of NAFLD (5% of the total variance).[14,15] A recent two-stage (discovery and replication) GWAS that included NAFLD patients characterized by liver biopsy confirmed the rs738409 variant in *PNPLA3* as a risk factor for the full histological spectrum in patients of European ancestry.[29] Likewise, this large GWAS confirmed important contributions from variants in *TM6SF2* (rs58542926) and *HSD17B13* (rs72613567), but not *MBOAT7* (rs641738), in the disease biology.[29]

Like many other complex diseases, NAFLD results from the interaction between genes and environmental factors.[5-7] Hence, in addition to individual genetic susceptibility, other important factors contribute to the phenotypic expression of NAFLD and NASH, including dietary patterns and food.

There have been attractive studies which focused on gene-diet interaction effect/s, for example, a recent study assessing a gene-diet interaction among rs738409, nutrient intake, and liver histology severity.[30] Vilar-Gomez et al.[30] showed that *PNPLA3* rs738409 G-allele might modulate the effect of specific dietary nutrients on the risk of fibrosis in patients with NAFLD.

Other studies have explored gene-gene interaction effects, which are also known as epistasis. For example, Vilar-Gomez et al.[31] found that the protection conferred by *HSD17B13* rs72613567 A-allele on severe histological outcomes may be limited to selected subgroups of individuals. Specifically, the protective effects of rs72613567 A-allele on the risk of inflammation and fibrosis seem to be notably stronger in women, persons aged 45 or older, individuals with diabetes, or those

with body mass index ≥35, even after adjusting for the other relevant confounders.[31]

Other human studies have explored the direct effect of the *PNPLA3* rs738409 on developing liver fibrosis in relation to liver histologic traits. Specifically, Vilar-Gomez et al.[32] recently reported that a large proportion of the indirect effect of rs738409 on fibrosis severity is mediated through portal inflammation.

Finally, recent studies have highlighted the influence of genetic variants, including variants influencing the risk and protection against NAFLD-histological severity (*PNPLA3*-rs738409, *TM6SF2*-rs58542926, *MBOAT7*-rs641738, and *HSD17B13*-rs72613567) and a variant influencing macronutrient intake (*FGF21*-rs838133), on the liver microbial DNA composition.[33] For example, Pirola et al.[33] found that members of the Gammaproteobacteria class were significantly enriched in carriers of the rs738409 and rs58542926 risk-alleles, including Enterobacter and Pseudoalteromonas genera, respectively.

GWAS ON NAFLD AND VARIANTS IN IMMUNE-RELATED LOCI

The analysis of the GWAS catalog using the EMBL-EBI dataset (EMBL's European Bioinformatics Institute) has shown interesting associations between variants in immune-related loci and NAFLD (Table 1). The human major histocompatibility complex on chromosome 6p21 has been associated with susceptibility to many liver diseases. GWAS confirmed the potential association of NAFLD with many variants in HLA genes and interleukin 36 alpha (*IL36A*) and beta (*IL36B*) (Table 1).

To obtain a more comprehensive view of the overlap between NAFLD and immune system-associated genes, we searched the literature with the query "NAFLD" and "immune system" using the web-based platform Genie (available at cbdm.mdc-berlin.de/tools/genie/).[34] Using a cutoff of 0.01 for abstracts and a false discovery rate <0.01 for genes, we retrieved 941/983 and 975/1,524 abstracts/genes, corresponding to NAFLD and the immune system, respectively. Two hundred fifty-eight genes were associated with both NAFLD and the immune system (Fig. 3A). As shown in Supplementary Figure 1, some of the 258 overlapping genes are expressed preferentially in cells of the immune system, for example, *MPO* (myeloperoxidase), a major component of neutrophil azurophilic granules. In contrast, certain genes, such as *C3* (complement C3), *SERPINA1* (serpin family A member 1, a serine protease inhibitor), or *KART18/19* (keratin 18 and 19, intermediate filament chain keratins), are expressed in different adult tissues, including liver, heart, ovary, lung, or colon (Supplementary Fig. 1). Only a few are expressed in any cells, for example, *KRT8*, *HSPD1* (heat shock protein family D member 1) or *HSPA5* (heat shock protein family D member 5, encoding a mitochondrial protein which may function as a signaling molecule in the innate immune system).

Both gene groups were significantly enriched in anti-apoptotic, cell communication, and signal transduction biological processes (Fig. 3B). As expected, the molecular function characterizing NAFLD-the immune system-shared genes are significantly similar (i.e., ligand-dependent nuclear receptor, chemokine, growth factor, cytokine, and receptor activities) (Fig. 3C). Finally, Figure 3D shows shared genes-associated transcription factors (TF). As novel findings, we found *BACH1*, which encodes a TF that belongs to the Cap'n'collar (CNC)

Table 1. GWAS catalog and associations between variants in immune-related loci and NAFLD susceptibility

Mapped loci	Variant ID	*P*-value	Study accession	Chromosome location
IL36A, IL36B	rs28946269	9×10^{-6}	GCST008468	2:113011237
HLA-DRB5, HLA-DRB9	rs7748270	4×10^{-12}	GCST90094908	6:32480822
HLA-DRB1, HLA-DQA1	rs5021727	3×10^{-9}	GCST90094908	6:32610856
HLA-DQA1, HLA-DRB1	rs9271325	2×10^{-8}	GCST90094908	6:32614736
HLA-DQA1, HLA-DRB1	rs9271406	2×10^{-8}	GCST90094908	6:32619811
HLA-DQA1	rs2213287	5×10^{-9}	GCST90094908	6:32637731
HLA-DQA1	rs9272699	2×10^{-8}	GCST90094908	6:32641452

GWAS, genome-wide association study; NAFLD, non-alcoholic fatty liver disease.
Source: https://www.ebi.ac.uk/ EMBL's European Bioinformatics Institute.

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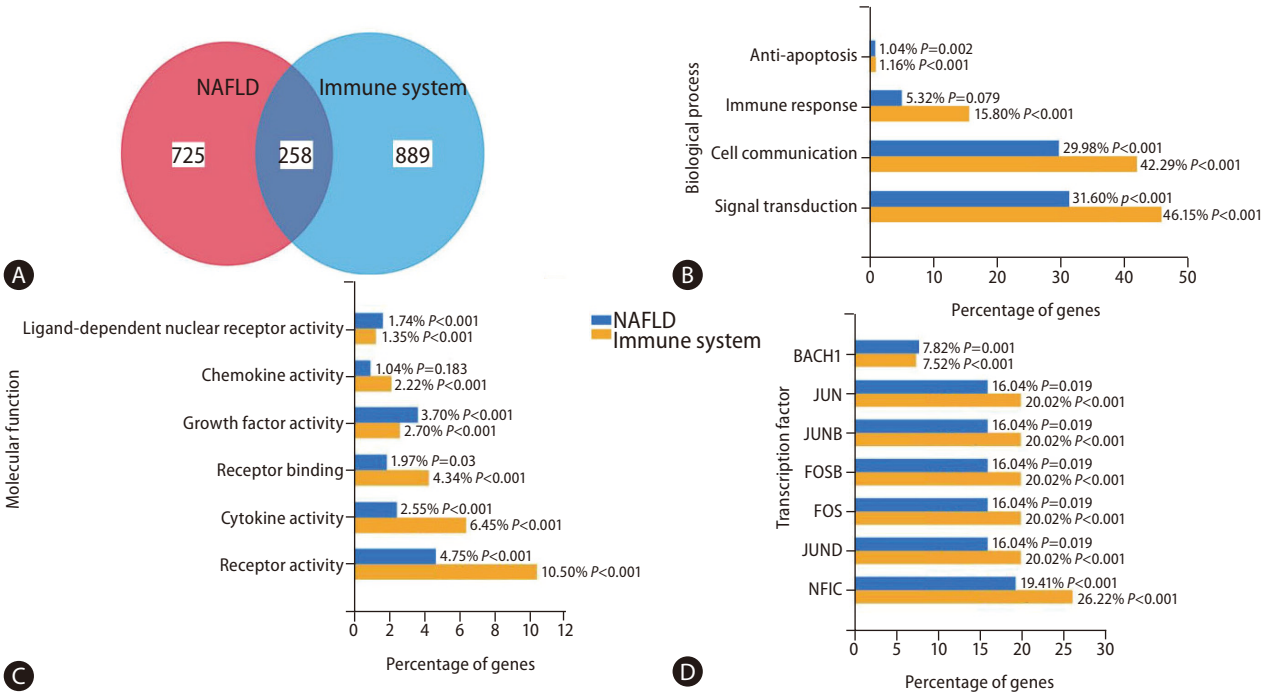

Figure 3. Overlapping between gene variants associated with NAFLD and the immune system. This figure provides a functional enrichment analysis of the genes and proteins associated with NAFLD and the immune system, the information of which has been extracted from heterogeneous genomic and proteomic resources using the FunRich program.[55,56] The literature was searched with the query "NAFLD" and "immune system" by using the platform Genie (available at https://edoc.mdc-berlin.de/11677/)[34] with a cutoff of 0.01 for abstracts and an FDR <0.01 for genes. Pathways were ranked according to the *P*-value, whereby $P<0.05$ was considered statistically significant. % indicates the percentage of altered genes in the whole pathway. (A) Venn diagram of the corresponding gene lists with overlapping genes obtained by the FunRich program, a user-friendly tool, which performs functional enrichment analysis on the generated datasets (available at http://www.funrich.org/).[55] (B) Enrichment of genes of both lists in Biological Processes. (C) Enrichment of genes of both lists in Molecular Function. (D) Associated transcription factors. NAFLD, non-alcoholic fatty liver disease; FDR, false discovery rate.

type of basic region leucine zipper factor family (CNC-bZip) associated with cancer metastasis.[35] On the other hand, we also found *NFIC*, whose encoded protein belongs to the *CTF/NF-I* family. These are dimeric DNA-binding proteins that function as cellular TFs and as replication factors for adenoviruses, which also play a role in cancer cell proliferation and metastasis through an epithelial-to-mesenchymal transition process.[36]

Finally, results from a recent study using multicellular liver culture that recapitulates many key features of NAFLD suggested a potential causal link between elevated interleukin 6 (IL6)/STAT3 activity and rs738409-mediated susceptibility to NAFLD.[37] Park et al.[37] showed that dampening IL6-STAT3 activity alleviated the rs738409-G risk allele-mediated risk of NAFLD. This effect was attributed to the elevated IL6-STAT3 activity in liver cultures carrying the rs738409 G-risk allele that increased NF-kB activity.[37] This finding has clinical implications. For instance, a network-based druggability assessment for STAT3, which examines the structure or the protein-protein interaction around the target, suggests that STAT3 is a good drug target presenting a ligand-based druggability score of 97%.[6] In addition, this finding is particularly relevant in light of the association between NAFLD-predisposing risk factors, including obesity and insulin-resistance, and *STAT3* gene variants.[38]

Interestingly, from the above-described approach of clustering NAFLD and the immune system-associated genes, we retrieved a long list of potential drugs to target the disease (data not shown). Among the obvious repurposed drug candidates, such as non-steroid anti-inflammatory drugs, statins, antidiabetic drugs, etc., auranofin emerged. Hwangbo et al.[39] reported that auranofin ameliorates the characteristics of NAFLD through the inhibition of NLRP3 inflammasome, and Lee et al.[40] recently found that auranofin attenuates hepatic

steatosis and fibrosis in NAFLD via NRF2 and NF-kappaB signaling pathways.

RISK ALLELES IN COMMON VARIANTS ASSOCIATED WITH NAFLD/ NASH AND GENE REGULATION OF IMMUNE SYSTEM: eQTLs

Activation of the immune system, including innate and or adaptive immune response, is an essential driver of the disease severity and progression.[3] While various immune-responsive cells are involved in the pathogenesis of NASH, including T cells and natural killer T cells, the classical effectors of NASH-linked inflammation are Kupffer cells and recruited macrophages.[3] In addition, the infiltrated immune cells play several roles in the liver of NASH patients, including the release of cytokines, chemokines, and eicosanoids, among other inflammatory factors.[3,41]

Analysis of genetic pathways in NASH has shown that the immune system is significantly enriched with the sub-pathway "innate immune system" and "cytokine signaling in the immune system".[7] However, much remains to be understood in how risk alleles modify the immune system.

The genomic tools, including GWAS complemented by expression quantitative trait locus (eQTL) analyses, are powerful instruments for understanding how disease-linked variants regulate the expression of quantitative molecular phenotypes across diverse tissues.

GWAS of complex diseases, including NAFLD and NASH, showed that some gene variants are implicated in the susceptibility of multiple traits—a phenomenon known as pleiotropy.[42] This feature involves not only the rs738409 variant in *PNPLA3* but also variants in *TM6SF2*, *HSD17B13*, and *MBOAT7* that are associated with diverse laboratory measurements related to hematological traits.[42]

In addition, the rs738409 has been shown to be associated with the soluble intercellular adhesion molecule 1 (sICAM-1) concentration in a large GWAS involving 22,435 healthy women from the Women's Genome Health Study.[43] ICAM-1 is an endothelium and cells of the immune system-derived inflammatory marker. This finding is particularly relevant, as previous studies demonstrated that NAFLD is associated with elevated circulating levels of sICAM-1 and abnormal liver expression of ICAM-1.[44] Furthermore, it was found that liver ICAM-1 expression levels are significantly correlated with liver lobular inflammatory infiltrate and the severity of necroinflammatory activity.[44]

Another important aspect is the exploration of the influence of genetic variation on gene expression across tissues and cell types. For example, Table 2 shows the associations of

Table 2. Variants in NAFLD/NASH-associated genes and regulation of gene expression in immune related loci

Tissue	Gene expression	Sample size	*P*-value
SNP ID: rs738409 hg19_coordinates: chr22:44324727 (*PNPLA3*)			
Adipose subcutaneous	*SAMM50*	385	$9.60E^{-07}$
Whole blood	*SAMM50*	5,257	$5.67E^{-106}$
Whole blood	*SAMM50*	31,300	$3.15E^{-18}$
Whole blood	*FAM89B*	31,300	$5.49E^{-06}$
SNP ID: rs58542926 hg19_coordinates: chr19:19379549 (*TM6SF2*)			
Whole blood	*CXCL9*	5,257	$3.13E^{-06}$
Whole blood	*CXCL16*	28,533	$5.71E^{-06}$
SNP ID: rs641738 hg19_coordinates: chr19:54676763 (TMC4/MBOAT7)			
Whole blood	*LILRP1*	5,417	$8.87E^{-06}$

Variant: rs738409, gene: *PNPLA3*. Variant: rs58542926, gene: *TM6SF2*. Variant: rs72613567, gene: *HSD17B13*. Variant: rs641738, gene: MBOAT4/TMC4.
SNP, single nucleotide polymorphism; PNPLA3, patatin-like phospholipase domain containing 3; TM6SF2, transmembrane 6 superfamily member 2; MBOAT7, membrane bound O-acyltransferase domain containing 7; TMC4, transmembrane channel-like 4; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis.
Information was retrieved from http://www.phenoscanner.medschl.cam.ac.uk/, a curated database holding publicly available results from large-scale genome-wide association studies.[45,46]

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major variants in NAFLD-NASH genes with gene expression levels in non-liver tissues, of which information has been extracted from PhenoScanner, a curated database holding publicly available results from large-scale genome-wide association studies.[45,46]

The rs738409 is associated with adipose tissue and blood expression levels of *SAMM50*, which plays a crucial role in the maintenance of the structure of mitochondrial cristae, the proper assembly of the mitochondrial respiratory chain complexes, and/or the maintenance of mtDNA.[47] In addition, the rs738409 is associated with whole blood expression levels of *FAM89B* (Family With Sequence Similarity 89 Member B), which negatively regulates TGFb-induced signaling—a key factor involved in the regulation of immune response.[48]

The rs58542926 in *TM6SF2* is associated with blood expression levels of *CXCL9 (*C-X-C Motif Chemokine Ligand 9)—a member of the chemokine superfamily that encodes secreted proteins involved in immunoregulatory and inflammatory processes, and expression levels of *CXCL16* (C-X-C Motif Chemokine Ligand 16), which is involved in several processes, including positive regulation of cell growth, response to interferon-gamma, and response to tumor necrosis factor.

The rs641738 in *MBOAT7* is associated with the whole blood expression levels of *LILRP1* (leukocyte immunoglobulin-like receptor pseudogene)—also known as leukocyte-expressed receptors of the immunoglobulin superfamily.

RISK ALLELES IN COMMON VARIANTS ASSOCIATED WITH NAFLD/NASH AND ITS RELATIONSHIP WITH IMMUNE SYSTEM CELLS TYPES

In the last few years, novel molecular approaches have allowed the differentiation between eQTLs in "bulk" samples of different tissues and "single cell" eQTLs. The difference is that eQTLs from bulk samples represent the average gene expression across all cells in a given tissue. Conversely, eQTLs using single-cell sequencing technology (scRNA-seq) allow the cell-specific gene expression signature (cell type-specific eQTLs).

Although technological advances illuminate the pathophysiology of NAFLD, how the major genetic variants associ-

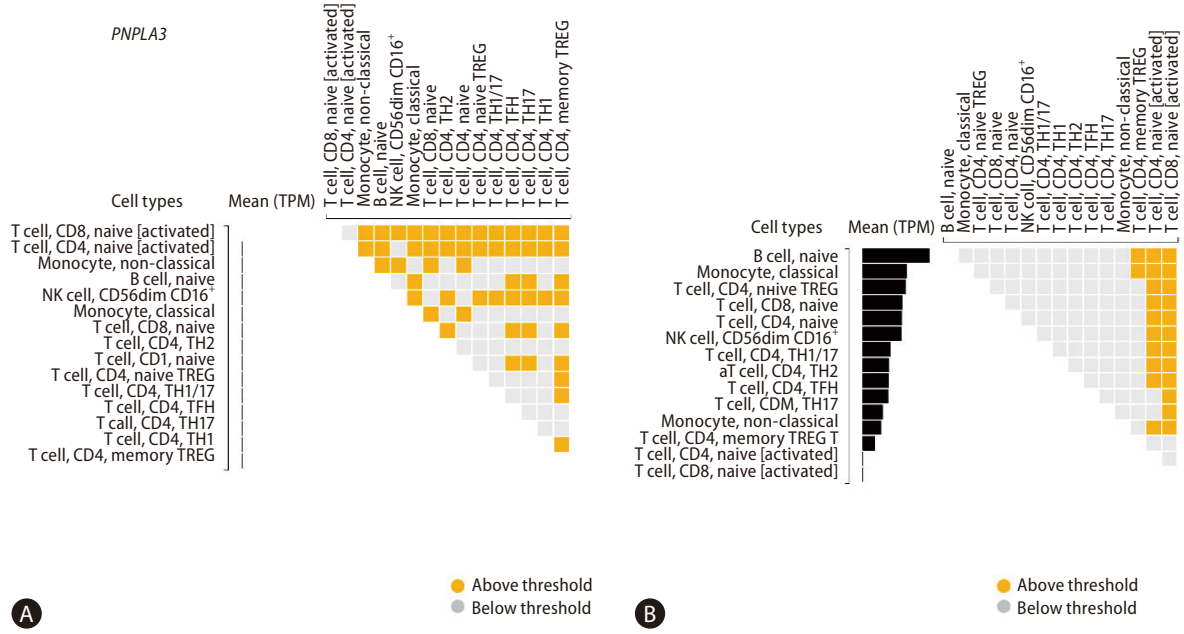

Figure 4. Gene expression levels of *PNPLA3* and *HSD17B13* in immune cells. Differential gene expressions of *PNPLA3* (A) and *HSD17B13* (B) across cell types as calculated by the DESeq package (version 1.6.3). Cells are sorted based on the median gene expression from the highest to the lowest. Squares in the upper diagonal matrix indicate results from pair-wise comparisons of two cell types on the x-axis and y-axis. The figure shows 2 log2 fold-change. Findings were retrieved from the DICE (Database of Immune Cell Expression, Expression quantitative trait loci [eQTLs] and Epigenomics) project (available at https://dice-database.org). PNPLA3, patatin-like phospholipase domain containing 3; HSD17B13, hydroxysteroid 17-beta dehydrogenase 13.

ated with the risk (rs738409) and protection (rs72613567) against NAFLD and NASH affect the gene expression of specific immune cells remains largely unknown. To gain further insight into this aspect, we explored the DICE database (database of immune cell expression, eQTLs, and epigenomics), which helped to reveal the effects of disease risk-associated genetic polymorphisms on specific immune cell types (https://dice-database.org).[49]

Figure 4 shows differential gene expressions of *PNPLA3* and *HSD17B13* across specific immune cell types. We found very modest levels of *PNPLA3* expression in T cell, CD8, naïve [activated], and T cell, CD4, naive [activated] (Fig. 4A). In addition, we explored the genetic variants directly associated with *PNPLA3* gene expression level (SNP located within +/– 1 Mb of the TSS) or eQTLs, and found three single nucleotide polymorphisms in chromosome 22 influencing T cell, CD4, memory TREG, including rs5766088, rs9626589, and rs9626589.

Conversely, we found significant levels of *HSD17B13* expression across a variety of immune cells, including B cell, naïve monocyte, classical T cell, CD4, naive TREGT cell, CD8, naïve T cell, CD4, naïve natural killer (NKO cell, CD56dim CD16+T cell), CD4, TH1/17T cell, CD4, TH1T cell, CD4, TH2T cell, CD4, TFHT cell, CD4, TH17 monocyte, non-classical, and T cell, CD4, memory TREG (Fig. 4B). More importantly, in addition to these cells being relevant effectors of cytotoxicity, these findings were also aligned with our previous results on the effect/s of the splice variant rs72613567 in *HSD17B13* on the liver transcriptome. Specifically, we found that the most signifi-

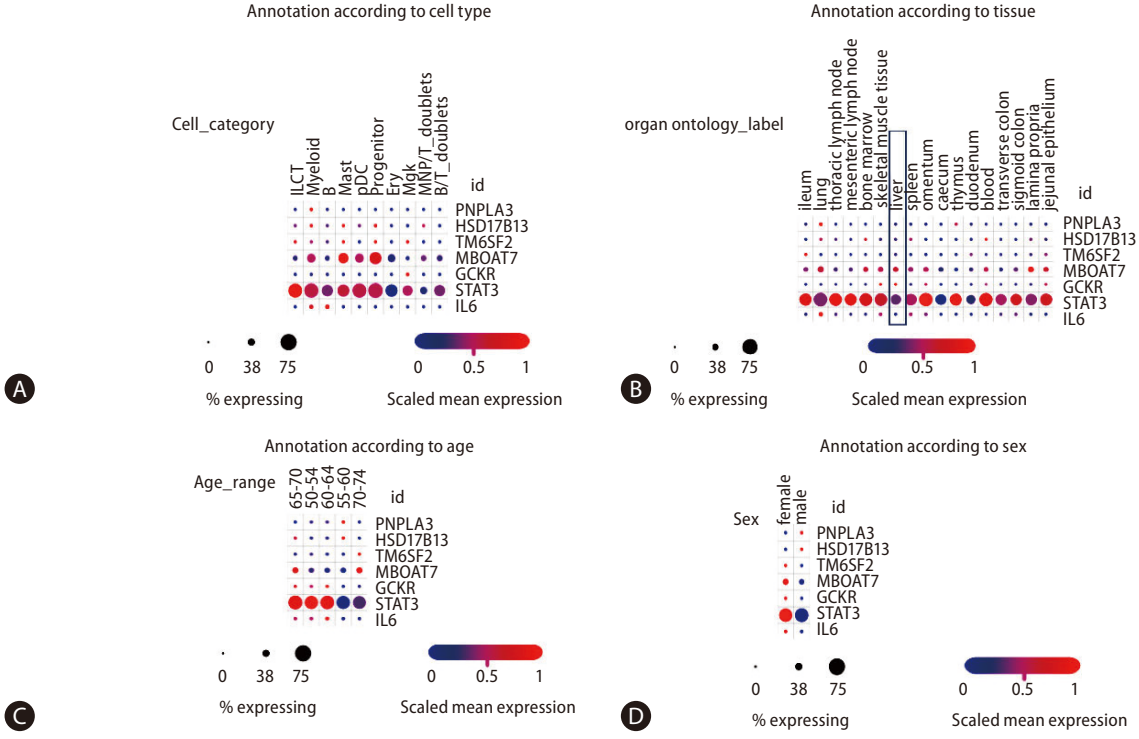

Figure 5. Analysis of NAFLD/NASH-risk alleles and cross-tissue immune cell expression. Information was retrieved from Single Cell Portal (available at https://singlecell.broadinstitute.org/single_cell/study/SCP1845/).[50] Panels depict annotation of cell population type (A), organ/tissue distribution (B), age (C), and sex of donors (D). Scaling is relative to each gene's expression across all cells in a given annotation selection (i.e., cells associated with each column label in the dot plot). Gene targets were arbitrarily selected, including major NAFLD/NASH-related loci and two immune-related genes (*IL6* and *STAT3*). ILCT: innate lymphoid cells, pDCs: plasmacytoid dendritic cells, which are a unique subset of dendritic cells specialized in secreting high levels of type I interferons, myeloid: myeloid cells are granulocytic and phagocytic leukocytes that traverse blood and solid tissues, B: B lymphocytes, also called B cells, mast: mast cells are immune cells of the myeloid lineage, progenitor: the common B- and T-cell progenitor can be found in the bone marrow, ery: *erythroid* cells, mgk: megakaryocytes/platelets, MNP/RT doublets cells: mononuclear phagocytes, B/T doublets: B and T cells stuck together as a "doublet." NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; IL6, interleukin 6; PNPLA3, patatin-like phospholipase domain containing 3; HSD17B13, hydroxysteroid 17-beta dehydrogenase 13; MBOAT7, membrane bound O-acyltransferase domain containing 7; GCKR, glucokinase regulator.

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cant changes in the liver gene expression are enriched by biological pathways related to the immune system, including antigen presentation and interferon-related processes, cytokine signaling, and signal transduction.[19]

More recent studies on multi-tissue single-cell transcriptomics have allowed a broader understanding of the genetic architecture of complex diseases concerning the cross-talk between genetic variants and immune cells.[50] Domínguez Conde et al.[50] profiled immune cell populations isolated from a wide range of donor-matched tissues, generating nearly 360,000 single cell transcriptomes. Using data from the study by Domínguez Conde et al.[50], which can be freely retrieved from the Single Cell Portal, we explored the distribution of *PNPLA3*, *HSD17B13*, *STAT3,* and *IL6* expressions across tissues and immune cell types using (Fig. 5). On the one hand, we observed that the expression levels of *PNPLA3* are generally very modest across cell types compared to *HSD17B13* levels, which present higher levels of expression in innate lymphoid cells, myeloid, mast, and progenitor cells, as well as megakaryocytes (Fig. 5A)—despite the relatively low percentage of gene-expressing cells. On the other hand, *MBOAT7* presents a relatively high level of expressions in myeloid, mast, and progenitor cells, with more than 50% of cells expressing the gene (Fig. 5A). As expected, *STAT3* presents not only very high levels of expression across diverse immune cells in all conditions, but also significant levels of expression in the liver tissue (Fig. 5B).

Remarkable differences in gene expressions across different age groups are also present in Figure 5C, the biological meaning of which remains unknown. However, these differences might explain differences in disease outcomes and sexual dimorphism (Fig. 5D).

CONCLUSION

Recent findings based on GWAS, single cells transcriptomics, and analysis of eQTLs may prime future studies that can help to understand the functional basis of shared loci between NAFLD and NASH and immune-mediated mechanisms of the disease severity.

Likewise, while translating GWAS, EWAS, and PHEWAS signals into clinical applications has been slow, genetic knowledge in NAFLD and NASH may significantly improve disease management and monitoring. The accumulated genetic knowledge is now being used to predict disease outcomes and personalized medicine in the field of NAFLD,[8,51,52] and to repurpose drugs and/or select potential actionable targets to treat the disease.[4,53,54]

Authors' contribution

C.J.P concept of the work, manuscript writing and approval. S.S. concept of the work, manuscript writing and approval.

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Conflicts of Interest

The authors have no conflicts to disclose.

SUPPLEMENTARY MATERIAL

Supplementary material is available at Clinical and Molecular Hepatology website (http://www.e-cmh.org).

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Review

Identification of high-risk subjects in nonalcoholic fatty liver disease

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Non-alcoholic fatty liver disease (NAFLD) is becoming the most common liver disease worldwide, and its burden is expected to increase due to the growing epidemic of obesity and diabetes. The key challenge among NAFLD patients is to identify those with advanced fibrosis (F3F4), who are at high risk of developing complications and will benefit from specialized management and treatment with new pharmacotherapies when they are approved. Liver biopsy appears unrealistic and unsuitable in practice, given the large number of high-risk patients and its well-known limitations. Non-invasive sequential algorithms using fibrosis-4 index as first-line test, followed by vibration-controlled transient elastography or patented blood test, are the best strategy for case finding of high-risk subjects. In fact, they are now recommended by several international guidelines, and should be used and disseminated to increase awareness among physicians beyond liver clinics where most NAFLD patients are seen. **(Clin Mol Hepatol 2023;29(Suppl):S196-S206)**

Keywords: Non-alcoholic fatty liver disease; Elastography; Vibration controlled transient elastography; FibroScan; Liver fibrosis

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) affects around one-fourth of the general population worldwide.[1] NAFLD encompasses a wide range of lesions, ranging from simple steatosis to non-alcoholic steatohepatitis (NASH), with faster liver fibrosis progression as well as the risk of developing cirrhosis and its complications, including hepatocellular carcinoma (HCC).[2] However, the vast majority of NAFLD patients will not progress, and only a minority, namely those with NASH and advanced fibrosis (F3, bridging fibrosis and F4, cirrhosis), are at the greatest risk of developing complications of chronic liver disease.[3] Patients with metabolic risk factors, particularly obesity and type 2 diabetes (T2DM), are at the highest risk of progressing to cirrhosis and HCC.[4] Due to the growing epidemic of obesity and diabetes, the burden of NAFLD is expected to increase.[5] Despite its high burden, NAFLD remains a largely under-recognized disease in primary care where most patients are seen. Additionally, the majority of NAFLD cases are asymptomatic with mild liver test abnormalities, making their identification a tough challenge for physicians in their daily clinical practice. As a result, less than 10% of patients diagnosed with NAFLD are referred to specialists.[6] Finally, there currently is no approved pharmacologic treatment for NAFLD. Therefore, the key challenge is to identify the minority of NAFLD patients with advanced fibrosis, who are at the greatest risk of developing complications, and will benefit from specialized management and treat-

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ment with new pharmacotherapies when they are approved.

For many years, liver biopsy has been considered the gold standard for the staging of liver fibrosis. However, it appears unrealistic and unsuitable, given the large number of high-risk patients and its well-known limitations.[7] Non-invasive strategies have been proposed as an alternative, and they have been an area of intensive research over the past decade.[8] These strategies include serum biomarkers of fibrosis and liver stiffness measurement (LSM), using either ultrasound- or magnetic resonance-based elastography techniques. Although none of these non-invasive tests can adequately discriminate NASH from simple steatosis in patients with NAFLD, they are now extensively used in liver clinics to detect advanced liver fibrosis and are recommended by international guidelines.[9-11] In this review, we discuss the performance, advantages, and limitations of non-invasive tests for identifying high-risk NAFLD patients.

WHO ARE THE HIGH-RISK NAFLD PATIENTS?

In NAFLD patients, NASH is the driver of fibrosis progression, but the presence of NASH without significant fibrosis is not associated with increased liver-related mortality or overall mortality.[12,13] probably due to the competing mortality risks of cardiovascular disease and non-liver related cancers in these patients. Several studies have reported that, besides the high rate of liver-related complication, the risk of all-cause mortality is clearly increased in NAFLD patients with advanced fibrosis.[14,15] In addition, two meta-analyses, based mostly on longitudinal retrospective studies, have shown that, the main prognosis driver for liver-related and overall mortality in NAFLD patients is the stage of liver fibrosis, namely advanced fibrosis.[16,17] These findings have been recently confirmed prospectively in the NASH CRN cohort (n=1,773 NAFLD patients), followed over a median period of 4.0 years (total: 8,120 person years).[18] Indeed, all-cause mortality increased with increasing fibrosis stages (0.32 deaths per 100 person-years for stage F0 to F2, 0.89 deaths per 100 persons-years for stage F3, and 1.76 deaths per 100 person-years for stage F4. Thus, it has now been well established that the risk of liver-related complications in NAFLD exponentially increases when transitioning to stage F3 and then F4. Advanced fibrosis is, therefore, the primary lesion to target when designing strategies to detect high-risk NAFLD patients who have the worse clinical outcomes.

Despite major efforts in the development of new drugs.[19] no pharmacologic treatment has yet been approved for NAFLD. The current consensus is that pharmacotherapy should be reserved for patients with NASH and at least significant fibrosis. At-risk NASH (or fibrotic NASH) is defined by the presence of NASH (NAFLD activity score ≥4 with one item of each, at least) and significant fibrosis (fibrosis stage ≥2).[20] Identification of these patients is important in tertiary referral centers, as they are the main target population for ongoing NASH phase 2 and 3 trials.

HOW TO IDENTIFY HIGH-RISK NAFLD PATIENTS?

Available non-invasive tools

Non-patented blood tests

The most commonly used non-patented blood tests are the fibrosis-4 index (FIB-4) and the NAFLD fibrosis score (NFS). The FIB-4 includes four simple parameters: age, platelets, and serum transaminases (aspartate aminotransferase [AST] and alanine aminotransferase [ALT]). The NFS includes seven parameters: age, body mass index (BMI), impaired fasting glucose/T2DM, AST, ALT, platelets, and albumin. The FIB-4 was initially developed for the non-invasive diagnosis of fibrosis in a set of 555 patients with HIV-chronic hepatitis C co-infection,[21] and then was also evaluated for the diagnosis of advanced liver fibrosis in NAFLD.[22] Contrary to FIB-4, the NFS has been developed specifically in NAFLD, in a large set including 480 patients.[23] Evidence for the accuracy of FIB-4 and NFS in NAFLD has now reached the level of meta-analysis, with area under the receiver-operating-characteristic curve (AUROC) at 0.76 for FIB-4 and 0.73 for NFS.[22] The results of these two tests were interpreted using two diagnostic thresholds, a lower to rule-out advanced liver fibrosis (FIB-4

Abbreviations:

ELF™, Enhanced Liver Fibrosis; GPs, general practitioners; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NFS, NAFLD fibrosis score; VCTE, vibration controlled transient elastography; T2DM, type 2 diabetes mellitus

<1.30, NFS <-1.455), and a higher to rule-in advanced liver fibrosis (FIB-4 >2.67, NFS >0.676). Meta-analyses have shown that the sensitivity for advanced liver fibrosis with the rule-out threshold of FIB-4 and NFS is acceptable, at around 75%, and specificity with the rule-in threshold is very good at 94%.[22,24]

Context of use, particularly in a clinical setting, is important when dealing with blood tests, knowing that NFS is not the best test for the screening of advanced liver fibrosis in patients with T2DM.[25-27] Also, age[28] and BMI,[29] included in the NFS formula, affect its performance in older patients with morbid obesity. By contrast, FIB-4, which is only affected by age, seems to be a better option in these populations. Both FIB-4 and NFS can be calculated for free through websites and smartphone applications. FIB-4, however, is the most popular and most studied non-patented blood fibrosis test due to its simplicity and the fact that serum transaminases and platelet count are largely prescribed by general practitioners (GPs) in their check-up for metabolic diseases. In large populations of unselected patients, at a threshold of 1.30, FIB-4 has the strong advantage to very easily rule-out a large proportion (60–80%) of the subjects evaluated.[30] Moreover, repeating FIB-4 measurement could evaluate the risk of liver-related complication[31] within time.

Patented blood tests

The most studied patented blood fibrosis tests in NAFLD include FibroTest®, FibroMeter™, and Enhanced Liver Fibrosis (ELF™) test.[7] These non-invasive tests combine indirect and direct markers of liver fibrosis, the latter being components of liver fibrosis or proteins directly involved in the processes of fibrogenesis and fibrolysis in the liver during chronic liver diseases. Recent meta-analyses evaluating the accuracy of these tests in NAFLD patients reported an AUROC for advanced liver fibrosis of 0.77 for FibroTest®,[32] 0.83 for ELF™,[33] and 0.89 for FibroMeter™.[34] Direct comparison performed in 417 patients with biopsy-proven NAFLD has found similar diagnostic accuracy between FibroMeter™ and ELF™.[35] Patented blood tests are more accurate than non-patented blood tests,[35,36] but their cost and limited availability limit their widespread application. Therefore, they are more suited when used as a second-line option, to further confirm the risk of advanced liver fibrosis suggested by the first-line non-patented blood fibrosis test.

Importantly, studies are concordant about the fact that negative predictive values (NPVs) for excluding advanced fibrosis are higher than the corresponding positive predictive values (PPVs). Thus, blood tests may be confidently used for first-line risk stratification to exclude advanced fibrosis. However, most of these studies have been conducted in tertiary referral centers where the pre-test probability of advanced fibrosis is higher (20–30%) than that in primary care (<5%), which could have a major impact in the accuracy results.[37]

Elastography

Elastography include ultrasound-based techniques, such as vibration-controlled transient elastography (VCTE) (FibroScan, Echosens, France), point shear wave elastography (pSWE), two-dimensional shear wave elastography (2D-SWE), and magnetic resonance elastography (MRE).[38] Among them, VCTE is the method with the largest amount of evidence.[7] Two large multicenter studies[39,40] reported high VCTE applicability (96–97%) in NAFLD patients. Moreover, the same cut-offs can be used without further adjustment for steatosis when the M and XL probes are used according to the appropriate BMI (30 kg/m^2). In a recent meta-analysis including 5,489 NAFLD patients in 37 studies, VCTE had excellent accuracy for diagnosing advanced fibrosis and cirrhosis, with AUROCs of 0.85 and 0.90, respectively.[22]

As for the remaining techniques, a recent systematic review of 82 studies (14,609 patients) and a meta-analysis of 70 studies (12,547 patients) showed that only MRE and pSWE had a specificity greater than 80% for the diagnosis of advanced fibrosis (89% and 86%, respectively).[41] Nonetheless, all evaluated techniques had a good diagnostic accuracy. The reported summary AUROC for diagnosing advanced fibrosis with VCTE, MRE, pSWE, and 2D-SWE were 0.85, 0.92, 0.89, and 0.72, respectively.[41] Although MRE had the best diagnostic accuracy, it remains a research tool due to its limited availability and cost. Moreover, pSWE/ARFI and 2D-SWE are not included in the current guidelines on the management of NAFLD due to the limited amount of data.[9-11] Taken together, these results suggest that VCTE is currently the technique with the highest level of evidence to confidently exclude advanced fibrosis and cirrhosis with a high negative predictive value (around 90%) in NAFLD patients.[7] For example, VCTE had a 94% to 100% NPV at a cut-off <8 kPa. On the other hand, the PPV did not exceed 64% at a cut-off >10 kPa. Finally, VCTE is a point-of care technique, available in most liver clinics worldwide, and is thus the technique of choice for the second-line test-

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ing of advanced fibrosis.

IDENTIFYING NAFLD PATIENTS WITH ADVANCED FIBROSIS

What is the best strategy?

The context of use is critical when using non-invasive tests, as it will strongly influence their diagnostic performance. The pretest probability of the target condition (advanced fibrosis) will impact PPV and NPV.[9] When dealing with patients in primary care, where the prevalence of advanced fibrosis is low (<5%), non-invasive tests are far better for ruling out (high NPV) rather than for diagnosing (high PPV) the presence of advanced fibrosis. This indicates the need for at least two tiers of non-invasive fibrosis tests for selecting patients from low-prevalence populations for further investigations and follow-up to reduce false positive results.[42] Therefore, using widely available, easy-to-obtain, and cheap blood tests (non-patented serum markers) as the first-line procedure followed, if positive, by a second-line confirmatory test (elastography or patented serum markers) seems the most appropriate strategy. The use of sequential algorithms is more effective than single tests in both low and high prevalence settings.[22,43]

Sequential strategies using blood tests followed by elastography

Several sequential strategies using non-invasive tests have been proposed to identify patients with advanced fibrosis in clinical practice.[9-11] The first algorithm proposed by the European Association for the Study of Liver (EASL) targets patients with high risk of NAFLD seen in primary care or diabetology clinics, using FIB-4 (single cutoff 1.3) followed by VCTE (single cutoff 8.0 kPa) (Fig. 1).[9] Patients with FIB-4 ≥1.3 are considered to be at intermediate-high risk of advanced fibrosis and should undergo VCTE, which may be performed before or after referral to liver specialist according to local availability and pathways. Finally, in patients with LSM ≥8.0 kPa, a third test (a patented blood test) can be performed, if available. In case of concordant results with VCTE, advanced fibrosis is highly likely. Otherwise, liver biopsy may be considered when results are discordant results or if a patented blood test is unavailable. Patients with FIB-4 <1.3 and/or LSM <8.0 kPa have a low risk of advanced fibrosis and can be monitored by their GP with repeated measurements during follow-up. The use of this algorithm in "real life" has been recently validated in a retrospective, multicenter French cohort of 1,051 patients with biopsy-proven NAFLD.[44] Compared with the performance of single non-invasive tests (NITs), agreement between two NITs (FIB4 and VCTE, VCTE and patented serum tests) increased specificity and PPV by 20%, thereby justifying the sequential use proposed in the EASL algorithm. The FIB-4/VCTE/FibroMeter™ and FIB-4/VCTE/FibroTest® algorithms performed similarly, providing 85% diagnostic accuracy and a liver biopsy requirement rate of only 10%.

Interestingly, in the same cohort of patients, the EASL algorithm was also able to predict the risk of liver-related events (LRE).[45] In patients with FIB-4 ≥1.3, the risk of LRE increased according to the VCTE results with adjusted hazard ratio of 3.9 (95% confidence interval [95% CI] 1.3–10.9) in those with 8.0<LSM<12.0 kPa and 12.4 (95% CI 5.1–30.2) in those with LSM ≥12.0 kPa. Finally, the utility of EASL algorithm has been examined in 467 patients with type 2 diabetes seen in primary care, independently from their transaminase levels.[46] Twenty of 440 (4.5%) patients were found to have advanced liver disease, compared to three patients who were previously identified through standard care (odds ratio 6.71, 95% CI 2.0–22.7; *P*=0.002). Alcohol and obesity were predictors of advanced disease, a finding consistent with previous studies.[47-49]

Other algorithms have been proposed since, including the American Gastroenterology Association (AGA) pathway[11] and the American Association of Clinical Endocrinology (AACE) algorithm.[10] The AGA pathway targets the same population as the EASL algorithm, and uses FIB-4 (dual cutoffs 1.3–2.67) followed by VCTE (dual cutoffs 8.0–12.0 kPa) (Fig. 2). Patients with FIB-4 in between 1.3 and 2.67 are considered as indeterminate risk, and should undergo VCTE. Patients with FIB-4 <1.3 and/or LSM <8.0 kPa are considered to be at low risk of advanced fibrosis, and can be monitored by their GP with repeated measurements during follow-up. Those with 8.0<LSM<12.0 kPa are considered at indeterminate risk, and should be referred to an hepatologist for liver biopsy or MRE. Those with FIB-4 ≥2.67 or LSM ≥12 kPa are considered at high risk, and should be referred to an hepatologist.

As for the AACE algorithm, it is very similar to the AGA pathway but consider the use of ELF™ (dual cutoffs 7.7–9.8)

as an alternative to VCTE in patients with FIB-4 in between 1.3 and 2.67 (Fig. 3). Patients with indeterminate risk (FIB-4 1.3–2.67 or LSM 8–12 kPa or ELF™ 7.7–9.8) and high risk (FIB-4 ≥2.67 or LSM ≥12 kPa or ELF™ ≥9.8) should be referred to an hepatologist for liver biopsy or MRE.

In summary, it is noteworthy that over the past year, guidelines from Hepatology, Gastroenterology, and Endocrinology International Societies recommended very similar sequential non-invasive strategies using the same tools and cutoffs. This will likely simplify the case finding and management of high-risk patients with NAFLD in clinical practice.

IDENTIFYING NAFLD PATIENTS WHO ARE AT RISK OF NASH

Several non-invasive scores combining serum and imaging biomarkers have been proposed to diagnose at-risk NASH patients. The first score is the FibroScan-AST (FAST), a continuous and composite score, combining controlled attenuation parameter (CAP), LSM by VCTE, and AST level.[50] The score ranges from 0 to 1 with a 0.35 rule-out cutoff (≥90% sensitivity) and a 0.67 rule-in cutoff (≥90% specificity). Patients with values between the two cutoffs are in the grey zone with indeterminate results. FAST had an AUROC of 0.85 for the detection of at-risk NASH patients in the pooled external validation cohort, with NPV of 94% for ruling out and PPV of 69% for ruling in at-risk NASH, respectively (Table 1). Overall, 60%

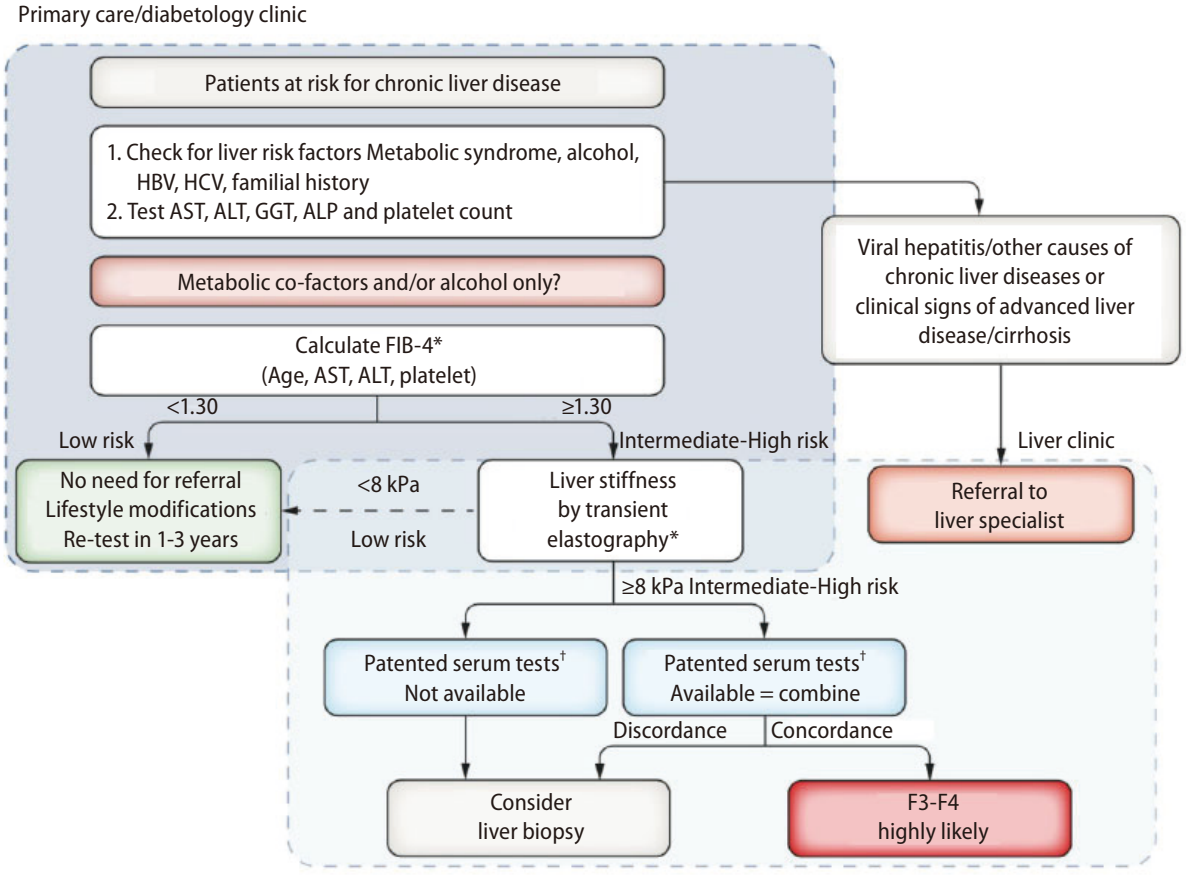

Figure 1. EASL algorithm. FIB-4 can be used in patients with metabolic co-factors and/or alcoholic liver disease to identify patients requiring referral to the liver clinic (FIB-4 >1.3). VCTE may be performed before or after referral to liver specialist according to local availability and pathways. Adapted from the article of EASL (J Hepatol 2021;75:659-689).[9] EASL, European Association for the Study of Liver; FIB-4, fibrosis-4; VCTE, vibration controlled transient elastography. *Transient elastography or FIB-4 may be performed before or after referral to liver specialist according to local availability and pathways. †Cut-offs to use: ELFTM 9.8 (NAFLD/ALD); FibroMeter 0.45 (NAFLD), Fibrotest 0.48 (NAFLD).

