FATTY LIVER DISEASE in the GENERAL POPULATION

REDEFINING, EARLY DETECTION AND DISEASE MANAGEMENT

LAURENS VAN KLEEF

Fatty liver disease in the general population

Redefining, early detection and disease management

Laurens Adriaan van Kleef

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Fatty Liver Disease in the General Population

Redefining, early detection and disease management

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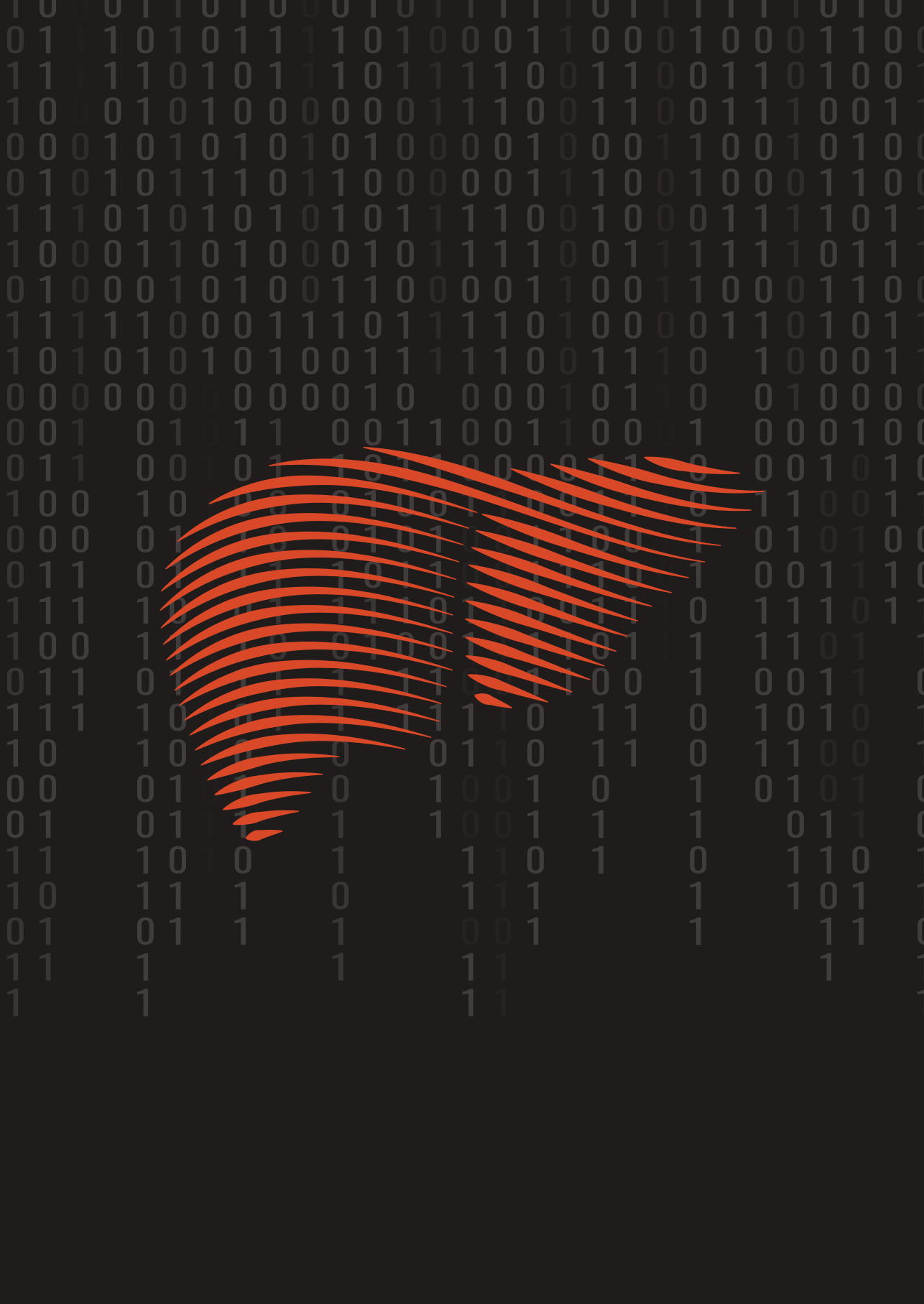
TABLE OF CONTENTS



CHAPTER 1

Introduction

CHAPTER 1.1

General introduction

Disease burden of fatty liver disease

Fatty liver disease is the accumulation of fat in the liver, affecting over 5% of hepatic cells.[1] It has become increasingly prevalent over the last decades, subsequent to the rapid increase of adiposity and metabolic dysfunction.[2, 3] Studies estimated the global prevalence of fatty liver disease to be exceeding 25%, thereby it is now the most common chronic liver disease.[3, 4] Focusing only on data published in 2016 and onwards, the prevalence has even increased to 33.8%, indicating the ongoing extent of the fatty liver disease pandemic.[3]

Fatty liver disease encompasses a spectrum of diseases, as shown in **Figure 1**. The initial phase is steatosis which can progress into steatohepatitis, which is histologically characterised by lobular inflammation and hepatocyte ballooning.[5-7] Steatosis and steatohepatitis can induce liver injury, resulting in scar tissue accumulation. This scar tissue is known as fibrosis and has several stages (F1 - F3). As scar tissue further accumulates, this can develop into end-stage liver disease: cirrhosis (F4). Typically, 10-20% of individuals with fatty liver disease will develop fibrosis, and of them, 20% will develop cirrhosis.[8-10] This last stage of fatty liver disease is strongly associated with hepatocellular carcinoma.[11] However, of particular concern, not only those with cirrhosis are at increased risk of primary liver cancer, but also individuals with steatohepatitis and fibrosis.[12]

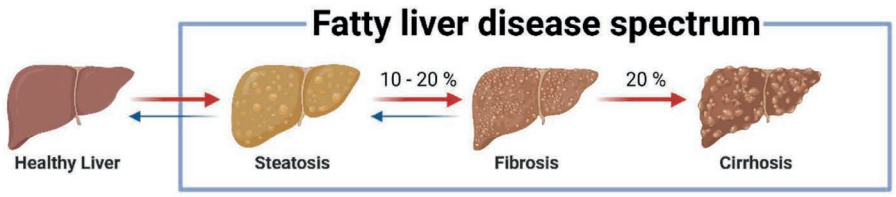


Figure 1: Overview of the fatty liver disease spectrum.

Exposure duration to steatosis and steatohepatitis is one of the main risk factors for progression towards more severe stages of fatty liver disease. For example, it takes up to 14 years to develop one stage of fibrosis, with only half of that among individuals with steatohepatitis.[13] Fortunately, steatosis and steatohepatitis are reversible. After the regression of fatty liver disease, the risk of advanced liver disease attenuates. Interestingly, even fibrosis is not permanent and can regress in the years after steatosis regression in contrast to prior beliefs.[14, 15]

Although the risk of cirrhosis may be relatively small, given the high prevalence of fatty liver disease, the absolute numbers with end-stage liver disease are particularly worrisome. Notably, fatty liver disease has become the second leading indication for liver transplantation in the United States and similar trends are expected in Europe.[16, 17] Besides the clinical burden, NAFLD also has an enormous economic burden. Currently, €35 billion is spent annually in France, Germany, Spain, and the United Kingdom combined and $103 billion in the United States, related to the direct healthcare costs of fatty liver disease.[18]

While other major causes of death are declining in Europe, there is a growth of years of working life lost to liver cirrhosis and primary liver cancer.[19] In fact, there has been a 25% increase in deaths due to chronic liver disease and a 70% increase in primary liver cancer since 1990.[20] In order to turn the tides, the European association for the study of the liver (EASL) and Lancet started a collaboration and provided a set of recommendations for clinicians but also for policymakers to reduce the burden of liver disease, focusing on the reduction of alcohol consumption and improvements of metabolic health.[21]

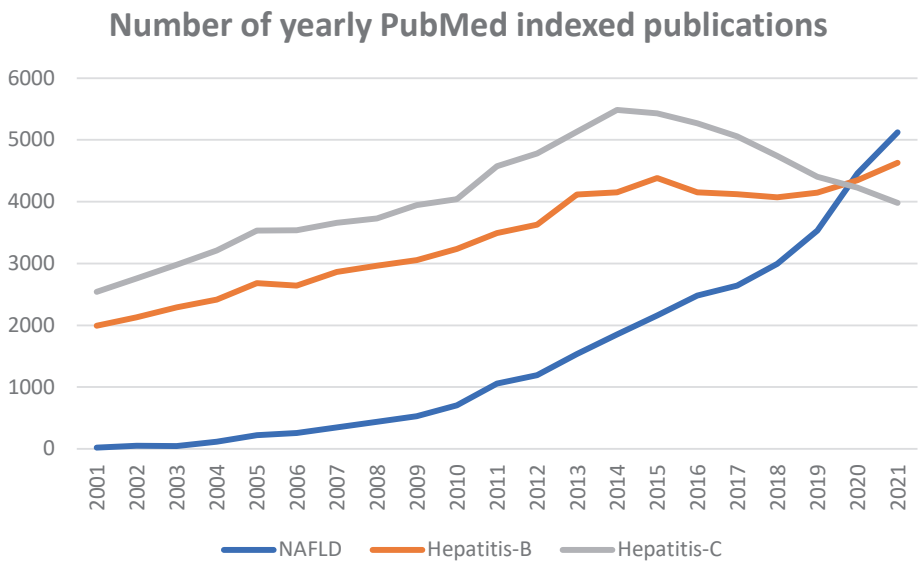


Figure 2: Yearly PubMed-indexed publications for NAFLD, Hepatitis-B and Hepatitis-C for the last 20 years.

Altogether, fatty liver disease has emerged as a global health concern and, consequently, has become an important research topic. This importance is illustrated by the number of peer-reviewed publications per year regarding non-alcoholic fatty liver disease (NAFLD) on PubMed each year, which rapidly expanded from less than 100 in 2003 to over 5.000 in 2021, exceeding other major hepatology domains such as hepatitis B or hepatitis C since 2020 (**Figure 2**).

Aetiology

A classical dichotomy in fatty liver disease is alcohol-related fatty liver disease (ARLD) and non-alcoholic fatty liver disease (NAFLD). In a real-world setting, however, the difference seems to be more gradual (**Figure 3**).[22] Although alcohol indeed induces hepatic steatosis, there is also a more devastating hepatoxic effect of its metabolites (e.g., acetaldehyde), resulting in a different disease course compared to NAFLD.[23] Therefore, excessive alcohol consumption has been an exclusion criteria for NAFLD, together with other secondary causes for steatosis, such as viral hepatitis and specific steatosis-inducing drugs.[1] However, the cut-off for daily alcohol consumption is arbitrary and the impact of alcohol on steatogenesis can be considered a gradual effect. Potential interactions between alcohol consumption and other causes for steatosis on adverse outcomes have not yet been unravelled. Commonly applied cut-offs to define ARLD range between 10-20 grams per day for female and 20-30 grams per day for male.[1, 24, 25]

Figure 3: Alcohol consumption in fatty liver disease. The gradient reflects an increase in daily alcohol consumption. It is arbitrary where NAFLD ends and ARLD starts.

A common denominator – although not required – of this selected group by the NAFLD criteria is the presence of metabolic dysfunction. For example, dyslipidemia, obesity, metabolic syndrome and diabetes are present in 69.1%, 51.3%, 42.5% and 22.5%, respectively.[4] Moreover, the mean BMI of 26.2 kg/m^2 in Asians and 29.9 – 31.0 kg/m^2 in non-Asians indicates that the vast majority of NAFLD patients are overweight, which illustrates the close relationship between metabolic dysfunction and fatty liver disease.[3] Hence, fatty liver disease is considered to be the hepatic expression of the metabolic syndrome.[26]

Nomenclature

Over the past years, there has been increasing support for redefining and renaming NAFLD. One of the main concerns with the conventional definition is the exclusion-based design that does not acknowledge fatty liver disease's primary drivers. Furthermore, it does not allow for the co-existence of 'NAFLD' and moderate-to-excessive alcohol consumption in individuals with metabolic dysfunction, while the latter may have caused or attributed to the disease.[27] Moreover, from the patients' perspective, the term 'non-alcoholic' is not informative and links the disease with alcohol which can be stigmatising and misleading.[28] Therefore, a new definition, metabolic dysfunction-associated fatty liver disease (MAFLD), has been proposed by a group of experts in 2020.[29] The differences have been outlined in **Figure 4**. This novel inclusion-based diagnosis requires the presence of steatosis together with metabolic dysfunction and, importantly, does not exclude individuals with secondary causes for steatosis. This metabolic dysfunction was defined as either overweight, diabetes, or a combination of at least two minor criteria, such as hypertension, dyslipidemia or high waist circumference. The specific focus of this thesis is NAFLD and MAFLD, rather than ARLD.

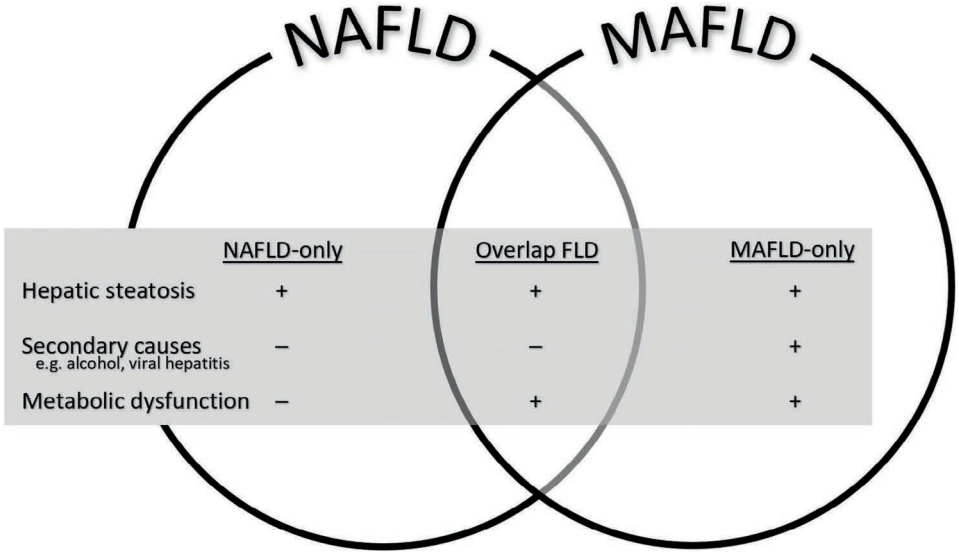


Figure 4: Differences between the definitions of NAFLD and MAFLD

Pathophysiology

Insulin resistance has a crucial role in the pathogenesis of fatty liver disease and the activated pathways may explain both hepatic and extrahepatic comorbidity and mortality.[30-32] Insulin resistance results in increased glucose production in liver cells (gluconeogenesis), inhibits glucose uptake in muscle cells, and stimulates lipolysis in fat cells.[31, 33, 34] Subsequently, there is an influx of free fatty acids in the liver, which inhibits the clearance of insulin and stimulates the synthesis of triglycerides and VLDL, which all contribute to hepatic steatosis.[35]

Another consequence of hepatic steatosis, free fatty acid overload and hyperglycemia is the increase in oxidative stress, accompanied by pro-inflammatory cytokine release. This cascade induces liver cell injury, apoptosis, and activated macrophages (Kupffer cells). In this process, hepatic stellate cells transform into myofibroblasts, in which form they produce more extracellular matrix than is degraded.[35-37] Interestingly, mitochondrial dysfunction co-occurs with the hepatic stellate cell transformation and activation. There is even evidence that mitochondrial dysfunction drives or accelerates fibrogenesis.[38] Since mitochondrial dysfunction plays a role in fibrosis throughout the body, therapies for fibrotic diseases are expected to find a breakthrough in mitochondria.[39]

The potential adverse effects might not be limited to liver injury. The effects of oxidative stress and pro-inflammatory state may contribute to an increased risk of comorbidity and all-cause mortality among individuals with fatty liver disease.[40] A range of diseases have been linked to fatty liver disease, among others, cardiovascular disease, kidney function impairment and neurocognitive decline as well as all-cause mortality.[32, 41-45] Furthermore, several malignancies are more frequent among those with fatty liver disease.[46] Whether this increased risk of comorbidity and mortality is driven by fatty liver disease or explained by shared risk factors and common pathophysiological changes remains unclear for most of these associations.

Non-invasive tests

Fortunately, most individuals with fatty liver disease will not progress to advanced liver disease and do not encounter any symptoms. Therefore, it is particularly challenging to identify those with more advanced fatty liver disease, which is the population that could benefit from hepatologist consultation. Although liver biopsy

is the gold standard for assessing steatohepatitis and fibrosis, it is an invasive approach and one can encounter serious complications.[47] As the actual risk of advanced liver disease in patients with fatty liver disease is relatively low, it is only recommended to perform liver biopsy in the presence of other signs of advanced liver disease.[24] In the current era, several non-invasive tests have become available to assess liver health and aid clinicians in selecting patients for further work-up, which may then include liver biopsy.

Table 1. Summary of biomarker-based non-invasive tests for fibrosis

	Details	AUC
FIB-4	Age, AST, Platelets, ALT	0.76
NFS	Age, BMI, impaired fasting glucose/diabetes, AST, ALT, Platelet, Albumin	0.73
AST/ALT ratio	AST ALT	0.64
APRI	AST, Platelets	0.70
FibroTest	α2 macroglobulin, Haptogloublin, GGT, Age, Bilirubin, Apolipoprotein A1	0.77
ELF-test	Type III procollagen peptide, hyaluronic acid and tissue inhibitor metalloproteinase-1	0.81

Abbreviations: ALT, Alanine transaminase; AST, aspartate aminotransferase; APRI, AST platelet ratio; AUC, area under the receiver operating characteristic curve; BMI, body mass index; ELF, enhanced liver fibrosis; FIB-4, fibrosis-4 index; NFS, NAFLD fibrosis score.

Biochemical assessment of liver fibrosis generally includes alanine transaminase (ALT), aspartate aminotransferase (AST) and, gamma-glutamyl transpeptidase (GGT), together with markers of liver function (INR, albumin) and portal hypertension (platelet count). Several research groups have combined these (or a subset of these) parameters into a biomarker-based non-invasive test to improve prediction accuracy (**Table 1**).[48-52] The most commonly used are FIB-4 (AUC 0.76) and NAFLD fibrosis score (NFS) (AUC 0.73) as they seem to have better accuracy as compared to AST/ALT ratio (AUC 0.64) and AST to platelet ratio (APRI) (AUC 0.70).[53] With the identification of novel promising biomarkers for fibrosis (e.g. hyaluronic acid, tissue inhibitor of metalloproteinases-1 [TIMP-1], type III collagen and a range

of globulins), new algorithms were developed to predict fibrosis. FibroTest and enhanced liver fibrosis test (ELF-test), for example, are encouraging new algorithms that seem to have better diagnostic performance (AUC 0.77 and 0.81).[54, 55] However, the tests are costly and patented, limiting their widespread use.

Table 2. Summary of imaging-based non-invasive tests for fibrosis

	Details	AUC
VCTE	Vibration-controlled transient elastography	0.85
pSWE	Point shear wave elastography	0.86
2D SWE	Two-dimensional shear wave elastography	0.75
MRE	Magnetic resonance elastography	0.92

Abbreviations: 2D-SWE, 2-dimensional shear wave elastography; AUC, area under the receiver operating characteristic curve; MRE, magnetic resonance elastography; pSWE, pulse shear wave elastography; VCTE, vibration controlled transient elastography.

The other group, imaging-based non-invasive tests (**Table 2**), assesses the presence of fibrosis by measuring liver stiffness. As fibrogenesis continues, scar tissue will accumulate, resulting in increased stiffness.[56] However, it should be noted that stiffness may also increase by inflammation and congestion (either due to biliary, portosystemic or central venous cause).[57-59] A technique enabling the quantification of liver stiffness is elastography, which can be part of ultrasound (pulse shear wave elastography [pSWE], vibration controlled transient elastography [VCTE] or 2-dimensional SWE [2D SWE]) or magnetic resonance imaging (MRI) with elastography assessment (MRE). These techniques have good accuracy (AUC ±0.85 and 0.92, respectively) and outperform traditional biomarker-based non-invasive tests regarding accuracy.[53, 60, 61] While MRE is still not widely available, elastography is increasingly common on regular ultrasound devices, increasing the availability of imaging-based biomarkers to clinicians.

Despite several good tests being available for the non-invasive assessment of fibrosis, options are scarce for steatohepatitis. Notably, the available non-invasive tests actually focus on the simultaneous presence of steatohepatitis and fibrosis (**Table 3**). Examples are the NIS-4 and MACK-3 score, which are biomarker-based algorithms that have good accuracy (both AUC 0.80).[62, 63] However, it should be

noted that elastography alone had similar accuracy limiting its additional value besides the assessment of fibrosis. The Fibroscan-AST (FAST)-score is a combination of liver stiffness, controlled attenuation parameter (CAP) and serum AST concentrations and was also designed to predict steatohepatitis with fibrosis. This score yielded similar results (AUC 0.74 – 0.95) as biomarker-based algorithms.[64] An MRI-based variant of the FAST-score, MAST-score, yielded a slightly better AUC of 0.86 in the derivation and 0.93 in the validation cohort.[65] These tests should be further validated and their value over the non-invasive assessment of fibrosis should be evaluated.

Table 3. Summary of non-invasive tests for steatohepatitis

	Details	AUC
NIS-4	miR-34a-5p, alpha-2 macroglobulin, YKL-40, HbA1c	0.80
MACK-3	AST, HOMA-IR, CK18	0.80
FAST-score	Liver stiffness, CAP and AST	0.85
MAST-score	MRI PDFF, MRE, AST	0.86 – 0.93

Abbreviations: AST, aspartate aminotransferase; AUC, area under the receiver operating characteristic curve; CAP, controlled attenuation parameter; HOMA-IR, homeostatic Model Assessment of Insulin Resistance; MRE, Magnetic Resonance Elastography; MRI PDFF, Magnetic Resonance Imaging Proton Density Fat Fraction

Given that the options are plenty, the EASL has composed a working group on non-invasive tests for liver disease. In their 2021 update, they recommended assessing fibrosis among those with risk factors for chronic liver disease (which included metabolic dysfunction) with FIB-4 or transient elastography if available. On the other hand, adopting non-invasive tests to assess steatohepatitis seemed premature.[66] Interestingly, confirmation with liver biopsy of fibrosis as assessed by elastography is no longer recommended in case patented tests are also indicative of fibrosis. This underscores the important role non-invasive tests have taken in the field of hepatology.

Disease management

There is an urgent need for pharmaceutical treatment, especially to treat steatohepatitis, but also in the prevention and treatment of fibrosis. Despite the

lack of available treatment, guidelines already recommend only treating patients with advanced disease, thus at least in the presence of steatohepatitis or fibrosis.[1, 24] At this moment, several novel agents are being investigated, with over 100 ongoing clinical trials (at time of printing this thesis). The most promising and best investigated novel drugs to date target the peroxisome proliferator-activated receptors (PPAR) or farnesoid X receptor (FXR).[67, 68] Although FXR is predominantly involved in the metabolism of bile acids while PPAR targets fatty acids, they both play an important role in lipid and lipoprotein metabolism. Interestingly, FXR can upregulate PPAR, hence a synergistic effect of dual FXR and PPAR therapy is also being investigated.[69]
In addition to these novel developed drugs, several studies showed benefits of anti-diabetic (e.g. sodium-glucose transport protein-2 [SGLT2] inhibitors and glucagon-like peptide-1 [GLP-1] receptor agonists) or lipid-lowering treatment (statins) for the severity of fatty liver disease.[70-73] However, the evidence is segmented and inconclusive. Therefore, current guidelines do not recommend the prescription of these drugs beyond their original indication.[1, 24] Nonetheless, proper treatment of diabetes and dyslipidemia in patients with fatty liver disease may be beneficial in preventing cardiovascular complications as well as NAFLD disease progression.

As there is currently no pharmaceutical treatment approved to treat fatty liver disease, disease management focuses on weight loss and improvements in metabolic health. Fortunately, fatty liver disease progression may halt when metabolic health improves. For example, NAFLD and fibrosis can even regress if a 5-10% weight reduction is accomplished.[74, 75] In a study context with typically strict monitoring, several lifestyle-intervention programs turned out to be very effective in preventing or treating fatty liver disease.[74, 76] However, the long-term outcomes of these interventions are directly related to the adherence to this new lifestyle, which turned out to be often suboptimal, limiting its applicability in clinical practice.[77, 78]

Current guidelines recommend structured programs aimed at lifestyle changes towards a healthy diet and habitual physical activity, a diet adjusted according to the macronutrient composition of the Mediterranean diet, as well as a 7-10% weight loss among overweight fatty liver disease patients.[1] Although the aims of these recommendations are clear, achieving them in real-world settings appears to be difficult.

Summary

Fatty liver disease has become the most prevalent chronic liver disease exceeding 25% and although most patients will not encounter any symptoms, fatty liver disease is expected to be the leading cause of advanced liver disease in the coming decades. The proposed name and definition change of NAFLD into MAFLD seems appropriate but requires further investigation. Moreover, an important challenge to overcome is the identification of individuals with fatty liver disease who require further attention and may benefit from pharmaceutical treatment when available. Last, given the coherence with metabolic dysfunction and lifestyle, future studies should focus on how they can contribute to specific evidence-based recommendations for future guidelines.

CHAPTER 1.2

Aims and outline

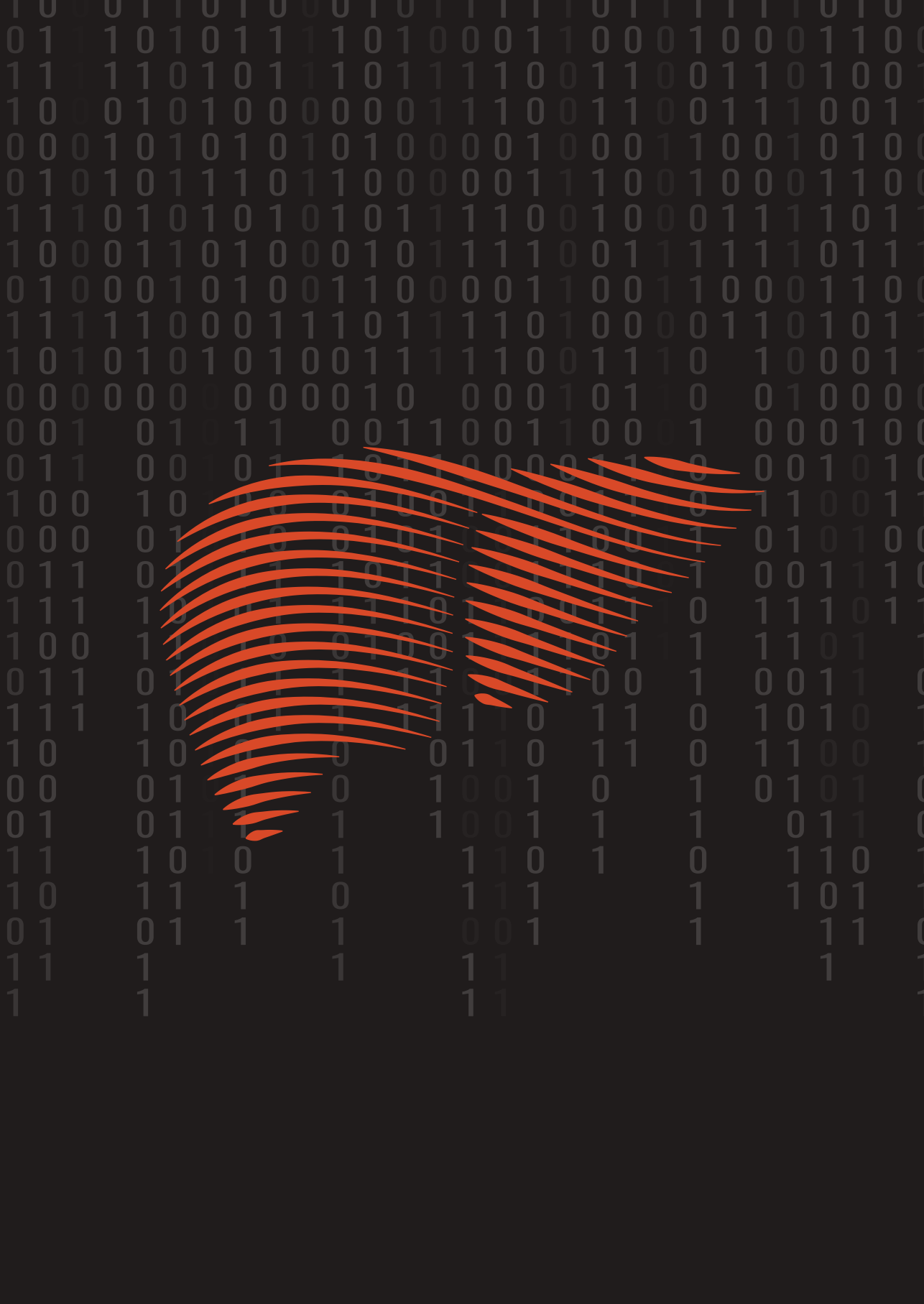
This thesis aims to investigate the renaming and redefining of NAFLD, discuss considerations in screening for advanced liver disease and identify potential improvements in fatty liver disease management.

Chapter 2 is about how to define fatty liver disease. Specifically, **Chapter 2.1** addressed the differences in patient characteristics, liver stiffness and fibrosis using both MAFLD and the conventional NAFLD definition in the general population. **Chapter 2.2** aims to comprehensively pool the available data on the transition from NAFLD to MAFLD to study the non-overlapping groups further. **Chapter 2.3** focuses on applying the MAFLD-criteria among patients with chronic hepatitis B, which was an exclusion criteria for the conventional NAFLD criteria. **Chapter 2.4** finally investigates the potential interactions between MAFLD and alcohol consumption in relation to mortality risk.

Chapter 3 is about considerations in early detection of (advanced) liver disease. Specifically, **Chapters 3.1 and 3.2** are about the clinical relevance of fatty liver disease in the elderly and the prognostic consequences of high liver stiffness in an elderly general population in relation to cardiovascular traits. **Chapter 3.3** addressed the relation between arrhythmias with liver stiffness and fatty liver disease and further investigated the impact of signs of venous congestion on liver stiffness. In **Chapter 3.4**, we tested the clinical applicability of the new EASL guideline on non-invasive tests for the identification of individuals at risk for advanced liver disease.

Chapter 4 is about treatment and prevention. **Chapter 4.1** focuses on different intensities and duration of physical activity and the potential benefits regarding fatty liver disease. These outcomes may aid clinicians in providing specific, evidence-based information to their patients. **Chapter 4.2** sheds further light on the potential benefits and mechanism of action of statin use across the entire fatty liver disease spectrum. In this multidimensional study, we performed a cross-sectional investigation of a general population cohort and a NAFLD-patient cohort, a meta-analysis, and an experimental study.

Chapter 5 is our response to the letters received on the studies of **chapters 2.4, 3.1, and 3.3,** illustrating the attention it received and the discussion it has triggered in the scientific community.



CHAPTER 2

Redefining and renaming NAFLD

CHAPTER 2.1

Application of metabolic dysfunction associated fatty liver disease improves detection of high liver stiffness: The Rotterdam Study

Laurens A. van Kleef*, Ibrahim Ayada*, Louise J.M. Alferink, Qiuwei Pan, Robert J. de Knegt

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ABSTRACT

Background & aims: Recently, metabolic dysfunction associated fatty liver disease (MAFLD) has been introduced and was defined as hepatic steatosis with either overweight, diabetes and/or a combination of other metabolic risk factors. We investigated the application of the novel MAFLD criteria as compared to non-alcoholic fatty liver disease (NAFLD).

Methods: We performed a cross-sectional analysis within The Rotterdam Study, a large prospective population-based cohort. Participants who attended the liver ultrasound and transient elastography program between 2009 – 2014 were eligible for inclusion. Subsequently, individuals with viral hepatitis, alcohol intake >60 grams/day, missing alcohol data and/or missing body mass index (BMI) were excluded. According to their NAFLD and MAFLD status based on metadata and ultrasound, participants were allocated in overlap fatty liver disease (FLD), NAFLD-only, MAFLD-only or no-FLD. Fibrosis was defined as liver stiffness ≥8.0 kilopascal.

Results: In our analysis, 5.445 participants were included, 1.866 (34.3%) had MAFLD and 1.604 (29.5%) had NAFLD. This resulted in 1.547 (28.4%) individuals with overlap-FLD, 319 (5.9%) with MAFLD-only, 57 (1.0%) with NAFLD-only and 3.522 (64.7%) with no-FLD. MAFLD-only was strongly associated with fibrosis (adjusted OR 5.30, $p<0.001$) and log-transformed liver stiffness (adjusted beta 0.116, $p<0.001$), opposing NAFLD-only in which no cases of fibrosis were identified and no association with liver stiffness (adjusted beta 0.006, $p=0.90$) was found.

Conclusions: FLD is highly prevalent in the general population. However, not NAFLD-only, but MAFLD-only was associated with fibrosis and higher liver stiffness independent of demographic and lifestyle factors. We believe using the novel MAFLD criteria will help improve the identification and treatment of FLD patients at risk for fibrosis.

INTRODUCTION

Fatty liver disease (FLD) is increasingly common with an estimated adult prevalence of 25% worldwide. It has become one of the leading causes of cirrhosis and hepatocellular carcinoma in the Western world.[4] The global rise of this disease and its burden on healthcare outcome(s) has followed a worrisome and rapid increase in obesity and metabolic disorders.[2, 79] Hepatic complications among individuals with FLD are relatively uncommon (0.77 deaths per 1000 person-years), compared to cardiovascular disease-specific mortality (4.79 per 1000 person-years).[4] And therefore, it is challenging to identify patients with advanced liver disease. Albeit the low relative risk, absolute numbers of advanced FLD (driven by the sheer amount of individuals with FLD) have made FLD one of the leading causes for liver transplantation.[80] Because individuals with FLD have a significant cardiovascular risk, adequate multidisciplinary attention is key.[81, 82] This includes focussing on improved lifestyle, treatment of hypertension, diabetes and lipid disorders, even in case of secondary causes of steatosis such as excessive alcohol consumption and steatogenic drug use.[83, 84]

Given the above, a novel entity of metabolic dysfunction associated fatty liver disease (MAFLD) was proposed allowing the co-existence of secondary causes of steatosis. This new definition comprises hepatic steatosis with diabetes, overweight or at least two minor metabolic abnormalities.[29] The rationale for changing this definition is to acknowledge the primary drivers of NAFLD instead of ruling out other causes. Moreover, this new definition provides guidance for screening and treating metabolic comorbidity by medical and lifestyle interventions.[27] Last, from the patients' perspective the current term 'non-alcoholic' is not informative, suggesting the linkage with alcohol which can be stigmatizing and misleading.[28]

The shift from NAFLD to an aetiology and inclusion-based definition of MAFLD has not yet been extensively studied, especially in European populations. Some recent publications already showed the usefulness and applicability of the MAFLD criteria in identifying individuals with impaired liver health and increased cardiovascular risk.[85-88] However, those were hampered by limited sample size, had no access to transient elastography and/or lacked up-to-date data from the general population

that reflects the current extent of the fatty liver disease pandemic. And as yet, the association of MAFLD and liver stiffness in the general population needs to be determined. Therefore, this study addresses the differences in patient characteristics, liver stiffness and fibrosis using both the MAFLD and the conventional NAFLD definition.

PARTICIPANTS AND METHODS

Participants

We performed a cross-sectional investigative analysis within The Rotterdam Study, a large ongoing prospective population-based cohort. Citizens aged over 45 years and living in the Rotterdam suburb of Ommoord, were eligible to participate and invited periodically for assessment. The department of Gastroenterology & Hepatology joined in 2009 and introduced a liver ultrasound and transient elastography program (Fibroscan, Echosens, France). The rationale of the Rotterdam Study and detailed information were provided previously.[89]

Participants who attended the liver ultrasound program between March 2009 and June 2014 were eligible for inclusion. In line with previous studies, participants that had a major risk factor for fibrosis, other than fatty liver disease, were excluded.[85] This comprised of >60 grams of daily alcohol consumption and viral hepatitis based on hepatitis B surface antigen or anti-hepatitis C (Roche Diagnostics, GmbH). Additionally, participants were excluded in case of (1) missing food frequency questionnaire (FFQ) data for the last two visits while drinking ≥4 days a week (since excessive alcohol could not be ruled out), or (2) missing BMI in the presence of steatosis and no other MAFLD inclusion criteria for persisting uncertainty about MAFLD diagnosis.

NAFLD diagnosis

NAFLD was defined as steatosis in the absence of well-known secondary causes of steatosis, comprising of steatogenic drug use (i.e. amiodarone, corticosteroids and methotrexate) and excessive alcohol consumption defined as >20 grams daily in female or >30 grams in male on either the FFQ or the home interview.[1]

MAFLD diagnosis

According to Eslam et al,[29] MAFLD was defined as steatosis in combination with metabolic dysfunction. This comprises either, overweight (BMI ≥ 25 kg/m^2), type 2 diabetes mellitus (defined as antidiabetic drug use or fasting plasma glucose ≥7.0 mmol/L), or a combination of at least two of the following metabolic abnormalities: (1) waist circumference ≥102 cm for male and ≥88 cm for female, (2) blood pressure ≥130/85 mmHg or antihypertensive drug use, (3) plasma triglycerides ≥1.70 mmol/L or lipid-lowering drug treatment, (4) high-density lipoprotein cholesterol (HDL-C) <1.0 mmol/L for men and <1.3 mmol/L for women or lipid-lowering drug treatment, (5) prediabetes defined as fasting plasma glucose 5.6-6.9 mmol/L or (6) homeostatic model assessment of insulin resistance (HOMA-IR) of ≥2.5. The last minor MAFLD criterium, C-reactive protein level > 2 mg/L, could not be applied for this data was unavailable.

We refer to original-MAFLD when the entire cohort was included, regardless of viral hepatitis, alcoholic liver disease or missing alcohol data. When excluding cases of viral hepatitis, alcoholic liver disease and missing alcohol data, we refer to this as modified-MAFLD.

Subgroups

To study differences carefully, we allocated the participants in subgroups based on their NAFLD and modified-MAFLD status, resulting in the following groups (1) neither NAFLD nor MAFLD, hereafter referred to as 'no-FLD' (2) both NAFLD and MAFLD hereafter referred to as 'overlap-FLD', (3) NAFLD without impaired metabolic health and as a result no MAFLD inclusion, hereafter referred to as 'NAFLD-only', and last, (4) MAFLD, but no NAFLD due to presence of secondary causes of steatosis (excessive alcohol or steatogenic drug use), hereafter referred to as 'MAFLD-only'.

Liver ultrasound and liver stiffness

A single experienced sonographer performed the liver ultrasounds on a Hitachi Hi Vision 900 (PvW). Steatosis was defined dichotomously on hyperechoic liver parenchyma in comparison with the kidney cortex or spleen.[90] Images were saved digitally and reassessed on request by a hepatologist with over 10 years of experience in liver sonography. Liver stiffness measurement (LSM) was performed using transient elastography (FibroScan, EchoSens, Paris, France). At least 10

measurements were obtained with the M or XL probe. Measurements were considered unreliable and were discarded in case of an interquartile range >30%, together with a LSM ≥ 7.1 kilopascal (kPa), according to the Boursier criteria.[91] Subsequently, hepatic fibrosis was defined as LSM ≥ 8.0 kPa.[92]

Additional covariates

During each visit, research assistants measured anthropometrics, including waist circumference. Trained interviewers administered the questionnaires to ensure that questions were correctly interpreted and were completed accurately. Medication use was extracted from the pharmacy's register to obtain accurate information on prescriptions of the participants. Blood samples were collected during fasting state. Glucose, blood lipids, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (GGT), alkaline phosphatase and platelet count were assessed by automatic enzyme procedures and insulin with automatic immunoassay (Roche, Diagnostic GmbH, Mannheim, Germany). HOMA-IR was calculated with glucose (mmol/L) multiplied by insulin (mmol/L) divided by 22.5.[93] The metabolic syndrome was defined according to the ATP-III criteria.[94]

Statistical analysis

Study characteristics were described with normally distributed variables provided as mean ± standard deviation (SD) and non-normally distributed variables as median with 25^{th}-75^{th} percentile (P25-P75). ANOVA was used to study differences in normally distributed continuous data, Kruskal-Wallis for non-normally distributed continuous data and Chi-squared test for categorical data. Logistic regression and linear regression were used to assess the association for the different subgroups (MAFLD-only, NAFLD-only, overlap-FLD and no-FLD) and fibrosis or liver stiffness. In multivariable analysis, adjustments were made for demographics (age and sex), education level (low, moderate or high) and intoxications (smoking [current/former or never] and alcohol consumption [grams/day]) and were selected based on prior research in this cohort.[95] Natural log transformation was applied to non-normally distributed variables before being added to the models. The no-FLD participants functioned as the control group. In a sensitivity analysis, this control group was narrowed by excluding participants with secondary causes for steatosis without meeting the MAFLD criteria. And last, the control group was replaced by participants without steatosis.

In an additional analysis the same associations were investigated for original-MAFLD, modified-MAFLD and NAFLD. The association between metabolic comorbidity and fibrosis was studied among participants with (both modified and original) MAFLD and the role of concomitant excessive alcohol was explored. All analyses were performed in R version 4.0.3 (The R Foundation for Statistical Computing, Vienna, Austria).

Ethics

The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus MC (registration number MEC 02.1015) and by the Dutch Ministry of Health, Welfare and Sport (Population Screening Act WBO, license number 1071272-159521-PG). The Rotterdam Study Personal Registration Data collection is filed with the Erasmus MC Data Protection Officer under registration number EMC1712001. The Rotterdam Study has been entered into the Netherlands National Trial Register (NTR; www.trialregister.nl) and into the WHO International Clinical Trials Registry Platform (ICTRP; www.who.int/ictrp/network/primary/en/) under shared catalog number NTR6831. All participants provided written informed consent to participate in the study and to have their information obtained from treating physicians. All authors had access to the study data and reviewed and approved the final manuscript.

RESULTS

In The Rotterdam Study, 5.967 participants underwent liver ultrasound between March 2009 and June 2014, among them 522 were excluded, leaving 5.445 participants for analysis (**Figure 1**). Baseline characteristics are provided for the included and excluded participants in **supplementary table 1**. For the included participants, the mean age at study visit was 69.7 (SD 9.1) years, they were predominantly female (58.5%) and of European ancestry (97.4%). Metabolic comorbidities (i.e. hypertension, dyslipidemia and (pre)diabetes) were common, resulting in a 42.0% prevalence of the metabolic syndrome. Hepatic steatosis was present in 35.5% (n=1.931). Reliable LSM were available in 72.7% (n=3.957) of the participants, and among them 6.0% (n=239) had fibrosis. The sensitivity and specificity for MAFLD to detect fibrosis was 59.4% and 69.9% respectively which was comparable to NAFLD (after exclusions for excessive alcohol and steatogenic

drug use) 55.7% and 69.2%. The positive and negative predictive value for MAFLD was 11.3% and 96.4% and for NAFLD after exclusions 8.8% and 96.7%.

MAFLD identifies more individuals with fatty liver disease

NAFLD was diagnosed in 1.604/5.445 (29.5%) participants, who represent 1.604/4.635 (34.6%) of the participants without secondary causes of steatosis. Modified-MAFLD was present in 1.866/5.445 (34.3%) participants. Diagnosis of modified-MAFLD was based on steatosis together with overweight (n=1.740/1.866), diabetes (n=469/1.866) and/or metabolic comorbidity (n=1.691/1.866). Among the participants with modified-MAFLD, 87% had > 1 MAFLD criteria, and 22% had all MAFLD criteria. **Supplementary table 2** provides

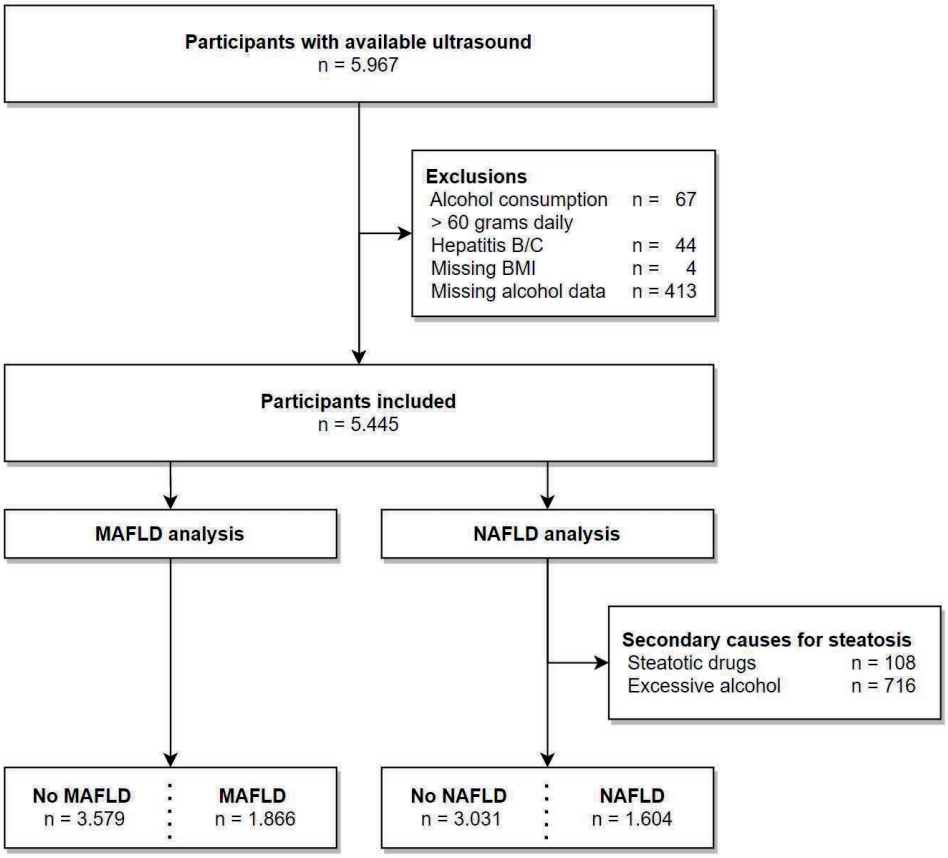

Figure 1 Flowchart of exclusions, NAFLD and MAFLD diagnosis. Participants can have multiple exclusion criteria or secondary causes for steatosis.

the descriptive characteristics of the (modified and original) MAFLD and NAFLD populations, but no statistical tests were performed between them, given the extensive overlap. The individual associations between the original-MAFLD, modified-MAFLD and NAFLD with fibrosis and liver stiffness are presented in **supplementary table 3**. In general, associations for original-MAFLD were significant and comparable with modified-MAFLD and observed effect sizes were more pronounced in the MAFLD groups, compared to NAFLD.

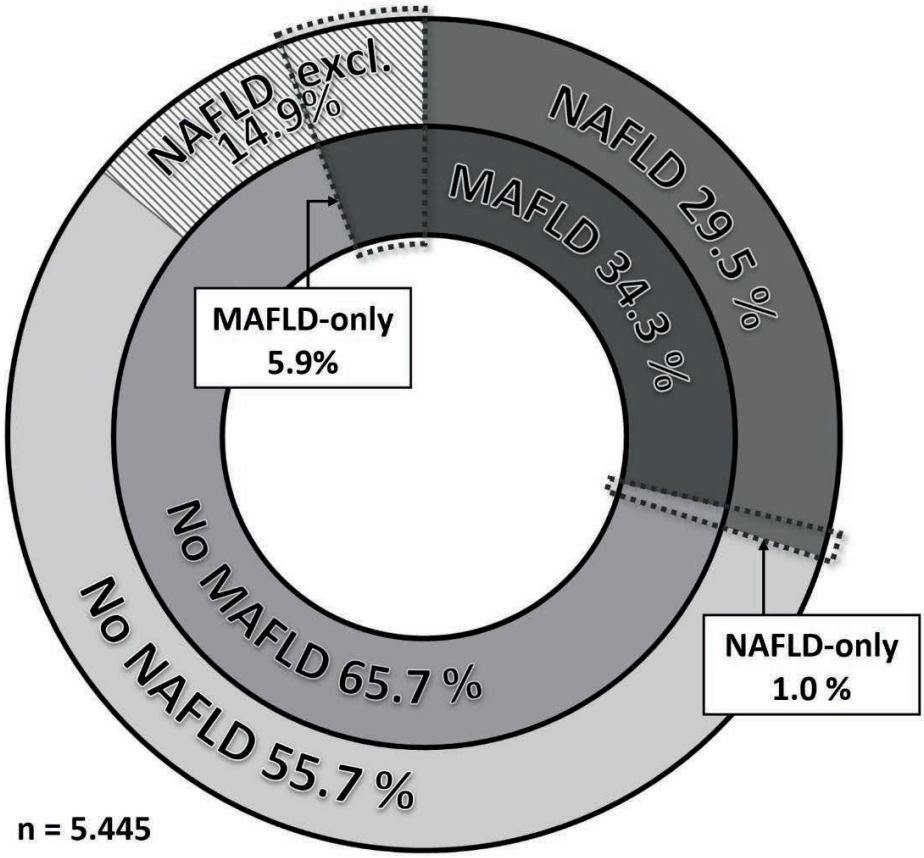

Figure 2 The inner circle represents MAFLD and the outer circle NAFLD. Non-overlapping groups are highlighted. MAFLD-only was present in 319 (5.9%) and NAFLD-only in 57 (1.0%) participants. NAFLD-exclusion criteria were excessive alcohol and steatogenic drug use.

Table 1: Characteristics of the MAFLD-only and NAFLD-only group, compared to no-FLD

Variable	Overlap-FLD n = 1547	MAFLD-only (A) n = 319	NAFLD-only (B) n = 57	No-FLD (C) n = 3522	*P* A vs B	*P* A vs C	*P* B vs C
Demographics							
Age (years)	70.0 (8.5)	68.8 (7.7)	68.5 (9.4)	69.7 (9.4)	0.815	0.082	0.334
Male, n (%)	641 (41.4)	166 (52.0)	26 (45.6)	1426 (40.5)	0.453	<0.001	0.518
European ancestry, n (%)	1323 (97.6)	294 (99.7)	51 (98.1)	3029 (97.1)	0.691	0.017	1.000
Education, n (%)					0.811	0.044	0.360
Low	847 (55.5)	130 (41.3)	25 (43.9)	1602 (45.9)			
Intermediate	424 (27.8)	118 (37.5)	22 (38.6)	1069 (30.6)			
High	255 (16.7)	67 (21.3)	10 (17.5)	818 (23.4)			
Current/former smoking, n (%)	1054 (68.3)	260 (81.5)	33 (58.9)	2088 (62.6)	<0.001	<0.001	0.451
Excessive alcohol intake*, n (%)	0 (0.0)	288 (90.3)	0 (0.0)	428 (12.2)	<0.001	<0.001	0.009
Physical examination							
High waist circumference†, n (%)	1132 (73.3)	230 (72.1)	0 (0.0)	1072 (30.4)	<0.001	<0.001	<0.001
BMI (kg/m^2)	30.4 (4.3)	29.8 (4.2)	23.4 (1.3)	26.2 (3.7)	<0.001	<0.001	<0.001
Comorbidity							
Hypertension, n (%)	1296 (83.8)	271 (85.0)	26 (45.6)	2416 (68.6)	<0.001	<0.001	<0.001
Diabetes, n (%)	406 (26.9)	63 (20.1)	0 (0.0)	361 (10.4)	<0.001	<0.001	0.019
Metabolic syndrome, n (%)	1040 (68.6)	218 (69.6)	0 (0.0)	984 (28.5)	<0.001	<0.001	<0.001
Biochemistry							
AST (U/L)	25 [21, 29]	26 [22, 31]	23 [21, 26]	24 [21, 28]	0.004	<0.001	0.351
ALT (U/L)	21 [16, 28]	23 [18, 29]	18 [15, 25]	17 [14, 22]	0.001	<0.001	0.250

Table 1: Characteristics of the MAFLD-only and NAFLD-only group, compared to no-FLD (continued)

Variable	Overlap-FLD n = 1547	MAFLD-only (A) n = 319	NAFLD-only (B) n = 57	No-FLD (C) n = 3522	*P* A vs B	A vs C	B vs C
GGT (U/L)	28 [20, 39]	34 [24, 50]	20.50 [15, 28]	21 [16, 31]	<0.001	<0.001	0.400
Alkaline phosphatase (U/L)	70 [59, 82]	67 [55, 79]	68 [60, 81]	68 [58, 80]	0.171	0.013	0.727
Platelets (10^9/L)	272 (66)	264 (67)	276 (51)	268 (69)	0.225	0.318	0.425
HDL-C (mmol/L)	1.31 (0.34)	1.44 (0.42)	1.61 (0.43)	1.55 (0.44)	0.004	<0.001	0.310
Triglycerides (mmol/L)	1.58 [1.20, 2.11]	1.54 [1.15, 2.13]	1.01 [0.75, 1.34]	1.16 [0.91, 1.53]	<0.001	<0.001	0.002
HOMA-IR	1.60 [1.09, 2.41]	1.35 [0.98, 1.98]	0.79 [0.53, 1.05]	0.82 [0.58, 1.18]	<0.001	<0.001	0.232
Transient elastography							
Liver stiffness (kPa)	5.2 [4.1, 6.4]	5.1 [4.2, 6.6]	4.9 [3.9, 5.3]	4.6 [3.8, 5.7]	0.015	<0.001	0.931
Fibrosis‡, n (%)	108 (10.4)	34 (14.9)	0 (0.0)	97 (3.7)	0.015	<0.001	0.399

Data is presented as mean (SD), median [P25-P75] or n and percentage. P-values are calculated using analysis of variance, Kruskal-Wallis or Chi-squared test. *Daily alcohol consumption >30 grams for male and >20 grams for female. † Waist circumference > 102 cm for male and > 88 cm for female. ‡Defined as liver stiffness ≥ 8.0 kPa. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; GGT, gamma- glutamyl transpeptidase; HOMA-IR, homeostatic model assessment of insulin resistance; MAFLD, metabolic dysfunction associated fatty liver disease; NAFLD, non-alcoholic fatty liver disease.