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of patients could be correctly classified and avoid a liver biopsy. It should be acknowledged that performances of FAST may vary according to the prevalence of at-risk NASH patients in the applied population. For instance, in a USA cohort with a 12% prevalence, FAST had an AUROC of 0.86, allowing to classify 78% of patients, whereas in another cohort from Turkey with a 57% prevalence, its AUROC was 0.74, with 43% of patients correctly classified.[50]

The second score, the magnetic resonance imaging (MRI)-AST (MAST) score, is based on the FAST concept, but using MRI (PDFF and MRE) instead of VCTE.[51] MAST had an AUROC of 0.93, a NPV of 98% for ruling out and a PPV of 50% for rul-

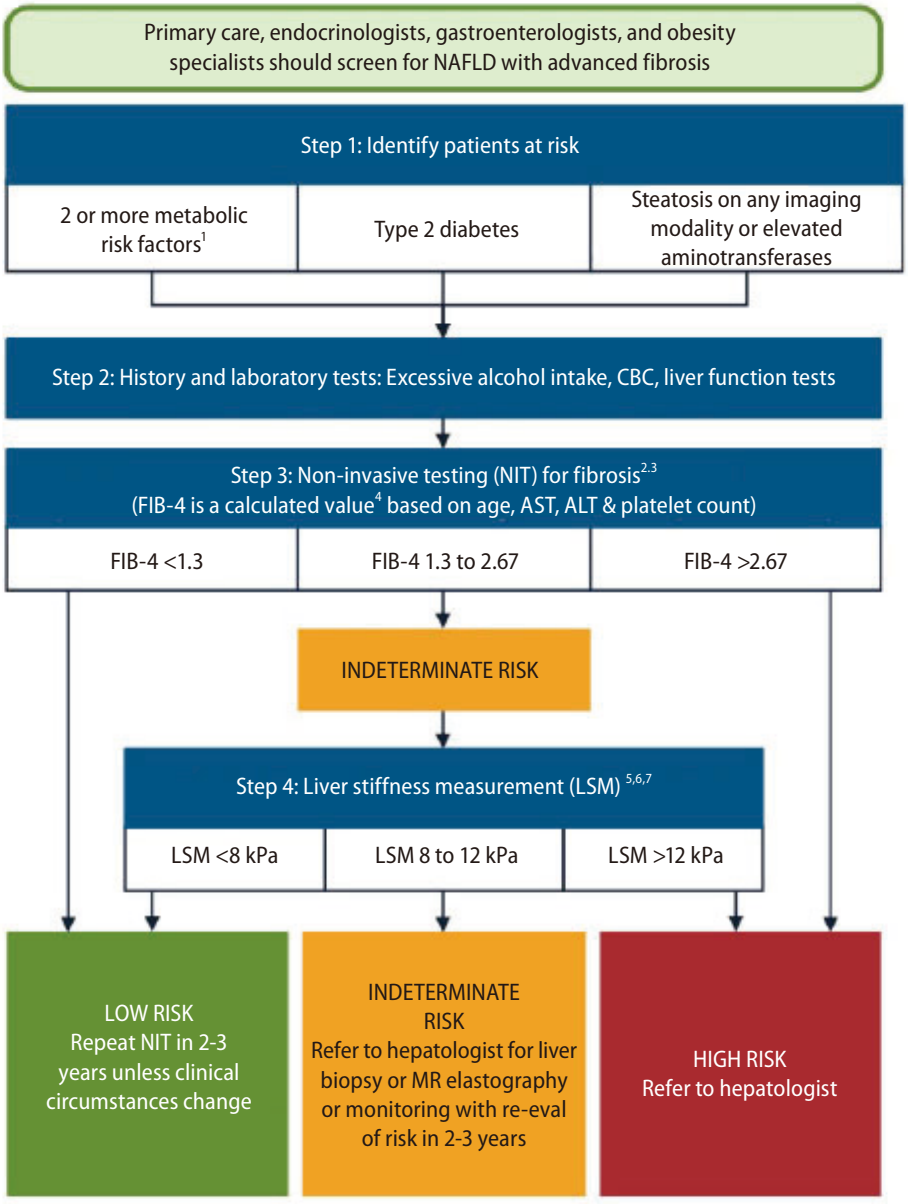

Figure 2. AGA pathway. FIB-4 (dual cutoffs 1.3–2.67) is used as first-line followed by VCTE (dual cutoffs 8.0–12.0 kPa). Adapted from the article of Kanwal et al. (Gastroenterology 2021;161:1657-1669).[11] AGA, American Gastroenterology Association; FIB-4, fibrosis-4; VCTE, vibration controlled transient elastography; NAFLD, non-alcoholic fatty liver disease; AST, aspartate aminotransferase; ALT, alanine aminotransferase; MR, magnetic resonance.

ing in at-risk NASH, respectively (Table 1). Overall, 70% of patients could be correctly classified and avoid a liver biopsy. Finally, the MRE combined with FIB-4 (MEFIB) index, a categorical score combining MRE and FIB-4, has been proposed, but with a different primary objective of detecting F2-4 fibrosis in NAFLD.[52] When evaluated for at-risk NASH, MEFIB had an AUROC of 0.77, a NPV of 93% for ruling out and a PPV of 55% for ruling in at-risk NASH, respectively (Table 1). Overall, 57% of patients could be correctly classified.

Head-to-head comparison of FAST, MAST, and MEFIB showed conflicting results. One study suggested that MAST outperformed FAST,[51] whereas another study suggested that MEFIB outperformed both MAST and FAST.[53] These results deserve several comments. First, one of the challenges with these scores was dealing with patients in the grey zone. Interestingly, in the study comparing the three scores,[53] the grey zone of MAST was significantly smaller than that of FAST and MEFIB (8.5% vs. 40.1% and 24.7%, respectively; $P<0.001$). As a result, the number of patients correctly classified as at-risk NASH was higher with MAST than with MEFIB and FAST (69.4% vs. 57.4% and 45.3%, respectively).[54] Second, when compared independently from the developers in a large cohort of T2DM patients with NAFLD, MAST and FAST outperformed MEFIB, and MAST allowed to correctly classify the largest number of patients.[55] However, high cost and limited availability may compromise widespread application of MRI-based scores. Further studies are needed.

CONCLUSIONS AND FUTURE PERSPECTIVES

The high-risk population in NAFLD patients is now well-identified (i.e., patients with advanced fibrosis), and simple non-invasive tools are available for case finding. Algorithms based on these non-invasive tools are effective and recommended by several international guidelines, but are mostly validated thus far in tertiary referral liver centers. The next step is to implement these algorithms beyond the liver clin-

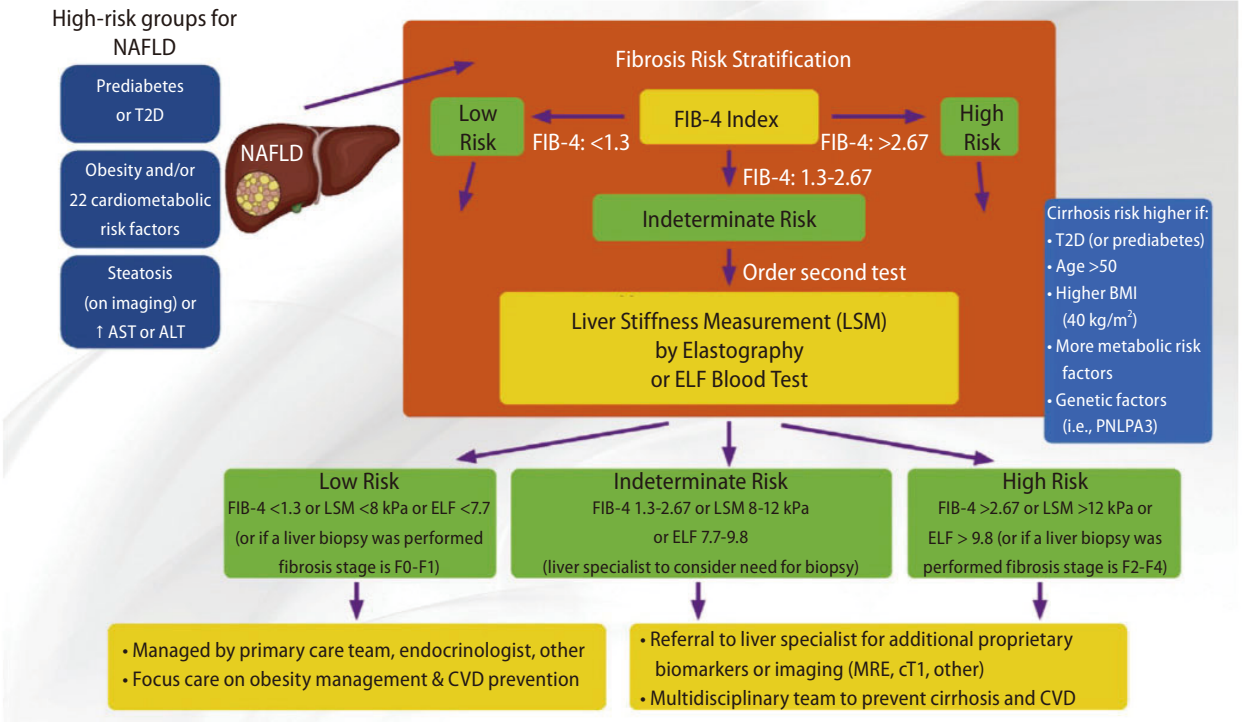

Figure 3. AACE algorithm. FIB-4 (dual cutoffs 1.3–2.67) is used as first-line followed by VCTE (dual cutoffs 8.0–12.0 kPa). ELF™ (dual cutoffs 7.7–9.8) can be used as an alternative to VCTE in patients with FIB-4 in between 1.3 and 2.67. Adapted from the article of Cusi et al. (Endocr Pract 2022;28:528-562).[10] AACE, American Association of Clinical Endocrinology; FIB-4, fibrosis-4; VCTE, vibration controlled transient elastography; ELF™, Enhanced Liver Fibrosis; NAFLD, non-alcoholic fatty liver disease; AST, aspartate aminotransferase; ALT, alanine aminotransferase; T2D, type 2 diabetes; BMI, body mass index; MRE, magnetic resonance elastography; CVD, cardiovascular disease.

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ics, particularly in primary care and diabetes clinics where most NAFLD patients are seen. The main barrier against is the lack of awareness among physicians managing these patients. Indeed, NAFLD remains largely unknown outside the fields of hepatology and gastroenterology, and is overlooked by most physicians.[56] As a result, less than 10% of NAFLD patients are referred to a specialist and opportunities for early interventions are missed, particularly in those with advanced fibrosis.[6] In addition, NAFLD is absent from nearly all national and international strategies and policies for non-communicable diseases, including obesity.[57] Therefore, dissemination of guidelines on the use of non-invasive tests and multidisciplinary approaches are critical to increase awareness and to improve management of NAFLD patients.

Authors' contribution

CS and LC contributed to the literature review and manuscript preparation.

Conflicts of Interest

Christiane Stern: Consluting fees from Echosens and stock options from Gilead. Laurent Castera: lecture fees for Echosens and Novo Nordisk, consultancies for Echosens, Madrigal, Novo Nordisk, MSD, Pfizer, and Sagimet.

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Table 1. Diagnostic performances of FAST, MAST, and MEFIB scores for the diagnosis of "at-risk" NASH patients

Score	AUROC	Number	At-risk NASH	Rule-out cutoff	N	Se	Sp	NPV	Grey zone (n)	Rule-in cutoff	N	Se	Sp	PPV	CC
FAST (50)	0.85	1,026	27%	<0.35	51%	0.89	0.64	0.94	30%	>0.67	19%	0.92	0.49	0.69	60.3%
MAST (51)	0.93	244	11.5%	<0.165	65%	0.89	0.72	0.98	18%	>0.242	17%	0.75	0.90	0.50	72.5%
MEFIB (53)	0.77	563	31.4%	MRE <3.3 kPa & FIB-4 <1.6	41%	0.91	0.56	0.93	25%	MRE ≥3.3 kPa & FIB-4 ≥1.6	34%	0.60	0.78	0.55	57.4%

NASH, non-alcoholic steatohepatitis; AUROC, area under the ROC curve; Se, sensitivity; Sp, specificity; NPV, negative predictive value; PPV, positive predictive value; AST, aspartate aminotransferase; FAST, FibroScan-AST; MRI, magnetic resonance imaging; MAST, MRI-AST; MRE, magnetic resonance elastography; FIB-4, fibrosis-4; MEFIB, MRE combined with FIB-4. Correctly classified (CC)=true negative for rule-out cutoff+true positive for rule-in cutoff/total.

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Review

Hepatocellular carcinoma surveillance in patients with non-alcoholic fatty liver disease

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Non-alcoholic fatty liver disease (NAFLD) may progress to cirrhotic or non-cirrhotic hepatocellular carcinoma (HCC), and is currently recognized as the fastest growing cause of HCC worldwide. Accordingly, professional society guidelines recommend HCC surveillance in patients with cirrhosis from any etiology, and some may consider it beneficial in subgroups with non-cirrhotic NAFLD at higher risk for HCC. Notably, patients with NAFLD-related HCC are more likely to have HCC diagnosed at more advanced stages and have poorer outcomes when compared to other etiologies, and suboptimal effectiveness of HCC surveillance programs is a major culprit. In this review, we summarize the current guidelines for HCC surveillance and discuss its benefits versus potential harms for NAFLD patients. We also address the unique challenges of HCC surveillance in NAFLD, including higher proportion of NAFLD-related HCC without cirrhosis, poor recognition of at-risk patients, lack of consensus regarding the value of surveillance in non-cirrhotic NAFLD, subpar effectiveness of surveillance tools related to NAFLD phenotype, and preponderant surveillance underuse among NAFLD patients. Finally, we examine the effectiveness of currently used surveillance tools (i.e., ultrasound and alpha fetoprotein) and outline future perspectives including emerging risk stratification tools, imaging surveillance strategies (e.g., abbreviated magnetic resonance imaging protocols), blood-based biomarkers (e.g., GALAD and circulating tumor DNA panels), and interventions to improve surveillance adherence. **(Clin Mol Hepatol 2023;29(Suppl):S207-S219)**

Keywords: Liver neoplasm; Non-alcoholic fatty liver disease; Early detection of cancer; Population surveillance; Hepatocellular carcinoma

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) encompasses a spectrum of conditions including simple steatosis, which is usually benign in nature, and non-alcoholic steatohepatitis (NASH), which may progress to cirrhosis and hepatocellular carcinoma (HCC).[1,2] NAFLD has become the fastest growing cause of HCC in Western countries over the past two decades.[3-6] When compared to other etiologies of chronic liver disease (CLD) such as hepatitis C virus and alcoholic liver disease, patients with NAFLD-related HCC are more likely to be diagnosed at later stages and have a worse prognosis.[7,8] There are several contributing factors including but not limited to suboptimal effectiveness of HCC surveillance programs.[7-9] HCC surveillance in patients with NAFLD is limited by unique challenges, including increased difficulty recognizing appropriate at-risk patients, a higher proportion of HCC occurring in the absence of cirrhosis compared to other etiol-

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ogies, unsatisfactory effectiveness of surveillance tools, underuse of HCC surveillance, and higher competing risk of non-HCC-related mortality (Fig. 1).[10-12] Herein, we will review the status of HCC surveillance in patients with NAFLD, explore areas of concern, and outline future perspectives.

GUIDELINES AND SUPPORTING EFFICACY DATA FOR HCC SURVEILLANCE

Multiple professional society guidelines including the American Association for the Study of Liver Disease (AASLD), European Association for the Study of the Liver (EASL), and Asian Pacific Association for the Study of the Liver (APASL) recommend HCC surveillance in at-risk individuals including subsets of chronic hepatitis B virus (HBV) infection and those with cirrhosis from any etiology (Table 1).[13-15]

The best data for HCC surveillance are derived from a large randomized controlled trial in patients with HBV infection, demonstrating a 37% reduction in HCC-related mortality.[16] However, it is unclear if these data apply to patients with cirrhosis, particularly those with NAFLD etiology, given differences in body habitus, liver heterogeneity, and hepatic steatosis that may impact surveillance effectiveness. When a

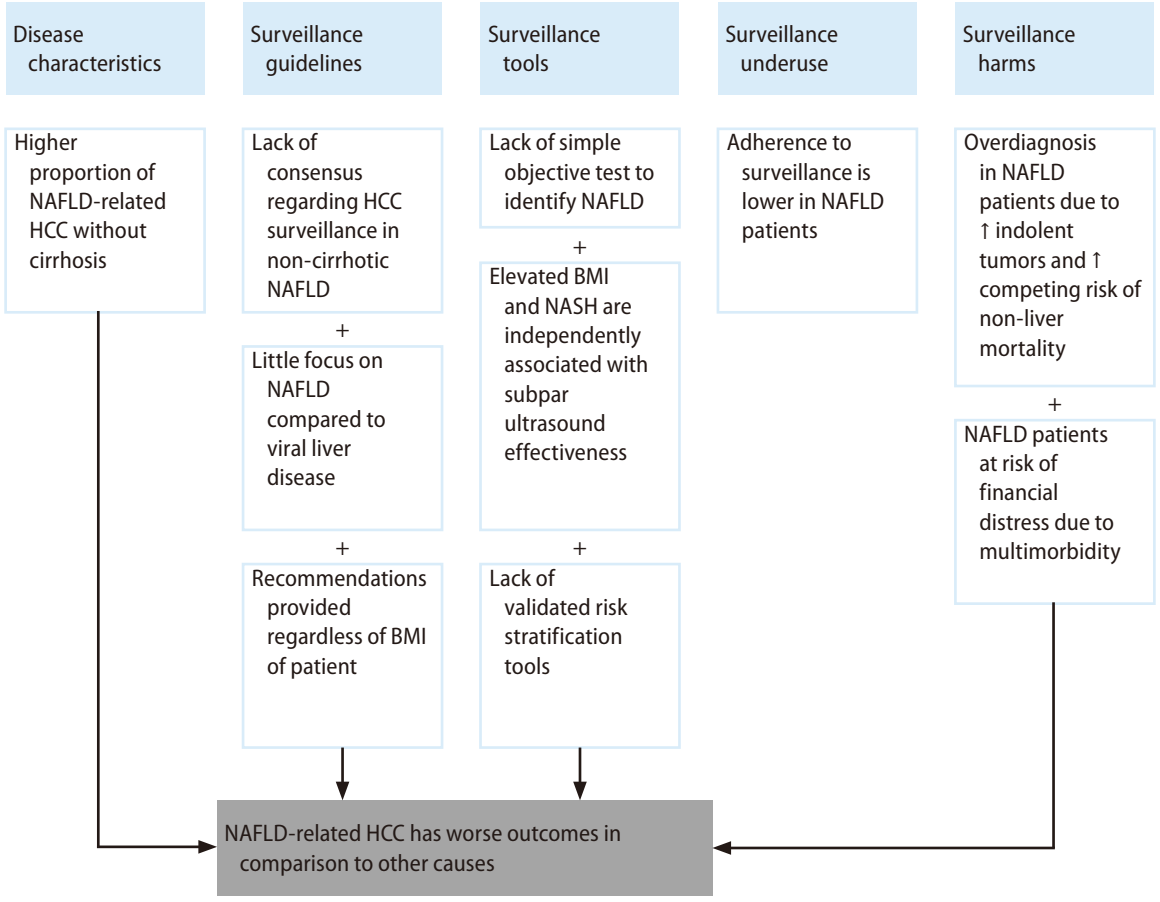

Figure 1. Unique challenges of HCC surveillance in NAFLD patients. NAFLD, non-alcoholic fatty liver disease; HCC, hepatocellular carcinoma; BMI, body mass index; NASH, non-alcoholic steatohepatitis.

Abbreviations:

AASLD, American Association for the Study of Liver Disease; AFP, alpha fetoprotein; AMRI, abbreviated magnetic resonance imaging; APASL, Asian Pacific Association for the Study of the Liver; AUC, area under the receiver operating characteristic curve; BMI, body mass index; CI, confidence interval; CLD, chronic liver disease; ct-DNA, circulating tumor DNA; CT, computed tomography; DCP, des-carboxy-prothrombin; EASL, European Association for the Study of the Liver; GALADUS, GALAD with ultrasound; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HR, hazard ratio; LI-RADS, Liver Imaging Reporting and Data System; miRNA, microRNA; MRI, magnetic resonance imaging; mt-HBT, multitarget HCC blood test; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; OR, odds ratio; RR, relative risk

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randomized clinical trial of HCC surveillance was attempted in Australia, it had to be closed due to low enrollment due to poor patient and provider acceptance of the no-surveillance arm. We are therefore forced to rely on level II case-control and cohort studies, instead of level I randomized controlled trial data. Meta-analyses of these studies demonstrate a consistent association between HCC surveillance and improved clinical outcomes.[17] A recent systematic review and meta-analysis identified 59 relevant studies between January 2014 and July 2020, including a total of 145,396 patients.[17] HCC surveillance was associated with improved early-stage HCC detection (relative risk [RR], 1.86; 95% confidence interval [CI], 1.73–1.98), curative therapy receipt (RR, 1.83; 95% CI, 1.69–1.97), and reduced mortality (hazard ratio [HR], 0.67; 95% CI, 0.61–0.720) after adjusting lead-time bias.[17]

Although clinical benefits were consistent in the subgroup of studies with >20% NAFLD patients, there were only two studies specifically examining the association between HCC surveillance and clinical outcomes in patients with NAFLD-related cirrhosis. Lo and colleagues reported a significant as-

Table 1. Professional society recommendations for HCC surveillance

Professional society	At-risk population	Surveillance tests	Frequency of surveillance	Notes
American Association for the Study of Liver Diseases (AASLD)	Patients with cirrhosis except Child-Pugh C unless awaiting liver transplantation	Ultrasound ± AFP	Every 6 months	CT or MRI are suggested if suboptimal liver visualization with ultrasound
National Comprehensive Cancer Network (NCCN)	Patients with cirrhosis	Ultrasound ± AFP	Every 6 months	
US Department of Veterans Affairs	Patients with cirrhosis	Ultrasound + AFP	Every 6–12 months	
American Gastroenterological Association (AGA)	Patients with cirrhosis	Ultrasound ± AFP	Every 6 months	Non-cirrhotic NAFLD patients with advanced (F3) fibrosis should be considered for HCC screening
European Association for the Study of the Liver (EASL)	Patients with cirrhosis, Child-Pugh A and B, or Child-Pugh C awaiting transplantation	Ultrasound	Every 6 months	HCC surveillance may be justified in patients with F3 fibrosis based on individual risk stratification
European Society of Medical Oncology (ESMO)	All cirrhotic patients as long as liver function and comorbidities allow curative or palliative treatments	Ultrasound ± AFP	Every 6 months	
British Society of Gastroenterology (BSG)	Patients with cirrhosis, Child-Pugh A and B with controlled ascites, or Child-Pugh C awaiting transplantation	Ultrasound + AFP	Every 6 months	
Asia-Pacific Association for the Study of the Liver (APASL)	Patients with cirrhosis	Ultrasound + AFP	Every 6 months	
Japanese Society of Hepatology (JSH)	Patients with cirrhosis	Ultrasound + AFP + AFP-L3 + DCP	Every 6 months in high-risk patients; every 3–4 months in extremely high-risk patients	

HCC, hepatocellular carcinoma; AFP, alpha fetoprotein; CT, computed tomography; MRI, magnetic resonance imaging; NAFLD, non-alcoholic fatty liver disease; AFP-L3, lens culinaris-agglutinin-reactive fraction of AFP; DCP, des-carboxy-prothrombin.

sociation with early-stage HCC detection (69.6% vs. 30%, *P*=0.001) whereas Aby et al. failed to find an association with curative treatment receipt (45.5% vs. 51.7%, *P*=0.70).[18,19] The authors of the meta-analysis identified increased data in NAFLD as an area of need, particularly given unique challenges of HCC surveillance in NAFLD patients, as discussed below in detail.

There has been increasing recognition that benefits of HCC surveillance must be weighed against potential harms. Based on an established taxonomy, cancer screening harms are classified as physical, psychological, or financial harms.[20] Physical harms extend beyond complications and includes aspects such as pain or radiation exposure from surveillance and diagnostic testing for positive or indeterminate results. Psychological harm can occur through the whole screening process, including apprehension of a positive result, anxiety or depression caused by receipt of an abnormal result, overestimation of the likelihood of a diagnosis, and distress related to being labeled with a diagnosis.[21] Financial harm may also result from direct costs of screening and diagnostic evaluation as well as indirect costs such as co-pays and transportation as well as opportunity costs from missed work.[22]

The above systematic review did not identify any studies examining financial or psychological harms, although the proportion of patients experiencing physical harms due to false positive or indeterminate results ranged from 8.8% to 27.5% across four applicable studies, with most harms being mild in severity. No studies rigorously evaluated etiology-specific differences in surveillance harms, although existing data suggest similar risk in NAFLD patients than those with viral cirrhosis. Subsequently, a multi-center mixed-methods study highlighted patients with true and false positive results could be associated with increased psychological harms, including depression and anxiety. Patients also reported financial harms, including indirect costs from aspects such as transportation and parking and opportunity costs from missed work. Financial harms appeared to be higher in those with multiple comorbidities, which may be pertinent for patients with NAFLD; however, the study was underpowered to evaluate etiology-specific differences in risk of financial and psychological harms. Notably, these harms appeared to be milder than those observed in other cancer screening programs and did not result in decisional regret to undergo HCC surveillance.

Other potential harms, including overdiagnosis, have been well studied in other cancers but there are few data for HCC surveillance overall, including in those with NAFLD. Overdiagnosis may relate to several factors including misclassification of premalignant lesions as cancer, detection of indolent tumors, or high competing risk of mortality.[23,24] Although HCC has traditionally been regarded as a uniformly aggressive cancer, recent data suggest that one-fourth to one-third of tumors may be indolent with slower tumor growth patterns.[25,26] Across studies, patients with non-viral liver disease had more indolent growth patterns than those with viral etiologies, suggesting greater risk for length time bias and overdiagnosis. Further, overdiagnosis may be particularly relevant for patients with NAFLD given higher comorbidity burden.[27]

Summary

HCC surveillance is recommended in patients with cirrhosis from any etiology. This practice is supported by cohort studies showing associations with increased early cancer detection and improved overall survival, although there are fewer data specifically in patients with NAFLD-related cirrhosis. HCC surveillance is associated with physical, financial, and psychological harms as well as risk of overdiagnosis, although existing data suggest these may be mild in severity. Continued data are needed to better define the overall value of HCC surveillance in patients with NAFLD cirrhosis.

IDENTIFICATION OF THE AT-RISK NAFLD POPULATION

HCC surveillance is currently recommended in all patients with cirrhosis from any etiology, including those with NAFLD-related cirrhosis. Cost-effectiveness analyses have suggested that HCC surveillance is cost-effective if the annual HCC incidence exceeds 0.8% in patients with compensated cirrhosis and exceeds 0.2% in patients without cirrhosis.[28,29] The annual incidence of HCC in patients with cirrhosis has traditionally been ~2–3% per year, although higher estimates have been reported in Asian cohorts with higher proportions of patients with active viral hepatitis. Several studies have shown that the annual incidence of HCC is lower in the setting of non-viral liver disease, with annual HCC incidence estimates ranging from 0.7% to 2.6% in patients with NAFLD cirrhosis[30,31] and from 0.01% to 0.13% in patients with non-cirrhotic

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NAFLD.[32,33]

While there is general agreement about the application of surveillance programs in patients with NAFLD cirrhosis, there is a lack of consensus regarding the value of HCC surveillance in non-cirrhotic NAFLD. The AASLD guidelines restrict surveillance recommendations to those with cirrhosis,[34,35] whereas a clinical practice update from the American Gastroenterological Association recommends surveillance in patients with F3 fibrosis[36] and EASL guidelines suggest HCC surveillance might be justified in F3 fibrosis patients based on individual risk stratification.[14] This debate has been contentious and noteworthy given growing literature demonstrating a substantial risk of developing HCC in the absence of cirrhosis in NAFLD patients compared to patients with other etiologies of liver disease.[37] Indeed, the proportion of NAFLD-related HCC patients without evidence of cirrhosis at diagnosis ranges from 46.2% to 54%, as compared to 2.8% to 22% of patients with HCC related to other etiologies.[7,38] A meta-analysis of existing literature reported a pooled proportion of 38.0% for non-cirrhotic HCC in NAFLD patients, compared to 14.2% for other liver disease etiologies, with an odds ratio of 2.61 (95% CI, 1.27–5.35).[39] However, cohort studies suggest the annual incidence of HCC in non-cirrhotic NAFLD falls below the cost-effectiveness threshold. An analysis of the Veterans Affairs administrative database found an annual HCC incidence of only 0.008 per 100 person-years among 292,366 persons with non-cirrhotic NAFLD, although this group was heterogeneous regarding baseline fibrosis level. A subsequent prospective multicenter study involving 1,773 NAFLD patients included in the NASH clinical research network (1,237 patients with stage F0–F2; 369 stage F3; and 167 stage F4) found the incidence of HCC per 100 person-years was 0.04 for patients with F0–F2 fibrosis, compared to 0.34 for F3 fibrosis and 0.14 for F4 fibrosis.[40] A meta-analysis of 18 studies with 470,404 patients found a pooled annual incidence of 0.03 per 100 person-years (95% CI, 0.01–0.07) in non-cirrhotic NAFLD, compared to 3.78 per 100 person-years (95% CI, 2.47–5.78) in those with cirrhosis.[41]

Overall, these data suggest that HCC surveillance is unlikely to be cost effective in non-cirrhotic NAFLD, outside of additional risk stratification tools. An in-depth discussion of HCC risk stratification in patients with NAFLD is beyond the scope of this review. However, in brief, several risk models incorporating clinical risk factors, genetic factors, and molecular factors have been proposed, with most not yet having been sufficiently validated for routine use in clinical practice.[42-45] If sufficiently validated, these risk models may facilitate a more individualized precision screening approach to targeted HCC surveillance to those at the highest risk.[46,47] While we await validated models to better risk stratify patients with non-cirrhotic NAFLD and identify subgroups who may benefit from HCC surveillance, consistently observed risk factors such as male sex, older age, and increasing number of metabolic syndrome components may help identify individuals with non-cirrhotic NAFLD who have higher HCC risk. Further, prior data clearly highlight the direct relationship between fibrosis stage and HCC risk, with F3 fibrosis posing significantly higher risk than F0-F2 fibrosis.

Summary

HCC surveillance in patients with NAFLD is currently restricted to those with cirrhosis. Surveillance is not cost-effective in broader non-cirrhotic patient populations based on current data, although it may be considered in individual patients with F3 fibrosis who are deemed to be at higher risk of developing HCC. There are several emerging risk stratification tools to accurately identify subgroups with non-cirrhotic NAFLD with sufficient risk to warrant surveillance, although these are currently insufficiently validated to be used in clinical practice.

SURVEILLANCE TOOLS IN PATIENTS WITH NAFLD

Ultrasound-based surveillance

Semi-annual ultrasound has been the standard of care strategy for HCC surveillance in at-risk groups for over 15 years.[14,48] Advantages of this strategy include widespread availability, low cost, non-invasiveness, and absence of patient exposure to ionizing radiations or contrast media.[35] Results from a meta-analysis showed that the pooled sensitivity and specificity of ultrasound alone for any-stage HCC detection in patients with cirrhosis were 84% and 91%, respectively, whereas the pooled sensitivity drops to 47% for those with early-stage HCC.[49] The suboptimal sensitivity of ultrasound for early-stage HCC contributes to a substantial number of patients diagnosed at later tumor stages, leading to worse

overall survival.[37]

The effectiveness of HCC detection by ultrasound is further limited in obese individuals and those with non-viral etiologies of liver disease. A retrospective study aimed at evaluating ultrasound accuracy for HCC diagnosis in obese patients demonstrated that sensitivity was 77% in patients with body mass index (BMI) <30 kg/m^2 and 21% in patients with BMI ≥30 kg/m^2.[50] Ultrasound is also not as sensitive for early HCC detection in NAFLD patients when compared to other etiologies of CLD, mainly due to subcutaneous fat accumulation as well as liver fatty infiltration which both hamper visualization. A retrospective cohort study involving 941 patients with cirrhosis, in which ultrasounds were independently reviewed by three abdominal radiologists, demonstrated that obesity, non-viral etiologies of liver disease including NAFLD, and Child Pugh class B cirrhosis were all independently associated with worse ultrasound quality for evaluation of liver lesions.[51] A subsequent study using the ultrasound Liver Imaging Reporting and Data System (LI-RADS) visualization score validated these associations as well as demonstrated that obesity and non-viral liver disease etiologies were associated with persistent poor visualization over time.[52] These data are important given that poor ultrasound visualization is associated with worse test performance, with LI-RADS visualization score B (moderate limitations) being associated with increased odds of false positive results (odds ratio [OR], 1.60; 95% CI, 1.13–2.27) and LI-RADS visualization score C (severe limitations) being associated with significantly higher odds of false negative results (OR, 7.94; 95% CI, 1.23–51.2). Ultrasound sensitivity exceeded 75% for those with LI-RADS visualization scores of A or B, compared to only 27.3% in those with a visualization score of C. Results from another single-center study with 352 patients similarly found that ultrasound sensitivity was significantly lower in obese patients compared to non-obese patients (76% vs. 87%, respectively, P=0.01) and in NAFLD patients compared to those with other etiologies (59% vs. 84%, respectively, P=0.02).[53] Overall, these data suggest that ultrasound visualization and other patient factors (e.g., presence of obesity, liver disease etiology, and Child Pugh class) may identify a subgroup of patients who would benefit from alternative surveillance modalities.

Role of alpha fetoprotein (AFP) in surveillance

AFP is the only biomarker to complete all five phases of biomarker validation.[54] Although it has insufficient accuracy to be used in isolation, there is accumulating evidence suggesting that adding AFP to ultrasound-based surveillance significantly improves test performance,[12,22] with a sensitivity of approximately 97% for any-stage HCC detection and 63% for early-stage detection.[49] There was a small trade-off in specificity, decreasing to 84%, although this was felt to still exceed the accepted threshold for a false positive rate, and the overall diagnostic odds ratio was similar if not higher for the two tests in combination. Recent data have demonstrated decreasing trends in AFP levels among HCC patients, in parallel with a shift in epidemiology to increasing non-viral cases, suggesting that the optimal threshold for AFP in patients with NAFLD may be lower than the traditional cut-off of 20 ng/mL.[55,56] Further, there are data suggesting that longitudinal measurements of AFP, examining changes over time instead of single threshold assessments, may increase test performance, although there are fewer data for this approach in patients with NAFLD than viral etiologies.[57,58]

Alternative imaging-based surveillance tools

While contrast-enhanced ultrasound can be used as a second-line diagnostic tool for HCC once a focal hepatic lesion is detected on conventional ultrasound, there are no strong data to demonstrate that this strategy would increase test performance for surveillance and early HCC detection. Further, logistical concerns such as need for repeat contrast injections may make this impractical for surveillance.[15,35]

Other imaging modalities such as computed tomography (CT) scan or magnetic resonance imaging (MRI) are increasingly being considered for HCC surveillance. Results from a prospective cohort study (the Prospective Intra-individual Cohort Study to Compare Gadoxetic Acid [Primovist®]-Enhanced Magnetic Resonance Image and Ultrasonography for the Surveillance of Early Stage Hepatocellular Carcinoma in Patients at High-Risk study, NCT01446666) suggested that MRI-based screening had a significantly higher sensitivity and specificity than ultrasound for early-stage HCC in high-risk patients with cirrhosis.[59] The trial performed concurrent ultrasound and MRI in 407 patients for 1.5 years, over which time 43 were diagnosed with HCC. MRI had significantly higher sensitivity for early-stage HCC detection (85.7% vs. 26.2%) as well as higher specificity (97.0% vs. 94.4%). However, the study was largely limited to patients with HBV-related

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cirrhosis, and these results have yet to be validated in broader patient populations including those with NAFLD cirrhosis. Other potential barriers including radiologic capacity and patient concerns such as claustrophobia may limit uptake so would need to be considered when estimating effectiveness of an MRI-based strategy. A cost-effectiveness analysis suggested an MRI-based strategy could be cost-effective in patients with annual incidence rate of HCC is >1.81%,[59] but not those with lower annual incidence, such as those with NAFLD cirrhosis.[2,30,31] Instead, it may be best reserved for patient subgroups, such as those with inadequate ultrasound visualization.[36]

To address the potential concerns about cost-effectiveness, several investigators have proposed abbreviated MRI (AMRI) protocols, in which selected sequences are performed and in-scanner time is reduced from approximately 45 minutes to 15 minutes. Potential protocols include non-contrast MRI protocols, dynamic contrast-enhanced protocols, and hepatobiliary phase AMRI, with each demonstrating promising performance in case-control studies. A meta-analysis of studies examining AMRI performance reported sensitivities of 69% and 86% for HCC lesions <2 cm and those ≥2 cm, and AMRI having higher sensitivity than that of ultrasound (82% vs. 53%).[60] A post-hoc analysis of the PRIUS study simulating AMRI by selecting MRI sequences similarly reported that AMRI had significantly higher sensitivity than that of ultrasound (86.0% vs. 27.9%, P<0.001), albeit with a higher false positive rate (4.4% vs. 3.7%).[61]

Another study, in which low dose two-phase CT (arterial phase and 3-minute delayed phase) and ultrasound were concurrently performed in a cohort of 139 patients with cirrhosis, similarly found that two-phase CT had significantly higher sensitivity for early-stage HCC (83.3% vs. 29.2%, P<0.001) and specificity (95.6% vs. 87.7%, P=0.03) compared to ultrasound-based surveillance. However, concerns regarding contrast exposure and cumulative radiation exposure may limit broader uptake as a surveillance modality.[35]

Biomarker-based surveillance tools

Growing evidence suggests that novel biomarkers could play a role to improve HCC surveillance in NAFLD patients. GALAD, consisting of Gender, Age, AFP-L3, AFP, and des-carboxy-prothrombin (DCP), is a promising biomarker panel with extensive phase II biomarker data, including in patients with NAFLD.[12,62,63] The largest study to evaluate GALAD is a multi-center case-control study examining GALAD in 6834 patients with CLD with (n=2,430) and without (n=4,404) HCC.[64] In this study, GALAD achieved a sensitivity and specificity of 60.6–80.2% and 88.6–95.8% for early HCC detection, respectively. However, this study included majority patients with viral hepatitis so unclear if these results would apply to those with NAFLD. A single-center cohort analysis suggested performance may be further improved by combining GALAD with ultrasound (GALADUS score), which resulted in an area under the receiver operating characteristic curve (AUC) of 0.98.[62] In a subsequent case-control study including NAFLD-related patients with and without HCC, the diagnostic performance of GALAD proved to be excellent for HCC detection.[65] Indeed, GALAD accurately detected HCC at any tumor stage with a significantly better performance than each individual biomarker, including AFP, AFP-L3, or DCP (AUC: 0.96 vs. 0.88, 0.86, and 0.87, respectively; P<0.001 for each). GALAD performance was independent of cirrhosis, as similar AUCs were obtained for patients with and without cirrhosis (AUC: 0.93 and 0.98, respectively). However, recent phase III data suggested lower diagnostic performance when evaluated in cohort analyses.[66]

There are other novel candidate biomarkers that could be of added value for HCC surveillance including methylated circulating tumor DNA (ct-DNA), microRNAs (miRNAs), long non-coding RNAs, exosomes, epigenetics, and lipidomics. Indeed, methylated ct-DNA released from cancer cells could be harvested in "liquid biopsies" and used as potential non-invasive biomarkers to detect HCC at an early stage.[67] In an international case-control study, the performance of a novel multitarget HCC blood test (mt-HBT) incorporating DNA methylation biomarkers (HOXA1, TSPYL5, and B3GALT6), AFP, and patient sex was clinically validated in an independent sample including 156 HCC patients.[68] In this study, mt-HBT detected early-stage tumors with 82% sensitivity, which was significantly higher than AFP (40%; P<0.001) and GALAD (71%; P=0.03). Notably, early-stage sensitivity was stable across subgroups, including sensitivities of 85% and 77% in patients with BMI values <30 kg/m^2 and those with BMI ≥30 kg/m^2, respectively as well as across all examined liver disease etiologies, making mt-HBT a potentially valuable tool for surveillance in patients with NAFLD. A recent network meta-analysis suggested similar efficacy of mt-HBT compared to ultrasound and AFP for early-stage HCC detection,[69]

although the authors noted the strength of data differed for the two modalities. In another multicenter validation study HelioLiver Test, another ct-DNA biomarker panel, yielded a sensitivity of 76% (95% CI, 60–87%) for early-stage HCC, significantly higher than AFP and GALAD.[70] Both ct-DNA assays are undergoing prospective evaluation at this time, so we anticipate validation in the near future.

There are several other biomarkers that have early phase II biomarker data, although a comprehensive review of these biomarkers is beyond the scope of this review. For example, HCC patients have elevated levels of liver-specific miRNAs including miR-106b-3p, miR-101-3p and miR-1246 when compared to healthy subjects, suggesting the potential utility of these biomarkers for early HCC detection in high-risk patients.[71] Specific hydroxymethylated genes are also associated with HCC in the absence of elevated AFP, suggesting a role of these epigenetically modified genes as potential biomarkers.[72] Similarly, Lewinska and colleagues identified a serum lipidome that was able to accurately distinguish NAFLD patients with HCC from controls without HCC.[73]

Although these blood-based biomarkers have promising early data, most have only been evaluated in phase II case-control studies but not validated in phase III or phase IV cohort studies.[54] Phase II studies are subject to selection bias and spectrum bias, potentially overestimating biomarker performance, highlighting the importance of subsequent validation. Further, much of the data for these biomarkers has been derived in patients with viral hepatitis, highlighting a need for increased data in emerging patient populations, including those after sustained virological response or NAFLD.

Summary

Ultrasound alone has insufficient sensitivity for early detection of HCC, which can be improved by using in combination with AFP. Emerging imaging surveillance strategies (e.g., MRI) and blood-based biomarkers (e.g., GALAD and ct-DNA panels) have promising early data suggesting high accuracy, although these require further validation prior to routine use in clinical practice.

SURVEILLANCE INTERVAL

Most guidelines recommend HCC surveillance in at-risk individuals every 6 months,[22] as it has a better sensitivity than a 6–12 months interval,[74] and a similar sensitivity but higher specificity and lower cost than a 3 months interval.[75] There are no data suggesting that HCC surveillance intervals should

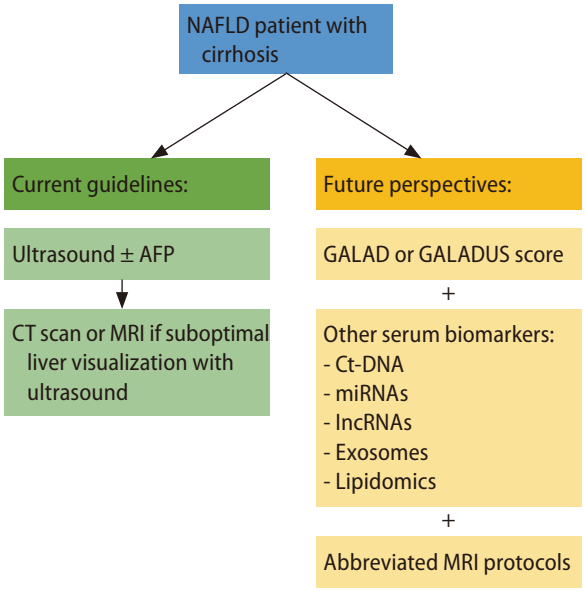

Figure 2. Current and future perspectives for HCC surveillance in NAFLD patients. NAFLD, non-alcoholic fatty liver disease; AFP, alpha fetoprotein; CT, computed tomography; MRI, magnetic resonance imaging; GALADUS, GALAD with ultrasound; ct-DNA, circulating tumor DNA; miRNA, microRNA; lncRNA, long non-coding RNA.

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be tailored to liver disease etiology.

Summary

HCC surveillance should be performed at semi-annual intervals in at-risk patients, including those with NAFLD cirrhosis.

SURVEILLANCE UNDERUSE

Adherence to the HCC surveillance programs is often suboptimal.[37] A systematic review showed that the HCC surveillance was performed in only 24% of patients with cirrhosis.[76] There was geographic variation in surveillance receipt, with the lowest receipt among studies from the United States, compared to those from Europe and Asia (17.8% vs. 43.2% and 34.6%, respectively; $P<0.001$). Subgroup analyses also demonstrated higher surveillance use among subspecialty care studies, compared to center-based and population-based studies (73.7% vs. 29.5% and 9.8%, respectively). Most notably, surveillance underuse is particularly concerning among NAFLD patients, as this one of the most consistent correlates for surveillance underuse across studies. In fact, studies suggest up to half of NAFLD-related HCC cases are not detected through surveillance.[7]

There are many patient- and provider-level barriers to HCC surveillance, contributing to HCC surveillance underuse.[77-79] Provider-level barriers to surveillance include time constraints in clinic, inadequate knowledge about guidelines, and difficulty identifying at-risk patients.[80] As discussed above, identification of at-risk patients with NAFLD can be particularly problematic for providers. Patient-reported barriers include challenges with the scheduling process, transportation difficulties, and cost of testing.[81,82] These data highlight the need for interventions targeting the surveillance process at multiple levels to increase optimal adherence.[77] Surveillance adherence can be improved through a variety of interventions including patient or provider education, electronic medical record reminder systems, automated recall systems via radiology, or population health programs using mailed outreach.[83-87] Most studies suggest similar efficacy of interventions across patient subgroups, including liver disease etiology, although few have performed rigorous moderator analyses.

Summary

HCC surveillance is underused in clinical practice, including in patients with NAFLD, related to patient and provider-reported barriers. Several multi-level interventions are efficacious for increasing surveillance utilization.

CONCLUSION AND FUTURE PERSPECTIVE

NAFLD is the now fastest growing cause of HCC worldwide, so it is critical to understand practices that can maximize survival for patients with NAFLD-related HCC (Fig. 2). In that vein, surveillance has been associated with significantly improved early tumor detection and survival. However, effectiveness of surveillance in clinical practice among patients with NAFLD has been limited by poor recognition of at-risk patients, suboptimal test effectiveness for early tumor detection, and surveillance underuse. Emerging risk stratification tools, imaging and blood-based surveillance strategies, and interventions to increase surveillance implementation all offer hope for improvements.

Authors' contribution

Drafting of the manuscript (Seif El Dahan); Critical revision of the manuscript for important intellectual content (all authors); Obtained funding (Singal); Study supervision (Singal).

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Conflicts of Interest

Amit Singal has served as a consultant or on advisory boards for Bayer, Wako Diagnostics, Exact Sciences, Roche, Glycotest, and GRAIL. None of the authors have any relevant conflicts of interest.

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Review

Preventive strategy for nonalcoholic fatty liver disease-related hepatocellular carcinoma

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The incidence of hepatocellular carcinoma (HCC) associated with nonalcoholic fatty liver disease (NAFLD) has been increasing worldwide, including Asia. Most patients with NAFLD-related HCC are at a much-advanced stage and older age at the time of diagnosis than those with virus-related HCC because they have not undergone HCC surveillance. This review provides an overview of the mechanism of hepatocarcinogenesis in NAFLD, preventive strategies for NAFLD-related HCC, and strategies for the surveillance of patients with NAFLD. **(Clin Mol Hepatol 2023;29(Suppl):S220-S227)**

Keywords: Hepatocellular carcinoma; Nonalcoholic fatty liver disease; Surveillance; Prevention

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is currently the leading cause of chronic liver disease in Korea, with an estimated prevalence of 20–30% among general population.[1] NAFLD is regarded as the hepatic manifestation of the metabolic syndrome and is also closely associated with diabetes, hyperlipidemia, obesity, and hypertension. Considering the trend of obesity in Korea,[2] NAFLD may become more prevalent in the near future and may become an important etiology of chronic liver disease and liver cancer. As the prevalence of metabolic syndrome has notably increased,[3] the prevalence of NAFLD has doubled in the last two decades to 30%. Although simple steatosis is often regarded as a non-progressive condition, 20–30% of patients with nonalcoholic fatty liver progress to chronic liver disease (nonalcoholic steatohepatitis [NASH]), which is characterized by hepatocyte injury, lobular inflammation, and fibrosis, and can result in liver cirrhosis (LC) (F4) in 20% of NASH patients with advanced fibrosis (F3) over 2 years.[4,5] NAFLD and NAFLD-related hepatocellular carcinoma (HCC) have received relatively little attention because cardiovascular events are the most common cause of death among patients with NAFLD. However, with the increase in the prevalence of metabolic syndrome and the decrease in the population with chronic hepatitis B or C worldwide, NAFLD, especially NASH, has increasingly become an important etiology of HCC.[6]

Hepatocarcinogenesis in patients with NAFLD and NASH is complex and not fully understood. Although the progression to cirrhosis occurs before the development of HCC in the majority of chronic liver diseases, this is not always the case with NAFLD-related HCC, because HCC may develop even if cirrhosis is not definitively present.[7] The rate of NASH-associated hepatocarcinogenesis is approximately 1.5–2.6% per year.[6]

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PATHOGENESIS: PROPOSED MECHANISMS

Obesity and diabetes, which are two important risk factors for NAFLD, increase the risk of HCC.[8] The pathogenesis of HCC in patients with NAFLD (Fig. 1) is also independent of the presence of liver cirrhosis. Among patients with NAFLD, HCC may develop even in the absence of advanced hepatic fibrosis and histological inflammation.

The association between obesity and HCC among patients with NAFLD has also been proven for HCC in a previous study in the United States, which included more than 900,000 persons. The individuals were stratified according to their body mass index (BMI). The relative risk of mortality of HCC was 4.52 and 1.90 in patients with obesity grade II and I, respectively.[9] A study from Korea with 700,000 participants also confirmed an increased risk (relative risk, 1.56) of HCC in patients with BMI >30 kg/m^2.[10] A persistent, low-grade inflammatory response due to obesity and an abundance of adipose tissue are thought to be key factors in hepatocarcinogenesis.[11] Increased levels of leptin, a proinflammatory, proangiogenic, and profibrogenic cytokine that promotes growth by activating the Janus kinase pathway,[12] are a result of obesity. Adiponectin, an anti-inflammatory cytokine, is decreased in obesity.[13-15] Lipotoxicity, which results from lipid accumulation in the liver, causes the development of reactive oxygen species, endothelial reticulum stress, and saturated and monounsaturated free fatty acids. Free fatty acids can disrupt cellular signaling pathways causing changes in gene transcription.[16] By activating numerous carcinogenic pathways, insulin and insulin-like growth factor may aid in the development of primary liver cancer.[17]

Insulin resistance and hyperinsulinemia also increase toxic metabolites in hepatocytes.[18] Hyperglycemia modifies the cell vasculature, leading to defects in endothelial cells. Endothelial damage leads to impaired fibrinolytic capacity, increased growth factor production, increased levels of adhesion molecules and inflammatory cytokines, increased reactive oxygen species, and enhanced cellular permeability.[19] Insulin resistance also leads to hyperinsulinemia, which triggers the production of free fatty acids and reactive carbonyl compounds in adipose tissue.[20] Advanced glycation end-products in hepatocytes aggravate oxidative stress and DNA damage, which are the probable consequences of hepatocarcinogenesis.[21]

The alteration of the gut microbiota in patients with NAFLD also leads to hepatocarcinogenesis.[22] The level of lipopolysaccharide, which is the main component of the outer membrane of gram-negative bacteria, increases with obesity. Interestingly, further evidence of the role of lipopolysaccharide in hepatocarcinogenesis is derived from the finding that gut sterilization and lipopolysaccharide removal reduce HCC development in the chronically damaged liver.[23,24]

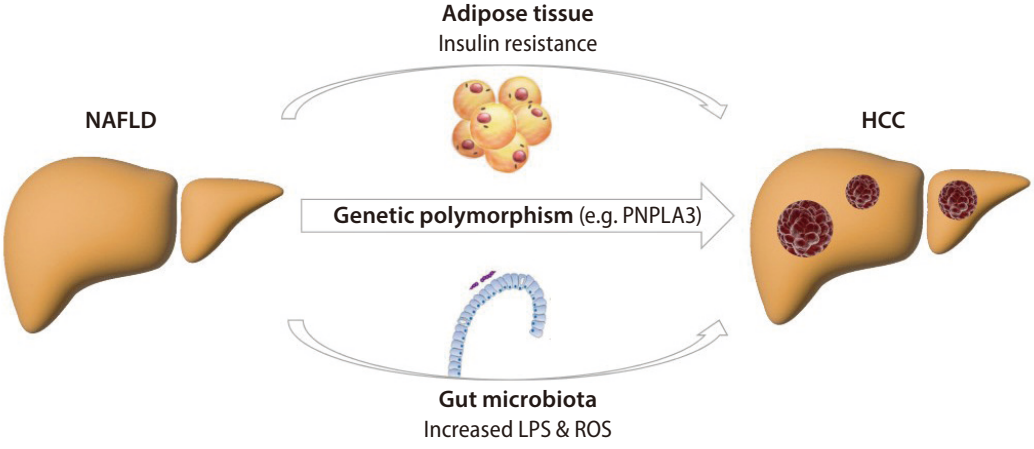

Figure 1. Pathogenesis of hepatocellular carcinoma in patients with nonalcoholic fatty liver disease. NAFLD, nonalcoholic fatty liver disease; HCC, hepatocellular carcinoma; LPS, lipopolysaccharide; ROS, reactive oxygen species.

Abbreviations:

BMI, body mass index; CI, confidence interval; HCC, hepatocellular carcinoma; HR, hazard ratio; LC, liver cirrhosis; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis

The development of HCC in NAFLD may also be influenced by genetic variation. The minor allele of *PNPLA3* rs738409 c.444C>G (encoding the I148M variant) has been linked to hepatocarcinogenesis. This polymorphism provides an elevated risk in the absence of potentially confounding covariates such as age, sex, coexisting diabetes, obesity, and cirrhosis.[25,26]

PREVENTION OF NAFLD-RELATED HCC

Several risk factors associated with hepatocarcinogenesis in the NAFLD population may be reduced by lifestyle interventions or chemoprevention; however, the benefits of these approaches are likely to extend beyond risk factor modification. Changes in lifestyle and management of metabolic risk factors may help prevent HCC. Further epidemiological studies are required to tailor screening strategies, particularly in noncirrhotic populations with NAFLD.

Weight reduction

The primary treatment for the majority of patients with NAFLD is weight reduction. However, weight loss has not been directly proven to reduce the incidence of NAFLD-related HCC. Previous clinical studies have demonstrated that weight loss positively influences NAFLD activity, with some data indicating the possibility of hepatic fibrosis regression. Weight reduction for all patients with NAFLD is recommended, especially those who are overweight (BMI >25 kg/m^2) or obese (BMI >30 kg/m^2), because weight loss at a rate of 0.5–1.0 kg/week can lead to improvement in biochemical tests, serum insulin levels, and liver histology.[27-30] Weight reduction of 5–7% leads to lower intrahepatic fat content in NAFLD patients, and weight loss of 7–10% is necessary to ameliorate hepatic inflammation and fibrosis.[1]

The following are the behavioral adjustments for obese patients: (1) consuming a low-calorie, low-fat diet; (2) regular participation in moderate physical activity; and (3) regular checking of body weight and abdominal circumference.

Physical activity

HCC risk reduction has recently been found in the European Prospective Investigation into Cancer and Nutrition cohort study among subjects who engaged in at least 2 hours of intense exercise each week with a hazard ratio (HR) of 0.5, independent of body weight and other common risk factors for HCC.[31] A meta-analysis of 14 prospective studies also indicated a considerably decreased risk of liver cancer in those with high physical activity compared to those with low physical activity (HR, 0.75; 95% confidence interval [CI], 0.63–0.89).[32]

Dietary modification

Among dietary patterns, higher adherence to the Mediterranean diet substantially lowered the risk of HCC (odds ratio, 0.51[33]; HRs, 0.62[34] and 0.68[35]). The Mediterranean diet is also recommended by European and Korean guidelines for NAFLD.[1,36]

Coffee is a dietary component that has shown potential for the treatment of both NAFLD and HCC. People who drank coffee at least twice a day had a considerably decreased incidence of HCC than non-drinkers (HR, 0.40; 95% CI, 0.20–0.79).[37] A meta-analysis of six Japanese cohort studies corroborated this finding, with a pooled relative risk estimate of 0.50 (95% CI, 0.38–0.66) for frequent coffee drinking vs. no coffee consumption.[38]

It has also been proposed that dietary antioxidants (vitamins C and E, as well as selenium) may help reduce hepatocarcinogenesis.[39] This may particularly important given that patients with NASH have been shown to have vitamin E and D insufficiency,[40] and that vitamin D deficiency may play a role in hepatocarcinogenesis.[41]

PHARMACOLOGIC PREVENTION

Several pharmacological treatments have been reported to modify risk variables and carcinogenic pathways in NAFLD-associated HCC, indicating their potential use in preventive pharmacological strategies. In this section, the pharmacological treatments that have been shown to prevent HCC are reviewed. There are few studies that have verified the chemopreventive effect only on NAFLD patients. Therefore, clinicians should be careful in interpreting the routine use of drugs such as metformin and statin as prophylactic therapy in patients with NAFLD.

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Aspirin

In large prospective population-based observational studies, aspirin and other antiplatelet medications have been shown to lower the risk of HCC.[42-44] Most studies have found that aspirin might exert a hepatitis B virus (HBV)-specific chemopreventive effect on HCC development. However, recent studies have also suggested that aspirin might have a preventive effect on NAFLD-related HCC.

A recent pooled analysis of two prospective United States cohort studies (the Nurses' Health Study and the Health Professionals Follow-up Study) analyzed 133,371 participants. This study reported that regular, long-term aspirin use was associated with a reduction in HCC risk in a dose-dependent manner, which was apparent after ≥5 years of use. Interestingly, similar associations were not found with non-aspirin nonsteroidal anti-inflammatory drugs.[43] The analysis of this study was not limited to those with NAFLD, but considering that one dominant HCC risk factor in the Unites States is NAFLD, it can be accepted as a significant result. A prospective study of 361 patients with biopsy-proven NAFLD also reported that daily aspirin use was associated with a significantly lower risk of advanced fibrosis compared to nonregular aspirin use (adjusted HR, 0.63; 95% CI, 0.43–0.85).[45] A recent systematic review and meta-analysis analyzing 19 observational studies also supported the preventive effect of aspirin on HCC development.[46]

The ideal dose and duration of aspirin for preventing HCC incidence are still uncertain, and the chemopreventive impact of other nonsteroidal anti-inflammatory medications other than aspirin on HCC is unknown. Future studies are needed to determine the chemopreventive effects of aspirin in NAFLD and NASH patients.

Metformin

Metformin suppresses hepatic fat formation and glucose excretion by activating adenosine monophosphate-activated protein kinase; it also reduces tumor necrosis factor expression. In a subanalysis of a meta-analysis[47] analyzing 37 studies, a substantial decrease in HCC risk in diabetic patients was observed among metformin users in terms of HCC incidence (78%) and death (77%). Another meta-analysis of 10 studies that included 22,650 HCC cases among 334,307 diabetic individuals found that metformin treatment was associated with a 41% decrease in HCC incidence.[48] Metformin appears to have antitumoral effects via several pathways by decreasing the level of insulin-like growth factor-1, suppressing c-Jun N-terminal kinase/p38 mitogen-activated protein kinase, human epidermal growth factor receptor-2, and nuclear factor-κB pathways, activating AMP-activated protein kinase, inhibiting the mammalian target of rapamycin pathway, and decreasing the endogenous production of reactive oxygen species.

Statins

The protective impact of statins on HCC development is most likely due to their anti-inflammatory characteristics, which are mediated through Janus kinase inhibition.[49] Several clinical studies have found that statins are useful in lowering the risk of HCC.[50-52] A recent meta-analysis of 24 studies found that statin users had a 46% lower risk of HCC, indicating that statins might be used in chemoprophylaxis.[53] A subanalysis of another meta-analysis found that using lipophilic statins (atorvastatin, pitavastatin, lovastatin, fluvastatin, simvastatin) was linked with a considerably lower risk of HCC when compared to hydrophilic statins (rosuvastatin, pravastatin) (27% vs. 51%).[54] Lipophilic statins have higher lipid solubility and membrane permeability, allowing them to have cholesterol-dependent effects on HCC development.[55]

SURVEILLANCE STRATEGY FOR NAFLD

The annual incidence of HCC in individuals with NAFLD-related cirrhosis is greater than 1.5%.[56,57] If liver cirrhosis is clinically suspected among patients with NAFLD, HCC surveillance is recommended.[58-60] Since NAFLD-related LC patients may lose weight when they progress to LC, the etiology of cryptogenic LC should not be judged based on BMI alone. Non-invasive modalities to diagnose advanced fibrosis such as transient elastography might be a good tool to discriminate those high-risk population.[1] As shown in the previous systemic review,[61] the incidence of HCC was quite low in subjects with early liver fibrosis (F0–2), 2.7% at 10 years and 23 per 100,000 person-years. However, patients with early liver fibrosis are more prone to develop HCC if they have other risk factors (obesity, metabolic syndrome, diabetes, etc.) and also HBV or hepatitis C virus infection in terms of metabolic-asso-

ciated fatty liver disease. Therefore, a surveillance strategy for NAFLD patients should be individualized.[58,62]

Although some evidence suggests that HCC can develop in livers without cirrhosis or steatohepatitis, surveillance should be carefully planned. Owing to the lack of robust data on the noncirrhotic population, it is difficult to develop evidence-based, cost-effective surveillance strategies for the NAFLD population. Clinical trials are needed to address the issue of surveillance in NAFLD, particularly in noncirrhotic persons.[63]

Abdominal ultrasonography is the primary tool used for HCC surveillance. However, it might be difficult to accurately execute this procedure in overweight or obese patients.[64,65] Computed tomography or magnetic resonance imaging can be used instead.

CONCLUSION

Weight loss, dietary changes, and increased physical activity continue to be the cornerstones of HCC prevention in patients with NAFLD. The impact of lifestyle factors and chemopreventive agents may differ between NAFLD-associated hepatocarcinogenesis and hepatocarcinogenesis due to other etiologies, taking into account the heterogeneity of the NAFLD and NASH populations. A better understanding of the underlying pathophysiological mechanisms and disease phenotypes may enable focused preventive interventions for NAFLD-associated HCC in the future. New insights into the etiology, pathogenesis, and surveillance of HCC in patients with NAFLD may enable the development of therapeutic and preventive strategies.

Authors' contribution

Study conceptualization: YC and JWP; Drafting of the manuscript: YC; Critical revision of the manuscript: BHK, YC, and JWP

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Conflicts of Interest

The authors have no conflicts to disclose.

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Review

Surveillance of the progression and assessment of treatment endpoints for nonalcoholic steatohepatitis

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Nonalcoholic steatohepatitis (NASH) is an aggressive form of nonalcoholic fatty liver disease (NAFLD) characterized by steatosis-associated inflammation and liver injury. Without effective treatment or management, NASH can have life-threatening outcomes. Evaluation and identification of NASH patients at risk for adverse outcomes are therefore important. Key issues in screening NASH patients are the assessment of advanced fibrosis, differentiation of NASH from simple steatosis, and monitoring of dynamic changes during follow-up and treatment. Currently, NASH staging and evaluation of the effectiveness for drugs still rely on pathological diagnosis, despite sample error issues and the subjectivity associated with liver biopsy. Optimizing the pathological assessment of liver biopsy samples and developing noninvasive surrogate methods for accessible, accurate, and safe evaluation are therefore critical. Although noninvasive methods including elastography, serum soluble biomarkers, and combined models have been implemented in the last decade, noninvasive diagnostic measurements are not widely applied in clinical practice. More work remains to be done in establishing cost-effective strategies both for screening for at-risk NASH patients and identifying changes in disease severity. In this review, we summarize the current state of noninvasive methods for detecting steatosis, steatohepatitis, and fibrosis in patients with NASH, and discuss noninvasive assessments for screening at-risk patients with a focus on the characteristics that should be monitored at follow-up. **(Clin Mol Hepatol 2023;29(Suppl):S228-S243)**

Keywords: Nonalcoholic steatohepatitis; Noninvasive diagnosis; Disease progression; Risk stratification; Treatment efficacy

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a heterogeneous and silently progressive disease that affects roughly one-third (32%) of the global population.[1,2] With an alarming increase in both worldwide prevalence and incidence, NAFLD has become one of the most common causes of chronic liver diseases in the majority of industrialized areas.[3,4] Compared with nonalcoholic fatty liver (NAFL), which is characterized by bland steatosis, nonalcoholic steatohepatitis (NASH) is a more progressive phenotype of NAFLD characterized by hepatocyte injury, inflammation, and scarring. It has been estimated that around 25% of NAFLD patients will develop NASH, and 20% of patients with NASH will develop cirrhosis

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and hepatocellular carcinoma (HCC) in 20 to 30 years from disease onset.[5] In the past decade, liver-specific and overall mortality rates of NASH have been increasing rapidly, especially in the patients with obesity, type 2 diabetes mellitus (T2DM), and metabolic syndrome.[6] Early identification and targeted treatment for NASH are urgently needed to improve patient outcomes.

Currently, diagnosis and evaluation of the severity of NASH is still based on liver biopsy-proven histopathological assessment and scoring, and is therefore reliant on invasive liver biopsy. The main scoring systems for NASH consider liver fibrosis, inflammation, and steatosis.[7,8] Although a number of noninvasive tests and predictive models have been developed to characterize fibrosis in NASH patients, their diagnostic performance and clinical application can be improved. Since there are still no NASH-specific drugs that have been approved by major drug administration agencies worldwide, lifestyle interventions including dietary changes and exercise, with the purpose of 10% weight loss, are the most effective approaches for the management of fibrotic NASH and underlying cardiometabolic comorbidities.[9] Liver biopsy, the current "gold" standard for the diagnosis of NASH, is essential for both patient enrollment and efficacy assessment of phase 2b trials of drugs currently under development in addition to all phase 3 trials.[10,11]

Accurate evaluation of the severity of NASH and the risk of progression to liver cirrhosis and HCC is essential for screening at-risk NASH patients and determining treatment responses (including NASH remission and cirrhosis prevention) to novel NASH drugs in clinical trials. In the present review, we discuss approaches used for the surveillance of the progression of NASH and assessment of treatment endpoints.

RISK OF NASH PROGRESSION

Fibrosis

Liver fibrosis is recognized as a determinant of liver-related morbidity and mortality in patients with NAFLD/NASH.[12] Previous studies have shown that significant fibrosis (≥F2) and advanced fibrosis (≥F3) are independently associated with overall mortality, liver transplantation, and liver-specific mortality in patients with NAFLD.[13] In one study, patients with fibrotic NAFLD had a lower survival rate after liver transplantation than those with non-fibrotic NAFLD, regardless of the presence of NASH.[14] A recent meta-analysis demonstrated that the risk of liver-related mortality, all-cause mortality, and requirement for a liver transplant increased with poorer biopsy-confirmed fibrosis stage.[15] According to the Finnish population-based FINRISK and Health 2000 studies with a median follow-up of 12.1 years, the crude incidence of liver-related outcomes in NAFLD was 0.97/1,000 person-years, and outcomes were associated with noninvasive fibrosis stage.[16] Moreover, HCC risk was highest with cirrhosis, followed by noncirrhotic fibrosis and comorbid T2DM in a biopsy-proven NAFLD cohort.[17] Correspondingly, NASH patients with compensated cirrhosis may have fewer liver-related complications if fibrosis regression is evident, which presents as a decrease in NAFLD fibrosis score (NFS), liver stiffness measurements, and hepatic collagen and alpha-smooth muscle actin expression.[18] In addition, most of novel drugs in phase 3 clinical trials targeting NASH also target fibrosis with stage ≥F2 to prevent fibrosis progression and liver-related events. Therefore, identifying NASH patients with significant fibrosis or advanced fibrosis can be used to identify populations at high risk for progression to liver cirrhosis and HCC.[19]

Abbreviations:

NASH, nonalcoholic steatohepatitis; NAFLD, nonalcoholic fatty liver disease; NAFL, nonalcoholic fatty liver; HCC, hepatocellular carcinoma; T2DM, type 2 diabetes mellitus; NFS, NAFLD fibrosis score; α-SMA, alpha-smooth muscle actin; BMI, body mass index; NAS, NAFLD activity score; ML, machine learning; AI, artificial intelligence; WSI, whole-slide images; SHG, second-harmonic generation; q-FPs, quantify fibrosis-related parameters; qFIBS, qFibrosis, qInflammation, qBallooning, and qSteatosis; FLI, fatty liver index; HSI, hepatic steatosis index; LAP, lipid accumulation product; MRS, magnetic resonance spectroscopy; DSI, Dallas Steatosis Index; FIB-4, fibrosis-4; AST, aspartate aminotransferase; APRI, AST to Platelet Ratio Index; HA, hyaluronic acid; PIIINP, amino-terminal propeptide of type III procollagen; TIMP-1, tissue inhibitor of metalloproteinase-1; NPV, negative predictive value; PPV, positive predictive value; CK18, cytokeratin 18; hs-CRP, hypersensitive C-reactive-protein; AC, attenuation coefficient; BSC, back scatter coefficient; TE, transient elastography; CAP, controlled attenuation parameter; MRI, magnetic resonance imaging; PDFF, proton density fat fraction; CT, computed tomography; LSM, liver stiffness measurement; ARFI, acoustic radiation force imaging; SSI, supersonic shear imaging; MRE, magnetic resonance elastography; MAST, MRI-aspartate aminotransferase; FAST, FibroScan-aspartate aminotransferase; HOMA, homeostasis model assessment; ADAMTSL2, A disintegrin, a metalloproteinase with thrombospondin motif like 2; OCA, obeticholic acid; CPA, collagen proportionate area; HRQoL, health-related quality of life; PRO, patient-reported outcomes; PROs, patient-reported outcomes

Inflammation: a trigger of fibrosis and carcinogenesis

Patients with simple steatosis are often considered to have a similar life expectancy to that of the general population, while patients with NASH are generally considered to have a lower life expectancy. In the presence of chronic inflammation, adipose tissue releases free fatty acids and toxic lipids, followed by fat accumulation, lipotoxicity, oxidative stress, and mitochondrial dysfunction in hepatocytes,[20] leading to liver fibrogenesis and carcinogenesis. It has been reported that up to one-third of NASH patients without effective intervention will develop advanced liver fibrosis or cirrhosis, and potentially HCC.[21] Although a previous study investigated the impact of fibrosis on the prognosis of NAFLD patients, persistent hepatocyte injury or chronic inflammation in the liver is one of the driving forces of disease progression and carcinogenesis.[22] A further study confirmed that fibrosis progression is faster in NASH than NAFL and that NASH patients are at higher risk for HCC than NAFL patients; NAFL patients progress one fibrosis stage per 14.3 years, while patients with NASH progress one fibrosis stage per 7.1 years.[23]

Metabolic dysfunction: cause or consequence?

Obesity is the most common cause of metabolic dysfunction, and is considered related to the epidemic of NAFLD. Overall obesity increases *de novo* lipogenesis and decreases β oxidation of free fatty acids and very low-density lipoprotein secretion, resulting in hepatocyte lipidosis and lipotoxicity. However, it should be noted that a large proportion of patients with NAFLD are lean or non-obese based on body mass index.[24,25] Approximately 8–19% of Asians with a body mass index (BMI) less than 25 kg/m^2 also have NAFLD,[26] and the prevalence of NAFLD in non-obese subjects has been found to be as high as 16%.[24] However, obesity as defined by BMI is only a crude measurement of obese status. Other anthropometric parameters might be useful for diagnosis of central obesity, occult obesity, and sarcopenic obesity. Central adiposity, sarcopenia, dyslipidemia, and insulin resistance are strongly associated with NASH and related fibrosis in a dose-dependent manner.[27] The progressive course of NASH is closely linked to an increasing number of metabolic comorbidities. T2DM has the strongest association with incident HCC in patients with NAFLD.[28-30] Metabolic syndrome is an independent predictor of all-cause, liver-specific, and cardiovascular mortality in patients with NAFLD.[31,32] In contrast, mortality of metabolically normal NAFLD patients is similar to that of patients without liver disease.[33-35] Thus, assessing metabolic dysfunction, including insulin resistance, may help define high-risk NASH patients.[36] In addition, accumulating evidence suggests that NAFLD has complex links with metabolic dysfunction; for example, NAFLD, especially NASH, is also associated with an increased risk of incident T2DM and atherosclerotic cardiovascular disease events.[37]

HISTOPATHOLOGICAL SURVEILLANCE FOR NASH

Liver biopsy is imperfect

Screening of high-risk patients and surveillance for the development of liver-related complications are urgently needed for the management of NASH given the chronic progressive nature of this disease. Several novel NASH pharmacological agents are currently under development, and monitoring the treatment response relies on accurate assessment in clinical trials. Histopathological assessment is considered the "gold" standard for the diagnosis and evaluating of NASH severity and fibrosis stage. However, liver biopsy is not feasible for repeated assessment due to its invasive nature. Furthermore, histological evidence from liver biopsies is only moderately accurate and requires additional validation, therefore more reliable techniques for accurate quantification of the severity of NASH and fibrotic stage are required.

Histological classification of NASH is currently performed using semiquantitative scoring systems. NAFLD activity score (NAS) which was developed by the NASH clinical research network, and the steatosis, activity, fibrosis scoring system developed by fatty liver inhibition of progression Pathology Consortium, are the two most widely used scoring systems.[7,38] Both systems identify the location and features of fibrosis, number of inflammatory foci, number of balloon cells, and percentage of parenchymal involvement of the steatosis. Assessment depends on manual and subjective judgment, resulting in intra- and inter-observer variability. Although liver biopsy is generally considered safe and is widely available, histological scoring is limited by sampling error and ordinal classification. Developing innovative methods based on ma-

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chine learning (ML), artificial intelligence (AI), and whole-slide images (WSI) may be a key to improve histopathological assessment.

Novel liver biopsy-based assessment tools

Second-harmonic generation (SHG) microscopy is highly sensitive to the collagen fibril/fiber structure, and has enabled the imaging of fibrillar collagen in various tissues. SHG-based novel technology has also been applied to assess hepatic fibrosis in chronic liver diseases.[39] HistoIndex as one of the SHG-based novel technologies for the assessment of hepatic steatosis has shown a good correlation with histopathologist scores,[40] and was applied in a phase 2 clinical trial (MGL-3196, Resmetirom) to evaluate dynamic changes in steatosis during treatment.[41] A model to quantify fibrosis-related parameters (q-FPs) was developed by Wang et al. to assess the characteristics of liver fibrosis in NAFLD. A model containing four q-FPs (number of collagen strands, strand length, strand eccentricity, and strand solidity) was established based on findings in 50 test subjects and validated in 42 validation subjects to facilitate continuous and quantitative evaluation of fibrosis.[42] Furthermore, a combination of qFibrosis, qInflammation, qBallooning, and qSteatosis (qFIBS index) was developed to allow quantitative assessment of the characteristics of NAS (lobular inflammation, ballooning, and steatosis) by using SHG and two-photon excitation fluorescence imaging technology. qFIBS was developed and then validated in a cohort of 219 patients with biopsy-proven NAFLD/NASH and showed a robust correlation with NAS and fibrosis stages.[43] Recently, qFIBS was applied in a phase 2 trial of tropifexor (NCT02855164), to assess the resolution of NASH and fibrosis. qFIBS was found to have sufficient sensitivity to evaluate regressive changes in septa morphology and a reduction in septa parameters in F3 patients, which cannot be captured by traditional scoring systems.[44]

Advances in machine-learning-based approaches are enabling histopathological monitoring of the progression and regression of NASH.[45] Digital WSI comprises scanning of hematoxylin-eosin -stained slides to quantify steatosis by assessing the steatosis proportionate area. Elastica van Gieson-stained slides can be scanned to quantify fibrosis by assessing the number of collagen and elastin fibers,[46-48] and is regarded as an automated, precise, objective and quantitative method to assess NASH. Assessment of ballooning cells, one of the most important features of NASH, is highly subjective. AI-based technology can be trained to reproducibly quantify ballooned hepatocytes and standardize the evaluation.[49] ML-based models have been used to assess NASH histological characteristics accurately in addition to treatment response. PathAI showed concordance with ordinal grades from pathologists in terms of three NAS components. In addition, PathAI detected improvements in the DELTA Liver Fibrosis score in fibrosis responders in the combination group (cilofexor+firsocostat) in the ATLAS study.[50] AI- and ML-based technologies are advancing rapidly and can potentially address the inadequacies of pathological assessment of fibrotic NASH.

NONINVASIVE MARKERS ARE MORE PRACTICAL THAN LIVER BIOPSY FOR MONITORING OF NASH

Given the increasing prevalence of NASH, the base of at-risk patients who need screening is large. Liver biopsy is a critical bottleneck in the diagnosis and monitoring of these patients. Thus, it is critical to develop accurate noninvasive tests, markers, and models to evaluate NASH severity and monitor drug efficacy. Based on these needs, researchers have developed several noninvasive assessment methods including serum biomarkers, elastography-based markers, imaging studies, genetic tests, and omics profiling.

Noninvasive tests are more acceptable for evaluation of steatosis degree and fibrosis stage than liver biopsy, and also improve screening compliance and monitoring of NAFLD. As histological assessment from liver biopsy is still imperfect, an ideal solution is to link clinical outcomes such as cirrhosis, HCC, and liver-related complications with novel noninvasive markers. Correlating the histological severity of NASH and fibrosis stages with quantified noninvasive markers is a feasible approach (Table 1).[51-57]

Serum biomarkers for assessment of steatosis

Currently, the most promising noninvasive diagnostic tools for hepatic steatosis are the fatty liver index (FLI), the hepatic steatosis index (HSI), the NAFLD-liver fat score, the visceral adiposity index, the lipid accumulation product (LAP), and the triglyceride×glucose index.[58] Most of these indexes have

Table 1. Surveillance markers for steatosis, steatohepatitis, and fibrosis in patients with NASH

Characteristics	Assessment	C-statistic
Steatosis	Controlled attenuation parameter (CAP)[51]	0.82
	Dallas steatosis index (DSI)[60]	0.82
	MRI-proton density fat fraction (PDFF)[52]	0.99
Steatohepatitis	Cytokeratin 18 (CK18)[53]	0.83–0.93
	NAFIC score[54,*]	0.85
	Corrected T1 (cT1)[109]	0.78
Fibrosis	Fibrosis-4 index (FIB-4)[55]	0.75 for SF 0.80 for AF 0.85 for cirrhosis
	Liver stiffness measurement (LSM)[88]	0.86 for SF 0.80 for AF 0.69 for cirrhosis
	NAFLD fibrosis score (NFS)[55]	0.83 for cirrhosis 0.73 for AF 0.72 for SF
	Aspartate aminotransferase (AST) to Platelet Ratio Index (APRI)[55]	0.70 for SF 0.75 for AF 0.75 for cirrhosis
	BARD score[56,†]	0.64 for SF 0.73 for AF 0.70 for cirrhosis
	Enhanced liver fibrosis (ELF) score[70]	0.79 for AF
	FiberMeter[70]	0.80 for AF
	Shear wave elastography (SWE)[57]	0.86 for AF 0.89 for SF 0.88 for cirrhosis
	Acoustic radiation force imaging (ARFI)[57]	0.77 for AF 0.84 for SF 0.84 for cirrhosis
	Magnetic resonance elastography (MRE)[88]	0.89 for SF 0.87 for AF 0.87 for cirrhosis

NASH, nonalcoholic steatohepatitis; AF, advanced fibrosis; SF, significant fibrosis.
[*]A scoring system using ferritin, fasting insulin, and type IV collagen 7S.
[†]A scoring system including body mass index, AST/ALT ratio, and diabetes.

been validated in biopsy-proven cohorts or magnetic resonance spectroscopy (MRS) results have been used as a reference. The accuracy of FLI, HSI, LAP, and the Zhejiang University index (ZJU) was evaluated in a general population by ultrasonography. Although FLI showed the highest C-statistic (0.85), the relatively low sensitivity of ultrasonography in detecting mild steatosis is of concern.[59] Although assessing steatosis grade is simpler than assessing inflammation or fibrosis, detecting >5% hepatic steatosis by circulating biomarkers alone is insufficient. Combinations of biomarkers would likely increase the accuracy of detecting steatosis. Dallas Steatosis Index (DSI), which consists of age, sex, diabetes, hypertension, race, BMI, serum triglycerides and alanine amiotransferase, was developed in the Dallas Heart Study of 737 patients with MRS-diagnosed liver fat. The C-statistic of DSI was found to be 0.82, but its diagnostic performance still needs external validation.[60] It should be noted that ultrasound tests are more widely available than blood-based tests. Serum proteins measured in these models are associated with metabolic disorders or insulin resistance and are not strictly specific

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to hepatic fat content, which may explain why these models have insufficient accuracy, especially in non-obese or lean subjects. The current serum-based noninvasive markers therefore have limited utility for surveillance.

Serum biomarkers for the assessment of liver fibrosis

Given that fibrosis is the major driver of liver-related outcomes in NAFLD, assessing fibrosis stage is essential for screening at-risk patients. The simple serum biomarker panel used in the fibrosis-4 index (FIB-4) and the aspartate aminotransferase (AST) to Platelet Ratio Index (APRI), originally developed for chronic viral hepatitis, could be applied in NASH patients. The cut-off value for FIB-4 is 2.67 and 1.30 to rule in and rule out advanced fibrosis in patients with NAFLD, respectively. NFS, developed from a liver biopsy-proven NAFLD cohort, has cut-off values of -1.455 and 0.676 to rule out or rule in advanced fibrosis.[61] BARD score (a scoring system include body mass index, AST/ALT ratio, and diabetes) was developed to diagnose advanced fibrosis by combining BMI, AST/ALT levels, and diabetic status.[62] Both FIB-4 and NFS are relatively easy to perform and are recommended for identification of NAFLD patients at low or high risk of advanced fibrosis. These tests have been widely used, and are available in primary health care units. However, due to the various etiologies of the cohorts who these makers were validated in, the accuracy of these tests needed to be improved when applied to NAFLD cohorts. In addition, models developed from biopsy-proven NAFLD cohorts often use higher cut-off values than those used for the general population. This leads to inferior diagnostic performance of NFS, FIB-4, and APRI in general population.[63,64]

Many biomarker tests, including those with patented markers, involve direct biomarkers of fibrogenesis or fibrinolysis from the extracellular matrix. Type III collagen and hyaluronic acid (HA) are common biomarkers. The amino-terminal propeptide of type III procollagen (PIIINP) can discriminate between regular and advanced fibrosis with a C-statistic of 0.82-0.84.[65] Enhanced liver fibrosis (ELF) test, a commercial panel of markers comprising serum HA, the PIIINP, and the tissue inhibitor of metalloproteinase-1 (TIMP-1), was first developed in children with NAFLD and validated in larger cohorts.[66,67] Recently, the ELF test was used to assess fibrosis improvement during aldafermin (NGM282) treatment.[68] Another type III collagen-based fibrosis algorithm including age, presence of diabetes, PRO-C3 (a marker of type III collagen formation), and platelet count (called ADAPT) showed better diagnostic performance than APRI, FIB-4 and NFS in predicting advanced fibrosis.[69] FibroMeter consists of age, weight, glucose, AST, ALT, ferritin, and platelets, and has been directly compared with ELF. ELF and FibroMeter had significantly higher C-statistics than NFS and FIB-4 in diagnosing advanced fibrosis, while the C-statistic did not differ significantly between ELF and FibroMeter.[70] FibroTest is a commercial panel with a C-statistic of 0.75–0.86 for significant fibrosis and 0.81–0.92 for advanced fibrosis.[71] FIBROSpect, which comprises alpha 2 macroglobulin, HA, and TIMP-1, is highly sensitive for advanced fibrosis (positive predictive value, PPV 92.5–94.7%), with a C-statistic of 0.86.[72] Hepamet was developed in 2,452 biopsy-proven NAFLD patients, and had a higher C-statistic than FIB-4 and NFS. Hepamet is unaffected by age, BMI or diabetes.[73] These tests, although more accurate at predicting advanced fibrosis, are expensive, and there is still a dearth of direct comparisons in the same cohorts. In general, biomarkers or models detecting advanced fibrosis have a relatively high negative predictive value (NPV) while the positive predictive value (PPV) requires improvement.

Serum biomarkers to assess steatohepatitis

Hepatocyte ballooning and inflammation are the most important features of steatohepatitis, but current biochemical and imaging measures cannot effectively distinguish NASH from NAFL. Serum ALT is not a sufficiently sensitive predictive marker for diagnosis of steatohepatitis as less than 30% of NASH patients have elevated ALT levels (>35 U/L). Use of ALT >2 times the upper limit of normal to diagnose NASH only has 50% sensitivity and 61% specificity.[74] Cytokeratin 18 (CK18) is released into the serum on initiation of apoptosis in the form of CK18-M30 and CK18-M65 fragments. Serum CK-18 has been the most widely investigated in the diagnosis of NASH. In one study, CK18 was thought to have potential predictive value for fibrosis, but showed a better correlation with ALT rather than with steatosis or fibrosis.[75] Another study involving repeated liver biopsy found that serum CK18 level was associated with NAS ≥5 (definite NASH) in patients with NAFLD.[76] Meta-analyses have confirmed that CK18 can predict steatohepatitis with a C-statistic around CK18 0.80 and sensitivity of 66–78%.[77,78] Index of NASH, which consists

of waist-to-hip ratio, triglyceride, ALT, homeostatic model assessment for insulin resistance (HOMA) and gender, was developed to diagnose steatosis[79] but showed low sensitivity in an external cohort, especially in non-obese subjects.[80] Although serum level of hypersensitive C-reactive-protein (hs-CRP) is included in the diagnosis of metabolic-dysfunction associated fatty liver disease[81] its diagnostic value in NASH requires further investigation. A recent study of 100 subjects observed an independent relationship between hs-CRP and NAFLD.[82] More direct evidence is required for use of hs-CRP as a diagnostic marker for NASH. Both single nucleotide polymorphisms and noncoding RNAs have been used to predict NASH. NASH Score (PNPLA3 genotype, AST, and fasting insulin) and circulating miR-122 have shown potential prognostic significance in NASH.[83,84] Unlike NASH-related fibrosis, there are currently no direct biomarkers for steatohepatitis. The available evidence indicates that use of a single biomarker to discriminate bland steatosis from NASH is unlikely to be successful.

Advances in imaging-based approaches

Ultrasonography is the most widely used imaging tool for identifying liver disease but lacks sensitivity. In patients with mild to moderate steatosis, the accuracy of ultrasonography is only around 50%.[85] Thus, quantitative ultrasound-based techniques are being developed to improve the diagnosis of hepatic steatosis. Attenuation coefficient (AC) and back scatter coefficient (BSC) have been shown to be correlated with the severity of hepatic steatosis. In a biopsy-proven study, AC and BSC achieved an accuracy of 61.7% and 68.3% in predicting steatosis grade, respectively, which are significantly higher accuracies that achieved with traditional ultrasonography.[85] Ultrasound-guided attenuation parameter has also showed excellent ability to distinguish steatosis grades (0.92, 95% confidence interval: 0.87–0.97) in non-B non-C chronic hepatitis subjects.[86] Transient elastography (TE) devices can be used to assess the controlled attenuation parameter (CAP) for liver fat quantification. CAP showed good sensitivity for detecting mild steatosis (S1) and excellent diagnostic accuracy in distinguishing S1, S2, and S3 in a study that used liver biopsy as the reference.[87,88] In terms of incidence and resolution of steatosis, CAP can also be used to assess dynamic changes.[89] Although the sampling error of CAP can be reduced by increasing the detection volume (3 cm^3), its accuracy is reduced by increasing amounts of subcutaneous adipose.

Among magnetic resonance imaging (MRI)-based biomarkers, MRS is sensitive to small amount of hepatic adipose and is recognized as the most accurate noninvasive method to quantify steatosis. MRS is often used as the reference when assessing other noninvasive markers.[59] However, advanced training is required to measure MRS, which has limited its widespread application. MRI-proton density fat fraction (PDFF) is more accessible than MRS in most tertiary health centers. MRI-PDFF can assess the fat content in the whole liver and also allow for the assessment of regions of interest. Multiple studies have proven a close agreement between fat content as assessed by MRI-PDFF and histological steatosis grade.[90,91] Liver fat content measured by MRS or MRI-PDFF changes over time, which could reflect dynamic changes in hepatic steatosis. MRI-PDFF can be used to determine absolute and relative liver fat content. MRI-PDFF was shown to have better diagnostic accuracy than CAP in a head-to-head comparison.[88]

Computed tomography (CT) can be used to assess liver fat content through the absolute attenuation of liver parenchyma value.[92] CT is more sensitive to moderate-to-severe steatosis than mild steatosis. The sensitivity for detecting grade ≥2 steatosis is more than 90%. Although CT is not routinely used to identify steatosis, it can be important in detecting incidental steatosis.

TE is the simplest and most commonly used noninvasive imaging tool for screening for fibrosis in clinics. The cut-off values of liver stiffness measurement (LSM) by TE for identifying advanced fibrosis varies with liver disease etiology. For NAFLD, a recent study determined a cut-off of 6.5 kPa to rule out advanced fibrosis and a cut-off of 12.1 kPa to rule in advanced fibrosis.[93] In a study of Asian NAFLD patients, the cut-off value to rule out advanced fibrosis was 7.9 kPa and the cut-off to rule in advanced fibrosis was 9.6 kPa.[94] LSM is sensitive to advanced fibrosis and cirrhosis, while its specificity for ruling out F1 and F2 fibrosis requires improvement. In addition, LSM can be affected by various factors including obesity, subcutaneous fat thickness, high ALT levels, and cholestasis.[95] Agile 3+ and Agile 4 are models that combine LSM with routine clinical parameters to identify advanced fibrosis and cirrhosis, respectively. Both Agile 4 and Agile 3+ showed better diagnostic performance, especially positive predictive value, than FIB-4 and LSM.[96] Acoustic radiation force imaging

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(ARFI) was developed from a chronic hepatitis C patient cohort to diagnose advanced fibrosis. The efficacy of ARFI, supersonic shear imaging (SSI), and TE was compared in a head-to-head study. Similar to TE, the application of ARFI and SSI in obese subjects is limited, and SSI showed higher accuracy than ARFI for diagnoses of F2 fibrosis.[97]

MRI machines can be equipped with magnetic resonance elastography (MRE) to assess liver stiffness. Both MRE and TE showed excellent diagnostic accuracy for diagnosing stage F2-F4 fibrosis with a C-statistic of greater than 0.90.[98] Several studies have reported that MRE is more accurate than TE.[88,98,99] MRE also has a higher success rate than TE at detecting fibrosis in obese patients (95.8% vs. 88.5%). In a recent meta-analysis, MRE had a higher C-statistic for detecting F≥2 and F≥3 but a similar performance to TE and shear wave elastography at detecting cirrhosis.[100] The combination of MRI with other imaging tests and biomarkers could increase diagnostic performance. MEFIB is the combination of MRE and FIB-4, and showed a relatively high PPV of 97.1% in diagnosing ≥stage F2 fibrosis.[101] The MRI-aspartate aminotransferase (MAST) score refers to the combination of MRI and NFS, FIB-4, and FibroScan-aspartate aminotransferase (FAST). MAST had a higher C-statistic than that of the components of this index, reducing the number of the patients in the "gray zone".[102]

DYNAMIC MONITORING AND PROGNOSIS RISK ASSESSMENT

Definition and biomarkers of at-risk NASH patients

Given the progressive nature of NASH, there are numerous efforts underway to develop novel drugs. Emerging treatments mostly target hepatic fibrosis and steatohepatitis-associated inflammatory activity. Patients who are at risk of disease progression should therefore be included in clinical trials and effective tests should be used to repeatedly assess the drug response. The Liver Forum defined the following NAFLD subgroups: NAFL, indeterminate NASH, NASH without fibrosis, NASH with early fibrosis, NASH with bridging fibrosis, compensated cirrhosis, and decompensated cirrhosis.[103] A number of biopsy-proven studies have showed that both fibrosis stage and NAS at baseline are correlated with a higher risk of increased fibrosis stage during follow-up. Recently, Harrison et al.[104] defined "at-risk NASH" patients as NAFLD patients with NAS ≥4 and fibrosis stage ≥2. Following this definition, several studies have offered noninvasive solutions to distinguish these patients from others.

MACK-3 is the combination of AST, HOMA, and CK18, and has shown high accuracy in at-risk NASH patients (NAS ≥4 and F ≥2).[105] Cut-off MACK-3 values of ≤0.134 and ≥0.550 can be used to rule out and rule in these patients who need more aggressive drug intervention, respectively.[106] The algorithm ADAPT mentioned previously is also effective at detecting at-risk patients.[107] A recent study compared the diagnostic performance of MEFIB, MAST, and FAST at detecting at-risk NASH patients. All three models provided utility in NAFLD risk stratification, while MEFIB showed better performance at detecting at-risk NASH than MAST and FAST.[108] Direct correlation with the severity of inflammation was previously regarded as the bottleneck of imaging tests, but currently corrected T1 (cT1) showed potential in predicting NASH. cT1 had better diagnostic accuracy (0.78 vs. 0.69) in identifying high-risk NASH than MRI-PDFF.[109] Furthermore, a protein-based signature of fibrosis could also serve as a diagnostic tool. A disintegrin, a metalloproteinase with thrombospondin motif like 2 (ADAMTSL2), and an 8-protein panel showed predictive value for at-risk NASH.[110]

Biomarkers of treatment response and clinical outcomes

The best clinical outcome to evaluate the efficacy of NASH treatment is liver-related morbidity and mortality, while the surrogate endpoint is histologic outcome. Current guidelines recommend histological NASH resolution without worsening of fibrosis or regression of fibrosis without worsening of NASH as the treatment endpoint in phase 3 trials of NASH.[11] The reliance on histologic outcomes for primary trial endpoints is a barrier to patient enrollment. There is an urgent need to develop accurate noninvasive markers that reflect drug-induced changes. Markers or algorithms that reflect disease severity or long-term prognosis could be utilized as surrogate endpoints for clinical trials of drugs targeting NASH (Fig. 1).