MAFLD was common in participants with NAFLD exclusion criteria

In our cohort, 1.547/5.445 (28.4%) had both NAFLD and modified-MAFLD, resulting in 96.4% of NAFLD individuals covered by the MAFLD criteria (**Figure 2**); 3.522/5.445 (64.7%) had neither NAFLD nor MAFLD (no-FLD). Lastly, two non-overlapping groups were identified as MAFLD-only and NAFLD-only. MAFLD-only was present in 319/5.445 (5.9%) of the participants. This MAFLD-only group is characterised by having steatosis, but not fulfilling the criteria of NAFLD because of excessive alcohol consumption (90%) and/or steatogenic drug use (11%). NAFLD-only was present in 57/5.445 (1.0%), and they did not comply with MAFLD, since no metabolic risk criteria were met. Of the participants with FLD (n=1.923), 80.4% had overlap-FLD, 16.6% had MAFLD-only and 3.0% had NAFLD-only.

MAFLD-only was associated with fibrosis

To further assess the difference between NAFLD and MAFLD, the non-overlapping groups were investigated. As a result of the differences in selection criteria, participants with MAFLD-only compared to NAFLD-only had more metabolic comorbidities (i.e. metabolic syndrome; p<0.001) and alcohol intake (p<0.001, **table 1**). No statistically significant differences for age, sex or education were found. However, MAFLD-only had higher AST (p=0.004), ALT (p=0.001), GGT (p<0.001) and liver stiffness (p=0.015) as compared to NAFLD-only. Moreover, fibrosis was common among MAFLD-only and not at all present in individuals with NAFLD-only (14.9% vs 0.0%, p=0.015, **table 1**).

Table 2: Association of MAFLD-only and overlap-FLD with fibrosis (LSM ≥ 8.0 kPa) compared to no-FLD

	Unadjusted			Adjusted		
	OR	95% CI	*P*	OR	95% CI	*P*
MAFLD-only	4.62	3.01 – 6.94	<0.001	5.30	3.12 – 8.89	<0.001
NAFLD-only		NA			NA	
Overlap-FLD	3.07	2.31 – 4.09	<0.001	3.29	2.44 – 4.42	<0.001

Results were obtained with logistic regression and given as OR and 95% CI for fibrosis as outcome, the reference group had no-FLD (cases = 97/2653). For NAFLD-only no cases (cases = 0/42) of fibrosis were observed, therefore logistic regression was not possible. In the overlap group (n=1034) were 108 cases and in the MAFLD only group (n = 228) 34. Multivariable analyses were adjusted for age, sex, alcohol consumption, smoking and education. Abbreviations: CI, confidence interval; FLD, fatty liver disease; LSM, liver stiffness measurement; MAFLD, metabolic associated fatty liver disease; OR, odds ratio.

Compared to no-FLD, MAFLD-only was associated with fibrosis (OR 4.62, p<0.001) and this was persistent after adjusting for age, sex, alcohol consumption, smoking and education level (aOR 5.30, p<0.001). Similar results were obtained for overlap-FLD (aOR 3.29, p < 0.001, **table 2**). This did not apply to NAFLD-only, since no cases of fibrosis were identified and logistic regression analysis was not possible. Subsequently, with linear regression (**table 3**), no association between NAFLD-only and (natural) log-transformed LSM could be demonstrated compared to no-FLD in multivariable analysis (beta 0.006, p=0.90). In contrast, this was clearly present for MALFD-only (beta 0.116, p < 0.001) and overlap-FLD (beta 0.106, p <0.001). Results from linear and logistic regression were consistent when the control group was replaced by participants without steatosis nor secondary causes for steatosis (control group A, n=2.262) or by participants without steatosis (control group B, n=2.647, **supplementary table 4**).

Table 3: Association of MAFLD-only, NAFLD-only and overlap-FLD with log transformed liver stiffness (kPa) compared to no-FLD

	Unadjusted			Adjusted		
	beta	95% CI	*P*	beta	95% CI	*P*
MAFLD-only	0.134	0.091 – 0.176	<0.001	0.116	0.072 – 0.159	<0.001
NAFLD-only	-0.002	-0.096 – 0.092	0.963	0.006	-0.083 – 0.095	0.900
Overlap-FLD	0.111	0.087 – 0.134	<0.001	0.106	0.083 – 0.128	<0.001

Results were obtained with linear regression and given as beta with 95% CI for (natural) log transformed liver stiffness (kPa) as outcome, the reference group had no-FLD (n = 2653). MAFLD-only, NAFLD-only and overlap FLD comprised 228, 42 and 1034 individuals with valid liver stiffness measurement. Multivariable analyses were adjusted for age, sex, alcohol consumption, smoking and education. Abbreviations: CI, confidence interval; FLD, fatty liver disease; LSM, liver stiffness measurement; MAFLD, metabolic dysfunction associated fatty liver disease; NAFLD, non-alcoholic fatty liver disease; OR, odds ratio.

Metabolic comorbidity is associated with fibrosis in participants with MAFLD

Participants with modified-MAFLD were categorised for the number of present inclusion criteria, comprising of overweight, diabetes mellitus and ≥2 minor metabolic comorbidities. Fibrosis prevalence increased from 8.3% and 8.9% for one and two criteria present to 20.5% for meeting all three MAFLD inclusion criteria. By logistic regression, fibrosis presence increased for having three (OR 2.43, p < 0.001) compared to having only one or two inclusion criteria. This result was persistent after adjusting for age, sex, education level, smoking status and alcohol

consumption (aOR 2.42, p < 0.001). One could argue that this association is driven mainly by the group of excessive alcohol consumers. But importantly, we observed that among participants with MAFLD, having all MAFLD criteria was associated with increased risk of fibrosis, regardless the presence of excessive alcohol consumption (aHR 2.30, 95%CI 1.49-3.53 without concomitant excessive alcohol consumption, compared to aHR 3.63, 95% CI 1.51-8.10 for concomitant excessive alcohol consumption), **supplementary table 5**. Similar results were obtained for the metabolic syndrome, which was also associated with an increased risk for fibrosis (aOR 1.86, p = 0.004), among individuals with MAFLD. Despite larger odds ratios among subjects with superimposed excessive alcohol consumption, no statistical significance was reached. This was in line with additional analysis: among subjects with MAFLD, higher log-transformed liver stiffness was observed for excessive alcohol consumption (beta 0.026, p = 0.344) but was not statistical significant.

DISCUSSION

In this large ongoing population-based cohort study, we examined the consequences of adopting the novel MAFLD criteria on identifying fibrosis and liver stiffness as compared to the conventional NAFLD definition. The prevalence of modified-MAFLD was higher than that of NAFLD in this elderly population (34.3% and 29.5% respectively) and among the participants with NAFLD, 96.4% did also comply with the MAFLD criteria. These results are consistent with other studies, which showed prevalences of 26-37% for MAFLD, [86-88, 96, 97] with >94% of the NAFLD cases also being identified with MAFLD.[87, 88, 97]

In our cohort, 1.0% was classified as NAFLD-only, i.e. having FLD without impaired metabolic health, thus not meeting the MAFLD definition. This implies that few participants are missed applying the MAFLD-criteria (most participants are in the overlap FLD, **figure 2**). A similar NAFLD-only prevalence was reported by Niriella et al[87] (1.3%) and Wong et al[88] (1.7%), whereas Lin et al[86] reported 4.7% of individuals having NAFLD-only in the NHANES III cohort. The difference in prevalence of the latter study as compared to ours may be explained by the younger population (43.7 years vs 69.7 years), which was notably less prone to hypertension (24.9% vs 73.6%). Besides, given the fact that NHANES III data were collected between 1988

and 1994, demographics, comorbidity and disease characteristics may have changed.

Interestingly, baseline characteristics of NAFLD-only were similar to those of the no-FLD population, an observation in agreement with previous literature.[86, 88] Moreover, multivariable analysis did not show an association with LSM for NAFLD-only, compared to no-FLD. This suggests that not including NAFLD-only with the novel MAFLD criteria does not impair good patient care, meaning not missing patients with an elevated LSM. However, it is essential to assess the long-term outcomes of this group with further follow-up studies before firm conclusions can be made. Given the good metabolic health of the NAFLD-only group, genetic predisposition needs to be investigated. Variations in PNPLA3 and TM6SF2 for example, have been linked to severe steatosis, steatohepatitis and fibrosis even without overt metabolic comorbidities, driven by impaired hepatic lipid metabolism.[98-100] However, false-positive results from abdominal ultrasound should be considered, given the imperfect test characteristics compared to the golden standard, liver biopsy.[101]

MAFLD-only was common, with a prevalence of 5.9% in this cohort, and representing even 16.6% of the participants with FLD. Identification of this MAFLD-only group is important since it was significantly associated with higher liver stiffness and fibrosis, independent of alcohol consumption, similar to previous reported results.[85, 88] Therefore, adapting the MAFLD criteria will enable better identification of individuals at risk for having fibrosis, which is an important predictor for liver-related events, including hepatocellular carcinoma.[102]

Fibrosis prevalence was higher among participants with MAFLD that had more metabolic comorbidity, for example, when all MAFLD inclusion criteria or the metabolic syndrome were present. A similar approach was reported by Yamamura et al, showing an association with fibrosis and metabolic abnormalities, in Japanese participants with FLD.[85] These consistent findings suggest that metabolic comorbidity along with steatosis, are the main drivers of fibrogenesis. This observation supports the call for intensifying multidisciplinary management of MAFLD and lifestyle programs to improve metabolic health and alcohol awareness in addition to careful assessment of liver health by a hepatologist, regardless of the presence of secondary causes of steatosis.[84]

Although this is the first large ongoing European cohort investigating MAFLD with access to detailed metabolic health data alongside liver ultrasound and transient elastography, there are some limitations that need mentioning. First, our cohort of participants is an ageing cohort with a mean age of 69.7 years and is predominantly Caucasian. Therefore, it may not be entirely representative of the whole population. In particular, our results may not be generalizable to a multi-ethnic, younger population. Second, this is a cross-sectional study and causal relations and long-term outcomes could therefore not be studied. This is of particular concern for the NAFLD-only group which had no cross-sectional association with fibrosis, but might be at risk in the long-term. A third and unavoidable factor is that 96.4% of the participants with NAFLD had overlap-FLD. As a consequence, the only comparison between NAFLD and MAFLD was possible using the non-overlapping "only" groups with relatively small numbers. This might have resulted in insufficient statistical power, especially in the NAFLD-only group (n=57). However, the effect sizes found in this group were very small, and might not have clinical relevance, even if it was significant. Fourth, although liver ultrasound is the diagnostic modality mostly used in the assessment of liver steatosis, it should be noticed that liver ultrasound has limited sensitivity for detecting mild steatosis.[101] Fifth, LSM is a non-invasive approach to assessing the presence of liver fibrosis and has a strong correlation with histologically staged liver fibrosis. Nonetheless, the gold standard for both steatosis and fibrosis remains liver biopsy, despite being invasive and prone to sampling error. [103, 104] Sixth, in our cohort, we had no information on C-reactive protein, which is one of the minor metabolic inclusion criteria for MAFLD diagnosis.[29] Despite missing this information, we already had a low rate of NAFLD-only, it is therefore unlikely that this had a major impact on our results. Seventh, the Rotterdam Study was not designed to study alcohol consumption specifically. Therefore, additional studies focusing on alcohol consumption are required in larger cohorts with more detailed alcohol data to further elucidate the potential synergistic risk with metabolic dysfunction. Last, since chronic viral hepatitis and alcoholic liver disease were excluded for modified-MAFLD (to allow for a fair comparison between MAFLD and NAFLD), these results might not be entirely generalizable to the entire MAFLD population. However, comparable associations were found for original-MAFLD and modified-MAFLD, indicating that the impact of this selection on our results is limited.

In conclusion, 96.4% of the participants with NAFLD were also identified with the novel MAFLD criteria (i.e overlap-FLD). NAFLD without impaired metabolic health, NAFLD-only, was present in only 1.0%. It had similar characteristics as no-FLD and was not associated with liver stiffness. Hence, the usage of the new MAFLD definition does not seem to lead to the exclusion of patients with FLD at risk for fibrosis. In contrast, 5.9% of our cohort had MAFLD-only, representing 16.6% of the participants with FLD. This MAFLD-only group was associated with both higher LSM and more prevalent fibrosis, which is an important predictor of hepatic complications. Moreover, among the participants with MAFLD, metabolic comorbidity (e.g. metabolic syndrome) was associated with fibrosis, which underlines the importance of this new definition. It also encourages adequate multidisciplinary treatment and lifestyle interventions between disciplines. To identify the MAFLD-only group, which would not have been identified by using the NAFLD criteria, we recommend to consider using the novel MAFLD criteria.

SUPPLEMENTARY FILES

Supplementary table 1: Participants' characteristics for inclusions and exclusions.

	Inclusions n = 5445	**Exclusions** n = 522
Demographics		
Age (years)	69.7 (9.1)	69.3 (9.5)
Male, n (%)	2259 (41.5)	296 (56.7)
European ancestry, n (%)	4697 (97.4)	446 (97.2)
Education, n (%)		
Low	2604 (48.3)	252 (48.8)
Intermediate	1633 (30.3)	140 (27.1)
High	1150 (21.3)	124 (24.0)
Current/former smoking, n (%)	3608 (66.4)	422 (81.3)
Excessive alcohol intake[*], n (%)	716 (13.1)	71 (65.1)[§]
Physical examination		
High waist circumference[†], n (%)	2434 (44.7)	219 (42.0)
BMI (kg/m^2)	27.6 (4.4)	27.2 (4.4)
Comorbidity		
Hypertension, n (%)	4009 (73.6)	386 (74.1)
Diabetes, n (%)	830 (15.5)	73 (14.5)
Metabolic syndrome, n (%)	2242 (42.0)	228 (45.0)
Biochemistry		
AST (U/L)	24 [21, 28]	25 [22, 29]
ALT (U/L)	19 [15, 24]	19 [15, 25]
GGT (U/L)	23 [17, 34]	27 [20, 42]
Alkaline phosphatase (U/L)	69 [58, 81]	67 [57, 79]
Platelets (10^9/L)	269 (68)	261 (67)
HDL-C (mmol/L)	1.48 (0.43)	1.55 (0.50)
Triglycerides (mmol/L)	1.28 [0.98, 1.73]	1.26 [0.92, 1.68]
HOMA-IR	1.00 [0.67, 1.57]	1.01 [0.67, 1.50]
Transient elastography		
Liver stiffness (kPa)	4.8 [3.8, 5.9]	4.9 [4.1, 6.1]
Fibrosis[‡], n (%)	239 (6.0)	30 (7.6)

Data is presented as mean (SD), median [P25-P75] or n and percentage. *Daily alcohol consumption >30 grams for male and >20 grams for female. [†] Waist circumference > 102 cm for male and > 88 cm for female. [‡]Defined as liver stiffness ≥ 8.0 kPa. [§]Available in 109 participants. Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; GGT, gamma- glutamyl transpeptidase; HOMA-IR, homeostatic model assessment of insulin resistance; MAFLD, metabolic dysfunction associated fatty liver disease; NAFLD, non-alcoholic fatty liver disease.

Supplementary table 2: Participants' characteristics for original-MAFLD, modified-MAFLD and NAFLD

	Original-MAFLD n = 2057	**Modified-MAFLD** n = 1866	**NAFLD** n = 1604
Demographics			
Age (years)	69.6 (8.4)	69.7 (8.4)	69.9 (8.5)
Male	930 (45.2)	807 (43.2)	667 (41.6)
European ancestry	1784 (97.9)	1617 (97.9)	1374 (97.6)
Education			
Low	1067 (52.6)	977 (53.1)	872 (55.1)
Intermediate	600 (29.6)	542 (29.4)	446 (28.2)
High	362 (17.8)	322 (17.5)	265 (16.7)
Current/former smoking	1473 (71.8)	1314 (70.6)	1087 (68.0)
Excessive alcohol intake*	328 (17.1)	288 (15.4)	0 (0.0)
Physical examination			
High waist circumference†	1496 (72.8)	1362 (73.1)	1132 (70.7)
BMI (kg/m^2)	30.2 (4.3)	30.3 (4.3)	30.1 (4.4)
Comorbidity			
Hypertension	1731 (84.2)	1567 (84.0)	1322 (82.4)
Diabetes	512 (25.4)	469 (25.7)	406 (25.9)
Metabolic syndrome	1387 (68.8)	1258 (68.8)	1040 (66.2)
Biochemistry			
AST (U/L)	25 [21, 29]	25 [21, 29]	25 [21, 29]
ALT (U/L)	22 [17, 29]	22 [17, 28]	21 [16, 28]
GGT (U/L)	29 [21, 42]	29 [21, 41]	28 [20, 39]
Alkaline phosphatase (U/L)	69 [58, 81]	69 [58, 81]	70 [59, 82]
Platelets (10^9/L)	269 (66)	271 (66)	272 (66)
HDL-C (mmol/L)	1.34 (0.37)	1.33 (0.36)	1.32 (0.35)
Triglycerides (mmol/L)	1.6 [1.2, 2.1]	1.6 [1.2, 2.1]	1.6 [1.2, 2.1]
HOMA-IR	1.5 [1.1, 2.3]	1.6 [1.1, 2.4]	1.6 [1.1, 2.4]
Transient elastography			
Liver stiffness (kPa)	5.2 [4.2, 6.4]	5.2 [4.1, 6.4]	5.1 [4.1, 6.3]
Fibrosis‡	160 (11.3)	142 (11.3)	108 (10.0)

Data is presented as mean (SD), median [P25-P75] or n and percentage. Data is presented for the entire cohort, original and modified MAFLD and NAFLD. *Daily alcohol consumption >30 grams for male and >20 grams for female. † Waist circumference > 102 cm for male and > 88 cm for female. ‡Defined as liver stiffness ≥ 8.0 kPa. Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; GGT, gamma- glutamyl transpeptidase; HOMA-IR, homeostatic model assessment of insulin resistance; MAFLD, metabolic dysfunction associated fatty liver disease; NAFLD, non-alcoholic fatty liver disease.

Supplementary table 3: Association of original-MAFLD, modified-MAFLD and NAFLD with fibrosis (LSM ≥ 8.0 kPa) and liver stiffness compared to those without.

	cases / n	Unadjusted			Adjusted		
		OR/beta	95% CI	*P*	OR/beta	95% CI	*P*
Fibrosis (LSM ≥ 8.0)							
Original MAFLD	160/1413	3.31	2.58 – 4.27	< 0.001	3.45	2.66 – 4.50	< 0.001
Modified MAFLD	142/1262	3.40	2.60 – 4.45	< 0.001	3.57	2.71 – 4.72	< 0.001
NAFLD	108/1076	2.82	2.11 – 3.79	< 0.001	3.02	2.24 – 4.10	< 0.001
Log LSM (kPa)							
Original MAFLD	1413	0.118	0.097 – 0.139	< 0.001	0.110	0.090 – 0.130	< 0.001
Modified MAFLD	1262	0.115	0.095 – 0.138	< 0.001	0.109	0.088 – 0.130	< 0.001
NAFLD	1076	0.107	0.083 – 0.130	< 0.001	0.105	0.082 – 0.127	< 0.001

Results were obtained with linear and logistic regression and given as OR and 95% CI for fibrosis and beta with 95% CI for natural log transformed LSM, the reference group had either no original MAFLD (cases: 109/2938), no modified MAFLD (cases: 97/2695) or no NAFLD (cases: 86/2262). Multivariable analyses were adjusted for age, sex, alcohol consumption, smoking and education. Abbreviations: CI, confidence interval; FLD, fatty liver disease; LSM, liver stiffness measurement; MAFLD, metabolic associated fatty liver disease; OR, odds ratio.

Supplementary table 4: Association of MAFLD-only, NAFLD-only and overlap-FLD with fibrosis (LSM ≥ 8.0 kPa) and log transformed LSM compared to participants without FLD nor secondary causes for steatosis (A) and no steatosis (B)

	Control	Unadjusted			Adjusted		
		OR/beta	95% CI	*P*	OR/beta	95% CI	*P*
Fibrosis (LSM ≥ 8.0)							
MAFLD-only	A	4.43	2.87 – 6.71	< 0.001	5.42	3.03 – 9.53	< 0.001
	B	4.61	3.00 – 6.93	<0.001	5.27	3.10 – 8.85	< 0.001
NAFLD-only	A		NA			NA	
	B		NA			NA	
Overlap-FLD	A	2.95	2.20 – 3.97	< 0.001	3.15	2.33 – 4.28	< 0.001
	B	3.07	2.31 – 4.08	<0.001	3.28	2.44 – 4.42	<0.001
Log LSM (kPa)							
MAFLD-only	A	0.134	0.091 – 0.177	<0.001	0.128	0.080 – 0.176	<0.001
	B	0.134	0.091 – 0.176	<0.001	0.117	0.072 – 0.161	<0.001
NAFLD-only	A	-0.002	-0.096 – 0.092	0.969	0.009	-0.080 – 0.098	0.838
	B	-0.002	-0.096 – 0.092	0.964	0.006	-0.083 – 0.095	0.897
Overlap-FLD	A	0.111	0.087 – 0.135	<0.001	0.109	0.086 – 0.132	<0.001
	B	0.111	0.087 – 0.134	<0.001	0.106	0.083 – 0.128	<0.001

Results were obtained with linear and logistic regression and given as OR and 95% CI for fibrosis and beta with 95% CI for natural log transformed LSM. For NAFLD-only no cases of fibrosis were observed, therefore logistic regression was not possible. Control group A (cases = 86/2262) had no-FLD and no secondary causes for steatosis, control group B (cases = 97/2647) had no steatosis. Multivariable analyses were adjusted for age, sex, alcohol consumption, smoking and education. Abbreviations: CI, confidence interval; FLD, fatty liver disease; LSM, liver stiffness measurement; MAFLD, metabolic associated fatty liver disease; OR, odds ratio.

Supplementary table 5: Association of metabolic comorbidity with fibrosis (LSM ≥ 8.0 kPa) in participants with MAFLD and the impact of excessive alcohol consumption in these associations.

	MAFLD	Unadjusted			Adjusted		
		OR	95% CI	*P*	OR	95% CI	*P*
All MAFLD criteria	Modified	2.43	1.66 – 3.51	< 0.001	2.42	1.62 – 3.58	< 0.001
	Original	2.49	1.74 – 3.53	< 0.001	2.46	1.69 – 3.56	< 0.001
Metabolic syndrome	Modified	1.84	1.22 – 2.85	0.005	1.76	1.15 – 2.77	0.012
	Original	1.81	1.23 – 2.73	0.003	1.86	1.24 – 2.86	0.004
All MAFLD criteria X excessive alcohol consumption							
All MAFLD criteria (-) and excessive alcohol (-)	Modified		Reference			Reference	
	Original		Reference			Reference	
All MAFLD criteria (+) and excessive alcohol (-)	Modified	2.47	1.62 – 3.74	< 0.001	2.30	1.49 – 3.53	< 0.001
	Original	2.47	1.63 – 3.71	< 0.001	2.31	1.50 – 3.53	< 0.001
All MAFLD criteria (-) and excessive alcohol (+)	Modified	1.56	0.92 – 2.57	0.085	1.30	0.75 – 2.18	0.339
	Original	1.43	0.86 – 2.31	0.154	1.13	0.66 – 1.87	0.650
All MAFLD criteria (+) and excessive alcohol (+)	Modified	4.11	1.75 – 8.91	< 0.001	3.63	1.51 – 8.10	0.002
	Original	4.02	1.79 – 8.40	< 0.001	3.59	1.56 – 7.70	0.002

Supplementary table 5: Association of metabolic comorbidity with fibrosis (LSM ≥ 8.0 kPa) in participants with MAFLD and the impact of excessive alcohol consumption in these associations. (continued)

	MAFLD	Unadjusted			Adjusted		
		OR	95% CI	*P*	OR	95% CI	*P*
Metabolic syndrome X excessive alcohol consumption							
Metabolic syndrome (-) and excessive alcohol (-)	Modified		Reference			Reference	
	Original		Reference			Reference	
Metabolic syndrome (+) and excessive alcohol (-)	Modified	2.08	1.30 – 3.46	0.003	1.94	1.20 – 3.27	0.009
	Original	2.13	1.34 – 3.55	0.002	2.03	1.26 – 3.41	0.005
Metabolic syndrome (-) and excessive alcohol (+)	Modified	2.39	0.95 – 5.48	0.048	1.87	0.69 – 4.54	0.186
	Original	2.42	1.05 – 5.25	0.029	1.68	0.66 – 3.91	0.243
Metabolic syndrome (+) and excessive alcohol (+)	Modified	2.65	1.42 – 4.95	0.002	2.23	1.18 – 4.25	0.014
	Original	2.43	1.32 – 4.50	0.004	2.07	1.10 – 3.91	0.023

Results were obtained with logistic regression and given as OR and 95% CI for fibrosis as outcome. Multivariable analyses were adjusted for age, sex, alcohol consumption (unless part of analysis), smoking and education. The modified MAFLD comprised all included MAFLD participants with valid LSM after exclusions for missing alcohol data, alcoholic liver disease and viral hepatitis (fibrosis = 142/1262). The original MAFLD comprised all MAFLD participants with a valid LSM prior to exclusions (fibrosis = 160/1413). Abbreviations: CI, confidence interval; LSM, liver stiffness measurement; MAFLD, metabolic associated fatty liver disease; OR, odds ratio.

CHAPTER 2.2

Systematically comparing epidemiological and clinical features of MAFLD and NAFLD by meta-analysis: focusing on non-overlap groups

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ABSTRACT

Background & aims: The applicability of the novel MAFLD definition has been studied in numerous cohorts and compared to NAFLD. No consensus has been reached on which definition is preferred. Therefore, this meta-analysis aims to compare the epidemiological and clinical features of NAFLD and MAFLD in the general and non-general population.

Methods: We searched Medline, Embase and Web of Science for studies comparing MAFLD to NAFLD. Based on MAFLD and NAFLD status, the following subgroups were investigated for liver health: overlap fatty liver disease, NAFLD-only and MAFLD-only. Data were pooled using random-effects models.

Results: We included 17 studies comprising 9.808.677 individuals. In the general population, MAFLD was present in 33.0% (95%CI 29.7 – 36.5%) and NAFLD in 29.1% (95%CI 27.1 – 31.1%). Among those with fatty liver disease, 4.0% (95%CI 2.4 – 6.4%) did not meet the MAFLD criteria but had NAFLD (NAFLD-only) and 15.1% (95%CI 11.5 – 19.5%) was exclusively captured by the novel MAFLD definition (MAFLD-only). Notably, this MAFLD-only group was at significantly increased risk for fibrosis (RR 4.2; 95%CI 1.3 – 12.9) and had higher ALT (mean difference: 8.0U/L, 95%CI 2.6 – 13.5) and AST (mean difference: 6.4 U/L, 95%CI 3.0 – 9.7), compared to NAFLD-only. Similar results were obtained among the non-general population.

Conclusions: MAFLD and NAFLD are highly prevalent in the general population, with considerable overlap between them. However, compared to NAFLD, significantly more individuals were additionally identified by MAFLD than were missed. Importantly, by using the MAFLD criteria, more individuals with liver damage were identified. Therefore, the novel MAFLD definition is superior to NAFLD on a population level.

INTRODUCTION

The global prevalence of fatty liver disease (FLD) is estimated to be over 25%, making it the most common liver disease worldwide. It has now become one of the leading causes of cirrhosis and hepatocellular carcinoma in the Western world.[4] The global magnitude of this disease and its burden on healthcare outcomes have followed an alarming and rapid increase in obesity and metabolic disorders.[2]

To acknowledge this direct relation of FLD with metabolic dysfunction, a novel definition has been recently proposed as metabolic dysfunction associated fatty liver disease (MAFLD).[27, 29] This new call for change in nomenclature and definition focuses on the presence of metabolic comorbidity (obesity/overweight, diabetes or ≥ 2 metabolic risk abnormalities) alongside steatosis, rather than the arbitrary exclusion of secondary causes of steatosis such as viral hepatitis and excessive alcohol consumption.

The transition from the conventional definition of NAFLD, which emphasises the alcohol stigma, to an aetiology and inclusion-based definition of MAFLD has been explored in numerous cohorts.[85-88, 97, 105-116] In general, almost all cases of NAFLD are identified by MAFLD and similar prevalence rates of NAFLD and MAFLD are observed. The differences between NAFLD and MAFLD become more evident in the two uniquely identified groups. First, the MAFLD-only group, which is characterised by steatosis, metabolic dysfunction, and secondary causes for steatosis. Second, the NAFLD-only group, which has steatosis in the absence of metabolic dysfunction (**Supplementary Figure 1**). Investigating these groups is required because the exclusion of NAFLD-only is a major concern in the transition towards MAFLD. The available literature suggests that those with MAFLD-only are at increased risk for both hepatic and extra-hepatic comorbidities and even mortality.[87, 97, 106, 112, 116-119] On the contrary, the NAFLD-only group, seems to have no apparent liver injury despite steatosis. However, given the low prevalence of especially NAFLD-only, most studies lacked power to compare MAFLD-only directly to NAFLD-only.

Therefore, this meta-analysis aims to systematically compare the epidemiological and clinical features between NAFLD and MAFLD with a particular focus on the non-

overlapping subgroups. These results might facilitate reaching consensus as to whether or not to adapt the new MAFLD definition.

METHODS

Data sources and searches

We conducted a systematic search in Medline, Embase and Web of Science; the last search was performed on the 17th of September 2021. Search terms were MAFLD and NAFLD or their affiliated terms and keywords. The search was performed in collaboration with the medical library of the Erasmus University Rotterdam, the Netherlands and the full search strategy can be found in **Supplementary Table 1**.

Study selection

Articles were screened by two authors (I.A. and L.K.) independently and included if they met the following criteria: adult population with data available regarding the presence of both NAFLD and MAFLD, either in the general population or from selected cohorts. Exclusion criteria were non-human studies, duplicates, non-original data or abstracts. Two independent investigators (I.A. and L.K.) screened titles and abstracts and subsequently full texts of potentially eligible articles found by the search strategy. In case identical cohorts were described in multiple studies, only the study with the most detailed and best extractable data was included. The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flowchart was used to create an overview of the data screening process and the PRISMA NMA checklist (**Supplementary Table 2**) as guidance for reporting on all the required aspects of a meta-analysis.[120, 121]

Outcome measures

Primary outcomes were NAFLD and MAFLD prevalence in the general population. Secondary outcomes were the prevalence of NAFLD-only, MAFLD-only and overlap fatty liver disease among those with either NAFLD or MAFLD, for both the general and non-general population. Non-general population cohorts were either cohorts from outpatient clinics[107, 109, 111] or a group that underwent colonoscopy with consequently a health check.[114] Last, to assess liver injury, MAFLD was compared to NAFLD, and MAFLD-only to NAFLD-only regarding alanine aminotransferase (ALT),

aspartate aminotransferase (AST), fibrosis and liver stiffness. Studies were included if at least one of the outcomes of interest was available.

Data extraction and quality assessment

Data extraction was performed independently by two authors (I.A. and L.K.). The Joanna Briggs Institute (JBI) checklist for cross-sectional studies reporting prevalence data were used for quality assessment[122]. Discrepancies were resolved by mutual discussion among authors (I.A. and L.K.).

Data synthesis and analysis

All analyses were performed in R version 4.0.4 (The R Foundation for Statistical Computing, Vienna, Austria). P-values of < 0.05 were considered statistically significant. Analyses were performed using *meta* version 4.18-2 and *metafor* version 3.0-2. Prevalences were pooled with inverse variance methods and random-effects models. Confidence intervals (CI) were calculated using Clopper Pearson. Statistical heterogeneity was assessed using the I^2 statistic. Funnel plots and Egger tests were performed for analysis concerning the primary outcomes. For analysis including at least five studies, the excessive influence of individual studies was assessed by excluding one study at a time. Analyses were performed separately for the general and non-general population.

RESULTS

Study selection and quality assessment

Our search yielded 439 unique articles, and we identified 38 potentially relevant articles based on title and abstract, of which 21 were excluded, leaving 17 studies for analysis, comprising 9.808.677 individuals (**Figure 1**). For the included articles, study characteristics' are described in **Table 1** and patient characteristics' in **Supplementary Table 2**. It is worth noting that multiple studies have used either the same American NHANES III cohort (from the same inclusion years)[117, 118, 123] or Japanese SAGA cohort[85, 116] with identical or highly overlapping populations. Therefore, to prevent overrepresentation, we only included one study of the same cohort in each (sub)analysis. In four published studies[85, 86, 106, 116] describing two identical cohorts, we have used different studies for primary[116, 124] and secondary outcomes,[85, 106] based on the availability of data and sample size.

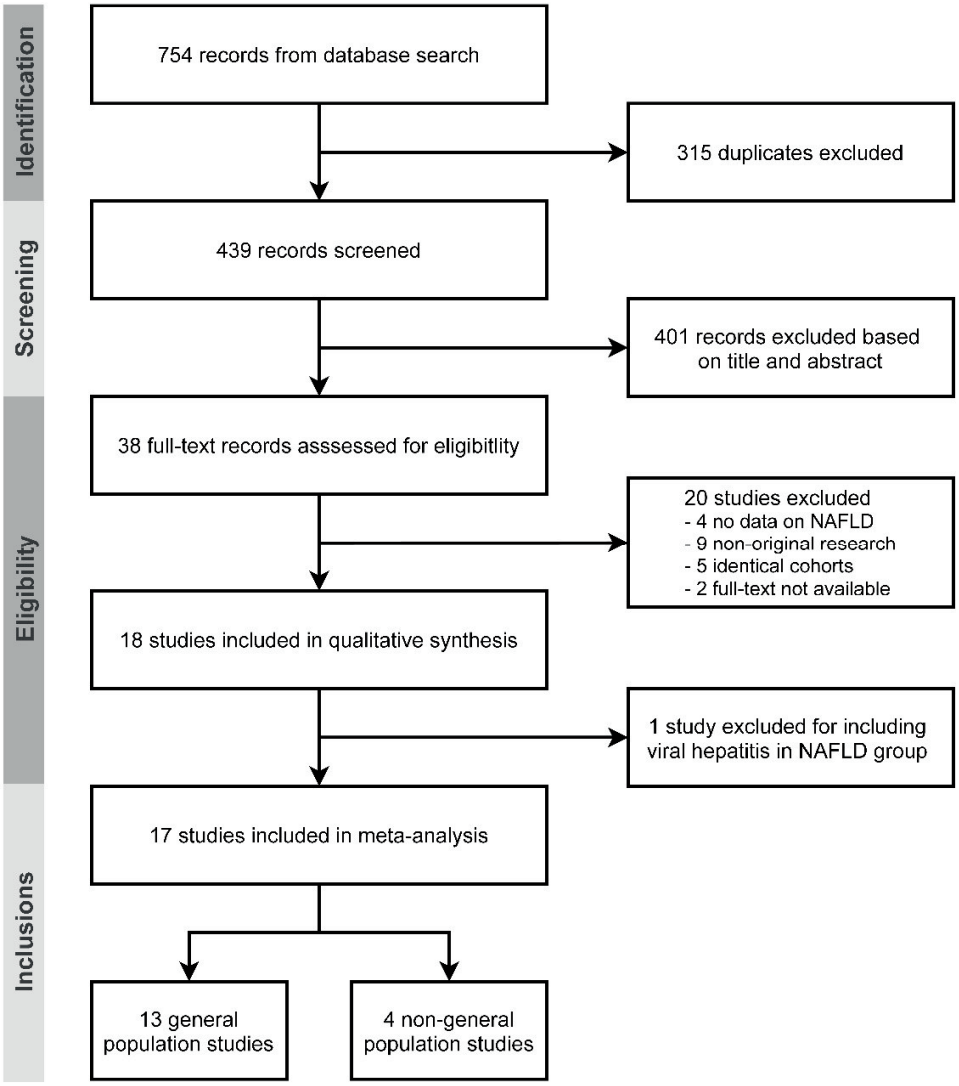


Figure 1: Selection of studies. Flowchart of literature search and study identification. Note that several studies used the same NHANES or SAGA cohort, therefore studies providing the clearest extractable data have been used for this meta-analysis.

Table 1: Study characteristics of included studies

Author	Country	n	Steatosis assessment	Fibrosis assessment	Quality assessment*
General population					
Ciardullo et al. 2021	USA	1,710	CAP	VCTE	8/9
Fujii et al. 2021	Japan	2,254	US	VCTE	8/9
Lee et al. 2020	S. Korea	9,584,399	Fatty Liver Index	N.A.	7/9
Liang et al. 2021	China	6,873	US	N.A.	8/9
Lin et al. 2020	USA	13,083	US	Non-invasive scores	8/9
Nguyen et al. 2021	USA	2,997	US	Non-invasive scores	8/9
Niriella et al. 2021	Sri Lanka	2,985	US	N.A.	7/9
Tsutsumi et al. 2021	Japan	2,160	US	Non-invasive scores	7/9
van Kleef et al. 2021	The Netherlands	5,445	US	VCTE	8/9
Wang et al. 2021	China	152,139	US	N.A.	8/9
Wong et al. 2020	Hong- Kong	922	H-MRS	VCTE	8/9
Yamamura et al. 2020	Japan	765	US	2D SWE	8/9
Yu et al. 2021	China	30,633	US	N.A.	8/9
Non-general population					
Fukunaga et al. 2021	Japan	124	US	N.A.	8/9
Guerreiro et al. 2021	Brazil	1,233	Biopsy	Biopsy	8/9
Huang et al. 2021	Taiwan	175	Biopsy	Biopsy	7/9
Zheng et al. 2020	China	780	Biopsy	Biopsy	8/9

Abbreviations: H-MRS, proton magnetic resonance spectroscopy; VCTE, vibration controlled transient elastography; CAP, controlled attenuation parameter; US, ultrasonography; N.A., non-applicable; SWE, shear wave elastography. *Joanna Briggs Institute (JBI) checklist for cross-sectional studies reporting prevalence data and JBI checklist for cross-sectional studies were used for quality assessment.

MAFLD-only is more prevalent than NAFLD-only

Based on studies among the general population, [85-88, 97, 105, 108, 110, 112, 113, 115, 116] we found a MAFLD prevalence of 33.0% (95%CI 29.7 – 36.5), compared to a NAFLD prevalence of 29.1% (95%CI 27.1 – 31.1, **Figure 2**). Among those with fatty liver disease, 79.9% (95%CI 75.3 – 83.9) met both NAFLD and MAFLD definitions (overlap-FLD), 4.0% (95%CI 2.4 – 6.4) did not meet the novel MAFLD criteria but had NAFLD (NAFLD-only) and 15.1% (95%CI 11.5 – 19.5, **Figure 3**) was additionally captured by the novel MAFLD definition (MAFLD-only).[85, 87, 88, 97, 105, 106, 108, 110, 112, 113] Among the non-general population,[107, 109, 111, 114] MAFLD-only seems to be even more frequent (27.4%, 95%CI 13.0 - 48.9), while the prevalence of NAFLD-only remains low (5.0%, 95%CI 1.7 – 13.7, **Supplementary Figure 2**).

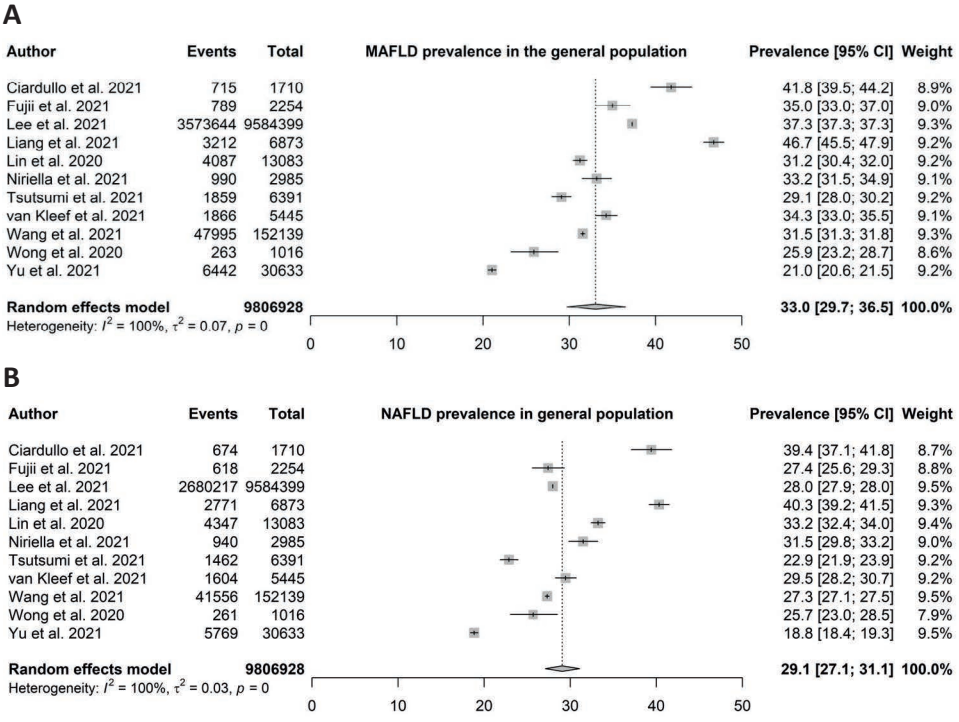


Figure 2. MAFLD (A) and NAFLD (B) prevalence in the general population. Data is provided as pooled prevalence of MAFLD (A) and NAFLD (B) by random effects models and was extracted from general population studies.

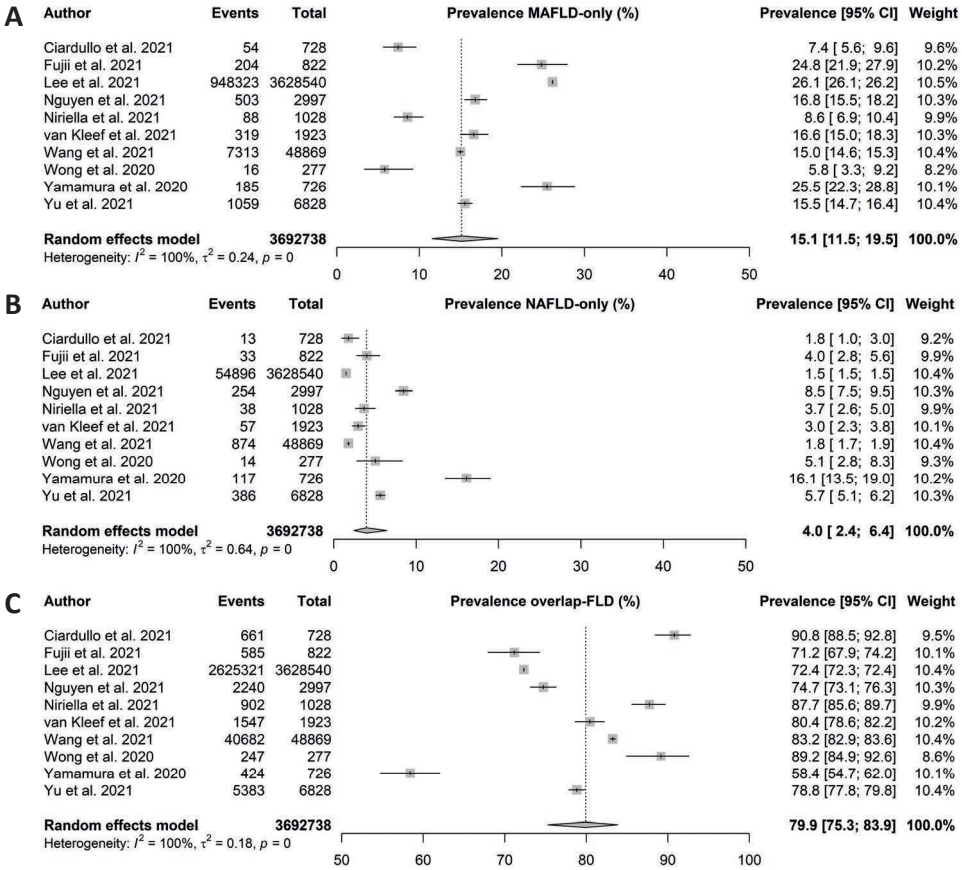


Figure 3. MAFLD-only (A), NAFLD-only (B) and overlap-FLD (C) prevalence in the general population with FLD. Data is provided as pooled prevalence of MAFLD-only (A), NAFLD-only (B) and overlap-FLD (C), by random effects models and was extracted from general population studies.

MAFLD-only is at highest and NAFLD-only at lowest risk for fibrosis

Five studies among the general population reported data on fibrosis within the non-overlapping groups, either based on elastography [85, 88, 105, 108] or FIB-4 > 2.67.[106] The highest pooled prevalence of fibrosis among the general population was found for MAFLD-only (10.2%, 95%CI 6.7 – 15.2), followed by overlap-FLD (4.9%, 95%CI 1.6 – 14.0) and no-FLD (3.2%, 95%CI 2.2 – 4.5). Notably, fibrosis was least common among the NAFLD-only population (2.2%, 95%CI 1.1 – 4.4, **Figure 4**). The relative risk (RR) of fibrosis in the MAFLD-only group was significantly higher compared to NAFLD-only in the general population (RR: 4.2; 95%CI 1.3 – 12.9, **Figure 5**). In line with these results, higher liver stiffness was reported for MAFLD-only compared to

NAFLD-only in the general population by individual studies.[85, 88, 105] Pooled results also indicate higher liver stiffness among those with MAFLD-only compared to NAFLD-only, however, statistical significance was not reached (mean difference 1.59 kPa, 95% CI -0.08 – 3.25, **Figure 5D**).

Next, within the general population, we compared the prevalence of fibrosis in MAFLD-only and NAFLD-only with overlap-FLD. We observed a non-significant RR of 1.8 (95% CI 0.5 – 5.9) for MAFLD-only and 0.6 (95% CI: 0.1 – 3.7) for NAFLD-only versus overlap-FLD (**supplementary Figure 3C and F** respectively), based on three studies.[105, 106, 108] No meta-analysis was performed for comparing NAFLD-only to no-FLD, since only two studies reported fibrosis data for no-FLD.[105, 108] (The no-FLD data is shown in **Figure 4D**).

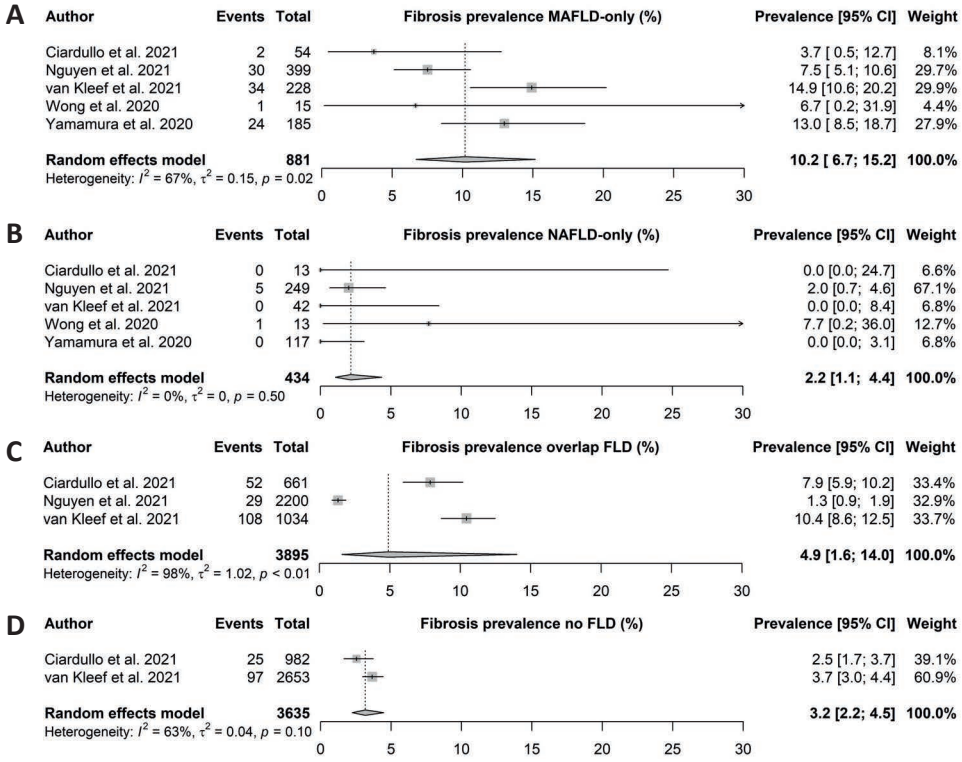


Figure 4. Fibrosis prevalence for MAFLD-only (A), NAFLD-only (B), overlap-FLD (C) and no-FLD (D) in the general population. Data is provided as pooled prevalence of fibrosis in MAFLD-only (A), NAFLD-only (B), overlap-FLD (C) and no FLD (D), by random effects models and was extracted from general population studies.

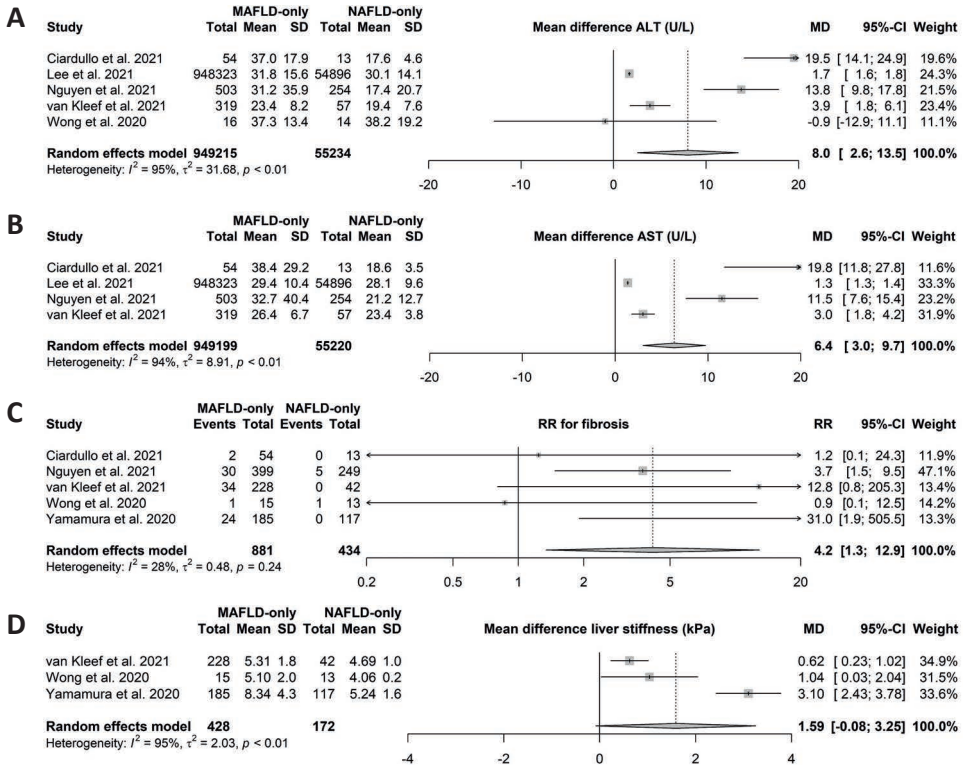


Figure 5. Mean differences of ALT (A), AST (B), relative risk for fibrosis (C) and mean difference of liver stiffness (D) for MAFLD-only compared to NAFLD-only in the general population. Data is provided as mean difference in U/L for ALT (A) and AST (B) and kPa for liver stiffness (D) for MAFLD-only compared to NAFLD-only. Relative risk for fibrosis was calculated and shown in panel C. Random effects models were applied and NAFLD-only was used as a reference.

MAFLD is associated with higher ALT and AST levels compared to NAFLD

Seven cohort studies among the general population reported data on ALT and AST levels for MAFLD and NAFLD.[85, 86, 88, 97, 105, 110, 113] Despite the considerable overlap between NAFLD and MAFLD (± 80%), we observed slightly higher levels of ALT (mean difference 1.1 U/L; 95%CI 0.6 – 1.6) and AST (mean difference 0.8 U/L; 95% CI 0.4 – 1.2) among those with MAFLD compared to NAFLD (**Supplementary Figure 4**). These differences became more evident in the non-overlapping groups of MAFLD-only and NAFLD-only. Based on five studies comparing MAFLD-only to

NAFLD-only, higher levels of ALT and AST were observed for MAFLD-only,[88, 97, 105, 106, 108] resulting in a mean difference of 8.0 U/L (95%CI 2.6 – 13.5) for ALT and 6.4 U/L (95%CI 3.0 – 9.8) for AST (**Figure 5**). Only one study reported AST and ALT levels within the non-overlapping groups for the non-general population, in which no significant differences were observed given the limited sample size and high standard deviations.[109]

In addition to a direct comparison of MAFLD-only to NAFLD-only, we also compared MAFLD-only to overlap-FLD (**Supplementary Figure 3**). Based on four studies,[97, 105, 106, 108] MAFLD-only was significantly associated with higher levels of ALT (mean difference 3.6 U/L; 95%CI 1.9 – 5.3) and AST (mean difference 4.5 U/L; 95%CI 2.2 – 6.8). On the other hand, for NAFLD-only compared to overlap-FLD, lower levels of ALT (mean difference -4.3 U/L; 95%CI -9.4 – 0.8) and AST (mean difference -1.5 U/L; 95%CI -4.8 – 1.8) were found, but these mean differences were not statistically significant.

Finally, three studies reported ALT and AST levels in the NAFLD-only and no-FLD population.[97, 105, 108] One described significantly higher ALT and AST among those with NAFLD-only compared to no-FLD defined by fatty liver index.[97] This is in contrast to other studies that have used abdominal sonography, reporting significantly lower AST and no differences or lower ALT [105, 108] within NAFLD-only versus no-FLD population. After pooling these results, no significant differences were found for ALT and AST levels between NAFLD-only and no-FLD (**Supplementary Figure 5**).

Bias assessment and sensitivity analysis

Funnel plots and Egger tests for the primary outcomes indicated no publication bias on the MAFLD (Egger: p = 0.94) and NAFLD (Egger: p = 0.45) prevalence (**Supplementary Figure 6**). Last, the excessive influence of individual studies on meta-analysis with at least five studies was assessed by excluding one study at a time. With this approach we found similar results and no excessive influence of any of the studies.