Some noninvasive markers reflect dynamic changes associated with histological changes. Imaging-based tests have the best potential to be surrogates of histological assessment of

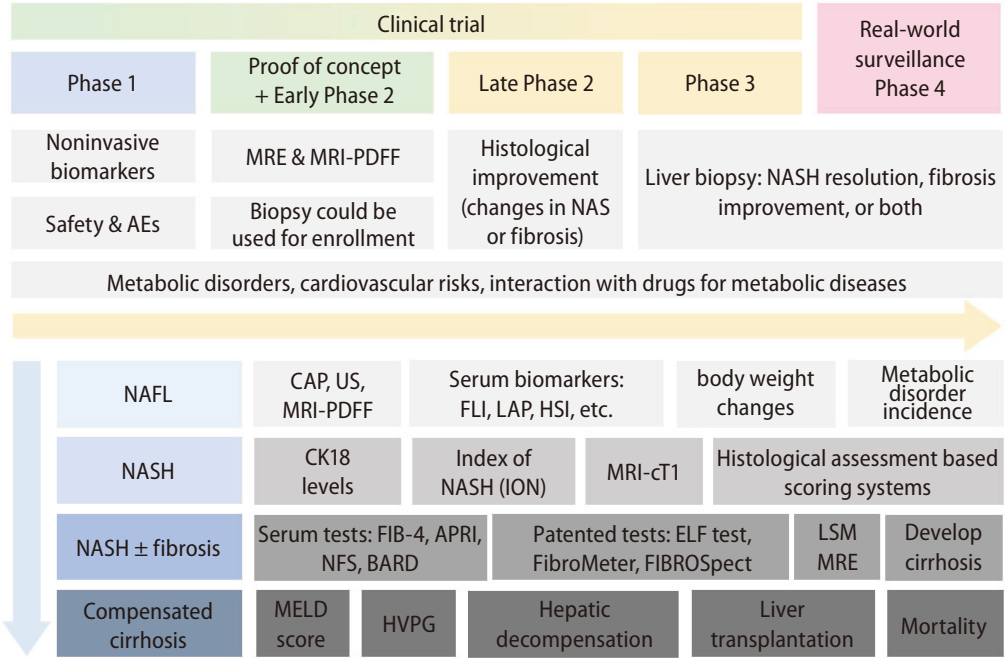

Figure 1. Evaluation approaches for different trial phases and different stages of NASH. Specific sets of evaluation tools should be used for different phases of NASH. Different assessments are also required for patients with different stages of NASH. MRE, magnetic resonance elastography; MRI-PDFF, magnetic resonance imaging-proton density fat fraction; NASH, nonalcoholic steatohepatitis; NAS, NASH activity score; AEs, adverse events; CAP, controlled attenuation parameter; US, ultrasound; CK18, Cytokeratin 18; cT1, corrected T1; FLI, fatty liver index; HSI, hepatic steatosis index; FIB-4, fibrosis-4 index; LAP, lipid accumulation product; NFS, NAFLD fibrosis score; ELF test, enhanced liver fibrosis test; LSM, liver stiffness measurement; MELD score, model for end-stage liver disease score; HVPG, hepatic venous pressure gradient.

steatosis grade and fibrosis stage.[111] As early as in the FLINT trial of obeticholic acid (OCA), MRI-PDFF was used as a surrogate marker of steatosis. Taking a 30% relative reduction in MRI-PDFF as an endpoint, OCA was better than the placebo in achieving the goal. In addition, non-responders also showed less histological improvement than responders (19% vs. 50%, respectively).[112] Patented ELF and PIIINP were also used as serum markers of treatment efficacy in the PIVENS Trial. ELF showed a significant correlation with advanced fibrosis in patients with NASH, but not with longitudinal changes in fibrosis.[113] As mentioned above, ML-based methods can be used to translate histological characteristics into continuous variables. For instance, collagen proportionate area (CPA) as assessed by digital image analysis may offer a more granular assessment of fibrosis than routine histological analysis. Small changes detected by CPA might be missed when comparing fibrosis stages.[114,115] Furthermore, ML-based histological assessment is worth evaluation as a surrogate endpoint in clinical trials.

MEASUREMENT OF HEALTH-RELATED QUALITY OF LIFE AND EXTRAHEPATIC OUTCOMES

NASH patients often have concomitant extrahepatic diseases, such as obesity, dyslipidemia, hypertension, T2DM, cardiovascular disease, and chronic kidney disease. In obese NASH patients, the diagnostic accuracy of noninvasive markers needs to be improved.[116] Our research group investigated the diagnostic value of metabolic disorders in NASH fibrosis.[36] Insulin resistance has been proven to play an essential role in the development of steatohepatitis and fibrosis. Although treatment may benefit comorbidities in NASH patients, there is insufficient evidence to use an improvement in metabolic comorbidities as a trial endpoint. Compared with cirrhotic patients, non-cirrhotic NASH patients are likely to have a higher incidence of cardiovascular disease.[117] In this case, metabolic-related events should be closely monitored, while longer follow-up periods are required to observe liver-related outcomes.

NAFLD not only increases the risk for development of he-

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patic and extrahepatic outcomes, but impairs health-related quality of life (HRQoL). In comparison with healthy controls, patients with NAFLD have decreased HRQoL scores and impaired patient-reported outcomes (PRO) that are worse than those of patients with other chronic liver diseases.[118] Changes in HRQoL and PRO scores in NAFLD are associated with hepatic disease severity and its improvement after effective treatment. The HRQoL score declines in order from NAFL to NASH, then advanced fibrosis, and cirrhosis in patients with NAFLD. Histological improvement such as reduction of steatosis degree, remission of NASH, decreased NAS, and regression of fibrosis stage after multiple new drugs trial for NASH can improve PRO and HRQoL scores. Therefore, evaluation and monitoring of HRQoL and PRO in NAFLD patients should be encouraged in routine diagnosis and treatment. PRO and HRQoL should be regarded as primary endpoints for the management of NASH and NASH-related cirrhosis.

SUMMARY

The increasing prevalence of NASH is associated with a large health economic burden globally that is characterized by excess mortality, adverse clinical outcomes, and poor patient-reported outcomes (PROs). Since there are still no effective drugs for NASH treatment, clinical trials of novel drugs have been ongoing over the past decade. NASH encompasses a heterogeneous collection of metabolic disorders and slowly progressing features of liver diseases. The challenge in monitoring NASH lies in developing techniques that allow dynamic assessment. Many noninvasive markers and algorithms to evaluate NASH severity and the efficacy of treatment have been developed. A number of serum markers, imaging modalities, and noninvasive algorithms are currently under investigation. Nevertheless, the diagnostic performance, accessibility, and cost-effectiveness of most of these modalities require improvement. Furthermore, the monitoring of NASH should also include PROs and extrahepatic diseases, especially metabolic disorders. Comprehensive but individualized surveillance should be available for each patient. We are convinced that given more efforts and cooperation among healthcare systems, researchers, pharmaceutical companies and NASH patients, advances can be made in monitoring and evaluation systems that will improve the management and prognosis of NASH patients.

Authors' contribution

Shi YW and Fan JG contributed to the study concept and design; Shi YW and Fan JG contributed to drafting the manuscript; Fan JG contributed to critical revision of the manuscript for important intellectual content; both authors confirmed critical revision of the manuscript for important intellectual content.

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Conflicts of Interest

The authors have no conflicts to disclose.

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Review

Eating, diet, and nutrition for the treatment of non-alcoholic fatty liver disease

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Nutrition and dietary interventions are a central component in the pathophysiology, but also a cornerstone in the management of patients with non-alcoholic fatty liver disease (NAFLD). Summarizing our rapidly advancing understanding of how our diet influences our metabolism and focusing on specific effects on the liver, we provide a comprehensive overview of dietary concepts to counteract the increasing burden of NAFLD. Specifically, we emphasize the importance of dietary calorie restriction independently of the macronutrient composition together with adherence to a Mediterranean diet low in added fructose and processed meat that seems to exert favorable effects beyond calorie restriction. Also, we discuss intermittent fasting as a type of diet specifically tailored to decrease liver fat content and increase ketogenesis, awaiting future study results in NAFLD. Finally, personalized dietary recommendations could be powerful tools to increase the effectiveness of dietary interventions in patients with NAFLD considering the genetic background and the microbiome, among others. **(Clin Mol Hepatol 2023;29(Suppl):S244-S260)**

Keywords: NAFLD; Mediterranean diet; Intermittent fasting; Calorie restricted diet; Precision medicine

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the fastest-growing and most prevalent liver disease worldwide, contributing essentially to liver-related morbidity and mortality.[1] Being a prototype of so-called "non-communicable diseases", the increasing prevalence of NAFLD, but also obesity, is regarded as closely related to changes associated with modern-day lifestyle including increased calorie intake, reduced physical activity, and sedentary behavior[2] that result in a mismatch between a decreased energy expenditure and an increased energy intake.[3,4] Among other factors,[4] this seems to be largely driven by socioeconomic factors leading to a rise in ubiquitous, cheap, and energy-dense food of low dietary quality. In the absence of approved pharmacological treatments, lifestyle and especially dietary interventions are even more important to counteract the growing burden of NAFLD.[5] Here, we provide a concise overview of different nutritional strategies in NAFLD, especially in overweight and obese patients (Fig. 1), and summarize our current understanding of the interplay between NAFLD and our diet to facilitate personalized nutritional advice in these patients.

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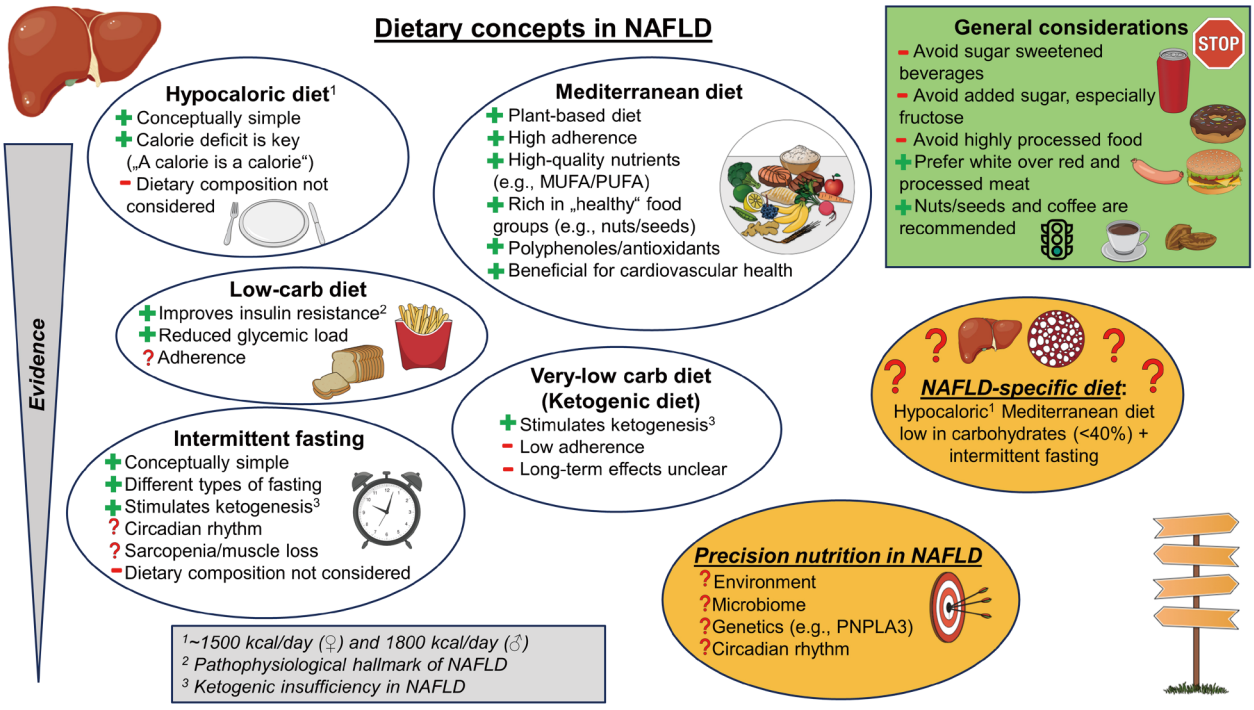

Figure 1. Overview of dietary concepts in NAFLD highlighting evidence, pathophyisological considerations and open questions. NAFLD, non-alcoholic fatty liver disease; MUFA, mono-unsaturated fatty acids; PUFA, poly-unsaturated fatty acids.

Current guideline recommendations

In brief, current European,[6,7] American,[8] Asian,[9] and Korean[10] guidelines highlight the importance of two essential concepts to treat NAFLD in overweight and obese individuals: (I) Weight loss aiming at a reduction of 7–10% in body weight, and (II) energy restriction aiming at a calorie deficit of approximately 500–1,000 kcal/day. On top of these established recommendations, the ideal macronutrient composition is currently a matter of debate: While the American society highlights uncertainties regarding long-term (histological) endpoints that preclude recommendations in favor of one type over another, a dietary composition in accordance to the Mediterranean dietary (MD) is generally advised by European and Asian societies[6,7,9] given clear signals towards beneficial effects beyond the macronutrient composition (see chapter Mediterranean Diet [MD]). Also, the latter advise avoiding added fructose, mostly via consumption of sugar-sweetened beverages (SSB). Importantly, both weight loss and a calorie deficit might be only achieved in combination with an increase in physical activity and exercise that ultimately lead to an increased energy expenditure.[5] Thus, a combined "lifestyle"-approach should always be preferred, and tailored to the individual patient to increase long-term adherence achieving a durable improvement in energy metabolism ("eat less, move more").[6,8]

Outcomes in nutritional research

To make use of dietary recommendations in clinical practice, one must take the endpoints that have been investigated in the respective studies into account. With this regard, di-

Abbreviations:

BMI, body mass index; CAP, controlled attenuation parameter; DNL, *de-novo* lipogenesis; HCD, high-carbohydrate diet; IF, intermittent fasting; IHLC, intrahepatic lipid content; LCD, low-carbohydrate diet; LSM, liver stiffness measurement; MD, Mediterranean diet; MUFA, mono-unsaturated fatty acids; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PUFA, poly-unsaturated fatty acids; RCT, randomized controlled trial; SFA, saturated fatty acids; SSB, Sugar-sweetened beverages; TRF, time-restricted feeding

etary recommendations for NAFLD are especially complex given the variety of clinical endpoints: (I) Improvement of liver histology including regression of fibrosis or resolution of non-alcoholic steatohepatitis (NASH);[11-13] (II) changes in quantitative parameters assessing liver fat content (i.e., hepatic steatosis) such as the intrahepatic triglyceride content/ intrahepatic lipid content (IHLC) assessed via magnetic resonance spectroscopy,[14,15] controlled attenuation parameter (CAP) assessed by transient elastography,[16,17] or scores combining laboratory values such as the fatty-liver-index;[18,19] (III) quantitative assessment of liver fibrosis using magnetic resonance elastography[20] or transient elastography-based liver stiffness measurement (LSM);[16-18,21] (IV) transaminases (aspartate aminotransferase [AST]/alanine transaminase [ALT]) as a surrogate for hepatic inflammation;[22] and (V) changes in metabolic parameters such as fasting blood glucose, insulin resistance, serum lipids but also body weight that do not specifically address changes in the liver. Especially regarding liver fat content, one has to consider its transiency and that presumed association with clinical endpoints are predominantly driven by hepatic fibrosis (e.g., cardiovascular diseases[23]) including mortality.[24] Also, combined scores such as the fatty-liver-index have not been developed for metric assessment of liver fat, making absolute changes in these scores uninterpretable.[25] At the same time, levels of ALT/AST have numerously been described as inadequate to portray disease severity and hepatic fibrosis in NAFLD.[26-28] Finally, studies using histological data are scarce.[11-13] While they would be urgently needed, they are reasonably limited given the invasiveness of liver biopsy. With this regard, trials focusing on accepted surrogates of hepatic fibrosis (such as magnetic resonance elastography or LSM) should be strongly encouraged in future nutritional intervention studies.

CALORIE RESTRICTION & HYPOCALORIC DIET

Clear evidence suggests that dietary calorie restriction is able to improve numerous metabolic parameters beyond its effect on liver-related outcomes (e.g., reviewed in [29]). Focusing on NAFLD, several studies have shown that a total energy deficit (~500 kcal/day resulting in ~1,500 kcal/day for women and ~1,800 kcal/day for men) leads to a decrease in body weight, transaminase levels, total body fat, visceral fat, and IHLC, regardless of how it is achieved.[15,22,30,31] An important study by Kirk et al.[32] (2009) reported similar changes in body weight, body composition, and IHLC after 7% of weight loss (i.e., after around 11 weeks) following a hypocaloric low-carbohydrate diet (LCD) vs. a high-carbohydrate diet (HCD) despite short-term effects in favor of LCD (i.e., after 48 hours).[32] Again, studies associating the degree of weight loss with the extent of histological improvement[11] and improvement of metabolic parameters[33] strongly favor a dose-dependent effect of nutritional/lifestyle interventions beyond macronutrient composition.[34] Interestingly, a recent meta-analysis of observational studies including >100,000 individuals has shown that the only difference between NAFLD and controls was a higher calorie intake while the macronutrient composition did not significantly differ.[35] Finally, evidence highlighting the importance of calorie reduction originates from the observation that LCD (as discussed in chapter Low-Carbohydrate Diet [LCD]) are only successful in reducing IHLC when integrated into a hypocaloric diet approach, but fail to decrease or even increase IHLC if carbohydrate restriction occurs at the expense of increased fat intake in an isocaloric manner.[36,37]

LOW-CARBOHYDRATE DIET (LCD)

On top of calorie restriction, increasing evidence suggests a diet low in carbohydrates to be especially fruitful for patients with NAFLD. On a population-based level, data from America show that intake of potato chips, potatoes, and SSB were the dominant factors associated with weight gain[38] paralleling the global increase in obesity and NAFLD in recent years, thereby clearly suggesting a certain role of a western diet typically high in carbohydrates for the surge in obesity and NAFLD. On the short term, Browning et al.[39] (2011) reported a favorable reduction in IHLC after a hypocaloric LCD (8% carbohydrates [C], 33% protein [P], 59% fat [F]) compared to a hypocaloric diet (50% C, 16% P, 34% F), as did Kirk et al.[32] (2009) after 48 hours. However, one has to note that reductions in IHLC were comparable after 7% weight loss,[32] supported by Haufe et al.[30] (2011) who also showed comparable reductions in IHLC after 6 months. Nevertheless, an increase in total energy expenditure by about ~50 kcal for every 10% decrease in the contribution of carbohydrates to total energy intake has been postulated,[40] together with a decrease in ghrelin and leptin levels contributing to de-

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creased appetite and satiety[41] following a LCD independently of body mass index (BMI). Importantly, these changes might be linked to an increase in ketogenesis and favorable changes in gut microbiota, which were even observed after an isocaloric LCD.[42] Another randomized controlled trial (RCT) aiming at maintained weight in adolescents reported a decrease in IHLC after 8 weeks only following an LCD (<25% C, 25% P, >50% F), but not a HCD (55% C, 25% P, 20% F).[43] In summary, benefits from an LCD seem to include a favorable glucose metabolism (reduced insulin resistance,[20] reduced basal glucose production[32]) independent of changes in IHLC, even in patients with established type-2 diabetes mellitus.[44] However, improvements of BMI, HDL and triglyceride profiles must be balanced with potential consequences of an LCD (i.e., high in dietary fat) such as elevated LDL and total cholesterol levels in the long-term.[45,46] Finally, both low carbohydrate consumption (<40% of total energy intake) and high carbohydrate consumption (>70%) were associated with higher overall mortality in unselected patients (i.e., a U-shaped relationship),[47] questioning long-term beneficial effects of LCD, but especially very-low-carbohydrate-diets (i.e., ketogenic diets).

Carbohydrate-insulin-model vs. energy-balance-model

Hypotheses discussing explanations for additional beneficial effects of an LCD on top of a hypocaloric diet be generated from the current discussion on two theories trying to explain energy metabolism in obesity: the carbohydrate-insulin-model and the energy-balance-model.[48,49]

The carbohydrate-insulin-model focuses on the influence of dietary carbohydrates on the human body. Specifically, an increase in carbohydrates (i.e., high glycemic load) leads to increased insulin secretion (i.e., hyperinsulinemia) that promotes energy storage in adipose tissue, exacerbating hunger and lowering energy expenditure, all together promoting weight gain in a generally anabolic state.[50] By further stimulating glucose uptake, suppressing the release of fatty acids from adipose tissue, and promoting fat and glycogen production, hyperinsulinemia following carbohydrate intake induces a vicious cycle that "*offers an explanation for why average BMI in many countries increased in the late 20th century as public health guidelines recommended replacement of dietary fat with carbohydrates, and consumption of high-glycemic-load foods increased substantially*".[51] Thus, the carbohydrate-insulin-model considers the high glycemic load as the starting point promoting anabolism including an anabolic hormonal profile, leading to "deposition" of substrates, leaving less energy for the brain (especially in the late postprandial period[52,53]) in turn inducing hunger and appetite.[48]

Considering that insulin resistance is regarded a hallmark of NAFLD progression closely linked to inflammation, oxidative stress, and disease progression,[54-56] an additional benefit of a LCD in NAFLD is reasonable from a pathophysiological perspective. Here, insulin resistance directly correlates with hepatic *de-novo* lipogenesis (DNL),[57] which has been shown to significantly contribute to IHLC in lean individuals without NAFLD (~11%), but being even more pronounced in obese individuals (~19%) and obese NAFLD patients (~38%). Most importantly, Luukkonen and colleagues[58] (2022) just recently described insulin resistance as an independent pathophysiological trait in NAFLD next to the genetic predisposition, being amplified if both factors are present. Considering this importance of insulin resistance in NAFLD, an increased DNL during carbohydrate overfeeding,[59-61] an increased DNL in NAFLD,[57,62] and the efficacy of LCD especially in hyper-insulinemic patients,[40] LCD could offer a "way out" of this vicious cycle. Here, Cohen and colleagues[63] (2021) could already demonstrate a reduction of DNL within 8 weeks of dietary sugar restriction in adolescents.

In summary, specific beneficial aspects include the above-mentioned increase in energy expenditure,[40,64] increase in satiety,[41] lower insulin and ghrelin action in adipose tissue, higher glucagon action in non-adipose sites, and increased leptin sensitivity in the muscle.[51]

The competing model to this theory is the energy-balance-model that considers the increased availability of (cheap and energy-dense) food as the starting point for obesity.[49] Specifically, the brain regulates body weight in response to external signals from our food environment that stipulate hormonal signals controlling food intake, but also energy partitioning within the body.[49] Importantly, proponents of this model argue against the simplistic approach of the carbohydrate-insulin-model neglecting that several variables in the food environment influence energy intake and energy partitioning. For example, energy expenditure and energy intake are dynamically interrelated by physiological counteracting mechanisms (e.g., adaptive thermogenesis corresponding to a reduced energy expenditure if energy intake is

decreased[65]) that are nearly impossible to look at in an isolated fashion.[66] While data supporting a lower energy expenditure following low-fat diets exist, authors claim that these differences are so small that "a calorie is a calorie".[66] Also, one must acknowledge that evidence from meta-analysis is currently lacking that an LCD (favoring the carbohydrate-insulin-model) is more effective than a low-fat diet if calorie restriction is achieved (favoring the energy-balance-model).[37]

MEDITERRANEAN DIET (MD)

Looking beyond the macronutrient composition, it seems that the dietary composition is still relevant for the effect of a given diet on metabolic parameters. Here, a dietary composition according to the MD has been most consistently associated with improved phenotype of NAFLD.[67] Specifically, the MD has been defined "primarily a plant-based diet characterized by a high ratio of monounsaturated fatty acids (MUFA) to saturated fatty acids (SFA) with total fat accounting for 30–40% of daily energy consumption".[68]

Next to improvement in metabolic dysregulation[69] and prevention of cardiovascular diseases,[70] adherence to the MD has been inversely associated with NAFLD prevalence[71] and severity,[72-74] reduction in liver fat content,[14,18,19,75-77] and LSM.[18,78] For instance, adherence to a low-carbohydrate MD (over 6 months) improved NAFLD (assessed by ultrasound).[77] However, the inverse association between adherence to MD and decrease in liver fat content might be largely mediated (i.e., driven) by a decrease in BMI[74] emphasizing the central role of adipose tissue-liver crosstalk when studying liver-related outcomes.[79]

Despite these promising results, the dietary composition of MD was heterogeneous across different studies and often combined with calorie restriction, thereby complicating direct comparison. Nevertheless, the best evidence that adherence to a MD on top of a hypocaloric diet is beneficial for NAFLD comes from studies from Israel. Gepner and colleagues[80,81] demonstrated that an LCD in combination with a MD achieved the greatest reduction in visceral adipose tissue and IHLC compared to an iso-caloric HCD. Interestingly, this effect was achieved despite only moderate weight loss, again supporting favorable effects of MD beyond calorie restriction.[80] Recently, the "DIRECT PLUS" RCT demonstrated a successful (and durable) weight loss and decrease in IHLC following a hypocaloric MD after 18 months.[14] What is even more interesting, the addition of dietary polyphenols (green tea and Mankai) further amplified these beneficial effects on IHLC (–38% relative change compared to –17% in the MD-only group).[14]

Specifically, several aspects seem to explain the success of the MD: First, one must consider that the MD is by itself characterized by a reduced carbohydrate intake (~approx. 40% of calorie intake), thereby mimicking favorable effects of a LCD on liver fat.[82] Second, the MD is low in food types that show clear harmful effects on NAFLD (such as Red and processed meat and SSB, as discussed in chapter Sugar sweetened beverages [SSB]), and rich in those that are considered beneficial (such as olive oil, nuts, legumes, seeds, whole grains, and vegetables).[67] Third, the MD is rich in molecules/compounds that are generally regarded as "healthy". Most prominently, polyphenols including flavonoids exhibit antioxidative effects reducing mortality in the general population,[83,84] but also inhibit DNL, suppress the activation of hepatic stellate cells, and reduce carcinogenesis in animal models.[85] Carotenoids (i.e., lipid-soluble phytochemical) exert similar antioxidative properties[86] but are also discussed to decrease lipid accumulation, insulin resistance, oxidative stress, and inflammation in the liver.[87] Fourth, it still seems clear that the quality of ingested nutrients matters.[36] For example, 4 studies have shown favorable changes in IHLC if energy from fat is derived from MUFA and poly-unsaturated fatty acids (PUFA) compared to SFA following an isocaloric[88] or hypercaloric diet.[89-91] Also, an isocaloric diet high in MUFA was superior in reducing IHLC compared to isocaloric control diets despite unchanged body weight.[92,93] Finally, adherence to MD seems to be easier than to other diets (e.g., HCD), which has been demonstrated by the recent CORDIOPREV study reporting adherence to the MD in 7 of 8 patients over a period of 7 years, given that patients are supported by dieticians.[94] For the first time ever, a significant reduced incidence of major cardiovascular events in patients with coronary artery disease following a MD without energy restriction participating in this RCT was reported, further advocating this dietary composition.[94]

FOOD GROUPS

Numerous food groups have repeatedly been associated

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with NAFLD.[95] Among them, red meat and SSB have shown the strongest negative impact on NAFLD prevalence and will be further discussed, while nuts and seeds seem to be protective.[95,96]

Sugar sweetened beverages (SSB)

Dietary fructose intake—mostly via SSB and high-fructose corn syrup—is one of the food groups with the strongest evidence supporting harmful effects on multiple health outcomes, including NAFLD.[97] From a physiological point of view, fructose metabolization is nearly exclusively limited to hepatocytes.[98] By bypassing the rate-limiting step of glycolysis catalyzed by phosphofructokinase, fructose not only provides more substrate to DNL than glucose, but also occurs independent of insulin and the energy status of the cell,[98] leading to an energy mismatch and subsequently promoting oxidative stress and insulin resistance.[99,100] Also, a roughly 100% first-pass effect following oral ingestion of fructose has been observed,[101] suggesting metabolism in the liver directly upon consumption. Keeping this "fructose-processing burden" in mind, the harmful effect of significant and/or long-lasting fructose consumption on the liver seem reasonable.

In brief, several meta-analyses have tried to dissect the effect on glycemic control,[102] metabolic syndrome,[103] or NAFLD.[104] When fructose was substituted for other calories, no effect was evident regarding glycemic control compared. In contrast, a clearly harmful effect was observed when SSB were consumed on top of the usual diet (i.e., as excess calories):[102] SSB showed a dose-dependent (increasing) effect on the prevalence of metabolic syndrome, while fruit juices showed a U-shaped relationship with protective effects at moderate doses.[103] Finally, a study on NAFLD found that addition of SSB (as ~30% excess energy) led to a significant increase in IHLC,[104] while the beneficial effect when cutting down on fructose-containing sugars was less clear. However, all 3 available meta-analyses highlight the interaction with food sources (i.e., where excess fructose comes from) as an essential modifier of these effects, with SSB being the least favorable. Also, healthy individuals and/or adolescents seem to respond less to fructose supplementation[105] or restriction.[106]

In individual studies, SSB have been associated with higher NAFLD prevalence,[107-110] presence of NASH[111] and even a higher degree of fibrosis.[112] Recently, 4 RCT investigated the effect of fructose restriction on liver-related outcomes: Geidl-Flueck et al.[113] (2021) demonstrated a 2-fold increase in hepatic fatty acid-secretion rates in healthy men ingesting fructose/sucrose group vs. glucose sirup, Schwimmer et al.[114] (2019) reported a decrease in IHLC after 8 weeks of restricting free sugars, Simons et al.[115] (2021) showed a significant decrease in IHLC after 6 weeks of a fructose-restricted diet in NAFLD, and Khodami et al.[116] (2022) reported on an improvement of insulin resistance, steatosis, and fibrosis surrogates in NAFLD patients similarly restricting free sugars.

From a pathophysiological perspective, dietary fructose promotes DNL, impairs fatty acid oxidation, and triggers hepatic inflammation, thereby clearly fueling hepatic insulin resistance (reviewed in [99]). Also, epigenetic changes occur,[117] and the role of the microbiome, metabolizing fructose to acetate being an additional substrate for DNL—is being increasingly understood.[118] Despite incompletely understood, dietary fructose even seems to increase nutrient absorption via improving survival of intestinal cells and increasing intestinal villus length.[119]

Thus, although data regarding a long-term comparison between glucose and fructose consumption are lacking,[36] available data clearly suggests that fructose consumption should be cut down to a minimum in patients with NAFLD.

Red and processed meat

Numerous studies within the last years have demonstrated a negative impact of red and especially processed meat on the prevalence of NAFLD. While some studies pointed towards a general association of meat with NAFLD,[73,120,121] more recent observational longitudinal studies[122-124] and cross-sectional studies[125,126] have linked high consumption of only red meat to an increased prevalence of NAFLD.[95] Of note, white meat (i.e., chicken or turkey) did not show any significant associations,[122] while processed meat of any type is still unfavourable.[123,125] Translating these associations into macronutrient composition, they are especially driven by animal protein since consumption of plant-based protein did not show a comparable association.[121,127] However, the harmful effects of high meat consumption on liver fat might be largely driven by a parallel increase in BMI,[128] as also shown for the MD. Nevertheless, selected studies have even reported an increased risk of fibrosis in NAFLD patients with high red/processed meat consumption.[123]

On a molecular basis, the diet-dependent acid-load seems to be an driving factor for these associations by inducing a low-grade metabolic acidosis[129,130] leading to a disturbance in acid-base-homeostasis.[131] Also, red meat contains a considerable amount of SFA and cholesterol, which have been shown to boost insulin resistance[132] and drive hepatic lipid storage.[133] Next, heme iron[134,135] and nitrate (added for preservation) contribute considerably to the harmful effects of red or processed meat, potentially via increased oxidative stress.[136] Finally, modification of the intestinal microbiota including the metabolism of certain components of red meat into harmful compounds (such as trimethylamine-N-oxide) seems to contribute to these negative effects.[137,138] Focusing on processed meat, cooking meat at high temperatures for a long duration can form heterocyclic amines, which induce unfavorable health effects including an increased risk of cancer[139] and chronic diseases, again mainly driven by an increased oxidative stress.[140]

INTERMITTENT FASTING (IF)

Several types of "intermittent fasting" (IF) have gained increasing popularity in recent years. In brief, "time-restricted feeding" (TRF) involves calorie intake only during a pre-specified time window (usually for 4–10 hours). With regard to timing, a recent study applying TRF on healthy individuals indicates a certain benefit in glycemic control when feeding is restricted to the time between 06:00–15:00 vs. during the mid of the day (11:00–20:00).[141] "Alternate day fasting" describes a mode of TRF in which fasting periods over 36 hours are followed by ad-libitum food consumption over the next 12 hours (i.e., every 2nd day, e.g., from 06:00–18:00). Finally, the 5:2 diet involves calorie restriction only on 2 non-consecutive days during the week, on which calorie intake is usually restricted to 500–600 kcal/day. This periodic calorie restriction is believed to provoke several physiological changes contributing to health benefits (reviewed in [142,143])—among others, it might counteract the disruption of circadian rhythm being associated with the development of NAFLD and metabolic syndrome.[144,145]

Stimulated by the success of Stekovic et al.[146] (2019) demonstrating significant improvement of metabolic parameters after 4 weeks and 6 months, an increasing number of studies have elucidated the beneficial effects of IF on health outcomes. Lately, an umbrella review of meta-analyses of RCT studying obesity-related outcomes reported beneficial outcomes for BMI, body composition, serum lipids, glucose homeostasis, and blood pressure.[147]

Focusing on NAFLD, 5 studies have so far specifically investigated IF in this patient population. Johari and colleagues[21] applied a modified alternate-day calorie restriction (i.e., 70% calorie restriction on fasting day, ad-libitum eating on non-fasting day) to demonstrate an improvement in ALT levels as well as LSM and ultrasound-based steatosis.[21] Another study showed a decrease in BMI and triglyceride levels following 12 weeks of ADF or time-restricted feeding (energy intake only during an 8 hours-window each day) despite no changes in LSM.[148] Holmer et al.[17] (2021) compared the 5:2 diet (<500/600 kcal/day on fast-days) with an LCD in patients with NAFLD. This diet was associated with a significant improvement in liver fat as assessed by MRI or CAP, as well as improvement in BMI and insulin resistance compared to a control diet, among others. However, no differences were observed compared to the LCD diet. Kord Varkaneh et al.[149] (2022) also compared the 5:2 diet over 12 weeks with a control group, and observed improvements of metabolic parameters including LSM and CAP. Finally, Xiao and colleagues[150] (2022) studied 60 NAFLD patients with type 2 diabetes mellitus randomized to 5:2 diet or liraglutide over 24 weeks, and found comparable metabolic improvement including a decrease in CAP in both groups. In addition to these studies, certain data exist on the effect of Ramadan fasting on the liver. Again, aside from the improvement in metabolic serum parameters including glucose homeostasis,[151] non-invasive scores of fibrosis and markers of subclinical inflammation improved in NAFLD patients.[152] Also, Ramadan fasting reduced the gene expression of "fat-mass-and-obesity-associated protein" (FTO) in overweight/obese individuals,[153] which has been associated with obesity[154] despite lower calorie intake.[155]

However, it is currently a matter of debate whether IF (i.e., time-dependent calorie restriction) is more effective[156] or equally effective[157,158] than continuous calorie restriction (e.g., hypocaloric diet), and whether it is effective if no calorie restriction/dietary counselling is applied.[159] In the setting of type-2 diabetes mellitus,[160] close monitoring of diabetes medication and blood glucose is needed due to concerns about hypoglycemia[161] although TRF has also been shown to be effective and safe in overweight/obese patients with type-2 diabetes mellitus. At the same time, sarcopenia might

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be an issue due to fasting inducing protein catabolism and muscle loss.[162-164]

An often discussed effect of IF is an increase in ketogenesis (reviewed in [165]). In brief, the production of ketone bodies (mainly acetoacetate and β-hydroxybutyrate) from fatty acids serves as an alternative energy supply from the liver to peripheral tissues when carbohydrates are unavailable, therefore being pronounced during fasting or starvation.[166] At the same time, ketogenesis represents an alternative lipid disposal pathway metabolizing acetyl-CoA derived from β-oxidation. While NAFLD is characterized by an abundance of substrates that need to be metabolized by the liver inducing oxidative stress, DNL is upregulated[57,58,62,167] and ketogenesis downregulated, leading to an exhausted mitochondrial capacity.[168] Thus, on top of the direct beneficial effects of ketone bodies including antioxidative and anti-inflammatory functions (discussed in [169,170]), IF (but also very-low-carbohydrate-diets) could reverse this so-called "ketogenic insufficiency" that has been observed in NAFLD[171] by increasing hydrolysis of IHLC partitioning fatty acids towards ketogenesis, thereby improving mitochondrial redox state.[20] Additional beneficial effects of fasting might include the simulation of the peroxisome proliferator-activated receptor alpha (PPARα) /fibroblast growth factor 21 (FGF21) signaling[172] involved in regulating fatty acid metabolism.[173]

PRECISION NUTRITION IN NAFLD

"Precision nutrition" aims at tailoring personalized dietary recommendations to individuals considering not only lifestyle and socioeconomic factors, but also incorporating data on the metabolome,[174] microbiome and the genetic background.[175] Here, a huge effort is being made towards personalized medicine[176] and deeper understand the interactions between our diet and our environment. Although few studies have focused on patients with NAFLD, data from unselected cohorts focusing on clinical endpoints closely related to NAFLD are indeed astonishing. Here, Zeevi and colleagues[177] (2015) demonstrated that large interpersonal variability exists in the postprandial glycemic response to identical meals. Together with a follow-up study by their group again showing heterogenous glycemic responses to sourdough or white bread,[178] these data indicate that often neglected factors such as the microbiome significantly influence the effectiveness of a given dietary intervention. Also, data from the PREDICT1 study support the central role of the gut microbiome explaining more variance in post-prandial triglyceride and insulin levels than the macronutrient composition of the ingested meals itself.[179] Exemplary looking at individual substrates, the beneficial effects of resveratrol on liver fat are discussed to be mediated by changes in the microbiome.[180]

When trying to understand the influence of our genes on dietary responses, data show that they are highly relevant for our postprandial glucose response alone explaining ~50% of the variance.[179] Looking at individual single nucleotide polymorphisms (reviewed in [181]), the PNPLA3 rs738409 G-allele has been best studied as a modifier for the dietary response. An early study in Hispanic children indicated a significant positive correlation between IHLC and dietary carbohydrates only in homozygous carriers of the G-allele.[182] Also, following an LCD, the improvement in IHLC and insulin sensitivity was highest in G/G-carriers.[183,184] Similarly, two studies confirmed significantly larger changes in hepatic fat on a low n-6:n-3 PUFA ratio diet in homozygous carriers of the PNPLA3 risk allele.[185,186] Finally, dietary carbohydrates, but also polyphenols and PUFA were associated with significant fibrosis on histology only in carriers of the PNPLA3 G-allele (G/C or G/G).[187] At the same time, our genes might not only influence our response to a certain diet, but also generally determine our macronutrient content. Here, it is believed that our genetic background explains up to 40% of our macronutrient intake[188] with SNPs in FGF21 (increased carbohydrate[189] or protein intake[190]) and FTO (increased protein intake[191,192]) being mostly studied, the latter potentially allowing greater weight loss during dietary/lifestyle interventions.[193,194]

CONCLUSION

In summary, nutritional research und understanding the influence of diet on disease severity is one of the most complex aspects in the management of NAFLD patients. While being highly efficient when done consequently, evaluating the effects of dietary interventions is challenging as they impact on the whole metabolism, and specific (beneficial) effects on the liver are hard to detangle. While this makes firm conclusions and guideline recommendations difficult, this must not be misinterpreted as a limitation of dietary interventions per se. Currently, many roads seem to be leading to

Rome as long as a calorie deficit is achieved and energy expenditure is increased. However, a hypocaloric diet, low in dietary carbohydrates, potentially including IF could be a diet tailored to successfully "treat" NAFLD, awaiting further study results. Also, increasing evidence suggests that a dietary composition according to the MD provides additional benefits for NAFLD patients beyond calorie restriction. On the other hand, personalized dietary recommendations might be necessary to make use of the full potential of dietary interventions in NAFLD.

Authors' contribution

Georg Semmler: Conceptualization, Writing- Original draft, Writing- Reviewing and Editing. Christian Datz: Conceptualization, Writing- Original draft, Writing- Reviewing and Editing. Michael Trauner: Conceptualization, Writing- Original draft, Writing- Reviewing and Editing.

Conflicts of Interest

The authors have nothing to disclose regarding the work under consideration for publication. The following authors disclose conflicts of interests outside the submitted work: GS received travel support from Gilead. CD is part of the scientific advisory board of SPAR Österreich AG. MT received grant support from Albireo, Almylam, Cymabay, Falk, Gilead, Intercept, MSD, Takeda and Ultragenyx, honoraria for consulting from Albireo, Boehringer Ingelheim, BiomX, Falk, Genfit, Gilead, Hightide, Intercept, Janssen, MSD, Novartis, Phenex, Pliant, Regulus and Shire, speaker fees from Bristol-Myers Squibb, Falk, Gilead, Intercept and MSD, as well as travel support from AbbVie, Falk, Gilead, and Intercept. He is also co-inventor of patents on the medical use of 24-norursodeoxycholic acid.

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Review

The effects of moderate alcohol consumption on non-alcoholic fatty liver disease

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Non-alcoholic fatty liver disease (NAFLD) is accepted as a counterpart to alcohol-related liver disease because it is defined as hepatic steatosis without excessive use of alcohol. However, the definition of moderate alcohol consumption, as well as whether moderate alcohol consumption is beneficial or detrimental, remains controversial. In this review, the findings of clinical studies to date with high-quality evidence regarding the effects of moderate alcohol consumption in NAFLD patients were compared and summarized. **(Clin Mol Hepatol 2023;29(Suppl):S261-S267)**

Keywords: Non alcoholic fatty liver disease; Moderate alcohol consumption; Alcohol related liver disease

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a chronic liver disease characterized by serial progression from isolated steatosis to steatohepatitis, fibrosis, and cirrhosis.[1] NAFLD is associated with the metabolic conditions of insulin resistance, type 2 diabetes, and obesity.[2] Mirroring the obesity epidemic, the global prevalence of NAFLD among adults is estimated to be 23–25%, and has become a major global concern as a dominant cause of chronic liver disease with increases in obesity and type 2 diabetes.[3–5] In particular, as the proportion of young patients is increasing, the burden of disease is expected to rise, and long-term management strategies are needed.[6,7]

NAFLD is defined as hepatic steatosis occurring in over 5% of hepatocytes without excessive use of alcohol, viral hepatitis, or autoimmune liver disease. NAFLD is considered the counterpart of alcohol-related liver disease (ARLD).[8-10] NAFLD and ARLD share a common pathophysiological basis involving gut dysbiosis and subsequent changes. In addition, single nucleotide polymorphisms in patatin-like phospholipase domain-containing 3 (PNPLA3), transmembrane 6 superfamily member 2 (TM6SF2), membrane bound O-acyltransferase domain containing 7 (MBOAT7), and 17-β hydroxysteroid dehydrogenase 13 gene (HSD17B13) are significant genetic risk factors for NAFLD and ARLD.[11-15] These two entities are difficult to distinguish because both histologically include a certain degree of steatosis, lobular inflammation, and ballooning.[16] However, NAFLD and ARLD are distinguished by excessive alcohol consumption based on history taking and questionnaires, however, the amount of safe alcohol consumption accepted as “non-alcoholic” is disputed. In previ-

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ous studies, conflicting evidence on whether moderate alcohol consumption is protective or detrimental for development of NAFLD was reported.[17,18]

In this review, the clinical results to date on the effects of moderate alcohol consumption in NAFLD patients were compared and summarized.

DEFINITIONS FOR MODERATE ALCOHOL CONSUMPTION

The effects of alcohol on patients appear over a long period of time, and because randomized control trials are difficult to perform, the effects can only be estimated using observational studies. Several definitions for significant alcohol consumption to date exist (Table 1).

The definition of moderate alcohol consumption adopted by most guidelines and previous studies is <21 units of alcohol per week for males and <14 units of alcohol per week for females. Some researchers adopt other definitions based on their needs,[26-28] however, many experts recommend the above definition for comparison and objectivity of studies.[29,30] One unit of alcohol is usually 10 mL of pure alcohol but standard drink definitions vary worldwide from 8–20 g of alcohol.[31] Therefore, the definition used should be confirmed when reviewing previous research.

DETERMINING WHETHER MODERATE ALCOHOL DRINKING IS BENEFICIAL OR DETRIMENTAL

Although alcohol is a carcinogen with a well-known dose-risk relationship,[32,33] meta-analyses based on many previous studies have published results that moderate alcohol consumption showed a protective effect against NAFLD (Table 2).

Notably, Sookoian et al.[28] suggested that moderate alcohol consumption is associated with a significant protective effect

Table 1. International definitions of clinically significant alcohol consumption

Organizations	Definitions
NIAAA[19] (1 standard drink=14 g)	Heavy alcohol use: Male: >14 standard drinks/week Female: >7 standard drinks/week
WHO[20]	Low risk: Male <40 g/day, Female <20 g/day Medium risk: Male 40–60 g/day, Female 20–40 g/day High risk: Male >60 g/day, Female >40 g/day
NICE thresholds for liver cirrhosis assessment[21]	Male: 50 units/week, Female: 35 units/week
AASLD[8], AACE[2], AGA[22]	Male: >21 standard drinks/week, Female: >14 standard drinks/week (over a 2-year period preceding baseline liver histology)
EASL–EASD–EASO[9]	Male: >30 g/day, Female: >20 g/day
EASL patient guideline[23] (1 unit equals 8 g of alcohol)	Male: >21 units/week, Female: >14 units/week
APASL[24]	Male: two standard drinks per day (i.e., 140 g ethanol per week) Female: one standard drink per day (i.e., 70 g ethanol per week)
China[25] (during the past 12 months)	Male: >210 g/week, Female: >140 g/week
KASL[10]	Male: >210 g/week, Female: >140 g/week

NIAAA, National Institute on Alcohol Abuse and Alcoholism; WHO, World Health Organization; NICE, National Institute for Health and Care Excellence; AASLD, American Association for the Study of Liver Diseases; AACE, American Association of Clinical Endocrinology; AGA, American Gastroenterological Association; EASL, European Association for the Study of the Liver; EASD, European Association for the Study of Diabetes; EASO, European Association for the Study of Obesity; APASL, Asian Pacific Association for the Study of the Liver; KASL, Korean Association for the Study of the Liver.

Abbreviations:

NAFLD, non-alcoholic fatty liver disease; ARLD, alcohol-related liver disease; BMI, body mass index; OR, odds ratio; CI, confidence interval; HR, hazard ratio; FIB-4, fibrosis-4 index; HCC, hepatocellular carcinoma; SES, socio-economic status; AUDIT, Alcohol Use Disorders Identification Test; CAGE, Cut, Annoyed, Guilty, and Eye; HiAlc Kpn, high-alcohol-producing Klebsiella pneumoniae; ALT, alanine transaminase

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against NAFLD (Table 2). Body mass index (BMI) was not a statistically significant confounding factor in meta-regression analysis (slope=0.01, P<0.44) but moderate alcohol consumption was more protective in women than men (53% in women, 30% in men). This result was consistent with the odds of having steatohepatitis (odds ratio [OR]=0.501, 95% confidence interval [CI]: 0.340–0.740, P<0.0005, I^2=0%) without heterogeneity.[28] Cao et al.[26] showed similar results. In pooled ORs for the prevalence of NAFLD, low- and moderate-risk alcohol consumption consistently showed a protective effect regardless of sex or BMI (≥25 vs. <25). A similar conclusion was presented in a recent meta-analysis. The risk of alcohol consumption in advanced fibrosis in patients with NAFLD was evaluated in recent meta-analyses. In Wijarnpreecha et al.[27] and Wongtrakul et al.[34], moderate alcohol consumption was associated with a lower risk of advanced fibrosis and steatohepatitis with lower-to-intermediate heterogeneity, although their definitions of alcohol consumption differed (Table 2). Furthermore, NAFLD patients with moderate alcohol consumption had a lower mortality risk than lifelong abstainers (hazard ratio [HR]=0.85, 95% CI: 0.75–0.95, I^2=64%).

Despite the above results, alcohol consumption does not guarantee a protective effect against the progression of cirrhosis.[35-37] In a large NAFLD cohort study in Korea, patients with low fibrosis-4 index (FIB-4) progressed to intermediate or high FIB-4 with light alcohol drinking (<10 g/day, adjusted HR=1.06, 95% CI: 0.98–1.16) and moderate alcohol drinking (10 to <20 g/day for women, 10 to <30 g/day for men, adjusted HR=1.29, 95% CI: 1.18–1.40).[38] In a recent NAFLD cohort study, moderate amounts of alcohol intake in NAFLD patients increased the risk of type 2 diabetes and of advanced fibrosis with the synergistic effect of insulin resistance.[39,40] The longitudinal association between moderate use of alcohol (≤2 drinks/day) and histology findings on follow-up liver biopsy more than 1 year apart were evaluated in a previous study; non-drinkers had a greater mean reduction in steatosis grade (0.49 reduction) than moderate drinkers (0.30 reduction, P=0.04) and moderate drinkers had significantly lower odds of steatohepatitis resolution compared with nondrinkers (adjusted OR=0.32, 95% CI: 0.11–0.92, P=0.04).[41]

Alcohol is also a well-known primary cause for developing hepatocellular carcinoma (HCC).[42,43] In a previous meta-analysis, the dose-risk curve indicated a linear relationship with the amount of alcohol consumed, estimated excess risk of 46% for 50 g/day and 66% for 100 g/day.[44] Furthermore, in a

Table 2. Comparison of previous meta-analyses in which the effects of moderate alcohol consumption in NAFLD patients were assessed

Author	Year	Search	Number of included studies	Primary outcome	Participants (n)	Definition of moderate alcohol consumption	Pooled OR (95% CI)	Heterogeneity (I^2)
Sookoian et al.[28]	2014	Unknown	8 studies	NAFLD prevalence	43,175	<40 g/day	0.684 (0.580–0.806)	NA
Cao et al.[26]	2016	Without restriction	13 cross-sectional studies, 2 cross-sectional following longitudinal studies, 1 cohort study	NAFLD prevalence	76,608	WHO definition	Light: 0.76 (0.72–0.80) Moderate: 0.75 (0.70–0.80)	66% 82.7%
Wijarnpreecha et al.[27]	2021	February 2019	6 cross-sectional studies	Prevalence of advanced liver fibrosis	8,936	<28 g/day for males <14 g/day for females	Modest drinkers vs. non-drinkers: 0.51 (0.35–0.75)	47%
Wongtrakul et al.[34]	2021	October 2020	14 cross-sectional or cohort studies	Prevalence of steatohepatitis	14,435	210 g/week for males 140 g/week for females	Steatohepatitis: 0.59 (0.45–0.78) Advanced fibrosis: 0.59 (0.36–0.95)	12% 75%

OR, odds ratio; CI, confidence interval; NAFLD, non-alcoholic fatty liver disease; WHO, World Health Organization.

meta-analysis, the risk of HCC was reported to decrease after alcohol cessation by 6% to 7% a year.[45] In another meta-analysis, Wongtrakul et al.[34] narrowed the analysis target to only NAFLD patients with moderate alcohol consumption, showing a significant HR of 3.77 (95% CI: 1.75–8.15, I^2=0%) for developing HCC.

Several disadvantages should be considered when interpreting the conflicting research results discussed above. Previous meta-analyses had several inherent limitations due to the design of the included studies. Almost all studies were cross-sectional in design, thus limiting establishment of causality of the observed factors associated with selection bias and reverse causality issues.[46] Even if the researchers used a well-designed survey tool such as Alcohol Use Disorders Identification Test and Cut, Annoyed, Guilty, and Eye, the results may be associated with recall bias. Population surveys can underestimate alcohol consumption by approximately 40–50%.[47] Drinking patterns as well as quantity can have an effect. For example, binge drinking affects lipid profile and liver function tests and aggravates liver fibrosis compared with non-binge drinking.[48,49] In several studies, moderate alcohol drinkers tended to have higher socio-economic status (SES) and were less obese than lifelong abstainers which may confound the association between alcohol consumption and NAFLD through interference from the interaction between NAFLD and obesity.[50,51]

POTENTIAL CONFOUNDING FACTORS REMAIN UNMEASURED

Gut microbiota

Confounding factors may exist that are not identified through history-taking or blood tests in routine clinic visits. In recent studies, consumption of alcohol and alcohol produced by the gut microbiome were shown to affect development of NAFLD. When blood alcohol concentration increases without significant alcohol consumption, autobrewery syndrome can be suspected. Some microbiota, particularly Proteobacteria (especially *Klebsiella pneumoniae* and *Escherichia coli*) can ferment dietary sugars into ethanol.[52] Engstler et al.[53] reported that patients with NAFLD, even children, have increased blood ethanol levels due to endogenously produced ethanol. Recently, Yuan et al.[54] found high-alcohol-producing *K. pneumoniae* (HiAlc Kpn) in the gut microbiome of up to 60% of NAFLD patients. When clinically isolated HiAlc Kpn was transferred into mice via fecal microbiota transplant, the recipient mice were observed to have NAFLD.[54] In another *in vivo* study using proteome and metabolome analyses, researchers showed that HiAlc Kpn catabolizes carbohydrates via the 2,3-butanediol fermentation pathway and a potential causative agent of NAFLD.[55] Therefore, the fecal microbiome in NAFLD patients should be considered a confounding factor.

Types of alcoholic beverages

Whether beer or wine is safer than liquor or distilled spirits regarding NAFLD has been questioned. The Centers for Disease Control and Prevention (CDC) revealed the amount of alcohol consumed is the most influential factor rather than the type of alcoholic drink.[56] In a cross-sectional study utilizing data from the NHANES III conducted in the United States from 1988 to 1994, suspected NAFLD (alanine transaminase >43 IU/L) was observed in 3.2% and 0.4% among 7,211 non-drinkers and 945 moderate wine drinkers (alcohol consumption <10 g/day), respectively, and the adjusted OR was 0.15 (95% CI: 0.05–0.49).[57] In a recent study in which the association between fibrosis and type and pattern of alcohol consumption in a biopsy-proven NAFLD cohort was evaluated, moderate (<70 g/week) alcohol consumption, particularly wine in a non-binge manner, was associated with lower fibrosis in NAFLD patients. In an animal study using a NAFLD mouse model fed a high-fat diet, extended-maceration wine improved glucose tolerance and reduced hepatic fat accumulation. Pomace also improved insulin sensitivity and reduced hepatic triglycerides.[58]

Recently, a randomized controlled trial was announced to evaluate the effects of beer on human gut microbiota. Marques et al. recruited 22 healthy men in Portugal who were assigned to drink 1 can of alcoholic or non-alcoholic lager each day for 4 weeks. Intestinal microbial diversity improved as determined based on the Shannon index,[59] indicating that drinking beer once a day can improve intestinal microbiome diversity regardless of alcohol content. That result is simultaneously consistent and contradictory to previous studies in which the effects of beer on the microbiome were investigated. In a study in Mexico, an increase in gut microbiome diversity, especially the relative abundance of

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Bacteroidetes, was observed in healthy men and women who consumed 355 mL of non-alcoholic beer a day for 30 days. However, the same improvement was not observed in a separate group who drank 355 mL of beer with 4.9% alcohol content.[60] The above positive effects of fermented alcoholic beverages are presumably due to polyphenols, although additional evidence is needed.

CONCLUSION

Clinical data have not conclusively proven the effects of moderate alcohol consumption and the amount of safe alcohol consumption for NAFLD patients has not been determined. Moderate alcohol consumption in patients with NAFLD has various effects and conflicting results have been reported. Unregulated factors such as sex, age, ethnicity, obesity, comorbidities, genetic factors, incomplete study design, unclear endpoints, economic and social aspects, and underreporting alcohol use confound the results. Based on the basic medical principle of "first, do no harm", recommending moderate drinking to NAFLD patients, especially those with comorbid diseases or advanced liver fibrosis, is premature. Additional longitudinal studies are expected to demonstrate the interactions between moderate alcohol consumption, effect of type/pattern of alcohol use, and SES based on NAFLD stage.

Authors' contribution

HO, WS, and YKC contributed to the design and drafting of the manuscript.

Conflicts of Interest

The authors have no conflicts to disclose.

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Review

Pharmacological advances in the treatment of nonalcoholic fatty liver diseases : focused on global results of randomized controlled trials

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Nonalcoholic fatty liver disease (NAFLD) is the most common cause of liver disease globally, and its prevalence is rapidly increasing. Nonalcoholic steatohepatitis (NASH), a progressive form of NAFLD, is characterized by hepatocellular injury, inflammation, and fibrosis. Patients with NASH or severe fibrosis should be treated according to international NAFLD guidelines. Currently, regulatory agencies have not approved any pharmaceutical treatment for NAFLD. Vitamin E and pioglitazone are efficacious for NASH resolution; however, their benefits must be weighed against the reported risks. In a phase 2 trial, a glucagon-like peptide-1 agonist commonly used for diabetes and obesity was found to improve liver histology in patients with NASH. Furthermore, therapeutic agents targeting NASH pathogenesis, including bile acid signaling, insulin resistance, and lipid metabolism, are in various phases of clinical development. In this article, we review the benefits and drawbacks of current pharmacotherapy and the efficacy of upcoming treatments for NASH. **(Clin Mol Hepatol 2023;29(Suppl):S268-S275)**

Keywords: Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; Treatment; Drugs; Clinical trials

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) affects approximately one-quarter of the adult population worldwide, making it the most common liver disease.[1] Nonalcoholic steatohepatitis (NASH), the progressive form of NAFLD, is characterized by hepatic triglyceride accumulation, hepatocyte injury, and lobular inflammation.[2] NASH is associated with accelerated fibrosis progression to cirrhosis and increased morbidity and mortality from liver disease.[3] More than 20% of patients with NASH will develop cirrhosis during their lifespan.[4] NASH is the leading indication for liver transplant in the United States,[5] and it is expected to become the most common cause of hepatocellular carcinoma in developed countries.[6]

Patients with NAFLD should be encouraged to lose weight by following a hypocaloric diet and engaging in physical activity.[2,7] In patients with NASH who are overweight or obese, more than 10% of weight loss due to lifestyle modification is associated with NASH resolution and fibrosis regression.[8,9] Weight loss also leads to a reduction of liver fat content in non-obese patients with NAFLD.[10] However, only a small percentage of patients achieve substantial weight loss, and long-term lifestyle changes are difficult to implement.[8,11] Therefore, patients with NASH require a practical therapeutic approach.

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Currently, there are no licensed drugs specifically approved for the treatment of NASH. In clinical practice, vitamin E and pioglitazone are efficacious for biopsy-proven NASH.[12] Furthermore, glucagon-like peptide 1 (GLP-1) agonists, which are commonly prescribed medications for diabetes and obesity, have the potential to ameliorate NASH.[13] The field of NASH treatment is rapidly evolving owing to the rising disease incidence and scarcity of current treatment options. Because the underlying mechanism of NASH is complex, NASH treatments are being developed for a wide range of targets, including oxidative stress, insulin resistance, apoptosis, bile acids, lipid metabolism, and hepatic inflammation and fibrosis. In this article, we review and summarize the efficacy and safety of current treatment options, based primarily on representative data from randomized controlled trials (RCTs), as well as emerging therapies that may enter clinical practice in the future.

CURRENT PHARMACOLOGIC THERAPIES

Vitamin E (alpha-tocopherol)

The imbalance between the reactive oxygen species' production and scavenging capacity causes oxidative stress.[14] Excess hepatic lipid causes reactive oxygen species over-production, accelerating the transition from NAFLD to NASH.[14] Vitamin E shows antioxidant properties by increasing specific enzymes and anti-fibrotic actions by regulating the inflammatory response.[15] In phase 3 PIVENS trial, patients with NASH without diabetes who received high dose vitamin E (800 IU/day; n=84) for 96 weeks showed a more statistically significant histological improvement, defined as ≥2 point reduction in the NAFLD activity score, than the placebo group (n=83) (43% vs. 19%).[12] The proportion of NASH resolution in the vitamin E group was also higher (36% vs. 21%). Recent prospective trials involving patients with NASH and diabetes, found that a combination treatment of vitamin E (800 IU/day) and pioglitazone is more efficacious than a placebo in terms of NASH resolution and steatosis improvement.[16] No prospective randomized studies have reported improved liver fibrosis and reduced liver-related death.[16] The international NAFLD guidelines suggest vitamin E supplementation for patients with NASH without diabetes (Table 1).[2,7,17] Unfortunately, although controversial, long-term administration of vitamin E is likely to raise the incidence of prostate cancer and hemorrhagic stroke.[18]

Pioglitazone

Pioglitazone, a peroxisome proliferator-activated receptor (PPAR)-γ agonist, reduces insulin resistance in the adipose tissue, muscle, and liver. Several prospective trials reported that patients with or without diabetes who received pioglitazone

Table 1. Summary of current NASH medications recommended by international guidelines

Drugs	Mechanism	Population	Guidelines (level of recommendation)
Vitamin E	Anti-oxidant	Non-diabetic patients with biopsy-proven NASH	AASLD 2018* EASL 2016 (B2) KASL 2021 (B1) AACE 2022 (Grade B, high strength of evidence)
Pioglitazone	PPAR-γ agonist	Diabetic patients with biopsy- proven NASH	AASLD 2018* EASL 2016 (B2) KASL 2021 (B1) AACE 2022 (Grade A, high strength of evidence)

NASH, nonalcoholic steatohepatitis; PPAR, peroxisome proliferator-activated receptor; AASLD, American Association for the Study of Liver Diseases; EASL, European Association for the Study of the Liver; KASL, Korean Association for the Study of the Liver; AACE, American Association of Clinical Endocrinology.
*The level and length of recommendations were not presented in the AASLD guidance 2018.

Abbreviations:

NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; GLP-1, glucagon-like peptide 1; RCTs, randomized controlled trials; PPAR, peroxisome proliferator-activated receptor; SAF, steatosis, activity, fibrosis; THR-β, thyroid hormone receptor beta; MRI, magnetic resonance imaging; ASK1, apoptosis-signal regulating kinase 1; MAPK, mitogen-activated protein kinase

(30 or 45 mg/day) showed more histological improvement in NASH than those who received placebo.[12,19,20] Cusi et al.[21] conducted a single-center study in which patients with prediabetes/diabetes and histologically confirmed NASH were randomly administered either pioglitazone (45 mg/day; n=50) or placebo (n=51). Pioglitazone treatment reduced NAFLD activity score by at least 2 points (58% vs. 17%) and resolved NASH (51% vs. 19%). A meta-analysis of eight RCTs found pioglitazone is efficacious for NASH resolution (odds ratio [OR] 3.22), improvement of advanced fibrosis (OR 3.15), and reversal of fibrosis (OR 1.66).[22] Thus, regardless of the diabetes status, pioglitazone is indicated for biopsy-proven patients with NASH (Table 1).[2,7,17] It is important to note that weight gain, fluid retention, and increased risk of fracture and bladder cancer are side effects of pioglitazone.

GLP-1 agonists

GLP-1 agonists affect glucose regulation by enhancing glucose-dependent insulin release, suppressing postprandial glucagon levels, and slowing gastric emptying. GLP-1 agonist is the mainstay treatment of obesity and diabetes because of their significant therapeutic benefits in weight loss, glycemic control, and improvements in the cardiometabolic system.[23] Although the underlying mechanisms of GLP-1 agonists on NASH have not been fully explained, considerable weight loss induced by GLP-1 agonists may lead to subsequent disease improvement. A phase 2 RCT with 320 biopsy-confirmed patients with NASH found that the semaglutide group (0.4 mg once daily for 72 weeks) had a higher proportion of disease resolution than the placebo group (59% vs. 17%).[24] Even though the treatment group had a lower rate of liver fibrosis progression (4.9% vs. 18.8%), there were no significant differences in the proportion of patients whose fibrosis stage improved. The American Association of Clinical Endocrinology guidelines recommend the use of GLP-1 agonist in patients with histology-proven NASH and diabetes.[17] A phase 3 ESSENCE trial involving 1,200 patients with NASH and F2-F3 fibrosis is currently investigating the efficacy of semaglutide at a dose of 2.4 mg once-weekly for NASH resolution and fibrosis improvement (NCT04822181; Table 2). The most common side effects among patients that receive GLP-1 agonist are gastrointestinal symptoms, such as nausea, vomiting, and diarrhea. GLP-1 agonists may increase the risk of acute pancreatitis, gallbladder disease, and biliary disease. Although GLP-1 agonists are currently used as subcutaneous injections in clinical protocols, oral formulations with improved tolerability are being developed.

Recently, advances have been made in developing glucagon-containing co-agonists to enhance the efficacy of GLP-1 agonists. A glucagon-stimulated increase in energy expenditure augments the effect of GLP-1-induced weight loss.[25] Cotadutide is a dual-receptor agonist with balanced GLP-1 and glucagon action. In phase 2 PROXYMO trial, 74 obese patients with biopsy-proven NASH and F1-F3 fibrosis were randomized to receive once-daily subcutaneous injections of cotadutide (300 μg or 600 μg) or placebo.[26] Cotadutide was associated with dose-dependent reductions in hepatic fat compared to the placebo. In the ongoing phase 3 PROXYMO-ADV trial, cotadutide is expected to show efficacy in treating NASH (Table 2).

FUTURE PHARMACOLOGIC THERAPIES

Obeticholic acid

The farnesoid X receptor is a nuclear receptor activated by bile acids that is abundant in the liver and intestines. It regulates bile synthesis, conjugation, and transport,[27,28] and plays a role in lipid and glucose metabolism.[28] The farnesoid X receptor activation can help reduce hepatic inflammation and fibrosis.[29,30]

Obeticholic acid is a potent and selective farnesoid X receptor agonist. In the interim analysis of phase 3 REGENERATE trial, 931 biopsy-proven patients with NASH and fibrosis stages F2-F3 were randomly assigned to receive obeticholic acid 25 mg daily (n=308), obeticholic acid 10 mg daily (n=312), or placebo (n=311) (Table 2).[31] At 18 months, the obeticholic acid group improved liver fibrosis by at least one stage with no worsening of NASH in a dose-dependent manner (23% vs. 18% vs. 12%, respectively), with no difference in the proportion of NASH resolution (12% vs. 11% vs. 8%, respectively). Indeed, in NASH phase 3 trials, obeticholic acid was the first agent to show a significant improvement in fibrosis. Mild to moderate pruritus was the most common adverse event, affecting up to 51% of patients treated with obeticholic acid 25 mg. Furthermore, nearly 17% of the obeticholic acid group experienced an early increase in low-density lipoprotein cholesterol, which returned to baseline

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Table 2. Current status of emerging drugs from phase 3 clinical trials of nonalcoholic steatohepatitis

Drug	Target	Population	Study name	Status
Obeticholic acid	Farnesoid X receptor agonist	NASH with F2-F3 fibrosis	REGENERATE	Ongoing
Lanifibranor	Pan-PPAR agonist	NASH with F2-F3 fibrosis	NATiV3	Ongoing
Resmetirom	Thyroid hormone receptor-beta agonist	NASH with F1-F3 fibrosis	MAESTRO-NASH	Ongoing
Semaglutide	Glucagon-like peptide-1 (GLP-1) agonist	NASH with F2-F3 fibrosis	ESSENCE	Ongoing
Cotadutide	dual GLP-1 and glucagon receptor agonist	NASH with F2-F3 fibrosis	PROXYMO-ADV	Ongoing
Obeticholic acid	Farnesoid X receptor agonist	NASH with compensated LC	REVERSE	Halted
Elafibranor	PPAR-alpha and -delta agonist	NASH with F1-F3 fibrosis	RESOLVE-IT	Halted
Selonsertib	Apoptosis signal-regulating kinase inhibitor	NASH with F3 fibrosis	STELLAR-3	Halted
Selonsertib	Apoptosis signal-regulating kinase inhibitor	NASH with compensated LC	STELLAR-4	Halted
Cenicriviroc	Inhibitor of CC chemokine receptors 2 and 5	NASH with F2-F3 fibrosis	AURORA	Halted
Aramchol	Fatty acid bile acid conjugate	NASH with F1-F3 fibrosis	ARMOR	Suspended*

PPAR, peroxisome proliferator-activated receptor; NASH, nonalcoholic steatohepatitis; LC, liver cirrhosis.
*Starting the double-blind part of phase 3 trial is delayed due to the formulation of Aramchol Meglumine.

levels at the end of the study. In contrast, in the recent REVERSE trials of 919 randomized patients with compensated NASH cirrhosis, obeticholic acid did not improve fibrosis (11.1% vs. 11.9% vs. 9.9% in obeticholic acid 10 mg vs. obeticholic acid 10 mg titrated to 25 mg vs. placebo, respectively).[32] The US Food and Drug Administration has not yet approved obeticholic acid as a NASH treatment due to its uncertain long-term benefit and safety risks.

pan-PPAR agonist

PPARs are a nuclear receptor family with three isotypes that regulate glucose and lipid metabolism, inflammatory cell activation, and fibrotic processes.[33] Three PPAR isotypes have been identified: PPAR-α, PPAR-β/δ, and PPAR-γ. PPAR-α is an essential regulator of fatty acid oxidation that suppresses inflammation by reducing reactive oxygen species formation. PPAR-β/δ stimulates hepatic glucose utilization and *de novo* lipogenesis. PPAR-γ regulates adipocyte differentiation and insulin sensitization.

Lanifibranor (IVA337), a pan-PPAR agonist, demonstrated higher efficacy in terms of improvement of insulin sensitivity, macrophage activation, and reduction of liver fibrosis than single or dual PPAR agonists.[34,35] In 2021, the results of phase 2b trials comparing lanifibranor 1,200 mg (n=83), lanifibranor 800 mg (n=83), or placebo (n=81) for 24 weeks in patients with biopsy-proven NASH were published.[36] The proportion of patients who met the primary endpoint, a decrease of at least 2 points in the SAF-activity score (the activity component of the Steatosis, Activity, Fibrosis [SAF] scoring system that includes hepatocytes ballooning and inflammation), was higher among those who received lanifibranor 1,200 mg than the placebo group (55% vs. 33%). The outcomes favored lanifibranor 1,200 mg over placebo for improvement in the fibrosis stage of at least one without worsening of NASH (48% vs. 29%). Fewer than 10% of patients in the lanifibranor group reported diarrhea, weight gain, and peripheral edema as common adverse effects. An ongoing phase 3 study of lanifibranor for NASH and F2-F3 fibrosis (NATiV3) is also expected to reveal similar results (Table 2).

In contrast, a phase 3 RCT of the dual PPARα–PPARδ agonist elafibranor (RESOLVE- IT) was halted because it failed to meet the predefined primary surrogate efficacy endpoint, NASH resolution without fibrosis worsening the interim analysis.[37]

Thyroid hormone receptor β-agonist

The thyroid hormone regulates glucose and lipid metabolism, in addition to fatty acids oxidation.[38,39] A selective thyroid hormone receptor beta (THR-β) agonist has been developed to improve liver-specific action while minimizing negative effects on the cardiac and skeletal systems, which are predominantly mediated by THR alpha. Resmetirom, an oral THR-β agonist, was studied in a phase 2 RCT involving 125 overweight or obese adults with biopsy-confirmed NASH

and stages 1–3 fibrosis.[40] Resmetirom treatment for 36 weeks resulted in a significant reduction in hepatic fat measured using magnetic resonance imaging (MRI)-proton density fat fraction compared with placebo (-37% vs. -9%). An ongoing phase 3 MAESTRO-NAFLD1 trial is evaluating the impact of resmetirom on liver histology in patients with NASH and stage 2–3 fibrosis (Table 2). The preliminary results showed that resmetirom was efficacious for hepatic fat assessed using MRI-proton density fat fraction.[41] The most prevalent side effects were mild gastrointestinal symptoms, including diarrhea and nausea.

Selonsertib

Apoptosis-signal regulating kinase 1 (ASK1) is a member of the mitogen-activated protein kinase (MAPK) family.[42] ASK1 is activated in response to oxidative stress and promotes hepatic inflammation and apoptosis, leading to liver fibrogenesis via MAPK downstream signaling. Hence, ASK1 is considered a treatment target for NASH.[43] Selonsertib is a first-in-class small-molecule ASK1 inhibitor with antifibrotic and anti-inflammatory effects. Based on the success in phase 2 trials of selonsertib in patients with NASH and F2-F3 fibrosis,[44] phase 3 RCTs comparing selonsertib 18 mg, selonsertib 6 mg, and placebo were subsequently conducted in patients with NASH and bridging fibrosis (F3, STELLAR-3; n=802) or compensated cirrhosis (F4, STELLAR-4; n=877) (Table 2).[45] The STELLAR-3 trial did not reveal significantly different fibrosis improvement without worsening of NASH between groups (10% vs. 12% vs. 13%, respectively). Moreover, fibrosis improvement was not observed in STELLAR-4 patients with cirrhosis (14% vs. 13% vs. 13%, respectively). In phase 2b ATLAS trial with 392 patients with NASH and F3-F4 fibrosis, selonsertib combination therapy revealed unfavorable outcomes in reversing fibrosis.[46] Although selonsertib is no longer being investigated, ASK1 may still be a viable candidate if more effective inhibitors are discovered.

Other NASH therapies in clinical trials

The novel medications that have entered phase 3 development stage include armachol (a bile acid and fatty acid analog)[47] and cenicriviroc (inhibitor of CC chemokine receptors 2 and 5) (Table 2).[48] Moreover, a large number of additional agents with diverse mechanisms for targeting the pathogenesis of NASH are in phase 2 development.[49]

CONCLUSIONS

Since the PIVENS study with vitamin E and pioglitazone on NASH resolution was successful in 2010, NASH has been extensively investigated to identify optimal medications. Large-scale RCTs have yielded promising results for farnesoid X receptor, GLP-1, and pan-PPAR agonists in improving hepatic inflammation and fibrosis. However, several obstacles must be overcome before they are approved by the US Food and Drug Administration for NASH treatment: 1) while liver biopsy remains the gold standard for diagnosis in clinical trials, further studies are needed to develop easy-to-use panels of serum and imaging-based biomarkers for noninvasive patient selection and treatment response; 2) given the complex pathophysiology of NASH and modest treatment response rates to individual drugs, it is highly likely that a combination treatment will also be required; and 3) the external validity of the RCT results should be confirmed, especially for real-world patients with NASH with more significant comorbidities. We believe that numerous drugs added to the pipeline of novel therapies could increase the chances of successful treatment of NASH and more completely reverse disease progression in affected patients in the future.

Authors' contribution

Study concept and design: Jihyun An and Joo Hyun Sohn; Data analysis and interpretation: Jihyun An and Joo Hyun Sohn; Wrote the paper: Jihyun An and Joo Hyun Sohn; All authors have read and approved the final version of the manuscript.

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Conflicts of Interest

The authors have no conflicts to disclose.

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Review

Bariatric surgery for non-alcoholic fatty liver disease: Indications and post-operative management

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The prevalence of obesity and metabolic consequences such as nonalcoholic fatty liver diseases (NAFLD) has become a crucial health problem. Lifestyle modifications, especially weight loss, effectively reduces liver injury in NAFLD patients. However, adherence to lifestyle changes is very low in the clinical setting. Bariatric surgery can improve metabolic components and cause long-term weight loss. Therefore, bariatric surgery could serve as an attractive treatment option for NAFLD patients. This review integrates data about the benefits of bariatric surgery on NAFLD but also describes the potential pitfalls. **(Clin Mol Hepatol 2023;29(Suppl):S276-S285)**

Keywords: NAFLD; Bariatric surgery; Alcohol use disorder

INTRODUCTION

The global prevalence of obesity has grown dramatically in the last 20 years and has become rapidly a public health issue.[1] The obesity epidemic led to a massive increase in cases of non-alcoholic fatty liver disease (NAFLD). NAFLD represents a spectrum of disease, consisting of non-alcoholic fatty liver (NAFL), nonalcoholic steatohepatitis (NASH), liver fibrosis, cirrhosis and eventually the development of hepatocellular carcinoma (HCC). Recently published data by Harrison et al showed a prevalence of NAFLD, NASH and significant fibrosis in asymptomatic middle-aged Americans of 38%, 14% and 6% respectively, with the highest prevalence in Hispanics (55%) and those with obesity (57%) and diabetes mellitus (70%).[2] Also in Asia the prevalence is increasing with an estimation of NAFLD prevalence of 20–30% in Korea.[3] Recently published data by Lee et al.[4] showed that even in young Korean men in their early 20s, the NAFLD prevalence consistently increased from 2015 to 2021, respectively from 10.6% to 16.4%. Data from the European Liver Transplant Registry (ELTR) and United Network for Organ Sharing (UNOS) demonstrate that NASH cirrhosis and NAFLD-related HCC are the fastest growing indication for liver transplant in recent years.[5,6]

Despite the increasing prevalence of NAFLD and NASH cirrhosis, there are still no Food and Drug Administration (FDA)-approved pharmacotherapies which halt progression in the spectrum of the disease and reduce liver-related complications in patients with NAFLD.

Lifestyle modification with reduced intake of calories combined with increased activity is still the cornerstone of NAFLD treatment. The main driver of NAFLD improvement is the amount of actual weight loss, while the type of diet seems to be less important. Prospective trials comparing various diets

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are lacking high-quality data. This is nicely summarized in a narrative review by Hydes et al.[7]. The authors concluded that the data only supports reducing saturated fat, refined carbohydrates and red and processed meats in the diet.

It has been shown that a weight reduction of at least 7–10% with conservative lifestyle modification is necessary to resolve NASH and to improve liver fibrosis.[8,9] This was clearly demonstrated in a prospective cohort study with paired liver biopsies in 261 patients. All patients who lost more than 10% of their weight had a 90% complete resolution of their NASH as well as an improvement of fibrosis in 45%.[8] We published data from a prospective study in children and adolescents admitted for severe obesity at a tertiary center (Zeepreventorium, De Haan, Belgium). NAFLD on ultrasound was present in 71.1% of these children. A total of 32.8% of patients had at least fibrosis grade 2, including 10.3% with transient elastography of 9 kPa or greater, compatible with significant fibrosis. All children and adolescents underwent intensive lifestyle therapy encompassing caloric restriction, physical activity, education on a healthy lifestyle, and psychosocial support. After 6 months, the median body weight loss was 16.0%. A significant improvement of steatosis was seen and more importantly, fibrosis improved in 75.0% of the study population (Fig. 1).[10]

Although weight loss reduction works, only 5–10% of patients will achieve the target weight loss with structured lifestyle interventions at 1 year and fewer than half of these patients maintain the weight loss 5 years later.[11] Therefore, bariatric surgery could be a therapeutic approach in selected obese patients afflicted with NAFLD.

BARIATRIC SURGERY MECHANISMS

The history of weight loss surgery dates back to 1953 and innovation has continued for years thereafter.[12] A variety of procedures of BS have been developed. Techniques that rely predominantly on malabsorption by deriving digestive juices to the very distal part of the ileum (biliopancreatic diversion [BPD], duodenal switch) lead to large weight loss with severe long-term complications as a consequence, and extreme malabsorptive techniques such as the jejunoileal bypass are therefore abandoned. The most commonly applied techniques currently worldwide are the Roux-en-Y-gastric bypass (RYGB) and sleeve gastrectomy (SG). Very low mortality and morbidity rates are associated with these two procedures performed laparoscopically.[13]

The mechanistic effects of BS are complex whereby weight loss due to malabsorption or restriction is not the only mode of action responsible for the potential effects on the liver. Alterations in gut hormone signaling, in bile acid levels and in adipose tissue (AT) inflammation will affect insulin signaling independently of weight loss. Acceleration of gastric emptying in SG and RYGB alters the enterohormonal balance, such as a markedly increased secretion of the gut peptides glucagon-like peptide (GLP-1) and peptide YY.[14] Both RYGB and SG increase the total circulating bile acid pool which play a role as metabolic signaling molecules.[15] BS also causes a reversal of the AT inflammation, and alters the endocrine functions of the AT, such as an increase of adiponectin and a decrease of serum leptin levels. All these physiological changes can contribute to the beneficial effects on the liver (Fig. 2).[16]

BENEFITS OF BARIATRIC SURGERY IN NAFLD PATIENTS

Bariatric surgery induces long-term excess weight loss up to 30% and remission of diabetes mellitus with reducing cardiovascular and cancer-related mortality, the two most frequent causes of death in patients with NASH.[17-21] Patients with obesity who meet the criteria for BS, namely body mass index (BMI) >40 kg/m^2 or ≥35 kg/m^2 and at least one or more obesity-related co-morbidities, frequently have features of NAFLD or NASH. Studies reported the presence of NAFLD and NASH in morbidly obese adults prior to weight loss surgery in 80.2% to 90% and 14.4%, respectively.[22-25] Despite the high prevalence of NAFLD/NASH in patients undergoing bariatric

Abbreviations:

NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; HCC, hepatocellular carcinoma; ELTR, European Liver Transplant Registry; UNOS, United Network for Organ Sharing; BS, bariatric surgery; BPD, biliopancreatic diversion; RYGB, Roux-en-Y-gastric bypass; SG, Sleeve gastrectomy; AT, adipose tissue; EWL, excess weight loss; BMI, body mass index; AGB, adjustable gastric banding; CI, cumulative incidence; HVPG, hepatic venous pressure gradient; LT, liver transplantation; AUD, alcohol use disorder

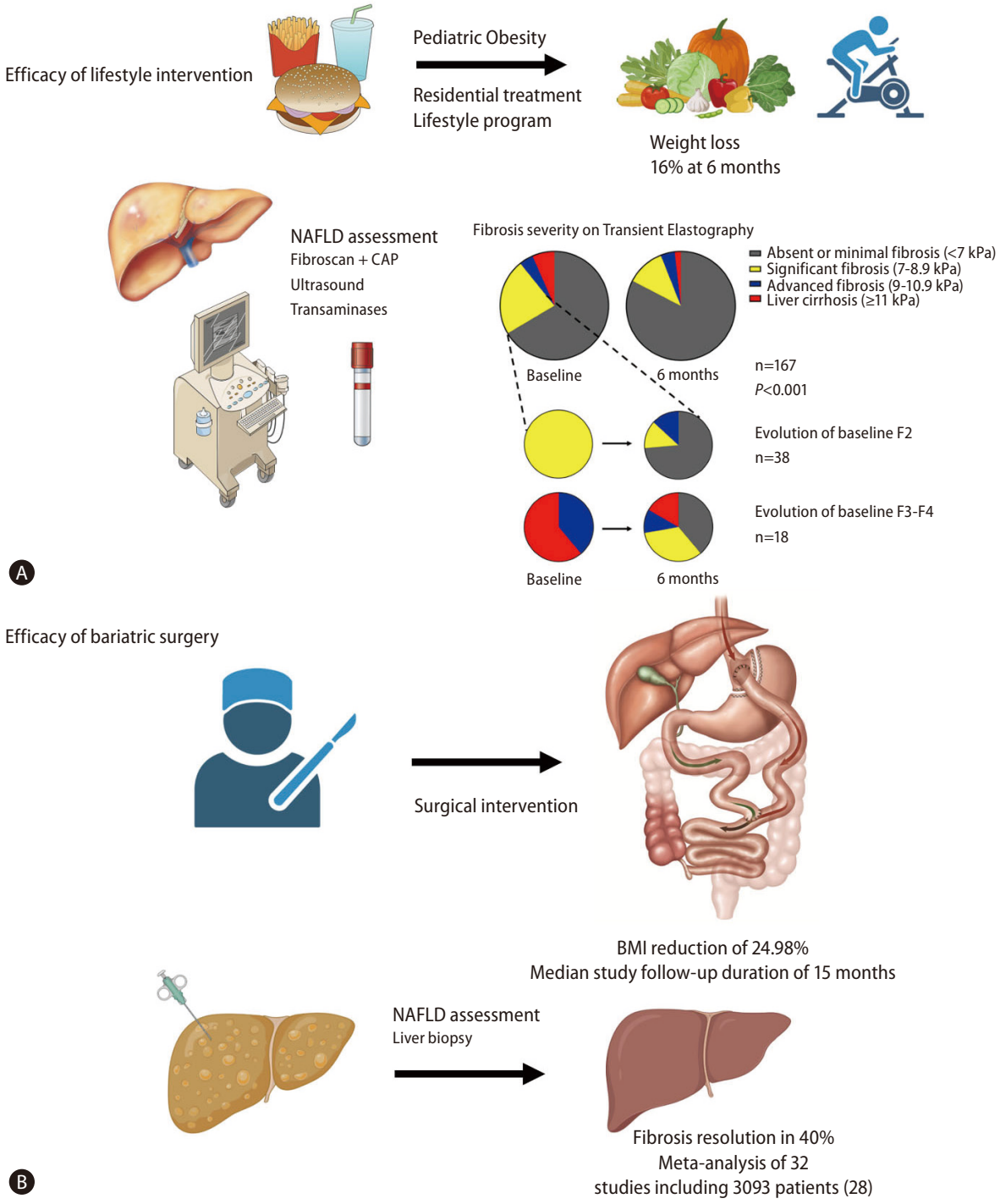

Figure 1. Weight loss interventions for the treatment of NAFLD. (A) Lifestyle intervention for pediatric NAFLD. NAFLD was assessed at baseline and after 6 months in 167 patients. Evidence of liver fibrosis was present in 56 patients. After treatment, fibrosis improved in 75% of patients. Figure adapted from the article of Lefere et al. (Clin Gastroenterol Hepatol 2022;20:2317-2326.e4).[10] (B) Bariatric surgery for NAFLD. In a meta-analysis of studies comparing liver biopsy before and after bariatric surgery, complete resolution of fibrosis was observed in 40% of patients.[28] NAFLD, nonalcoholic fatty liver disease; BMI, body mass index; CAP, controlled attenuation parameter.

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surgery, this co-morbidity is not consistently determined as an indication. Also screening for fatty liver disease is not routinely done in the preoperative period, nor screening to stage liver fibrosis by liver biopsy or non-invasive markers at

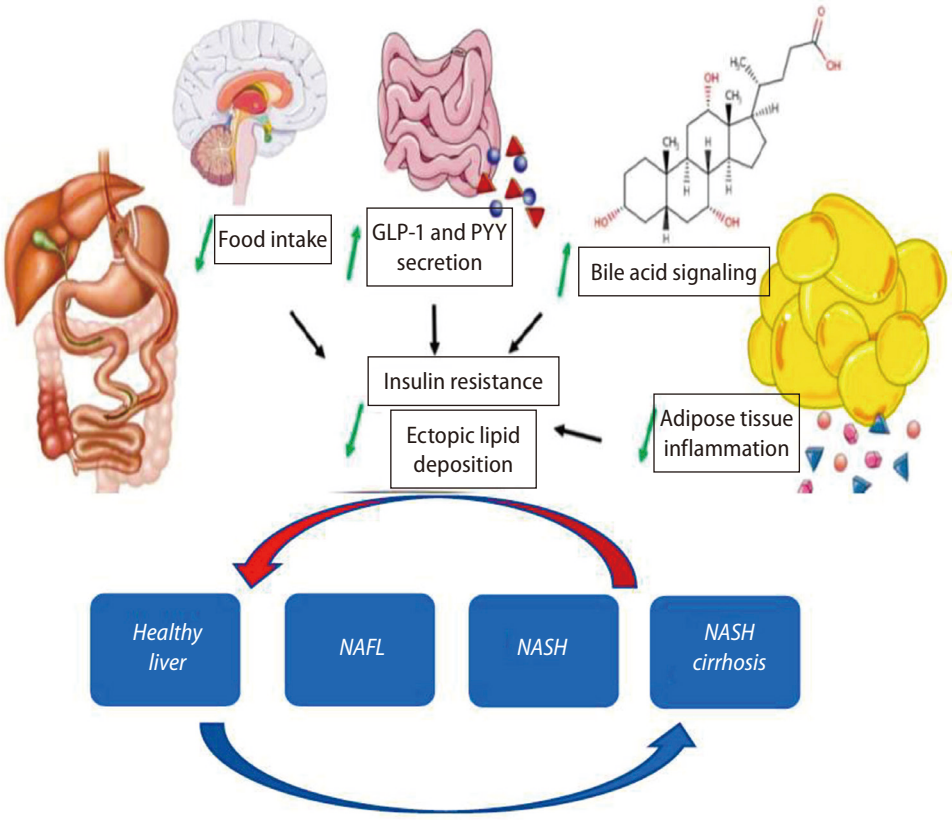

Figure 2. Mechanisms of resolution of non-alcoholic fatty liver disease (NAFLD) after bariatric surgery. Factors including regulation of food intake and food preferences, gut hormone secretion, bile acid signaling and visceral adiposity and adipose tissue inflammation. The potential for reversal of cirrhosis is still debated. GLP-1, glucagon-like peptide; PYY, peptide YY; NAFL, nonalcoholic fatty liver; NASH, nonalcoholic steatohepatitis.

Table 1. Potential indications for bariatric surgery in NASH patients

Indication	Recommend surgical method	Expected improvement
Obese patients (BMI ≥35 kg/m^2) with NASH fibrosis and comorbidities, or obese patients with NASH fibrosis who otherwise meet BS criteria (BMI >40 kg/m^2)	RYGB or SG	-Significant lower risk for major adverse liver and cardiac events[32] -Resolution of steatosis (from 66 to 88%) -Resolution of inflammation and ballooning (from 50 to 84%) -Resolution of fibrosis (from 40 to 68%)[26-31]
NASH cirrhosis and no significant portal hypertension (HVPG <10 mmHg)	SG	-Prevention of decompensation[36] -Improvement of liver transplant candidacy[45] -Increased survival after liver transplantation[47]
Liver transplant recipients with obesity and NAFLD or NASH	SG	-Prevention of recurrence of NASH and fibrosis progression[50,51] -Improvement of metabolic risk factors with better graft survival

NASH, non-alcoholic steatohepatitis; BMI, body mass index; BS, bariatric surgery; HVPG, hepatic venous pressure gradient; NAFLD, non-alcoholic fatty liver disease; RYGB, Roux-en-Y-gastric bypass; SG, Sleeve gastrectomy.

the time of surgery.

In the absence of randomized controlled trials, several prospective and retrospective cohort studies and meta-analyses represent that sustained weight loss is associated with a reduction in steatosis, inflammation and fibrosis after BS (Fig. 1).[26-31] In a recent meta-analysis, twenty-one studies (12 RYGB, 3 adjustable gastric banding [AGB], 2 SG, 1 vertical banded gastroplasty, 3 multiple procedures) enrolling 2,374 patients were included. The pooled proportion of patients who had improvement of steatosis was 88%, steatohepatitis improved in 59% and fibrosis improved or resolved in 30% of patients.[30] Another systematic review and meta-analysis of 32 cohort studies in obese patients, including comparison of 3,093 liver biopsy results before and after BS, confirmed resolution of steatosis in 66%, inflammation in 50%, ballooning degeneration in 76% and fibrosis in 40% of the patients.[28] These beneficial findings suggested in prior systematic reviews and meta-analyses are supported by a prospective long-term follow-up study with consecutive liver biopsies at 1 and 5 years after BS. One year after surgery, NASH resolved in 85% of patients.[29] Similar results were obtained after 5 years BS, indicating the durability of the response. Resolution of NASH without worsening of liver fibrosis was achieved in 84% of patients. Fibrosis regressed gradually and improved in 70% of patients compared to baseline fibrosis after 5 years. Importantly, in patients with advanced fibrosis (stage 3 fibrosis) at baseline, fibrosis improved in 68% and disappeared in 45% of patients at 5 years.[31] Limited weight loss and less improvement of insulin resistance following BS were associated with the persistence of NASH.

A recent retrospective cohort study conducted by Aminian et al.[32] was the first to demonstrate a significant lower risk for major adverse liver and cardiac outcomes in the bariatric surgery group compared with nonsurgical management in patients with biopsy-proven NASH and obesity. Specifically, the cumulative incidence (CI) of major liver outcomes at 10 years was 2.3% in the BS group versus 9.6% in the nonsurgical group. Regarding major adverse cardiac events, CI at 10 years was 8.5% in the bariatric surgery group and 15.7% in the nonsurgical group.[32]

Besides preventing liver fibrosis is the development of NAFLD-related malignancies another aspect to consider. Retrospective cohort studies have shown that the adjusted CI of NAFLD-related malignancy (including HCC) is lower in patients who underwent BS vs. not.[33] Lastly the benefits of BS extend beyond the liver to affect diseases of other organ systems, specifically the risks of cardiovascular illnesses, stroke and renal failure.[20]

During the last year, endoscopic bariatric therapies have become popular to treat obesity and metabolic conditions. Most of these techniques induce restrictive and metabolic effects. As most studies show that both RYGB and SG improve NAFLD with similar effects,[34] endoscopic bariatric techniques could also serve as an option to induce weight loss. Data are currently limited because relatively small sample size in studies, but endoscopic bariatric therapies appear to be effective on NAFLD.[35] These techniques need to be further investigated in the field of fatty liver diseases. BS may be an effective treatment for obese patients (BMI ≥35 kg/m^2) with NASH fibrosis or obese patients with NASH fibrosis who otherwise meet BS criteria (BMI >40 kg/m^2).

BARIATRIC SURGERY IN CIRRHOSIS AND CONTEXT OF LIVER TRANSPLANTATION

Bariatric surgery in cirrhosis

Obesity is a strong predictor of decompensation in patients with compensated cirrhosis of various etiologies, independent of other predictors such as albumin or portal hypertension.[36] Increased mortality, poor survival after liver transplantation and increased risk of bacterial infections and sepsis related death are correlated with BMI levels >35 kg/m^2. Weight loss should therefore be an important therapeutic goal also in patients with compensated cirrhosis.

Data from BS in cirrhotic patients are mostly coming from retrospective analyses of incidental findings at the time of surgery, with a prevalence between 0.5% and 1.5%.[37] In two US nationwide database studies, the in-hospital mortality rate after BS is slightly higher in patients with compensated cirrhosis versus those without cirrhosis (0.9% and 0.6% vs. 0.3% and 0.1%) and markedly increased in patients with decompensated cirrhosis (16.3% and 19.4%).[38,39] Bariatric surgery is therefore absolutely contra-indicated in patients with decompensated cirrhosis.

The type of surgery is one of the criteria that should be considered in balancing the risks and benefits of BS in patients with compensated cirrhosis. A systematic review of the outcome of 122 patients with compensated cirrhosis under-

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going BS showed that mortality related to BS was only observed in BPD and RYGB in 20 and 3.9% respectively. No mortality was observed with SG and AGB.[40] Stable liver function and no progression to liver dysfunction was observed in a small cohort of compensated cirrhotic patients with SG after 10-year follow-up.[41] Currently, a laparoscopic SG is the preferred procedure and seems feasible in compensated cirrhotic patients. Another advantage of SG is the gradual weight loss, absence of malabsorption, and the preservation of endoscopic access to the biliary tree.

A recently published AGA clinical practice guideline for BS in cirrhosis suggests that BS can be considered in selected patients with compensated cirrhosis (Child-Pugh A, model for end-stage liver disease [MELD] score <12) but should only be performed after careful evaluation and management of extrahepatic comorbidities, and after assessing the grade of portal hypertension. It is necessary to exclude those with a history of decompensated cirrhosis or those with significant portal hypertension which could be assessed by an upper endoscopy (presence of varices) or measurement of hepatic venous wedge pressure gradient (>10 mmHg).[42]

Portal hypertension is indeed another criteria to balance the risk of BS in cirrhotic patients. A recent study assessed the prognostic role of hepatic venous pressure gradient (HVPG) in cirrhotic patients undergoing elective extrahepatic surgery. The authors showed that HVPG of more than 16 mmHg is associated with a higher 1-year mortality and a very high risk of death (44%) was seen in the presence of HVPG >20 mmHg.[43] Whether this also applies specifically to BS requires further study, but it is reasonable to follow this guidance and severe portal hypertension (>16 mmHg) must be considered a contraindication for BS. Low-risk for surgery is seen in patients with HVPG less than 10 mmHg.[44]

Bariatric surgery before and after liver transplantation (LT)

Treating obesity before liver transplantation can reduce the risk of decompensation on the waiting list and comorbidities, peri-operative and post-operative.[45,46] Takata et al.[45] showed improved LT candidacy in several patients as the BMI dropped. In a systematic review of five studies with the intention of improving LT candidacy, 78% of patients could be listed, and the rate of major and minor complications was 2% and 8%, respectively.[47] Also, in liver transplant candidates, the preferable type of surgery is SG as previously discussed in the section of BS and cirrhosis.

Long-term weight gain and the development of metabolic syndrome are the main concerns post-liver transplant. Recurrent NAFLD/NASH after transplantation is very common, ranging from 10 to 100% and 4 to 28%.[48,49] Probably, the outcomes of NASH cirrhosis liver transplant recipients are not as good as previously thought and this is due to the development of metabolic risk factors. It has been shown that NASH transplant recipients have a 10-year graft survival of 61%, which is significantly lower than other liver diseases.[48] Sleeve gastrectomy is the most performed procedure in this patient group, with the advantage of lack of malabsorption and no interference with immune suppressive drugs. Optimal timing of BS needs to be defined, because delaying too long can cause rapid fibrosis in the graft and reduce patient survival. Reported series described an interval from LT to BS ranging between 27 and 70 months.[50,51]

Post-operative management

Monitoring liver function

Due to rapid weight loss during the first few months after surgery, hepatic damage can occur with increasing liver enzymes. Liver function is, however expected to return to normal within a year with a reduction in AST, ALT, and GGT levels already observed 6 months post-surgery.[52] Also, Nickel et al.[53] showed an increase in liver transaminases 1 month after surgery, but normalization within one year was observed. Contributing factors to the short-term elevation of enzymes are the rapid metabolic changes and slow adaptation of liver function after surgery.