DISCUSSION

In this meta-analysis, MAFLD (33.0%) and NAFLD (29.1%) were highly prevalent in the general population, underscoring the magnitude of the fatty liver disease pandemic. Among the fatty liver disease population, only 4.0% had steatosis in the absence of metabolic dysfunction (NAFLD-only) and would therefore be missed if the MAFLD definition would be implemented. On the other hand, the MAFLD definition identifies an unneglectable and a significantly larger group of MAFLD-only (15.1%), which is of particular interest since they had significantly higher AST and ALT levels, and higher risk for fibrosis (RR 4.2) compared to NAFLD-only.

This meta-analysis shows that the MAFLD-only subgroup had the highest prevalence rate of fibrosis (10.2%), which would be missed when applying the conventional NAFLD criteria. Identifying these individuals is of utmost importance since fibrosis is a strong predictor for hepatic and extra-hepatic comorbidity and all-cause mortality.[125] Moreover, a recent model from a joint international study predicted a substantial increase of (initially asymptomatic) hepatic fibrosis among the FLD population.[8] In light of this accumulating disease burden, adapting the MAFLD criteria might help improve the identification of individuals with fibrosis, who are especially prone to adverse outcomes.[124] In this group, liver health might improve and disease progression may be halted after lifestyle changes and appropriate treatment of metabolic diseases.[126] Moreover, when pharmacological treatment for fatty liver disease becomes available, this group might benefit the most from these developments. Therefore, using the MAFLD criteria may enable better management of those at risk for adverse outcomes.

Given the differences in the definition of NAFLD and MAFLD, the MAFLD-only group comprises predominantly individuals with excessive alcohol consumption and viral hepatitis, which are well-known risk factors for liver dysfunction. Therefore, as expected, this group was at the highest risk for impaired liver health, as seen by higher risk for fibrosis and higher mean ALT and AST. Importantly, given the high global prevalence of fatty liver disease, one might have multiple etiologies for liver injury, especially in the presence of metabolic dysfunction. Therefore, the simultaneous presence of MAFLD and secondary causes for steatosis can result in a clinically relevant cumulative risk for liver damage. Our pooled results support

this theory, since MAFLD-only compared to overlap-FLD had significantly higher ALT (mean difference: 3.6 I/U) and AST (mean difference: 4.5 I/U) and a suggestively, but not significantly, higher RR (1.8) for fibrosis. These findings emphasise the concerns of excluding individuals with secondary causes of steatosis according to the NAFLD criteria. Moreover, in a biopsy-proven cohort, the presence of MAFLD was associated with liver-related events and hepatocellular carcinoma among the chronic hepatitis B population.[127] Therefore, identifying subjects with MAFLD in the presence of secondary causes for steatosis is essential to provide adequate multidisciplinary treatment.

Additionally, this meta-analysis revealed that only 4.0% of individuals with NAFLD had no metabolic dysfunction, thus not meeting the MAFLD criteria (NAFLD-only). Some studies have already suggested that this group might not have increased risk for liver damage,[85-88, 105, 106, 108] which is supported by our meta-analysis showing that the NAFLD-only group has the lowest pooled prevalence of fibrosis (2.2%), significantly lower than its counterpart MAFLD-only (10.2%).Therefore, missing out on NAFLD-only appears not to be a significant issue when adapting the novel MAFLD criteria. However, it should be stated that despite combining all available evidence, only 434 individuals were included in the NAFLD-only group, among them 6 had fibrosis, of which 1 had no fibrosis after repeating LSM and the remaining 5 were based on FIB-4 > 2.67 and might not be actual cases. Thus, given that the MAFLD-only group is considerably larger and at increased risk for fibrosis compared to NAFLD-only, adapting the MAFLD criteria would be superior to NAFLD in detecting advanced liver disease on a population level.

Shifting from NAFLD to MAFLD will lead to missing out on the small NAFLD-only group. Despite proving that this group was at a significantly lower risk for liver dysfunction than MAFLD-only, their actual risk for liver dysfunction remains to be determined. In this meta-analysis, we observed a non-significantly decreased risk of fibrosis (RR 0.6) for NAFLD-only compared to overlap-FLD. This is in contrast to a recent study, which suggested that a subgroup of NAFLD-only with severe steatosis was actually at similar risk for fibrosis as MAFLD.[128] However, it should be noted that hepatitis B was highly prevalent (>80%) and not an exclusion criteria for their NAFLD-only group. However, in the NAFLD-only group of this meta-analysis, no significant differences for ALT and AST could be demonstrated compared to overlap-FLD, and the results of individual studies varied widely. Therefore, with the

available data, it seems that those with NAFLD-only are at lower risk for impaired liver health than overlap-FLD, but these findings need to be validated in additional studies.

For investigating the risk of NAFLD-only, a comparison to no-FLD is required. However, insufficient studies reported details about their no-FLD group hampering further investigation of this critical question. Therefore, we encourage future studies to report the fibrosis prevalence accurately for all different subgroups. If NAFLD-only is indeed not at increased risk for adverse outcomes, one could even argue that this group might not have fatty liver disease at all, and was falsely identified as fatty liver disease by imperfect test characteristics of abdominal ultrasound (sensitivity 85%, specificity 94%)[101] or have other drivers for steatosis (e.g. variations in PNPLA3 and TMS6F2) which have been linked to steatosis but not to metabolic syndrome.[98, 100, 129]

Overall, our meta-analysis supports the transition from NAFLD to MAFLD. In clinical practice, this would enable the presence of MAFLD together with i.e., hepatitis B or excessive alcohol consumption. However, the optimal management of patients with multiple etiologies which could result in cumulative risk should be investigated in further studies. For example, one could argue that antiviral therapy should be considered earlier in those with steatosis, metabolic dysfunction and viral hepatitis in addition to stricter monitoring and treatment of metabolic diseases. Increased awareness regarding the novel MAFLD criteria among (primary) health care professionals is needed to allow for the identification of the at risk group of MAFLD-only. Lastly, the change to MAFLD would also question the need for follow-up of the NAFLD-only group. Although, this group seemed to be at lowest risk for liver damage, additional research is needed with adequate follow-up before discharging this group safely from further monitoring.

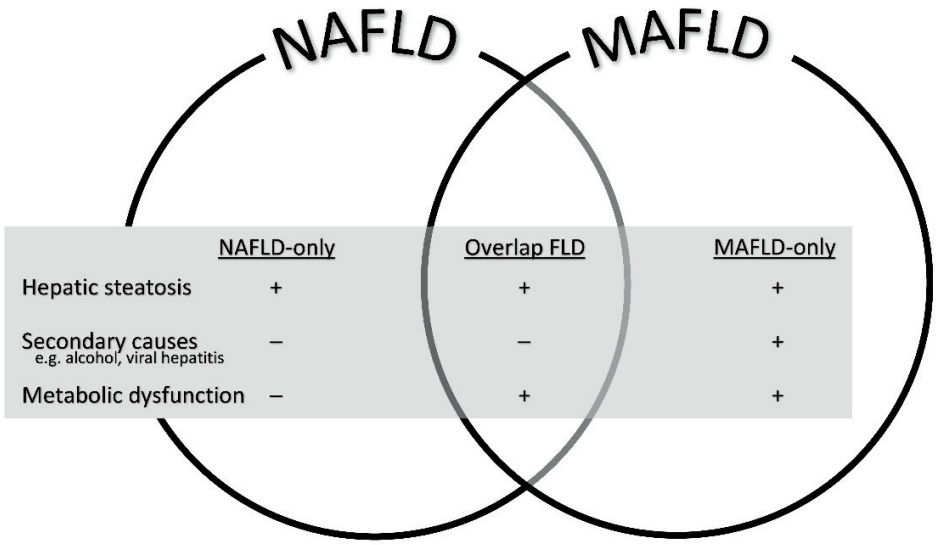
Although this is the first meta-analysis combining the available evidence comparing MAFLD to NAFLD, which included data from the United States, Latin America, Asia and Europe, the following limitations need mentioning. First, limited studies are currently available, resulting in substantial heterogeneity, given the different cohort origins, era of data collection and diagnostic criteria for steatosis and fibrosis. Moreover, following the limited number of studies, publication bias could not be accurately assessed for all sub-analyses and was only ruled out for the

primary outcomes (MAFLD and NAFLD prevalence). Likewise, insufficient studies and data were available for clinically relevant subgroup analysis, particularly when assessing MAFLD-only, NAFLD-only and overlap FLD. Second, although results from the non-general population were consistent with those in the general population, even fewer studies were published among this population and results should be interpreted with caution. Third, the MAFLD-only prevalence is directly affected by viral hepatitis and excessive alcohol consumption, given the design of the NAFLD exclusion criteria. Consequently, variations in regional prevalence of viral hepatitis and excessive alcohol consumption will influence the to be expected benefits of transitioning towards MAFLD. Fourth, this study could not investigate the risk for fibrosis in the MAFLD-only group per NAFLD exclusion criteria (viral hepatitis, excessive alcohol consumption, steatogenic drug use). Further studies are required to investigate if these subgroups are equally at risk for fibrosis.

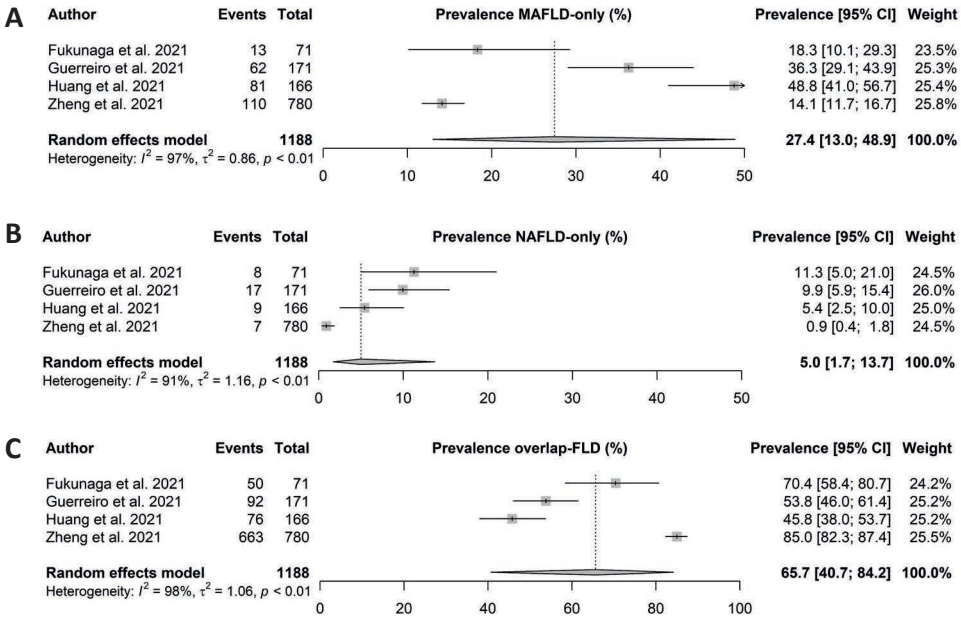
Conclusion

By adopting the novel MAFLD criteria, significantly more individuals were additionally identified with fatty liver disease. Importantly, this extra included group (MAFLD-only) was at significantly increased risk for fibrosis (RR 4.2) and had higher ALT and AST than NAFLD-only. This indicates that on a population level, the novel MAFLD definition is superior to NAFLD, and pledges to adapt the MAFLD criteria. However, future studies should further assess the NAFLD-only group and emphasise the consequences of missing out on this group.

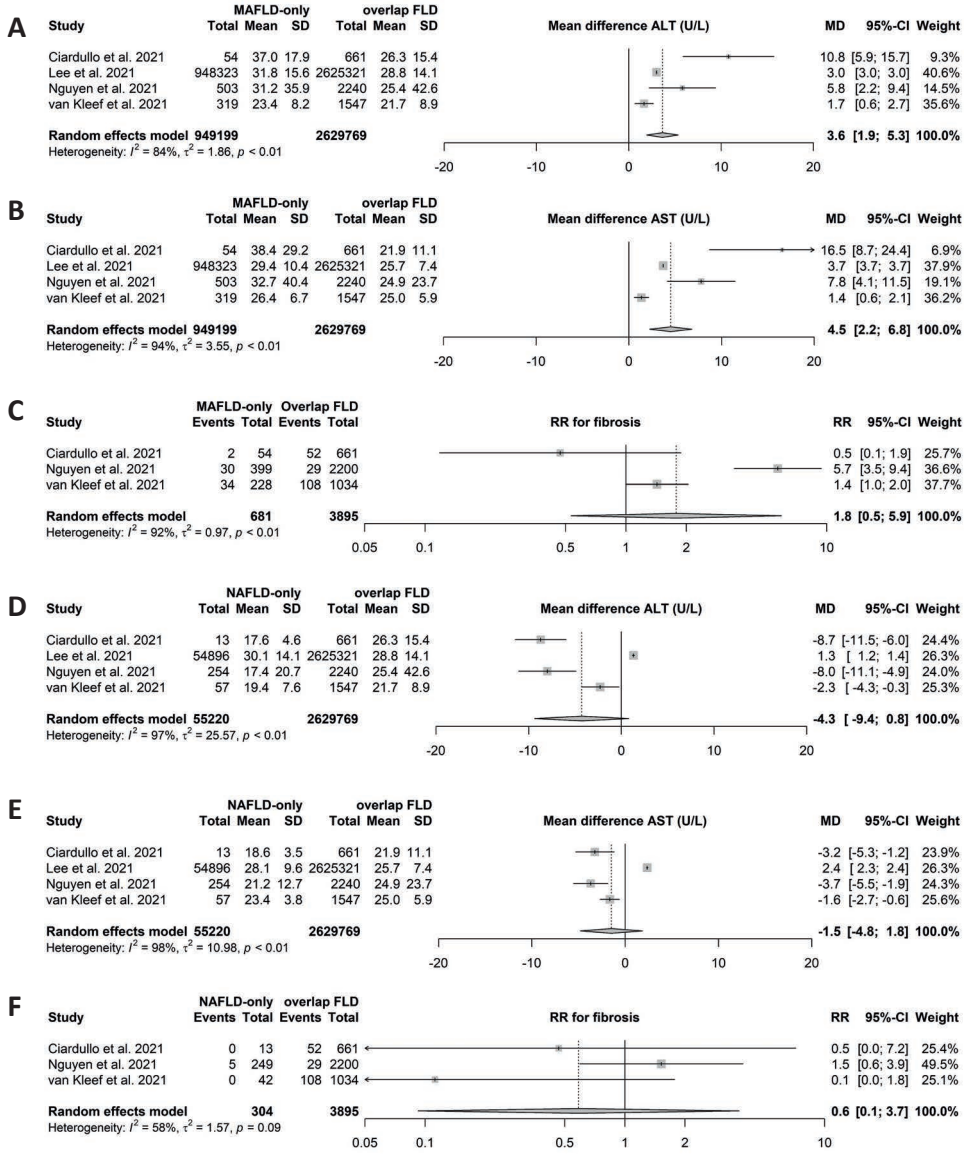
SUPPLEMENTARY FILES



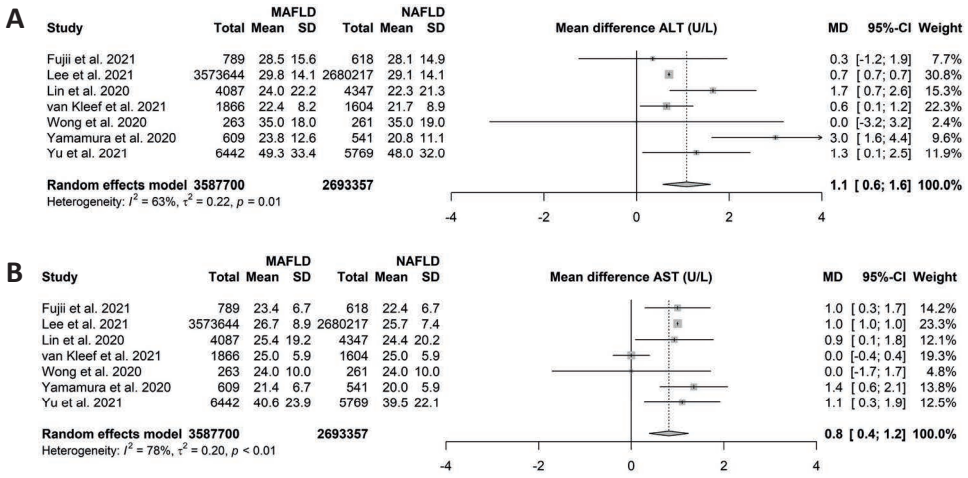
Supplementary figure 1: Characteristics of the different groups identified by the novel MAFLD criteria and the conventional NAFLD criteria. Abbreviations: NAFLD, non-alcoholic fatty liver disease; FLD: fatty liver disease; MAFLD, metabolic dysfunction associated fatty liver disease



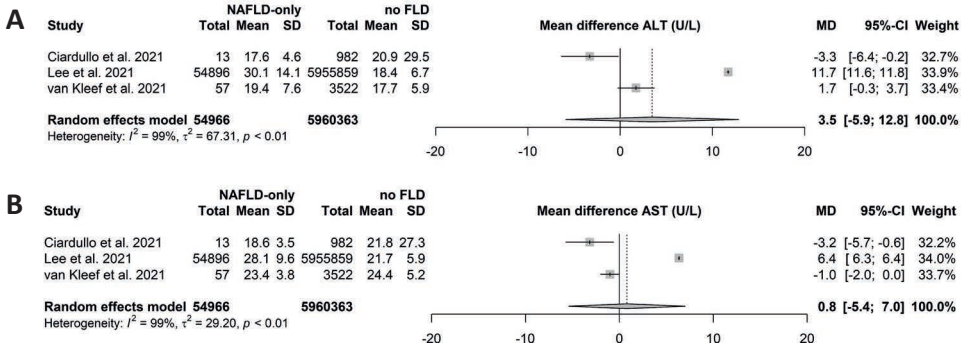
Supplementary figure 2: MAFLD-only (A), NAFLD-only (B) and overlap-FLD (C) prevalence in the non-general population with FLD. Data is provided as pooled prevalence of MAFLD-only (A), NAFLD-only (B) and overlap-FLD (C), by random effects models and was extracted from non-general population studies.



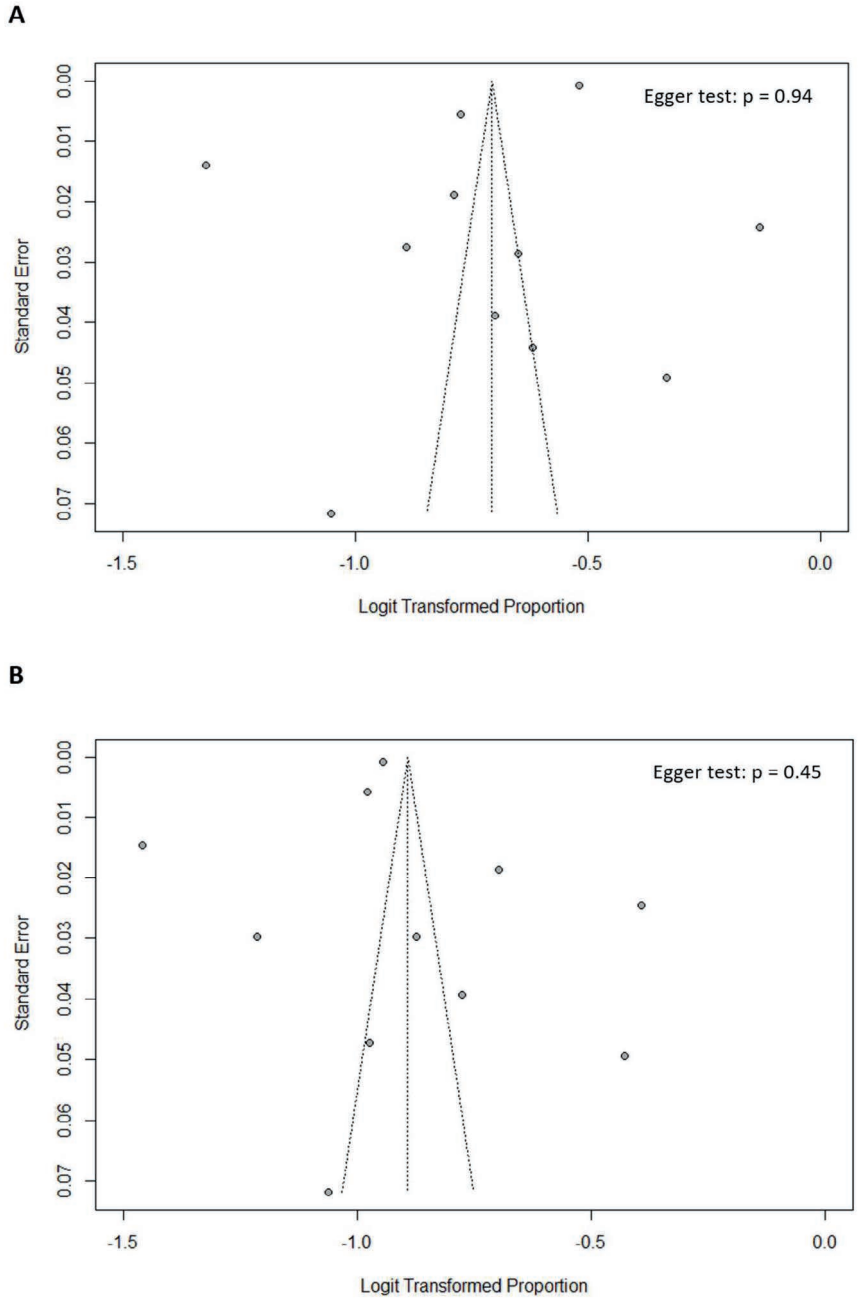
Supplementary figure 3: ALT, AST and relative risk of fibrosis for MAFLD-only and NAFLD-only compared to overlap FLD in the general population. Data is provided as mean difference in U/L for ALT (A and D) and AST (B and E) for MAFLD-only or NAFLD-only compared to overlap-FLD. Relative risk for fibrosis was calculated and shown in panel C and F. Random effects models were applied and overlap-FLD was used as a reference.



Supplementary figure 4: Mean differences of ALT (A) and AST (B) for MAFLD compared to NAFLD in the general population. Data is provided as mean difference in U/L for ALT (A) and AST (B) for MAFLD compared to NAFLD. Random effects models were applied and NAFLD was used as a reference.



Supplementary figure 5: **ALT and AST for NAFLD-only compared to no FLD in the general population.** Data is provided as mean difference in U/L for ALT (A) and AST (B) for NAFLD-only compared to no FLD. Random effects models were applied and NAFLD-only was used as a reference.



Supplementary figure 6: Assessment of publications bias for primary outcomes, MAFLD prevalence (A) and NAFLD prevalence (B)

Supplementary table 1A: Outcomes of database search

Database searched	via	Years of coverage	Records	Records after duplicates removed
Embase	Embase.com	1971 - Present	239	234
Medline ALL	Ovid	1946 - Present	166	26
Web of Science Core Collection*	Web of Knowledge	1975 - Present	349	179
Total			**754**	**439**

*Science Citation Index Expanded (1975-present) ; Social Sciences Citation Index (1975-present) ; Arts & Humanities Citation Index (1975-present) ; Conference Proceedings Citation Index- Science (1990-present) ; Conference Proceedings Citation Index- Social Science & Humanities (1990-present) ; Emerging Sources Citation Index (2015-present)

Supplementary table 1B: Exact search per database

embase.com

('metabolic fatty liver'/de OR (MAFLD* OR ((metabolism OR dysmetabolism OR metabolic* OR metabolic-dysfunction* OR metabolic-associat* OR metabolic-dysfunction-associat* OR metabolic-syndrome*-associat*) NEXT/1 (fatty-liver OR fld))):ab,ti) AND ('nonalcoholic fatty liver'/de OR (nonalcohol* OR non-alcohol* OR nafld*):Ab,ti)

Medline ALL Ovid

((MAFLD* OR ((metabolism OR dysmetabolism OR metabolic* OR metabolic-dysfunction* OR metabolic-associat* OR metabolic-dysfunction-associat* OR metabolic-syndrome*-associat*) ADJ (fatty-liver OR fld))).ab,ti.) AND (Non-alcoholic Fatty Liver Disease / OR (nonalcohol* OR non-alcohol* OR nafld*).ab,ti.)

Web of Science Core Collection

TS=(((MAFLD* OR ((metabolism OR dysmetabolism OR metabolic* OR metabolic-dysfunction* OR metabolic-associat* OR metabolic-dysfunction-associat* OR metabolic-syndrome*-associat*) NEAR/1 (fatty-liver OR fld)))) AND ((nonalcohol* OR non-alcohol* OR nafld*)))

Supplementary table 2: Demographic, anthropometric and clinical variables

Author	Age(years)	Male	BMI(kg/m^2)	Diabetes	Hypertension	Viral Hepatitis
General population						
Ciardullo et al. 2021	48.5	48.6	28.3	8.9	30.6	Hep B: 26.0 Hep C: 4.0
Fujii et al. 2021	53.0	60.2	25.3	13.9	29.1	Excluded
Lee et al. 2020	50.0	48.5	Unspecified	9.1	24.2	4.6
Liang et al. 2021	61.6	42.4	24.9	20.4	73.5	Hep B: 5.3
Lin et al. 2020	43.7	46.8	27.3	15.1	24.9	Unspecified
Nguyen et al. 2021	44.2	54.2	28.2	15.0	51.2	12.9
Niriella et al. 2021	53.0	36.4	Unspecified	29.5	56.6	Unspecified
Tsutsumi et al.2021	51.0	67.1	25.1	7.4	37.2	Excluded
van Kleef et al. 2021	69.2	41.5	27.4	15.2	73.6	Excluded
Wang et al. 2021	48.9	81.1	N.E.	8.8	41.3	Hep B: 2.0
Wong et al. 2020	48.0	42.7	22.8	5.8	15.6	Hep B: 4.9
Yamamura et al.2020	54.0	46.1	24.1	11.6	33.6	Excluded
Yu et al. 2021	45.6	63.5	23.5	6.9	Unspecified	Unspecified
Non-general population						
Fukunaga et al.2021	59.0	80.6	23.1	17.7	25.0	Excluded
Guerreiro et al.2021	56.0	45.2	N.E.	52.1	62.0	24.0
Huang et al.2021	51.3	38.0	27.7	26.0	37.0	56.0
Zheng et al. 2020	42.5	75.0	26.8	34.0	54.0	38.0

Type of viral hepatitis was only shown if provided by original article. Abbreviations: Hep B, hepatitis B; Hep C, hepatitis C; N.E., not extractable. Data is presented as means or as percentage.

CHAPTER 2.3

Metabolic dysfunction-associated fatty liver disease increases risk of adverse outcomes in patients with chronic hepatitis B

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JHEP Rep. 2021 Aug 8;3(5):100350

ABSTRACT

Background & aims: A recent consensus document has defined metabolic dysfunction associated fatty liver disease (MAFLD) as hepatic steatosis together with overweight, diabetes and/or a combination of other metabolic risk factors. The clinical relevance of this novel diagnosis is unknown among patients with chronic hepatitis B (CHB). We studied the association between MAFLD (with or without steatohepatitis) and adverse clinical outcomes in patients with CHB.

Methods: We performed a retrospective long-term follow-up cohort study at two tertiary hospitals on CHB patients that underwent liver biopsy. Biopsies were reassessed for steatosis, fibrosis degree, and steatohepatitis presence. Associations with event-free hepatocellular carcinoma (HCC)-free and transplant-free survival were explored.

Results: In our cohort, 1.076 patients were included, median follow up was 9.8 years (P25-P75: 6.6-14.0) and 107 events occurred in 78 patients, comprising death (n=43), HCC (n=36), liver decompensation (n=21) and/or liver transplantation (n=7). MAFLD was present in 296 (27.5%) patients and was associated with reduced event-free (aHR 2.00, 95%CI 1.26 – 3.19), HCC-free (aHR 1.93, 95%CI 1.17 – 3.21) and transplant-free survival (aHR 1.80, 95%CI 0.98 – 3.29) in multivariable analysis. Among patients with MAFLD, the presence of steatohepatitis (p=0.95, log-rank test) was not associated with adverse outcomes.

Conclusion: The presence of MAFLD in CHB patients was associated with an increased risk for liver-related clinical events and death. Among patients with MAFLD, steatohepatitis did not increase the risk of adverse outcomes. Our findings highlight the importance of metabolic dysfunction in patients with CHB.

INTRODUCTION

Chronic hepatitis B (CHB) is the most common form of chronic viral hepatitis, with an estimated 3.9% prevalence globally.[130] As a consequence of liver cirrhosis and hepatocellular carcinoma (HCC), CHB results in an estimated 887.000 deaths annually.[131] The available antiviral agents effectively suppress HBV DNA and reduce but not eliminate the risk of adverse clinical outcomes.[132] The persistent risk of adverse outcomes may be partially attributable to the presence of co-existing liver diseases such as fatty liver disease. Chronic hepatitis B infection is very much endemic in the Asia-Pacific region, where the prevalence of non-alcoholic fatty liver disease (NAFLD) has also increased rapidly over the last decennia and now matches or even exceeds prevalence in Europe.[4, 133, 134] NAFLD is expected to become the leading cause of liver-related morbidity and the main indication for liver transplantation globally [4, 135] and already accounts for 21.5% of the transplantations in the United States.[80]

With the prevalence of NAFLD rapidly increasing in the regions where HBV infection is most common, a large population is potentially at risk for having two concomitant liver diseases, which may result in a synergistic effect on the risk of HCC, cirrhosis and death. Indeed, (severe) steatosis has been linked to more advanced liver fibrosis and a higher risk of HCC in patients with CHB.[136-138] A previous study from our group also suggested that the presence of non-alcoholic steatohepatitis (NASH) was an important driver of this association.[139] However, assessment of the role of NAFLD in patients with CHB is complex since it requires exclusion of secondary causes of steatosis (one of which may be HBV infection), and since the assessment of steatohepatitis may be challenging in patients with alternative causes of liver inflammation such as active viral hepatitis.

Recently, a transition from NAFLD to metabolic dysfunction associated fatty liver disease (MAFLD) was introduced at an international expert consensus meeting.[29] Hence, diagnosis is no longer based on exclusion of secondary causes for steatosis and/or presence of steatohepatitis, but the focus has shifted towards positive diagnostic criteria based on a combination of significant hepatic steatosis and presence of (components of) the metabolic syndrome. While this rather practical approach underlines the importance of metabolic dysfunction in the pathogenesis

of fatty liver disease, the clinical relevance of this novel classification is yet unknown. In the NHANES III cohort, the novel definition of MAFLD yielded a similar prevalence when compared to the conventional NAFLD criteria,[86] and similar observations were made in cohorts from Hong Kong and Japan.[85, 88] Moreover, among patients with MAFLD, CHB was associated with more inflammation and fibrosis.[140] However, the impact of superimposed MAFLD on long-term clinical outcomes, and the clinical significance of the concomitant presence of biopsy-proven steatohepatitis, is still unclear.

Therefore, in this study, we aimed to investigate the association between MAFLD (with or without steatohepatitis) and adverse clinical outcomes in patients with CHB.

PATIENTS AND METHODS

Patients

This was a multicentre retrospective cohort study comprising all hepatitis B surface antigen (HBsAg) positive patients who underwent liver biopsy between 2005 – 2016 at the Toronto Centre for Liver Disease in Toronto, Canada and between 1985 – 2012 at the Erasmus University Medical Centre in Rotterdam, The Netherlands.[139] Baseline assessment was set at date of liver biopsy.

Biopsy assessment

All liver biopsies were reassessed by three dedicated, experienced, tertiary centre histopathologists for the presence of steatosis (positive if >5%, according to the Brunt classification),[141] degree of fibrosis (based on METAVIR score),[142] inflammatory activity,[143] and presence of ballooning. Steatohepatitis was defined as the combined presence of steatosis, inflammatory activity and ballooning. Moreover, for sensitivity analysis, steatohepatitis was based on NAFLD activity score (NAS) ≥ 3,[6] as used previously.[139]

Follow-up and endpoints

Data regarding antiviral treatment and events, defined as HCC, liver decompensation, liver transplantation and all-cause mortality, were collected from the electronic medical records or local registries through February 2018. The

primary endpoint for this study was event-free survival, with liver transplant-free survival and HCC-free survival assessed as secondary endpoints.

Patient classification

Patients with >5 % steatosis or steatohepatitis on liver biopsy were classified as having fatty liver disease. Patients with fatty liver disease were classified as MAFLD in the presence of either a body mass index ≥25 kg/m^2 (non-Asians) or ≥23 kg/m^2 (Asians) or diabetes mellitus. Thereafter, we re-assessed the charts of non-overweight non-diabetes patients with fatty liver disease for the presence of ≥ 2 minor metabolic health comorbidities (such as hypertension and dyslipidemia).[29] Patients with fatty liver disease, without sufficient data for assessment of MAFLD (defined as missing data on BMI in the absence of other MALFD criteria) were excluded (n=13). Patients within the MAFLD group were further classified as MAFLD with and MAFLD without steatohepatitis based on the biopsy results.

Statistical analysis

Cohort characteristics were described with normally distributed variables presented as mean, SD and non-normally distributed variables as median ± 25th – 75th percentile (P25-P75). Distribution was assessed visually and by skewness and kurtosis. ANOVA was used to study differences for normally distributed continuous data, Kruskal-Wallis for non-normally distributed continuous data and Chi-squared test for categorical data. Kaplan Meier analysis with log-rank statistics was used for survival analysis. Cox proportional hazard models were applied for multivariable analysis. Multivariable models were adjusted for age, sex, hepatitis B envelope antigen (HBeAg) serostatus, advanced fibrosis and antiviral treatment based on factors previously identified as predictors of adverse outcomes in this cohort.[139] All analyses were performed in R 4.0.3 with the Survival package 3.2-3. A *P*-value of < 0.05 was considered statistically significant.

Ethics

This study was conducted according to the principles set forth in the Declaration of Helsinki. The requirement for informed consent was waived and the individual institutional review boards gave the necessary approval. All authors had access to the study data and reviewed and approved the final manuscript.

RESULTS

This cohort comprised 1.089 patients, of whom 13 patients were excluded due to insufficient data for MAFLD classification. Among the remaining 1.076 patients, fatty liver disease was detected in 346 (32%), of whom 296 (86%) had MAFLD (**figure 1**). MAFLD diagnosis was predominantly (96.3%) based on the presence of steatosis with overweight and/or diabetes. Among patients with MAFLD, 134/296 (45%) had steatohepatitis and 156/296 (53%) had NAS ≥ 3.

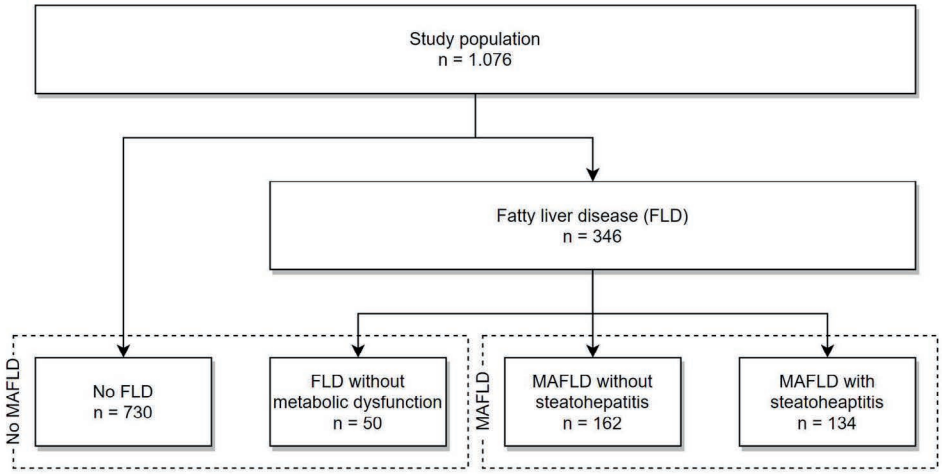

Figure 1 Flowchart study population. MAFLD, metabolic dysfunction associated fatty liver disease; FLD, fatty liver disease.

At study enrolment, the median age was 38.6 years, 66% was male and the majority (57%) had Asian ethnicity. In the overall cohort, 52% were overweight/obese, 5% had diabetes, and 11% had hypertension and/or hyperlipidemia. The majority of patients had elevated alanine aminotransferase (ALT) (70%) at the time of biopsy. Patients with MAFLD were significantly older, were more frequently male and more often had advanced fibrosis (**Table 1**). Characteristics for MAFLD with or without steatohepatitis are shown in **supplementary table 1**.

Table 1: patient characteristics

Variable	No MAFLD n = 780	MAFLD n = 296	*p*
Age (years)	36.7 (13.2)	43.6 (11.7)	<0.001
Female, n (%)	310 (39.7)	59 (19.9)	<0.001
Race, n (%)			0.573
Caucasian	216 (27.7)	92 (31.1)	
Asian	448 (57.4)	168 (56.8)	
African/Black	96 (12.3)	30 (10.1)	
Other	20 (2.6)	6 (2.0)	
Overweight*, n (%)	279 (35.8)	278 (93.9)	<0.001
Hypertension/hyperlipidemia, n (%)	47 (6.0)	76 (25.7)	<0.001
Diabetes, n (%)	20 (2.6)	34 (11.5)	<0.001
ALT (IU/L)	52 [33, 95]	53 [38, 80]	0.409
Elevated ALT†, n (%)	512 (68.5)	216 (75.8)	0.027
HBeAg-positive, n (%)	385 (49.5)	91 (30.7)	<0.001
HBV DNA (log IU/mL)	5.71 (2.63)	4.82 (2.64)	<0.001
Hepatic activity (A2-4), n (%)	394 (50.5)	153 (51.7)	0.782
Advanced fibrosis (F3-4), n (%)	197 (25.3)	94 (31.8)	0.041

Data is presented as mean (SD), median [P25-P75] or n and percentage. *BMI > 25 kg/m2 (non-Asians) or > 23 kg/m2 (Asians), †exceeding local ULN

Median follow-up was 9.8 years (P25-P75: 6.6-14.0), resulting in 11.729 person-years of follow-up. Overall, 107 events occurred in 78 patients, comprising death (n=43), HCC (n=36), liver decompensation (n=21) and/or liver transplantation (n=7). The number of events per group is shown in **supplementary table 2**.

MAFLD is associated with impaired event-free and HCC-free survival

The presence of MAFLD was associated with a significantly decreased event-free survival in the overall population (p<0.001, **figure 2A**), which was consistent in patients with (**figure 3A**) and without (**figure 3B**) advanced fibrosis at study enrolment. Additionally, MAFLD was associated with reduced HCC-free survival (p<0.001, **figure 4A**) and transplant-free survival (p<0.001, **figure 4B**).

Similar results were obtained in multivariable analysis (**table 2**), where MAFLD was independently associated with a reduced event-free survival (aHR 2.00, 95%CI 1.26 – 3.19), HCC-free survival (aHR 1.93, 95%CI 1.17 – 3.21) and transplant-free survival (aHR 1.80, 95%CI 0.98 – 3.29), adjusted for age, sex, HBeAg serostatus, advanced fibrosis and antiviral treatment. Additional adjusting for ALT, ethnicity or medical

centre did not result in significant changes in the described associations. Findings were consistent when only liver-related outcomes were assessed: MAFLD increased the risk of incident HCC (aHR 1.96, 95%CI 1.00 – 3.86, p = 0.049) and of a composite endpoint comprising only liver-related events (decompensation, HCC or liver transplant; aHR 2.19, 95%CI 1.26 – 3.83, p = 0.006).

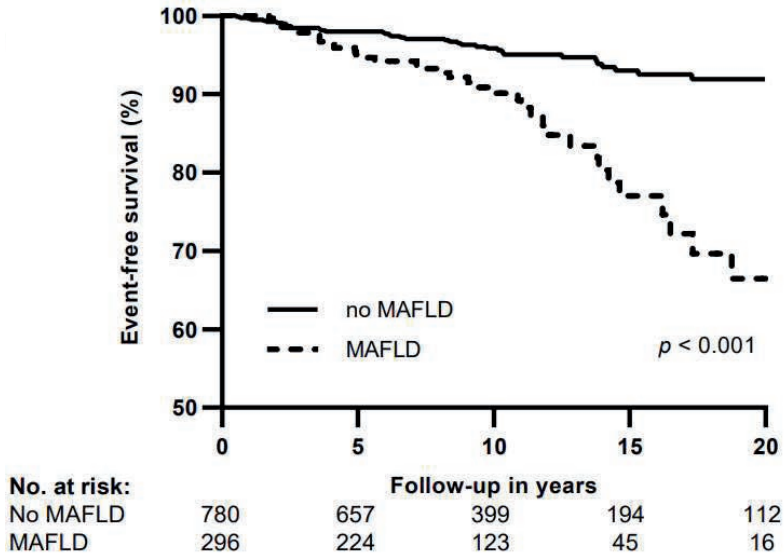

Figure 2 Association of MAFLD with event-free survival. MAFLD, metabolic dysfunction associated fatty liver disease.

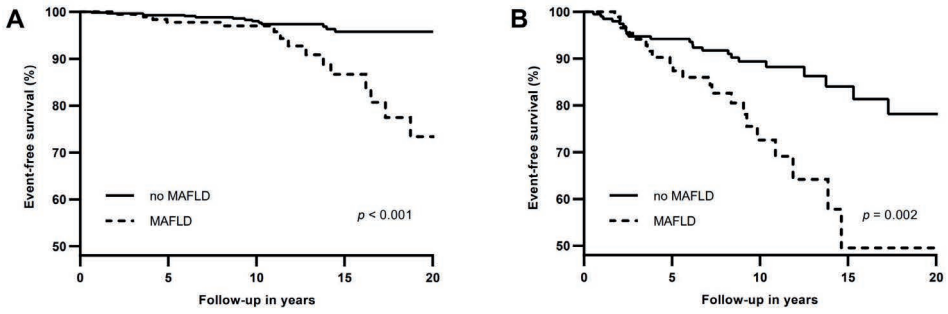

Figure 3. Association of MAFLD for event-free survival in (A) patients without and (B) with advanced fibrosis. MAFLD, metabolic dysfunction associated fatty liver disease. Advanced fibrosis: METAVIR F3-F4

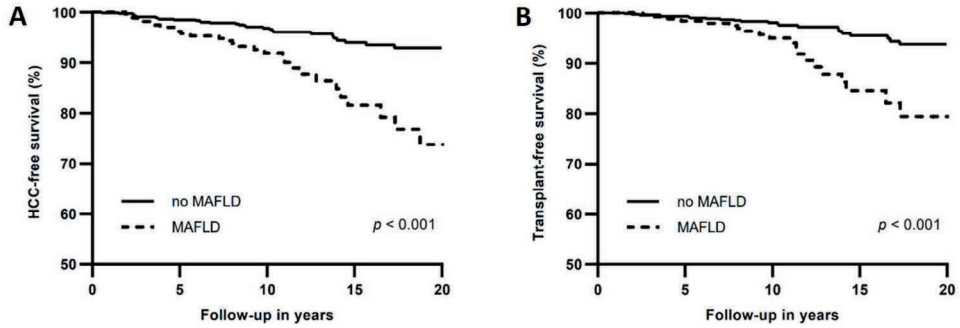

Figure 4. Association of MAFLD with (A) HCC- and (B) transplant-free survival. HCC, hepatocellular carcinoma; MAFLD, metabolic dysfunction associated fatty liver disease.

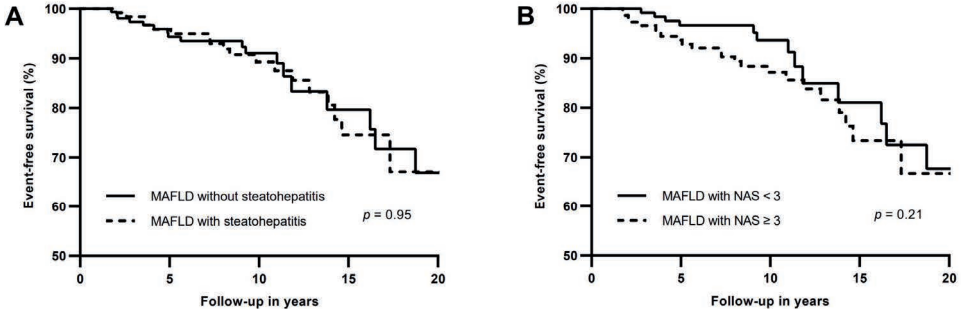

Figure 5. Event-free survival in patients with MAFLD according to presence of (A) steatohepatitis and (B) NAS ≥ 3. MAFLD, metabolic dysfunction associated fatty liver disease; NAS, NAFLD activity score.

Table 2: MAFLD and adverse outcomes

Outcome	HR	95% CI	*P*
Clinical event*			
Unadjusted	3.01	1.91 – 4.73	< 0.001
Multivariable†	2.00	1.26 – 3.19	0.003
HCC / transplant / Death			
Unadjusted	3.04	1.85 – 4.98	< 0.001
Multivariable†	1.93	1.17 – 3.21	0.011
Transplant / Death			
Unadjusted	2.82	1.56 – 5.09	< 0.001
Multivariable†	1.80	0.98 – 3.29	0.058

Results were obtained with Cox-proportional hazards analysis and given as HR with 95% CI. *Clinical event: decompensation, HCC, transplant or death. †Adjusted for age, sex, HBeAg serostatus, advanced fibrosis and antiviral treatment; HCC, hepatocellular carcinoma; HR, Hazard rate.

Similar outcomes in patients with MAFLD with or without steatohepatitis
Event-free survival was similar for patients with MAFLD irrespective of the presence of steatohepatitis (p = 0.95, **figure 5A**) or the presence of NAS ≥ 3 (p = 0.21, **figure 5B**). These results were consistent in multivariable analysis: no associations with adverse outcomes were found for steatohepatitis (p = 0.91) and NAS ≥ 3 (p = 0.38) among patients with MAFLD.

Fatty liver disease without metabolic dysfunction is not associated with adverse outcomes
Among 346 patients with fatty liver disease, 50 had no metabolic risk factors and therefore did not comply with the MAFLD criteria. Event-free survival was similar in these patients compared to patients without signs of fatty liver disease (p=0.56). Similar results were obtained in multivariate analysis (aHR 0.77, 95%CI 0.26 – 2.29).

DISCUSSION

In this large multi-ethnic multicentre cohort study, the presence of MAFLD was independently associated with impaired event-free, HCC-free and transplant-free survival in patients with CHB. Among patients with MAFLD, the concomitant presence of steatohepatitis did not increase the risk of adverse outcomes.

Various recent studies have shown that the obesity pandemic has spread outside the Western World to regions endemic for hepatitis B, leading to an increased prevalence of fatty liver disease in the CHB population.[4, 133, 134] The clinical relevance of concomitant steatosis in patients with CHB has long been debated, since several studies, including a meta-analysis, could not identify steatosis as a risk factor for adverse outcomes.[144] One of the reasons for these contradictory results could be the steatogenic effect of HBV infection. Several potential molecular pathways leading to steatosis are identified for hepatitis B.[145-147] Interestingly, CHB patients with steatosis may also have up to three times increased rates of HBsAg clearance.[136] This complex interplay can result in underestimating the steatogenic effect of HBV and subsequently complicate research into the clinical relevance of fatty liver disease in patients with CHB.

Importantly, recent studies have shown higher rates of significant fibrosis in patients with CHB and steatosis.[148] Our study confirms this association using biopsy-based fibrosis assessment: patients with MAFLD were significantly more likely to have advanced fibrosis at the time of study enrolment. Furthermore, a recent study from our group identified NASH as a risk factor for clinical events in patients with CHB and advanced fibrosis.[139] An important limitation of using NASH as a predictor of adverse outcomes is the requirement for liver biopsy, which is invasive and associated with a risk of severe complications. Besides, the considerable inter-observer variability reported in previous studies is a major concern.[149] Furthermore, assessment of NASH may even be more complicated in patients with CHB, since many histopathological hallmarks of steatohepatitis may also be accounted for by the presence of concomitant HBV-associated inflammation.

Given the contrasting findings regarding the importance of steatosis and the major limitations of using biopsy-proven NASH for risk stratification in HBV, using the novel MAFLD criteria to identify patients at higher risk of adverse outcomes could be of major clinical relevance.

In our cohort, the vast majority of patients with fatty liver disease (86%) also complied with MAFLD criteria, predominantly due to being overweight (94%). In our study, superimposed MAFLD was associated with a significantly impaired event-free, HCC-free and transplant-free survival, but also with incident HCC or liver-related events. This emphasises that impaired event-free survival in this study is not only driven by increased mortality, which may be partly attributable to cardiovascular disease, but also by the increased risk of liver-related events.

The identification of MAFLD as a risk factor for decreased event-free survival and increased risk of HCC is in line with previous studies showing that metabolic comorbidity (e.g. diabetes and metabolic syndrome) is a risk factor for adverse outcomes in patients with CHB.[138, 150, 151] This raises the question of whether MAFLD, even in the absence of advanced fibrosis, is an indication for HCC surveillance. Additionally, future studies should assess whether MAFLD may be a contributing factor to the persistently elevated risk of HCC observed in patients otherwise adequately treated for CHB.[152]

Among the patients with MAFLD in our cohort, 45% had concomitant steatohepatitis. Importantly, steatohepatitis in these patients was not associated with impaired event-free survival, despite being an important predictor for adverse outcomes in a population not stratified for MAFLD.[139] Results were consistent for concomitant presence of NAS ≥ 3. This indicates that the disease burden of fatty liver disease is not limited to patients with NASH, but extends to patients with MAFLD without steatohepatitis. Moreover, these findings suggest that when using the novel MAFLD definition, liver biopsy may not be essential for prognostic assessment of steatohepatitis in patients with both MAFLD and CHB, but could be replaced by thorough metabolic assessment.

Another interesting observation in our cohort is that 14% of patients with fatty liver disease did not comply with the MAFLD criteria. This might either reflect HBV-associated steatosis or so-called lean fatty liver disease. These patients were not at increased risk for adverse outcomes compared to CHB patients without fatty liver disease. These findings further underscore the importance of metabolic dysfunction, rather than fatty liver disease itself, with adverse outcomes.

Given the importance of metabolic health in the CHB population, we recommend a multidisciplinary approach for disease management. This includes screening and treatment of metabolic comorbidities and providing lifestyle intervention programs. Moreover, to prevent disease progression as a result of CHB, the role of early antiviral treatment in this population is up for debate. Whether regression of MAFLD, improvements in metabolic health or early treatment are beneficial on liver-related outcomes in patients with CHB has yet to be determined.

Although this is one of the largest biopsy-controlled, multi-ethnic cohorts with CHB patients to date, spanning over 20 years of follow-up, there are some limitations. First, this is a retrospective cohort study, and data on metabolic comorbidities were not systematically collected. Although we have excluded patients with steatosis with missing insufficient data for classification as MAFLD or no MAFLD, our approach might potentially have misclassified few lean, non-diabetic patients with fatty liver disease as non-MAFLD if multiple minor metabolic dysfunctions were present but not assessed. However, such misclassification would not impact any of our findings, as it would have resulted in including at-risk patients in the control group, causing bias towards finding no difference in adverse event risk. Secondly,

while the long duration of follow-up is an important strength of our cohort, it should be appreciated that patients without MAFLD at baseline might have developed this during follow-up. This issue would only have mitigated the observed differences in our cohort and are therefore unlikely to have had a significant impact on our findings. Furthermore, given the long follow-up duration, patients may have received various forms of antiviral therapy over time. While we adjusted for having received antiviral therapy during follow-up, not all effects of antiviral therapy may be captured by these analyses. However, since patients with MAFLD had more often elevated ALT, they would have been managed more aggressively, making it unlikely that undertreatment of patients with concomitant fatty liver disease has influenced the results of our study. Next, diagnosis of steatohepatitis in patients with CHB is challenging. Current guidelines define NASH based on the presence of steatosis with (lobular) inflammation and ballooning. Since patients with MAFLD have steatosis by definition, only inflammatory activity and ballooning can be used for classification. In our group of CHB patients with MAFLD and data on inflammatory patterns, 99% had lobular inflammation. Inflammatory activity, therefore, does not have significant discriminatory value in this context. This indicates that ballooning is the main discriminating factor in diagnosing steatohepatitis in patients with CHB and MAFLD. These limitations in defining steatohepatitis among patients with CHB may account for the absence of a significantly increased risk of adverse events among MAFLD patients with steatohepatitis when compared to those without. Finally, alcohol use is a well-recognised risk factor for liver disease progression. Patients with known alcoholic liver disease were excluded from this cohort, but a subset of our patients reported (previous) alcohol use. Adding (previous) alcohol use to our models did not influence any of the reported associations.

In conclusion, our study shows that MAFLD is independently associated with impaired event-free, HCC-free and transplant-free survival in patients with CHB. Among patients with MAFLD, the concomitant presence of steatohepatitis did not influence the risk of adverse outcomes. Our findings provide the first evidence for the clinical usefulness of the novel MAFLD criteria in CHB and highlight the importance of metabolic health in these patients.