Assessment of liver function after BS requires performing routine liver tests including bilirubin, transaminases, GGT, INR, and albumin at months 3, 6, and 12 and afterwards every 1–2 years, if normal findings at 12 months.[44]

Strict follow-up of weight loss and supplementation of vitamins and trace elements should be performed even more carefully in patients with known liver disease to avoid further progression in those with pre-cirrhotic stages and to prevent decompensation in those with cirrhosis.

Ideally, the presence and severity of liver disease should be carefully assessed prior to BS. In the general NASH population, a liver stiffness measurement (LSM) cut-off of less than 8 kPa can reliably exclude advanced fibrosis and cirrhosis with

a 94 to 100% negative predictive value.[54] Values above 12 to 15 kPa have a high positive predictive value (ranging from 80 to 90%) to detect advanced fibrosis or cirrhosis. Follow-up with noninvasive LSM measurements is not routinely done but data are present where a significant reduction in LSM could be observed in the majority of patients 6 months after surgery. Patients with an intraoperative diagnosis of significant fibrosis or cirrhosis should be referred to liver specialists for further evaluation because of the need for hepatocellular carcinoma screening and close monitoring to prevent episodes of decompensation.

Liver failure after bariatric surgery

BS procedures with a marked malabsorptive component, such as jejunoileal bypass or BPD, were proven to cause life-threatening complications including acute liver failure in up to 10% of patients, and should therefore be abandoned.[55] Liver failure following RYGB and SG is rarely reported. Mahawar et al.[56] reported 10 cases of liver failure after RYGB. Four out of the 10 reports were seen in cirrhotic patients, 2 had extended limb RYGB, 1 distal RYGB and 2 had early or late complications.[56] Extended limb or distal version of RYGB can behave like biliopancreatic diversion with higher potential for malabsorption.

The pathogenesis of liver failure after BS remains poorly understood. Potential contributing factors include rapid weight loss, which increases fatty acid delivery to the liver, and macro- and micronutrient malnutrition. Protein malnutrition plays a pivotal role in liver disease progression. The European practice guidelines on nutrition in chronic liver disease suggests that the optimal daily protein intake should not be lower than the recommended 1.2 to 1.5g/kg.[57] Liver transplantation needs to be considered if reversal of BS is not possible due to severe liver decompensation.

Alcohol use after bariatric surgery

Several studies have suggested that the incidence of alcohol consumption increases over the postoperative period of BS, predominantly in the second postoperative year, with a high prevalence, ranging from 12 to 20%.[58,59] Ibrahim et al.[59] reported an identical risk after RYGB and SG in the second year, although some cohorts described a lower prevalence in restrictive procedures such as SG. Additional studies are needed to clarify the importance of the type of surgery.

BS affects the pharmacokinetics of alcohol with higher peak alcohol concentrations and a greater feeling of drunkenness. Other potential mechanisms involved in post-bariatric alcohol use disorder (AUD) are still debated. Alterations in secretion of gut hormones like incretins and ghrelin, bile acid alterations, vagal nerve signaling, and changes in gut microbiota might impact the central nervous system processes and increase the sensitivity for alternative rewards such as alcohol.[16]

We recently published single-center data showing that 6% of 188 patients transplanted for alcoholic liver disease between 2008 and 2018 had a history of BS. These patients were significantly younger and presented with more severe decompensated liver disease.[60] Similarly, a recent study reported that a history of BS is increasingly common in patients presenting with acute alcoholic hepatitis. Although BS patients were younger at presentation, survival was similar.[61] In a retrospective observational analysis of obese adults based on insurance claims, women had undergone BS had twofold increased risk of alcoholic cirrhosis and alcohol misuse compared to women without prior surgery.[62]

BS patients should therefore be educated about the possible risks of alcohol use which can lead rapidly to the development of alcoholic cirrhosis, and surgeons should be reluctant to perform BS in patients with a history of AUD.

CONCLUSION

The major impact of NASH on the risk of cirrhosis and hepatocellular carcinoma highlights the urgent need for effective therapies to reverse the disease. Weight loss is the cornerstone in the treatment of NAFLD but difficult to reach and to keep long-term the target goals with only conservative lifestyle changes.

Obese patients with NASH fibrosis could benefit from BS. There is evidence that BS is safe, improves steatosis, inflammation and fibrosis score and reduces the risk for mortality from cardiovascular disease and NAFLD-associated HCC. Patients with cirrhosis need to be carefully selected by a multidisciplinary team of specialists to assess of the risk and the choice of type of surgery (Table 1).

Severe malnutrition related to excessive rapid weight loss after BS and de novo alcohol misuse are the most important contributors for the deterioration of liver function after BS. Prevention and early recognition of alcohol misuse pre- and

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post-surgery is a major unmet need.

Authors' contribution

Conceptualization and writing: AG, SL.

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Conflicts of Interest

The authors have no conflicts to disclose.

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Review

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Liver transplantation for non-alcoholic fatty liver disease: indications and post-transplant management

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Non-alcoholic fatty liver disease (NAFLD) is currently the fastest growing indication to liver transplantation (LT) in Western Countries, both for end stage liver disease and hepatocellular carcinoma. NAFLD/non-alcoholic steatohepatitis (NASH) is often expression of a systemic metabolic syndrome; therefore, NAFLD/NASH patients require a multidisciplinary approach for a proper pre-surgical evaluation, which is important to achieve a post-transplant outcome comparable to that of other indications to LT. NAFLD/NASH patients are also at higher risk of post-transplant cardiovascular events, diabetes, dyslipidemia, obesity, renal impairment and recurrent NASH. Lifestyle modifications, included diet and physical activity, are key to improve survival and quality of life after transplantation. A tailored immunosuppressive regimen may be proposed in selected patients. Development of new drugs for the treatment of recurrent NASH is awaited. **(Clin Mol Hepatol 2023;29(Suppl):S286-S301)**

Keywords: NAFLD; NASH; Liver transplantation; Cardiovascular risk; Metabolic syndrome

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is currently the fastest growing indication to liver transplantation (LT) both in United States and Europe.[1,2] NAFLD is the hepatic expression of a systemic metabolic dysfunction. Indeed, NAFLD is commonly associated to cardiovascular (CV) disease, obesity, glucose impairment and dyslipidemia, which make more challenging the management of NAFLD patients in the transplant setting (Fig. 1). The term metabolic-associated fatty liver disease (MAFLD) was recently proposed to better characterize the metabolic dysfunction associated fatty liver disease,[3] launching the debate on potential change in diagnosis, development of new therapies and improved clinical management.

MAFLD

MAFLD is defined by the evidence of hepatic steatosis

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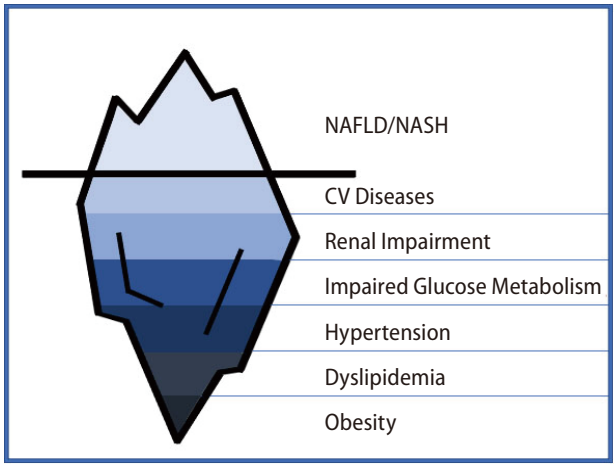

Figure 1. Management of NAFLD in the liver transplant setting. NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; CV, cardiovascular.

(based on histologic, radiologic or blood test findings), associated with at least one of the following three criteria: overweight/obesity, type 2 diabetes mellitus (DM), and evidence of metabolic dysregulation.[4] Metabolic dysregulation is in turn defined by the presence of at least two of the following criteria: waist circumference ≥102/88 cm in Caucasian men/women and ≥90/80 cm in Asian men/women; blood pressure ≥130/85 mmHg or the use of specific treatment, triglycerides ≥150 mg/dL or the use of specific treatment, high-density lipoprotein ≤40/50 mg/dL in men/women or the use of specific treatment, pre-diabetes, reactive C protein (RCP) ≥2 mg/dL and insulin resistance index (HOMA-IR) ≥2.5.[4] The definition of MAFLD does not imply the absence of significant alcohol consumption or other causes of liver injury,[4] but these patients should be defined as having dual etiology fat-

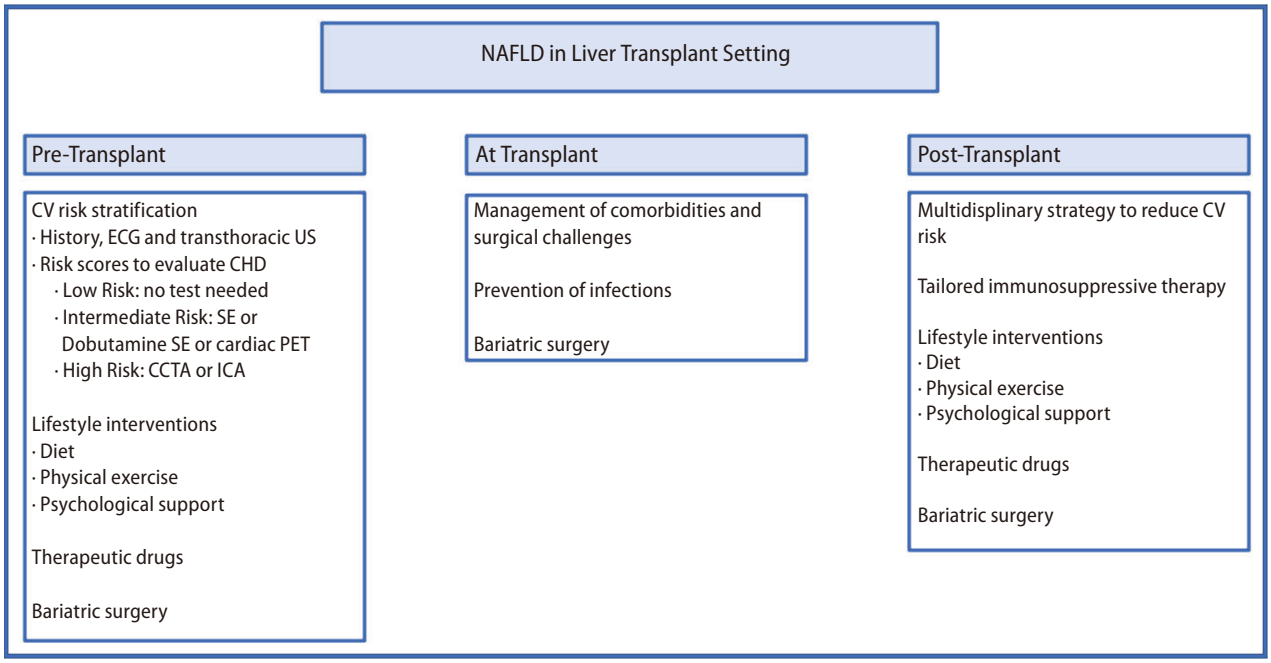

Figure 2. Management of NAFLD in the liver transplant setting. NAFLD, non-alcoholic fatty liver disease; CV, cardiovascular; ECG, electrocardiogram; US, ultrasound; CHD, coronary heart disease; SE, stress echocardiography; cardiac PET, cardiac positron emission tomography; CCTA, coronary computed tomography angiography; ICA, invasive coronary angiography.

Abbreviations:

NAFLD, non-alcoholic fatty liver disease; LT, liver transplantation; ESLD, end stage liver disease; HCC, hepatocellular carcinoma; NASH, non-alcoholic steatohepatitis; CV, cardiovascular; MAFLD, metabolic-associated fatty liver disease; HDL, high-density lipoprotein; BMI, body mass index; ACLF, acute on chronic liver failure; CAD, coronary artery disease; CAD-LT, coronary artery disease in liver transplantation; CACS, coronary artery calcium scoring; CHD, coronary heart disease; ESC, European Society of Cardiology; CCTA, coronary computed tomography angiography; ICA, invasive coronary angiography; FFR, fractional flow reserve; ECG, electrocardiogram; TTE, transthoracic echocardiography; SE, stress echocardiography; SRTR, Scientific Registry of Transplant Recipients; IS, immunosuppressive; BS, bariatric surgery; PROCAM, Prospective Cardiovascular Münster; SCORE, Systematic Coronary Risk Evaluation Project; ACEi, ACE inhibitors; ARB, angiotensin receptor blockers; ARNI, aldosterone antagonists, angiotensin receptor-neprilysin inhibitors; BB, b-adrenergic receptor blockers; EF, ejection fraction; CCBs, calcium channel blockers; CsA, cyclosporine; TAC, tacrolimus; MMF, mycophenolate mofetil; mTOR, mammalian target of rapamycin; SO, sarcopenic obesity; ELTR, European Liver Transplant Registry; QoL, quality of life

Table 1. Practical advices for the management of complications before and after liver transplantation for NASH

Clinical issues	Management	
	Before transplant	After transplant
Diabetes mellitus	Diet and physical activity Continuous blood glucose monitoring	Modification of IS regimen (early tapering of steroids, minimization of CNIs, utilization of mTOR inhibitor, and/or MMF)[61] If no response: Metformin is the first line therapy if eGFR >30 min/mL[62] GLP-1 receptor agonists, SGLT2 inhibitors and DPP-4 inhibitors are alternatives to metformin[63,64] Insulin in patients not responder to other treatments
Dyslipidemia	Diet and physical activity Statins (use lower dose in CHILD B and with caution in CHILD C patients)	Diet and physical activity If no response: Statins are the first line therapy, preferring hydrophilic ones66 Fibrates are useful in monotherapy in patients not tolerating statins, or in combination to statins if hypertriglyceridemia is associated Ezetimibe might be a therapy in association to statins67 Fish oil could be considered in patients with hypertriglyceridemia68
Obesity	Diet and physical activity[70] Bariatric surgery/endoscopy	No drugs are approved in the post-transplant setting Bariatric surgery: sleeve gastrectomy is preferred over Roux-en-Y bypass[70] Bariatric endoscopy as a potential and growing new approach[73]
CV events	Risk stratification Specific algorithm to assess CV diseases	Diet and physical activity Treatment of CV risk factors: Type 2 DM, dyslipidemia, arterial hypertension, obesity, tobacco use and renal impairment. Patients with known CV risk: cardiac examination and BNP testing every month, echocardiography every 6 months[38] Patients without known CV risk: echocardiography every 12 months[38] If systolic dysfunction: ACEi, ARB, aldosterone antagonists, ARNI, BB[79]
Arterial hypertension	Diuretics, particularly in patients with ascites Non selective Beta-Blockers (carvedilol>propranolol) ACE inhibitors, if not impaired renal function Calcium Channels Blockers	Modification of IS therapy to a CNIs sparing strategy First line treatment is Calcium Channels Blockers[62] Second line treatment is Beta-Blockers[62] ACE inhibitors have to be used carefully in the immediate post-transplant setting[62]
Renal impairment	Treatment of comorbidities: hypertension, diabetes and obesity Avoid nephrotoxic drugs	Treatment of comorbidities: hypertension, diabetes and obesity Modification of IS therapy, reducing CNIs or shift to mTOR inhibitors.
NASH	Diet and physical activity FXR agonist, GLP-1 receptor agonists, orlistat and lipogenesis inhibitor	Diet and physical activity No drugs can be recommended in the post-transplant setting Ongoing trial on FXR agonist, GLP-1 receptor agonists, orlistat and lipogenesis inhibitor[86-90]
Sarcopenia	Dietician counselling Controlled physical activity to preserve residual motility	Dietician counselling Physical activity to improve muscle mass

NASH, non-alcoholic steatohepatitis; CNIs, calcineurin inhibitors; mTOR, mammalian target of rapamycin; MMF, mycophenolate mofetil; eGFR, Estimated Glomerular Filtration Rate; CV, cardiovascular; DM, diabetes mellitus; bnp, B-type natriuretic peptide; ACEi, ACE inhibitors; ARB, angiotensin receptor blockers; ARNI, Angiotensin Receptor Neprilysin Inhibitor; BB, b-adrenergic receptor blockers; FXR, farnesoid X receptor; GLP-1, glucagon-like peptide 1; IS, immunosuppressive; SGLUT 2, Sodium-GLUcose Transporter 2; DPP-4, Dipeptidyl peptidase-4; ACEi, Angiotensin-converting enzyme inhibitors.

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ty liver disease.[5] The term MAFLD may improve patients characterization and help to identify individuals at higher risk for future adverse events and mortality. Indeed, Kim et al.[6] recently found a strong association between MAFLD and all-cause and cause-specific mortality, whereas NAFLD per se is not related to all-cause and cause-specific mortality. Specifically, patients who met the definition of MAFLD but not of NAFLD, had a 1.7-fold higher risk of all-cause mortality (hazard ratio [HR] 1.66; 95% confidence interval [CI] 1.19–2.32; *P*=0.003) and a 24% higher CV mortality (HR 1.24; 95% CI 1.01–1.51; *P*=0.041). Changing the nomenclature from NAFLD to MAFLD could focus on the metabolic underpinning and adjust the management of these patients, including in a transplant setting.

INDICATIONS TO LIVER TRANSPLANTATION IN PATIENT WITH NAFLD/NASH

Currently, approximately 25% of the global population is affected by NAFLD and up to 25% of these individuals have non-alcoholic steatohepatitis (NASH),[7] with an alarming growth of incidence in young population.[8] The estimated incidence of NAFLD and NASH in 2030 are 101 million and 27 million, respectively. A recent analysis reported an increment trend of 168% for decompensated cirrhosis, 178% for liver-related death and 137% for hepatocellular carcinoma (HCC), between 2015 and 2030.[9] Similarly, a modelling study predicted an increased rate of HCC cases of 117% in France and 88% in UK.[9] LT is the only lifesaving approach for NASH-related end stage liver disease (ESLD) and non-resectable HCC.[10] It is therefore not surprising that NAFLD is rapidly growing as indication for LT and is currently the second leading cause for LT in USA, accounting for 21.5% of performed transplants in adults during 2018.[1] An exponential growth has also been seen in Europe, going from 1.2% in 2002 to 8.4% in 2016.[2] Patients transplanted for NASH have more frequently HCC than non-NASH patients, 39.1% vs. 28.9% respectively (*P*<0.001), are older (median: 60 vs. 55 years, *P*<0.001) and with higher body mass index (BMI) (mean: 32.6 vs. 25.8 kg/m^2, *P*<0.001).[11] The reason why HCC seems to be more prevalent as indication to LT in NASH than in non-NASH patients has not yet been thoroughly understood. Proposed mechanisms include the presence of a chronic systemic inflammatory environment, genetic polymorphisms as PNPLA3 and TM6SF2, greater iron absorption, gut dysbiosis, increased lipid storage with lipotoxicity, insulin resistance and higher insulin-like growth factor (IGF) levels.[12,13] In addition, NASH patients are often obese, thus making more difficult to perform ultrasound screening of HCC.

Notably, a significant proportion of HCC in patients with NAFLD/NASH may arise in a non-cirrhotic liver. In an Italian multicenter study on 756 patients with HCC, Piscaglia et al.[14] showed that 46.2% of NAFLD-HCC occurred in a pre-cirrhotic liver. Similar results have been reported by independent cohort in Germany and Japan (41.7% and 49%, respectively).[15,16]

ACUTE ON CHRONIC LIVER FAILURE

Acute on chronic liver failure (ACLF) is defined as an "Acute decompensation of cirrhosis (ascites, hepatic encefalopathy [HE], gastrointestinal [GI] bleed and/or infection) associated with organ failure (OF) and high 28-day mortality (>15%)".[17,18] In a recent study based on National Inpatient Sample (NIS) database, Axley et al.[19] showed that NASH cirrhosis is the most rapidly growing etiology causing hospital admission for ACLF, with an increase of 63%, from 3.5% in 2006–2008 to 5.7% in 2012–2014 (*P*<0.001). In this series, infection was the most common precipitating event in ACLF (80%). Compared with non-NASH ACLF, these patients required a longer hospitalization though inpatient mortality was lower. A retrospective study based on the Veteran Health estimated an incidence of ACLF (based on European Association for the Study of the Liver - chronic liver failure criteria [EASL-CLIF] criteria) among NASH cirrhosis patients of 3.4/1,000 (95% CI, 2.9–4.0), confirming bacterial infections as the most common precipitant factor. Among individuals with ACLF grade 3, in NASH patients, kidney failure was the most common organ failure, although NASH and hepatitis C etiology shared the highest rates of circulatory failure.[20] Growing evidence suggests that patients with ACLF grade 3 should be evaluated for LT and may achieve an excellent outcome after transplant,[21] provided that they are appropriately selected.[22] Pre-transplant evaluation is important in NAFLD/NASH patients due to their increased CV and systemic risk. Importantly, NASH was not associated to an increased risk of post-transplant mortality in patients undergoing transplantation for ACLF.[21,22]

PRE-TRANSPLANT EVALUATION

Metabolic syndrome, DM, and CV diseases that are often present in patients with NASH should be considered at time of LT evaluation, as they are important causes of death after LT and may be an absolute or relative contraindication to transplantation (Fig. 2).[23] The CV issues in patients with NASH may act synergistically with the cardiac alterations associated with cirrhosis (e.g., cirrhotic cardiomyopathy, prolonged QTc).[24] Adequate risk stratification of coronary artery disease (CAD) is essential to improve post-transplant survival. CAD is present in approximately 25%[25] of LT candidates, and patients with NASH or renal dysfunction are more likely to have a higher burden of CAD and critical coronary artery stenosis.[26,27] Worldwide, there is considerable variability in how LT programs assess cardiac risk, as models used to predict cardiovascular risk in the general population have not been validated in patients with liver disease. Regardless of the risk stratification approach used, a dedicated cardiology and anesthesia team must be involved in selecting candidates for LT.[28] As a first approach, it is necessary to obtain a medical history and search for the presence of CAD risk factors to determine the need for screening and the choice of the type of investigations. Traditional CV risk factors: male sex, hypertension, hyperlipidemia, smoking, age >60 years, left ventricular hypertrophy, previous CV disease or diabetes have been identified as the main risk factors associated with significant coronary artery stenosis in LT candidates.[29,30] So far, only three clinical risk scores have been proposed to stratify cardiac risk in LT candidates:

· CArdiovascular Risk in Orthotopic Liver Transplantation (CAR-OLT)[31]: a prognostic model designed to predict the overall 1-year risk of death or hospitalization for a significant CV event; however, it has not yet received external validation and does not estimate long-term CV risk.

· Cardiac arrest risk index[32]: a point-based model to predict cardiac arrest and ventricular arrhythmias within 30 days after transplantation.

· CAD-LT (coronary artery disease in liver transplantation)[33]: effectively stratifies pre-LT risk for significant CAD and thus can guide more targeted evaluation of candidates with less number of tests and faster waiting list inclusion.

Troponin-I and RCP, appear to have high sensitivity in predicting cardiac risk in liver transplant candidates, but more studies are needed before they can be used in clinical practice.[34,35] Current studies have revealed that coronary artery calcium scoring has a negative predictive value of 95–100% for significant coronary heart disease (CHD).[36,37] Therefore, the most recent American Society of Transplantation guidelines proposed its use in the risk stratification of LT candidates.[23] Non-invasive stress testing (e.g., dobutamine stress echocardiography, myocardial perfusion imaging and CV magnetic resonance) have been validated to detect CAD in general but are suboptimal for patients with ESLD.[28] According to the current European Society of Cardiology[38] guidelines, non-invasive testing should be offered to patients with more than two risk factors for CAD and poor functional status. Invasive coronary angiography is the gold-standard test to identify significant CHD in the general population, but currently, in LT candidates, studies are inconclusive and not able to predict the impact of asymptomatic pre-LT CV abnormalities on long-term outcomes.[39,40] Coronary computed tomography angiography (CCTA) is a non-invasive test valid for assessing the risk of CHD in LT candidates, although no studies are comparing it with invasive coronary angiography (ICA) in this population.[41] CCTA alone does not provide a functional assessment of coronary stenosis, which can be obtained by integrating this examination with fractional flow reserve obtained from computed tomography in this population.[42]

The most recent guidelines, published in October 2022 by the American Transplant Society,[28] recommend the following algorithm:

· Cardiac physical examination, electrocardiogram (ECG), and resting trans-thoracic echocardiography (TTE) (with measurement of myocardial strain and bubble study to assess pulmonary hypertension and intracardiac and extracardiac leads) for all LT candidates without CHD.

· In LT candidates at low risk of significant CHD (age <40 years, able to achieve ≥4 metabolic equivalents (METs), no NASH or diabetes, no CHD risk factors), if initial ECG and resting TTE are normal, additional cardiac stress testing may not be necessary.

· In intermediate-risk liver transplant candidates, non-invasive exercise testing may be considered (stress echocardiography [SE] is preferred; dobutamine SE if patient cannot exercise. Positron emission tomography as an alternative if available).

In LT candidates at high risk of significant CHD (diabetes, NASH, or ≥2 other CHD risk factors), coronary anatomic imaging (CCTA or ICA) is mandatory.

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· ICA should be the last procedure performed in the evaluation before listing for liver transplantation after the patient has already been considered an acceptable transplant candidate.

Lifestyle modifications are recommended to improve clinical outcomes after transplantation. Obese patients should lose weight through a low-calorie diet and adequate physical activity.[23] Weight loss in this patient population must be carefully controlled and managed by experts to avoid loss of muscle mass and subsequent sarcopenia, which is a known risk factor that increases post-transplant mortality and worsens patient prognosis (Table 1).[43,44]

WAITING-LIST MANAGEMENT

A recent analysis on patients from OPTN (Organ Procurement and Transplantation Network)/UNOS (United Network for Organ Sharing) registry showed that, in comparison to patients with alcoholic liver disease (ALD), the risk of 90-day and 1-year waitlist mortality was significantly higher in NASH patients (P=0.042 and P=0.008).[45] Model for End-Stage Liver Disease-Na (MELD-Na) score, Chronic Kidney Disease (CKD) stage >3 and hyponatremia were significantly associated to mortality. Nagai and colleagues also demonstrated that 90-day Delta MELD-Na was lower in Alcoholic Liver Disease (ALD) patients than in NASH patients, suggesting that NASH patients may have a faster disease progression. When considering patients with HCC as indication to LT, NASH patients showed a higher risk of 1-year waitlist mortality compared to HCC-ALD; however, an explanation could be that NASH patient were older.[45] Another study based on UNOS registry data from 2002 to 2016 found a higher unadjusted cumulative incidence of exclusion from wait list (WL) for mortality and deterioration in NAFLD patients compared to patients with other indications to LT, but when adjusted for confounder factors, waitlist mortality was similar between NASH and non-NASH patients.[46] In fact, by analyzing data from the Scientific Registry of Transplant Recipients (SRTR) from 2002 to 2016, Younossi et al.[47] found no significative difference in terms of outcome during the waiting-list (transplant vs. drop out) between different etiologies. Young et al.[48] demonstrated that patients with NASH-HCC are less likely to have exception to MELD on WL and, as a result, they are less likely to receive LT than patients waitlisted for other etiologies. Another factor that may contribute to disparities in HCC exception is the better hepatic function in NASH-HCC patients at diagnosis and the slower progression of cirrhosis compared with Hepatitis C Virus (HCV)-HCC patients,[48] which results in lower MELD score. As a consequence, NASH-HCC patients have significantly higher rates of primary surgical resection and lower rates of LT when compared with HCV-HCC patients,[49] leading to lower likelihoods to receive LT and longer WL times. Furthermore, NASH patients—including those with a low MELD score, were more frequently delisted or died due to CV complications. It thus seems that the MELD score does not fully represent the clinical condition of NASH patients. New prognostic scores to better stratify the risk of short-term deterioration and mortality of patients with NASH are expected.

POST-TRANSPLANT MANAGEMENT

Early complications

It is estimated that about 40% of all deaths occurring in the first 30 days post-transplant are due to CV complications. Transplant operation is technically more challenging in obese patients; this is reflected by increased operative time, major operative transfusion requirements, increased surgical complications, such us hepatic arterial injury or malposition, inferior vena cava injury and uncontrolled bleeding, and higher rate of operative revision.[50] Consequently, obesity and diabetes mellitus together increased the 30-day risk of post-surgery complications, such as wound infections, sepsis, renal failure, and prolonged mechanical ventilation with extent of hospital stay.[51-53] NASH patients have more short-term mild complications, such as persisting ascites, pleural effusion, dyspnea, fever, electrolyte disturbance, abnormal liver enzymes or wound infections, while moderate severe complications were not significantly different between NASH and non-NASH patients. Mortality and graft survival at 90-days after LT were similar with patients transplanted for non-NASH cirrhosis.[54] Therefore, although the higher percentage of early complications, short-term graft and patient outcomes between NASH and non-NASH patients are comparable.

Late complications

Diabetes, hypertension, dyslipidemia, renal impairment and NASH have a key role as risk factors for the development of CV events after LT (Table 1).[55] In particular, NASH patients have a higher mortality rate for cardio- and cerebro-vascular complications than non-NASH patients and such difference is particularly significant during the first year after LT.[45] Recently, a Spanish Group showed that the introduction of a post-transplant multidisciplinary approach achieved by a multi-professional team, including the figures of hepatologist, endocrinologist and advanced practice nurses, decreased the incidence of CV events from 14% to 6%, acting on prevention and early detection of CV risk factors.[56]

Diabetes mellitus

Prevalence of diabetes mellitus in NAFLD prior to LT is between 33% and 66%.[57] Male gender, ethnicity, family history, older age, BMI >30 kg/m^2, HCV infection, and the use of immunosuppressive (IS) drugs, tacrolimus and corticosteroids, are risk factors for the development of post-transplant diabetes.[58,59] The gold standard for the diagnosis of diabetes after LT is the oral glucose tolerance test, whereas glycated haemoglobin might be used for monitoring, keeping in mind that in liver disease patients it could be falsely low due to anemia and splenomegaly. Diabetes Mellitus (DM) severely influences the prognosis of transplanted patients leading to higher 10-years mortality, increased CV events and greater infections rate.[60,61]

At present there is no specific therapeutical indications for DM in LT recipients. A first step in the management of post-LT diabetes is modification of immunosuppression treatment.[62] Metformin is the most used treatment in general population with DM and could be safely prescribed as first line treatment in transplanted recipients with Estimated Glomerular Filtration Rate (eGFR) >30 mL/min, with no drug interaction with calcineurin inhibitors (CNIs).[63] Promising results are expecting from the new antidiabetic drugs, such as agonist of GLP-1 receptor and SGLT2 inhibitors, which both have not only cardioprotective and nephroprotective benefits, but also effects on weight loss.[64,65] However specific interactions with immunosuppressive drugs need to be further investigated.

Dyslipidemia

Lipid metabolism impairment has a post-LT prevalence between 45% and 71%. Risk factors for the development of dyslipidemia are IS therapy, diabetes, high BMI, and individual predisposition.[66] Dyslipidemia after LT seems not to respond to life-style changing and is associated with a higher need of pharmacological therapy than in the pre-transplant setting.[24] Among statins, the hydrophilic ones should be preferred as they are not metabolized by cytochrome P 450-3A4,[67] thus not interfering with IS drugs. Pravastatin has not interaction with CNIs and it is the most used in the setting of LT. Ezetimibe in monotherapy is not useful but it could have a potential role in association with statins.[68] Fish oil are preferred to fibrates for the treatment of isolated hypertriglyceridemia.[69]

Obesity

There is an increased prevalence of obesity both in transplant candidates and recipients. Patients, especially NASH ones, should be counseled before and after LT regarding consequences of obesity. Low diet, lifestyle modifications, and physical activity are mandatory especially after LT.[70,71] However, they are not always successful to prevent further increase in body weight as reported by Diwan et al.[72] who showed superiority of sleeve gastrectomy vs. dietary intervention in total body weight loss after LT. Among techniques, sleeve gastrectomy is always preferred over the Roux-en-Y gastric bypass for multiple reasons, firstly because it guarantees endoscopic access to the biliary system for the treatment of eventual post-transplant biliary strictures and secondly for malabsorption concern.[24,73] However, there is not consensus about which is the best time for bariatric surgery (BS), if before, simultaneously or after LT. The Mayo Clinic experience found that BS in contemporary with LT is a safe option, however restricted selection criteria of patients are mandatory.[72,73] Small case series are reported about BS after LT, some with complications due to peritoneal adhesions.[74,75] Further studies should be focused on new endoscopic bariatric techniques that are undoubtedly less invasive and are showing promising results in patients with NAFLD.[76]

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Cardiovascular events

CV disease is the most common extrahepatic cause of death in transplant recipients, independently from the underlying etiology, with a cumulative incidence of up to 30.3% within 8 years from LT.[34] Over the past decade, the increasing transplant indication for NASH and the older age of LT candidates, combined with the known metabolic effects of IS drugs, have contributed to the increased risk of CV disease in LT recipients. Patients transplanted for NASH have higher risk of dying from CV complications than patients transplanted for other reasons.[77] A recent study reported that the CV event rate 5 years after LT was approximately 40% in NASH patients and only 5–10% in non-NASH recipients.[78] This finding was not confirmed by a meta-analysis of 119,327 patients, that, surprisingly, showed no difference in complications rates between NASH and non-NASH patients.[79] Interestingly, no differences in overall survival and graft survival were observed between the two groups in either study.[78,79] In clinical practice, the Prospective Cardiovascular Münster Score (PROCAM)[80] and the Systematic Coronary Risk Evaluation Project (SCORE)[81] may be useful for rapid risk stratification of CHD after LT, but validated scores for predicting heart failure are not available. The first step in reducing the rate of cardiac events is to prevent and treat the CV risk factors, namely: diabetes, dyslipidemia, arterial hypertension, obesity, tobacco use and renal impairment. In patients with known cardiac disease prior to transplantation, monthly cardiac physical evaluation and B-Type Natriuretic Peptide (BNP) testing may be considered. Studies on the exact timing for echocardiography screening after LT are lacking; annual and semiannual screening in low- and high-risk patients, respectively, might be appropriate. In patients with severe CHD before LT, the use of statins may result in a survival benefit (HR 0.25; 95% CI 0.12–0.49; $P<0.001$).[39] Aspirin should be considered for secondary prophylaxis, whereas there is no evidence for its use in primary prevention.[77] In LT recipients with systolic dysfunction, as in the general population, anti-remodeling therapy, such as ACE inhibitors (ACEi), angiotensin receptor blockers (ARB), aldosterone antagonists, angiotensin receptor-neprilysin inhibitors (ARNI) and b-adrenergic receptor blockers (BB), may improve ejection fraction and relieve heart failure symptoms. However, they have no effect on diastolic dysfunction.[82] A case by case multidisciplinary team discussion, which includes hepatologist, surgeon, cardiologist, interventional cardiologist and anesthesiologist, is required to properly assess the individual CV risk after liver transplantation and to successfully prevent and treat CV events. A strict collaboration with primary care physician, dietician, psychologist and transplant hepatologist is advisable after liver transplantation to prevent weight gain, improve physical function and ameliorate adherence to lifestyle changes, thus reducing modifiable CV risk factors.

Arterial hypertension

Seventy per cent of patients after LT are affected by arterial hypertension.[83] As previously mentioned for diabetes, CNIs sparing strategy should be always adopted to prevent and further reduce blood pressure when hypertension occurs. Calcium channel blockers (AST to Platelet Ratio Index [APRI], Fibrosis-4 [FIB-4]), are the first line treatment due their effect on arterial renal vasodilatation opposed to the mechanism of CNIs and reducing systemic vascular resistance.[63] Beta-blockers could be used as a second line option.[63] ACE-inhibitors should be not used in the first period after LT due to the risk of hyperkalemia and metabolic acidosis, but they should be considered in patients with concomitant chronic kidney disease and diabetes mellitus.[63]

Renal impairment

NAFLD/NASH transplanted patients are particularly at risk of developing renal impairment because of their frequent comorbidities (hypertension, diabetes, and obesity) associated to the well-known risk due to the use of CNI-based immunosuppression regimen. There are not precise guidelines for the treatment of renal disease after liver transplantation, however the efforts should be directed to the prevention and treatment of metabolic dysfunction and tailoring of IS therapy.

Recurrent NASH

In patients transplanted for NASH, post-transplant features of hepatic steatosis are present in up to 78–88% of cases,[78,84] while NASH is less common, ranging from 4% to 41%.[84] Risk factors for the development of post-transplant NAFLD are similar to the pre-transplant setting, which include obesity, hypertension, and diabetes.[85] Patients usually develop recur-

rent NAFLD/NASH in the first 5 years after liver transplantation.[86] Once NASH occurs, 11–14% patients may develop cirrhosis within 5 years after LT.[87] Liver biopsy is the gold standard for the diagnosis of NAFLD/NASH. Less invasive techniques, such as magnetic resonance imaging (MRI), controlled attenuation parameter (CAP), magnetic resonance proton density fat fraction, serologic methods (AST to Platelet Ratio Index [APRI], Fibrosis-4 [FIB-4]), transient elastography, and magnetic resonance elastography, have been proposed but require validation.[88] Current guidelines are not specific for the management of recurrent NAFLD/NASH after liver transplantation. The first therapeutic approach should include weight loss and dietician counselling. Regarding medical therapy, there are no drugs that can be recommended in post-LT setting, since clinical trials did not include transplanted patients. In pre-transplant population, obeticholic acid, a FXR agonist, has been associated to histological improvement[89,90]; the same effect has been proved with Pioglitazone, that also reduces the chronic inflammatory environment.[91] Aramchol, a lipogenesis inhibitor, and liraglutide, a GLP1-receptor agonist, have been associated to a reduction in liver fat and steatohepatitis.[92,93] GLP1-receptor agonists and orlistat may also have a role in reducing NAFLD/NASH fibrosis.[94] Further data in recurrent NASH are awaited.

MANAGEMENT OF IMMUNOSUPPRESSION AND RISK OF REJECTION

IS treatment constitutes one of the most critical factors impacting outcomes after liver transplantation. The introduction of CNIs—cyclosporine (CsA) and tacrolimus (TAC)—reported a reduction in acute rejection rates and improvements in short-term patient and graft survival.[95] Long-term survival, in contrast, is most impacted by renal, CV, and metabolic toxicity secondary to medication use, especially CNIs and glucocorticoids,[96-98] in particular in predisposed patients such as those undergoing LT for NASH. The goal of the world's LT experts is to reduce the toxicity of immunosuppression by tailoring therapy basing on individual patient characteristics. Steroids are obesogenic drugs that induce glucose intolerance, hypertension and hyperlipidemia. Their clinical use is short-lived in clinical practice, which limits their potential collectivizing effects. CNIs are associated with developing all components of the metabolic syndrome as a consequence of the inhibition of insulin secretion and increased insulin resistance. They, therefore, present a pro-diabetogenic action, more associated with TAC than with CsA, which, on the other hand, presents a more significant prolipidemic effect. The nephrotoxic effect of CNIs is also known to occur due to renal and systemic vasoconstriction mediated by this family of drugs, which is responsible for the onset of arterial hypertension. In patients transplanted for NASH, the strategy should be to early reduce or withdraw the steroids,[24] introducing alternative immunosuppressive drugs with a lower impact on the metabolic profile. From OPTN/SRTR 2019 Annual Data Report, it was found that 75% of patients were treated with the dual regimen consisting of CsA and mycophenolate mofetil (MMF), and the MMF was reported to be used in 45% as maintenance therapy at 1- and 2-years after LT.[95] Patients treated with MMF combined with reduced-doses of CNIs had lower CV risk and reduced renal function impairment than those treated with a regimen containing only standard-dose of tacrolimus plus corticosteroids.[99] However, there still needs to be a consensus on the ideal minimization regimen. Newer mammalian target of rapamycin (mTOR) inhibitors[100] are associated with an increased risk of post-LT dyslipidemia, whereas they are neutral concerning diabetes mellitus and hypertension. Moreover they are associated with a reduction in body weight, a lower frequency of cardiac events and, compared with CNIs, are associated with a more favorable renal profile.[24] mTOR inhibitors, combined with CNIs, are associated to a prolonged long-term survival in patients transplanted for HCC.[101] In NASH patients, the use of drugs with less impact on the metabolic-cardiovascular profile, being the only modifiable factor, is the best strategy to reduce post-LT complications and improve outcomes.

SARCOPENIA

Up to 20% of NASH patients are estimated to be affected by sarcopenia.[102] A synergic overlap between pathophysiology of these two conditions resulted in an increased risk of NAFLD development when sarcopenia is present and vice versa.[103,104] Pre-LT sarcopenia has been associated with increased risk of adverse outcomes after liver transplantation, such as higher risk of bacterial infection and mortality.[105] Specific data regarding sarcopenia and NASH are still needed, however patients affected by sarcopenia and NASH are

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found to have an increased risk of insulin resistance, atherosclerosis and CV disease.[103,106] Metabolic alterations associated with cirrhosis may reverse after liver transplantation; however, few data on the assessment of body composition after LT are available. In 2013, Tsien et al.[107] investigated the potential role of post-transplant sarcopenia evaluating changes in body mass composition in prospective cohort of transplanted patients. Among 53 Patients (7.5% affected by NASH disease), 41 (77%) experienced a decreased in abdominal wall muscles and 43% an increase in fat area in a medium follow-up of 19.3±9 months. However only patients who experienced post-transplant sarcopenia had 3.1-fold increased risk of developing DM (P=0.05, 95% CI 1.01–9.38), with no evidence in decreased overall survival.[107] A review published in 2013 showed that, despite conflicting and few data with different methods of muscle mass assessment, further reduction of skeletal muscle mass has been observed up to one year after liver transplantation.[108] Possible explanations have been proposed including persistence of hypermetabolism soon after LT, IS drugs, mostly mammalian target of rapamycin (mTOR) inhibitors and corticosteroids, length of hospitalization and occurrence of post-transplant infections that tend to be more frequent in patients with pre-LT sarcopenia resulting in an increased risk of muscle mass depletion.[105,109,110] Subsequently, Jeon et al.[111] in retrospective cohort of 145 patients who underwent LT reported that all patients with pre-transplant sarcopenia remain sarcopenic soon after LT and 15% of patients with normal muscle mass pre-transplant developed sarcopenia de novo post-LT. Although there was an increased trend of mortality soon after LT in newly developed sarcopenia, these finding were not confirmed at 6 months from LT, when sarcopenia resulted not to be a predictor of death.[111] Similar findings have been reported by Bhanji et al.[112] who assessed the skeletal muscle mass in two hundred and ninety-three patients 7 month after LT (interquartile range 4.8–12 months). Ninety-eight patients (61%) resulted to be affected by post-LT sarcopenia, both with newly developed sarcopenia (25/98) and persistent sarcopenia (73/98). There was no difference in survival between post-LT sarcopenic patients (both *de novo* and persistent) and non-sarcopenic patients. It has been postulated that patients with post-LT sarcopenia resulted to be less affected by metabolic liver disease before LT (2.7% vs. 12.2% P=0.002). However, in contrast with these findings, Carias et al.[113], which retrospectively evaluated changing on body composition after LT in a cohort of 207 adult patients (21.7% with NASH), found that, at multivariate logistic regression analysis, NASH etiology is an independent predictor of sarcopenic obesity development (P=0.014; 95% CI: 1.44–25.26, OR 6.03). Sarcopenic obesity (SO) is defined as the contemporary presence of sarcopenia in the contest of obesity.[114] The prevalence of SO in the context of cirrhosis ranges between 20% and 35%.[115] At present, studies on SO are limited and mostly focused on pre-transplant period, but a meta-analysis on the role of SO in liver transplantation reported an increased risk of death at least two times higher in SO vs. not SO patients both at short- and long-term follow-up.[116] Indeed the original aim of the meta-analysis was to assess the role of SO in patients with NASH after LT, but Hegyi et al.[116] were not able to perform the analysis due to lack of data. Data about the impact of post-LT sarcopenia continues to be scarce as recently highlighted by a review of Ooi et al.[105] who showed that upon 35 studies on sarcopenia in the setting of liver transplantation only 6 focused on the potential role of sarcopenia and SO after LT. Further data are needed on body composition's changes in post-transplant period to ensure better management of these patients in order to guarantee better outcomes.

SURVIVAL AFTER TRANSPLANTATION

Liver transplantation represents the only life-saving therapy in patients with ESLD. In an analysis by Haldar et al.[11] on data from the European Liver Transplant Registry (ELTR) of patients transplanted between January 2002 and December 2016, NASH was not an independent predictor of patient or graft survival. However, older recipient age (61–65 years: HR 2.07; 95% CI 1.39–3.08; >65 years: HR 1.72; 95% CI 1.10–2.71; relative to ≤45 years), MELD score >23 (HR 1.48; 95% CI 1.04–2.30; relative to ≤11) and BMI either ≤18.5 kg/m^2 (HR 4.29; 95% CI 1.01–18.21; 18.5–25 kg/m^2: HR 2.24; 95% CI 1.27–3.96) or >40 kg/m^2 (HR 1.96; 95% CI 1.16–3.32; relative to 25–30 kg/m^2) were independent predictors of post-LT mortality. A systematic review with meta-analysis[117] evaluated the variables associated with patient and graft survival in individuals with NASH-related liver disease, showing that recipient age >65 years, pre-transplant DM, MELD >23, functional status, HCC, dialysis prior to LT, hepatic encephalopathy and time/year of LT were predictors of mortality after transplantation. As previously described in patients transplanted for other etiologies

of ESLD, increased patient mortality was associated with older age of the recipient (HR=2.07, 95% CI: 1.71–2.50, I^2=0, τ_2=0, *P*=0.40) and pre-transplant DM (HR=1.18, CI 95%: 1.08–1.28, I^2=0, τ_2=0, *P*=0.76). No difference in term of patient and graft survival rates were found between NAFLD/NASH and non-NAFLD/NASH patients transplanted for HCC.[11] Likewise, post-transplant HCC recurrence rates have been shown to be similar between NASH and non-NASH aetiologies, 13.3% vs. 14%, respectively (*P*=0.879). Median time to HCC recurrence did not change between the two groups, 22.6 vs. 13.3 months (*P*=0.274).[118] NASH and obesity may be associated with a reduced quality of life,[119] however no specific studies investigating quality of life (QoL) in NASH transplanted patients are yet available.

CONCLUSION

NAFLD/NASH has now become one of the most common indication for liver transplantation worldwide. Multidisciplinary management of NASH and NASH-associated comorbidities may mitigate morbidity and mortality in patients with NASH both before and after liver transplantation. Patients selection is crucial to achieve post-transplant survival comparable to other etiologies of liver disease. In transplant recipients, diet, physical activity, and adjustment of IS therapy are key for prevention of NASH recurrence. In the future, an improved risk stratification in NASH candidates for transplantation and new drugs for the treatment of NASH recurrence are expected.

Authors' contribution

S.B., F.D.A., M.G., A.Z., M.G., G.G., M.S. writing—original draft preparation, F.P.R., P.B. writing—review and editing. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors have no conflicts to disclose.

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Review

Non-alcoholic fatty liver disease: the pathologist's perspective

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Non-alcoholic fatty liver disease (NAFLD) is a spectrum of diseases characterized by fatty accumulation in hepatocytes, ranging from steatosis, non-alcoholic steatohepatitis, to cirrhosis. While histopathological evaluation of liver biopsies plays a central role in the diagnosis of NAFLD, limitations such as the problem of interobserver variability still exist and active research is underway to improve the diagnostic utility of liver biopsies. In this article, we provide a comprehensive overview of the histopathological features of NAFLD, the current grading and staging systems, and discuss the present and future roles of liver biopsies in the diagnosis and prognostication of NAFLD. **(Clin Mol Hepatol 2023;29(Suppl):S302-S318)**

Keywords: Non-alcoholic fatty liver disease; Biopsy; Diagnosis; Prognosis; Histology

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a condition in which there is fatty infiltration in the liver in the absence of secondary causes, including significant alcohol consumption. The morphological spectrum of NAFLD encompasses "simple" steatosis, non-alcoholic steatohepatitis (NASH), and cirrhosis. Histological evaluation by liver biopsy plays an important role in the diagnosis of NAFLD and NASH, and in excluding the possibility of other diseases. Another role of the liver biopsy is prognostication, as the histological parameters may potentially provide important information for identifying groups of NAFLD patients at risk for developing cirrhosis, liver failure and hepatocellular carcinoma (HCC). Grading and staging systems, such as the NAFLD activity score (NAS) and Steatosis-Activity-Fibrosis (SAF) scores, are currently widely used to assess disease severity and prognosis, and also to evaluate response to treatment in both the

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practical setting and clinical trial setting. In this review, we summarize the histopathological features of NAFLD, the grading and staging systems, and the recent advances in ancillary tool development for the accurate diagnosis and prognostic prediction of NAFLD.