SUPPLEMENTARY FILES

Supplementary table 1: characteristics of patients with MAFLD according to the presence of steatohepatitis

Variable	MAFLD without steatohepatitis n = 162	MAFLD with steatohepatitis n = 134	*p*
Age (years)	43.3 (11.2)	43.9 (12.1)	0.680
Female, n (%)	35 (21.6)	24 (17.9)	0.518
Race, n (%)			0.198
Caucasian	47 (29.0)	45 (33.6)	
Asian	99 (61.1)	69 (51.5)	
African/Black	12 (7.4)	18 (13.4)	
Other	4 (2.5)	2 (1.5)	
Overweight*, n (%)	154 (96.2)	125 (98.4)	0.453
Hypertension/hyperlipidemia, n (%)	37 (22.8)	39 (29.1)	0.274
Diabetes, n (%)	14 (8.6)	20 (14.9)	0.132
ALT (IU/L)	50 [36, 77]	56 [41, 82]	0.084
Elevated ALT†, n (%)	106 (69.7)	110 (82.7)	0.016
HBeAg-positive, n (%)	57 (35.2)	34 (25.4)	0.090
HBV DNA (log IU/mL)	5.15 (2.70)	4.40 (2.50)	0.016
Hepatic activity (A2-4), n (%)	71 (43.8)	82 (61.2)	0.004
Advanced fibrosis (F3-4), n (%)	46 (28.4)	48 (35.8)	0.215

Data is presented as mean (SD), median [P25-P75] or n and percentage. *BMI > 25 kg/m2 (non-Asians) or > 23 kg/m2 (Asians) †exceeding local ULN

Supplementary table 2: Prevalence of events for MAFLD and no MAFLD patients

Variable	No MAFLD n = 780	MAFLD n = 296
≥ 1 event	42 (5.4)	36 (12.2)
Decompensation	12 (1.5)	9 (3.0)
Hepatocellular carcinoma	18 (2.3)	18 (6.1)
Liver transplant	4 (0.5)	3 (1.0)
All-cause death	25 (3.2)	18 (6.1)

Data is presented as n and percentage.

CHAPTER 2.4

MAFLD and excessive alcohol consumption are both independent risk factors for mortality

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ABSTRACT

Background & aims: MAFLD often co-occurs with excessive alcohol consumption, while its prognostic value in this group remains unclear. We aimed to study the mortality risk of MAFLD in relation to excessive alcohol consumption and its potential interactions.

Methods: We analyzed individuals aged 25 – 74 enrolled in the NHANES III cohort with available steatosis and alcohol data. Individuals with viral hepatitis, BMI <18.5 and missing data on age or follow-up were excluded, leaving 12.656 participants for analysis with a median follow-up of 22.9[20.9-24.8] years. MAFLD was defined as steatosis on ultrasound in the presence of metabolic dysfunction. Daily alcohol intake of ≥10 grams in females and ≥20 grams in males was considered excessive alcohol consumption. We quantified mortality risk with multivariate Cox regression for MAFLD and excessive alcohol consumption. Models were adjusted for age, age squared, sex, race, marital status, education and smoking.

Results: MAFLD was present in 31% and excessive alcohol consumption in 13% and were both independently and simultaneously associated with increased mortality risk in fully adjusted models (aHR 1.21, 95%CI 1.13 – 1.30 and aHR 1.14, 95%CI 1.04 – 1.26, respectively). Similarly, MAFLD was associated with increased mortality risk in individuals with and without excessive alcohol consumption. Participants with both MAFLD and excessive alcohol consumption (4.0%) expressed the highest mortality risk (aHR 1.47, 95%CI 1.28 – 1.71). Results were consistent using the initial ten years of follow-up, a stringent definition of excessive alcohol, and propensity score weighting.

Conclusions: MAFLD increases mortality risk independent of excessive alcohol consumption. This underscores the importance of MAFLD, even in patients with excessive alcohol consumption.

INTRODUCTION

Since the recent introduction of the novel Metabolic dysfunction Associated Fatty Liver Disease (MAFLD) criteria, several research groups have investigated its potential.[29, 86, 88, 105] The additionally identified group with MAFLD (but not Non-Alcoholic Fatty Liver Disease [NAFLD]) is characterised by metabolic dysfunction with steatosis and also includes the presence of secondary causes for steatosis such as viral hepatitis or excessive alcohol consumption. On an important note, the difference between NAFLD and MAFLD is not solely based on the use of alcohol or the presence of viral hepatitis, but also the presence of lean-NAFLD without metabolic risk factors. The latter patients do not comply with MAFLD criteria.[29]

There is now emerging evidence that the prognosis of patients with viral hepatitis could be negatively affected by MAFLD.[127, 153] However, in non-endemic regions like Europe and North America, viral hepatitis accounts only for a rather small proportion of the MAFLD-only group, and additionally identified individuals with fatty liver disease have mostly excessive alcohol consumption.[105, 106, 108] It needs to be stressed that next to alcohol use, these patients have metabolic dysfunction and may likely be not the same group of patients as those with alcoholic liver disease (without MAFLD). Nevertheless, various research groups have attributed the excess mortality or fibrosis risk of this group predominantly to excessive alcohol consumption and did therefore not support the transition to MAFLD.[154, 155] Just shortly after the important de-stigmatization steps taken in the field of alcoholic liver disease,[156] this point of view yet again poses a large and growing group of individuals at the risk of stigmatization. To date, it remains to be elucidated whether the prognosis of MAFLD patients with excessive alcohol consumption is predominantly dependent on their alcohol intake or mainly affected by metabolic dysfunction. We, therefore, aimed to study the mortality risk of MAFLD patients in relation to alcohol use.

PARTICIPANTS AND METHODS

This study was performed within the National Health and Nutrition Examination Survey (NHANES). The NHANES was designed to study individuals' health and nutritional status throughout the United States. In short, from all members of the

sample, extensive data on health and nutrition were collected by interview, physical examination, and a battery of clinical measurements and tests. Detailed information regarding the procedures and rationale have been described elsewhere.[157] Individuals that were part of NHANES III (1988-1994) with available data on steatosis and alcohol were eligible for inclusion. Exclusion criteria were viral hepatitis, BMI < 18.5, missing data on age at baseline and lack of follow-up.

Alcohol

Excessive alcohol consumption was defined as ≥ 10 grams per day for female and ≥ 20 grams per day for male based on interview data in which participants were asked about their drinking habits over the past year, in line with previous studies in the NHANES.[154] According to United States standards, alcoholic drinks counted as 14 grams of alcohol each. In addition, a more stringent definition of excessive alcohol consumption was used in the additional analysis (≥ 20 grams per day for female and ≥ 30 grams per day for male), which has been suggested to be the limit that alcohol can induce steatosis.[1]

Liver assessment

Participants aged 25 – 74 years underwent gallbladder ultrasound (Toshiba Sonolayer SSA-90A), which images were recorded and reassessed in 2009 and 2010 for the presence and grade of hepatic steatosis as described extensively elsewhere.[157-159]

MAFLD was defined as steatosis (irrespective of the gradation) combined with metabolic dysfunction. This comprises either overweight (BMI ≥25 kg/m^2), type 2 diabetes mellitus (defined as antidiabetic drug use, fasting plasma glucose ≥7.0 mmol/L, HBA1c > 6.4% or based on oral glucose tolerance test [OGTT]), or a combination of at least two of the following metabolic abnormalities: (1) waist circumference >102 cm for male and >88 cm for female, (2) blood pressure ≥130/85 mmHg or antihypertensive drug use, (3) plasma triglycerides ≥1.70 mmol/L or lipid-lowering drug treatment, (4) high-density lipoprotein cholesterol (HDL-C) <1.0 mmol/L for men and <1.3 mmol/L for women or lipid-lowering drug treatment, (5) prediabetes defined as fasting plasma glucose 5.6-6.9 mmol/L, HbA1c 5.7-6.4% or matching OGTT, (6) homeostatic model assessment of insulin resistance (HOMA-IR) of ≥2.5, (7) or C-reactive protein (CRP) level > 2 mg/L.[29]

Follow-up and mortality data

Data on vital status was obtained from the national death index and made available in the public use files provided by the National Center for Health Statistics (NHCS) which contained complete data until the 31st of December 2015.[160]

Covariates

Research assistants systematically collected data on age, race, marital status and smoking. Blood samples were taken, which were analyzed for triglycerides, HDL-C, and CRP. An oral glucose tolerance test was performed in which prior to the test, glucose and insulin were measured, and the blood glucose levels were reassessed two hours after consuming 75 grams of glucose.

Statistical analysis

First, we quantified mortality risk for the presence of MAFLD and excessive alcohol consumption (in one multivariate model) with multivariate Cox proportional hazards analysis and adjusted the results for age, age squared and sex in model 1 and additionally for race, marital status, education and smoking status in model 2. Next, we assessed the mortality risk for MAFLD stratified for the presence of excessive alcohol consumption. Moreover, mortality risk was quantified for the four mutually exclusive groups based on MAFLD and excessive alcohol status: MAFLD–/Alc–, MAFLD+/Alc –, MAFLD–/Alc– and MAFLD+/Alc+. In sensitivity analyses, we focused on the 10-year mortality and used stringent definitions of excessive alcohol consumption.

Finally, to address imbalances by an alternative approach, we performed a sensitivity analysis using propensity score weighting to adjust for baseline differences with regard to age, sex, marital status and education to ascertain that these factors did not bias the results. This method is known as inverse probability treatment weighting (IPTW). For this method, a propensity score was constructed based on the probability of being in the MAFLD– or MAFLD+ group using the aforementioned covariates. Next, patients were weighted by the inverse of this propensity, which was stabilised prior to the analysis by using the estimated marginal means of the calculated propensity. Then the weights were inspected across the groups for comparability and possible extreme outliers. Finally, the weights were used in the Cox proportional hazards analysis performing the same

analysis as in the primary analysis. Analyses were performed in in R version 4.0.4 (Foundation for Statistical Computing, Vienna, Austria), using the *survival* package 3.2-10 and SAS version 9.4. *P*-values < 0.05 were considered statistically significant.

Ethics

Participants of the NHANES III provided informed consent. This study was conducted according to the principles as set forth in the Declaration of Helsinki.

Table 1: Participants' characteristics stratified for excessive alcohol and MAFLD status

	MAFLD – / Alc – n = 7.610	**MAFLD + / Alc –** n = 3.399	**MAFLD – / Alc +** n = 1.146	**MAFLD + / Alc +** n = 501
Demographics				
Age (years)	38.7 [28.5, 55.4]	49.9 [37.4, 63.3]	36.9 [27.4, 49.8]	44.8 [34.7, 58.8]
Male	3226 (42.4)	1553 (45.7)	706 (61.6)	360 (71.9)
Race				
Hispanic	2024 (26.6)	1278 (37.6)	276 (24.1)	208 (41.5)
Black	2383 (31.3)	779 (22.9)	364 (31.8)	95 (19.0)
White	2871 (37.7)	1215 (35.7)	482 (42.1)	179 (35.7)
Other	332 (4.4)	127 (3.7)	24 (2.1)	19 (3.8)
College	2538 (33.6)	792 (23.4)	367 (32.3)	121 (24.3)
Current smoking	2100 (27.6)	699 (20.6)	654 (57.1)	221 (44.1)
MAFLD criteria				
BMI ≥ 25	3932 (51.7)	3039 (89.4)	491 (42.8)	438 (87.4)
Diabetes	648 (8.8)	969 (29.1)	69 (6.2)	101 (20.4)
Metabolic dysfunction	3624 (47.6)	3040 (89.4)	462 (40.3)	435 (86.8)
Biochemistry				
AST (U/L)	18 [15, 22]	20 [17, 26]	20 [17, 25]	24 [19, 33]
ALT (U/L)	13 [10, 18]	17 [12, 26]	14 [10, 19]	21 [15, 36]
HDL-C (mmol/L)	1.35 (0.37)	1.16 (0.34)	1.52 (0.49)	1.31 (0.45)
Triglycerides (mmol/L)	1.13 [0.81, 1.62]	1.79 [1.23, 2.59]	1.11 [0.80, 1.65]	1.80 [1.18, 2.79]
HbA1c (%)	5.4 (0.9)	6.0 (1.5)	5.2 (0.6)	5.5 (1.0)

Data is presented as mean (SD), median [P25-P75] or n and percentage. Abbreviations: AST, aspartate aminotransferase; ALT, alanine aminotransferase; BMI, body mass index; HbA1c, glycated haemoglobin; HDL-C, high density lipoprotein cholesterol.

RESULTS

We included 13.225 participants of the NHANES III cohort (1988 – 1994) with available alcohol and liver ultrasound data. Of them, 283 were excluded for viral hepatitis, 272 for BMI < 18.5, 3 for missing data on age, and 11 for lack of follow-up, leaving 12.656 participants for analysis. The median age of the population used for analysis was 41.6 years [30.3 – 58.4], 46% was male and metabolic dysfunction was highly prevalent (e.g. overweight 62%, diabetes 15% and ≥2 minor metabolic dysfunction criteria 60%). MAFLD was present in 31% and excessive alcohol consumption in 13%, resulting in the following distribution of mutually exclusive groups: MAFLD–/Alc– (60.1%), MAFLD+/Alc– (26.9%), MAFLD–/Alc+ (9.1%) and MAFLD+/Alc+ (4.0%). Detailed baseline characteristics of these groups are available in **Table 1**.

In this cohort, 3.804 participants died during the median follow-up of 22.9 [20.9 – 24.8] years, resulting in a mortality rate of 14.4 per 1.000 person-years. Of these deaths, 31.3% (n=1.193) occurred in the initial ten years of follow-up (mortality rate: 9.8 per 1.000 person-years).

Table 2: Mortality risk for the presence of MAFLD and excessive alcohol consumption

	HR	95% CI	*P*
Model 1			
MAFLD	1.18	1.11 – 1.26	>0.001
Excessive alcohol	1.19	1.09 – 1.31	>0.001
Model 2			
MAFLD	1.21	1.13 – 1.30	>0.001
Excessive alcohol	1.14	1.04 – 1.26	0.007

Results were obtained with Cox proportional hazards and given as HR with 95% CI for all cause mortality as outcome (3.804/12.656). MAFLD and excessive alcohol consumption were simultaneously added in the multivariate model. Excessive alcohol consumption was defined as ≥10 grams and ≥20 grams per day in female and male. Results were adjusted for age, age squared, sex (model 1) and in addition for race, marital status, education and smoking (model 2). Abbreviations: CI, confidence interval; HR, hazard ratio; MAFLD, metabolic dysfunction associated fatty liver disease.

MAFLD (aHR 1.21, 95%CI 1.13 – 1.30) and excessive alcohol consumption (aHR 1.14, 95%CI 1.04 – 1.26) were independently and simultaneously associated with increased mortality in fully adjusted models (**Table 2**). Furthermore, after stratification for excessive alcohol status, MAFLD increased mortality risk in both individuals with and without excessive alcohol consumption (HR 1.41, 95%CI 1.17 – 1.71 and HR 1.19, 95%CI 1.10 – 1.27, respectively). Similarly, by introducing an interaction term between MAFLD and excessive alcohol consumption, we could not demonstrate loss of effect for these components in relation to mortality risk (aHR for effect modification 1.14, 95% 0.94 – 1.38).

Further investigating the impact of alcohol and MAFLD on mortality using the four mutually exclusive groups, we demonstrated in the age and sex-adjusted models that the mortality risk for MAFLD+/Alc+ (HR 1.45, 95%CI 1.25 – 1.67) equals the product of MAFLD+/Alc– (HR 1.18, 95%CI 1.10 – 1.26) and MAFLD–/Alc+ (HR 1.17, 95%CI 1.04 – 1.32). In fully adjusted models, similar mortality risk as in the unadjusted models was observed for MAFLD+/Alc– and MAFLD+/Alc+, whereas MAFLD–/Alc+ was no longer associated with increased mortality risk **Table 3**.

Table 3: Mortality risk for the four mutually exclusive groups based on MAFLD and excessive alcohol status

	HR	95% CI	*P*
Model 1			
MAFLD – Alc –		**reference**	
MAFLD + Alc –	1.18	1.10 – 1.26	>0.001
MAFLD – Alc +	1.17	1.04 – 1.32	0.011
MAFLD + Alc +	1.45	1.25 – 1.67	>0.001
Model 2			
MAFLD – Alc –		**reference**	
MAFLD + Alc –	1.19	1.11 – 1.28	>0.001
MAFLD – Alc +	1.08	0.96 – 1.23	0.203
MAFLD + Alc +	1.47	1.28 – 1.71	>0.001

Results were obtained with Cox proportional hazards and given as HR with 95% CI for all cause mortality as outcome (3.804/12.656). Excessive alcohol consumption was defined as ≥10 grams and ≥20 grams per day in female and male. Results were adjusted for age, age squared, sex (model 1) and in addition for race, marital status, education and smoking (model 2). Abbreviations: CI, confidence interval; HR, hazard ratio; MAFLD, metabolic dysfunction associated fatty liver disease.

Next we used a more stringent definition of excessive alcohol consumption (≥20 and ≥30 grams per day in female and male). The distribution of the mutually exclusive groups was as follows: MAFLD–/Alc– 64.9%, MAFLD+/Alc– 28.5%, MAFLD–/Alc+ 4.3% and MAFLD+/Alc+ 2.3%. With this definition, the mortality risk for excessive alcohol consumption was more pronounced (aHR 1.24, 95%CI 1.10 – 1.41), whereas the effect of MAFLD remained stable (aHR 1.21, 95%CI 1.13 – 1.29). Following this trend, the MAFLD–/Alc+ group was at increased mortality risk in contrast to the results obtained with the more lenient definition as mentioned before (**Table 4**). Moreover, there was again no effect modification of mortality risk for MAFLD and excessive alcohol consumption (aHR 1.00, 95%CI 0.78 – 1.28). Similarly to the results with this stringent definition, including alcohol abstinence as a confounder increased the effect size of excessive alcohol consumption (aHR 1.35, 95%CI 1.21 – 1.51). Focussing on individuals with exceptional high alcohol intake (≥60 grams per day, n = 212), mortality risk estimates for MAFLD were similar to previous findings, but no longer significant (aHR 1.56, 95%CI 0.95 – 2.59).

Table 4: Mortality risk for MAFLD and excessive alcohol consumption using a stringent definition for excessive alcohol in fully adjusted models

	HR	95% CI	*P*
MAFLD	1.21	1.13 – 1.29	>0.001
Excessive alcohol	1.24	1.10 – 1.41	0.001
Excessive alcohol X MAFLD			
MAFLD – Alc –		**reference**	
MAFLD + Alc –	1.21	1.13 – 1.29	>0.001
MAFLD – Alc +	1.24	1.05 – 1.47	0.010
MAFLD + Alc +	1.50	1.24 – 1.80	>0.001

Results were obtained with Cox proportional hazards and given as HR with 95% CI for all cause mortality as outcome (3.804/12.656). MAFLD and excessive alcohol consumption were simultaneously added in the multivariate model. Excessive alcohol consumption was defined as ≥20 grams and ≥30 grams per day in female and male. Results were adjusted for age, age squared, sex, race, marital status, education and smoking. Abbreviations: CI, confidence interval; HR, hazard ratio; MAFLD, metabolic dysfunction associated fatty liver disease.

When only the first ten years of follow-up were taken into account, MAFLD was still independent of excessive alcohol consumption associated with increased mortality (aHR 1.14, 95%CI 1.01 – 1.28). Alcohol was only independently associated with ten year mortality if the stringent definition of excessive alcohol consumption was used (aHR 1.37, 95%CI 1.11 – 1.71) and not with the lenient definition (aHR 1.13, 95%CI 0.95 – 1.35). As a final sensitivity analysis, we performed a propensity score weighting analysis where patients were matched on age, sex, marital status and education level. By using the same cox proportional hazard analysis as stated in Table 2 and 3, the results were in line with our previous findings.

DISCUSSION

We investigated the role of MAFLD on mortality in relation to excessive alcohol consumption and demonstrated that the simultaneous presence of MAFLD and excessive alcohol consumption cumulatively increased mortality risk.

There is a large proportion of excessive alcohol consumption in individuals only selected by MAFLD and not by NAFLD, typically > 70 %.[105, 106, 108] Some studies indicated that their prognosis is not determined by MAFLD but rather by their alcohol intake.[154, 155] Our comprehensive investigation of the potential interactions between alcohol consumption and MAFLD provides evidence that MAFLD in fact has prognostic value regardless excessive alcohol consumption. First, we have shown that MAFLD and excessive alcohol were independent and simultaneous predictors for all-cause mortality. Second, MAFLD increases mortality risk in patients with and without excessive alcohol. Third, the mortality risk of individuals with both excessive alcohol consumption and MAFLD exceeds the risk observed for MAFLD+/Alc– and MAFLD–/Alc+ alone. Finally, we replicated this finding using IPTW, a sophisticated approach to account for imbalances in comparison groups. Altogether we have shown convincing evidence supporting the clinical relevance of MAFLD independent of excessive alcohol consumption.

There is no extensive data available yet on MAFLD and excessive alcohol consumption, but we previously reported increased liver stiffness in patients captured only by MAFLD independent of alcohol consumption.[105] Similarly, Yamamura et al., reported a high prevalence of fibrosis in both MAFLD patients with

(19.7%) and without (15.5%) modest alcohol consumption.[85] Moreover, Tsutsumi et al. demonstrated an increased risk of cardiovascular disease independent of alcohol consumption in patients with MAFLD.[116] Furthermore, our findings are in line with the evidence summarised by Idalsoaga et al. in their review on NAFLD and alcohol-related liver disease.[161] They concluded that NAFLD and alcoholic liver disease often coexist and that alcohol consumption, even within the arbitrary thresholds allowed for NAFLD, may contribute to disease progression. Within individuals with steatosis, Younossi et al., also demonstrated the relevance of alcohol consumption, particularly for higher thresholds of alcohol consumption.[162] Although most evidence originated from the NAFLD-era, they support the findings of this MAFLD-oriented paper.

Although the group of individuals with both MAFLD and excessive alcohol use had the highest risk of mortality, we found no effect modification between excessive alcohol consumption and MAFLD. The absence of effect modification means that there is a cumulative increase in mortality risk in case both MAFLD and excessive alcohol consumption are present. We, therefore, have to reject our hypothesis that the simultaneous presence of MAFLD and excessive alcohol consumption might result in synergistically increased mortality risk, as described recently.[163]

From another point of view, MAFLD might no longer be relevant among individuals with exceptionally high alcohol intake due to competing risks. Nonetheless, among participants with alcohol intake exceeding 60 grams per day, MAFLD seemed equally harmful. However, we note that confidence intervals were wide due to the limited number of participants drinking this much (n = 212), hampering us from firm conclusions.

Conflicting results have been obtained in the NHANES III cohort regarding the mortality risk of MAFLD.[117, 119, 154] These may be attributed to differences in design, specifically (1) adjusting for several MAFLD criteria, (2) not accounting for age as a non-linear risk factor, and (3) not considering mild hyperechogenicity as steatosis. For our aim, the interaction between MAFLD and excessive alcohol consumption, we adjusted primarily for demographics and social-economic status in the mortality risk of MAFLD. However, additional adjusting for BMI yielded again similar mortality risk for patients with and without excessive alcohol consumption, although attenuated.

Several studies have shown that the association between alcohol intake and mortality follows a J-shaped curve, in which those drinking moderately have the lowest mortality risk. This phenomenon was recently replicated in the NHANES cohort.[164] Although this non-linear association is debated and there is no safe limit of alcohol consumption,[165, 166] this J-shaped association might cause underestimating the mortality risk for excessive alcohol consumption. Nonetheless, in our study still a modestly increased mortality risk was observed for excessive alcohol consumption, which increased with a more stringent definition of excessive alcohol consumption. Similarly, by accounting for "abstainer bias" (which may drive the J-shaped curve) by additional adjusting the final models for alcohol abstinence, we demonstrated relatively larger effect sizes for excessive alcohol consumption (aHR 1.35, 95%CI 1.21 – 1.51) while the effect of MAFLD remained stable (aHR 1.21, 95%CI 1.13 – 1.29). This illustrates that the J-shaped association between alcohol consumption and mortality did not affect our conclusions regarding the clinical relevance of MAFLD.

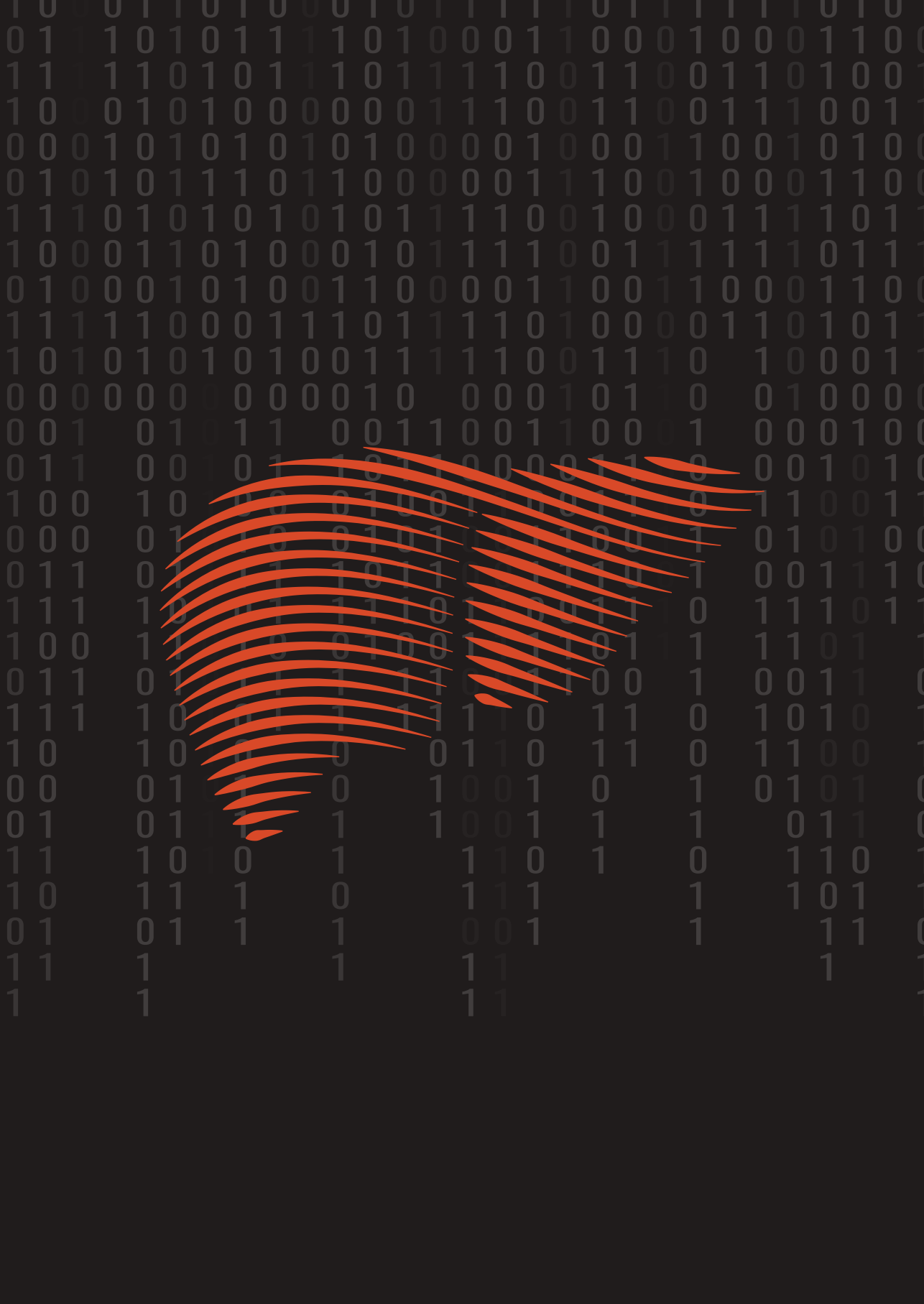
Interestingly, among the 494 individuals with excessive alcohol consumption in the absence of metabolic dysfunction, only 23% (n=112) had steatosis. Hence, among MAFLD patients with excessive alcohol consumption, the primary driver of steatosis is likely to be metabolic dysfunction and not excessive alcohol consumption. Therefore, MAFLD with excessive alcohol consumption should not be similarised nor seen as alcoholic liver disease.

This study's findings clearly showed the clinical relevance of MAFLD in patients with excessive alcohol consumption and, in that respect, support the transition from NAFLD towards MAFLD. The cumulative risk of MAFLD and excessive alcohol consumption for mortality illustrates that within the MAFLD spectrum, these individuals are at increased risk. Hence, MAFLD patients should not only be treated for metabolic traits, but their alcohol consumption should also be addressed and vice versa. Given the increased mortality risk of MAFLD patients with excessive alcohol consumption, we support a specific subgroup for these individuals – under the same umbrella term of MAFLD – as was proposed recently.[154]

Although this study decomposed the effects of MAFLD and excessive alcohol comprehensively and had a large sample size with a median follow-up of 23 years the following limitations need mentioning. First, coherent to the extensive follow-

up, baseline data from this cohort originated from 1988-1994 and the prevalence of MAFLD and excessive alcohol consumption might not reflect its current extent. Second, we did not use the provided weights to modulate the United States general population because we focused on the concept of interaction between MAFLD and excessive alcohol consumption in relation to mortality rather than estimating exact risks for the United States population. Third, in population studies, alcohol consumption is difficult to assess and often underreported. Hence, we applied a rather low cut-off for excessive alcohol data and replicated our findings using other cut-offs. Fourth, although the extended follow-up is one of the strengths of the NHANES cohort, one can argue the prognostic value of modifiable factors such as MAFLD and excessive alcohol consumption beyond a certain time point. Therefore we confirmed our main results using only the initial ten years of follow-up. Fifth, there was an imbalance between the four mutually exclusive groups in terms of age, sex and socioeconomic status. In addition to taking these factors into account in multivariate models, we performed propensity score weighting, which yielded similar results. Last, although mortality is the ultimate endpoint, we could not differentiate between all-cause mortality and liver-related mortality due to the restricted nature of this data. Moreover, no follow-up data were available on liver-specific data such as fibrosis stage or liver-related events. Additional studies should investigate whether, despite the absence of interaction for all-cause mortality, the risk of liver-related adverse outcomes is synergistically increased for the simultaneous presence of MAFLD and excessive alcohol consumption.

In conclusion, MAFLD increases mortality risk regardless of excessive alcohol consumption and there was no effect modification regarding mortality between MAFLD and excessive alcohol consumption. Therefore, MAFLD seems an important entity regardless of drinking habits. However, since mortality risks increase for the simultaneous presence of MAFLD and excessive alcohol consumption, we recommend a specific subgroup for the presence of excessive alcohol consumption, using MAFLD as the umbrella term.



CHAPTER 3

Considerations in early detection of liver disease

CHAPTER 3.1

Fatty liver disease is not associated with increased mortality in the elderly: a prospective cohort study

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ABSTRACT

Background & aims: Fatty liver disease has been associated with excess mortality. Screening for hepatic steatosis in patients with metabolic dysfunction is therefore recommended by several guidelines, despite a paucity of evidence on the clinical relevance of fatty liver disease in this specific subgroup.

Methods: We studied participants of an ongoing prospective cohort (the Rotterdam Study). Individuals aged ≥65 were enrolled from 2009 – 2014 and followed through 2018. Steatosis was assessed by ultrasound and liver stiffness by transient elastography. The association between hepatic steatosis and liver stiffness with mortality was assessed using Cox regression analysis adjusted for age, sex, education, smoking, individual components of the metabolic syndrome, heart failure, coronary heart disease, and stroke.

Results: We included 4.093 elderly participants (aged 74.4±6.6; 42.7% male), 36.8% had ultrasound-based steatosis. During the median follow-up of 6.9 years, 793 participants died (29.6 per 1.000 person-years). In the overall population, steatosis was not associated with mortality in multivariable analysis (aHR 0.87, 95%CI 0.73 – 1.03). Findings were consistent across a range of clinically relevant subgroups, including age categories, sex, metabolic syndrome, elevated liver enzymes and cardiac disease. Sensitivity analyses showed similar results for mortality beyond five years of follow-up, cancer-related and cerebro-cardiovascular mortality. Furthermore, among participants with steatosis, higher liver stiffness (aHR 1.04 per kPa, 95%CI 0.95 – 1.14) was not associated with mortality.

Conclusion: The presence of fatty liver disease was not associated with mortality in this cohort nor in a range of subgroups. This indicates that screening for fatty liver disease and/or fibrosis is unlikely to improve outcomes among the elderly population.

Introduction

Fatty liver disease has become the most common cause of chronic liver disease in many Western countries. Various studies have established the association between the presence of fatty liver disease and an increased risk of hepatocellular carcinoma as well as both liver and non-liver-related mortality.[44] Given the strong association between the metabolic syndrome and its components with fatty liver disease, screening for steatosis and/or advanced liver disease in patients with metabolic comorbidity is recommended by several guidelines.[1, 21, 66, 167, 168]

Screening strategies typically target individuals with clinical risk factors for fatty liver disease or biochemical signs suggestive of liver disease.[21, 66, 167, 168] Since the prevalence of metabolic comorbidities is rapidly increasing, an increasing number of patients will become eligible for hepatic assessment. This is particularly relevant in the elderly, because the majority will have at least one risk factor for fatty liver disease and among them screening appears to be challenging due to poor performance of non-invasive tests in this group.[169-171]

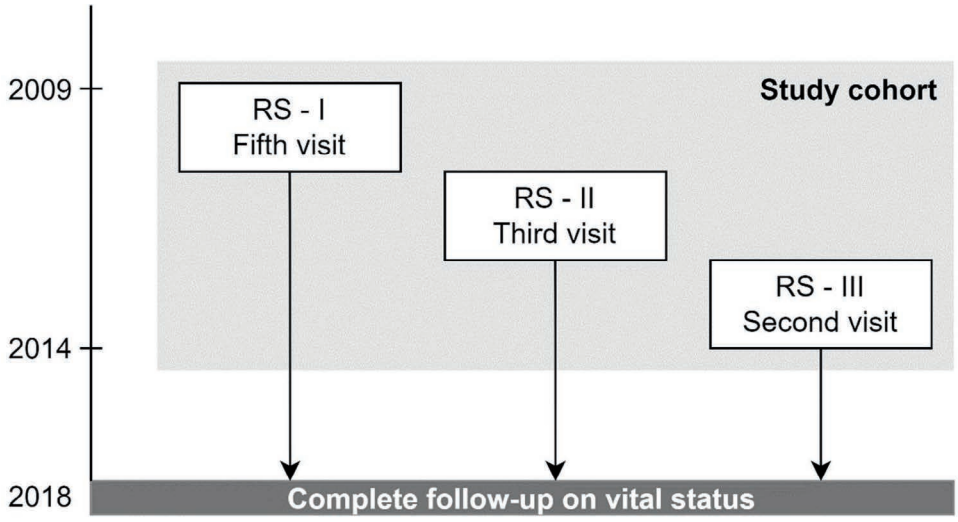
Current guidelines that support screening would therefore necessitate an assessment of hepatic steatosis in the majority of elderly persons,[21, 66, 167, 168] despite a paucity of evidence on the clinical relevance of fatty liver disease in this target population. Although fatty liver disease has been associated with mortality in several large studies, careful assessment of elderly subgroups in these cohorts revealed no association between the presence of fatty liver disease and excess mortality in this subset.[172-175] These studies, however, included only a limited number of elderly participants with fatty liver disease and these findings therefore warrant further exploration.

Therefore, we investigated the relationship between fatty liver disease and mortality in an elderly population.

PARTICIPANTS AND METHODS

The Rotterdam Study is a large, prospective, population-based cohort study, which commenced in 1989, enrolling adults aged 45 or above residing in the Ommoord

district of Rotterdam, the Netherlands. Since 2009 hepatic assessment was introduced as part of the regular visits. The rationale, study design, and recent findings have been summarised elsewhere.[89] For the current analysis, only participants visiting the research center between 2009 and 2014, aged ≥ 65, with available data on hepatic ultrasound were included (**Figure 1**).



Liver assessment	Subjects	Follow-up	Deaths
Abdominal ultrasound	4.093	6.9 year	793
Fatty Liver Index	3.912	6.8 year	743
Transient elastography	2.584	6.7 year	308

Figure 1: Overview of the Rotterdam Study subsets included in our study cohort. For the final cohort, RS-I, RS-II and RS-III data was combined. Data originated from visits in 2009 until 2014 and follow up was complete until 2018.

Liver stiffness and steatosis assessment

All enrolled persons underwent hepatic ultrasound by an experienced operator. Steatosis was assessed using established ultrasound criteria.[90] For sensitivity analyses, we also defined the presence of steatosis as a fatty liver index (FLI) ≥ 60.[176] Metabolic dysfunction-associated fatty liver disease (MAFLD) was defined as the presence of steatosis together with either overweight, diabetes or presence of ≥ 2

minor criteria.[29] Non-alcoholic fatty liver disease (NAFLD) was defined as the presence of steatosis in the absence of secondary causes comprising viral hepatitis, steatogenic drug use or excessive alcohol consumption (> 20 grams per day for female or >30 grams per day for male).[1] Participants were excluded from NAFLD analysis if secondary causes for steatosis were present or could not be ruled out, in line with recent publications.[105]

Liver stiffness was measured using transient elastography (FibroScan ®, EchoSens, France), according to the manufacturer's instructions. Only measurements that complied with the criteria described by Boursier et al. were considered valid (IQR < 30% in case of LSM ≥ 7.0 kPa).[91] Liver stiffness was subsequently categorised using a threshold of 8.0 kPa, which suggests fibrosis.[92]

Follow-up and mortality data

Mortality data were extracted from the municipal registries and was complete until the 1st of January 2018. Cause-specific mortality was obtained from medical records and complete until the 1st of January 2015. In addition to all-cause mortality, we also assessed the association between fatty liver disease and cancer-related mortality (comprising all neoplasms regardless of primary origin) and cerebro-cardiovascular mortality (comprising cerebrovascular, cardiovascular and vascular events).

Covariates

Blood samples were acquired at each study visit. Performed tests included liver biochemistry, serum glucose, Homeostatic Model Assessment for Insulin Resistance (HOMA-IR), and assessment of dyslipidemia. All individuals underwent anthropomorphic measurements, including waist circumference. Medication data were obtained from direct linkage with pharmacy databases, with actual use verified during an interview. According to the ATP-III criteria, the metabolic syndrome was present if the participants complied with at least three of the following subcomponents.[94] (1) (pre)diabetes, defined as fasting glucose > 5.6 mmol/L, anti-diabetic drug use or diagnosis of diabetes by health care professionals; (2) High waist circumference, defined as > 102 cm in males or > 88 cm in females; (3) Hypertriglyceridemia, defined as triglycerides ≥ 1.7 mmol/L and/or lipid-lowering drug use; (4) Hypo-HDL, defined as high-density lipoprotein (HDL) < 1.04 mmol/L in male or < 1.30 in female and/or lipid-lowering drug use; and

(5) hypertension, defined as either a systolic blood pressure ≥ 130 mmHg, diastolic blood pressure ≥ 85 mmHg and/or antihypertensive drug use.

Statistical analysis

Associations between baseline factors and mortality during follow-up were assessed using Cox proportional hazard regression. Associations between steatosis, NAFLD, MAFLD and all-cause mortality were first explored in the overall population. The fully adjusted model comprised education, smoking, alcohol, the individual components of the metabolic syndrome (hypertension, (pre)diabetes, high waist-circumference, hypo-HDL, hypertriglyceridemia), history of coronary heart disease, heart failure and stroke, based on previous research in this cohort and clinical relevance.[95] Next, we assessed the association between steatosis and all-cause mortality across various subgroups, including age categories, sex, presence of metabolic syndrome (and its individual components), presence of liver test abnormalities (according to local upper limit of normal), and history of cardiovascular disease (heart failure, stroke or coronary heart disease).

Furthermore, we assessed the impact of BMI on the investigated associations in two ways. First, we included BMI as a covariate besides the already included covariates. Second, we stratified the main analysis for BMI categories (< 25, 25-30 and ≥30 kg/m^2).

For additional sensitivity analyses, associations were further explored for mortality before and after five years of follow-up; and for cause-specific mortality: 1) cancer mortality; and 2) cerebro-cardiovascular mortality. Additionally, analyses were performed with the diagnosis of steatosis based on FLI instead of ultrasound. Finally, we assessed the association between liver stiffness (continuous and categorical) and mortality stratified for the presence of steatosis. Participants with a history of heart failure were excluded from these analyses as heart failure is associated with increased liver stiffness due to congestion.[57, 177]

Analyses were performed using R version 4.0.4 (Foundation for Statistical Computing, Vienna, Austria), using the *survival* package 3.2-10. A *P*-value of < 0.05 was considered statistically significant.

Ethics and participants involvement

The Rotterdam Study has been approved by the Medical Ethics Committee of Erasmus MC (registration number MEC 02.1015) and by the Dutch Ministry of Health, Welfare and Sport (Population Screening Act WBO, license number 1071272-159521-PG). The Rotterdam Study Personal Registration Data collection is filed with the Erasmus MC Data Protection Officer under registration number EMC1712001. The Rotterdam Study has been entered into the Netherlands National Trial Register (NTR; www.trialregister.nl) and into the WHO International Clinical Trials Registry Platform (ICTRP; www.who.int/ictrp/network/primary/en/) under shared catalog number NTR6831. All participants provided written informed consent to participate in the study and to have their information obtained from treating physicians. All authors had access to the study data and reviewed and approved the final manuscript. Participants were not involved in the research design and conduct.

RESULTS

We included 4.093 elderly participants; the mean age was 74.4±6.6 years, 98.1% was of European ancestry, 42.7% was male. Metabolic comorbidity was highly prevalent (e.g. diabetes 18.0%; BMI 27.6±4.2 kg/m^2, metabolic syndrome 54.7%). This resulted in 85.4% (n = 3.496) of participants necessitating hepatic assessment for the presence of metabolic dysfunction according to the 2021 EASL guideline on non-invasive tests.[29, 66] Among the included participants, 36.8% had steatosis and 7.1% liver stiffness ≥ 8.0 kPa. Additional baseline characteristics are shown in **Table 1**. During the median follow-up duration of 6.9 years, 793 deaths were recorded, yielding a mortality rate of 29.6 per 1.000 person-years. Among those with cause-specific mortality data (n=344/793), 39.2% died due to cancer and 30.5% died due to cerebro-cardiovascular events; only 1 participant died a liver-related death. MAFLD was present in 1459 of 4089 (35.7%) participants after excluding 4 participants for insufficient data for classification, and NAFLD was present in 1148 of 3225 (35.6%) participants after excluding 868 participants with secondary causes for steatosis (n = 611) and/or insufficient data on alcohol consumption (n = 266).

Table 1: Participants' characteristics

Variable	All n = 4093	steatosis n = 1508	No steatosis n = 2585
Demographics			
Age (years)	74.4 (6.6)	73.7 (6.0)	74.8 (6.9)
Male	1749 (42.7)	661 (43.8)	1088 (42.1)
Education			
Low	2131 (52.8)	853 (57.6)	1278 (50.0)
Medium	1200 (29.7)	418 (28.2)	782 (30.6)
High	705 (17.5)	211 (14.2)	494 (19.3)
Current/former smoking	2766 (67.7)	1076 (71.6)	1690 (65.5)
Excessive alcohol intake	499 (13.0)	227 (16.1)	272 (11.3)
Physical examination			
Waist circumference (cm)			
Male	99.2 (10.8)	105.0 (10.3)	95.6 (9.4)
Female	89.8 (11.9)	97.1 (10.8)	85.6 (10.3)
BMI (kg/m^2)	27.6 (4.2)	29.9 (4.3)	26.3 (3.6)
Comorbidity			
Hypertension	3585 (87.8)	1387 (92.3)	2198 (85.1)
Diabetes	720 (18.0)	408 (27.8)	312 (12.4)
Metabolic syndrome	2193 (54.7)	1092 (74.0)	1101 (43.4)
Coronary heart disease	463 (11.3)	178 (11.8)	285 (11.0)
Heart failure	208 (5.1)	76 (5.0)	132 (5.1)
Biochemistry			
AST (U/L)	25 [21, 29]	25 [22, 29]	24 [21, 28]
ALT (U/L)	18 [14, 24]	21 [17, 27]	17 [14, 21]
Fatty liver index > 60	1404 (35.9)	893 (62.1)	511 (20.6)
Transient elastography			
Liver stiffness (kPa)*	4.9 [4.0, 6.1]	5.3 [4.3, 6.7]	4.8 [4.0, 5.9]
Liver stiffness ≥8.0 kPa*	183 (7.1)	110 (12.4)	73 (4.3)

Data is presented as mean (SD), median [P25-P75] or n and percentage. *Comprises only valid measurements in participants without heart failure. Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index.

Steatosis is not associated with all-cause mortality in the elderly

In our cohort, the presence of hepatic steatosis was not associated with a higher risk of death in fully adjusted models (aHR 0.87; 95%CI 0.73 – 1.03, **Figure 2**). Similar results were obtained for MAFLD (aHR 0.87, 95%CI 0.73 – 1.03) and NAFLD (aHR 0.89, 95%CI 0.73 – 1.09). Findings were consistent using age and sex-adjusted

models or when the presence of steatosis was based on a fatty liver index ≥ 60. Furthermore, similar associations were observed for mortality during the initial five years of follow-up (aHR 0.85, 95%CI 0.67 – 1.08) compared to the follow-up beyond five years (aHR 0.89, 95%CI 0.70 – 1.14; **Supplementary Table 1**). Adding BMI to the final model did not affect our outcomes, but revealed that higher BMI - taking into account all other metabolic dysfunction criteria – was associated with reduced mortality risk (aHR 0.94 per kg/m^2, 95%CI 0.92 – 0.97).

Age and sex adjusted model					
	deaths / n	**HR**	**95% CI**	**HR-plot**	**P**
Assessed by ultrasound					
Steatosis	793 / 4093	0.93	0.80 – 1.08		0.342
MAFLD	791 / 4089	0.94	0.81 – 1.09		0.423
NAFLD	591 / 3225	0.95	0.79 – 1.13		0.543
Assessed by FLI					
Steatosis	743 / 3912	1.06	0.91 – 1.23		0.479
MAFLD	754 / 3944	1.05	0.90 – 1.22		0.554
NAFLD	553 / 3083	1.04	0.87 – 1.24		0.658
				0.5 1.0 2.0	
Fully adjusted model					
	deaths / n	**HR**	**95% CI**	**HR-plot**	**P**
Assessed by ultrasound					
Steatosis	793 / 4093	0.87	0.73 – 1.03		0.098
MAFLD	791 / 4089	0.87	0.73 – 1.03		0.113
NAFLD	591 / 3225	0.89	0.73 – 1.09		0.273
Assessed by FLI					
Steatosis	743 / 3912	1.00	0.82 – 1.23		0.978
MAFLD	754 / 3944	1.00	0.81 – 1.22		0.971
NAFLD	553 / 3083	1.04	0.83 – 1.32		0.721
				0.5 1.0 2.0	

Figure 2. Mortality risk among elderly participants for steatosis, MAFLD and NAFLD. Results were obtained with Cox regression analysis. The age and sex adjusted model was only adjusted for age and sex; the fully adjusted model was in addition adjusted for education, smoking, alcohol, the individual components of the metabolic syndrome (hypertension, (pre)diabetes, hypo-HDL, hypertriglyceridemia, and high waist circumference), heart failure, coronary heart disease and stroke. Abbreviations: CI, confidence interval; FLI, fatty liver index; HDL, high-density lipoprotein; HR, hazard rate; MAFLD, metabolic dysfunction associated fatty liver disease; NAFLD, non-alcoholic fatty liver disease.

Steatosis is not associated with an increased mortality risk – subgroup analysis

In line with the findings in the overall population, the presence of hepatic steatosis was not associated with increased mortality risk across a range of pre-specified subgroups, including age categories, sex, presence of diabetes or metabolic dysfunction, elevated liver enzymes, or history of cardiac disease in models adjusted for age and sex (**Supplementary Figure 1**) and in fully adjusted models (**Figure 3**). Interestingly, the presence of hepatic steatosis was actually associated with a mildly reduced mortality risk among patients with hypertension, (pre)diabetes and high waist circumference.

	Fully adjusted model				
	Events / n	HR	95% CI	HR-Plot	P
Entire population	793 / 4093	0.87	0.73 – 1.03		0.098
Age					
65-70	82 / 1313	1.03	0.63 – 1.70		0.895
70-75	112 / 1036	0.89	0.57 – 1.41		0.630
75+	599 / 1744	0.84	0.69 – 1.03		0.086
Sex					
Male	405 / 1749	0.91	0.72 – 1.15		0.432
Female	388 / 1956	0.82	0.64 – 1.04		0.104
Metabolic syndrome	449 / 2193	0.81	0.66 – 1.00		0.053
Hypertension	710 / 3585	0.81	0.68 – 0.97		0.023
(pre)diabetes	372 / 1799	0.79	0.63 – 1.00		0.049
Hypo-HDL	415 / 1866	0.81	0.65 – 1.02		0.076
Hypertriglyceridemia	398 / 1935	0.86	0.69 – 1.09		0.209
High waist circumference	335 / 1864	0.79	0.62 – 0.99		0.041
Elevated liver enzymes	201 / 918	0.90	0.66 – 1.23		0.491
Cardiovascular disease	263 / 713	0.89	0.66 – 1.20		0.440

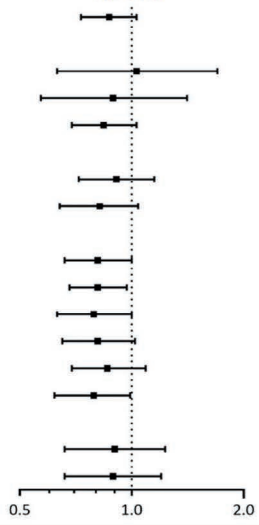


Figure 3. Mortality risk among elderly participants with steatosis: subgroup analysis. Results were obtained with Cox regression analysis. The fully adjusted model was adjusted for age, sex, education, smoking, alcohol, the individual components of the metabolic syndrome (hypertension, (pre)diabetes, hypo-HDL, hypertriglyceridemia, and high waist circumference), heart failure, coronary heart disease and stroke. Abbreviations: CI, confidence interval; HDL, high-density lipoprotein; HR, hazard rate.

Similarly, when analyses were stratified for BMI, we confirmed our previous findings that the absence of steatosis in patients with metabolic dysfunction (in this case obesity) could be suspicious. Multivariable models indicated that steatosis was associated with reduced mortality risk among the obese (HR 0.63, 95%CI 0.45 –

0.90), while it did not affect mortality in overweight (HR 0.99, 95%CI 0.79 – 1.26) and normal weight (HR 0.99, 95%CI 0.68 – 1.44).

No association between steatosis and cancer-related mortality or cerebro-cardiovascular mortality

In multivariable analysis, the presence of steatosis was not associated with cancer-related mortality (aHR 0.77, 95%CI 0.51 – 1.16) or cerebro-cardiovascular mortality (aHR 0.90, 95%CI 0.54 – 1.50). Of note, these HRs were similar to those observed for all-cause mortality (aHR 0.87; 95%CI 0.73 – 1.03).

Hepatic steatosis with liver stiffness ≥ 8.0 kPa is not associated with increased mortality

Valid liver stiffness measurements were available in a subset of participants (n = 2.584, age 72.7±6.6; median follow-up = 6.7 years, mortality rate 18.8 per 1000 person-years). Among those with steatosis, liver stiffness (aHR 1.04 per kPa, 95%CI 0.95 – 1.14) was not associated with mortality. Similar results were obtained among those without steatosis (aHR 0.98 per kPa, 95%CI 0.90 – 1.06). Even when those with both steatosis and liver stiffness ≥ 8.0 kPa were compared to those without steatosis and lower liver stiffness, no significant differences were observed in survival (aHR 1.11, 95%CI 0.65 – 1.89). Similar results were obtained when high liver stiffness was defined as 10.0 kPa.

DISCUSSION

In this large ongoing prospective cohort comprising community-dwelling elderly individuals with a median follow-up of 6.9 years, the presence of fatty liver disease was not associated with increased mortality. Consistent results were obtained across a range of clinically relevant subgroups. These findings indicate that hepatic assessment is unlikely to improve outcomes among the elderly.

Fatty liver disease is a widely accepted risk factor for liver-related morbidity and mortality based on the results of various large cohort studies.[4] As a result, several guidelines recommend hepatic assessment to screen for the presence of fatty liver disease, particularly in those with metabolic dysfunction.[1, 21, 66, 167, 168] Since most people aged 65 or over have at least one metabolic risk factor, up to an alarming

85% of our study population would opt for hepatic health assessment according to these guidelines. However, the clinical relevance of hepatic steatosis and fibrosis in this elderly population is currently unclear.

In the current study, fatty liver disease (steatosis, MAFLD and NAFLD) was not a risk factor for mortality. These findings align with a recent study, demonstrating that the clinical relevance of fatty liver disease attenuates as age increases.[175] Our results were consistent across all subgroups, across different periods of follow-up, and for both cancer-related and cerebrocardiovascular mortality. Furthermore, even patients with both steatosis and elevated liver stiffness (suggestive of liver fibrosis) were not at increased risk of death.

There are several explanations to account for the differences observed in our cohort when compared to previously published data. First, liver-related death is uncommon among community-dwelling elderly since the majority of patients developing end-stage liver disease do so at a younger age. For example, the average age for NASH is 40-50 years and NASH-cirrhosis 50-60 years,[167] in line with the mean age for NASH-related liver transplantation in the United States (59 years).[178] Second, the participants enrolled in this cohort, i.e., the community-dwelling elderly able to visit the research center may represent a healthy subset; a phenomenon related to survival bias. This is further illustrated by the rather low median liver stiffness, even among those with elevated liver stiffness, the median liver stiffness was only 9.2 kPa. This indicates that cirrhosis is rare in the elderly general population, unlike fibrosis.

Another potential confounder is weight loss. As described previously, only a minor decrease in body fat percentage results in rather large improvements of liver fat or hepatic triglycerides, even while adiposity persists.[179, 180] To further complicate matters, weight loss is an important predictor for impaired survival among the elderly. Weight loss might thus facilitate steatosis regression and also predict mortality. This might explain why the presence of steatosis was associated with a reduced risk of mortality in those with metabolic dysfunction (e.g. high waist circumference, hypertension and prediabetes) or obesity, in whom steatosis is expected and could be conspicuous when absent. In fact, additionally adjusting for BMI in the final models revealed that higher BMI (in light of all other confounders)

was associated with lower mortality risk. This phenomenon is in line with the so-called obesity paradox and concepts of reverse-causality.[181]

Our findings have very important clinical implications. Screening for fatty liver disease is recommended by a range of guidelines, especially among those with metabolic dysfunction.[1, 21, 66, 167] Such risk factors were present in up to 85% of this elderly study population, resulting in a vast number of community-dwelling elderly adults as potentially eligible for hepatological assessment. However, the current study indicates that such screening strategies may not be warranted in the elderly, as the first of Wilson and Jungner's criteria, namely that the condition should be an important health problem, is violated in elderly subjects.[182] Therefore, screening for fatty liver disease and/or fibrosis is unlikely to improve outcomes among the elderly and is not recommended.

It is, however, essential to note that our findings cannot be applied to younger populations, as in those cohorts the disease burden of fatty liver disease increased drastically over the past decades.[2, 119] For example, fatty liver disease is already one of the major indications for liver transplant in the United States.[80] Rather, our findings highlight that policies to limit the disease burden of fatty liver disease should focus on the young-to-middle-aged population and not the elderly.

This is the largest study to date on the association between fatty liver disease and mortality in the elderly, but the following limitations should be considered. First, this cohort is almost entirely of European ancestry (98%) and results should be confirmed among multi-ethnic populations. Second, the median follow-up is limited to 6.9 years. Nonetheless, 749 events occurred and given the large sample size a total of 26.765 person-years of follow-up was obtained. Moreover, in additional analyses, we observed no differences in hazard rates before and after five years of follow-up, suggesting the limited impact of the follow-up duration on our results. Third, one can argue that fatty liver disease was not associated with increased risk of mortality, for the multivariable models included many parameters closely related to fatty liver disease itself. However, it is unlikely that this affected any of our conclusions because results were consistent in additional analysis when only adjusted for age and sex. Fourth, since data on liver-related events are not available in this cohort, these could not be addressed in our analyses. Finally, the gold standard for assessing steatosis and fibrosis remains liver biopsy, which is

invasive and prone to sampling error.[103] However, since ultrasound-based diagnosis is operator-dependent, we confirmed our results through sensitivity analyses using a fatty liver index-based definition of steatosis. Unfortunately, both modalities cannot distinguish between different steatosis grades reliably. Therefore, additional research using controlled attenuation parameter (CAP) or MRI-PDFF to quantify steatosis severity by a continuous assessment is warranted to investigate the association between steatosis severity and mortality.

Conclusion

In this large cohort of adults aged ≥ 65 years, the presence of fatty liver disease was not associated with increased mortality, while a worrisome 85% of this group necessitated hepatic assessment according to recent guidelines. Findings were consistent across a range of clinically relevant subgroups. These findings do not support the currently recommended screening for fatty liver disease and/or fibrosis among the elderly population.

SUPPLEMENTARY FILES

Supplementary Table 1: Steatosis and survival among the elderly before and after five years of follow up

	Simple model			
	Events / n	**HR**	**95% CI**	**P**
0 - 5 years of follow up				
Steatosis	424 / 4093	0.91	0.74 – 1.12	0.378
MAFLD	422 / 4089	0.91	0.73 – 1.12	0.364
NAFLD	306 / 3225	0.91	0.71 – 1.17	0.474
> 5 years of follow-up				
Steatosis	369 / 3282	0.95	0.76 – 1.18	0.648
MAFLD	369 / 3281	0.98	0.78 – 1.22	0.833
NAFLD	285 / 2645	0.98	0.77 – 1.26	0.885
	Fully adjusted model			
	Events / n	**HR**	**95% CI**	**P**
0 - 5 years of follow up				
Steatosis	424 / 4093	0.85	0.67 – 1.08	0.177
MAFLD	422 / 4089	0.85	0.66 – 1.08	0.175
NAFLD	306 / 3225	0.87	0.65 – 1.16	0.339
> 5 years of follow-up				
Steatosis	369 / 3282	0.89	0.70 – 1.14	0.353
MAFLD	369 / 3281	0.90	0.70 – 1.16	0.414
NAFLD	285 / 2645	0.93	0.70 – 1.23	0.593

Results were obtained with Cox regression analysis. The age and sex adjusted model was only adjusted for age and sex; the fully adjusted model was in addition adjusted for education, smoking, alcohol, the individual components of the metabolic syndrome (hypertension, (pre)diabetes, hypo-HDL, hypertriglyceridemia, and high waist circumference), heart failure, coronary heart disease and stroke. Abbreviations: CI, confidence interval; HDL, high-density lipoprotein; HR, hazard rate.

Age and sex adjusted model					
	Events / n	**HR**	**95% CI**	**HR-Plot**	**P**
Entire population	793 / 4093	0.91	0.74 – 1.12		0.378
Age					
65-70	82 / 1313	1.26	0.81 – 1.95		0.303
70-75	112 / 1036	1.07	0.74 – 1.57		0.712
75+	599 / 1744	0.87	0.72 – 1.04		0.118
Sex					
Male	405 / 1749	0.97	0.79 – 1.20		0.784
Female	388 / 1956	0.88	0.71 – 1.10		0.259
Metabolic syndrome	449 / 2193	0.83	0.69 – 1.01		0.065
Hypertension	710 / 3585	0.89	0.76 – 1.04		0.148
(pre)diabetes	372 / 1799	0.84	0.68 – 1.04		0.104
Hypo-HDL	415 / 1866	0.85	0.69 – 1.04		0.114
Hypertriglyceridemia	398 / 1935	0.88	0.72 – 1.08		0.209
High waist circumference	335 / 1864	0.85	0.68 – 1.06		0.149
Elevated liver enzymes	201 / 918	0.92	0.70 – 1.23		0.582
Cardiovascular disease	263 / 713	0.93	0.72 – 1.21		0.663
				0.5 1.0 2.0	

Supplementary Figure 1. Mortality risk among elderly participants with steatosis: subgroup analysis Results were obtained with Cox regression analysis. Abbreviations: CI, confidence interval; HR, hazard rate.

CHAPTER 3.2

Liver stiffness is associated with excess mortality in the general population driven by heart failure: The Rotterdam Study

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Submitted

ABSTRACT

Background & aims: Elevated liver stiffness may reflect hepatic fibrosis but can also be secondary to venous congestion. We aimed to study the association between liver stiffness and mortality in the general population, stratified for heart failure (HF) and/or coronary heart disease (CHD).

Methods: We analyzed individuals enrolled in the ongoing prospective population-based Rotterdam Study who attended a visit between 2009 and 2014 with available liver stiffness data. Exclusion criteria were alcohol abuse, viral hepatitis, incomplete data on HF and unreliable liver stiffness measurements, leaving 4.153 participants (aged 67.5±8.4 years, 44.2% male) for analysis with a median follow-up of 6.0[5.1-7.0] years. The association between liver stiffness and mortality was assessed in the overall population and after stratification by HF/CHD, using Cox regression. Additionally, associations between HF, CHD, and echocardiographic characteristics and liver stiffness were quantified with linear regression.

Results: In the overall population, liver stiffness ≥8.0 kPa was associated with excess mortality (adjusted hazard ratio [aHR]:1.37, 95%CI:1.00 – 1.89). However, this association was driven by participants with heart failure (aHR 2.48, 95%CI 1.15 – 5.35), whereas no significant association was observed between liver stiffness and mortality in subjects without HF and/or CHD (aHR 1.07, 95%CI 0.70 – 1.64). Results were consistent when individuals with viral hepatitis, alcohol abuse or unreliable liver stiffness measurement were not excluded. Several cardiovascular characteristics were significantly associated with higher liver stiffness, including a previous diagnosis of HF, moderate to poor diastolic dysfunction, and right atrium diameter over 4.5 cm (effects ranging from +0.7 to +1.9 kPa, $p<0.05$).

Conclusion: In our cohort of community-dwelling elderly, high liver stiffness was associated with excess mortality, primarily explained by participants with HF. Moreover, (indicators of) HF were associated with increased liver stiffness.

INTRODUCTION

Liver stiffness assessment is an established non-invasive approach to rule out significant fibrosis among individuals with chronic liver disease.[1, 24] However, stiffness of the liver increases not only due to fibrosis but is also affected by inflammation and venous congestion.[57-59] Clinical or subclinical central venous congestion is often present in individuals with cardiovascular disease and has been associated with adverse outcomes.[183-185] Through its association with venous congestion, elevated liver stiffness has been identified as a predictor of short-term mortality in patients with acute heart failure (HF).[186, 187]

Liver stiffness measurements have been assigned an important role in the early detection of advanced liver disease in at-risk populations, and several groups are currently exploring its use for population-based screening for significant liver disease.[1, 66, 188] However, as elevated liver stiffness is not a specific tool for fibrosis, it might predominantly reflect central venous congestion, particularly among patients at high cardiovascular risk.[177]

We, therefore, aimed to study (1) the association between liver stiffness and mortality in relation to the presence of (signs) of HF and (2) study the association between liver stiffness and indicators of HF.

PARTICIPANTS AND METHODS

Study population. This study was performed within the Rotterdam Study, a large ongoing cohort established in 1989. Individuals aged ≥ 45 years old living in Ommoord, a suburb of Rotterdam, the Netherlands, were eligible to participate. Since 2009, the hepatology department has introduced abdominal ultrasound and transient elastography in the regular visits. The rationale and details of the Rotterdam Study have been extensively described recently.[89] For the current analyses, we enrolled participants who visited the research center between 2009 and 2014 with liver stiffness data **(Figure 1).** Exclusion criteria were lack of data on heart failure, unreliable liver stiffness measurement, alcohol abuse (≥60 gram per day) and viral hepatitis.

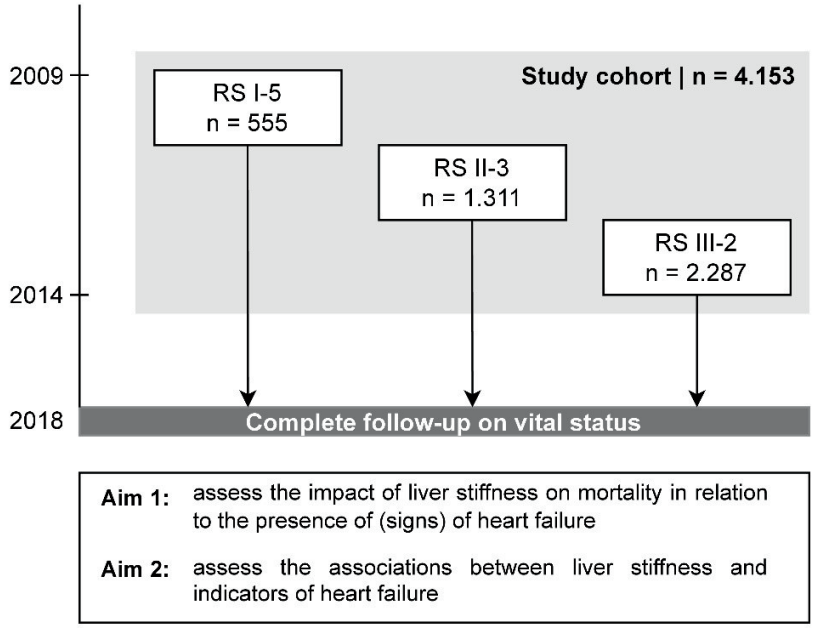

Figure 1: Overview of the aims and Rotterdam Study subsets included in our study. Three different Rotterdam Study cohorts that attended a visit between 2009 and 2014 were used for our aims and follow-up on vital status was complete until May 2018.

Hepatology assessment

Participants underwent abdominal ultrasound to assess hepatic steatosis based on hyper-echogenicity of the liver parenchyma. At the same visit, a liver stiffness measurement was performed (FibroScan ®, EchoSens, France). Measurements not meeting the reliability criteria of Boursier et al., were discarded.[91] Liver stiffness was considered high when ≥ 8.0 kPa according to cut-offs provided for research among the general population.[92]

Cardiovascular assessment

Data on cardiovascular diseases, including HF and coronary heart disease (CHD), were obtained during the study visits and from treating medical professionals. Diagnoses were verified by research physicians according to the definitions as outlined in the ESC guidelines.[189] Briefly, HF was defined as a combination of the presence of typical symptoms or signs of heart failure, such as breathlessness at rest or during exertion, ankle edema and pulmonary crepitations, confirmed by objective evidence of cardiac dysfunction or when two typical symptoms suggestive

of HF were present and at least one of the following: history of cardiovascular disease, positive response to initiated treatment for heart failure or objective evidence of cardiac dysfunction. CHD was defined as myocardial infarction or revascularisation (e.g. percutaneous coronary intervention or coronary artery bypass grafting). Detailed methodological information on the data collection and definitions used for cardiovascular diseases have been published previously.[190]

By transthoracic echocardiograms, systolic and diastolic function was assessed in several ways. For systolic function, we used fractional shortening, which was based on the left ventricular end-diastolic dimension (LVEDD) and left ventricular end-systolic dimension (LVESD) and defined as: (LVEDD – LVESD)/LVEDD * 100%. Additionally, the sonographers made a qualitative global assessment of systolic function based on the 2D echocardiogram. Diastolic function was assessed using the E/A ratio and mitral valve deceleration time. The peak E velocity was the early filling velocity occurring with mitral valve opening and the peak A velocity was the velocity occurring with contraction of the atrium.[191] The average of three cycles have been used to calculate the E/A ratio. The mitral valve deceleration time was the time between peak E and crossing of the wave when extrapolated with the baseline. The E/A ratio and mitral valve deceleration time were then combined for a qualitative assessment of diastolic dysfunction. Specifically, normal (E/A ratio 0.75-1.50 and deceleration time 150-280 ms), impaired relaxation E/A ratio < 0.75 and deceleration time > 280 ms) and restrictive (E/A ratio > 1.50 and deceleration time < 150 ms).[191] Diastolic dysfunction was considered indeterminate if only one of the two criteria for dysfunction were met.

Follow-up and mortality data

All-cause mortality data were obtained from local registries and clinical follow-up data. Verified information on all-cause mortality was available until May 2018.

Covariates

Prior to the study visit, a home interview was scheduled in which, among others, data on alcohol intake and smoking were collected. Blood samples were taken during each study visit and subsequent laboratory tests included liver biochemistry, serum glucose, serum lipids. Anthropometric measurements included length, weight and waist circumference. Medication data were obtained during the interview and linkage with electronic systems of pharmacies. Last, the metabolic

syndrome was defined according to the ATP-III criteria[94], and was present if at least three of the following components were present: 1) (pre)diabetes, defined as fasting glucose > 5.6 mmol/L, anti-diabetic drug use or diagnosis of diabetes by health care professionals; (2) High waist circumference, defined as > 102 cm in males or > 88 cm in females; (3) Hypertriglyceridemia, defined as triglycerides ≥ 1.7 mmol/L and/or lipid-lowering drug use; (4) Hypo-HDL, defined as high density lipoprotein (HDL) < 1.04 mmol/L in male or < 1.30 in female and/or lipid-lowering drug use; and (5) hypertension, defined as either a systolic blood pressure ≥ 130 mmHg, diastolic blood pressure ≥ 85 mmHg and/or antihypertensive drug use.

Statistical analysis

First, we assessed the associations between liver stiffness at baseline and all-cause mortality using Cox proportional hazard regression. Associations were explored in the entire cohort and in subgroups with (1) no CHD nor HF, (2) HF (3) CHD without HF at time of the study visit. Liver stiffness has been assessed dichotomously and on a continuous log-transformed scale. In model 1, analyses were adjusted for age and sex, in model 2 also for smoking, alcohol consumption and steatosis, and in model 3 also for the individual components of the metabolic syndrome (hypertension, (pre)diabetes, high waist-circumference, hypo-HDL, hypertriglyceridemia, [model 3]). In sensitivity analysis, we added excluded individuals for viral hepatitis or alcohol abuse and liver stiffness measurements regardless of their IQR.

To explore how cardiovascular health relates to liver stiffness, we assessed the associations between cardiovascular characteristics and liver stiffness cross-sectionally using linear regression among all included participants. Investigated parameters reflected several domains of cardiovascular disease and comprised systolic function (fractional shortening and qualitative assessment), diastolic function (E/A ratio and qualitative assessment) and markers of systemic venous congestion (right atrium diameter).

Analyses were performed in R version 4.0.4 (Foundation for Statistical Computing, Vienna, Austria), using the *survival* package 3.2-10. *P*-values < 0.05 were considered statistically significant.

Ethics

The Rotterdam Study has been approved by the Medical Ethics Committee of Erasmus MC (registration number MEC 02.1015) and by the Dutch Ministry of Health, Welfare and Sport (Population Screening Act WBO, license number 1071272-159521-PG). The Rotterdam Study Personal Registration Data collection is filed with the Erasmus MC Data Protection Officer under registration number EMC1712001. The Rotterdam Study has been entered into the Netherlands National Trial Register (NTR; www.trialregister.nl) and into the WHO International Clinical Trials Registry Platform (ICTRP; www.who.int/ictrp/network/primary/en/) under shared catalog number NTR6831. All participants provided written informed consent to participate in the study and to have their information obtained from treating physicians. All authors had access to the study data and reviewed and approved the final manuscript.

RESULTS

General characteristics. Between 2009 and 2014, liver stiffness was part of the standard examination and measured in 4.573 participants. After excluding 215 participants for unreliable measurements, 113 for incomplete data on HF, 57 for alcohol abuse and 35 for viral hepatitis, 4.153 participants remained for analysis. The mean age was 67.5±8.4 years, 44.2% was male and metabolic comorbidity was highly prevalent (46.7% metabolic syndrome, 13.8% diabetes). The median liver stiffness was 4.8 kPa [3.9 – 5.9] and was 8.0 kPa or higher in 6.2% (n = 256). CHD was present in 321 (7.7%) participants and HF in 97 (2.3%). Additional characteristics are available in **Table 1.** During the median follow-up of 6.0 [5.1 – 7.0] years, 373 deaths were recorded, resulting in an overall mortality rate of 15.1 per 1.000 person-years.

Liver stiffness is associated with mortality in individuals with heart failure but not in those without

In the overall study population, liver stiffness ≥ 8.0 kPa was associated with excess mortality in fully adjusted models (adjusted hazard ratio[aHR] 1.37, 95%CI 1.00 – 1.89) **Table 2**. Interestingly, this association was entirely driven by participants with HF (aHR 2.48, 95%CI 1.15 – 5.35) and disappeared after excluding participants with HF and/or CHD (aHR 1.07, 95%CI 0.70 – 1.64). Liver stiffness ≥ 8.0 kPa in participants

Table 1: Participants' characteristics

	Study population n = 4.153
General	
Age (years)	67.5 (8.4)
Male	1834 (44.2)
Current/former smoking	2759 (66.6)
Comorbidity	
Hypertension	2880 (69.4)
Diabetes	568 (13.8)
Metabolic syndrome	1909 (46.7)
Cardiovascular disease	
CHD – , HF –	3769 (90.8)
CHD + , HF +	34 (0.8)
CHD + , HF –	287 (6.9)
CHD – , HF +	63 (1.5)
Cardiovascular assessment	
Fractional shortening (%)	42.3 (5.0)
Qualitative systolic function	
Normal	3598 (87.1)
Fair	462 (11.2)
Moderate / poor	73 (1.8)
E/A ratio	0.95 (0.29)
Qualitative diastolic dysfunction	
Normal	2662 (65.8)
Impaired relaxation	71 (1.8)
Restrictive pattern	29 (0.7)
Indeterminate	1283 (31.7)
Right atrium diameter (cm)	3.4 (0.5)
Hepatic assessment	
Steatosis	1379 (33.2)
Liver stiffness (kPa)	4.8 [3.9, 5.9]
Liver stiffness ≥8.0 kPa	256 (6.2)

Data is presented as mean (SD), median [P25-P75] or n and percentage.
Abbreviations: CHD, coronary heart disease; HF, heart failure.

Table 2: Mortality risk for the presence of liver stiffness ≥ 8.0 kPa

	Events / n	HR	95% CI	P
Entire population	373 / 4153			
Model 1		1.44	1.06 – 1.96	**0.018**
Model 2		1.45	1.06 – 1.98	**0.020**
Model 3		1.37	1.00 – 1.89	0.054
Subgroup analysis				
CHD – , HF –	280 / 3769			
Model 1		1.18	0.80 – 1.76	0.405
Model 2		1.19	0.79 – 1.79	0.394
Model 3		1.07	0.70 – 1.64	0.755
HF +	42 / 97			
Model 1		2.09	1.08 – 4.06	**0.030**
Model 2		2.35	1.13 – 4.89	**0.023**
Model 3		2.48	1.15 – 5.35	**0.021**
CHD + , HF –	51 / 287			
Model 1		1.25	0.53 – 2.93	0.615
Model 2		1.19	0.50 – 2.84	0.690
Model 3		1.43	0.58 – 3.49	0.437

Results were obtained with Cox regression analysis. Model 1 was adjusted for age and sex, model 2 also for smoking, alcohol consumption and steatosis and model 3 also for the individual components of the metabolic syndrome (hypertension, (pre)diabetes, high waist circumference, hypo-HDL, and hypertriglyceridemia).
Abbreviations: CHD, coronary heart disease; CI, confidence interval; HF, heart failure; HR, hazard rate.

with CHD alone was not significantly associated with increased mortality risk despite a modest effect (aHR 1.43, 95%CI 0.58 – 3.49). Similar results were obtained when liver stiffness was assessed on a continuous log-transformed scale (aHR 2.20 per log(kPa) 95%CI 1.04 – 4.67) among participants with HF **Supplementary Table 1)**. In addition, results were consistent when individuals with viral hepatitis, alcohol abuse or unreliable liver stiffness measurements were not excluded.

Cardiovascular disease and function were associated with liver stiffness

There were clear associations between a range of cardiovascular characteristics and higher liver stiffness in our cohort **Table 3**. For example, the presence of HF with CHD (+1.9 kPa, $p < 0.001$) or without CHD (+1.7 kPa, $p < 0.001$), moderate to poor diastolic dysfunction (+0.7 kPa, $p = 0.004$) and right atrium diameter over 4.5 cm

(+0.7 kPa, p = 0.001) were associated with significantly higher liver stiffness levels. Interestingly, the presence of CHD in the absence of HF was not associated with liver stiffness (+0.0 kPa, p = 0.81). Similar patterns were observed when liver stiffness was assessed as a categorical variable using 8.0 kPa as cut-off (**supplementary Table 2**).

Table 3: Associations between cardiovascular characteristics and liver stiffness

	Beta	95% CI	p
Clinical assessment			
CHD – , HF –		**Reference**	
CHD + , HF +	1.89	1.23 – 2.56	**< 0.001**
CHD + , HF –	0.03	-0.22 – 0.28	0.813
CHD – , HF +	1.73	1.23 – 2.22	**< 0.001**
Systolic dysfunction			
Fractional shortening (%)	-0.02	-0.04 – -0.01	**< 0.001**
Qualitative systolic function			
Normal		**Reference**	
Fair	0.34	0.14 – 0.54	**0.001**
Moderate / poor	0.69	0.22 – 1.15	**0.004**
Diastolic dysfunction			
E/A ratio	0.45	0.23 – 0.66	**< 0.001**
Qualitative diastolic dysfunction			
Normal		**Reference**	
Impaired relaxation	-0.08	-0.56 – 0.40	0.734
Restrictive pattern	0.49	-0.22 – 1.20	0.175
Indeterminate	0.09	-0.05 – 0.22	0.199
Systemic venous congestion			
RA diameter (cm)	0.26	0.12 – 0.39	**< 0.001**
RA diameter > 4.5 cm	0.72	0.30 – 1.15	**0.001**

Results were obtained with linear regression analysis and adjusted for age, sex, smoking, alcohol consumption, steatosis and the individual components of the metabolic syndrome (hypertension, (pre)diabetes, high waist circumference, hypo-HDL and hypertriglyceridemia).

Abbreviations: CHD, coronary heart disease; CI, confidence interval; HF, heart failure; RA, right atrium.

DISCUSSION

In this study, we showed that increased liver stiffness was a predictor of all-cause mortality in the general population. Interestingly, this was primarily accounted for by individuals with heart failure; no association was observed between liver stiffness and mortality among subjects without a history of HF or CHD. Last, the presence of (signs of) heart failure was associated with an increase in liver stiffness.

Liver stiffness assessment has become an invaluable tool for stratifying the risk of hepatic fibrosis among patients with established liver disease. Based on these findings, several groups, such as LiverScreen, are currently exploring the use of liver stiffness assessment for early detection of significant liver disease in the general population.[188] In the current study, high liver stiffness was associated with increased mortality risk. However, this was explained by individuals with HF. In fact, there was no excess mortality among individuals with high liver stiffness without HF or CHD.

Our study may have important consequences for the ongoing liver stiffness-based screening programs that aim to detect advanced liver disease, because in our cohort of elderly individuals, the 2% of the population with heart failure, accounted for 10% of the cases with liver stiffness ≥ 8.0 kPa. Elevated liver stiffness in the general population may therefore often be attributed to cardiovascular disease, because screening programs for advanced liver disease typically target individuals with metabolic dysfunction who are both at risk for cardiovascular disease and fatty liver disease.[21, 66] Our findings highlight an important limitation of liver stiffness as a screening tool in the elderly, and suggest that if the goal is to screen for patients at risk for advanced liver disease additional measures should be undertaken to rule out heart failure first. If this cannot be done, referral to a cardiologist appears to be indicated in patients with elevated liver stiffness without other signs of chronic liver disease.

Our study confirms previous reports on higher liver stiffness among patients with cardiovascular disease.[192, 193] Although this may partially be attributed to the presence of liver fibrosis due to co-existing fatty liver disease, it is more likely that liver stiffness reflects venous congestion in this specific subgroup for several

reasons. First, we have excluded important causes for fibrosis, such as alcohol abuse and viral hepatitis. Second, we addressed several risk factors for fibrosis in multivariable models, such as steatosis and diabetes.[194] Third, there is emerging evidence on the impact of venous congestion on liver stiffness, which in specific subgroups may exceed the impact of fibrogenesis.[57, 186] This indicates that liver stiffness may have prognostic value, not only among those with decompensated heart failure,[186, 187] but also among non-hospitalised heart failure patients. Now that liver stiffness measurements are becoming readily available by the adoption of elastography on regular ultrasound devices, it would be interesting to see in future studies whether the adoption of liver stiffness in risk prediction models for patients with heart failure leads to improved accuracy and has clinical utility compared to currently available algorithms.

There is plenty of experience with elastography in the liver. However, this technique may also be applied to other structures. Recently, it has even been successfully used to assess the stiffness of inferior vena cava (IVC) in an experimental setting.[195] Using the stiffness of IVC as assessed by transient elastography, one bypasses the impact of fibrosis and hepatic inflammation. The results may then be more specific for venous congestion. However, additional research is required on whether the application of elastography for the IVC is reliable and has value over liver stiffness in cardiovascular disease.

Although this is one of the first studies assessing the impact of cardiovascular disease on liver stiffness in the general population and the potential consequences for future screening strategies, the following limitations should be considered.

First, this cohort comprised predominantly elderly participants of European ancestry and further research is warranted focusing on multi-ethnic and younger populations. Especially the impact of cardiovascular disease on liver stiffness on a population level might be different from this cohort. Second, this study had a limited median follow-up duration of 6.0 years. Nonetheless, our analyses comprised 24.650 person-years of follow-up given the large sample size. Third, excluding individuals with alcohol abuse and viral hepatitis could have attributed to liver stiffness not being a risk factor in individuals without HF. However, not excluding these individuals in sensitivity analysis did not increase mortality risk in the population without HF. Fourth, due to the cross-sectional design of the analysis

on the associations of (signs of) cardiovascular disease and liver stiffness, we could not investigate the direction of these associations. However, physiological mechanisms support that cardiovascular disease by venous congestion affects liver stiffness.

Conclusion

In this large population-based study, we demonstrated that high liver stiffness was associated with excess mortality, but this result was entirely driven by HF. Furthermore, a range of cardiovascular characteristics and heart failure were associated with an increase in liver stiffness. These findings highlight important limitations of elastography-based screening for advanced liver disease in low-prevalence populations.

SUPPLEMENTARY FILES

Supplementary table 1: Mortality risk for liver stiffness on a continuous log transformed scale

	Events / n	HR	95% CI	P
Entire population	373 / 4153			
Model 1		1.50	1.09 – 2.04	**0.012**
Model 2		1.52	1.10 – 2.10	**0.010**
Model 3		1.40	1.00 – 1.95	**0.049**
Subgroup analysis				
CHD – , HF –	280 / 3769			
Model 1		1.20	0.82 – 1.76	0.345
Model 2		1.23	0.83 – 1.82	0.298
Model 3		1.09	0.73 – 1.64	0.668
HF +	42 / 97			
Model 1		1.93	1.10 – 3.37	**0.021**
Model 2		2.17	1.14 – 4.15	**0.018**
Model 3		2.20	1.04 – 4.67	**0.040**
CHD + , HF –	51 / 287			
Model 1		1.13	0.47 – 2.74	0.787
Model 2		1.08	0.44 – 2.66	0.865
Model 3		1.21	0.47 – 3.13	0.699

Results were obtained with Cox regression analysis, the risk is expressed per 1 increase of log(liver stiffness). Model 1 was adjusted for age and sex, model 2 also for smoking, alcohol consumption and steatosis and model 3 also for the individual components of the metabolic syndrome (hypertension, (pre)diabetes, high waist circumference, hypo-HDL and hypertriglyceridemia).

Abbreviations: CHD, coronary heart disease; CI, confidence interval; HF, heart failure; HR, hazard rate.

Supplementary table 2: Associations between cardiovascular characteristics and liver stiffness ≥ 8.0

	OR	95% CI	p
Clinical assessment			
CHD – , HF –		**Reference**	
CHD + , HF +	3.05	1.30 – 7.12	**0.010**
CHD + , HF –	1.18	0.73 – 1.90	0.501
CHD – , HF +	2.72	1.37 – 5.40	**0.004**
Systolic dysfunction			
Fractional shortening (%)	0.98	0.95 – 1.00	**0.042**
Qualitative systolic function			
Normal		**Reference**	
Fair	1.51	1.06 – 2.16	**0.022**
Moderate / poor	1.31	0.61 – 2.81	0.490
Diastolic dysfunction			
E/A ratio	1.63	1.08 – 2.47	**0.019**
Qualitative diastolic dysfunction			
Normal		**Reference**	
Impaired relaxation	0.78	0.30 – 2.07	0.619
Restrictive pattern	1.89	0.51 – 7.06	0.344
Indeterminate	1.16	0.87 – 1.57	0.315
Systemic venous congestion			
Right atrium diameter (cm)	1.30	0.95 – 1.78	0.105
Right atrium > 4.5 cm	1.55	0.73 – 3.28	0.256

Results were obtained with logistic regression analysis and adjusted for age, sex, smoking, alcohol consumption, steatosis and the individual components of the metabolic syndrome (hypertension, (pre)diabetes, high waist circumference, hypo-HDL and hypertriglyceridemia).

Abbreviations: CHD, coronary heart disease; CI, confidence interval; HF, heart failure; OR, odds ratio.

CHAPTER 3.3

Liver stiffness not fatty liver disease is associated with atrial fibrillation: The Rotterdam Study

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ABSTRACT

Background & aims: Fatty liver disease has become the most prevalent chronic liver disease globally and is linked to cardiovascular disease, including arrhythmias. However, inconsistent findings are published on the association between fatty liver disease and atrial fibrillation, and the role of liver stiffness in this association remains unclear.

Methods: Within the Rotterdam Study, a large prospective ongoing cohort, participants attending the abdominal ultrasound program between 2009-2014 were included. Exclusion criteria were no atrial fibrillation data or >20% missing data across analysis variables. Steatosis was assessed by ultrasound, liver stiffness by transient elastography and atrial fibrillation by 12-lead electrocardiograms. Incident atrial fibrillation was based on medical records and was complete until 2014. Logistic and Cox regression were used to quantify associations between fatty liver disease and atrial fibrillation.

Results: We included 5.825 participants (aged 69.5±9.1, 42.9% male), 35.7% had steatosis, liver stiffness was available in 73.3%, and 7.0% had prevalent atrial fibrillation. Steatosis was not associated with prevalent atrial fibrillation in fully adjusted models (OR 0.80, 95%CI 0.62 – 1.03); findings were consistent for NAFLD and MAFLD. Liver stiffness was significantly associated with prevalent atrial fibrillation (OR 1.09 per kPa, 95%CI 1.03 – 1.16); however, this was only persistent among those without steatosis (OR 1.18 per kPa, 95%CI 1.08 – 1.29). Last, no associations were found between steatosis (HR 0.88, 95%CI 0.59 – 1.33, follow-up 2.1 [1.1-3.2] years) and incident atrial fibrillation.

Conclusions: Fatty liver disease was not associated with prevalent or incident atrial fibrillation, while liver stiffness was significantly associated with atrial fibrillation, especially among those without steatosis. This association might be driven by venous congestion instead of fibrogenesis, but this awaits further validation. We recommend assessing cardiovascular health in participants with high liver stiffness, especially in the absence of overt liver disease.

INTRODUCTION

Fatty liver disease has become the most common chronic liver disease, affecting over 25% of adults globally.[4] It ranges from simple hepatic steatosis to clinically relevant fibrosis and cirrhosis, which are significant drivers for advanced liver disease and hepatocellular carcinoma.[135] However, the disease burden of fatty liver disease is not limited to hepatic complications but extends to renal dysfunction, extrahepatic malignancies and cardiovascular morbidity.[41, 82, 196-198]

Atrial fibrillation is a highly prevalent heart rhythm disorder that has been suggested to be associated with fatty liver disease.[199] Several mechanisms driving this association are proposed, including systemic inflammation, dyslipidemia, increased insulin resistance, and renin-angiotensin system activation.[200-202] Moreover, liver stiffness, a transient elastography-based marker for liver fibrosis, may be an important parameter in this assumed association. However, the mechanism remains unclear and results have not yet been validated.[203]

Few studies investigated the association between fatty liver disease and atrial fibrillation, and results so far were inconsistent.[81, 204-207] These studies were hampered by biomarker-based assessment of fatty liver disease (instead of imaging), limited sample size, or failed to adjust for important confounders. Moreover, most of the studies have not assessed the role of liver stiffness in the association with atrial fibrillation.

Within the large prospective population-based Rotterdam Study, we investigate the association between fatty liver disease and liver stiffness with prevalent and incident atrial fibrillation. A defining feature of our study is the use of several fatty liver disease definitions and the availability of liver stiffness measurement, which altogether provides a thorough assessment of liver health.

METHODS

This analysis was embedded within the Rotterdam Study, a large prospective population-based cohort that commenced in 1989. Citizens of Ommoord, a district of Rotterdam, were selected based on zip code and were eligible to participate

when at least 40 years old. Participants are invited to the Rotterdam Study research center every four to six years. Since 2009, abdominal ultrasound and transient elastography have been part of the repeated assessments within the Rotterdam Study. The study design, principles, and recent findings of the Rotterdam study were published recently.[89]

We included participants that had visited the research center between March 2009 and June 2014 and had undergone abdominal ultrasound. Participants with no data on atrial fibrillation or >20% missing data for the included variables were excluded.

Hepatology assessment

Abdominal ultrasound was performed by a single sonographer (PvW) on a Hitachi Hi-Vision 900. Measurements included craniocaudal length of the spleen and hepatic vein diameter (measured 20 mm distal of the inferior vena cava (IVC)) and the assessment of steatosis, which was based on hyperechoic liver parenchyma compared to the kidney or spleen.[90] According to the European Association for the Study of the Liver (EASL) guidelines,[1] Non-alcoholic fatty liver disease (NAFLD) was defined as hepatic steatosis in the absence of secondary causes of steatosis comprising viral hepatitis (B or C), steatogenic drug use, and excessive alcohol consumption defined as ≥ 30 grams for males and ≥ 20 grams per day for females. In addition, participants were excluded for NAFLD if quantitative alcohol data was missing while reporting alcohol consumption frequency of ≥ 4 days a week; since we could not rule out excessive alcohol intake. Metabolic dysfunction associated fatty liver disease (MAFLD) was defined according to the novel criteria as steatosis together with overweight, diabetes or the presence of two minor metabolic dysfunction criteria.[29] Liver stiffness was measured with transient elastography (FibroScan, EchoSens, Paris, France) using the same device throughout the study period. At least ten individual measurements were required for a valid measurement with an interquartile range of ≤ 30% if liver stiffness exceeded 7.0 kPa.[91] High liver stiffness was defined as a valid liver stiffness measurement ≥ 8.0 kPa, based on prior research in the general population.[92]

Cardiovascular assessment

The 2020 ESC guidelines defined atrial fibrillation as abnormal electrocardiographic characteristics comprising irregular R-R intervals, absence of distinct repeating P waves and irregular atrial activations assessed on either a 30 second

tracing ECG or an entire 12-lead ECG.[208] In line with these guidelines, atrial fibrillation was checked for on a 10-seconds entire 12-lead electrocardiogram (ECG; Esaote, Biomedical, Florence, Italy) obtained from the regular visits and assessed by the Modular ECG analysis system. Two research physicians validated the automatic diagnosis of atrial fibrillation. In addition to the information from the regular study visits, data were obtained from treating physicians and used for assessing prevalent and incident atrial fibrillation. These diagnoses were confirmed by independent reading of the ECG by research physicians.[190] Follow up was complete until January 1st, 2014. Prevalent coronary heart disease (CHD) and heart failure (HF) were based on data obtained during study visits and from treating physicians. The definitions and procedures to obtain cardiovascular data in the Rotterdam Study have been described in detail previously.[190] IVC diameter was measured with an ACUSON Cypress 3V2c transducer during cardiac echocardiography.

Additional covariates

Research assistants and trained interviewers acquired participants' anthropometrics, alcohol consumption, smoking habits, and education level. Alcohol consumption was additionally derived from the self-completed food frequency questionnaire. Medication data were obtained from linkage with the participants' pharmacies.

Blood samples were collected while participants were fasting. Glucose, blood lipids, aspartate aminotransferase, and alanine aminotransferase were assessed by automatic enzyme procedures and insulin with automatic immunoassay (Roche, Diagnostic GmbH, Mannheim, Germany).

Diabetes was defined as fasting glucose ≥ 7.0 mmol/L**,** drug treatment for diabetes, or obtained from treating physicians' data. The metabolic syndrome was defined, conform the ATP-III criteria,[94] as at least three of the following components: (1) fasting glucose > 5.6 mmol/L or anti-diabetic drug use, (2) waist circumference > 102 cm for males and > 88 cm for females (3) triglycerides ≥ 1.7 mmol/L or statin use, (4) HDL-C < 1.04 mmol/l in male and < 1.30 in female or statin use and (5) hypertension based on either a systolic blood pressure ≥ 130, diastolic blood pressure ≥ 85 or antihypertensive drug use.

Statistical analysis

We imputed missing values of covariates included in the main models or additional analyses to reduce potential bias from missing data. This was performed with the R-package MICE 3.13.0 under the fully conditioned specification. We created fifty imputed datasets and analyses were performed in each dataset and consequently pooled using Rubin's rules to take into account the uncertainty of the imputed values. More information regarding the imputation procedure is available in **Supplementary Table 1.**

Participants' characteristics before imputation were described as n and %, mean and standard deviation (SD) or median and 25^{th} – 75^{th} percentile [P25-P75], according to the nature of the data. In addition, imputed data was compared to non-imputed data in **Supplementary Table 2.**

Logistic and linear regression was used to assess the associations between fatty liver disease and liver stiffness (continuous and ≥ 8.0 kPa) with prevalent atrial fibrillation. We used steatosis for our main analysis and results were verified by using NAFLD and MAFLD in additional analysis. We used three multivariable models, based on established risk factors of NAFLD and/or atrial fibrillation. Model 1, was only adjusted for age and sex. Model 2 was additionally adjusted for alcohol consumption, smoking, education level, prevalent heart failure, prevalent coronary heart disease, and the individual categorical components of the metabolic syndrome (high waist circumference, hypertension, hypo-HDL, hypertriglyceridemia and (pre)diabetes). Model 3 included covariates that could affect liver stiffness by other means than fibrosis (craniocaudal spleen length, inferior vena cava diameter, liver vein diameter, and ALT levels)[57-59] and was therefore only applied for the analysis assessing liver stiffness. Analyses were performed among the entire population and subsequently stratified for steatosis status. Moreover, in sensitivity analyses, participants with prevalent HF and/or CHD were excluded.

Next, in longitudinal analysis, Cox proportional hazards analysis was used to assess the impact of baseline fatty liver disease (MAFLD, NAFLD and steatosis) on the risk of incident atrial fibrillation. Baseline was set on the date of abdominal ultrasound, and in addition to the general exclusion criteria, we excluded participants with prevalent atrial fibrillation or lack of follow-up for atrial fibrillation. In line with the

cross-sectional analysis, results were adjusted for the covariates included in model 1 and model 2.

Last, to get further insight into which parameters might influence liver stiffness, associations with liver stiffness as outcome and prevalent atrial fibrillation, heart failure, IVC diameter, and liver vein diameter as exposure were explored with linear regression. These analyses were performed on the non-imputed data, as in particular IVC and liver vein diameter were frequently imputed. Results were adjusted for age, sex, alcohol consumption, smoking, education, high waist circumference, hypertension, hypo-HDL, hypertriglyceridemia, and (pre)diabetes. Similarly, the associations between prevalent atrial fibrillation with IVC and liver vein diameter as well as liver stiffness have been quantified using linear regression with the same model.

All analyses were performed in R version 4.0.3 (The R Foundation for Statistical Computing, Vienna, Austria). P-values of < 0.05 were considered statistically significant.

Ethics

The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus MC (registration number MEC 02.1015) and by the Dutch Ministry of Health, Welfare and Sport (Population Screening Act WBO, license number 1071272-159521-PG). The Rotterdam Study Personal Registration Data collection is filed with the Erasmus MC Data Protection Officer under registration number EMC1712001. The Rotterdam Study has been entered into the Netherlands National Trial Register (NTR; www.trialregister.nl) and into the WHO International Clinical Trials Registry Platform (ICTRP; www.who.int/ictrp/network/primary/en/) under shared catalogue number NTR6831. All participants provided written informed consent to participate in the study and to have their information obtained from treating physicians.

RESULTS

In this cohort study, 5.967 participants underwent abdominal ultrasound, of whom 22 participants were excluded for no atrial fibrillation data and 120 participants for

missing data across >20% of variables of interest, resulting in 5.825 participants for analysis (**Figure 1**). The mean age was 69.5 (SD 9.1) years, 42.9% (n = 2.499) was male and mean BMI was 27.5 kg/m^2 (SD 4.3). At baseline, steatosis was present in 35.7% (n = 2.079) and atrial fibrillation in 7.0% (n = 405). Among included participants, 73.3% (n = 4.270) had a valid liver stiffness measurement and 6.1% (n = 262) had liver stiffness ≥ 8.0 kPa. Additional characteristics are provided in **Table 1** and characteristics after imputation are provided in **Supplementary Table 2**. A direct comparison of participants with prevalent atrial fibrillation to those without is available in **Supplementary Table 3**.

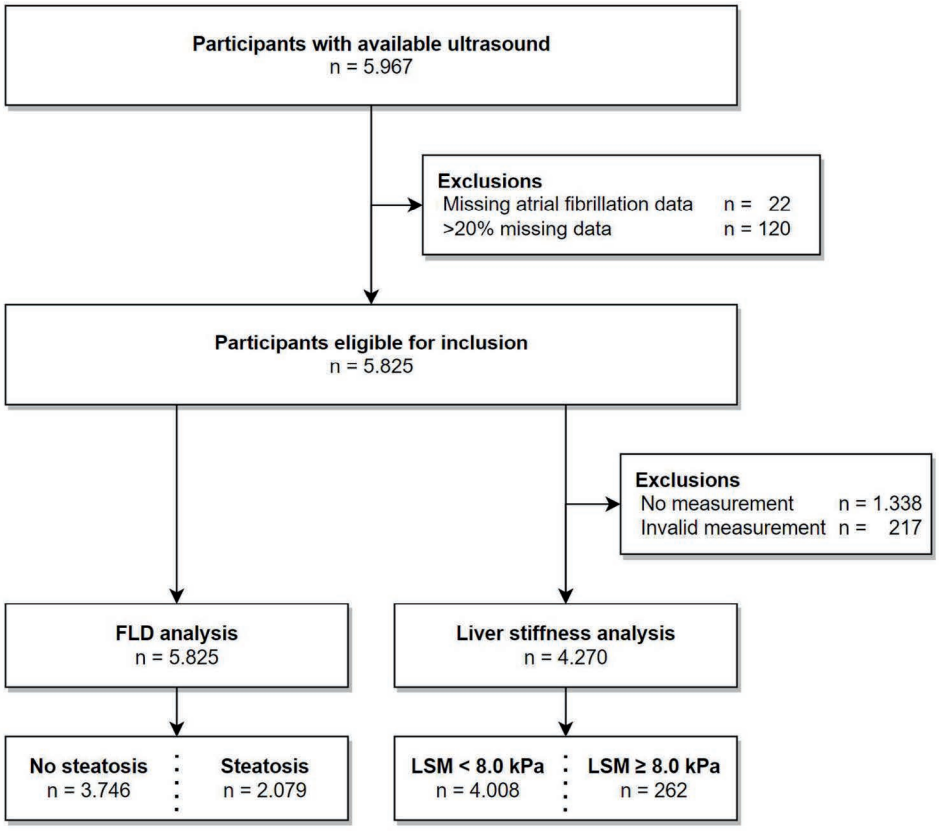

Figure 1. Participant selection. Abbreviations: FLD, Fatty liver disease; LSM, Liver Stiffness Measurement.

Table 1: Participants' characteristics

Variable	All n = 5825	Steatosis n = 2079	No steatosis n = 3746
Demographics			
Age (years)	69.5 (9.1)	69.4 (8.4)	69.6 (9.4)
Male	2499 (42.9)	942 (45.3)	1557 (41.6)
European ancestry	5036 (97.4)	1813 (98.0)	3223 (97.1)
Education			
Low	2776 (48.2)	1067 (52.0)	1709 (46.0)
Intermediate	1731 (30.0)	608 (29.7)	1123 (30.3)
High	1255 (21.8)	375 (18.3)	880 (23.7)
Current/former smoking	3936 (67.7)	1486 (71.7)	2450 (65.5)
Alcohol intake (gram/day)	7.3 (8.2)	8.2 (9.5)	6.8 (7.3)
Physical examination			
High waist circumference*	2584 (44.4)	1455 (70.0)	1129 (30.1)
BMI (kg/m^2)	27.5 (4.3)	29.9 (4.3)	26.2 (3.7)
Comorbidities			
Hypertension	4654 (80.0)	1798 (86.7)	2856 (76.3)
Diabetes	873 (15.2)	493 (24.1)	380 (10.3)
Metabolic syndrome	2897 (49.8)	1476 (71.2)	1421 (38.0)
Atrial fibrillation	405 (7.0)	135 (6.5)	270 (7.2)
Coronary heart disease	520 (8.9)	197 (9.5)	323 (8.6)
Heart failure	206 (3.5)	73 (3.5)	133 (3.6)
Biochemistry			
AST (U/L)	24 [21, 28]	25 [21, 29]	24 [21, 28]
ALT (U/L)	19 [15, 24]	22 [17, 28]	17 [14, 22]
HDL-C (mmol/L)	1.5 (0.4)	1.4 (0.4)	1.6 (0.4)
Triglycerides (mmol/L)	1.3 [1.0, 1.7]	1.6 [1.2, 2.1]	1.2 [0.9, 1.5]
Transient elastography			
Liver stiffness (kPa)	4.8 [3.9, 5.9]	5.1 [4.1, 6.4]	4.7 [3.8, 5.7]
Liver stiffness ≥8.0 kPa	262 (6.1)	155 (10.8)	107 (3.8)
Ultrasound			
Liver vein diameter (mm)	5.0 (1.4)	5.1 (1.4)	5.0 (1.4)
IVC diameter (mm)	17.9 (3.4)	17.6 (3.1)	18.0 (3.5)
Spleen length (cm)	9.7 (1.3)	10.0 (1.4)	9.6 (1.3)

Data is presented as mean (SD), median [P25-P75] or n and percentage. *Waist circumference >102 cm for male and >88 cm for female. †

Abbreviations: BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; HDL-C, high density lipoprotein cholesterol; IVC, inferior vena cava.

Table 2: Association of fatty liver disease with prevalent atrial fibrillation

	OR	95% CI	*P*
Steatosis			
Model 1	0.94	0.76 – 1.17	0.582
Model 2	0.80	0.62 – 1.03	0.082
NAFLD			
Model 1	0.85	0.65 – 1.10	0.205
Model 2	0.76	0.57 – 1.02	0.071
MAFLD			
Model 1	0.96	0.77 – 1.20	0.735
Model 2	0.81	0.62 – 1.04	0.097

Results were obtained with logistic regression and given as OR with 95% CI for prevalent atrial fibrillation as outcome. Atrial fibrillation was present in 405/5829 participants. Model 1 was adjusted for age and sex, model 2 in addition for alcohol consumption, smoking, education, high waist circumference, hypertension, hypo-HDL, hypertriglyceridemia, (pre)diabetes, CHD and HF. Abbreviations: CI, confidence interval; CHD cardiovascular disease; HF, heart failure; NAFLD, non-alcoholic fatty liver disease; OR, odds ratio.

Not fatty liver disease, but liver stiffness was associated with atrial fibrillation

Hepatic steatosis was not associated with higher prevalence of atrial fibrillation across all multivariable models (steatosis: $OR_{model\ 2}$ 0.80; 95%CI 0.62 – 1.03, **Table 2**) and similar results were obtained when steatosis was replaced by NAFLD or MAFLD. In a subset without prevalent CHD and/or HF fatty liver disease was consistently not associated with higher prevalence of atrial fibrillation (**supplementary Table 4**). On the other hand, liver stiffness ≥ 8.0 kPa was significantly associated with atrial fibrillation in fully adjusted models that included covariates affecting liver stiffness ($OR_{model\ 3}$ 2.08, 95%CI 1.33 – 3.25, **Table 3**). Furthermore, a similar association was observed for liver stiffness (continuous) in multivariable analysis ($OR_{model\ 3}$ 1.09 per kPa, 95%CI 1.03 – 1.16, **Table 3**). These results were consistent in a subset of participants without prevalent CHD and/or HF (**Supplementary Table 5**).

Liver stiffness was only associated with atrial fibrillation among those without steatosis

Associations between liver stiffness and prevalent atrial fibrillation were further examined in participants with steatosis (n = 1.440) and without steatosis (n =

Table 3: Association of liver stiffness with prevalent atrial fibrillation

	OR	95% CI	*P*
Liver stiffness ≥ 8.0 kPa			
Model 1	2.82	1.91 – 4.15	<0.001
Model 2	2.49	1.63 – 3.79	<0.001
Model 3	2.08	1.33 – 3.25	0.001
Liver stiffness (kPa)			
Model 1	1.15	1.10 – 1.21	<0.001
Model 2	1.12	1.07 – 1.18	<0.001
Model 3	1.09	1.03 – 1.16	0.002

Results were obtained with logistic regression and given as OR with 95% CI for prevalent atrial fibrillation as outcome. Atrial fibrillation was present in 209/4270 participants. Model 1 was adjusted for age and sex, model 2 in addition for alcohol consumption, smoking, education, high waist circumference, hypertension, hypo-HDL, hypertriglyceridemia, (pre)diabetes, CHD and HF, model 3 in addition for spleen size, IVC diameter, liver vein diameter and alanine aminotransferase. Abbreviations: CI, confidence interval; CHD cardiovascular disease; HF, heart failure; IVC, inferior vena cava; NAFLD, non-alcoholic fatty liver disease; OR, odds ratio.

2.830). For liver stiffness ≥ 8.0 kPa among the steatosis population, results were only significant in the age and sex-adjusted model ($OR_{model\ 1}$ 2.22, 95%CI 1.24 – 4.00) and no longer after including all confounders ($OR_{model\ 3}$ 1.68, 95%CI 0.82 – 3.47). This was in contrast to those without steatosis, for whom the association was statistically significant in all models ($OR_{model\ 3}$ 2.86, 95%CI 1.56 – 5.22, **Table 4**). Similarly, liver stiffness (continuous) was not associated with atrial fibrillation in multivariable analyses in the steatosis population ($OR_{model\ 3}$ 1.03 per kPa, 95%CI 0.95 – 1.11), while we observed a significant association in the no steatosis population in all models ($OR_{model\ 3}$ 1.18 per kPa, 95%CI 1.08 – 1.29, **Table 4**).

Fatty liver disease was not associated with incident atrial fibrillation

For the longitudinal analysis, we excluded 405 participants with prevalent atrial fibrillation and 356 participants without follow-up. During a median follow-up of 2.1 [1.1-3.2] years, 132 out of 5.064 individuals had incident atrial fibrillation (incidence rate 10.2 per 1000 person-years). Hepatic steatosis was not associated with incident atrial fibrillation ($OR_{model\ 2}$ 0.88; 95%CI 0.59 – 1.38) and similar results were obtained when steatosis was replaced by NAFLD or MAFLD (**Table 5**).

Table 4: Association of fibrosis and liver stiffness with prevalent atrial fibrillation stratified for steatosis

	Steatosis		No steatosis	
	OR	95% CI	OR	95% CI
Liver stiffness ≥ 8.0 kPa				
Model 1	2.22	1.24 – 4.00	3.62	2.12 – 6.19
Model 2	1.87	0.95 – 3.69	3.55	2.01 – 6.25
Model 3	1.68	0.82 – 3.47	2.86	1.56 – 5.22
Liver stiffness (kPa)				
Model 1	1.10	1.03 – 1.17	1.25	1.15 – 1.36
Model 2	1.05	0.98 – 1.13	1.23	1.12 – 1.34
Model 3	1.03	0.95 – 1.11	1.18	1.08 – 1.29

Results were obtained with logistic regression and given as OR with 95% CI for prevalent atrial fibrillation as outcome. 70/1440 participants with steatosis had atrial fibrillation and 139/2830 of those without steatosis. Model 1 was adjusted for age and sex, model 2 in addition for alcohol consumption, smoking, education, high waist circumference, hypertension, hypo-HDL, hypertriglyceridemia, (pre)diabetes, CHD and HF, model 3 in addition for spleen size, IVC diameter, liver vein diameter and alanine aminotransferase. Abbreviations: CI, confidence interval; IVC, inferior vena cava; OR, odds ratio.