DEFINITION AND DIAGNOSTIC CRITERIA

Steatosis, or fatty change, is the accumulation of fat droplets in the hepatocyte cytoplasm, and can be classified as macrovesicular or microvesicular based on the size of the lipid droplets (described in more detail in the subsequent section). NAFLD is defined as the presence of steatosis in ≥5% of hepatocytes, in the absence of significant alcohol use or other causes of steatosis, including viral hepatitis or drug/toxin-induced liver injury.[1-3] NASH is characterized by the presence of active injury, in the form of hepatocellular ballooning degeneration and lobular inflammation (mostly lymphocytic with some neutrophils), in addition to varying degrees of steatosis. Although there are slight differences in the definitions in various practice guidelines, the presence of hepatocellular ballooning is regarded as an important factor for the diagnosis of NASH; in fact, it is considered the *sine qua non* of steatohepatitis for practical purposes, and its presence differentiates NASH from simple steatosis.[1-3] Fibrosis is typically located in zone 3 with a perivenular and perisinusoidal pattern, and this feature is helpful in corroborating the diagnosis of NASH. Mallory-Denk body (MDB) formation, apoptotic hepatocytes (acidophilic bodies), and lipogranulomas are other histological features of NASH. NASH-cirrhosis is defined as cirrhosis associated with current or previous histological evidence of NAFL or NASH.[2,3]

Steatosis

The typical steatosis in NAFLD is of the macrovesicular pattern[4]. Macrovesicular steatosis is classically characterized by a large lipid droplet occupying the cytoplasm of a hepatocyte, pushing its nucleus to the periphery (Fig. 1).[5] It is also increasingly being recognized that the lipid droplets may vary in size as the triglycerides accumulate in the hepatocytes over time, and thus a range of lipid droplet sizes may occur. As such, the terms large, medium and small droplet steatosis have been used to describe this variance in lipid droplet sizes, and it is understood that these findings fall under the macrovesicular pattern of hepatic steatosis.

Of relatively more importance is the distinction of small droplet steatosis from microvesicular pattern of hepatic steatosis. Microvesicular steatosis is characterized by the cytoplasm of hepatocytes being filled with numerous tiny lipid droplets and the presence of a central nucleus.[6] While small droplet steatosis may morphologically mimic microvesicular steatosis, typical NAFLD will only show patches of small droplet steatosis accompanied by other areas of large and medium droplet steatosis (Fig. 1). For most pathologists, the terminology of microvesicular steatosis is more often preferred

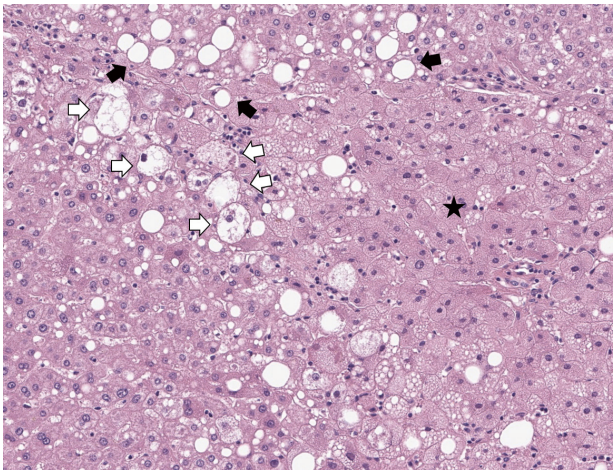

Figure 1. Steatosis. A combination of large and small droplet macrovesicular steatosis is seen in this example of non-alcoholic steatohepatitis. In large droplet macrovesicular steatosis, the fat droplet occupies more than half of the hepatocyte cytoplasm and pushes the nucleus to the edge of the cell (black arrows). Smaller droplets are also seen. A small patch of microvesicular steatosis is noted on the right (black star), characterized by innumerable tiny fat droplets in the hepatocyte cytoplasm. A few ballooned hepatocytes are also noted (white arrows) (H&E, original magnification ×200).

Abbreviations:

NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; HCC, hepatocellular carcinoma; NAS, NAFLD activity score; SAF, Steatosis-Activity-Fibrosis; Shh, sonic hedgehog; MDB, Mallory-Denk body; AIH, autoimmune hepatitis; NASH-CRN, non-alcoholic steatohepatitis clinical research network; FLIP, fatty liver inhibition of progression; ALD, alcoholic liver disease; SHG, Second-Harmonic Generation; TPEF, Two-Photon Excited Fluorescence; NLO, Non-Linear Optimal; CPA, collagen proportionate area; MRI-PDFF, magnetic resonance imaging-proton density fat fraction

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for instances whereby this pattern diffusely involves the liver. This is also important clinically as the differential diagnoses for microvesicular steatosis are distinctly different from macrovesicular steatosis (Table 1).

The steatosis of NAFLD typically begins in the perivenular region (zone 3), and is graded semiquantitatively as "mild", "moderate", or "severe", when 5–33%, 33–66%, or more than 66% of the hepatocytes are affected, respectively. A zone 1-predominant distribution of steatosis is rare in adults (~1%), while more commonly found in children and teenagers (~12%). An azonal distribution is more likely to be associated with ballooning degeneration, MDBs and advanced fibrosis.[7,8]

Hepatocellular ballooning

Hepatocyte ballooning is characterized as an enlarged hepatocyte with rarefied pale cytoplasm, usually with the presence of a large, hyperchromatic nucleus and a prominent nucleolus, indicating the presence of hepatocellular injury (Fig. 2).[4,6] The cytoplasmic changes reflect injury to the cytoskeleton of these hepatocytes, with loss of intact keratin 8 and 18 and increased detection of keratin fragments.[9] As the cytoskeleton injury progresses, the increased clumping of these keratin fragments contributes to MDB formation.[10]

It is worthwhile noting that in chronic cholestatic conditions, hepatocytes may also suffer from similar cytoskeleton injury resulting in morphological changes similar to ballooning. This is classically described as "feathery degeneration".[11] One may easily make the distinction by observing the adjacent steatotic or cholestatic changes, in order to decide which term to use. Mimics of ballooned hepatocytes include hydropic change of hepatocytes and microvesicular steatosis.

Ballooned hepatocytes exist in an "undead" state where they are unable to undergo apoptosis while releasing factors such as sonic hedgehog (Shh) to aid with tissue repair and healing. These ballooned hepatocytes were found to lack caspase 9—a protease critical for apoptosis.[12]

Ballooned hepatocytes are also associated with activation of the stress kinase c-Jun N-terminal kinase, which upregulates the hedgehog signaling pathway in the absence of apoptosis.[12-14] Prolonged hepatocyte lipotoxicity leads to persistent activation of the pathway. This is further exacerbated by the downregulation of protective enzymes such as HSP27, a protein with antioxidant properties that responds to cellular stress.[15]

In NAFLD, the activity of the hedgehog signaling pathway correlates with the severity of liver damage and fibrosis.[16] Analysis of a representative subset of subjects enrolled in the PIVENS clinical trial also found that response to treatment

Table 1. Differential diagnoses for macrovesicular and microvesicular steatosis[94]

Differentials for macrovesicular steatosis
Alcoholic liver disease
Non-alcoholic fatty liver disease
Other metabolic conditions, such as diabetes mellitus, growth hormone deficiency and hyperthyroidism
Genetic diseases, such as cystic fibrosis, PFIC1 mutations and Wilson disease
Malnutrition and related causes, including inflammatory diseases affecting the small bowel and gastrointestinal surgery
Differentials for microvesicular steatosis
Acute fatty liver of pregnancy
Alcoholic foamy degeneration
Genetic mitochondrial disease
Other genetic diseases, such as ornithine transcarbamylase deficiency, fatty acid oxidation disorders, and Wolman disease/cholesterol ester storage disease
Infections, including human herpes virus 8 and toxin of bacillus cereus
Toxins, including arsenic toxicity and industrial solvents
Medication effect, including linezolid, Reye syndrome, amiodarone, nucleoside analog reverse-transcriptase inhibitors used in human immunodeficiency virus treatment, valproate, high-dose tetracycline

PFIC1, progressive familial intrahepatic cholestasis type 1.

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corresponds to a greater decrease in Shh-producing hepatocytes.[17] Increased Shh is also associated with an increased risk of primary liver cancers, via the upregulation of cyclin B1 and cyclin-dependent kinase 1 mitotic proteins, as well as the induction of the epithelial-mesenchymal transition in malignant cells.[16]

Mallory-Denk bodies

MDBs, also known as Mallory hyaline in the past, are cytoplasmic aggregates that could be identified in some cases of steatohepatitis. MDBs appear as aggregates of hepatocytic keratins, K8 and K18, as well as ubiquitin and p62 in the cytoplasm.[18-21] The aggregates could be highlighted by immunohistochemical staining. Of note, MDB is not a specific histological feature for NAFLD, and is also observed in various inflammatory diseases, including alcoholic hepatitis and primary biliary cholangitis, and HCC.[22]

Lobular necroinflammation

Inflammatory cell infiltrations in the hepatic lobules are commonly seen in steatohepatitis.[23] The number of inflammatory cells may vary but are usually more accentuated in zone 3, in contrast to the portal/periportal distribution as seen in viral hepatitis. Mononuclear cells are the major constituent cells; some polymorphonuclear leukocytes and histiocytes are also present (Fig. 3). Microgranulomas, which represent macrophages engulfing lipid droplets, may be observed. Apoptosis of hepatocytes (acidophilic bodies) may be present, in accordance to the severity of inflammation.[24] Lobular inflammation may become less conspicuous in the cirrhotic stage of the disease.[25]

Other histological findings

Enlarged mitochondria, or megamitochondria, are detectable under light microscopy as eosinophilic inclusions in the

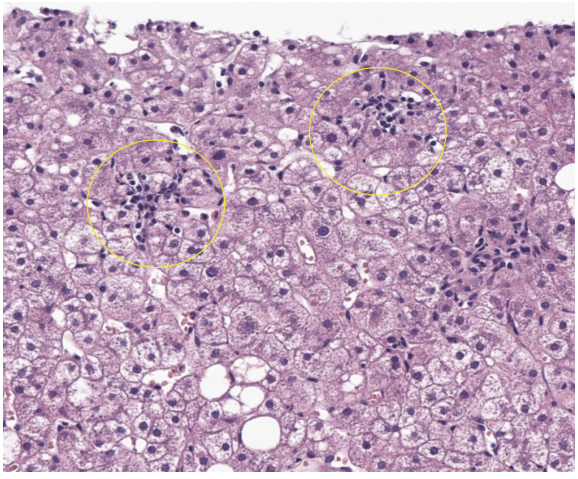

Figure 3. Lobular necroinflammation. Foci of lobular spotty necrosis are seen in this example of non-alcoholic steatohepatitis (yellow circles). The inflammatory cell infiltrations are mainly composed of mononuclear cells (H&E, original magnification ×200).

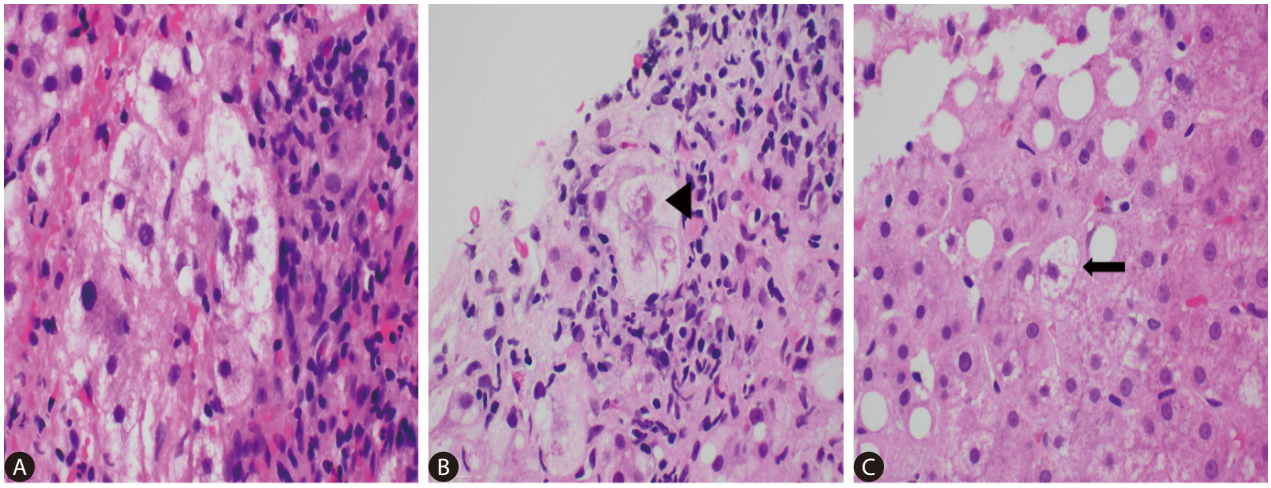

Figure 2. The many faces of ballooned hepatocytes (A–C: H&E, original magnification x400). (A) A cluster of classical ballooned cells. (B) Occasionally the cytoplasmic keratins aggregate to form tighter and more eosinophilic clumps, also known as Mallory-Denk bodies (black arrowhead). (C) A lonely non-classical ballooned cell (black arrow) which is similar in size to the adjacent non-ballooned hepatocytes.

cytoplasm. It has been proposed that megamitochondria signify the presence of cell injury or an adaptation process secondary to lipid peroxidation[4]. Glycogenated nuclei—clear intranuclear inclusions of hepatocytes—are associated with diabetes mellitus, and are more readily observed in NAFLD compared with alcoholic liver disease (ALD).[26]

Although the typical NASH histology is characterized by a lobular distribution of inflammation, there is often also a mild degree of portal mononuclear infiltration. In fact, portal inflammation that is moderate (but patchy) can be seen in the setting of severe NASH, NASH in the pediatric population or young adults, and also in the setting of disease resolution post-treatment.[27-29] However, when there is a significant amount of portal inflammation (diffuse, moderate/severe) that is disproportionate to the degree of lobular inflammation, one should consider the possibility of a concurrent disease, including chronic viral hepatitis and autoimmune hepatitis (AIH).[30] The differential diagnosis of NAFLD/NASH is discussed in more detail in a subsequent section.

Fibrosis

Hepatic fibrosis is caused by the excessive production, deposition, and net accumulation of extracellular matrix by activated hepatic stellate cells and other myofibroblasts.[4,31] In line with the preferential and initial deposition of steatosis in zone 3 of the hepatic lobule, the subsequent hepatocellular injury via the presence and accumulation of these lipotoxic lipids culminate in fibrosis commencing in the perivenular and zone 3 regions.[4,32-34]

The characteristic histologic pattern of fibrosis in NASH is the zone 3 pericellular and/or perisinusoidal pattern (often described as a "chicken-wire pattern"), resulting from the deposition of collagen and other extracellular matrix fibers around the hepatocytes (Fig. 4).[4] In advanced disease, the fibrosis extends to involve the portal and periportal (zone 1) regions, with subsequent central-portal bridging fibrosis and eventually cirrhosis.

In contrast, pediatric cases of NASH are more commonly associated with periportal fibrosis and the absence of perisinusoidal fibrosis.[4,35,36] This is due to the preferential and initial deposition of fat in the zone 1 region. As a result, the subsequent downstream hepatocellular injury and fibrosis are centered on zone 1 rather than zone 3.

ANCILLARY TESTS

Connective tissue stains

A good quality connective tissue stain is essential to identify hepatic fibrosis and especially crucial in detecting ear-

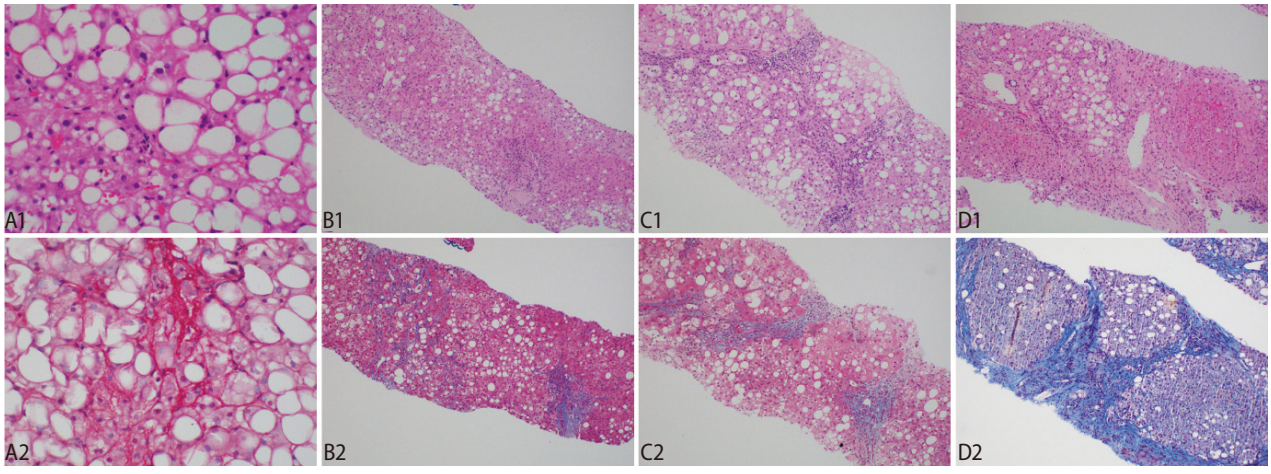

Figure 4. Stages of fibrosis and the utility of histochemical stains in accentuating the histological appearance. (A1, A2) The classical 'chicken-wire' appearance of pericellular fibrosis is accentuated with a Sirius Red histochemical stain, which reveals the collagen fibers in red. (B1, B2) Stage 2 fibrosis is the co-presence of pericellular fibrosis and also portal fibrosis. Masson Trichrome stain shows the blue pericellular collagen fibers on the left and the portal fibrosis on the right. (C1, C2) The fibrosis extends across the hepatic lobules and forms bridging fibrosis. (D1, D2) The presence of hepatocytic nodules heralds cirrhosis, with Masson Trichrome stain confirming the broad fibrous bands. Original magnification ×200 (A1, A2), ×40 (B1–D2); H&E (A1, B1, C1, D1), Sirius Red (A2), Masson Trichrome (B2, C2, D2).

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ly-stage fibrosis of NAFLD. Connective tissue stains widely used in liver pathology include trichrome, Sirius red and Gordon-Sweets reticulin stains.

Trichrome stain is the connective tissue stain of choice for the assessment of fibrosis in most laboratories because of its wide availability. However, a good trichrome stain requires proper optimization to avoid overstaining or understaining, which may lead to misinterpretation of the degree of fibrosis.[37] Although both trichrome and Sirius red stains are employed in computer-assisted morphometric quantitation of liver fibrosis,[38-40] Sirius red stain is shown to be superior to trichrome stain because of its highly detailed and contrasted staining of collagen fibers and high sensitivity in identifying early perivenular and pericellular fibrosis.[39,41] Nevertheless, both trichrome and Sirius red stains are equivalently good for routine daily practice. The choice between these two stains largely depends on personal preference and reagent availability. Gordon-Sweets reticulin stain primarily highlights type III collagen, and therefore it is used to assess hepatocyte cord thickness, reticulin framework integrity, and nodular architecture.[37] Although it can also evaluate fibrosis by highlighting type I collagen (the predominant collagen in hepatic fibrosis), it is less sensitive for the detection of early perivenular fibrosis.[41] Of note, reticulin loss may be focally present in areas of steatosis, which may lead to the erroneous interpretation of a well-differentiated hepatocellular neoplasm, especially when the tissue is sampled with the clinical impression of a "hepatic nodule".

Immunohistochemical stains

Cytokeratin 8/18 (CK8/18) is normally distributed in the cytoplasm with a strong intensity. In hepatocytes with ballooning, expression is loss or diminished in majority of the cytoplasm, and immunoreactivity is only retained in the MDBs.[19,21] Immunohistochemical staining for p62 and ubiquitin also highlights MDBs.[18,21] p62 is an autophagy substrate and a biomarker for the activity of autophagy, while ubiquitin is involved degradation of proteins.[42] Expression of Shh is identified in the hepatocytes of NAFLD. It was reported that hepatic Shh expression was associated with the degree of liver injury by histological evaluation and by circulatory biochemical profile.[43]

GRADING AND STAGING SYSTEMS

Grading and staging are histological markers of activity (severity of active necroinflammation) and chronicity (degree of fibrosis) of chronic liver disease, respectively. Scoring systems of grading and staging are utilized in chronic viral hepatitis to semiquantitatively evaluate disease severity and monitor disease progression.[44] They are useful in clinical management guideline development, pathology report standardization and histology assessment for clinical trials. Nevertheless, scoring systems for chronic viral hepatitis cannot be simply applied in NAFLD because they do not account for steatosis and ballooning degeneration, which are crucial in assessing disease activity in NAFLD. Additionally, they also do not consider perivenular and perisinusoidal fibrosis, which is the distinctive fibrosis pattern in NAFLD. Hence, the development of scoring systems designed for NAFLD is necessary to fill the gap. In 1999, the first scoring system for NAFLD was developed by Brunt et al.[32] It was derived from a cohort of 51 patients with NAFLD undergoing liver biopsy. The disease activity grade (0–3) was assigned according to a constellation of histological features composed of steatosis, lobular and portal inflammation, and ballooning degeneration. The fibrosis stage (0–4) was based on the fibrosis pattern of adult NAFLD from perivenular and pericellular fibrosis (stage 1), periportal fibrosis (stage 2), bridging fibrosis (stage 3) and cirrhosis (stage 4).

In 2005, the non-alcoholic steatohepatitis clinical research network (NASH-CRN) proposed the NASH-CRN scoring system, also known as the Kleiner scoring system,[7] based on a cohort of 50 NAFLD patients (32 adults and 18 children). In this system, the disease activity grade (NAS) is the unweighted sum of semiquantitative scores (0–8) for steatosis (0–3), ballooning degeneration (0–2), and lobular inflammation (0–3) (Table 2). The fibrosis stage (0–4) is similar to the Brunt fibrosis stage; however, the early fibrosis stage (stage 1) was refined and stratified into 1a (delicate pericellular fibrosis visualized by connective tissue stain only), 1b (dense pericellular fibrosis visualized by hematoxylin-eosin section) and 1c (portal/periportal fibrosis only). Stage 1c was added to represent the characteristic early fibrosis pattern among pediatric NAFLD patients. The NAS was demonstrated to be associated with the histological diagnosis of steatohepatitis: over 85% of patients with NAS ≥5 were diagnosed as steatohepatitis, whereas 99% of patients with NAS 0–2 were categorized as

Table 2. NAFLD Activity Score (NAS) and fibrosis stage by NASH-CRN[7]

NAS			
Score	Steatosis	Lobular inflammation	Ballooning degeneration
0	<5%	None	None
1	5–33%	<2 foci/20× field	Few
2	>33–66%	2–4 foci/20× field	Many
3	>60%	>4 foci/20× field	
Fibrosis score			
Score	Histological findings		
1a	Mild pericellular fibrosis (only seen on connective tissue stain)		
1b	Moderate pericellular fibrosis (readily seen on H&E)		
1c	Portal/periportal fibrosis without pericellular fibrosis		
2	Pericellular and portal/periportal fibrosis		
3	Bridging fibrosis		
4	Cirrhosis		

NAFLD, non-alcoholic fatty liver disease; NASH-CRN, non-alcoholic steatohepatitis clinical research network.

not diagnostic of steatohepatitis.[7,45] The NAS of 4 or more is used as one of the inclusion criteria in various clinical trials of NASH patients.[46,47] One should note that the primary objective of the NAS is to evaluate the overall histological changes. It has been repeatedly emphasized that the NAS should not be regarded as a numerical diagnostic criterion that substitutes the histological diagnosis of steatohepatitis.[7,48]

In 2012, Bedossa et al.[49] established a diagnostic algorithm and a scoring system from a cohort of 679 obese patients undergoing bariatric surgery. The fatty liver inhibition of progression (FLIP) algorithm classified a biopsy into either steatosis (without NASH) or NASH by semiquantification of steatosis, ballooning degeneration, and lobular inflammation. This algorithm improved the interobserver agreement in differentiating between steatosis and NASH (from moderate [kappa 0.54] to substantial [kappa 0.66]) among expert liver pathologists. Such an improvement was significantly more substantial among general pathologists (from fair [kappa 0.35] to substantial [kappa 0.61]).[50] The SAF score was the combination of semiquantitative scores of steatosis (S0–S3), activity (A0–A4; ballooning degeneration [0–2] and lobular inflammation [0–2]) and fibrosis (F0–F4) (Table 3). Although the NAFLD-CRN and SAF scoring systems are apparently similar, direct inter-translation between these two systems is not feasible.[51] It is noteworthy that there are several considerable differences. First, steatosis is not integrated into the activity score of the SAF compared to the NAS because the prognostication of steatosis in long-term outcomes and fibrosis progression remains controversial.[52-54] Second, the grading scheme for hepatocellular ballooning differs in the two systems—the NAFLD-CRN system assesses the quantity, while the SAF system evaluates the morphology of the ballooned cells (Tables 2, 3). Third, the NAFLD-CRN system grades lobular inflammation from 0 to 3 (0, none; 1: <2 foci/200× field; 2: 2–4 foci/200× field; 3: >4 foci/200× field), while the SAF system only grades lobular inflammation from 0 to 2 (0, none; 1: 1–2 foci/200× field; 3: >2 foci/200× field). Last but not least, both NAFLD-CRN and SAF systems have been externally validated by other groups but only the NAFLD-CRN system is currently widely used for clinical trials.[51,55,56]

Histological features in NAFLD apart from ballooning degeneration and lobular inflammation are also shown to have prognostic significance. Portal inflammation and MDBs are two histological parameters that have been consistently demonstrated to be associated with adverse clinical outcomes and fibrosis.[52-54,57] A more comprehensive but more complicated scoring system, the *expanded* NAS, has been proposed recently to provide a more accurate evaluation of the histological activity of NAFLD by incorporating portal inflammation and MDBs.[58] The clinical significance and applicability of the expanded NAS require further studies.

Any scoring system is inevitably subject to have intraobserver and interobserver variabilities. While the agreement in the evaluation of steatosis and fibrosis has been demonstrat-

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Table 3. Steatosis-Activity-Fibrosis (SAF) score and fatty liver inhibition of progression (FLIP) algorithm[50]

SAF score			
Steatosis	Steatosis		
S0	<5%		
S1	5–33%		
S2	>33–66%		
S3	>66%		
Activity A0-A4 (LI+BD)	Score	Lobular inflammation (LI)	Ballooning degeneration (BD)
	0	· None	· None
	1	· ≤2 foci/20× field	· Hepatocytes with a round shape and pale cytoplasm usually reticulated. Size is quite similar to that of normal hepatocytes
	2	· >2 foci/20× field	· Hepatocytes with a round shape and pale cytoplasm usually reticulated. Some cells are twice of the size of normal hepatocytes
Fibrosis	Histological findings		
F1a	Mild pericellular fibrosis (only seen on connective tissue stain)		
F1b	Moderate pericellular fibrosis (readily seen on H&E)		
F1c	Portal/periportal fibrosis without pericellular fibrosis		
F2	Pericellular and portal/periportal fibrosis		
F3	Bridging fibrosis		
F4	Cirrhosis		
FLIP algorithm			
Steatosis	Ballooning degeneration	Lobular inflammation	Diagnosis
1, 2, or 3	0	0, 1, or 2	NAFLD
1, 2, or 3	1 or 2	0	NAFLD
1, 2, or 3	1 or 2	1 or 2	NASH

ed to be substantial to almost perfect among different pathologists (kappa 0.79–0.80 and 0.54–0.84, respectively) and for the same pathologist (kappa 0.82–0.85 and 0.73–0.85, respectively), the agreement in the grading of ballooning degeneration and lobular inflammation is only fair to substantial among different pathologists (kappa 0.20–0.69 and 0.35–0.60, respectively) and for the same pathologist (kappa 0.66–0.72 and 0.60–0.70, respectively).[44,49,51,59] Computer-assisted image analysis may provide a more reliable way to minimize intraobserver and interobserver variabilities in the future.[59]

PEDIATRIC NAFLD

In the pediatric population, about half of NASH cases demonstrate the features of "type 2" NASH, characterized by moderate-to-severe steatosis with a panacinar distribution, portal inflammation, and portal fibrosis.[36] Hepatocyte ballooning and MDBs are less frequently seen compared to adults. This pattern is not restricted to children; "type 2" NASH has also been described in a subset of young adults.[29]

LOOKING AT NAFLD UNDER THE MICROSCOPE: APPLICATIONS IN UNIQUE SETTINGS AND DIFFERENTIAL DIAGNOSES

Identifying ballooned hepatocytes

As the presence of hepatocyte ballooning is the key to the histopathological diagnosis of NASH, it is of paramount importance that this is identified with confidence by pathologists. Although ballooned hepatocytes demonstrate the

characteristic appearance as described earlier, pathologists not infrequently encounter situations in which the hepatocyte in question demonstrates equivocal changes that fall short of a "classic" balloon cell (Fig. 2). Some of these "equivocal" balloon cells would belong to the "grade 1" ballooning of the SAF score, proposed by Bedossa et al.[49], while others could represent other changes with similar morphology, such as hydropic change of hepatocytes and microvesicular steatosis. In order to increase the accuracy of balloon cell identification, ancillary immunohistochemical stains such as CK8/18, ubiquitin, or Shh could be used. In addition, artificial intelligence (AI)-based technologies may have a role in the future.

Steatosis and steatohepatitis of other etiologies

Steatosis or steatohepatitis occurs in a variety of other settings, such as ALD, metabolic disorders (e.g., Wilson disease), chronic viral hepatitides, and drug/toxin-induced liver injury. Steatosis or steatohepatitis associated with ALD often demonstrate histological features that overlap with those of NAFL or NASH, respectively. Although ALD also commonly presents with macrovesicular steatosis in the perivenular zone, the general histological picture of steatohepatitis is more pronounced in ALD compared to NASH, with more abundant ballooned hepatocytes, MDBs, acidophil bodies, lipogranulomas, and neutrophilic infiltration[60]. Neutrophils may predominate in alcohol-related steatohepatitis, sometimes forming aggregates around ballooned hepatocytes ("neutrophilic satellitosis"). Alcoholic foamy degeneration and sclerosing hyaline necrosis are not features of NAFLD. The presence of cholestasis may help in the differential diagnosis between alcoholic steatohepatitis and NASH, as it is not a typical histological feature of the latter. The pattern of fibrosis is similar to that of NASH, with the zone 3-predominant perisinusoidal fibrosis that eventually progresses to bridging fibrosis and cirrhosis. Most importantly, the key distinguishing feature is the patient's history of alcohol consumption, and therefore clinicopathological correlation is necessary.[61]

Among the different viral hepatitis, steatosis has been described to be a common histological feature of chronic hepatitis C. However, the degree of steatosis in chronic hepatitis C alone should be at most mild, and in the presence of moderate or severe steatosis in patients with chronic hepatitis C, a co-existing cause of fatty liver should be investigated. Drug/toxin-induced liver injury may present as steatosis or even steatohepatitis ("drug-induced steatohepatitis, DISH"); examples of offending drugs include glucocorticoids, tamoxifen, irinotecan and amiodarone. As the histological features are most often similar to that of NAFL or NASH, the clinical information is the most important key to the diagnosis.

NAFLD with serum autoantibody positivity

Coexistence of AIH with NASH is not a rare occurrence; in such cases, there is a significant amount of portal lymphoplasmacytic infiltration and interface hepatitis in addition to the histological features of NASH. Correlation with the clinical findings, including elevated serum immunoglobulin G levels and positive autoantibodies, is important when contemplating the possibility of a combined AIH, as portal mononuclear cell infiltration with focal mild interface hepatitis may be encountered in NASH.[62] Moreover, serum autoantibody positivity has been identified in up to 34% of NAFLD patients in the absence of AIH, and no significant differences in the histology of NAFLD have been found according to serum autoantibody status.[63-65]

NAFLD in the post-liver transplantation setting

NAFLD may occur as a recurrent disease or *de novo* disease in the post-liver transplantation setting. In a study over a 10-year-period that analyzed 11 cases of recurrent disease and 80 *de novo* NAFLD in post-liver transplant patients, a higher prevalence of diabetes mellitus was observed in recurrent NAFLD.[66] Severe fibrosis and steatohepatitis were more readily observed in recurrent NAFLD versus *de novo* NAFLD. Interestingly, serial biopsies have demonstrated resolution of steatosis in 22.5% patients with *de novo* NAFLD but in none of the patients with recurrent NAFLD.[66]

Association of NAFLD with steatohepatitic HCC

Steatohepatitic HCC is associated with metabolic syndrome, a key driver of NAFLD. This HCC variant shows features resembling steatohepatitis within the tumor itself, including macrovesicular steatosis, balloon cells, intratumoral inflammation and intratumoral pericellular fibrosis.[67,68]

Salomao et al. demonstrated that their cohort with steatohepatitic HCCs had significantly higher numbers of metabolic

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syndrome risk factors (2.44 vs. 1.48, *P*=0.01) and higher percentage of patients with at least 3 metabolic syndrome components (50% vs. 22.5%, *P*=0.02).[69] However, this intuitive association has been challenged in another study by Yeh et al.[70] that evaluated 12 steatohepatitic HCCs arising in patients without metabolic syndrome. In this cohort, a subset of tumor showed loss of 9q12-q31-1 via genomic microarray analysis.

STATE-OF-ART AND FUTURE TRENDS

Role of digital pathology and AI

Due to the limitations of current methods to assess NAFLD and liver fibrosis, there is considerable interest in the use of AI to improve these systems for risk stratification, diagnosis, monitoring, and prognostication of NAFLD in patients.[71] AI can be integrated in AI-based digital pathology systems to assess NAFLD. Digital pathology is defined as the process of utilizing whole slide scanners for digitizing of histopathology slides, producing images that allow for quantitative analyses.[72] When combined with AI, these systems have the potential to diagnose and prognosticate NAFLD via automated processes.[73]

Taylor-Weiner et al.[74] developed a machine learning-based approach for the assessment of liver histology in NAFLD. For the assessment of the diagnostic features of NAFLD, the model's predictions were significantly correlated with the consensus NAS grades of pathologists' assessments—steatosis: ρ=0.66, lobular inflammation: ρ=0.54, hepatocellular ballooning: ρ=0.62. For the assessment of fibrosis, the model's predictions were also significantly correlated with the consensus staging of pathologists, with a weighted Cohen's kappa of 0.801 and 0.817 for the NASH CRN and the Ishak classifications respectively. This level of agreement is within the range of agreement between individual pathologists and the consensus staging by pathologists.

Machine learning models also enabled the identification and quantification of novel and complex parameters that are usually difficult to evaluate with conventional methods. The study identified the steatosis to hepatocellular ballooning ratio to be a significant parameter of NAFLD progression, where subjects with more hepatocellular ballooning and less steatosis at baseline were significantly more likely to experience a clinical event.[74]

The study also proposed the DELTA Liver Fibrosis Score—a machine learning-derived metric used to measure changes in the intra-sample distribution of fibrosis associated with disease progression or therapy. When a stringent DELTA Liver Fibrosis Score threshold was applied comparing images pre- and post-treatment, significant differences could be found in samples that previously did not demonstrate any significant difference using conventional pathologist staging methods. Therefore, the DELTA Liver Fibrosis Score could be a more sensitive method for assessing histological response to treatment, potentially being a useful tool in NAFLD clinical trials.[74]

Forlano et al.[75] developed an automated image analysis-based system to quantify steatosis, ballooning, inflammation, and fibrosis from the histological images of NAFLD patients. There was excellent concordance between manual annotations of histopathologists and the automated measurements, with an intraclass correlation coefficient of 0.95–0.99 for the four parameters measured. The fully automated model was described to be straightforward to install, not requiring specialized equipment, only requiring modest computational effort, and being able to produce results within 2 minutes.[75]

Second-Harmonic Generation (SHG) microscopy

SHG microscopy and Two-Photon Excited Fluorescence (TPEF) microscopy are both imaging techniques under the umbrella of Non-Linear Optimal microscopy techniques, which were described to produce images of good spatial resolution, depth of penetration, and excitation capability.[76] Both SHG and TPEF imaging can be performed regardless of the means of sample preparation—where both frozen and formalin-fixed paraffin-embedded tissues can be used without staining.[77]

In the liver, TPEF microscopy enables the visualization of the liver background and lobular organization, while SHG microscopy characterizes the morphology of collagen (Fig. 5).[78] Combined SHG/TPEF microscopy can localize and quantify fibrillar collagen in 2D and 3D, enabling the automated quantification of fibrosis.[79] These features tackle known limitations of traditional histological scores with semiquantitative grading systems such as inter- and intraobserver variation.[80]

Other than NAFLD, combined SHG/TPEF microscopy has been initially used to quantify fibrosis in other liver condi-

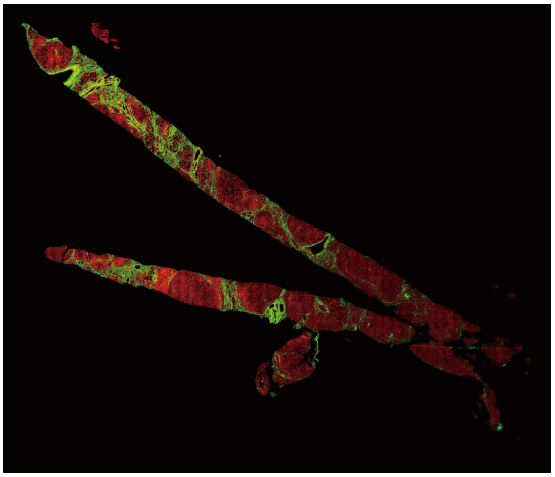

Figure 5. An example of a case of non-alcoholic fatty liver disease -cirrhosis seen by second harmonic generation/two-photon excitation fluorescence (SHG/TPEF) (SHG/TPEF microscopy, scanning power).

tions, especially chronic hepatitis B. Developed by Xu et al.[81], qFibrosis, a combined index based on 87 parameters, was first validated with core biopsies of chronic hepatitis B patients. qFibrosis was found to be able to reliably replicate the Metavir fibrosis staging by histopathologists, and was more sensitive in differentiating fibrosis stages compared to collagen proportionate area (CPA). qFibrosis was also described to have decreased sensitivity to sampling error, and can aid in the correction of intra- and interobserver bias.[81] For chronic hepatitis B patient post-antiviral therapy, qFibrosis was not only able to detect the changes observable by histopathologists, but could also detect and characterize subtle changes in fibrosis, potentially being more sensitive in evaluating changes in fibrosis.[82]

Following the successes of combined SHG/TPEF microscopy in chronic hepatitis B, several models in the same vein have been developed for NAFLD.

Quantifiable fibrosis-related parameter (q-FP)

Established by Wang et al.[83], the q-FP model was the first established SHG based model that quantified fibrosis-related parameters in NAFLD. The q-FPs included the geometric and textural features of collagen fibers, and the number of collagen fibers. The collagen fibers at defined regions such as the general liver section, perisinusoidal space, vessels, and vessel bridges were measured and characterized. Seventy of the q-FPs had inter- and intraobserver concordance ≥0.8 and were strongly related to the NAS fibrosis staging. Sixteen of these q-FPs with the strongest concordance were included in a principal component analysis model, differentiating any stage of fibrosis versus no fibrosis, and cirrhosis versus earlier fibrosis stages with an area under the curve (AUC) of 0.88 and 0.93 respectively. Four q-FPs—number of collagen strands, strand length, strand eccentricity, and strand solidity—were found to also be independently associated with fibrosis stages. These 4 q-FPs could model fibrosis along a continuous linear scale using desirability functions, with the obtained measurements being significantly correlated with actual fibrosis stage.

SHG B-index

Chang et al.[84] developed a SHG-based model, the SHG B-index, to scan and analyze the SHG properties of collagen in unstained liver tissue specimens of NAFLD patients, and is able to grade the severity of liver fibrosis. A total of 14 parameters that correlated strongly with the Brunt fibrosis staging classification were selected.

The SHG B-index had a high correlation with Brunt fibrosis staging, with an excellent ability to differentiate advanced fibrosis from no or mild fibrosis. However, between Brunt stages 0–2, the SHG B-index had a poorer discriminatory ability. The SHG B-index was also able to identify different fibrosis stages, with AUROCs of 0.853–0.985 for the prediction of mild fibrosis, significant fibrosis, bridging fibrosis, and cirrhosis.

The study also utilized Youden's index to derive optimal SHG B-index cut-off values to identify specific Brunt fibrosis groups. The cut-off value for advanced fibrosis had an overall diagnostic accuracy of 98.5% for prediction of the presence of bridging fibrosis, with a positive predictive value of 96.6% and a negative predictive value of 92.6%. This suggests that the SHG B-index has high accuracy for the discrimination of advanced fibrosis compared to milder stages of fibrosis. This is clinically important as bridging fibrosis is a clinically important feature that is associated with poor prognosis in NAFLD patients.

qFibrosis/qFIBS

Liu et al.[85] modified features of qFibrosis to compare the features of collagen and fibrosis in pediatric and adult NAFLD. The study found that there was more baseline collagen in livers of adult NAFLD, and a predominance of portal fibrosis in pediatric NAFLD compared to centrilobular fibrosis in adult

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NAFLD. qFibrosis was also able to detect subtle differences not apparent in histology, such as wider central vein lumens in pediatric NAFLD, possibly indicating the presence of increased portal-central vascular shunting. The same group expanded combined SHG/TPEF microscopy further to produce qFIBS, an algorithm that provides an automated quantitative assessment of histological features pertinent to NASH. qFIBS quantifies the four key histopathological features of NAFLD—fibrosis (qFibrosis), inflammation (qInflammation), hepatocyte ballooning (qBallooning), and steatosis (qSteatosis), with the goal of predicting the severity of NAFLD. Each parameter in qFIBS correlated well their corresponding histological counterparts, and could distinguish between different grades of the histological feature with an AUC between 0.813–0.939. qFIBS was also validated in both adult and pediatric NAFLD liver biopsy samples.[86]

Leow et al.[87] refined the qFibrosis algorithm further, including 26 new periportal parameters to produce an algorithm with a better discriminatory ability for F1 and F2 fibrosis according to the NAS. These new parameters are able to better compensate for limitations of previous AI-based SHG algorithms, where they are less discerning in discriminating between early stages of fibrosis. Having a better ability to discriminate between early fibrosis stages can play an important role in clinical trials— increasing the accuracy of patient enrollment, while more accurately monitoring treatment responses.[87]

Therefore, it can be seen that AI has great potential and could have a large role to play in multiple aspects of NAFLD.

The role of liver biopsy in clinical trials

Despite the large amount of resources invested into NAFLD clinical trials, no drug has been specifically approved for the treatment of NAFLD yet.[88,89] While the complex and multifactorial pathophysiology of NAFLD provides numerous potential targets for intervention, this complexity also hampers the ability to define clear, measurable, and objective clinical endpoints in clinical trials.[90]

Liver biopsies are still considered as the gold standard for the diagnosis and evaluation of NAFLD. The quality of the obtained sample can be affected by the method of procurement, location, type, and dimensions of the liver biopsy.[91] For the same sample, the intra- and interobserver variability of histopathologist evaluation could also affect the reported results. The limitations of the procurement and interpretation of liver biopsies could affect the enrollment of participants into clinical trials, as well as incorrectly assess the histological treatment responses in serial liver biopsies. In addition, the presence of co-morbidities such as type 2 diabetes, metabolic syndrome, and cardiovascular diseases, along with the lack of uniformity of confounders such as alcohol, diet, and physical activity also complicates the interpretation of NAFLD clinical trial results.[92,93]

Aside from key clinical endpoints such as liver-related mortality, liver transplantation, hepatic decompensation, and HCC, histological changes in serial liver biopsies have also been used as the main surrogate endpoints in clinical trials, especially for NAFLD patients without cirrhosis. Currently, meaningful endpoints that indicate an improvement in NAFLD include a reduction of the NAS ≥2 with ≥1-point reduction in either lobular inflammation or hepatocellular ballooning without worsening of fibrosis, resolution of NAFLD without worsening of fibrosis, and the improvement in liver fibrosis without worsening of NAFLD.[92,94] An improvement of fibrosis is defined as an improvement by at least 1 fibrosis stage using the Brunt criteria.

Other proposed surrogate endpoints include the use of non-invasive imaging and biochemical modalities, but these modalities are not validated for and have limited use in late-phase clinical trials. Magnetic resonance imaging-proton density fat fraction is a validated technique used in early-phase clinical trials to assess the extent of steatosis in each segment of the liver, and can detect small changes in steatosis better than histopathologist interpretation of liver biopsies. Liver stiffness can also be determined using elastography-based methods such as vibration-controlled transient elastography, magnetic resonance elastography, and shear wave elastography, but have not been validated to be used as surrogate endpoints in clinical trials.[92,95] Numerous serum biomarkers and algorithms have been investigated to prognosticate the severity of NAFLD. Acute-phase proteins, cytokines, and markers of oxidative stress and apoptosis have been evaluated in NAFLD patients but were found to have limited utility. Previously mentioned algorithms such as the NAFLD Fibrosis Score and FIB-4 have also been considered for use in clinical trials.[90] However, these algorithms only showed a modest ability to predict fibrosis, as well as lacking conclusive data on how these measures change in response to disease progression, thus not being suitable surrogate

endpoints for clinical trials.[95]

Unfortunately, there are also no clear endpoints for NAFLD clinical trials in the pediatric age group. This is contributed and complicated by the presence of knowledge gaps in pediatric NAFLD, as well as the numerous added limitations involved with conducting research in pediatric patients.[92]

CONCLUSION

Despite the remarkable advances in non-invasive biomarker development during the recent years, liver biopsy evaluation still has important roles in the setting of NAFLD diagnosis, such as confirmation or exclusion of the diagnosis, distinction of NASH from simple steatosis, assessment of disease severity and stage, and other histological alterations.[96] In fact, currently, only liver biopsy can provide simultaneous information on steatosis, inflammation, hepatocellular injury, fibrosis and concurrent liver disease. In addition, liver biopsy is essential in clinical trials, for confirming the presence of NASH, assessing and semiquantitating individual features and evaluating the effects of the therapeutic intervention. To overcome the current limitations of liver biopsy, such as the problem of inter/intraobserver variability, new diagnostic tools are being developed—with the recent burst of research on AI-based pathology tools and the increasing implementation of digital pathology into routine diagnostic practice, it will probably not be long before these new technologies will make their way into routine clinical care.

Authors' contribution

Conceptualization: WQL, AC, PM, RL, HK. Supervision: HK. Writing - original draft preparation: WQL, AC, PM, RL, KY, HK. Writing - review & editing: WQL, AC, PM, RL, HK. Approval of final version of manuscript: all authors.

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Conflicts of Interest

The authors have no conflicts to disclose.