Table 5: Association for FLD with incident atrial fibrillation

	HR	95% CI	*P*
Steatosis			
Model 1	0.95	0.66 – 1.37	0.793
Model 2	0.88	0.59 – 1.33	0.548
NAFLD			
Model 1	0.88	0.57 – 1.35	0.544
Model 2	0.86	0.53 – 1.38	0.522
MAFLD			
Model 1	0.98	0.68 – 1.42	0.912
Model 2	0.91	0.60 – 1.38	0.657

Results were obtained with cox regression and given as HR with 95% CI for incident atrial fibrillation as outcome. Incident atrial fibrillation occurred in 132/5064 participants. Model 1 was adjusted for age and sex, model 2 in addition for alcohol consumption, smoking, education, high waist circumference, hypertension, hypo-HDL, hypertriglyceridemia, (pre)diabetes, CHD and HF. Abbreviations: CI, confidence interval; CHD cardiovascular disease; HF, heart failure; HR, hazard ratio.

Table 6: Association of heart failure, IVC and liver vein diameter with liver stiffness

	beta	95% CI	*P*
All participants			
Heart failure	1.75	1.34 – 2.16	<0.001
IVC ø (per 5 mm)	0.13	0.04 – 0.23	0.005
Liver vein ø (per 5 mm)	0.58	0.35 – 0.81	<0.001
Steatosis			
Heart failure	2.95	2.10 – 3.79	<0.001
IVC ø (per 5 mm)	-0.03	-0.25 – 0.20	0.816
Liver vein ø (per 5 mm)	0.35	-0.17 – 0.87	0.188
No steatosis			
Heart failure	1.09	0.66 – 1.52	<0.001
IVC ø (per 5 mm)	0.19	0.10 – 0.29	<0.001
Liver vein ø (per 5 mm)	0.67	0.42 – 0.91	<0.001

Results were obtained with linear regression and given as beta with 95% CI with liver stiffness as outcome. Results were adjusted for age, sex, alcohol consumption, smoking, education, high waist circumference, hypertension, hypo-HDL, hypertriglyceridemia and (pre)diabetes. Abbreviations: IVC, inferior vena cava.

Liver stiffness is associated with IVC and liver vein diameter

Last we investigated the association between liver stiffness and parameters that could reflect or affect venous congestion. We observed higher liver stiffness among participants with heart failure (beta 1.75 kPa, 95%CI: 1.34 – 2.16), this was consistent among those with and without steatosis. Similarly, among participants with steatosis, higher liver stiffness was seen for larger IVC diameter (beta 0.19 kPa per 5 mm, 95% 0.10 – 0.29) and liver vein diameter (beta 0.67 per 5 mm, 95%CI 0.42 – 0.91, **Table 6**). However, this attenuated in the steatosis population and was no longer significant. Finally, atrial fibrillation was associated with an increased IVC (+ 1.9 mm, 95%CI 1.56 – 2.28) and liver vein diameter (+ 0.5 mm, 95%CI 0.33 – 0.63) as well as increased liver stiffness (+ 1.1 kPa 95% CI 0.83 – 1.39).

DISCUSSION

In this large population-based cohort study, fatty liver disease was not a risk factor for prevalent or incident atrial fibrillation. Liver stiffness, however, was associated with prevalent atrial fibrillation, which was only significant among those without

steatosis. This observation might be explained by hepatic congestion driven by (subclinical) venous congestion.

Current evidence regarding the association between fatty liver disease and atrial fibrillation is inconsistent.[81, 204-207, 209, 210] Interestingly, in their meta-analysis, Cai et al. investigated the effect of adjusting for cardiovascular risk factors and demonstrated weaker associations between fatty liver disease and atrial fibrillation after adjusting for cardiovascular risk factors (RR 1.19 vs RR 1.65).[209] Moreover, they demonstrated larger effect sizes in patient cohorts with typically more metabolic comorbidity, smaller studies, and when fatty liver disease was diagnosed by fatty liver index instead of imaging. These observed differences underscore the need for a well-defined cohort with an accurate steatosis assessment and accurate adjustment for relevant confounders.

In our large general population-based study, we did not identify abdominal ultrasound-based fatty liver disease as an independent risk factor for prevalent or incident atrial fibrillation among the elderly. Compared to other studies, our population is older, has more age-related comorbidity such as diabetes and hypertension, while the average BMI is one of the lowest reported in studies assessing NAFLD and atrial fibrillation. Since trends were more clear among morbid populations, this may have contributed to not demonstrating an association with NAFLD and atrial fibrillation.[202, 209] Nonetheless, this confirms that in the general population, the role of fatty liver disease in the development of atrial fibrillation is limited as suggested previously[209] or might not exist at all, especially after adjusting for confounders such as hypertension, dyslipidemia, (pre)diabetes, waist circumference and prevalent heart diseases.

Fatty liver disease is undisputedly associated with fibrogenesis, as reflected in higher liver stiffness. A few studies have already investigated the association between fibrosis or liver stiffness with atrial fibrillation. For example, an association was demonstrated between atrial fibrillation and liver stiffness in a rather small study (n = 76) among the Finnish elderly.[203] In addition, FIB-4 and APRI, markers for fibrosis, were associated with atrial fibrillation among patients with NAFLD.[211] However, using biomarker-based algorithms to assess fibrosis limits the possibility for accurate adjustment, given that those algorithms themselves include relevant predictors for atrial fibrillation (e.g. FIB-4 includes age).

Our study assessed liver stiffness in the entire cohort by transient elastography and demonstrated that liver stiffness was associated with prevalent atrial fibrillation. Interestingly, the association between liver stiffness and prevalent atrial fibrillation turned out to be substantially higher and only significant among those without steatosis. This indicates that fatty liver disease is unlikely to be the driver for higher liver stiffness among individuals with atrial fibrillation. Furthermore, it is up for debate whether higher liver stiffness was caused by fibrogenesis, since only among those without steatosis (and thus at lowest risk for fibrosis) an association with liver stiffness and atrial fibrillation was demonstrated. We note, however, that individuals without steatosis while having high liver stiffness might be "burnout NAFLD". However, among individuals that underwent CT-scan 4-5 years prior to liver stiffness measurement, we had only evidence for hepatic steatosis in 1 / 35 fibrosis patients without steatosis at the current visit. "Burnout NAFLD" is therefore unlikely to explain the association between liver stiffness and atrial fibrillation among those without steatosis.

Liver stiffness is not a direct measurement for fibrosis, but a derivative that could also be affected by portosystemic congestion, inflammation, cholestasis and central venous pressure.[57-59] The latter is of particular interest since individuals with atrial fibrillation have higher right atrium pressure,[212] which is associated with (subclinical) venous congestion in the liver.[213] Similarly, we demonstrated larger IVC and liver vein diameter among those with prevalent atrial fibrillation, indicating (subclinical) venous congestion. Our results support that subtle signs of congestion might increase liver stiffness, since not only heart failure but also larger diameter of IVC or hepatic veins were associated with higher liver stiffness. This suggests that the association between liver stiffness and atrial fibrillation could be explained by venous congestion, implicating reverse causality by (cardiovascular conditions causing) atrial fibrillation. Interestingly, results were consistent after excluding participants with coronary heart disease and heart failure, supporting an independent role of atrial fibrillation via venous congestion in the association with liver stiffness.

Venous congestion might eventually result in fibrosis since prolonged exposure to hepatic congestion can lead to hepatocyte atrophy by increased sinusoidal pressure, known as congestive hepatopathy.[214] Within our data, the association between atrial fibrillation and liver stiffness attenuated (but remained significant),

after additional adjustment for covariates reflecting venous congestion. This supports that the association between atrial fibrillation and liver stiffness is partially (but not fully) explained by venous congestion and allows a role for (advanced) fibrosis. However, IVC and liver veins are imperfect markers for subclinical venous congestion, and residual confounding should thus be considered. Therefore, further research, preferably with histological evidence in addition to liver stiffness measurements and objective measurements of systemic venous pressure, is warranted.

If the association between NAFLD and atrial fibrillation is predominantly driven by venous congestion, currently used cut-offs for liver fibrosis (e.g. ≥ 8.0 kPa) may need to be reassessed in patients with atrial fibrillation and other cardiovascular diseases that could result in venous congestion. Moreover, individuals with high liver stiffness in the absence of overt liver disease might benefit from cardiovascular assessment, given the apparent capability of cardiovascular disease to increase liver stiffness. These findings are especially relevant now that transient elastography is regularly applied among those without liver disease. For example, the novel EASL guideline on non-invasive tests recommends transient elastography to screen for advanced liver disease among those with metabolic dysfunction and intermediate-to-high FIB-4, which is highly common among the elderly.[66, 170] As the prevalence of cardiovascular disease increases by age, the specificity of liver stiffness to detect fibrosis will attenuate. Future studies should assess whether this, in specific subgroups, eventually leads to detecting more cardiovascular disease than liver disease and thus initial (or simultaneous) referral to a cardiologist seems indicated. Furthermore, our results suggest that future studies using liver stiffness as outcome should consider addressing the impact of cardiovascular disease on their results.

Although this is one of the most extensive studies investigating prevalent and incident atrial fibrillation with steatosis assessment by ultrasound and liver stiffness data, the following limitations need to be mentioned. First, our study population has a mean age of 69.5 years and is almost entirely of European ancestry (97.4%). Despite that at this age atrial fibrillation is increasingly prevalent, the generalizability of our results might be limited, especially to younger and multi-ethnic populations. Second, the results derived from the cross-sectional analysis could not be used to study causality. Moreover, in the subgroup analysis assessing

the association between liver stiffness and atrial fibrillation among individuals with steatosis, only 70 individuals had atrial fibrillation. Therefore, the final models in certain subgroups could have been overfitted and should be interpreted with caution. However, we used all three models throughout our cross-sectional analysis regarding liver stiffness to allow for a fair comparison between different subgroups. Third, our longitudinal analysis was hampered by a short follow-up duration and cases may have been missed since atrial fibrillation is often subclinical or paroxysmal. Fourth, the gold standard to assess steatosis and fibrosis is liver biopsy. However, since a biopsy is invasive and prone to severe complications, exposing a healthy cohort to these risks is unethical. Therefore, we used abdominal ultrasound and transient elastography to assess steatosis and fibrosis, which correlates strongly with histological findings.[101] However, we note that ultrasound has limited sensitivity in detecting mild steatosis.[1]

Conclusion

In conclusion, fatty liver disease was not associated with prevalent or incident atrial fibrillation in our large population-based study. In contrast, higher liver stiffness, in particular among those without steatosis, was associated with prevalent atrial fibrillation. Awaiting validation, our results indicate that this association could be driven by venous congestion instead of fibrogenesis. Since this study indicates that increased liver stiffness may result from conditions originally not linked with liver disease, further research is required to determine if the same liver stiffness cut-offs for fibrosis are applicable in participants with concomitant atrial fibrillation. As for now, we recommend to consider assessing cardiovascular health in participants with high liver stiffness, especially in the absence of overt liver disease.

SUPPLEMENTARY FILES

Supplementary table 1: details on the multiple imputation process

Software used	R version 4.0.3 with R-package MICE 3.13.0
Imputation method	Fully conditional specification
Maximum iterations	25
Imputed data sets created	50
Analysis variables	Age, alcohol consumption, alanine aminotransferase, education level, inferior vena cava diameter, liver vein diameter, metabolic syndrome, metabolic syndrome components (hypertension, (pre)diabetes, hypo-HDL, hypertriglyceridemia and high waist circumference), sex, smoking status, spleen size
Auxiliary variables	Alkaline phosphatase, aspartate aminotransferase, body mass index, diastolic blood pressure, food frequency questionnaire derived alcohol intake, gamma-glutamyl-transferase, Homeostatic Model Assessment for Insulin Resistance, height, diabetes mellitus type II, systolic blood pressure, waist circumference
Handling of variables	
Non-normally distributed	Predictive mean matching
Normally distributed	Linear regression
Binary/categorical	Logistic regression
Population	Imputation was performed on data of 5.825 participants after application of exclusion criteria.

Supplementary table 2: Participants' characteristics before and after imputation

	Before imputation	Missing	After imputation*
Demographics			
Age (years)	69.5 (9.1)	0 (0)	–
Male	42.9	0 (0)	–
European ancestry	97.4	657 (11.3)	Not imputed
Education		63 (1.1)	
Low	48.2		48.2
Intermediate	30.0		30.0
High	21.8		21.7
Current/former smoking	67.7	14 (0.2)	67.7
Alcohol intake (gram/day)	7.3 (8.2)	22 (0.4)	7.3 (8.2)
Physical examination			
High waist circumference†	44.4	1 (0.0)	44.4
BMI (kg/m^2)	27.5 (4.3)	1 (0.0)	27.5 (4.3)
Comorbidity			
Hypertension	80.0	8 (0.2)	80.0
Diabetes	15.2	86 (1.5)	15.2
Metabolic syndrome	49.8	9 (0.2)	49.8
Atrial fibrillation	7.0	0 (0.0)	–
Coronary heart disease	8.9	0 (0.0)	–
Heart failure	3.5	0 (0.0)	–
Biochemistry			
AST (U/L)	24 [21, 28]	1 (0.0)	24 [21, 28]
ALT (U/L)	19 [15, 24]	1 (0.0)	19 [15, 24]
HDL-C (mmol/L)	1.48 (0.43)	0 (0.0)	–
Triglycerides (mmol/L)	1.27 [0.97, 1.72]	0 (0.0)	–
Transient elastography			
Liver stiffness (kPa)	4.8 [3.9, 5.9]	1555 (26.7)	Not imputed
Liver stiffness ≥8.0 kPa	6.1	1555 (26.7)	Not imputed
Ultrasound			
Liver vein diameter (mm)	5.0 (1.4)	903 (15.5)	5.0 (1.4)
IVC diameter (mm)	17.9 (3.4)	710 (12.2)	17.9 (3.4)
Spleen length (cm)	9.7 (1.3)	1053 (18.1)	9.8 (1.3)

Data is presented as mean (SD), median [P25-P75] or n and percentage. *Imputed data is based on pooled data from 50 imputations with 25 iterations each. †Waist circumference >102 cm for male and >88 cm for female. – represents no missing data. Abbreviations: BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; HDL-C, high density lipoprotein cholesterol; IVC, inferior vena cava.

Supplementary Table 3: Participants' characteristics

Variable	No atrial fibrillation n = 5420	Atrial fibrillation n = 405	*p*-value
Demographics			
Age (years)	69.08 (8.91)	75.23 (8.94)	<0.001
Male	2268 (41.8)	231 (57.0)	<0.001
European ancestry	4674 (97.3)	362 (98.9)	0.096
Education			0.275
Low	2594 (48.4)	182 (45.2)	
Intermediate	1596 (29.8)	135 (33.5)	
High	1169 (21.8)	86 (21.3)	
Current/former smoking	3648 (67.5)	288 (71.5)	0.108
Alcohol intake (gram/day)	7.31 (8.13)	7.53 (8.66)	0.600
Physical examination			
High waist circumference*	2386 (44.0)	198 (48.9)	0.065
BMI (kg/m^2)	27.44 (4.28)	28.37 (4.79)	<0.001
Comorbidities			
Hypertension	4314 (79.7)	340 (84.4)	0.028
Diabetes	800 (15.0)	73 (18.5)	0.068
Metabolic syndrome	2646 (48.9)	251 (62.3)	<0.001
Coronary heart disease	434 (8.0)	86 (21.2)	<0.001
Heart failure	120 (2.2)	86 (21.2)	<0.001
Biochemistry			
AST (U/L)	24 [21, 28]	26 [22, 30]	<0.001
ALT (U/L)	19 [15, 24]	19 [15, 25]	0.327
HDL-C (mmol/L)	1.5 (0.4)	1.4 (0.4)	<0.001
Triglycerides (mmol/L)	1.3 [1.0, 1.7]	1.3 [1.0, 1.7]	0.449
Transient elastography			
Liver stiffness (kPa)	4.8 [3.8, 5.8]	5.9 [4.7, 7.1]	<0.001
Liver stiffness ≥8.0 kPa	222 (5.5)	40 (19.1)	<0.001
Ultrasound			
Liver vein diameter (mm)	5.0 (1.4)	5.5 (1.8)	<0.001
IVC diameter (mm)	17.7 (3.3)	19.5 (3.9)	<0.001
Spleen length (cm)	9.7 (1.3)	9.9 (1.3)	0.024

Data is presented as mean (SD), median [P25-P75] or n and percentage. *Waist circumference >102 cm for male and >88 cm for female. † Abbreviations: BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; HDL-C, high density lipoprotein cholesterol; IVC, inferior vena cava.

Supplementary table 4: Association of fatty liver disease with prevalent atrial fibrillation among participants without heart failure or coronary heart disease

	OR	95% CI	*P*
Steatosis			
Model 1	0.88	0.67 – 1.15	0.355
Model 2	0.73	0.54 – 0.98	0.038
NAFLD			
Model 1	0.88	0.64 – 1.19	0.399
Model 2	0.72	0.52 – 1.02	0.065
MAFLD			
Model 1	0.88	0.67 – 1.16	0.374
Model 2	0.72	0.53 – 0.97	0.033

Results were obtained with logistic regression and given as OR with 95% CI for prevalent atrial fibrillation as outcome. Atrial fibrillation was present in 262/5164 participants. Model 1 was adjusted for age and sex, model 2 in addition for alcohol consumption, smoking, education, high waist circumference, hypertension, hypo-HDL, hypertriglyceridemia, (pre)diabetes. Abbreviations: CI, confidence interval; IVC, inferior vena cava; NAFLD, non-alcoholic fatty liver disease; OR, odds ratio.

Supplementary table 5: Association of liver stiffness with prevalent atrial fibrillation among participants without heart failure or coronary heart disease

	OR	95% CI	*P*
Liver stiffness ≥ 8.0 kPa			
Model 1	2.41	1.47 – 3.95	<0.001
Model 2	2.55	1.54 – 4.22	<0.001
Model 3	2.33	1.39 – 3.91	0.001
Liver stiffness (kPa)			
Model 1	1.15	1.08 – 1.22	<0.001
Model 2	1.15	1.08 – 1.22	<0.001
Model 3	1.13	1.06 – 1.20	<0.001

Results were obtained with logistic regression and given as OR with 95% CI for prevalent atrial fibrillation as outcome. Atrial fibrillation was present in 143/3885 participants. Model 1 was adjusted for age and sex, model 2 in addition for alcohol consumption, smoking, education, high waist circumference, hypertension, hypo-HDL, hypertriglyceridemia and (pre)diabetes, model 3 in addition for spleen size, IVC diameter, liver vein diameter and alanine aminotransferase. Abbreviations: CI, confidence interval; IVC, inferior vena cava; NAFLD, non-alcoholic fatty liver disease; OR, odds ratio.

CHAPTER 3.4

Poor performance of FIB-4 in elderly individuals at risk for chronic liver disease – implications for the clinical utility of the EASL NIT guideline

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To the editor:

With the identification of novel risk factors for chronic liver disease, the number of patients potentially eligible for referral for hepatologist consultation has expanded rapidly. Hence, non-invasive tools for risk stratification and identification of patients at highest risk are essential. We, therefore, read with great interest the 2021 update of the "*EASL Clinical Practice Guidelines on non-invasive tests for evaluation of liver disease severity and prognosis"*. [66] Through the synthesis of available evidence and expert opinion, an algorithm was constructed to aid practitioners in identifying patients at the highest risk of significant liver disease. In the algorithm, a major role has been allocated to FIB-4 as an early stratification tool, despite uncertain diagnostic accuracy. [215, 216] We assessed the performance of the FIB-4-based risk stratification in participants enrolled in the Rotterdam Study, [89] a large, population-based cohort with available data on liver biochemistry, metabolic syndrome, alcohol consumption, and liver stiffness. Participants at risk for chronic liver disease were defined according to the EASL guideline as having either metabolic syndrome (based on the ATP-III criteria [94]) or excessive alcohol consumption (≥ 20 grams or ≥ 30 grams daily for females/males) or viral hepatitis. Participants with viral hepatitis were excluded from this analysis since they require referral regardless of FIB-4 outcomes. Next, we applied the first step of the algorithm, which selects participants with FIB-4 ≥ 1.3 for liver stiffness assessment, and investigated its performance to detect fibrosis based on liver stiffness ≥ 8.0 kPa. In sensitivity analysis, we investigated the algorithm in several subgroups and applied a validated age-dependent cut-off for FIB-4, raising the cut-off for participants ≥ 65 years from 1.3 to 2.0. [217]

We included 3.891 participants (aged 67.3±8.2, 44.2% male), of whom 6.0% had significant liver fibrosis based on liver stiffness. Among those considered at increased risk for chronic liver disease based on the presence of the metabolic syndrome and/or excessive alcohol consumption (n=1.875, 8.6% liver stiffness ≥ 8.0 kPa), 26 were excluded for concomitant viral hepatitis. Among the remaining subjects (n=1.849), 1.104 had intermediate-to-high FIB-4 (≥1.3) and 745 low FIB-4 (<1.3) scores, of whom 10.8% and 5.4% had liver stiffness of ≥ 8.0 kPa, respectively. Therefore, FIB-4 had poor discriminative performance (AUROC:

Table 1: Diagnostic performance of FIB-4 in elderly individuals at risk for chronic liver disease

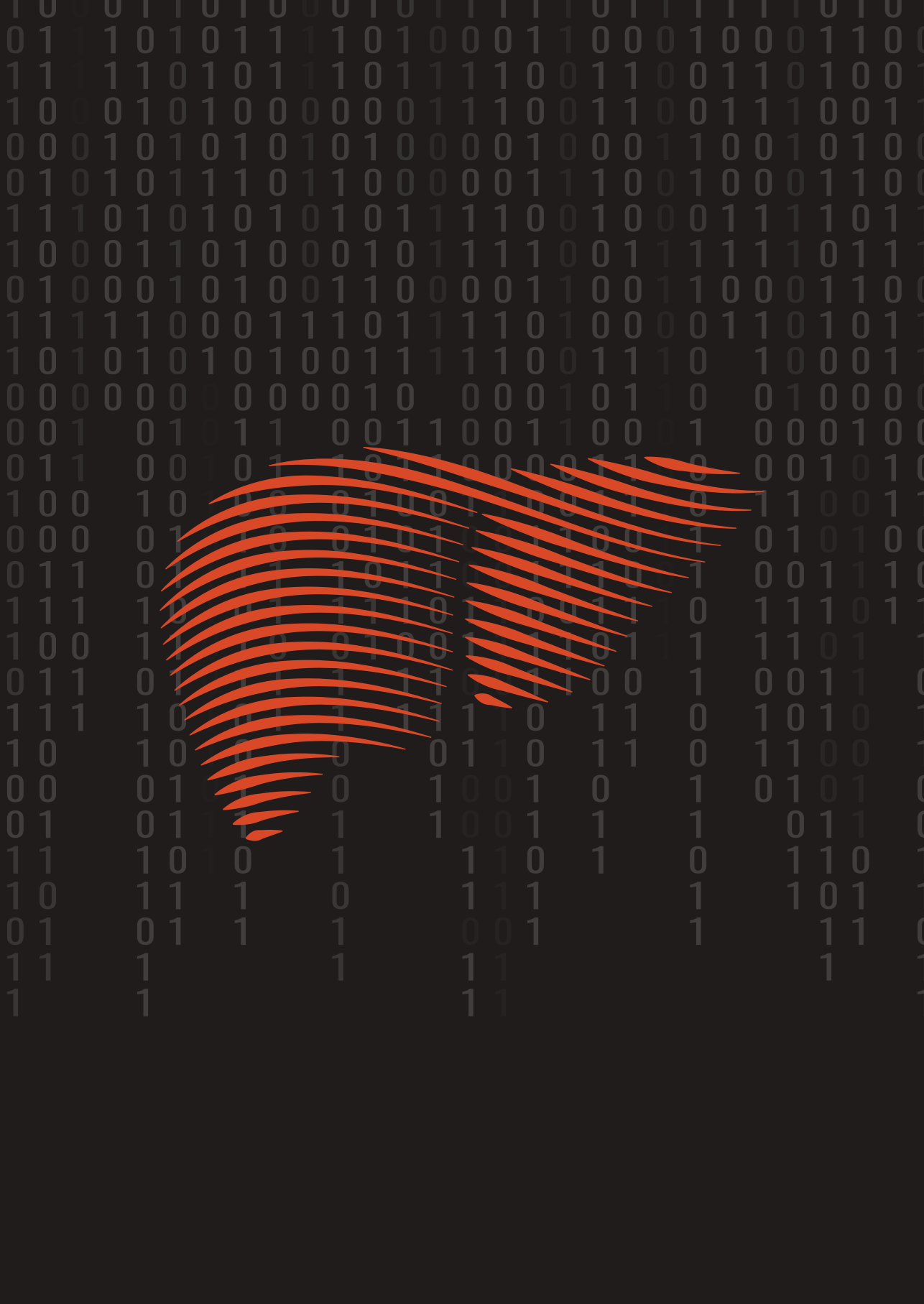
	n	LSM ≥ 8.0 kPa	Sens	Spec	NPV	PPV	Accuracy	DOR	Referral
Overall	1849	159	74.8%	41.7%	94.6%	10.8%	44.6%	2.13	59.7%
Inclusion criteria									
Metabolic	1263	116	72.4%	40.9%	93.6%	11.0%	43.8%	1.82	60.3%
Alcohol	320	16	81.3%	44.1%	97.8%	7.1%	45.9%	3.42	57.2%
Both	266	27	81.5%	42.7%	95.3%	13.8%	46.6%	3.28	59.8%
Liver enzymes									
normal	1340	65	69.2%	41.3%	96.3%	5.7%	42.7%	1.59	59.2%
elevated	509	94	78.7%	42.9%	89.9%	23.8%	49.5%	2.78	61.1%
Age									
< 65	669	40	47.5%	62.3%	94.9%	7.4%	61.4%	1.50	38.3%
≥ 65	1180	119	84.0%	29.5%	94.3%	11.8%	35.0%	2.20	71.9%
≥ 65*	1180	119	36.1%	76.2%	91.4%	14.5%	72.1%	1.81	25.1%
Diabetes									
Yes	395	62	75.8%	44.7%	90.9%	20.3%	49.6%	2.54	58.5%
No	1435	95	73.7%	41.4%	95.7%	8.2%	43.6%	1.98	59.6%
Steatosis									
Yes	921	114	73.7%	46.1%	92.5%	16.2%	49.5%	2.39	56.4%
No	928	45	77.8%	37.7%	97.1%	6.0%	39.7%	2.12	63.0%

*An age-dependent cut-off of FIB-4 (2.0 instead of 1.3) was applied for individuals ≥ 65 years.
Abbreviations: DOR, diagnostic odds ratio; LSM, liver stiffness measurement; NPV, negative predictive value; PPV, positive predictive value; sens, sensitivity; spec, specificity.

0.635) and missed 40/159 (25.2%) individuals at high risk for significant fibrosis despite selecting 59.7% of individuals for additional transient elastography, resulting in 74.8% sensitivity and 41.7% specificity (**Table 1**). Although the accuracy of FIB-4 varied somewhat across subgroups, the overall performance was consistently poor either due to low sensitivity and/or an unacceptably high number of individuals selected for referral for liver stiffness assessment. While the application of age-specific cut-offs decreased the number of subjects selected for a referral from 72% to 25%, this resulted in an unacceptable loss in sensitivity (from 84% to 36%). Moreover, of particular importance, participants with elevated liver enzymes, diabetes or steatosis were persistently at high risk (8-10%) for fibrosis, despite a low FIB-4. Although these are the groups at the highest risk for advanced fibrosis, we note that transient elastography also misclassifies more frequently

among those with diabetes and is not the gold standard to assess fibrosis.[53] However, consistent with our results, a similarly poor detection rate for FIB-4 to detect histological proven advanced fibrosis was obtained in a recent meta-analysis with over 5.000 NAFLD patients, resulting in a worrisome fibrosis prevalence of 15% despite a low FIB-4.[53]

Based on these findings, we conclude that the newly proposed FIB-4-based algorithm has poor clinical utility in elderly subjects in primary care. The high proportion of patients with clinical risk factors, combined with the poor diagnostic performance of FIB-4 would result in an unmanageably large number of referrals, whilst failing to identify over 25% of patients with fibrosis. These findings highlight the need for alternative non-invasive tools for the identification of patients at the highest risk of significant liver fibrosis and/or the availability of liver stiffness assessment tools outside liver clinics.



CHAPTER 4

Fatty liver disease management

CHAPTER 4.1

Objectively measured physical activity is inversely associated with Non-Alcoholic Fatty Liver Disease: The Rotterdam Study

Laurens A. van Kleef, Amy Hofman, Trudy Voortman, Robert J. de Knegt

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ABSTRACT

Background & aims: The disease burden of non-alcoholic fatty liver disease (NAFLD) increases rapidly, in line with the obesity pandemic. Physical activity has been linked to a lower risk of NAFLD. However, the impact of different intensities of activity and sedentary behaviour remains unclear, as well as if their effects on NAFLD are explained by metabolic health.

Methods: We performed cross-sectional analyses within the population-based Rotterdam Study cohort. Abdominal ultrasound and accelerometry data were collected between 2009 and 2014. NAFLD was defined as hepatic steatosis diagnosed by ultrasound, in the absence of secondary causes for steatosis: viral hepatitis, steatogenic drugs, and excessive alcohol. We categorised accelerometry data into sedentary time, light, moderate and vigorous physical activity.

Results: We included 667 participants (mean age 63.3±6.3 years, 53% female), 34.3% had NAFLD. Total physical activity was associated with lower NAFLD prevalence adjusted for demographics, lifestyle and socio-economic factors (OR 0.958 per 10 min/day, 95%CI 0.929 – 0.986). More intensive physical activity was more strongly associated with lower NAFLD prevalence: odds ratios for light, moderate, and vigorous physical activity were 0.931 (95%CI 0.882 – 0.982), 0.891 (95%CI 0.820 – 0.967) and 0.740 (95%CI 0.600 – 0.906) per 10 min/day, respectively. These associations were explained by metabolic health, in particular, HOMA-IR (proportion mediated: 0.59, $p < 0.001$) and waist circumference (proportion mediated: 1.08, $p < 0.001$). Beyond this indirect effect, no direct effect could be demonstrated ($p = 0.282 – 0.827$).

Conclusions: Physical activity at each intensity is inversely associated with NAFLD prevalence, with larger effects for higher intensities of physical activity. This association is mediated by better metabolic health, mainly lower insulin resistance and waist circumference. Physical activity should therefore be incorporated into NAFLD disease management and prevention programs.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) has become the most prevalent chronic liver disease in the western world and is associated with severe hepatic and extra-hepatic comorbidities and mortality.[4, 218] Moreover, the disease burden of NAFLD is expected to further increase in the following decades, as a result of the rapid increase in adiposity and metabolic syndrome.[2, 79] Weight loss and improvements in metabolic health are important targets in prevention and disease management; in fact, NAFLD and even fibrosis can regress if 5% weight reduction is accomplished.[74, 75] These beneficial effects are driven by improved insulin resistance, stimulation of fat metabolism and increased mitochondrial function.[74, 219] Many studies have therefore investigated the potential of dietary intake and composition in steatosis regression.[74, 220] In addition, previous studies demonstrated a beneficial association between physical activity and NAFLD to some extent.[221-226] However, specific advice on physical activity duration and intensity is lacking.

Most studies to date that have been investigating the association between physical activity and NAFLD were hampered by several challenges. First, physical activity measurement was often self-reported. This limitation might be resolved by the recent progression in technology that has resulted in compact devices, which can accurately measure physical activity time and intensity over longer periods on a large scale.[227] This type of continuous activity tracking is an objective approach and not subject to recall bias and differs significantly from conventional, self-reported information.[228] Second, using serological algorithms instead of imaging to define steatosis is of concern, as it lacks sensitivity and specificity. Third, these algorithms often include parameters (i.e. BMI, waist circumference, fasting glucose and HDL-C) that are directly linked with physical activity, which may therefore be mediators or confounders.[229, 230] Since such parameters are part of the algorithm, adjusting for and exploring the role of those variables in the association between physical activity and NAFLD is impossible, even in case of sufficient sample size and extensive data available. Therefore, it remains unclear whether physical activity is directly associated with NAFLD[223] or is effectuated by improvements in body composition and metabolic health.[224]

In this study, we investigated the association between objectively measured physical activity and ultrasound-based NAFLD with emphasis on different intensities of physical activity, sedentary behavior and the impact of metabolic health in these associations.

PARTICIPANTS AND METHODS

This is a cross-sectional study within The Rotterdam Study, an ongoing prospective population-based cohort study. All citizens aged 45 years and older living in Ommoord, a suburb in Rotterdam, were eligible to participate and repeatedly invited for study visits. Further details about the Rotterdam Study have been described in detail elsewhere.[89] The current study comprises all participants who visited the study site between March 2009 and June 2014 and participated in both the abdominal ultrasound and physical activity monitoring program. Exclusion criteria were secondary causes of liver steatosis, comprising excessive alcohol consumption, steatogenic drug use and hepatitis B or C.[1] In addition, participants were excluded if excessive alcohol intake could not be ruled out based on interview data and if food-frequency questionnaire (FFQ) data were unavailable.

NAFLD diagnosis

Abdominal sonography was performed by a single experienced sonographer (PvW) using a Hitachi Hi Vision 900. Hepatic steatosis was based on hyperechogenic liver parenchyma compared to the kidney cortex or spleen, according to Hamaguchi et al.[90] and was reassessed by an experienced hepatologist in abdominal ultrasound on request. Since secondary causes for steatosis were already excluded, NAFLD was diagnosed when liver steatosis was present.

Physical activity

A triaxial accelerometer (GeneActiv; Activinsights Ltd, Kimbolton UK) was used to assess physical activity duration, intensity and sedentary time. The mean time between abdominal ultrasound and physical activity assessment was 40 days, and >90% underwent physical activity assessment within three months after the abdominal ultrasound. The participants were requested to wear the device continuously on the non-dominant wrist for one week. Physical activity was measured relative to gravity (1 mg = 9.81 mm/s^2) with an interval of 20 ms, and

categorised based on intensity according to White et al.[231] into time spent in sedentary behaviour (< 48 mg), light (48-154 mg, e.g. walking), moderate (154-389 mg, e.g. cycling) and vigorous activity (> 389 mg e.g. running). Technical and statistical procedures have been described in detail previously.[228]

Covariates

At the study location, research assistants measured anthropometrics, which included waist circumference. Trained interviewers administered questionnaires at the participant's home to ensure completion and correct interpretation. Alcohol intake frequency and quantity were assessed during a home interview and with a validated self-administered FFQ. Excessive alcohol use was defined as >30 grams/day for males and >20 grams/day for females based on FFQ or interview data.[1] Based on the same FFQ, coffee consumption, total caloric intake, and an overall diet quality score were calculated.[232] Medication use is based on a digital linkage with the participants' pharmacy. Systemic corticosteroids, amiodarone, methotrexate and tamoxifen were defined as steatogenic drugs.

During fasting state, blood samples were collected from the participants. Glucose, high-density lipoprotein cholesterol (HDL-C), triglycerides, aspartate aminotransaminase (AST), alanine aminotransferase (ALT), and gamma-glutamyl transpeptidase (GGT) were analyzed by automatic enzyme procedures and insulin with automatic immunoassay (Roche, Diagnostic GmbH, Mannheim, Germany). HOMA-IR was based on glucose and insulin levels. Viral hepatitis was determined on hepatitis B surface antigen and anti-hepatitis C, analyzed by automatic immunoassay (Roche Diagnostic GmbH, Mannheim, Germany).

Metabolic factors were included both continuously and dichotomised according to the definition of the metabolic syndrome, following the ATP III criteria:[94] (1) fasting glucose > 5.6 mmol/L and/or anti-diabetic drug use, (2) waist circumference > 102 cm (males) or > 88 cm (females); (3) Triglycerides ≥ 1.7 mmol/L and/or lipid-lowering drug use, (4) HDL-C < 1.04 mmol/L in male or < 1.30 in female and/or lipid-lowering drug use; and (5) hypertension based on either a systolic blood pressure ≥ 130 mmHg, diastolic blood pressure ≥ 85 mmHg and/or antihypertensive drug use.

Statistical analysis

The study sample was characterised using descriptive statistics. Associations between physical activity (per 10 min/day) and NAFLD (yes/no) were studied with logistic regression analyses. As independent variables, we separately studied: total physical activity, light, moderate and vigorous physical activity and sedentary time. Potential confounders and mediators were visualised in a directed acyclic graph (supplementary figure 1). We adjusted for demographics (age and sex; model 1) and lifestyle and social-economic status (smoking, education and daily alcohol consumption; model 2). In a third model, we assessed whether the association of physical activity and NAFLD was explained by metabolic health, by additionally including all the components of the metabolic syndrome ((pre)diabetes, high waist circumference, abnormal triglycerides, abnormal HDL-C and hypertension; model 3). In additional analysis, model 2 was also adjusted for caloric intake, diet quality score and coffee consumption.

To assess which of the metabolic health components contributed most, the subcriteria of the metabolic syndrome were added individually to model 2, one at a time, instead of all combined. For this additional analysis, we used both the categorical metabolic syndrome components (yes/no) and continuous measures of metabolic health, comprising systolic blood pressure, triglycerides, HDL-C, HOMA-IR and waist circumference standardised for the upper limit of normal (ULN; 88 cm for female and 102 cm for male).

For one can debate whether the metabolic factors are only confounders or actually mediators, we performed a mediation analysis using the *Lavaan* package 0.6 – 9 to assess further the role of metabolic health in the pathway between physical activity and NAFLD. Investigated mediators were the individual continuous parameters of metabolic health: systolic blood pressure, triglycerides, HDL-C, HOMA-IR and standardised waist circumference. These factors were included one at a time and outcome of interest was the proportion of the total effect mediated by the particular metabolic health parameter.

To further investigate the associations of different intensities of physical activity with NAFLD, we analyzed them using a stepwise approach. First, we analyzed vigorous physical activity as an independent variable, and then we added moderate

physical activity (moderate-to-vigorous physical activity) and finally light physical activity (total physical activity).

Natural cubic splines were used to study non-linear effects of physical activity variables with the outcome with the Splines package 4.0.2. All analyses were performed in R version 4.0.3 (The R Foundation for Statistical Computing, Vienna, Austria). P-values of < 0.05 were considered statistically significant.

Ethics

The Rotterdam Study has been approved by the Medical Ethics Committee of Erasmus MC (registration number MEC 02.1015) and by the Dutch Ministry of Health, Welfare and Sport (Population Screening Act WBO, license number 1071272-159521-PG). The Rotterdam Study Personal Registration Data collection is filed with the Erasmus MC Data Protection Officer under registration number EMC1712001. The Rotterdam Study has been entered into the Netherlands National Trial Register (NTR; www.trialregister.nl) and into the WHO International Clinical Trials Registry Platform (ICTRP; www.who.int/ictrp/network/primary/en/) under shared catalog number NTR6831. All participants provided written informed consent to participate in the study and to have their information obtained from treating physicians. All authors had access to the study data and reviewed and approved the final manuscript.

RESULTS

Of the 1.033 participants that participated in the GeneActiv program, 888 had valid accelerometry data and attended the abdominal ultrasound. Of this group, in total 221 participants were excluded, 162 for the presence of secondary causes of steatosis and 59 for insufficient data on alcohol consumption, resulting in 667 participants for final analysis (**Figure 1**). Overall, the mean age of participants during the study visit was 63.3 years (SD 6.3), 53% were female, and the majority were of European ancestry (97.3%). Total physical activity on average comprised 61.9% light, 29.8% moderate and 8.2% vigorous activity. Metabolic comorbidity was common, resulting in a 40.5% prevalence of metabolic syndrome. NAFLD was present in 229 participants (34.3%). These participants were on average older

compared to the participants without NAFLD and had a higher prevalence of overweight, diabetes, hypertension and lipid disorders (**Table 1**).

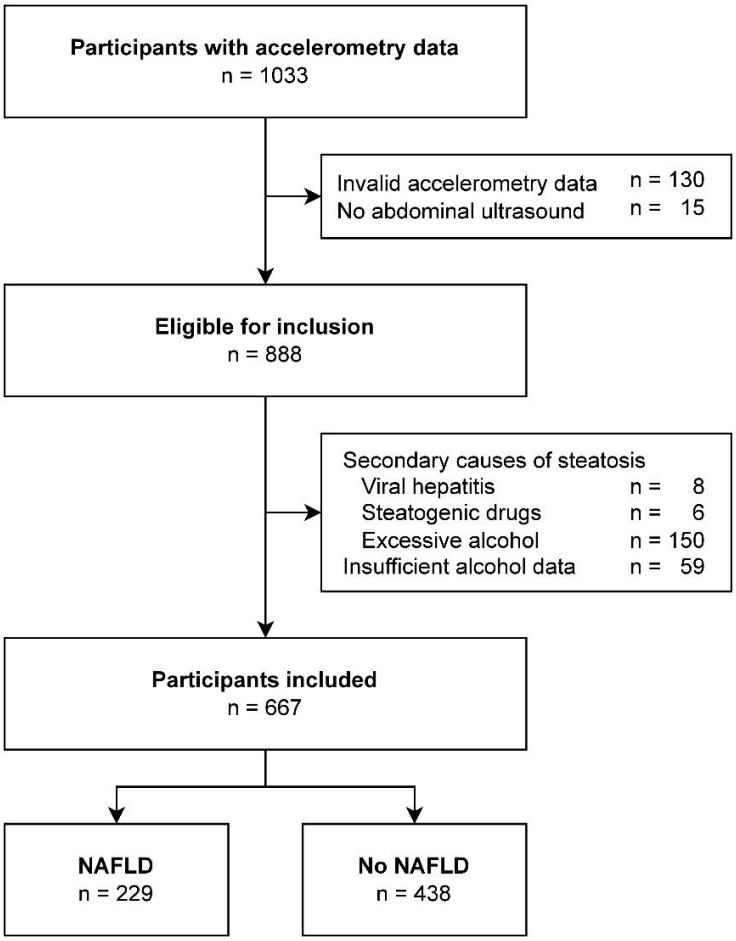


Figure 1. Flowchart of participant selection. Participants can have multiple exclusion criteria at once.

Table 1: Participants' characteristics

Variable	All n = 667	NAFLD n = 229	No NAFLD n = 438
Demographics			
Age (years)	63.3 (6.3)	64.0 (6.6)	62.9 (6.1)
Female	353 (52.9)	117 (51.1)	236 (53.9)
European ancestry	580 (97.3)	201 (97.6)	379 (97.2)
Education			
Low	276 (41.4)	103 (45.2)	173 (39.5)
Intermediate	190 (28.5)	68 (29.8)	122 (27.9)
High	200 (30.0)	57 (25.0)	143 (32.6)
Current/former smoking	460 (69.0)	166 (72.5)	294 (67.1)
Alcohol intake (g/d)	5.4 (5.5)	4.9 (5.6)	5.6 (5.4)
Coffee consumption (cups/day)[†]	2.8 (1.7)	2.7 (1.8)	2.8 (1.7)
Caloric intake (kcal/day)[‡]	2212 (737)	2169 (687)	2233 (760)
Diet Quality Score[‡]	7.1 (1.9)	6.8 (1.9)	7.2 (1.9)
Metabolic health			
BMI (kg/m^2)	27.7 (4.52)	30.3 (4.7)	26.4 (3.8)
Metabolic syndrome*	265 (40.5)	139 (62.3)	126 (29.2)
(pre)diabetes	255 (38.7)	135 (59.5)	120 (27.8)
High waist circumference	303 (45.5)	159 (69.7)	144 (32.9)
Abnormal triglycerides	188 (28.6)	80 (35.6)	108 (25.0)
Abnormal HDL-C	222 (33.8)	95 (42.2)	127 (29.4)
Hypertension	482 (72.4)	188 (82.5)	294 (67.1)
Physical activity			
Total PA (min/d)	246.7 (57.2)	236.8 (55.4)	251.9 (57.6)
Light PA (min/d)	152.8 (31.2)	148.1 (31.2)	155.3 (30.9)
Moderate PA (min/d)	73.6 (20.9)	70.1 (19.4)	75.5 (21.4)
Vigorous PA (min/d)	20.3 (8.8)	18.7 (7.8)	21.2 (9.2)
Sedentary time (min/d)	796.9 (81.4)	806.1 (86.8)	792.1 (78.2)
Biochemistry			
AST (U/L)	23 [20, 27]	24 [21, 28]	23 [20, 27]
ALT (U/L)	19 [15, 25]	21 [17, 30]	18 [14, 23]
GGT (U/L)	24 [17, 34]	28 [20, 38]	22 [16, 31]
HOMA-IR	1.0 [0.7, 1.6]	1.5 [1.0, 2.2]	0.8 [0.6, 1.2]

Data are presented as mean (SD), median [P25-P75] or n and percentage. *According to the ATP III criteria, defined as ≥ 3 sub-criteria. [†]Available in 481/667 [‡]and in 552/667 participants. Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; GGT, gamma glutamyl transpeptidase; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostatic model assessment of insulin resistance; NAFLD, non-alcoholic fatty liver disease; PA, physical activity.

Physical activity was associated with NAFLD

After adjustment for demographics (model 1), more physical activity was associated with a lower prevalence of NAFLD (OR 0.957, per 10 min/day, 95%CI 0.929 – 0.986, **Table 2**). No non-linear effects for physical activity time with NAFLD were observed using natural cubic splines (df = 2, p = 0.38). Findings were consistent after additional adjustment for lifestyle and socio-economic factors (model 2, OR 0.958, per 10 min/day 95%CI 0.929 – 0.986). Similar results were obtained when adjusting for caloric intake, diet quality score and coffee consumption in addition to model 2 (OR 0.962, per 10 min/day 95%CI 0.927 – 0.997), among a subset of participants with available dietary data (n = 480). For sedentary time, no statistically significant association was found after adjustment for covariates in model 2 (OR 1.019, per 10 min/day, 95%CI 0.999 – 1.041).

Table 2: Physical activity and sedentary time in relation to NAFLD

	OR	95% CI	*P*
Total physical activity (10min/day)			
Model 1: Demographics	0.957	0.929 – 0.986	0.004
Model 2: Lifestyle	0.958	0.929 – 0.986	0.004
Model 3: Metabolic health	0.986	0.953 – 1.019	0.405
Sedentary time (10min/day)			
Model 1: Demographics	1.019	0.998 – 1.040	0.074
Model 2: Lifestyle	1.019	0.999 – 1.041	0.068
Model 3: Metabolic health	1.006	0.984 – 1.029	0.588

Results were obtained with logistic regression and given as OR and 95% CI for NAFLD (yes/no) as outcome per 10 min/day higher PA or sedentary time. Model 1 was adjusted for age and sex, model 2 was additionally adjusted for education, smoking and alcohol, and model 3 was additionally adjusted for the individual categorical components of the metabolic syndrome. Abbreviations: NAFLD, non-alcoholic fatty liver disease; OR, odds ratio; CI, confidence interval.

All intensities of physical activity were inversely associated with NAFLD

When we examined physical activity intensities separately, we observed that all levels of intensity studied were related to lower NAFLD risk (model 2). Generally, more intensive physical activity was more strongly associated with a lower prevalence of NAFLD (**Table 3**). Adjusted for demographics, lifestyle and socio-economic factors (model 2) we found an OR of 0.931 (95%CI 0.822 – 0.982), 0.891 (95%CI 0.820 – 0.966), and 0.740 (95%CI 0.600 – 0.906) per 10 min/day for light,

moderate, and vigorous activity, respectively. Similar trends for larger effects with higher levels of intensity were observed when different summed combinations of levels of intensity were analyzed; i.e., only vigorous (OR 0.740, 95%CI 0.600 – 0.906), vigorous and moderate combined (OR 0.916, 95%CI 0.861 – 0.972), light, moderate and vigorous combined (total) (OR 0.958, 95%CI 0.929 – 0.986) per 10 min/day (**Supplementary table 1**).

Table 3: Physical activity per category of intensity in relation to NAFLD

	OR	95% CI	*P*
Vigorous PA (10min/day)			
Model 1: Demographics	0.733	0.595 – 0.895	0.003
Model 2: Lifestyle	0.740	0.600 – 0.906	0.004
Model 3: Metabolic health	0.908	0.716 – 1.144	0.416
Moderate PA (10min/day)			
Model 1: Demographics	0.891	0.820 – 0.966	0.006
Model 2: Lifestyle	0.891	0.820 – 0.967	0.006
Model 3: Metabolic health	0.964	0.877 – 1.058	0.441
Light PA (10min/day)			
Model 1: Demographics	0.931	0.882 – 0.981	0.008
Model 2: Lifestyle	0.931	0.882 – 0.982	0.009
Model 3: Metabolic health	0.976	0.919 – 1.037	0.433

Results were obtained with logistic regression and given as OR and 95% CI for NAFLD (yes/no) as outcome per 10 min/day higher PA. Model 1 was adjusted for age and sex, model 2 was additionally adjusted for education, smoking and alcohol, and model 3 was additionally adjusted for the individual categorical components of the metabolic syndrome. Abbreviations: CI, confidence interval; NAFLD, non-alcoholic fatty liver disease; OR, odds ratio; PA, physical activity.

Metabolic health mediated the association between NAFLD and physical activity

The association between total physical activity with NAFLD attenuated after adjusting for all the components of the metabolic syndrome (model 3, OR 0.986, 95%CI 0.953 – 1.019, **Table 2**), per 10 min/day. Similar results were obtained for the individual intensities (**Table 3**). The metabolic syndrome components were also studied one at a time, by adding them individually into model 2 (**Table 4**). The association of total physical activity and NAFLD attenuated most when adjusting for large waist circumference (OR 0.979, 95%CI 0.948 – 1.011) and pre-diabetes (OR 0.964, 95%CI 0.934 – 0.995), per 10 min/day. When we adjusted the models for

continuous measures of metabolic health, the attenuation became even more evident: HOMA-IR (OR 0.990, 95%CI 0.968 – 1.036) and standardised waist circumference (OR 1.002, 95%CI 0.968 – 1.036), per 10 min/day.

Table 4: Associations of total physical activity with NAFLD and additional adjustment for individual metabolic health parameters one at a time.

		OR	95% CI	*P*
Model 2		0.958	0.929 – 0.986	0.004
Additional adjustment for criteria of metabolic syndrome (y/n)				
	+ Hypertension	0.959	0.931 – 0.988	0.007
	+ Abnormal HDL-C	0.966	0.937 – 0.996	0.026
Model 2	+ Abnormal triglycerides	0.966	0.937 – 0.995	0.025
	+ High waist circumference	0.979	0.948 – 1.011	0.200
	+ (Pre)diabetes	0.964	0.934 – 0.995	0.023
Additional adjustment for continuous parameters of metabolic health				
	+ Systolic blood pressure (mmHg)	0.959	0.931 – 0.988	0.006
	+ HDL-C (mmol/L)	0.973	0.943 – 1.003	0.079
Model 2	+ Triglycerides (mmol/L)	0.971	0.942 – 1.001	0.062
	+ Standardized waist circumference*	1.002	0.968 – 1.036	0.919
	+ HOMA-IR	0.990	0.959 – 1.023	0.557

Results were obtained with logistic regression and given as OR and 95% CI for NAFLD (yes/no) as outcome per 10 min/day total PA. Shown variables were studied one at a time, by adding them individually into model 2. *Standardised for the upper limit of normal (88 cm for female and 102 cm for male). Model 2 was adjusted for age, sex, education, smoking and alcohol. Abbreviations: CI, confidence interval; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostatic model assessment of insulin resistance; NAFLD, non-alcoholic fatty liver disease; OR, odds ratio.

Last, we assessed the impact of those continuous measures of metabolic health (systolic blood pressure, triglycerides, HDL-C, HOMA-IR and standardised waist circumference) one at a time in mediation analysis. This revealed that the association of physical activity with NAFLD was mediated by metabolic health (**Figure 2, Supplementary table 2**). Likewise our conventional logistic regression models, HOMA-IR (proportion mediated: 0.59, $p < 0.001$) and standardised waist circumference (proportion mediated: 1.08, $p < 0.001$) explained most of the

observed effect. In this model even a slight positive effect estimate was observed for the direct effect of physical activity in relation to NAFLD with waist circumference as mediator, resulting in a proportion mediated effect exceeding 1.0. Beyond these indirect effects of HOMA-IR and waist circumference, no direct effect could be demonstrated (p=0.282 – 0.827). Systolic blood pressure was the only investigated parameter not being a mediator in this association (proportion mediated 0.03, p = 0.488).

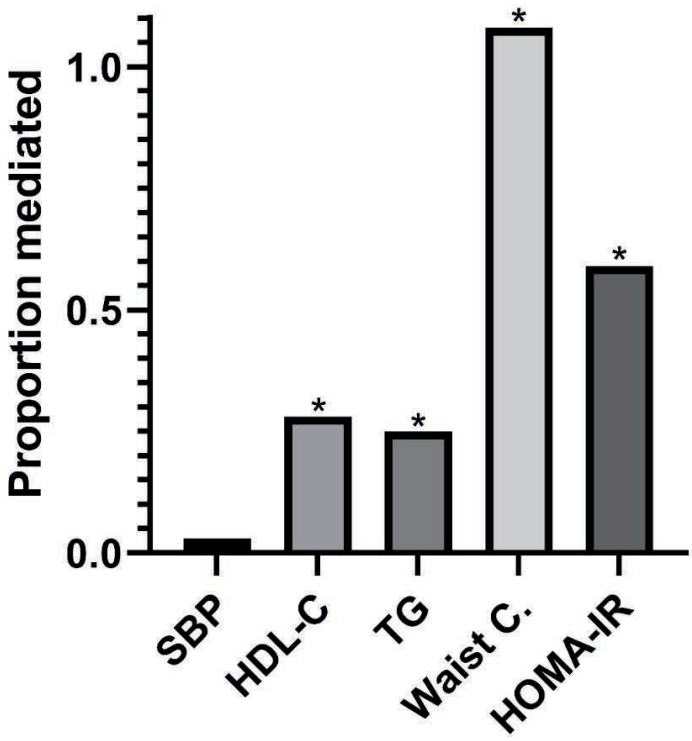


Figure 2. Proportion mediated of the total effect (OR 0.958 per 10 min total physical activity per day) for physical activity on NAFLD. The shown variables were included one at a time in mediation analysis and were adjusted for age, sex, education, smoking and alcohol consumption (model 2). In this analysis even a slight positive effect estimate was observed for physical activity in relation to NAFLD with waist circumference as mediator, resulting in a proportion mediated of 1.08. *Indicates the particular parameter mediated a significant proportion of the total effect. Abbreviations: HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostatic model assessment of insulin resistance; SBP, systolic blood pressure; TG, triglycerides; Waist C, waist circumference.

DISCUSSION

In this large population-based cohort study, physical activity measured with accelerometry was inversely associated with the presence of NAFLD, with larger effect sizes seen for more intensive activity. This association was mediated by improved metabolic traits, especially lower insulin resistance and waist circumference. More sedentary time was suggestively associated with higher NAFLD, but did not reach statistical significance.

Previous studies have observed an association between total physical activity and lower NAFLD, but were hampered by self-reported physical activity data, had no access to abdominal ultrasound and/or lacked accurate confounder adjustment.[221-226] We confirm this beneficial association of more physical activity with lower NAFLD in a large cohort with objective physical activity assessment, ultrasound-based NAFLD diagnosis, and adjustment for potential confounders. Furthermore, we add to the evidence that more intensive physical activity is more strongly associated with lower NAFLD prevalence. However, the beneficial effect was not exclusively observed for vigorous activity, but also in a lesser extent for moderate and light physical activity. We observed no significant association between sedentary time and NAFLD. To the best of our knowledge, this is the first study assessing different intensities of physical activity obtained with accelerometry in the association with NAFLD.

Studying the effect of intensity for this association is difficult since time spent in vigorous activity is undisputedly related to time spent in other intensities of physical activity. Therefore, the effect of vigorous activity could be reflected in the analysis investigating moderate and/or light activity alone. Hence, we did not only analyze the impact of different intensities per single category, but we additionally performed analyses with a composite physical activity duration, starting with vigorous activity alone and adding lower intensities resulting in moderate-to-vigorous, and total physical activity. This analysis confirmed that compositions with overall more intensive activity resulted in a larger effect size.

Despite that vigorous activity was associated most strongly with NAFLD, additional benefits were shown for light and moderate physical activity, which confirm

previous studies assessing non-vigorous activity and NAFLD. For example, George et al.[223] showed that a lifestyle intervention focusing on low to moderate-intensity physical activity improved metabolic health in patients with chronic liver disease. Moreover, benefits were found in other studies for non-vigorous physical activity regarding ALT, intrahepatic triglycerides, glucose management, weight, cardiovascular risk, and/or mortality, which are (or share) important risk factors for NAFLD.[230, 233-236] That not only vigorous activity is beneficial, but also less intensive forms of exercise is especially relevant for individuals currently not physically active or those who might not be able to exercise vigorously.

Physical activity is known to improve metabolic health and reduce mortality, with stronger associations found for more intensive physical activity.[234-236] For example, improved insulin resistance is a major benefit and is effectuated by energy consumption during the activity. This, in turn, aids in achieving or maintaining a healthy body composition, which is a key factor in insulin sensitivity.[237] Another consequence of physical activity is the enhancement of fatty acid metabolism in muscle tissue and increased muscle mass, resulting in an enhanced basal metabolic rate, which could not be achieved by dietary restrictions alone.[229, 237] For the metabolic syndrome, those effects are mainly reflected in (pre)diabetes and waist circumference. In our study, these two factors contributed most to the association between physical activity and NAFLD in both conventional and mediation analyses. In several other studies, the associations of physical activity with NAFLD attenuated as well after adjusting for (changes in) anthropometrics and glucose management.[221, 238, 239] Improvements of liver enzymes have been demonstrated by physical activity also in the absence of weight loss, but this was not adjusted for glucose management.[223, 240] Thus, these results support that the association of physical activity with liver health is not only a matter of weight or waist circumference, but is also affected and mediated by other metabolic health parameters, in particular glucose management.

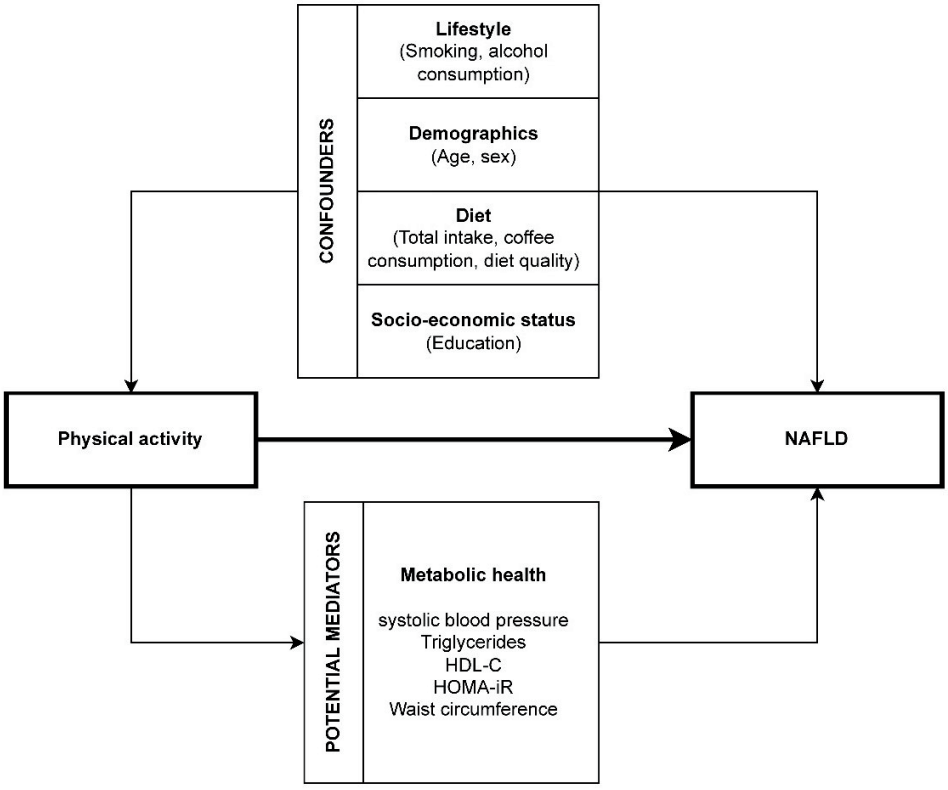
Evidence for the effects of physical activity at different intensities on NAFLD is relevant to improve current guidelines for disease management and prevention programs. For example, in a recent intervention study among patients with NAFLD, three individually tailored counselling sessions to increase low to moderate physical activity resulted in a 1 hour/week increase of physical activity in over 60% of the participants and improved glucose management and ALT levels.[223] Additional

studies are needed to investigate lasting effects, NAFLD regression rates, impact on fibrosis or liver stiffness, feasibility of implementation on a large scale in NAFLD patients and how more vigorous activity could be incorporated best. However, supported by our results, it seems feasible with a small intervention to achieve health benefits in patients with NAFLD focusing on physical activity.

Although this is one of the largest studies with accelerometric-based physical activity data and NAFLD diagnosis based on imaging, there are some limitations. First, this study had a cross-sectional design and the causality of relationships could therefore not be studied. For example, changes in physical activity habits in order to achieve weight loss in obese participants could have mitigated the associations. Second, although we adjusted for an extensive set of confounders, covering demographics, lifestyle and metabolic health, residual confounding cannot be ruled out. Further studies should investigate the potential interaction between diet, physical activity and NAFLD. Third, despite being an objective measurement and not prone to recall bias, accelerometry is yet not able to accurately recognise different types of exercise. Therefore, we were unable to investigate the role of resistance compared to aerobic physical activity. Fourth, despite that abdominal ultrasound is the most used diagnostic tool to assess steatosis, it is not the gold standard and lacks sensitivity, in particular for the detection of mild steatosis, compared to liver biopsy.[101] However, it is unethical to perform liver biopsy in healthy participants since it is an invasive intervention and is prone to severe complications.

In conclusion, we have demonstrated that physical activity is associated with a lower prevalence of NAFLD, which was mediated by better metabolic health, in particular lower waist circumference and better glucose management. Although we observed the strongest associations for vigorous physical activity, additional benefits were objectified for both moderate and light physical activity. This is especially relevant for those unable to reach vigorous physical activity, and indicates that increasing time spent in lower intensities of physical activity may already be beneficial in achieving or maintaining good liver health. Therefore, we recommend incorporating physical activity to its full extent in NAFLD disease management and prevention.

SUPPLEMENTARY FILES



Supplementary figure 1: Directed acyclic graph for the association between physical activity and NAFLD with confounders and potential mediators.

Supplementary table 1: Physical activity per summed combination of intensities in relation to NAFLD

Intensity	OR	95% CI	*P*
Vigorous PA (10min/day)			
Model 1: Demographics	0.733	0.595 – 0.895	0.003
Model 2: Lifestyle	0.740	0.600 – 0.906	0.004
Model 3: Metabolic health	0.908	0.716 – 1.144	0.416
Moderate+Vigorous PA (10min/day)			
Model 1: Demographics	0.915	0.861 – 0.971	0.004
Model 2: Lifestyle	0.916	0.861 – 0.972	0.004
Model 3: Metabolic health	0.972	0.907 – 1.041	0.421
Mild+Moderate+Vigorous PA (10min/day)			
Model 1: Demographics	0.957	0.929 – 0.986	0.004
Model 2: Lifestyle	0.958	0.929 – 0.986	0.004
Model 3: Metabolic health	0.986	0.953 – 1.019	0.405

Results were obtained with logistic regression and given as OR and 95% CI for NAFLD (yes/no) as outcome per 10 min/day higher PA. Model 1 was adjusted for age and gender, model 2 was additionally adjusted for education, smoking and alcohol, and model 3 was additionally adjusted for the individual categorical components of the metabolic syndrome. Abbreviations: CI, confidence interval; NAFLD, non-alcoholic fatty liver disease; PA, physical activity; OR, odds ratio.

Supplementary table 2: Mediation analysis for the association between physical activity and NAFLD by continuous parameters of metabolic health one at a time.

	Proportion mediated	*P* mediated effect	Proportion direct effect	*P* direct effect
Systolic blood pressure (mmHg)	0.03	0.488	0.97	0.009
HDL-C (mmol/L)	0.28	0.001	0.72	0.055
Triglycerides (mmol/L)	0.25	0.002	0.75	0.045
Standardised waist circumference*	1.08	< 0.001	-0.08	0.827
HOMA-IR	0.59	< 0.001	0.41	0.282

Proportion mediated of the total effect (OR 0.958 per 10 min total physical activity per day) for physical activity on NAFLD. The shown variables were included one at a time in mediation analysis and were adjusted for age, sex, education, smoking and alcohol consumption (model 2). In this analysis, even a slight positive effect estimate was observed for physical activity in relation to NAFLD with waist circumference as mediator, resulting in a proportion mediated of 1.08. *Indicates the particular parameter mediated a significant proportion of the total effect. Abbreviations: HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostatic model assessment of insulin resistance; SBP, systolic blood pressure; TG, triglycerides; Waist C, waist circumference.

CHAPTER 4.2

Dissecting the effects and mechanism of action of statin use on fatty liver disease: a multidimensional study

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ABSTRACT

Background & aims: Statin use could benefit non-alcoholic fatty liver disease (NAFLD) patients, but the evidence is segmented and inconclusive. This multidimensional study comprehensively investigated the potential benefits and mechanism-of-action of statins in NAFLD.

Methods: A cross-sectional investigation was performed within the Rotterdam Study (general population; n=4.576) and the PERSONS cohort (biopsy-proven NAFLD patients; n=569). Exclusion criteria were secondary causes for steatosis and insufficient data on alcohol, dyslipidemia or statin use. Associations of statin use with NAFLD (among the entire population), NASH and fibrosis (among NAFLD individuals) were quantified. These results were pooled with available literature in a meta-analysis. Last, we assessed statins' anti-lipid and anti-inflammatory effects in 3D cultured human liver organoids and THP-1 macrophages.

Results: Statin use was inversely associated with NAFLD in the Rotterdam study compared to participants with untreated dyslipidemia. In the PERSONS cohort, statin use was inversely associated with NASH but not with fibrosis. The meta-analysis included 7 studies and indicated a not significant inverse association between statin use and NAFLD (pooled-OR 0.69, 95%CI 0.46 – 1.01) and significant inverse associations with NASH (pooled-OR 0.59, 95%CI 0.44 – 0.79) and fibrosis (pooled-OR 0.48, 95%CI 0.33 – 0.70). In vitro, statins significantly reduced lipid droplet accumulation in human liver organoids and downregulated the expression of pro-inflammatory cytokines in macrophages.

Conclusion: Pooled results demonstrated that statin use was associated with a lower prevalence of NASH and fibrosis and might prevent NAFLD. These associations may be partially attributed to statins' anti-lipid and anti-inflammatory characteristics. Given their under-prescription, adequate prescription of statins may limit the disease burden of NAFLD.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a major health concern with an estimated prevalence exceeding 25% globally, driven by an alarming increase in obesity and metabolic disorders.[4] Despite major efforts, there is still an urgent need for effective treatment since novel pharmaceutical agents do not meet the required endpoints yet.

Interestingly, some studies have suggested that statins (HMG-COA reductase inhibitors) might effectively reduce the risk of NAFLD.[241, 242] Experimental and clinical studies have shown that the effects of statins go beyond their cardiovascular-protective ability and consist of anti-inflammatory, anti-thrombotic and anti-fibrotic properties and may thus inhibit progression from simple steatosis to fibrosis and non-alcoholic steatohepatitis (NASH).[243-245]

On the contrary, prescribing statins to patients with chronic liver disease often raises the issue of hepatotoxicity among clinicians, given that statins are metabolised in the liver by CYP450 isoenzymes.[246] However, one of the most common side effects of statins, asymptomatic transaminitis, is still relatively uncommon (around 3%), occurs in the first year of treatment initiation, is dose-dependent, and is usually self-limiting.[246] Moreover, statin use was safe even among those with NAFLD and elevated liver enzymes.[247] Lastly, a meta-analysis showed that the prevalence of transaminitis among patients using simvastatin or lovastatin is not significantly different from that of individuals using placebo.[248]

Convincing evidence regarding the safety of statins in NAFLD patients is available, but the evidence concerning their hepatoprotective effects is segmented and inconclusive.[249] Considering the complexity of addressing this question, this study took a multidimensional approach. We first comprehensively assessed the associations of statin use with NAFLD, NASH and fibrosis in a large general population cohort, a biopsy-proven NAFLD patient cohort, and a meta-analysis of pooling existing data. Finally, we experimentally tested the effects of statins on lipid accumulation and inflammatory gene expression in human liver organoids and macrophages, respectively, to explore potential mechanism-of-action.

Methods

To assess the potential multifaceted effects of statins on the NAFLD disease spectrum, we performed a multidimensional study comprising a cross-sectional investigation in a general population cohort and a NAFLD patient cohort, a meta-analysis, and finally an experimental exploration.

The Rotterdam Study is an ongoing population-based cohort.[89] Participants visiting the research center between 2009 and 2014 were eligible for inclusion (n = 5.967). Exclusion criteria were secondary causes for steatosis (n = 922 [excessive alcohol consumption, viral hepatitis and steatogenic drug use]) or insufficient data (n = 469) on alcohol consumption, dyslipidemia or statin use (supplementary table 1). NAFLD was defined as steatosis on abdominal ultrasound: hyperechoic liver parenchyma compared to kidney or spleen. Liver stiffness data was available for 72% of this cohort and fibrosis was defined as liver stiffness ≥ 8.0 kPa after discarding unreliable measurements according to the Boursier criteria (n = 141) and measurements among those with heart failure (n = 70).[91] Data regarding statin use was obtained by linkage with the participants' pharmacies and verified during an interview. Dyslipidemia was defined as either hypo-HDL or hypertriglyceridemia, applying cut-offs from the Adult Treatment Panel III criteria for the metabolic syndrome.[250]

In order to prevent distortion of those without dyslipidemia who are unlikely to benefit from statin use, we identified three subgroups based on statin use and dyslipidemia: statin use, non-treated dyslipidemia and no dyslipidemia. These groups were used to quantify the association of statin use with NAFLD by logistic regression. Subsequently, we selected participants with NAFLD to further investigate the potential benefits of statins on fibrosis. For this analysis, patients with NAFLD using statins were compared to NAFLD patients without statin use, regardless of dyslipidemia. Multivariable models were adjusted for age, sex, hypertension, (pre)diabetes, and high waist circumference. For sensitivity analyses, NAFLD was replaced by the newly introduced definition of MAFLD[29] and the cut-off for fibrosis was lowered to 7.0 kPa to increase statistical power.

The PERSONS (Prospective Epidemic Research Specifically Of NASH) cohort is a well-characterised cohort of biopsy-proven Chinese NAFLD patients, who visited the

First Affiliated Hospital of Wenzhou Medical University between 2016 and 2019.[251, 252] Patients with NAFLD and available metabolic health data who required liver biopsy due to abnormal liver imaging, abnormal liver function test, and/or abnormal fibrosis tests, were eligible for inclusion (n = 569). NASH was defined as the simultaneous presence of steatosis, lobular inflammation, and hepatocellular ballooning.[6] Fibrosis was defined as Brunt classification ≥ F2.[141] Data on statin use was obtained from the patient's healthcare system. Associations for statin use with NASH and fibrosis were quantified with logistic regression using the same multivariable models.

A meta-analysis was performed according to the PRISMA guidelines in order to comprehensively assess the potential associations of statins with NAFLD, NASH and fibrosis. The systematic literature search was performed on 10th of January 2022 in Medline, Embase and Web of Science, using NAFLD and statins with affiliated terms (detailed search and screening methods are included in supplementary methods and the full search strategy in supplementary table 2). Briefly, we included original studies that reported on the association between statin use and our primary outcomes in individuals with metabolic dysfunction. Primary outcomes were the presence of (1) NAFLD, (2) NASH, or (3) fibrosis. In addition, findings from the Rotterdam Study and PERSONS cohort in the current study, were also included. Odds ratios were extracted from fully adjusted models and pooled using generic inverse variance and random-effects models. Furthermore, we assessed excessive influence of our results and other individual studies on the pooled outcome by excluding one study at a time.

To assess the direct effects on lipid accumulation and to identify potential causal pathways, we tested simvastatin and lovastatin in 3D cultured primary human liver organoids as previously described.[253] Moreover, pathological inflammation is a crucial driver of NAFLD progression towards NASH, which is mainly mediated by macrophages.[254] Therefore, we tested the effects of simvastatin and lovastatin on the expression of inflammatory genes in cultured human THP-1 macrophages. Detailed experimental methods were described in supplementary methods.

Statistical analyses were performed in R version 4.0.4 (The R foundation for statistical computing, Vienna, Austria), SPSS v26.0 (IBM Corp., Armonk, N.Y., USA) and GraphPad Prism (version 8.0.2; GraphPad Software Inc., La Jolla, CA). P-values

of < 0.05 were considered statistically significant. The meta-analysis was performed using R-package *meta* version 4.18-2 and *metafor* version 3.0-2.

Ethics

The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus MC (MEC-02.1015) and by the Dutch Ministry of Health, Welfare and Sport (1071272-159521-PG) and has been entered into the WHO International Clinical Trials Registry Platform (NTR6831). The PERSONS cohort study was approved by the internal review board for ethics at the First Affiliated Hospital of Wenzhou Medical University (2016-246, 1 December 2016) and was registered in the Chinese Clinical Trial Registry (ChiCTR-EOC-17013562). All participants signed a written informed consent to participate in this study.

Results

We analyzed 4.576 participants from the Rotterdam Study (age: 69.9±9.2 year; male 41.0%, BMI 27.6±4.3 kg/m^2), of whom 1.591 (34.8%) had NAFLD. Among participants with NAFLD, valid liver stiffness measurement was available in 65.7% (n = 1.046), of whom 9.7% (n = 101) had fibrosis. In this cohort, 28.4% (n = 1.298) used statins, 24.3% (n = 1.110) had non-treated dyslipidemia and 47.4 % (n = 2.168) had no dyslipidemia. Of note, statin users had more metabolic comorbidity (e.g. diabetes [33% vs 14%] and hypertension [90% vs 74%]) compared to the non-treated dyslipidemia group. As expected, HDL (1.4 vs 1.2 mmol/L) and triglycerides (1.4 vs 1.9 mmol/L) levels were favourable in those on statin treatment (**Table 1**).

Importantly, statin treatment was inversely associated with NAFLD (OR 0.72, 95%CI 0.59 – 0.86; **Table 2**) compared to participants with non-treated dyslipidemia, adjusted for age, sex, hypertension, (pre)diabetes, and high waist circumference. However, participants without dyslipidemia (and not on statin therapy) had the lowest odds of NAFLD (OR 0.50, 95%CI 0.42 – 0.60). The association between statin use and fatty liver disease was consistent if NAFLD was replaced by MAFLD (**Table 2**). Among participants with NAFLD and reliable liver stiffness measurement (n = 1.046), statins were used in 32% and was not significantly associated with fibrosis (OR 0.65, 95%CI 0.40 – 1.07), in fully adjusted models. However, in a sensitivity analysis, the statistical power increased by using a more lenient definition of

Table 1: Participants' characteristics

	Part 1: Rotterdam Study			Part 2: PERSONS Cohort	
	Statin use n = 1.298	Untreated dyslipidemia n = 1.110	No dyslipidemia n = 2.168	Statin use n = 73	No statin use n = 496
Demographics					
Age (years)	71.9 (8.2)	69.0 (9.4)	69.2 (9.4)	47.9 (12.2)	41.5 (12.1)
Male	645 (49.7)	432 (38.9)	800 (36.9)	50 (68.5)	361 (72.8)
Metabolic health					
BMI (kg/m^2)	28.4 (4.2)	28.6 (4.6)	26.6 (4.1)	26.6 (3.1)	26.6 (3.4)
Diabetes	418 (33.0)	153 (14.0)	146 (6.9)	45 (61.6)	100 (20.2)
Hypertension	1163 (89.6)	819 (73.8)	1386 (63.9)	37 (50.7)	97 (19.6)
Biochemistry					
AST (U/L)	25 [21, 30]	24 [21, 28]	24 [21, 27]	31 [24, 45]	34 [25, 54]
ALT (U/L)	20 [16, 26]	19 [15, 26]	17 [14, 22]	40 [26, 62]	52 [29, 92]
Trigelycerides (mmol/L)	1.4 [1.0, 1.9]	1.9 [1.5, 2.2]	1.1 [0.9, 1.3]	1.9 [1.3, 2.7]	1.9[1.4, 2.4]
HDL (mmol/L)	1.4 (0.4)	1.2 (0.3)	1.7 (0.4)	1.03 (0.24)	1.02 (0.25)
Liver assessment					
NAFLD	554 (42.7)	524 (47.2)	513 (23.7)	73 (100)	496 (100)
NASH	–	–	–	41 (56.2)	351 (70.8)
Fibrosis*	54 (6.5)	42 (5.5)	84 (5.1)	14 (19.2)	91 (18.3)

Data is presented as mean (SD), median [P25-P75] or n and percentage. Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; HDL, high density lipoprotein; NAFLD, non-alcoholic fatty liver disease; NASH, on-alcoholic steatohepatitis. * Based on LSM ≥ 8.0 kPa (in Rotterdam Study) or biopsy (in PERSONS cohort)

fibrosis (liver stiffness ≥ 7.0 instead of 8.0 kPa), and in this analysis statin use was significantly associated with lower prevalence of fibrosis (OR 0.54, 95%CI 0.36 – 0.82; **Table 2**).

Table 2: Association for statin use with NAFLD, NASH and fibrosis in fully adjusted models

Rotterdam study			
	OR	**95% CI**	**P**
NAFLD	0.72	0.60 – 0.86	<0.001
MAFLD	0.73	0.62 – 0.87	<0.001
Fibrosis			
Stiffness ≥ 8.0 kPa	0.65	0.40 – 1.07	0.096
Stiffness ≥ 7.0 kPa	0.54	0.36 – 0.82	0.004

PERSONS Cohort			
	OR	**95% CI**	**P**
NASH	0.55	0.32 – 0.95	0.031
Ballooning	0.53	0.29 – 0.96	0.035
Lobular inflammation	0.64	0.33 – 1.26	0.197
Fibrosis	0.86	0.44 – 1.68	0.857

Results were obtained with logistic regression and adjusted for age, sex, hypertension, (pre)diabetes, and high waist circumference. The reference group for NAFLD and MAFLD analysis were participants with untreated dyslipidemia. NASH and fibrosis analyses were performed among individuals with NAFLD.

Next, we analyzed 569 patients with biopsy-proven NAFLD from the PERSONS cohort (age: 42.3±12.3 year; male 72.2%; BMI 26.6±3.3 kg/m^2), of whom 68.9% (n = 392) had NASH, and 18.5% (n = 105) fibrosis. Additional baseline characteristics are shown in **Table 1**. In this cohort 12.8% (n = 73) used statins, which was inversely associated with NASH (OR 0.55, 95%CI 0.32 – 0.95) but not with fibrosis (OR 0.86, 95%CI 0.44 – 1.68) in fully adjusted models (**Table 2**).

To further validate observed trends and our findings and to increase statistical power, we performed a meta-analysis. Our systematic search found 1766 unique articles, of which 6 were included for the analysis **(Figure 1**).[255-260] Combining the results obtained from our Rotterdam Study and PERSONS cohort, 5 studies were finally included for NAFLD, 3 for NASH and 7 for fibrosis analysis, respectively. Study characteristics, including the definitions of NAFLD, NASH and fibrosis, as well as

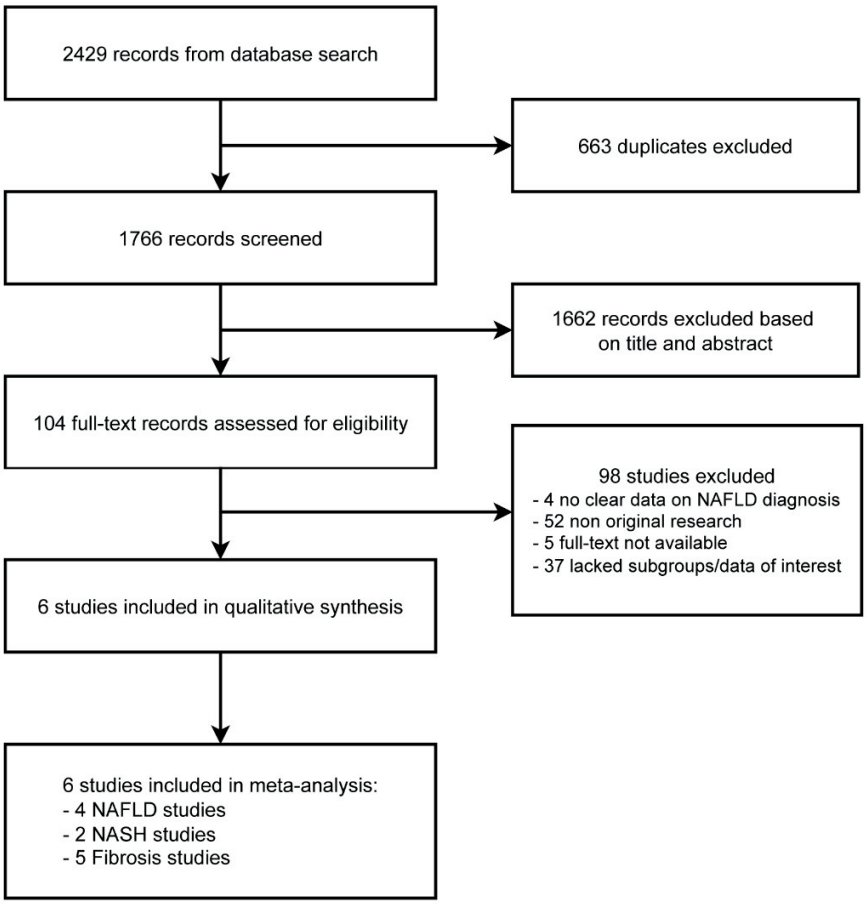


Figure 1. Selection of studies. PRISMA flowchart of literature search and study identification.

quality assessment of included studies are shown in **supplementary table 3** and **supplementary table 4**, respectively. Different multivariable models were used across the included studies, but all extracted odds ratios were accounted for age, sex and metabolic health (e.g. BMI, metabolic syndrome and/or diabetes). Pooled results indicated a not significant, but inverse association for statin use with steatosis (pooled OR 0.69, 95%CI 0.46 – 1.01; **Figure 2A**), a significant inverse association with NASH (pooled OR 0.59, 95%CI 0.44 – 0.79; **Figure 2B**) and a significant inverse association with fibrosis (pooled OR 0.48, 95% CI 0.33 – 0.70; **Figure 2C**), among those with metabolic dysfunction. Excessive influence analysis did not reveal a study, nor our own results, with a specifically large influence on the

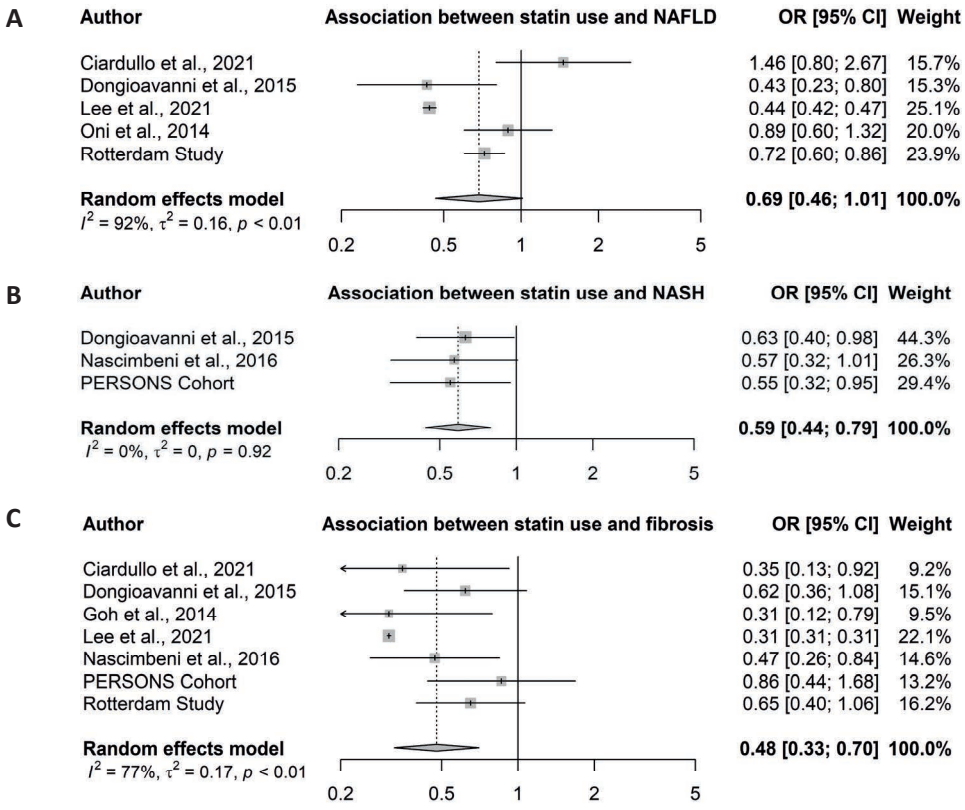


Figure 2. Forest plots showing the associations between statin use and the NAFLD disease spectrum. Random effects models were used to assess pooled odds ratio of the association between statin use and NAFLD (A), statin use and NASH (B), and statin use and fibrosis(C).

pooled odds ratios. Interestingly, in this excessive influence analysis, when excluding for example the studies of Ciardullo et al[255] or Oni et al[257], there was a significant inverse association for statin use with NAFLD (**supplementary Figure 1**).

To investigate whether statins have a direct effect on lipid accumulation, we tested simvastatin and lovastatin in our fatty liver model of primary human liver organoids mimicking steatosis.[253] We found that experimental treatment with simvastatin and lovastatin appears to attenuate the intracellular accumulation of lipid droplets. Although the effect on lipid droplet size was mild, we observed significant reduction of the average number of formed lipid droplets per viable cell with more prominent effects at higher concentrations of statins (**Supplementary figure 2**). Finally, we

tested the effects on inflammatory gene expression in human THP-1 macrophages since macrophage-driven inflammation is a driver of NAFLD disease progression. With a low concentration (0.1 µM) of simvastatin and lovastatin, significant inhibition on the expression of inflammatory genes such as CXCL10, IL-6, IL-12, and interferon-gamma was already observed (**Figure 3**).

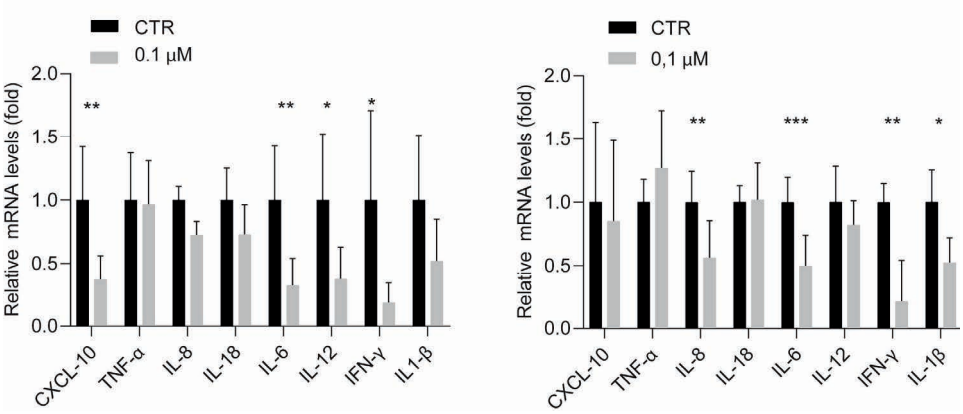


Figure 3. The effect of statin treatment on inflammatory gene expression in human macrophages. Human THP-1 monocytes were differentiated into macrophages and treatment with statin. Relative gene expression is represented as CTR (untreated) and as treated with simvastatin 0,1 µM (left panel, n = 8) and lovastatin 0.1 µM (right panel, n = 6). Data are presented as mean ± SD, *P < 0.05, **P < 0.01, ***P < 0.001

Discussion

In this multidimensional study, we comprehensively investigated the potential benefits of statin use on liver health within the NAFLD disease spectrum. Our pooled results demonstrated that among those with metabolic dysfunction, the use of statins was significantly associated with a lower prevalence of NASH and fibrosis and may also prevent NAFLD. These multifaceted beneficial effects are likely attributed to multiple mechanism-of-actions.

In the Rotterdam Study, a well-defined population-based cohort, we found a beneficial association between statin use and NAFLD prevalence compared to participants with untreated dyslipidemia. However, participants without dyslipidemia (and without statin use) were even at lower risk of NAFLD, indicating that statins could not entirely normalise the odds for NAFLD. Similar results were obtained when NAFLD was replaced by MAFLD, indicating that the beneficial effect

of statins can be extrapolated to the larger MAFLD population. Among those with NAFLD, statins may even be anti-fibrotic, however, this was only significant for a rather lenient definition of fibrosis using 7.0 kPa as threshold (which increased power). This anti-fibrotic property of statins has been shown in various experimental NASH models in which statins can inhibit the paracrine signalling between hepatocytes and hepatic stellate cells resulting in deactivation of these stellate cells, which in turn inactivates fibrogenesis.[261, 262]

In addition, among biopsy-proven NAFLD patients, we found that statins were inversely associated with NASH (OR 0.55). This adds to the evidence that statins may be hepatoprotective, in line with previous findings.[242, 255, 256] However, no benefit of statins on fibrosis was observed in this cohort. This finding was unexpected, given the beneficial association between statins and NASH which is a major predictor for fibrosis.[13] This may be explained by the relatively early stage of NAFLD disease, as supported by the younger population in the PERSONS cohort with better metabolic health, resulting in a lower prevalence of fibrosis than other biopsy-proven NAFLD patient cohorts.[263] Moreover, only 14 out of 73 NAFLD patients had fibrosis while using statins, therefore, this part of the study may be underpowered.

To account for different outcomes across available studies as well as ours and to increase statistical power, level of evidence and generalizability of results, we performed a meta-analysis. Statins seem to have a preventative effect on the presence of NAFLD, although this did not reach statistical significance (OR 0.69 95%CI 0.46-1.01), and further validation is warranted. Furthermore, the pooled odds ratios indicated a significant protective effect of statin use on the presence of NASH (OR 0.59) and fibrosis (OR 0.48). Interestingly, previous studies have shown larger effects on the development or progression of NAFLD for higher dosage and longer duration of statin use.[242, 256, 258] This dose-dependent effect in itself supports the efficacy of statins in NAFLD. Besides the evidence from cross-sectional studies, similar protective effects of statin use were observed in longitudinal data. For example, in a meta-analysis the risk for hepatocellular carcinoma and mortality was lower among biopsy-proven NAFLD patients using statins, further highlighting the potential benefits of statins in NAFLD disease management.[264]

In addition to the clear benefits of statins on cardiovascular outcomes, there is now emerging evidence indicating that statin use is hepatoprotective. Therefore, among individuals with NAFLD, statin treatment – which is safe and inexpensive – might be indicated regardless of dyslipidemia to reduce the risk of advanced liver disease. Currently 40-50% of patients with NAFLD do not receive statin treatment, while it is indicated.[265-267] Therefore, even prescribing statins according to recent guidelines[268] might reduce the disease burden of NAFLD, as physicians involved in the multidisciplinary treatment of NAFLD should be aware of the potential hepatoprotective effects of statins. However, additional research is required on whether statins are effective in preventing NAFLD among those without an indication.

To illuminate the protective findings on liver health and demonstrate causality, we explored statins' direct anti-lipid properties in 3D cultured human liver organoids. Interestingly, we found that the high statin-concentration of 10 µM could significantly inhibit the number of induced lipid droplets, while on the other hand, the average size of remaining lipid droplets increased. Although this seems counterintuitive, this indicates a stronger inhibitory effect of statins on smaller lipid droplets than on larger ones. Whether prolonged exposure to statin treatment also results in the inhibition of these larger lipid droplets needs to be investigated in further studies. Last, while most in vitro experiments have used statin in the concentration of 1-50 µM, the reported serum concentrations in humans are much lower.[269] Therefore, the experimental concentration of 10 µM may not reflect clinical practice. Nonetheless, a short experimental exposure to a high concentration of statins may be a proxy for chronic use of statins. This might explain that in several cohort studies the hepatoprotective effects were only relevant after six months of treatment[242] and were stronger with higher cumulative dosage.[258] This direct anti-steatosis effect might be explained by downregulating LDL, activating sterol regulatory element-binding proteins (SREBPs) and peroxisome proliferator-activated receptor alpha (PPARα) alongside increased β-oxidation, but further research is warranted to identify involved pathways.[270, 271]

Last, we investigated the possible anti-inflammatory properties in monocytes differentiated macrophages, since macrophages have a crucial role in NASH.[272] We demonstrated that the rather low concentration of 0,1 µM already downregulated the expression of inflammatory genes in macrophages such as CXCL10, IL-6, IL-8 IL-

12, IL-1beta and IFN-gamma. Other studies have shown as well that statins may exert anti-inflammatory effects by inhibiting RhoA/Rho-kinase, a small GTPase that induces oxidative stress.[273] Supporting our experimental findings, pooled data indicated that several inflammatory markers in serum of patients with metabolic syndrome were significantly decreased (e.g. CRP, IL-6 and IL-1).[274] Furthermore, mRNA expression of IL-6 is increased in animal models of NASH.[272] Interestingly, our study showed a decrease in expression of these inflammatory genes by statins. These anti-inflammatory properties might partially contribute to the hepatoprotective effect of statins.

The following limitations need to be mentioned. First, this study had a cross-sectional design and there was no data on the duration of statin therapy as well as treatment compliance, which limits the ability to investigate causality. Nonetheless, the additional experimental results show potential mechanisms that support causality. Second, our meta-analysis was based on only 3-7 studies, which did not allow for subgroup analysis and assessment of publication bias. Moreover, in the analysis concerning NAFLD and fibrosis there was substantial heterogeneity, particularly driven by a specific study[258], as this study used FLI and BARD score to define NAFLD and fibrosis. However, in excessive influence analysis, results were consistent after excluding this specific study indicating its limited impact on our conclusions. Third, the beneficial effect of statins may be partially explained by reverse epidemiology as there may be historical reluctance in prescribing statins to those with liver disease. However, under-prescription of preventive medicine is a general concern ranging from 22-70%,[275] similar to the reported 40-50% under-prescription of statins in NAFLD and NASH patients.[265-267] Therefore, the impact of this phenomenon on our results is limited. Although we have provided an experimental proof-of-concept regarding statins' possible mechanisms-of-action, further in depth investigation in additional experimental models are warranted.

In summary, pooled results demonstrated that statin use was associated with lower odds of NASH and fibrosis. Moreover, statin use might also prevent NAFLD in patients with metabolic dysfunction. In our experimental models, statins inhibited lipid synthesis and downregulated the expression of pro-inflammatory cytokines, which may partially explain the clinical benefits. This emerging evidence for the hepatoprotective properties of statins should be considered in the disease management of NAFLD.

SUPPLEMENTARY FILES

Supplementary table 1: exclusion criteria

	Rotterdam Study
Eligible for inclusion	**5.967**
NAFLD exclusion criteria	
Viral hepatitis	44
Excessive alcohol	780
Steatogenic drug use	98
Incomplete data	
Alcohol consumption	408
Dyslipidemia	54
Statin use	7
Total participants excluded	**1.391**
Participants included	**4.576**

Abbreviations: NAFLD, non-alcoholic fatty liver disease

Supplementary table 2A: database search and outcomes

	With duplicates	Without duplicates
Embase.com	1482	1124
Medline ALL Ovid	415	415
Web of Science SCI-EXPANDED & SSCI	406	180
Cochrane CENTRAL Register of Trials	126	47
Total	**2429**	**1766**

Supplementary table 2B: Exact search performed per database

Embase.com

('hydroxymethylglutaryl coenzyme A reductase inhibitor'/exp OR (((hydroxymethylglutaryl* OR hydroxyl-methyl-glutaryl* OR hmg) NEAR/3 (coenzyme-A OR coa) NEAR/3 (inhibitor*)) OR statin* OR atorvastatin* OR bervastatin* OR cerivastatin* OR compactin* OR crilvastatin* OR dalvastatin* OR fluindostatin* OR glenvastatin* OR lovastatin* OR pitavastatin* OR pravastatin* OR rosuvastatin* OR simvastatin* OR tenivastatin*):ab,ti) AND ('nonalcoholic fatty liver'/exp OR (((nonalcohol* OR non-alcohol*) NEAR/3 (fatty-liver* OR hepatosteatos* OR steatohepatit*)) OR ((nonalcohol* OR non-alcohol*) NEAR/3 (liver* OR hepat*) NEAR/3 (steatos*)) OR nafld OR nash):ab,ti) NOT [conference abstract]/lim AND [english]/lim NOT ([animals]/lim NOT [humans]/lim)

Medline ALL Ovid

(exp Hydroxymethylglutaryl-CoA Reductase Inhibitors / OR (((hydroxymethylglutaryl* OR hydroxyl-methyl-glutaryl* OR hmg) ADJ3 (coenzyme-A OR coa) ADJ3 (inhibitor*)) OR statin* OR atorvastatin* OR bervastatin* OR cerivastatin* OR compactin* OR crilvastatin* OR dalvastatin* OR fluindostatin* OR glenvastatin* OR lovastatin* OR pitavastatin* OR pravastatin* OR rosuvastatin* OR simvastatin* OR tenivastatin*).ab,ti.) AND (Non-alcoholic Fatty Liver Disease / OR (((nonalcohol* OR non-alcohol*) ADJ3 (fatty-liver* OR hepatosteatos* OR steatohepatit*)) OR ((nonalcohol* OR non-alcohol*) ADJ3 (liver* OR hepat*) ADJ3 (steatos*)) OR nafld OR nash).ab,ti.) AND english.la. NOT (exp animals/ NOT humans/)

Web of Science SCI-EXPANDED & SSCI

TS=(((((hydroxymethylglutaryl* OR hydroxyl-methyl-glutaryl* OR hmg) NEAR/2 (coenzyme-A OR coa) NEAR/2 (inhibitor*)) OR statin* OR atorvastatin* OR bervastatin* OR cerivastatin* OR compactin* OR crilvastatin* OR dalvastatin* OR fluindostatin* OR glenvastatin* OR lovastatin* OR pitavastatin* OR pravastatin* OR rosuvastatin* OR simvastatin* OR tenivastatin*)) AND ((((nonalcohol* OR non-alcohol*) NEAR/2 (fatty-liver* OR hepatosteatos* OR steatohepatit*)) OR ((nonalcohol* OR non-alcohol*) NEAR/2 (liver* OR hepat*) NEAR/2 (steatos*)) OR nafld OR nash))) AND DT=(article) AND LA=(english)

Cochrane CENTRAL Register of Trials

((((hydroxymethylglutaryl* OR hydroxyl-methyl-glutaryl* OR hmg) NEAR/3 (coenzyme-A OR coa) NEAR/3 (inhibitor*)) OR statin* OR atorvastatin* OR bervastatin* OR cerivastatin* OR compactin* OR crilvastatin* OR dalvastatin* OR fluindostatin* OR glenvastatin* OR lovastatin* OR pitavastatin* OR pravastatin* OR rosuvastatin* OR simvastatin* OR tenivastatin*):ab,ti) AND ((((nonalcohol* OR non-alcohol*) NEAR/3 (fatty-liver* OR hepatosteatos* OR steatohepatit*)) OR ((nonalcohol* OR non-alcohol*) NEAR/3 (liver* OR hepat*) NEAR/3 (steatos*)) OR nafld OR nash):ab,ti)

Supplementary table 3: Overview of included studies

Author	Study characteristics			Patients characteristics				Hepatic assessment		
	Population	Country	n	Age (years)	Male (%)	BMI (kg/m^2)	Diabetes (%)	NAFLD	NASH	Fibrosis
Goh et al., 2014	Diabetes	USA	220	52	31	37	100	Biopsy	Biopsy	Biopsy
Oni et al., 2014	Metabolic syndrome	Brazil	1.277	43*	79*	25*	N.E.	US	–	–
Dongiovanni et al., 2015	NASH + diabetes	Europe (multi-center)	1.059	42	52	34.2	27	Biopsy	Biopsy	Biopsy
Nascimbeni et al., 2016	Gastric bypass	Italy	346	53	40	42	74	Biopsy	Biopsy	Biopsy
Ciardullo et al., 2021	Diabetes	USA	744	61	52.6	33.3	100	CAP ≥274	–	LSM ≥9.7
Lee et al., 2021	Diabetes	Korea	60.918	41*	71*	24.4*	100	FLI ≥60	–	BARD ≥2.0
Rotterdam Study	Dyslipidemia or NAFLD**	Netherlands	2.408	71	45	28.5	24	US	–	LSM ≥8.0
PERSONS Cohort	NAFLD	China	569	42	72	26.6	25	Biopsy	Biopsy	Biopsy

Abbreviations: NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; BMI, body mass index (kg/m^2); NE, not extractable.

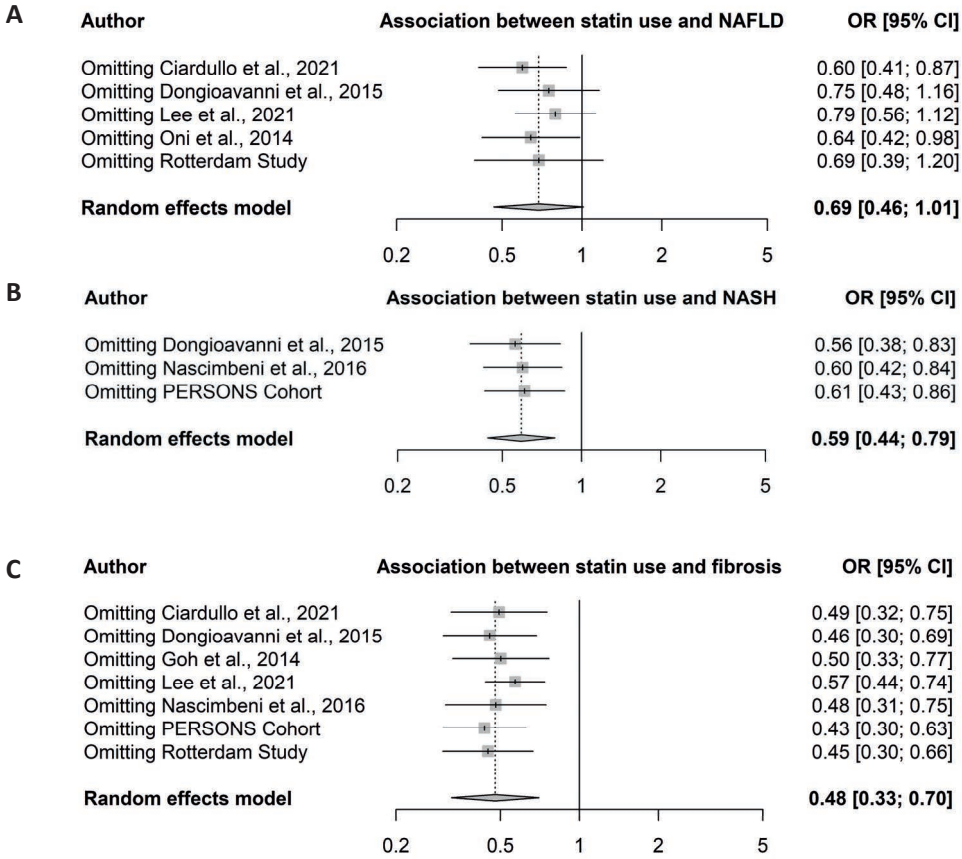
*based on whole study population rather than the included population with metabolic dysfunction in the meta-analysis

**Participants with dyslipidemia were assessed for the presence of NAFLD, and participants with NAFLD for the risk of fibrosis.

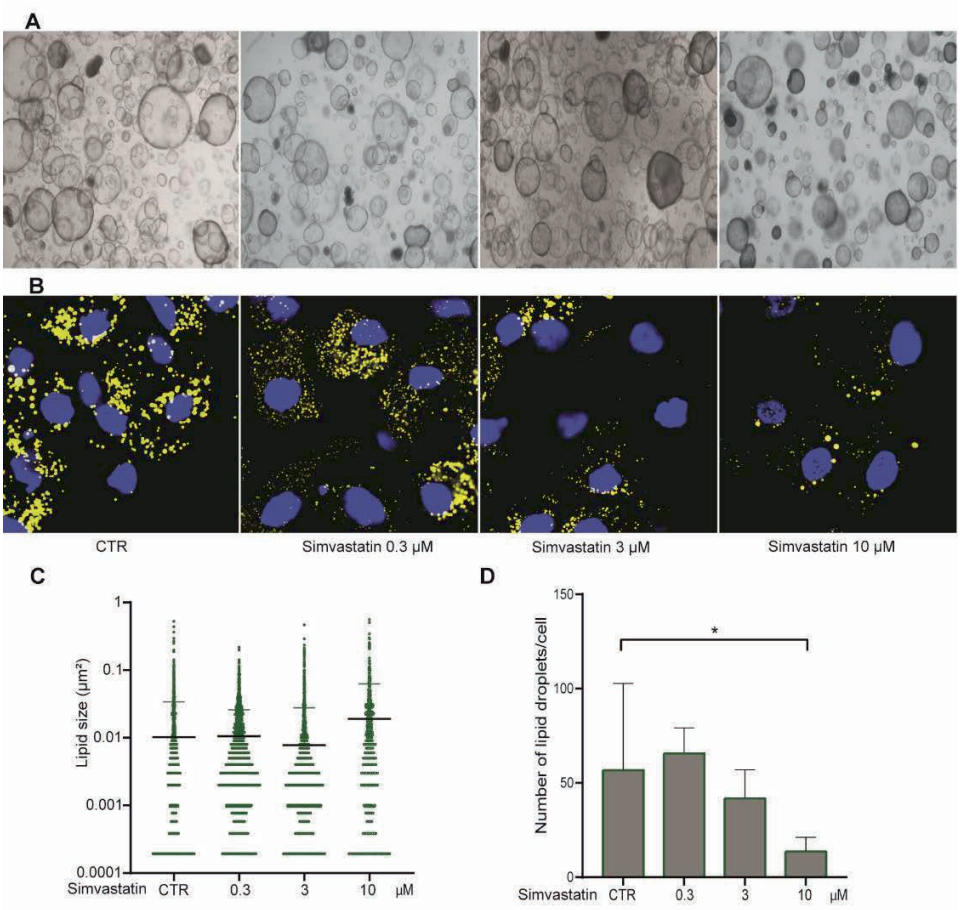
Supplementary Table 4: Quality assessment of studies included in meta-analysis

Authors	Year	Selection	Comparability	Outcome	Total
Ciardullo et al.[1]	2021	★★★★	★	★★★	8/9
Dongiovanni et al.[1]	2015	★★★★	★★	★★★	9/9
Goh et al.[1]	2014	★★★★	★	★	7/9
Lee et al.[2]	2021	★★★	★★	★★★	8/9
Nascimbeni et al.[1]	2016	★★★	★★	★★★	8/9
Oni et al.[1]	2014	★★★	★★	★★	7/9

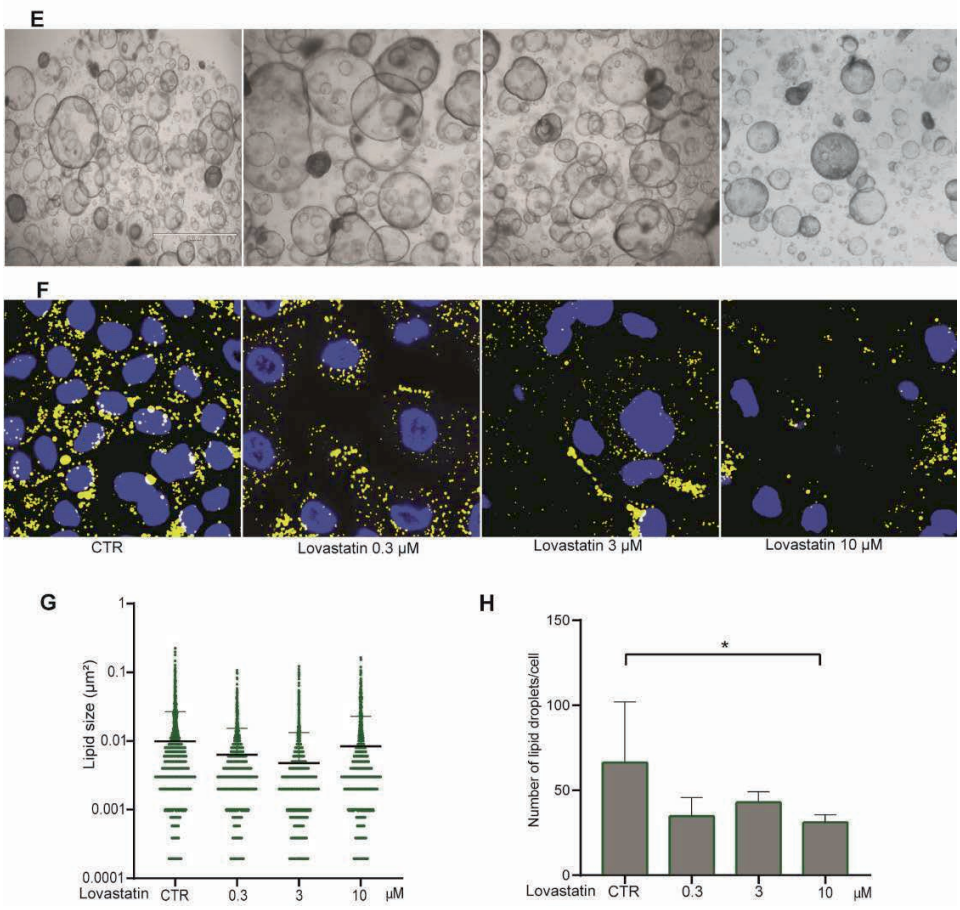
[1] The Newcastle-Ottawa quality assessment scale for cross-sectional cohort studies was used. [2] The Newcastle-Ottawa quality assessment scale for case-control studies was used.