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Original Article

The independent effect of exercise on biopsy-proven non-alcoholic fatty liver disease: A systematic review

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Non-alcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease worldwide. Although previous studies have demonstrated that exercise independently reduces hepatic steatosis measured by imaging modalities in NAFLD, the effect of exercise on histological endpoints remains unclear. We aimed to conduct a systematic review of the independent effect of exercise on hepatic steatosis, steatohepatitis, and liver fibrosis as measured by histological assessment or non-invasive tests (NITs) in biopsy-proven NAFLD. A systematic literature search of PubMed, Embase, and Web of Science databases was performed using keywords related to exercise, NAFLD, and biopsy. Articles were selected based on the following inclusion criteria: (1) involved human subjects with biopsy-proven NAFLD, (2) analyzed the independent effect of exercise, (3) assessed changes in hepatic steatosis, steatohepatitis, or liver fibrosis via either histological evaluation or NITs, and (4) were original research studies. We identified a total of six studies that analyzed the independent effect of exercise on histological endpoints in biopsy-proven NAFLD. Two randomized controlled trials (RCTs) did not detect significant histological improvement following exercise interventions, while other non-randomized interventional studies showed that exercise reduces hepatocyte ballooning and liver fibrosis. In addition, five studies assessed NIT outcomes, collectively demonstrating that exercise improves hepatic steatosis measured by magnetic resonance imaging-based techniques but not serum biomarkers for steatohepatitis and liver fibrosis. Additional large RCTs and meta-analyses are warranted to investigate the independent effect of exercise on histological and clinical outcome endpoints in NAFLD. **(Clin Mol Hepatol 2023;29(Suppl):S319-S332)**

Keywords: Non-alcoholic fatty liver disease; Fatty liver; Exercise

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD), recently redefined as metabolic-associated fatty liver disease (MAFLD),[1,2] has emerged as the most common etiology of chronic liver disease worldwide and is a leading cause of cirrhosis and hepatocellular carcinoma.[3,4] The global prevalence of NAFLD is projected to increase from 25% to over half of the adult population by the year 2040.[5,6] NAFLD represents a spectrum of liver disease ranging from non-alcoholic fatty liver (NAFL) with bland steatosis to non-alcoholic steatohepatitis (NASH), a condition characterized by liver inflammation and hepatocellular damage that may cause progressive fibrosis leading to cirrhosis. Currently there is no approved pharmacological

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therapy for the treatment of NAFLD. As such, lifestyle modifications including exercise, diet, and weight reduction remain the cornerstone of NAFLD management.[7,8]

An increasing number of randomized controlled trials (RCTs) and meta-analyses in the past decade have assessed the impact of exercise on NAFLD independent of other lifestyle interventions.[9-14] The vast majority of these studies, however, focus on the effect of exercise on imaging-based measures of hepatic steatosis. Given that only a few studies involve biopsy-proven NAFLD, limited evidence is available to address the impact of exercise on NASH resolution and liver fibrosis, the two primary regulatory endpoints for NASH drug development. Thus, we conducted a systematic review to (1) summarize the literature on the independent effect of exercise on hepatic steatosis, steatohepatitis, and liver fibrosis as measured by histological assessment or non-invasive tests (NITs) in biopsy-proven NAFLD, and (2) highlight the need for additional research centered on analyzing histological and clinical outcomes associated with exercise interventions.

METHODS

We conducted a systematic literature search using PubMed, Embase, and Web of Science databases from inception to October 10, 2022 to identify original research studies on the independent effect of exercise on hepatic steatosis, steatohepatitis, or liver fibrosis measured by histological assessment or NITs in human subjects with biopsy-proven NAFLD (Fig. 1). The search was performed based on the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines[15] using the following keywords: (exercise, physical activity, physical endurance, physical exertion, physical training, endurance exercise, endurance training, aerobic exercise, aerobic training, walking, jogging, running, treadmill, swimming, resistance exercise, resistance training, progressive resistance, weight training, weight lifting, muscle exercise, muscle training, strength training, interval training, high-intensity interval, or HIIT) and (non-alcoholic fatty liver disease, fatty liver, hepatic steatosis, NAFLD, non-alcoholic steatohepatitis, steatohepatitis, or NASH) and (biopsy, histology, histologic, histological, histopathology, histopathologic, or histopathological).

After removing duplicates, we included articles that met the following inclusion criteria: (1) involved subjects with biopsy-proven NAFLD, (2) analyzed the independent effect of exercise, (3) assessed changes in hepatic steatosis, steatohepatitis, or liver fibrosis via either histological evaluation or NITs, and (4) were primary research studies. Reference lists of each included paper were then manually reviewed to identify additional eligible studies.

RESULTS

Our literature search yielded a total of nine studies, including seven interventional studies and two observational reports, that investigated the independent effect of exercise on hepatic steatosis, steatohepatitis, or liver fibrosis in biopsy-proven NAFLD (Fig. 1). Six studies evaluated histological endpoints, and five studies assessed NIT outcomes.[16-24] The participant demographics of included studies are shown in Table 1. Protocols and results of interventional studies measuring histological and NIT endpoints are summarized in Tables 2 and 3.

Impact of exercise on biopsy-proven NAFLD assessed by histological evaluation

Two of the six studies that assessed histological endpoints were RCTs, neither of which reported statistically significant histological improvement following exercise interventions.[16,17] Hickman et al.[16] randomly assigned 21 adults with NAFLD, 18 of whom had biopsy-proven NASH, to six months of either circuit-based resistance exercise without dietary changes or dietary-induced weight loss (DIWL). The exercise intervention consisted of three moderate-intensity sessions per week, starting with one circuit (12 minutes) per session

Abbreviations:

NAFLD, non-alcoholic fatty liver disease; NITs, non-invasive tests; RCTs, randomized controlled trials; MAFLD, metabolic-associated fatty liver disease; NAFL, non-alcoholic fatty liver; NASH, non-alcoholic steatohepatitis; PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analyses; DIWL, dietary-induced weight loss; NAS, NAFLD activity score; LFDE, low-fat diet plus exercise; MFDE, moderate-fat/low-processed-carbohydrate diet plus exercise; NRCT, non-randomized controlled trial; BMI, body mass index; MET, metabolic equivalent; OR, odds ratio; CI, confidence interval; MRI-PDFF, magnetic resonance imaging-proton density fat fraction; NFS, NAFLD fibrosis score; FIB-4, fibrosis-4; CK-18, cytokeratin 18; HTGC, hepatic triglyceride content; FAST, Fibroscan-AST

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during the first week and then a gradual increase to five circuits (60 minutes) per session by the fifth week. Supervision was offered to participants but not strictly required for the exercise intervention, resulting in an attendance rate of 90% for supervised sessions. The DIWL group achieved significant weight loss (mean −9.7%) while the exercise group did not. Post-intervention liver biopsies were performed in 14 participants (11 with NASH), revealing a significant decrease in both steatosis severity and NAFLD activity score (NAS) in the DIWL but not the exercise group. Neither group experienced significant change in lobular inflammation, hepatocyte ballooning, or fibrosis stage. Within the NASH-only cohort, two of the three participants in the DIWL group achieved NASH resolution while two of the eight participants in the exercise group achieved NASH resolution but this difference was not statistically significant (P=0.49).[16]

In another RCT, Eckard et al.[17] reported that a combination of aerobic exercise and resistance training did not result in significant histological improvement. Fifty-six subjects with NAFLD, including 36 with biopsy-proven NASH, underwent one of four interventions for six months: (1) low-fat diet plus exercise (LFDE), (2) moderate-fat/low-processed-carbohydrate diet plus exercise (MFDE), (3) exercise only, or (4) standard of care with basic nutrition and exercise education. Exercise intervention consisted of supervised moderate-intensity aerobic and resistance training sessions lasting

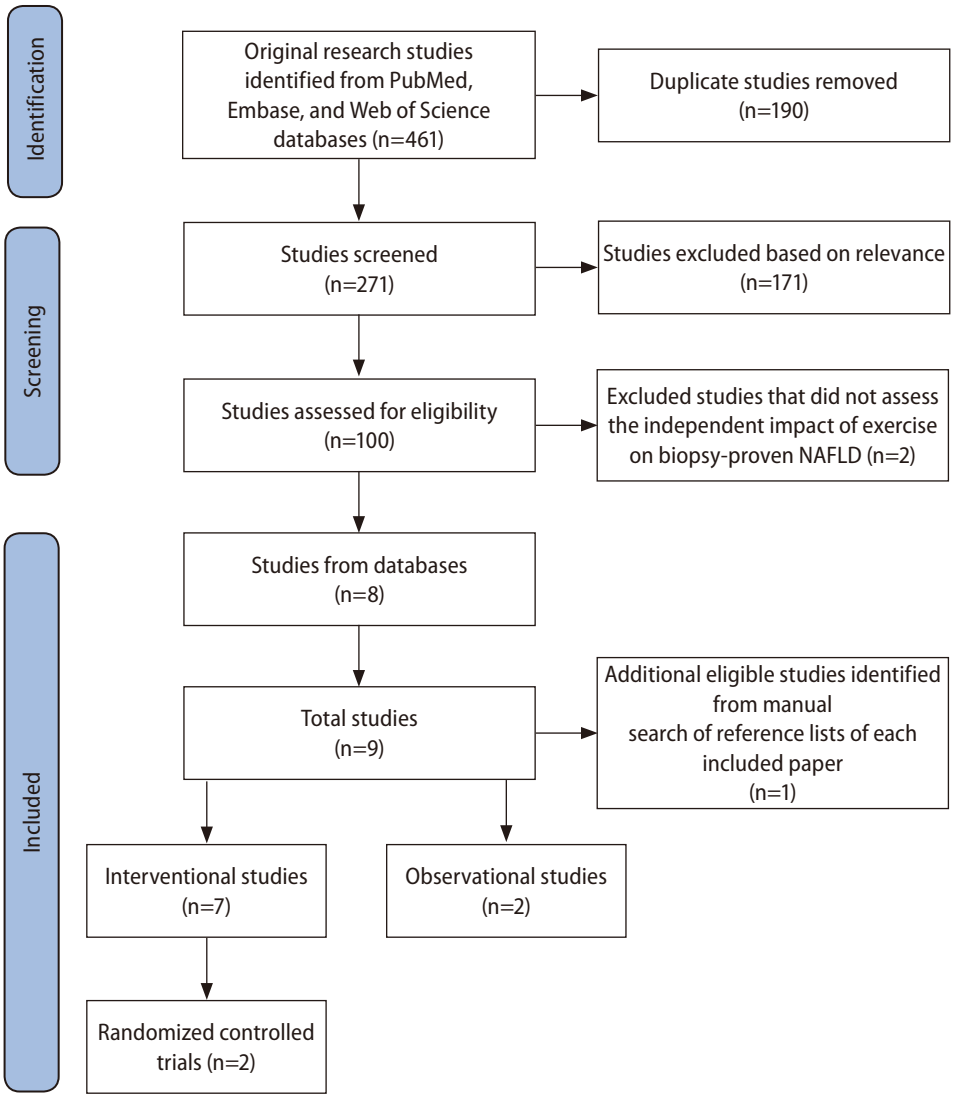

Figure 1. Identification, screening, and inclusion of studies for review. NAFLD, non-alcoholic fatty liver disease.

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Table 1. Participant demographics of included studies

Study number	Author	Study design	Mean age	Sex (% female)	Mean BMI	Biopsy-proven NAFLD in total (n)	Biopsy-proven NAFLD in exercise group (n)
1	Hickman et al.[16]	RCT	48	38	34	21	9
2	Eckard et al.[17]	RCT	50	39	35	56	9
3	Naimimohasses et al.[18]	NRCT	58	60	35	50	16
4	O'Gorman et al.[19]	NRCT*	60	71	36	24	16
5	Rezende et al.[22]	RCT	55	100	33	40	19
6	Stine et al.[23]	RCT	50	61	35	28	18
7	Houghton et al.[24]	RCT	54	n.r.	33	24	12
8	Kistler et al.[20]	Cross-sectional	48	63	n.r.	813	n.a.
9	Lahelma et al.[21]	Cross-sectional	51	65	40	100	n.a.

BMI, body mass index; NAFLD, non-alcoholic fatty liver disease; RCT, randomized controlled trial; NRCT, non-randomized controlled trial; n.r., not reported; n.a., not applicable.
*Non-randomized controlled trial design for non-invasive test endpoints; uncontrolled trial design for histological endpoints.

20–60 minutes each and occurring four to seven days per week. None of the four groups achieved significant weight loss following their interventions. While both the LFDE and MFDE cohorts experienced a significant decrease in NAS and the LFDE cohort achieved a significant improvement in Brunt grade, the exercise only group did not experience a significant change in either NAS or Brunt grade. None of the groups experienced significant change in fibrosis stage. Among the 36 participants with NASH, 19 (53%) saw an improvement in either Brunt grade or fibrosis including nine (25%) who had resolution of NASH. However, the authors did not report the distribution of patients with NASH across the four groups and did not distinguish NASH from NAFL as an endpoint, thereby preventing assessment of the independent impact of exercise on NASH. In addition, results for individual components of the Brunt grading system (steatosis, lobular inflammation, and hepatocyte ballooning) were not reported.[17]

Two additional interventional studies evaluated the impact of exercise on histological endpoints in NAFLD.[18,19] Naimimohasses et al.[18] conducted a non-randomized controlled trial (NRCT) comparing exercise and diet interventions among 31 subjects. The exercise group participated in two supervised and one to three unsupervised aerobic exercise sessions per week, with each session lasting 21–42 minutes at 40–75% heart rate reserve, while the diet group followed a moderately hypocaloric Mediterranean diet. After 12 weeks of intervention, the exercise and diet groups experienced significant mean weight reductions of 2 kg and 7 kg, respectively. Upon histological evaluation, the exercise intervention elicited a significant improvement in both hepatocyte ballooning (P=0.02) and fibrosis (P=0.04) but not steatosis (P=0.50), lobular inflammation (P=0.50), or NAS (P=0.09). In contrast, the dietary intervention significantly reduced both steatosis and NAS but not fibrosis, hepatocyte ballooning, or lobular inflammation.[18]

The exercise-induced histological changes reported by Naimimohasses et al.[18] were concordant with those found by O'Gorman et al.[19] in an uncontrolled interventional trial of similar study design. Sixteen participants with biopsy-proven NAFLD underwent a 12-week exercise intervention consisting of two supervised and one to three unsupervised moderate-to-vigorous aerobic exercise sessions per week, with each session lasting 21–42 minutes at 40–75% heart rate reserve.

The exercise intervention led to significant reduction of body mass index (BMI), although none of the participants

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Table 2. Protocols and results of interventional studies measuring histological outcomes

Study number	Exercise and control group interventions	Mean change in weight or BMI	Exercise protocol				Histological outcomes
			Intervention duration (weeks)	Frequency (sessions per week)	Session duration	Intensity	
1	EG: circuit-based resistance exercise (n=13) DG: dietary-induced weight loss (n=8)	Weight: EG: "Statistically insignificant" (exact change n.r.) DG: −9.7% BMI: EG: 0 kg/m^2 DG: −3 kg/m^2	24	3	Initially 1 circuit (12 min), gradually increased to 5 circuits (60 min) by week 5	"Moderate" (50% of 1-RM)	EG: Steatosis: not improved (P=0.12) Lobular inflammation: not improved (P=0.77) Hepatocyte ballooning: not improved (P=0.34) NAS: not improved (P=0.29) Fibrosis: not improved (P=1.0) DG: Steatosis: improved (P=0.04) Lobular inflammation: not improved (P=0.17) Hepatocyte ballooning: not improved (P=0.50) NAS: improved (P=0.05) Fibrosis: not improved (P=0.50)
2	EG: aerobic and resistance exercise (n=9) DG: LFDE (n=12); MFDE (n=9) CG: No intervention (n=11)	EG: +0.1 lb (95% CI −3.6 to +0.6) LFDE: −0.2 lb (−3.7 to +3.2) MFDE: −3.0 lb (−6.6 to +0.6) CG: −2.5 lb (−6.0 to 1.0)	24	4–7	20–60 min	"Moderate"	EG: Brunt grade*: not improved (mean −0.2; 95% CI −0.7 to +0.3) NAS: not improved (−0.8; −1.8 to +0.3) Fibrosis: not improved (−0.4; -1.5 to +0.6) LFDE: Brunt grade: improved (−0.8; −1.4 to −0.3) NAS: improved (−1.3; −2.2 to −0.5) Fibrosis: not improved (−0.7; −1.6 to 0.3) MFDE: Brunt grade: not improved (−0.2; −1.1 to +0.6) NAS: improved (−1.2; −2.0 to −0.5) Fibrosis: not improved (−0.1; −1.3 to +1.1) CG: Brunt grade: not improved (−0.4; −1.4 to +0.6) NAS: not improved (−0.4; −1.4 to +0.6) Fibrosis: not improved (+0.4; −0.3 to +1.1)

Table 2. Continued

Study number	Exercise and control group interventions	Mean change in weight or BMI	Exercise protocol				Histological outcomes
			Intervention duration (weeks)	Frequency (sessions per week)	Session duration	Intensity	
3	EG: aerobic exercise (n=16) DG: moderately hypocaloric Mediterranean diet (n=15)	Weight: EG: –2 kg (*P*=0.0005) DG: –7 kg (*P*<0.0001) BMI: EG: –1.1 kg/m^2 (*P*<0.0001) DG: –1.9 kg/m^2 (*P*=0.0002)	12	3–5	Initially 21 min, gradually increased to 42 min over 12 weeks + 5–7 min warm-up and cool-down	"Individualized" (40–75% HR reserve)	EG: Steatosis: not improved (*P*=0.50) Lobular inflammation: not improved (*P*=0.50) Hepatocyte ballooning: improved (*P*=0.02) NAS: not improved (*P*=0.09) Fibrosis: improved (*P*=0.04) DG: Steatosis: improved (*P*=0.004) Lobular inflammation: not improved (*P*=0.38) Hepatocyte ballooning: not improved (*P*=0.11) NAS: improved (*P*=0.01) Fibrosis: not improved (*P*=0.50)
4	EG: aerobic exercise (n=16)	EG: –2.1 kg/m^2 (*P*<0.001)	12	3–5	Initially 21 min, gradually increased to 42 min over 12 weeks + 5–7 min warm-up and cool-down	"Moderate to vigorous" (40–75% HR reserve)	EG: Steatosis: not improved (*P*=1.0) Lobular inflammation: not improved (*P*=0.74) Hepatocyte ballooning: Improved (*P*=0.02) NAS: not improved (*P*=0.17) Fibrosis: improved (*P*=0.03)

BMI, body mass index; EG, exercise group; DG, diet group; n.r., not reported; kg, kilogram; m, meter; min, minute; RM, repetition maximum; NAS, non-alcoholic fatty liver disease activity score; CG, control group; LFDE, low-fat diet plus aerobic and resistance exercise; MFDE, moderate-fat/low-processed-carbohydrate diet plus aerobic and resistance exercise; lb, pound; CI, confidence interval; HR, heart rate

*Individual histological components of the Brunt grading system, including steatosis, lobular inflammation, and hepatocyte ballooning, were not reported.

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Table 3. Protocols and results of interventional studies measuring non-invasive test outcomes

Study number	Exercise and control group interventions	Mean change in weight or BMI	Exercise Protocol				Non-invasive test outcomes	
			Intervention duration (weeks)	Frequency (sessions per week)	Session duration	Intensity	Imaging-based	Serum-based
3	EG: aerobic exercise (n=16) DG: hypocaloric Mediterranean diet (n=15) CG: No intervention (n=14)	Weight: EG: –2 kg (P=0.0005) DG: –7 kg (P<0.0001) CG: 0 kg (P=0.99) BMI: EG: –1.1 kg/m^2 (P<0.0001) DG: –1.9 kg/m^2 (P=0.0002) CG: +0.5 kg/m^2 (P=0.14)	12	3–5	Initially 21 min, gradually increased to 42 min over 12 weeks + 5–7 min warm-up and cool-down	"Individualized" (40–75% HR reserve)	Transient elastography: EG: Steatosis: improved (P=0.003) Fibrosis: improved (P=0.004) DG: Steatosis: improved (P=0.004) Fibrosis: improved (P=0.02) CG: Steatosis: not improved (P=0.21) Fibrosis: not improved (P=0.22)	EG: FAST: not improved (P=0.08) FIB-4: not improved (P=0.14) DG: FAST: improved (P=0.004) FIB-4: not improved (P=0.23) CG: FAST: improved (P=0.04) FIB-4: improved (P=0.03)

Table 3. Continued

Study number	Exercise and control group interventions	Mean change in weight or BMI	Exercise Protocol				Non-invasive test outcomes	
			Intervention duration (weeks)	Frequency (sessions per week)	Session duration	Intensity	Imaging-based	Serum-based
4	EG: aerobic exercise (n=16) CG: no intervention (n=8)	EG: –2.1 kg/m2 (*P*<0.001 within EG, *P*=0.04 compared to CG) CG: exact change n.r.	12	3–5	Initially 21 min, gradually increased to 42 min over 12 weeks + 5–7 min warm-up and cool-down	"Moderate to vigorous" (40–75% HR reserve)	Transient elastography: EG: Steatosis: improved (*P*=0.006) Fibrosis: improved (*P*=0.03) CG: Steatosis: not improved (*P*=0.75) Fibrosis: not improved (*P*=0.08)	n.r.
5	EG: aerobic exercise (n=19) CG: no intervention (n=21)	EG: –0.55 kg/m^2 (*P*=0.06) CG: –0.25 kg/m^2 (*P*=0.34)	24	2	Initially 30 min, increased every 8 weeks until maximum of 50 min + 5 min warm-up and cool-down	Ranged from ventilatory anaerobic threshold up to 10% below respiratory compensation point	Transient elastography: no significant difference in change in steatosis and fibrosis between EG and CG (*P*-value n.r.)	n.r.

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Table 3. Continued

Study number	Exercise and control group interventions	Mean change in weight or BMI	Exercise Protocol				Non-invasive test outcomes	
			Intervention duration (weeks)	Frequency (sessions per week)	Session duration	Intensity	Imaging-based	Serum-based
6	EG: aerobic exercise (n=18) CG: no intervention (n=10)	Weight: EG: –2.5kg CG: +1.5kg (p<0.01 between EG and CG) BMI: No difference between 2 groups (exact change n.r.)	20	5	30 min	"Moderate" (HR corresponding to 45–55% VO2 peak)	MRI-PDFF EG: Steatosis: improved (*P*=0.02) CG: Steatosis: not improved (*P*=0.94) EG vs. CG: Difference in change in steatosis: *P*=0.01	No difference between EG and CG in change in NFS, FIB-4, APRI, AST/ALT, ADN, or CK-18
7	EG: aerobic and resistance exercise (n=12) CG: no intervention (n=12)	Weight: EG: +1 kg (*P*=0.12) CG: +1 kg (*P*=0.15) BMI: EG: 0 kg/m^2 (*P*=0.12) CG: +1 kg/m^2 (*P*=0.18)	12	3	45–60 min	Aerobic exercise: 16–18 on Borg RPE (very hard) Resistance exercise: 14–16 on Borg RPE (hard)	MRS: EG: Steatosis: improved (*P*=0.04) CG: Steatosis: not improved (*P*=0.08) EG vs. CG: Difference in change in steatosis: *P*=0.02	No difference between EG and CG in change in AST/ALT, NFS, ELF, or CK-18

BMI, body mass index; EG, exercise group; DG, diet group; CG, control group; kg, kilogram; m, meter; min, minute; HR, heart rate; FAST, Fibroscan-AST score; FIB-4, Fibrosis-4 index; n.r., not reported; MRI-PDFF, magnetic resonance imaging-proton density fat fraction; NFS, NAFLD fibrosis score, APRI, AST-to-platelet ratio; ADN, adiponectin; CK-18, cytokeratin 18; RPE, rating of perceived exertion; MRS, magnetic resonance spectroscopy; ELF, enhanced liver fibrosis test.

achieved the recommended ≥7% weight loss for improving histological outcomes in NAFLD.[7] Exercise significantly reduced hepatocyte ballooning (P=0.02) and liver fibrosis (P=0.03) but not steatosis (P=1.0), lobular inflammation (P=0.74), or NAS (P=0.17). Thirteen subjects in the exercise group had biopsy-proven NASH but the study did not report separate results for the NASH cohort or the number of subjects who experienced NASH resolution, and was limited by the lack of a control group.[19]

Two observational studies evaluated the association between exercise intensity and liver fibrosis in biopsy-proven NAFLD.[20,21] In a retrospective cross-sectional study of 813 subjects with biopsy-confirmed NAFLD enrolled in the NASH Clinical Research Network, Kistler et al.[20] found that participants who engaged in ≥75 minutes of vigorous-intensity exercise (metabolic equivalent [MET] value≥6) had significantly decreased odds of having NASH (odds ratio [OR] 0.65; 95% confidence interval [CI] 0.43–0.98) and those who participated in ≥150 minutes of vigorous-intensity exercise had significantly decreased odds of having advanced fibrosis (OR 0.53; 95% CI 0.29–0.97) in multivariate logistic regression analysis adjusting for age, sex, BMI, education, income, and glucose. However, neither moderate-intensity exercise (MET value 3–5.9) nor total volume of exercise was significantly associated with NASH or degree of fibrosis.[20] In another cross-sectional study of 100 participants with biopsy-proven NAFLD, Lahelma et al.[21] demonstrated that increased amount of moderate-to-vigorous activity (MET value>3)—measured by a combination of accelerometer readings and self-report questionnaires—was independently associated with decreased risk of NAFLD fibrosis (OR 0.94; P=0.02).[21] Of note, these studies were limited by cross-sectional study design and self-reported physical activity data potentially leading to misclassification bias.[20,21]

In sum, a total of six studies have analyzed the independent effect of exercise on histological endpoints in biopsy-proven NAFLD, including two RCTs, one NRCT, one uncontrolled trial, and two cross-sectional reports. Notable heterogeneity existed between studies in exercise type, frequency, and duration as well as in supervision level and distinction of NASH from NAFL. Studies similar in design reported concordant histological changes: the two RCTs did not detect significant histological improvement after six-month exercise intervention, whereas reduction of hepatocyte ballooning and fibrosis was reported in the NRCT and uncontrolled trial, both of which implemented an aerobic exercise intervention with nearly identical duration, frequency, and intensity.[16-21]

Impact of exercise on biopsy-proven NAFLD assessed by non-invasive tests

Since the advent of NITs for hepatic steatosis and fibrosis, three RCTs to date have studied exercise-induced changes in non-invasive biomarkers of hepatic steatosis, steatohepatitis, or liver fibrosis in biopsy-proven NAFLD.[22-24] In the first such study published, Rezende et al. used transient elastography as a NIT for liver steatosis and fibrosis. The authors randomly assigned 40 post-menopausal women to 24 weeks of either semiweekly supervised aerobic exercise sessions each lasting 30–50 minutes or no exercise. Neither group achieved a significant reduction in BMI. Aerobic exercise did not significantly improve hepatic steatosis or fibrosis score compared to the non-exercising control group. Of note, the frequency of exercise in this study design was lower compared to that of other study exercise protocols. In addition, steatosis severity was unable to be measured in 30% of study participants due to large body habitus. Nonetheless, this is the only RCT to use transient elastography to analyze the independent effect of exercise on biopsy-proven NAFLD.[22]

In another RCT involving noninvasive biomarkers, Stine et al.[23] compared changes in both liver steatosis quantified by magnetic resonance imaging-proton density fat fraction (MRI-PDFF) and serum biomarkers for liver fibrosis and NASH between exercise and standard of care in 28 participants with biopsy-proven NASH. Exercise intervention consisted of 20 weeks of five 30-minute supervised moderate-intensity aerobic exercise sessions per week. Significantly greater weight loss was observed in the exercise group compared to control group, although there was no significant difference in change in BMI. Exercise significantly decreased MRI-PDFF compared to standard of care (P=0.01). Moreover, forty percent of exercise subjects achieved at least a 30% relative reduction in MRI-PDFF—a commonly cited threshold for surrogate histological response[25]—compared to 13% of control participants (P<0.01). Changes in serum markers for liver fibrosis and NASH, including NAFLD fibrosis score (NFS), fibrosis-4 (FIB-4) index, AST-to-platelet ratio, AST-to-ALT ratio, and exploratory biomarkers adiponectin and cytokeratin 18 (CK-18), were not significantly different between the exercise and

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control group.[23]

Similarly, Houghton et al.[24] investigated the effect of exercise on both hepatic triglyceride content (HTGC) measured by magnetic resonance spectroscopy and serum biomarkers for liver fibrosis and NASH compared to standard of care in 24 participants with biopsy-confirmed NASH. The 12-week exercise intervention in this RCT consisted of a combination of supervised aerobic and resistance exercise three sessions per week, 45–60 minutes per session. Neither the exercise nor control group experienced significant change in weight or BMI. The exercise group achieved significant improvement in HTGC but not in AST-to-ALT ratio, NFS, enhanced liver fibrosis test, or CK-18 relative to the control group.[24]

Two additional NRCTs have investigated the effect of exercise on biopsy-proven NAFLD measured by NITs.[18,19] In the same NRCT as described above, Naimimohasses et al.[18] reported significant improvements in hepatic steatosis and fibrosis scores measured by transient elastography in both the exercise and diet groups after 12 weeks, but not in a standard of care control group. When compared to dietary modification, the exercise intervention led to a greater reduction in both steatosis (13.8% vs. 12.5% reduction) and fibrosis (27.6% vs. 20.8% reduction), although the authors did not state if these differences were statistically significant. For other measured serum NITs, the exercise group did not experience significant change in either the Fibroscan-AST (FAST) score or FIB-4 index, while the diet group achieved a significant improvement in the FAST score but not in the FIB-4 index. Interestingly, the control group saw significant reduction in both the FAST score and FIB-4 index.[18]

In the same study as described above, O'Gorman et al.[19] used transient elastography to measure serial hepatic steatosis and fibrosis scores in two non-randomized groups: (1) 16 participants with biopsy-proven NAFLD (13 with NASH) who underwent a 12-week aerobic exercise program, and (2) eight subjects with biopsy-proven NAFLD (six with NASH) who underwent standard of care. When compared to baseline measurements within the exercise group, both hepatic steatosis and fibrosis scores significantly improved one week following the completion of the exercise intervention, only steatosis score significantly improved three months following the intervention, and neither steatosis nor fibrosis score significantly improved 12 months following the intervention. The authors also assessed group-by-time interactions between the exercise and control groups and found that the change in steatosis was significantly greater in the exercise group at one week following the intervention but not at three or 12 months. No significant difference in the change in fibrosis was observed between the two groups at any of the measured timepoints. Although the exercise and control groups were non-randomized and results for NAFL and NASH were not reported separately, this is the only study to assess whether exercise leads to sustained improvement in steatosis and fibrosis months after the conclusion of an exercise intervention in participants with biopsy-proven NAFLD.[19]

In summary, a total of five studies, including three RCTs and two NRCTs, have analyzed the independent effect of exercise on biopsy-proven NAFLD using NITs for hepatic steatosis, steatohepatitis, or liver fibrosis.[18,19,22-24] All but one study implemented aerobic exercise regimens, with duration of intervention ranging from 12 to 24 weeks.[18,19,22,23] Three studies relied on transient elastography and reported different effects of exercise on hepatic steatosis and fibrosis scores[18,19,22] while the remaining two studies used MRI-based modalities that detected significant improvement in hepatic steatosis following exercise interventions.[23,24] In addition, three studies assessed serum biomarkers and did not report significant exercise-induced changes, although these biomarkers served as secondary outcomes and therefore may have been underpowered.[18,23,24]

DISCUSSION

To our knowledge, this is the first systematic review to examine the independent effect of exercise on hepatic steatosis, steatohepatitis, or liver fibrosis measured by histological assessment or NITs in patients with biopsy-proven NAFLD. Large well-powered studies investigating the impact of exercise on biopsy-proven NAFLD are limited in number. Perhaps most notably, there is no RCT data demonstrating that exercise independently improves NASH or NASH-related fibrosis assessed by histological evaluation, in contrast to the numerous RCTs and meta-analyses confirming a causal relationship between exercise and reduction of imaging-based measures of hepatic steatosis.[9-14] Although four studies suggest that exercise may improve specific histological features such as fibrosis and hepatocyte ballooning, these have important methodologic limitations such as self-reported physical activity data, lack of a control group, or non-randomized study

design.[18-21] The two published RCTs reported that in the absence of weight loss or dietary modification, exercise failed to significantly improve histological markers of NAFLD. However, given the limited statistical power of these RCTs, an independent effect of exercise on biopsy-proven NAFLD cannot be excluded.[16,17]

Exercise has been proposed to independently target key metabolic and inflammatory pathways implicated in the development and progression of NAFLD.[26] For example, exercise may reduce hepatic steatosis by upregulating peroxisome proliferator-activated receptors and adiponectin levels which in turn improves insulin resistance and lipolysis.[27,28] Previous studies have also demonstrated the inhibitory effect of exercise on inflammatory mediators, such as interleukin-1 beta and tumor necrosis factor-a, involved in the pathogenesis of hepatocellular injury and fibrosis.[27,29] Understanding whether exercise in the absence of other lifestyle modifications adequately achieves histological improvement not only holds important clinical implications in the current management of NAFLD but is also relevant for the interpretation of future clinical trials evaluating novel investigational therapies. Carefully designed and adequately powered RCTs are needed to address the independent effects of exercise form, duration, and intensity on histological endpoints.

The challenges of conducting trials involving histological evaluation should be acknowledged, especially with regards to limited study recruitment and loss of follow-up associated with serial liver biopsies. In cases where histological assessment is unfeasible, NITs for NAFLD may serve as alternative endpoints. To date, five studies including three RCTs have used imaging or serum NITs in biopsy-proven NAFLD. These studies demonstrated that while exercise significantly reduces MRI-quantified hepatic steatosis, its effect on steatosis and fibrosis estimated by transient elastography remains unclear.[22-24] The different findings between these two imaging techniques may be explained by greater accuracy of MRI-based modalities in detecting steatosis and fibrosis compared to transient elastography.[30,31] Serum markers of fibrosis and exploratory biomarkers for NASH were also studied as secondary outcomes in three studies, and were not found to be significantly improved by exercise.[18,23,24] As the prevalence of NITs for detecting liver fibrosis and diagnosing NASH increases in the clinical setting, future studies involving exercise interventions should too incorporate commonly used NITs to improve applicability of findings.

Although physical activity is associated with lower all-cause mortality in NAFLD and reduced risk of hepatocellular carcinoma in the general population,[32,33] there are no studies published to date that have investigated the effect of exercise on key clinical endpoints in NASH, including progression to cirrhosis and liver-related mortality. The lack of literature on these endpoints is unsurprising as measuring these outcomes often require long-term follow-up potentially leading to high attrition rates. In addition, studies have not shown sustained benefits of exercise on hepatic steatosis and fibrosis in NAFLD following the completion of exercise interventions.[19,34] Given the difficulty of implementing strictly supervised exercise programs for a prolonged duration, establishing methods of transitioning exercise interventions to the community setting to promote long-term exercise adherence may benefit patients with NAFLD. Furthermore, exercise is also only one subset of physical activity, and other types of physical activity, known as non-exercise activity thermogenesis, may be considered as additional interventions.

We acknowledge several limitations of our systematic review, including the lack of meta-analysis and formal risk-of-bias assessments of eligible studies. In addition, exploring the relationship between exercise and other outcomes such as inflammatory markers, metabolic alterations, and cardiorespiratory fitness fell outside the scope of our review. Nonetheless, we demonstrated the need for larger interventional trials to investigate the independent effect of exercise on hepatic steatosis, steatohepatitis, and liver fibrosis as well as key clinical endpoints in biopsy-proven NAFLD.

Authors' contribution

GC, BB, AD, and JL contributed to the conceptualization and methodology of the review. GC conducted the literature search, extracted and interpreted data, and drafted the manuscript. GC, BB, AD, and JL critically revised the manuscript. JL supervised the study. All authors approved the final version of the manuscript.

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Conflicts of Interest

The authors have no conflicts to disclose.

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Types of Manuscripts

Contributions may be submitted as original articles, review articles, editorials and special topics. Special topics cover guidelines, meeting reports and hepatology issues elsewhere. Review articles, editorials and special topics are invited by the editorial board. However, authors who are interested in contributing reviews can submit reviews and are subjected to peer review. Letters to the editor may be subjected to peer review and undergo editing for clarity and brevity.

Ethical Conduct of the Study and the Report

All investigations involving human participants must be conducted according to the ethical guidelines of the Declaration of Helsinki, and be approved by the institutional review board. For studies involving animal experimentation, author(s) must provide assurance that all the animals received humane care according to the criteria outlined in the NIH "Guide for the Care and Use of Laboratory Animals". The author must state that the use of animals (means all mammals and birds) in the manuscript was approved by the institutional Animal Ethical Committee (AEC) in accordance to the article 14th of Korean Animal Protection Law, or equivalent, in the paper. It must be clearly stated that animal use has complied to the article 13th of Korean Animal Protection Law (The principles of animal use) and the relevant institutional polices in the manuscript. Copies of the protocol approved by institutional AEC or equivalents, must be available for review by the editor if necessary.

The corresponding author must give written assurance that neither the submitted material nor portions thereof have been published previously or are under consideration for publication elsewhere. Any material that could constitute prior or concurrent publication of similar data by any one of the authors should be submitted with the manuscript. It is assumed that the corresponding author speaks for his or her co-authors and certifies that all the listed authors meaningfully participated in the study and that they have seen and approved the final manuscript.

Authors should acknowledge any commercial affiliation or consultancy that could be constructed as potential conflicts of interest under a heading "Conflict of Interest statement" prior to the references.

For the policies on the research and publication ethics not stated in this instructions, 'Good Publication Practice Guidelines for Medical Journals (https://www.kamje.or.kr/board/view?b_name=bo_publication&bo_id=7&per_page=)' or 'Guidelines on good publication (http://www.publicationethics. org.uk/guidelines)' can be applied.

Ensure correct use of the terms sex (when reporting biological factors) and gender (Identity, psychosocial or cultureral factors), and, unless inappropriate, report the sex and/or gender of study participants, the sex of animals or cells, and describe the methods used to determine sex and gender.

If the study was done involving an exclusive population, for example in only one sex, authors should justify why, except in obvious cases, (e.g., prostate cancer).

Authors should define how they determined race or ethnicity and justify their relevance.

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The manuscript should be written in A4 (21×30 cm) paper in double space texts by leaving 3 cm space in the right, left, top and bottom sides at 10 point fonts.

Original articles

Original articles describing clinical and basic studies in the field of hepatology. Manuscripts are expected to be well-organized and clearly written. They should not exceed 6,000 words, including the abstract, references, tables, and figure legends. No more than 8 figures and tables, with a maximum of 6 panels per figure. It is permitted for you to submit additional methodological details, non-essential figures or portions of your manuscript as supplementary material for online publication only. References cited in the main text may not be listed in the supplementary materials. The only references be listed in the supplement are those cited exclusively in the supplement. References should not exceed a maximum of 50.
Original article must arranged as follows: (1) title page (2) abstract (250 words or less with a list of 5 or less key words), (3) introduction, (4) materials and methods (or patients and methods), (5) results, (6) discussion, (7) acknowledgements, (8) conflict of interest statement (9) references, (10) tables, and (11) figure legends.
In case of submission of original articles (not applicable for reviews, editorials, and letters), authors should summarize the contents of the article in a concise, pictorial form designed to easily understand main findings of the work described in the article. Graphical abstracts should be submitted as a separate JPG or TIFF files at the online submission step of file upload. The submission of the graphical abstract is mandatory when submitting an original article. Graphical abstracts should be provided as an image with a minimum size of 531 × 531 pixels (height × width) using a minimum resolution of 600 dpi. When submitting a larger image, please make sure to use the same ratio. Also, please note that your image will be scaled proportionally to fit in the available window, which is a rectangle with a size of 200 × 500 pixels.

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Review articles on selected topics of interest for the readers of ***the Clinical and Molecular Hepatology*** and will be solicited by the Editors. Review articles are expected to be clear, concise and updated. The maximum length is 5,000 words. The inclusion of a maximum of 8 high quality tables and/or colored figures to summarize critical points is highly desirable.

Editorials

This section consists of invited brief editorial comments on articles published in ***the Clinical and Molecular Hepatology***. The length of an editorial should not exceed 1,500 words and 1 table or 1 figure is allowed. References should not exceed a maximum of 20.

Letters to the editor

Letters to the editor should be related to a recent article published in ***the Clinical and Molecular Hepatology*** within previous two years. Letters to the editor must arranged as follows: (1) title page, (2) body (3) references (maximum of 15), and (4) a maximum number of 1 tables or figures is allowed. The length of an letter to the editor should not exceed 800 words, and the maximum number of authors is 6. Abstract is not required.

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The correspondence consists of replies on editorials from the authors of the original publication in ***the Clinical and Molecular Hepatology***. The length of an correspondence should not exceed 1,500 words and 1 table or 1 figure is allowed. References should not exceed a maximum of 15. Correspondence letters are not usually peer reviewed, but we might invite replies from the authors of the original publication.

Special topics

Special topics should be no longer than 800 words with 10 or less references.

Snapshot

Snapshot consists of a large single page figure with schematic diagrams and tables that graphically summarize current knowledge about a particular subject within the field of hepatology. A detailed figure legend which includes all relevant information can be included and may be incorporated into the main figure. The figure is accompanied by a short summary article that should not exceed a maximum of 600 words. References should not exceed a maximum of 10. The snapshot should contain a descriptive title.

1. Title page

Provide a concise title. List the full names of all authors and their institutional affiliation. In a multi-authored work involving more than a single institution, indicate individual affiliation by means of superscript Arabic numbers. Indicate a change of address in a similar fashion. List the footnotes to the title page. Provide the contact information for the corresponding author (name, address, telephone number, fax number, e-mail address and Orcid ID), and running title (Less than 50 characters). All abbreviations should be explained in this page (e.g. AFP, alpha fetoprotein; ALT, alanine aminotransferase). ***The Clinical and Molecular Hepatology*** employs a system to screen plagiarism (CrossRef). When submitting your manuscript to this journal, you accept that your manuscript may be screened for plagiarism against previously published material.

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Abstract of original articles must contain 250 words or less and must be organized as follows: Background/Aims, Methods, Results, and Conclusions. Three to Five keywords should be provided at the end of the abstract.

3. Highlight

Authors of original articles are requested to include "Highlights" which consist of three to four sentences summarizing the originality and main findings of the article. "Highlights" should not exceed 100 words in total. Highlights must be organized in a box and placed after the end of the abstract. The authors are encouraged to include the "Highlights" with initial article submission. When submitting a revised manuscript, the submission of the "Highlights" is mandatory.

4. Introduction

Provide the minimum background information that will orient the general reader. Do not engage in a literature review.

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Provide a level of detail such that another investigator could repeat the work. For methods that are used without significant modification, citation of the original work will suffice. Identify and provide references for all the statistical methods used.

6. Results and discussion

Present the major findings of the study in graphical form if practicable. Do not illustrate minor details if their message is adequately conveyed by simple descriptive text. Mention all the tables and figures. In the discussion, concisely present the implications of the new findings for the field as a whole, minimizing any reiteration of the results and avoid repetition of material in the introduction; keeping a close focus on the specific topic of the paper.

7. Acknowledgements

An acknowledgement of persons who made a genuine assistance and provided special reagents may be included. Grant and financial support related with the work should be specifically stated.

8. Authors' contribution

Based on the ICMJE guidelines for authorship criteria, how each author has contributed to the paper should be clarified (e.g, Conception or design of the work, Data collection, Data analysis and interpretation, Drafting the article, Critical revision of the article, and Final ap-

proval of the version to be published).

9. References

References should be numbered in the order they are cited, and the number of reference should be marked in the text by means of a superscript Arabic numerical. Only literature that is published or in press (with the name of the publication) may be numbered and listed; abstracts and letters to the editor may be cited. Cite the names of all authors when there are six or less; when seven or more list the first six followed by et al.

Articles in journals

1. Kim YS, Jung ES, Hur W, Bae SH, Choi JY, Song MJ, et al. Noninvasive predictors of nonalcoholic steatohepatitis in Korean patients with histologically proven nonalcoholic fatty liver disease. Clin Mol Hepatol 2013;19:120-130.
2. Chung C, Iwakiri Y. The lymphatic vascular system in liver diseases: its role in ascites formation. Clin Mol Hepatol 2013;19:99-104.

Literature in press

An online article that has not yet been published in an issue can be cited by its Digital Object Identifier (DOI). The DOI will remain valid and allow an article to be tracked even after its allocation to an issue.
Wong GL. Management of chronic hepatitis B patients in immunetolerant phase: what latestguidelines recommend. Clin Mol Hepatol. 2018 Jan 22. doi: 10.3350/cmh.2017.0068.

Book chapters

1. Gumucio JJ, Berkowitz CM. Structural organization of the liver and function of the hepatic acinus. In: Kaplowitz N, ed. Liver and Biliary Diseases. Vol I. 2nd ed. Baltimore: Williams & Wilkins, 1992:2-17.

Abstract or Article in a supplement

1. Cho YJ, Lee SH, Kim BH, Yang SK, Jo YH, Lee DH. Characteristics of hepatocellular carcinoma with reference to ages in Korean patients [Abstract]. Hepatology 1998;28:246A.
2. Bellentani S, Marino M. Epidemiology and natural history of non-alcoholic fatty liver disease (NAFLD). Ann Hepatol 2009;8 (Suppl 1):S4-S8.

Websites

1. Ontario Chronic Disease Prevention Alliance (OCDPA). Economic cost of chronic disease in Canada 1995-2003. OCDPA web site, <http://www.ocdpa.on.ca/OCDPA/docs/OCDPA_EconomicCosts.pdf>. Accessed 7 Sep 2011.

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Please include an alphabetical list of all non-standard abbreviations used within the manuscript. Please do not abbreviate unless a term is used more than five times in a paper. In this case, the abbreviation should be spelled out, in its first use in the text with the abbreviated form in parentheses, and it should also be listed on the footnote page. Abbreviations used in figures or tables should be defined in the legend.

12. Drug names

Use generic names. The proprietary name may be mentioned in parenthesis. The names and locations (city and state or country) of manufacturers should be included in parentheses when mentioning proprietary drugs, tools, instruments, software, etc.

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Prepare tables on individual sheets of paper, double spaced and numbered consecutively with Arabic numerals in the order of their appearance in the text. The title of tables should be written concisely in clauses and phrases. The first letter of the table title starts with a capital letter. Explain all abbreviations and symbols such as *, †, ‡, §, ‖, **, ††, ‡‡, §§. Do not duplicate the material presented in a figure.

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Number the figures with Arabic numerals in the order they are mentioned in the text. Provide a title (this should not appear on the figure itself) and sufficient explanation to render the figure intelligible without reference to the text. For any copyrighted material, indicate that permission has been obtained (see Permissions, above). Figure legends should be typed consecutively on a separate sheet of paper.

15. Figures

Illustrations should be sharp and clear. Figure files can be uploaded in the JPG or TIFF formats which authors prefer at a final resolution of not less than 300 dpi. Microscopic pictures should be explained according to the staining method and scaled by the power of magnification. Authors are charged for color figures.

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The journal utilizes blind peer-review in evaluating manuscripts for publication. Submitted papers will be reviewed by at least two referees, and decisions will be available in approximately one months. With respect to the revision and resubmission of manuscripts, it is the journal's policy to allow a couple of resubmission only, which should be received within 2 months from the time of receipt of the initial review letter. In general, a manuscript requiring more than a couple of revision or returned beyond 2 months will be handled as a new submission. The journal does not have article submission charges.

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Please read this checklist carefully to ensure that your manuscript is complete and in compliance with the CMH Guide for Authors.

1) General Format	Yes	No
[1] Did you have the title page, abstract, the text (introduction, materials and methods, results, and discussion), acknowledgements, conflict of interest statement, references, tables, and legends for figures?	☐	☐
[2] Is the manuscript double-spaced in an A4-size paper?	☐	☐
[3] The manuscript of special topics should not be longer than 800 words.	☐	☐
[4] The number of authors for letters to the editor must not exceed 6.	☐	☐

2) Abstract	Yes	No
[1] Abstract must contain 250 words or less and must be organized as follows: Backgrounds/Aims, Methods, Results, and Conclusions.	☐	☐
[2] Five or less key words should be provided at the end of the abstract.	☐	☐

3) Introduction, Methods, Results, Discussion, Acknowledgements, Conflict of Interest Statement, References	Yes	No
[1] Identify the committee(s) approving the study protocol and include a statement of compliance with ethical regulations.	☐	☐
[2] An acknowledgement of persons who made a assistance and provided special reagents may be included. Grant and financial support related with the work should be specifically stated.	☐	☐
[3] Please state any conflicts of interest.	☐	☐
[4] All citations in the paper have a complete and accurate reference in the reference list. The number of references in special topics should be 10 or less.	☐	☐

4) Tables and Figures	Yes	No
[1] Prepare tables on individual sheets of paper, double spaced and numbered consecutively with Arabic numerals in the order of their appearance in the text.	☐	☐
[2] Explain all abbreviations and symbols.	☐	☐
[3] Figure legends should be typed consecutively on a separate sheet of paper.	☐	☐
[4] Figures should be supplied in the JPG or TIFF format at a final resolution of 600 dpi or higher.	☐	☐