Supplementary figure 1: Excessive influence analysis in which we omitted one study at a time for the associations between statin use and NAFLD (A), NASH (B) and fibrosis (C).



Supplementary figure 2 A-D. Effects of simvastatin on lipid accumulation in 3D cultured human liver organoids. (A) Bright field images representing morphology organoids treated with LPO only (CTR) or with LPO and different concentrations of simvastatin (20 x magnification). (B) Confocal images of lipid droplets (yellow, stained by AdipoRed) and nuclei (blue, stained by Hoechst) (2000x magnification at room temperature). (C) Effect of simvastatin on the size of the induced lipid droplets; 2565, 3301, 2902, 866 lipids were captured for CTR, 0.3 µM, 3 µM and 10 µM respectively. (D) Effect of simvastatin on the number of induced lipid droplets per nucleus (n = 4). Data are presented as mean ± SD, *P < 0.05.



Supplementary figure 2 E-F. Effects of lovastatin on lipid accumulation in 3D cultured human liver organoids. (E) Bright field images representing morphology of organoids treated with LPO only (CTR) or with LPO and different concentrations of lovastatin (20 x magnification). (F) Confocal images of lipid accumulation (yellow, stained by AdipoRed) and nuclei (blue, stained by Hoechst) (2000 x magnification at room temperature). (G) Effect of lovastatin on the size of induced lipid droplets; 1772, 1686, 2390, 1728 lipids were captured for CTR, 0.3 µM, 3 µM and 10 µM respectively. (H) Effect of lovastatin on the number of induced lipid droplets, normalised for the amount of cells (n = 4). Data are presented as mean ± SD, *P < 0.05.

Supplementary methods

Study selection

Articles were screened and included by I.A. if they met the following criteria: adult population with data available regarding the presence of NAFLD and usage of statins, conducted from cohort studies from selected patients or populations. Exclusion criteria were non-human studies, duplicates, non-original data or abstracts. Investigator I.A. screened titles and abstracts and subsequently full texts of potentially eligible articles found by the search strategy. The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flowchart was used to create an overview of the data screening process and the PRISMA NMA checklist (supplementary figure 1) as guidance for reporting on all the required aspects of a meta-analysis.

Data extraction and quality assessment

Data extraction was performed independently by two authors (I.A. and L.v.K). Discrepancies were resolved by mutual discussion among authors (I.A. and L.v.K). The Newcastle-Ottawa quality assessment scale for cross-sectional studies was used for quality assessment, which can be found in **supplementary table 4**.

Liver organoids culture

Organoids capture some of the key multicellular, anatomical and even functional hallmarks of real organs, thus have an advantage compared to classical cell lines. Studies have demonstrated that organoids can be used to model organ development and disease. Primary organoids are cultured from tissue stem cells in 3D structure, thus retaining characteristics of the tissue of origin. Therefore, human intrahepatic cholangiocyte organoids (ICO) were used for this model. Organoids were cultured in matrigel with Advanced DMEM/F12 (Life Technologies, cat.no.12634-010), adding 1 M HEPEs (Lonza, cat. no. 17-737E), ultraglutamine (Lonza, cat. no. BE17-605E/U1) and penstrep as the basic culture medium, supplied with 1:50 B27 supplement (minus vitamin A), 1:100 N2 supplement, 1 mM N-acetylcysteine, 10 mM nicotinamide, 50 ng/ml EGF, 100n g/ml FGF-10, 50 ng/ml HGF, 5 µM A83-01, 10 µM forskolin, 10 nM gastrin and 10% R-spondin1 (produced by 293T-H-RspoI-Fc cell line). [276] The organoids were cultured for approximately 1 week in which the medium was refreshed every 72 hours. When the appropriate size was reached, statin treatment was initiated for 96 hours. The use of human

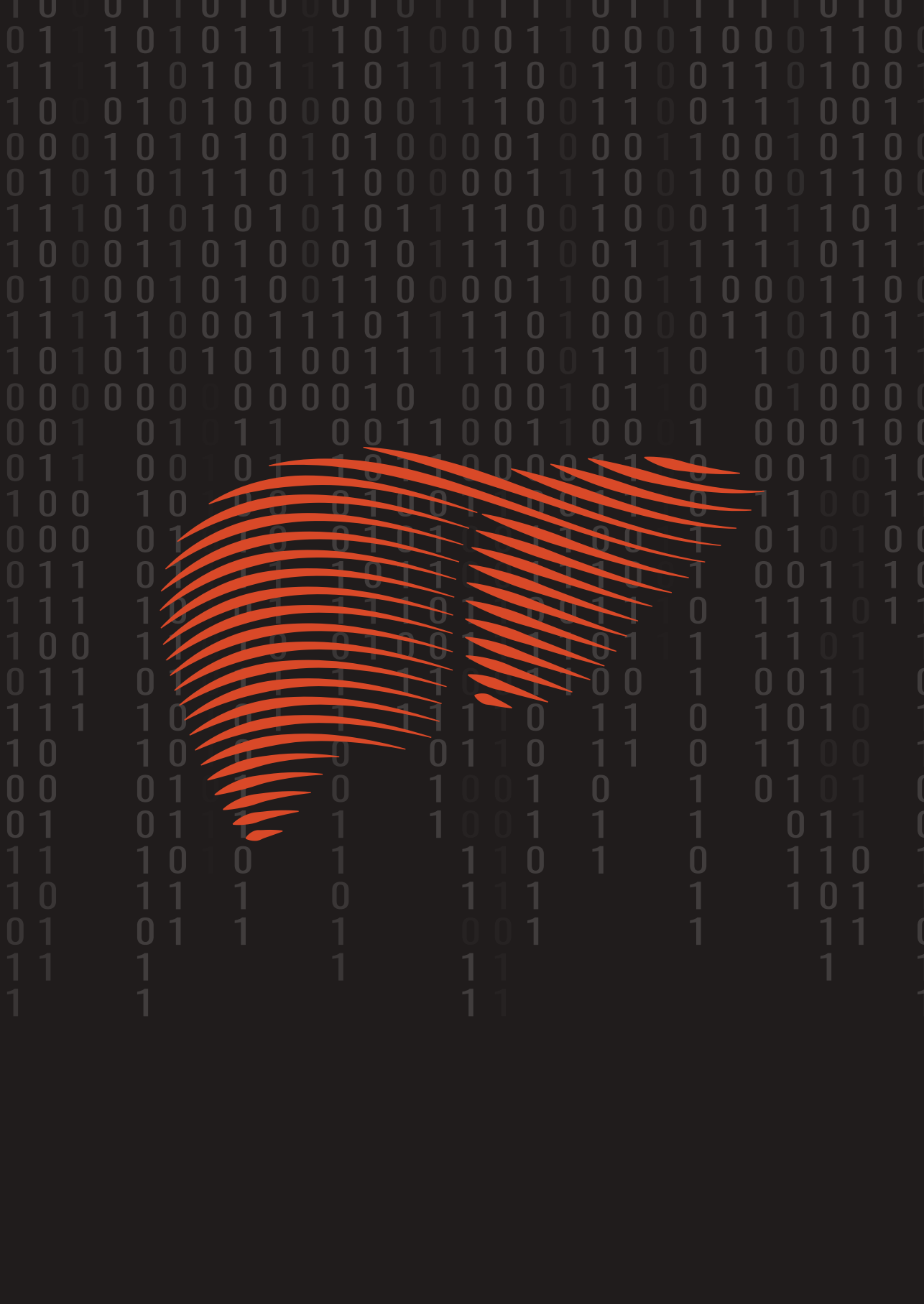
liver tissues for research purposes was approved by the Medical Ethical Council of Erasmus MC and informed consent was given (MEC-2014-060).

Staining of lipid droplets

Firstly induction of lipid synthesis was initiated by adding lactate, pyruvate and octanoic acid to the organoid expansion medium.[253, 277] Lactate and pyruvate are physiological derivates of both gluconeogenesis and de novo lipogenesis. Octanoic acid is a medium chain fatty acid which induces triglycerides accumulation. Lipids and nuclei were subsequently stained with AdipoRed (Lonza, cat.no.PT-7009) and Hoechst 33342 (Life Technologies, cat.no.H3570) after disrupting the matrigel construction, followed by spinning down in order to remove excess matrigel. The stained organoids were then incubated for 20 minutes in a dark incubator at 37 degrees Celsius. Next, we washed and spun down once with 1 X PBS, followed by adding the organoids, including an anti-fading medium on glass slides. Images were captured by confocal microscope Zeiss LSM510meta and Leica SP5, and quantified with ImageJ software. Analysis was performed by splitting the individual colour channel for lipids and threshold converting to 8-bit. The acquired images were measured with particles and their surface areas in pixels, then converted to square micrometers for lipids areas.[253]

Culturing of THP1-cells

Human monocytic cell line (THP-1) was cultured in RPMI 1640 medium (ThermoFisherScientific, Waltham, MA, USA), complemented with 10% (v/v) inactivated fetal bovine serum (FBS) with 100 IU/mL of penicillin and 100 mg/mL of streptomycin. For macrophage differentiation, THP-1 cells were treated with 20 ng/mL of phorbol 12-myristate 13-acetate (PMA) at 37°C for 48 hours. Then, cells were cultured for another 24 hours in RPMI 1640 medium without PMA. Treatment with statins was sustained for 24 hours followed by qRT-PCR. Gene expression of cytokines including CXCL10, TNF alpha, IL8, IL18, IL6, IL12, IFN gamma and IL1beta were quantified by qRT-PCR. GAPDH was used as a housekeeping gene for the normalisation of gene expression.



CHAPTER 5

Authors' replies

CHAPTER 5.1

Authors' response – Letter to the editor: focus on MAFLD, even if patients have a history of excessive alcohol consumption

Laurens A. van Kleef, Robert J. de Knegt, Willem Pieter Brouwer

Hepatology. 2022 Aug 25. Epub ahead of print

To the editor:

We appreciate the interest of Jiang et al. in our recently published manuscript that demonstrated MAFLD and excessive alcohol consumption were both independent predictors of all-cause mortality.[278, 279]

They suggested that MAFLD could be a mediator in the association between excessive alcohol consumption and all-cause mortality; therefore, the risk of alcohol consumption might be underestimated in our study. Although we like this suggestion of mediation, there is no evidence provided to strengthen this theory further. Moreover, we note that the mortality risk of excessive alcohol consumption did not increase (aHR remained 1.14) when MAFLD as a variable was left out of the analysis. Hence, we feel that it is unlikely that we underestimated the mortality risk of excessive alcohol consumption. As there was no (indirect) evidence of mediation and interaction in our study or other literature, we did not proceed with more differentiating methods to further quantify mediation and moderation simultaneously.[280]

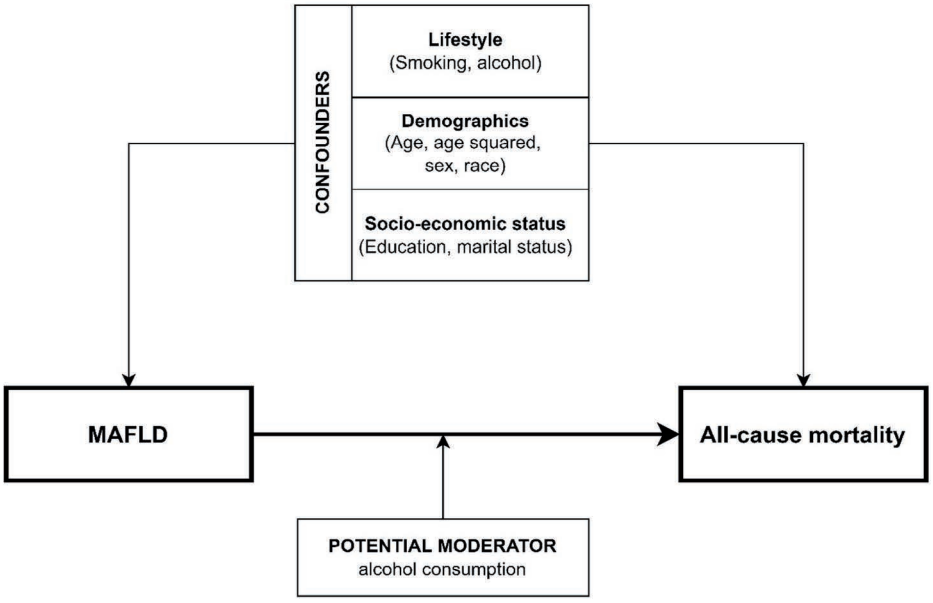


Figure 1: Directed acyclic graph of covariates with MAFLD as exposure and all-cause mortality as outcome.

Second, the authors were interested in our thought process of selecting variables included in our models. The directed acyclic graph (DAG) has now been provided in this letter, illustrating the exposures, confounders, potential moderators and outcome. Because of the limited number of events in the smallest group, we were restricted in the number of covariates that could be included to prevent potential overfitting. Variables were selected based on clinical relevance and previous studies in the NHANES cohort investigating mortality and fatty liver disease, and the contribution to goodness of fit was assessed by the Akaike information criterion (AIC).[119, 154]

Finally, multistate models were proposed to unravel further the risk of liver-related mortality and non-liver-related mortality for different states: "healthy", "excessive alcohol consumption", and "MAFLD". An important state lacking in this suggestion is the presence of both MAFLD and excessive alcohol consumption. However, unfortunately, liver-related death data is not publicly available in the NHANES cohort. Yet, based on previous studies,[154] only few liver-related deaths were recorded in this cohort. Therefore, it is unlikely that with this data, definitive answers can be provided on liver-related death. Hence, scientists should collaborate and unite cohorts to successfully perform multistate models and further unravel non-liver-related and liver-related mortality risk in individuals with excessive alcohol consumption, MAFLD or both.

CHAPTER 5.2

Authors' response – Reflection on the no-association between the presence of fatty liver disease and mortality in the elderly

Laurens A. van Kleef, Milan J. Sonneveld, Robert J. de Knegt

Hepatology. 2022 Jul 21. Epub ahead of print

To the editor:

We appreciate the interest of Ning et al. in our study demonstrating steatosis not being associated with excess mortality in community-dwelling elders.[281, 282] Their main concern is using 8.0 kPa as the cut-off for identifying individuals at high-risk of fibrosis. This cut-off was based on recommendations outlined in the 2021 EASL guideline on non-invasive tests.[66] However, we performed extensive additional analyses, including liver stiffness as a continuous variable and applying a higher cut-off (10.0 kPa), both of which yielded similar results.

Notably, liver stiffness values typically associated with cirrhosis (e.g. ≥15.0 kPa) are rare among community-dwelling elderly without heart failure. In fact, in our population, only 0.2% exceeded this threshold. While, at first glance, this might seem to contrast results from histology-proven NAFLD cohorts, it should be noted that such populations do not reflect the general population but represent a selection referred for further evaluation. This distinction is crucial for our conclusions, and results should not be generalised to elderly patients with steatosis and fibrosis, already under specialist care.

Another potential issue highlighted is the generalizability toward non-European populations. Our study suggests that steatosis in the last decades of life does not impair life expectancy, probably due to high competing mortality risk of non-liver-related origin. Given the global differences in (liver-related) comorbidities and life expectancy, careful external validation of our findings is warranted. It is likely that our cut-off for defining elders may need to be reassessed in other populations and may be subject to change because of the ongoing increase in life expectancy. Additional studies that include other ethnicities and additional age categories should further elucidate this important topic.

Furthermore, the authors mentioned potential bias by healthcare interventions after steatosis assessment. Besides no drugs being approved for steatosis, we note that participants in our population-based cohort were not referred to health care professionals in case of steatosis. Moreover, in the Netherlands (like many countries), there is a strict cardiovascular-risk management program comprising treating metabolic health factors typically associated with steatosis. Hence, lifestyle

recommendations and pharmaceutical interventions were independent of incidental findings during study visits.

Finally, steatosis not increasing mortality risk in the elderly aligns with the peak age of death due to chronic liver disease in Europe, which is the late-40s and early-50s. This contrasts smoking-related and other obesity-related mortality, which typically occurs decades later.[21] This indicates that elderly individuals with chronic liver disease are likely to be relatively healthy survivors, supporting our hypothesis that screening for fatty liver disease in an elderly community-dwelling population is unlikely to improve their outcomes.

CHAPTER 5.3

Authors' response – Letter to the editor: Fatty liver and mortality in the elderly

Laurens A. van Kleef, Milan J. Sonneveld, Robert J. de Knegt

Hepatology. 2022 Aug 11. Epub ahead of print

To the editor:

We thank Sun et al. for their interest in our manuscript, in which we showed that fatty liver disease (FLD) in an elderly population was not associated with increased mortality.[281, 283]

Sun et al. expressed several potential concerns.

The first concern is about the potential interplay between metabolic syndrome, cardiovascular disease and FLD. These factors are interrelated and adjusting for these factors may attenuate individual effect estimates. To overcome this issue, we performed extensive analyses, using both a simple (age-and-sex adjusted) and fully adjusted model, all of which yielded similar results.

Secondly, they cited a study reporting excess mortality in biopsy-proven NAFLD patients, and suggested that these findings conflict with our results.[44, 284] However, two critical differences in study design account for these contrasting findings. First, the Swede nationwide cohort's mean age was 22 years lower than in our cohort. Furthermore, our study was performed in the general population, whereas the quoted study selected only biopsy-confirmed NAFLD patients. The Swedish study, therefore, reflects a younger hospital-based population that required liver biopsy and cannot be compared to community-dwelling elderly.

The third concern is based on a study reporting different risks of liver-related death among men and women across age strata, suggesting that these differences may have influenced our findings.[285] First of all, it is important to note that we studied mortality risk among patients with FLD versus those without, whereas the referenced study reported absolute cause-specific mortality rates in NAFLD patients without a non-NAFLD comparator. Interestingly, Lin et al. reported that just <6% of deaths in elderly NAFLD patients were attributable to liver disease, and <1% among NAFLD patients without cirrhosis. Importantly, these estimates may even be inflated because patients were enrolled based on ICD-codes, thus reflecting a subset of patients who have already undergone liver health evaluation, unlike most NAFLD patients in the general population. Nevertheless, we performed additional stratified analyses for sex and age categories, which yielded consistent

results. This underscores that our findings are robust and, taken together with the study from Lin et al., highlight the importance of other causes of death amongst (elderly) patients with FLD.

Their final concern is the lack of liver histology in our cohort. Since population-based studies of community-dwelling subjects using liver biopsy are not feasible, non-invasive methods are the only option. Although misclassification of steatosis and fibrosis may occur through noninvasive methods, liver ultrasound has shown good specificity for hepatic steatosis and reflects clinical practice and guideline recommendations.[1]

In conclusion, we feel that our findings consistently show that among the community-dwelling elderly, ultrasound-based presence of FLD is not associated with excess mortality.

CHAPTER 5.4

Authors' response – Screening for fatty liver disease and fibrosis in the elderly population: a call for action

Laurens A. van Kleef, Milan J. Sonneveld, Robert J. de Knegt

Hepatology. 2022 Aug 11. Epub ahead of print

To the editor:

We appreciate the interest of Li et al. in our recently published manuscript on fatty liver disease (FLD) and mortality risk in the elderly.[281, 286]

In our study comprising 4.093 community-dwelling elderly, hepatic steatosis was not associated with excess mortality. Based on these findings, we concluded that widespread screening for FLD in elderly individuals (≥65 years) is unlikely to improve their outcomes. At first glance, this finding might seem counter-intuitive, as several previous studies have shown higher mortality risk among patients with FLD.[44, 284] In their letter, Li et al. refer to a recent study comprising a large cohort of patients with biopsy-proven NAFLD, which was associated with excess mortality, progressively increasing with worsening histology.[44] It is, however, important to note that the findings from this study cannot be applied to our study population. First, the patients enrolled in the study by Simon et al. were on average 22 years younger than those enrolled in our study. Furthermore, they studied patients who underwent liver biopsy, and their findings are therefore reflective of a hospital-based population of patients with an indication for liver biopsy. Most NAFLD patients, however, are not referred for hepatologist consultation and do not undergo histological assessment. This distinction is especially relevant for the community-dwelling elderly that we studied, as they have apparently remained free from liver-related complications despite the presence of FLD, and are unlikely to die of de novo liver-related complications during follow-up.

Another concern mentioned by Li et al. was a potential risk of type 2 statistical error due to a limited number of fibrosis cases and a relatively short follow-up of 6.9 years. While we concede that the number of patients with potential liver fibrosis (based on liver stiffness ≥8.0 kPa) was relatively limited, a follow-up duration of almost 7 years is considerable in this elderly population. However, our findings should preferably be further validated in more extensive programs like LiverScreen.[188]

A third concern mentioned by Li et al. is that the prevalence of diabetes might be lower in our cohort when compared to other studies.[287] However, the overall prevalence of diabetes in our overall cohort was 18.0%, with a prevalence of 27.8%

among patients with FLD, aligning with the diabetes prevalence in NAFLD patients reported by Ng et al.(25.6%).

Taken together, we feel that our findings are sufficiently robust to support our conclusion that FLD is not associated with excess mortality among community-dwelling elderly. These findings do not support widespread screening for fatty liver disease in this population.

CHAPTER 5.5

Authors' response – Liver stiffness, fatty liver disease and atrial fibrillation in the Rotterdam Study: some issues

Laurens A. van Kleef, Maryam Kavousi, Robert J. de Knegt

J Hepatol. 2022 Aug 18:S0168-8278(22)02996-8.

To the editor:

We thank Tsai et al. for their interest in our recent publication demonstrating that liver stiffness was associated with atrial fibrillation, whereas fatty liver disease was not.[177, 288] First, the authors mentioned in their letter the low sensitivity of a single 10-second ECG for the diagnosis of atrial fibrillation. We agree that the diagnostic accuracy of a 10-second ECG alone is generally poor. Therefore, in this study, we defined atrial fibrillation using a comprehensive approach comprising not only the 10-second 12-lead ECG at the study visit but also objective data obtained from other health care professionals by linking electronic medical records with our study database. To prevent arbitrariness in the diagnosis of atrial fibrillation, research physicians, supervised by an experienced cardiologist, confirmed each diagnosis by an independent reading of the ECG. Based on our study's prevalence of atrial fibrillation, this approach seems to detect most participants with atrial fibrillation.

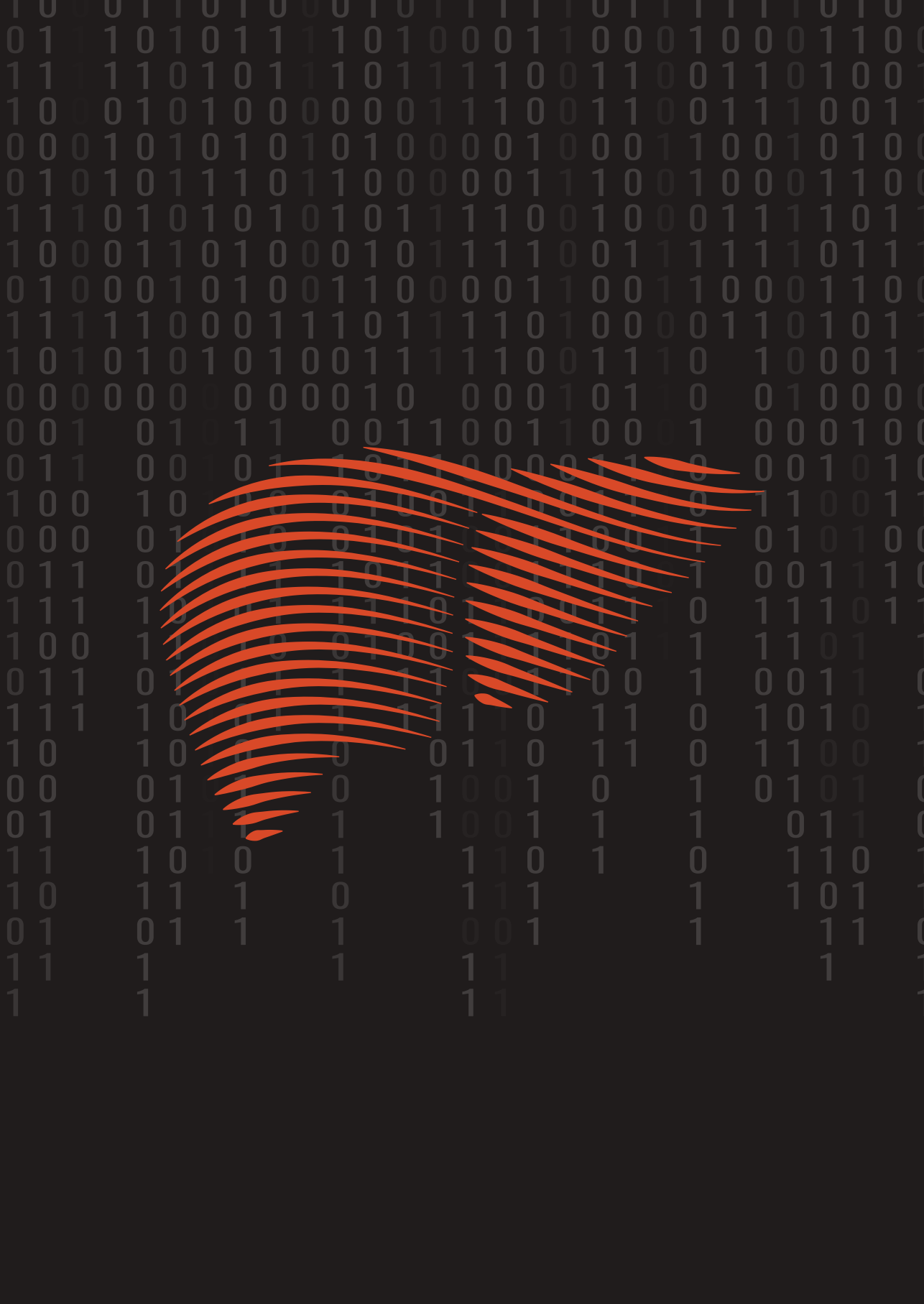
Notably, this comprehensive approach may explain the relatively high atrial fibrillation prevalence in this elderly population compared to the study by Feinberg et al., mentioned by the authors.[289] We would like to note that this study originates from 1995 while there is evidence that the prevalence of atrial fibrillation has increased in the last decades, either due to better sensitivity of diagnostic strategies or an increase in prevalence of risk factors for atrial fibrillation.[290] Moreover, recent studies have a larger sample size, and the prevalence seems more accurate for our data collection period. Indeed, a study among over 200.000 Swedes demonstrated that the prevalence of atrial fibrillation based on ICD-10 codes was 4.2% among those 60-69 years and 9.7% among those 70-79 years old. We would like to note that the population in Sweden is comparable to the Netherlands and that these findings perfectly align with our results.

Secondly, they mentioned the potential issue of not evaluating risk factors for atrial fibrillation in this study, such as obesity and BMI, as well as thyroid disorders, valvular disease and chronic obstructive pulmonary disease. Unfortunately, because of overfitting, we could not specifically investigate all potential covariates in the associations and we endorse future studies investigating the potential impact of these conditions on the association between atrial fibrillation and liver stiffness. Regarding BMI, it was a deliberate choice not to utilise BMI because of the

increasing concerns about the predictive ability of BMI in elderly populations. Sarcopenia, a condition common in the elderly, could result in favourable BMI levels while only muscle mass is lost and not fat mass whilst being a risk factor for NAFLD.[291] Waist circumference is a better proxy for metabolic health and is therefore used in this study.[292] Nonetheless, we repeated our primary analysis with additional adjustment for BMI and the results remained consistent. Moreover, we would like to note that waist circumference has a better predictive value for atrial fibrillation compared to BMI in men, whereas BMI was a slightly better predictor in women.

The final comment by the authors is about the controlled attenuation parameter (CAP). First of all, we note that our data originates from 2009 – 2014, when CAP was not yet available on commercial devices. However, validating these findings using CAP would be interesting, which may have better accuracy in detecting mild steatosis.[293] Yet, it seems highly unlikely that missing out on some individuals with only mild steatosis resulted in a complete distortion of associations. Last, the Fibroscan-AST (FAST) score was mentioned; this is a composite of liver stiffness, CAP and AST to predict the presence of NASH-fibrosis.[64] This score is likely to encounter the same issues as liver stiffness alone and could not discriminate between venous congestion and NASH-fibrosis. Because of the important role attributed to liver stiffness in this algorithm, validation of the diagnostic accuracy of the FAST score is required in populations at risk of venous congestion.

In conclusion, we believe that the potential issues mentioned by Tsai et al. have not had any impact on our conclusion that atrial fibrillation was associated with liver stiffness and not with fatty liver disease. However, additional studies are warranted to investigate the exact impact of the association between atrial fibrillation and fatty liver disease on current elastography-based risk stratification algorithms.



CHAPTER 6

General discussion

CHAPTER 6.1

Summary

SUMMARY

Chapter 2 of this thesis investigates the consequences of renaming and redefining NAFLD towards MAFLD. NAFLD is defined as steatosis in the absence of secondary causes such as alcohol and viral hepatitis. On the other hand, MAFLD is defined as steatosis in the presence of metabolic dysfunction.

In **Chapter 2.1**, we investigated the consequences of adapting MAFLD among 5.445 participants of The Rotterdam study. We found that MAFLD was more common than NAFLD (34.3% versus 29.5%) and MAFLD being able to identify 96.4% of the participants with NAFLD. Importantly, participants missed by the NAFLD criteria, but included by the MAFLD criteria (MAFLD-only 5.9%) had higher liver stiffness and more frequent fibrosis (OR 5.3) in fully adjusted models. This was in contrast to participants with NAFLD that did not comply with the MAFLD criteria (NAFLD-only 1.0%), in which no cases of fibrosis were identified and no association with liver stiffness was established.

As limited numbers hampered a direct comparison of NAFLD-only to MAFLD-only in this study, we compared the epidemiological and clinical features of NAFLD and MAFLD by meta-analysis in **Chapter 2.2**. We included 17 studies comprising 9.808.677 individuals and found that MAFLD was more common than NAFLD (33.0% vs 29.1%). Significantly more individuals were additionally identified by MAFLD (MAFLD-only) than were missed (NAFLD-only). Notably, this MAFLD-only group was at significantly increased risk for fibrosis (RR 4.2), had higher ALT (+ 8.0 U/L) and higher AST (+ 6.4 U/L) compared to NAFLD-only. This indicates that the novel MAFLD definition is superior to NAFLD on a population level and pledges to adapt the MAFLD criteria.

In **Chapter 2.3,** we investigated the clinical relevance of the novel MAFLD criteria among patients with chronic hepatitis B, who were previously excluded from NAFLD diagnosis. In this multicenter cohort study comprising 1.076 chronic hepatitis B patients with a median follow-up of 9.8 years, superimposed MAFLD was associated with reduced event-free (HR 2.00, 95%CI 1.26 – 3.19), HCC-free (HR 1.93, 95%CI 1.17 – 3.21) and transplant-free (HR 1.80, 95%CI 0.98 – 3.29) survival in fully adjusted models. Among patients without metabolic dysfunction, steatosis did not increase the risk of adverse outcomes. Our findings highlight the importance of metabolic dysfunction in patients with chronic hepatitis B.

Chapter 2.4 finally focuses on potential interactions between MAFLD and excessive alcohol consumption concerning adverse outcomes. Our findings in 12.656 participants (22.9 years follow-up, 3.804 deaths) from a prospective population-based cohort indicate that both MAFLD and excessive alcohol intake are independent risk factors for all-cause mortality without effect modification. Similarly, MAFLD was associated with equally increased mortality risk in individuals with and without excessive alcohol consumption. Results were consistent using the initial ten years of follow-up, a stringent definition of excessive alcohol, and propensity score weighting. These findings underscore the importance of MAFLD, even in patients with excessive alcohol consumption.

Chapter 3 focuses on considerations in early detection of liver disease. Guidelines recommend screening for liver disease among individuals with metabolic dysfunction, targeting predominantly elderly individuals. Therefore we studied in **Chapter 3.1** the association between fatty liver disease and mortality among the elderly. Our findings in 4.093 elderly participants (6.9 years follow-up, 793 deaths) from a prospective population-based cohort indicate that fatty liver disease was not associated with mortality (aHR 0.87, 95%CI 0.73 – 1.03). Findings were consistent across a range of clinically relevant subgroups, beyond five years of follow-up and for both cancer-related and cerebro-cardiovascular mortality. Furthermore, among participants with steatosis, higher liver stiffness was not associated with mortality. Therefore, screening for and follow-up of elderly patients with fatty liver disease is unlikely to improve outcomes and should not be advised.

Chapter 3.2 of this thesis investigated the association between liver stiffness and clinical outcomes in relation to the presence of heart failure. Among 4.266 participants of the Rotterdam Study, liver stiffness ≥ 8.0 kPa was associated with excess mortality (aHR 1.38, 95%CI 1.00 – 1.89). However, this association was entirely driven by participants with heart failure (aHR 2.69, 95%CI 1.28 – 5.64), whereas no association was observed between liver stiffness and mortality in subjects without heart failure (aHR 1.09, 95%CI 0.72 – 1.65). Furthermore, a range of cardiovascular characteristics and heart failure were associated with an increase in liver stiffness. These findings highlight important limitations of elastography-based screening for advanced liver disease in low-prevalence populations.

Within 5.825 participants of the Rotterdam Study, we demonstrated in **Chapter 3.3** that not fatty liver disease (OR 0.80, 95%CI 0.62 – 1.03) but liver stiffness (OR 1.09

per kPa, 95%CI 1.03 – 1.16) was associated with prevalent atrial fibrillation; however, this was only persistent among those without steatosis (OR 1.18 per kPa, 95%CI 1.08 – 1.29). Interestingly, both atrial fibrillation and liver stiffness were associated with subclinical signs of venous congestion, indicating that the association between atrial fibrillation and liver stiffness may be driven by venous congestion and not solely by fibrogenesis. Therefore, we recommend assessing cardiovascular health in participants with high liver stiffness, especially in the absence of overt liver disease.

In **Chapter 3.4**, we evaluated the newly developed screening strategy for fatty liver disease, as proposed by the EASL non-invasive test workgroup. Almost 50% of the 3.891 participants opt for FIB-4 assessment based on the presence of metabolic syndrome or excessive alcohol consumption. However, we demonstrated that applying FIB-4 in this population at risk for chronic liver disease had poor discriminative value (sensitivity 75%, specificity 42%, diagnostic odds ratio 2.1), with similarly poor performance among groups at even higher risk of fibrosis. Furthermore, despite the referral rate of 60%, 25% of participants with high liver stiffness were still missed. These findings highlight the need for alternative non-invasive tools for the identification of patients at the highest risk of significant liver fibrosis and/or the availability of liver stiffness assessment tools outside liver clinics.

Chapter 4 aims to investigate existing treatment and prevention targets. In **Chapter 4.1**, we focused on the impact of objectively measured physical activity on NAFLD among 667 participants of The Rotterdam Study. We found that physical activity was significantly associated with lower NAFLD prevalence. Importantly, all intensities of physical activity were beneficial. However, as expected, stronger associations were observed for higher intensities of physical activity. These beneficial associations were entirely mediated by better metabolic health, particularly waist circumference and HOMA-IR. Therefore, we recommend incorporating physical activity to its full extent in NAFLD disease management and prevention, even for those unable to reach vigorous activity.

In **Chapter 4.2,** we demonstrated that statin use in the general population (Rotterdam Study) was associated with a lower risk of NAFLD compared to individuals not using statins while indicated. Furthermore, statin use among individuals was associated with lower liver stiffness. Similarly, among biopsy-proven NAFLD patients (PERSONS cohort), statin use was associated with a lower

risk of NASH. Our meta-analysis confirmed these results and indicated lower risk of NAFLD (OR 0.69, 95%CI 0.46 – 1.01), NASH (OR 0.59, 95%CI 0.44 – 0.79) and fibrosis (OR 0.48, 95%CI 0.33 – 0.70). Finally, our experimental study showed that statins downregulated pro-inflammatory cytokines, which may explain the anti-NASH and anti-fibrotic properties. Now that there is emerging evidence for the hepatoprotective properties of statins, they should be considered in the disease management of NAFLD, especially in the presence of an existing indication.

CHAPTER 6.2

Discussion

GENERAL DISCUSSION

Fatty liver disease is the most common chronic liver disease, currently estimated at a global prevalence of 33%, with a steady increase of 0.7% per year.[3] The disease burden of fatty liver disease is expected to increase accordingly. Moreover, since metabolic dysfunction and fatty liver disease affect children and adolescents as well, an increasing number of patients are now being exposed to excess liver fat from a young age. Prolonged exposure to fatty liver disease may express an even higher risk of complications, particularly (decompensated) liver cirrhosis and hepatocellular carcinoma (HCC). This increasing disease burden is particularly worrisome because countries are poorly prepared for this public health challenge, with none of the 102 investigated countries scoring over 50% on the preparedness index.[294]

In this thesis, we investigated how to manage and potentially overcome the fatty liver disease pandemic. Moreover, we aimed to provide guidance for further research and policymakers. First, we investigated how redefining and renaming NAFLD could contribute to better identifying patients at risk for advanced liver disease. Secondly, we addressed potential concerns of (factors of) risk stratification algorithms to detect advanced liver disease to identify patients that may benefit from specialist care and pharmaceutical intervention when available. Third, we investigated known potential targets in disease prevention and management.

Redefining and renaming NAFLD: Where do we stand?

In late 2020 a group of international experts proposed metabolic dysfunction-associated fatty liver disease (MAFLD) as the replacement for non-alcoholic fatty liver disease (NAFLD).[29] This new name and definition no longer excluded secondary causes for steatosis but required the presence of metabolic dysfunction. This renaming and redefining should reduce stigma and acknowledges the close relationship with metabolic health.

Since the publication of the novel MAFLD criteria, several stakeholders soon published their views on the newly proposed MAFLD definition. For example, patients, nurses, and healthcare professionals favoured changing NAFLD into

MAFLD.[295-298] Even polls on Twitter were held, and almost two-thirds of the respondents were in favour of the name change.[299] However, it was not all a warm welcome for MAFLD, as several others pointed out that a name change is premature because the pathophysiology of fatty liver disease was (and currently is) not completely understood.[300, 301] Moreover, in contrast to what the term MAFLD implicates, a proportion of NAFLD patients are lean, and typically being metabolic relatively healthy, which will be missed with this definition.[302, 303]

After the new MAFLD definition had been proposed, numerous research groups, including our group from the Rotterdam Study, started investigating the consequences of the potential transition from NAFLD towards MAFLD.[85, 86, 88, 105] We, alongside others, revealed in **chapter 2.1** that regarding patient selection, only few NAFLD patients did not comply with the novel MAFLD criteria (NAFLD-only, due to absence of metabolic dysfunction). In contrast, a substantial number of extra patients were identified (MAFLD-only, due to concomitant secondary causes). Importantly, this additionally identified group typically had worse liver biochemistry and a higher risk of fibrosis, which could partially be explained by their secondary causes for steatosis such as alcohol consumption, viral hepatitis or steatogenic drug use.[97, 105, 106] To overcome distortion by these secondary causes on the overall outcomes we still excluded participants with viral hepatitis and exceptionally high alcohol intake (≥60 grams alcohol per day) for the main analysis of our study. Interestingly, this modified MAFLD-only group was still at increased risk for liver fibrosis, even after additional adjusting for alcohol consumption, highlighting the clinical relevance of this newly identified group.[105]

In order to further investigate whether MAFLD was associated with adverse outcomes in patients that were previously excluded, we investigated MAFLD among populations with chronic hepatitis B (CHB) and excessive alcohol consumption. We have shown in **chapter 2.3** that not steatosis, but MAFLD was associated with incident HCC and event-free survival in patients with CHB. Several research groups have confirmed these findings and showed that MAFLD was associated with HCC and excess mortality in CHB populations.[112, 304, 305] Similarly, we demonstrated **in chapter 2.4** the importance of MAFLD among individuals with excessive alcohol consumption on all-cause mortality. Notably, MAFLD and excessive alcohol consumption were both independent risk factors and resulted in cumulative increased risk of all-cause mortality.[278] Since viral hepatitis and

excessive alcohol consumption account for the vast majority of MAFLD-only patients, these findings support that MAFLD is a valid and clinically relevant diagnosis regardless of secondary causes for steatosis. [105, 106, 108]

In late 2021, already 17 studies were published that compared NAFLD to MAFLD, yet consensus was not reached.[85-88, 97, 105-116] Since meta-analyses increase the level of evidence, we pooled these individual studies on the consequences of transitioning from NAFLD towards MAFLD, to facilitate the ongoing debate in **chapter 2.2**.[306] We showed that of those with fatty liver disease, ±80% were captured both by the NAFLD and MAFLD definition, ±5% did not comply with the novel MAFLD definition, and ±15% was additionally identified with MAFLD (**Figure 1**).[307]

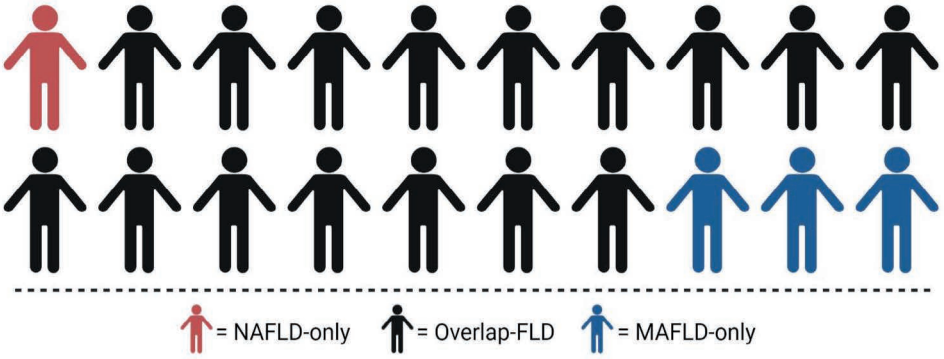


Figure 1: Distribution of NAFLD and MAFLD. Among those with fatty liver disease, generally, ±5% did not comply with the novel MAFLD criteria and ±15% had no NAFLD because they were excluded for secondary causes of steatosis.

This was the first time the non-overlapping groups, particularly the NAFLD-only group, were large enough for further comparison. Comparing these non-overlapping groups is essential because most of the discussion focuses on the unique groups and obviously not on the patients identified by both definitions. We confirmed in this meta-analysis that MAFLD-only was at a significantly higher risk for advanced liver disease than their counterpart NAFLD-only, supporting the transition to MAFLD on a population level. Moreover, NAFLD-only was also at lower risk for fibrosis and had better liver biochemistry when compared to overlap-FLD. These findings indicate that it is safe to miss out on individuals with NAFLD-only. This claim is further supported by longitudinal studies indicating that individuals

with NAFLD-only were not at increased risk of mortality,[119, 154] similar to our findings in a CHB population.[127]

At the same time, another meta-analysis by Lim et al. regarding NAFLD and MAFLD focused on the characteristics of these patients.[308] They demonstrated that MAFLD patients were of similar age, more often male and had more metabolic dysfunction, in line with the selection criteria of the MAFLD definition. However, we expressed our concerns with their interpretation of the non-overlap between NAFLD and MAFLD.[309] They entirely attributed the non-overlap as MAFLD failing to identify NAFLD, whilst we have shown that it works both ways. In fact, NAFLD fails to capture individuals with fatty liver disease at least three times as often as MAFLD (**Figure 1**).[105, 307]

Currently, the MAFLD definition is endorsed by a range of stakeholders supported by a paramount of data.[310] Despite MAFLD being endorsed by several associations of the study for the liver,[25, 311-315] the EASL and AASLD have not yet taken position because MAFLD might not be the perfect term either. During the NAFLD renaming consensus meeting in Chicago, in which we participated, it became clear that there is a need for an overarching term that could include all patients with steatosis. Moreover, from a patients' perspective, including the term fatty could still be stigmatizing. Therefore, several other suggestions have been made, such as steatotic liver disease and lipid-mediated liver disease. Within these overarching terms, metabolic dysfunction (as defined in MAFLD) could be one of the subtypes. Other – not per se mutually exclusive – subtypes could be alcohol-related, drug-induced and cryptogenic. An important benefit of this classification system is that the old NAFLD population can easily be identified, and knowledge on biomarker performance and drug utility could still be applied while, in the meantime, future studies could validate it for new subgroups. Consensus is yet to come and may take several more years. However, important steps have been made in acknowledging that alcohol consumption is a relevant additional disease in “NAFLD”.

Recommendations and future perspectives

There is paramount evidence for MAFLD being superior on a population level compared to NAFLD. However, consensus is crucial and as long as the EASL and AASLD have not taken position, future general population studies should not only use NAFLD or MAFLD but verify the outcomes in the other group. Since MAFLD is

more homogenous and more prevalent, statistical power is generally superior, which potentially gets the entire scientific field to the next level. However, in case of inconsistent results in NAFLD and MAFLD, one should be aware of the non-overlapping groups NAFLD-only and MAFLD-only, which may explain the differences. Particularly when assessing hepatic outcomes, instead of extrahepatic comorbidity, one might still prefer excluding viral hepatitis and alcoholic liver disease in sensitivity analysis to avoid the excessive influence of these individuals on the overall outcomes.

In the end, I believe categorising fatty liver disease based on the presence of metabolic dysfunction (either using MAFLD or a subgroup within an overarching term) better describes the disease and directs health care professionals to potential treatment targets. Finally, using MAFLD as an umbrella term (or a broader term), several clinically relevant subgroups, such as MAFLD with excessive alcohol consumption, diabetes and/or obesity, should be identified and further studied.

Considerations in early detection of liver disease: is screening justified?

Early detection of advanced liver disease in patients with NAFLD or metabolic dysfunction, regardless of the established diagnosis of liver disease, is widely advocated and supported by several guidelines.[1, 21, 66, 167, 168] Of the recent guidelines on NAFLD, only the AASLD NAFLD guidance paper does not recommend early detection in patients with metabolic dysfunction since no pharmacological treatment is available.[24]

To prevent a sprawl of screening programs and ensure effectiveness, strict criteria for population-based screening have been developed. They all need to be met before screening can be implemented and is beneficial for the population. This set of criteria provided by Wilson and Jungner is depicted in **Table 1**.[182] In light of potential population-wide screening for advanced liver disease in the general population, our findings described in **chapter 3** will be discussed in light of two criteria: (1) The condition sought should be an important health problem and (2) There should be a suitable test.

Table 1: Wilson and Jungner Criteria[182]

The condition sought should be an important health problem
The natural history of the condition, including development from latent to declared disease, should be adequately understood
There should be a recognizable latent or early symptomatic stage
There should be a suitable test or examination
The test should be acceptable to the population
There should be an agreed policy on whom to treat as patients
There should be an accepted treatment for patients with recognised disease
Facilities for diagnosis and treatment should be available
The cost of case-finding should be economically balanced in relation to possible expenditure on medical care as a whole
Case-finding should be a continuing process and not a "once and for all" project

"The condition sought should be an important health problem"

Although fatty liver disease is highly prevalent and associated with hepatic and extra-hepatic comorbidity and mortality,[44] we provided evidence in **chapter 3.1** that fatty liver disease is unlikely to be an important health problem in the elderly.[281] In fact, we have shown that among individuals aged ≥ 65 years, fatty liver disease was not associated with impaired survival. Interestingly, in individuals with metabolic dysfunction, the presence of fatty liver disease was even associated with improved survival. This phenomenon is in line with the so-called obesity paradox, an example of reverse causation.[181] Given the rather provocative conclusions, this study received several letters.[282, 283, 286] One of them questioned the consequences for elderly patients already under specialist care with advanced fibrosis. Indeed, we would like to note that our findings in the general population cannot be extrapolated to these patients as they are likely to be at increased risk of liver and non-liver-related mortality. Moreover, findings obtained in NAFLD patients that underwent biopsy cannot be extrapolated to the general population, as these patients reflect a highly selected subset of individuals that had an indication for referral and subsequent biopsy. Importantly, in **chapter 3.2** we also demonstrated that the increased mortality risk in individuals with high liver stiffness was entirely attributed to heart failure and not to liver disease. In fact, high liver stiffness in the absence of heart failure was not associated with mortality.

Recommendations and future perspectives

Although the presence of fatty liver disease and high liver stiffness is an important condition in younger populations,[2, 119] it seems unreasonable to screen for fatty liver disease in the elderly based on our findings. However, additional studies are warranted to further investigate from when on fatty liver disease has no consequences for mortality and should not be screened for, especially considering increasing life expectancy. Moreover, the benefit of monitoring elderly individuals with fatty liver disease and otherwise well-treated concomitant metabolic dysfunction can be debated and therefore warrants further investigation. Finally, the cost-effectiveness of screening and monitoring should be evaluated for several subgroups and be taken into account for future guidelines.

"There should be a suitable test"

In the last decades, plenty of non-invasive tests such as FIB-4 index, ELF test and elastography have become available to assess the presence of fatty liver disease and fibrosis. However, despite the numerous non-invasive tests available, it remained challenging to identify individuals with advanced liver disease due to limited accuracy and low prevalence of advanced liver disease.[53]

In 2021, the EASL updated their guideline on the use of non-invasive tests and a major role was allocated to the FIB-4 index in their referral strategy.[66] Among individuals with metabolic dysfunction, who require diagnostic work-up according to this new guideline, we showed in **chapter 3.4** that the FIB-4-based risk stratification algorithm had poor performance.[170] Almost 60% of elderly participants required referral to a hepatologist for additional transient elastography or liver biopsy, while the algorithm still missed out on 25% of cases with elevated liver stiffness. In a multi-cohort study, the FIB-4 applied in a non-selected population had equally poor performance.[171] Interestingly, they suggested that waist circumference had better diagnostic accuracy than the FIB-4.

Even though transient elastography has the best diagnostic accuracy and has an outstanding high negative predictive value, there are some important concerns with this technique. Especially in populations without liver disease, high liver stiffness is unlikely to reflect fibrosis. We have shown in **chapter 3.3** that atrial fibrillation and (signs of) heart failure was associated with increased liver stiffness, which could be due to liver injury, but is more likely to be venous congestion.[177]

Recommendations and future perspectives

As of yet, it seems premature to use transient elastography as a screening tool for advanced fatty liver disease, especially in the elderly in whom cardiovascular disease and subclinical venous congestion can distort the interpretation of high liver stiffness. This phenomenon should be taken into account, and the simultaneous assessment of steatosis (by controlled attenuation parameter [CAP]) may direct health care providers on whether the high liver stiffness is caused by fatty liver disease or cardiovascular disease, although other liver disease may need to be ruled out first. Additional studies are warranted to investigate under what conditions and in which subgroup liver stiffness measurements can be used as a screening tool for advanced liver disease. In case differentiating between venous congestion and liver fibrosis cannot be done conveniently in these screening programs, it would be interesting to see whether it is feasible to screen simultaneously for advanced liver disease and (subclinical) venous congestion.

Fatty liver disease management: consequences for future guidelines

Prevention of fatty liver disease and inhibiting disease progression are essential to managing the fatty liver disease pandemic, especially since no pharmaceutical options have been approved yet. Given the coherence between metabolic dysfunction and fatty liver disease, management of fatty liver disease focus predominantly on diet, physical activity and proper treatment of metabolic comorbidities. The recommendations of the EASL, AASLD and APASL guidelines on fatty liver disease have been summarised in **Table 2**.[1, 24, 25] The APASL is the first to have a dedicated guideline on MAFLD. Remarkably, the EASL and AASLD have not updated their guideline since 2016 and 2018, respectively, while since their release over 15.000 new articles have been published on fatty liver disease.

Diet

An extensive discussion of the role of diet in fatty liver disease is beyond the scope of this thesis. Nonetheless, new findings will briefly be touched upon because dietary recommendations are one of the pillars of fatty liver disease management.

Table 2. Overview of international guidelines on the management of fatty liver disease

	EASL 2016	AASLD 2018	APASL 2020
Lifestyle	Combining dietary improvements with exercise is more effective than either modality alone		
Diet	500-1000 kcal deficit per day aimed at weight loss of up to 1 kg per week		
Weight loss	• 7-10% in overweight patients	• 7-10% in NASH and fibrosis • 3-5% in simple steatosis	• 7-10% in overweight patients • 3-5% in lean patients
Diet plan	• Hypocaloric • Mediterranean diet	• Hypocaloric • No specific dietary plan	• Hypocaloric • Mediterranean diet
Fructose	Avoid processed food and items high in fructose	No statement	Avoid processed food and items high in fructose
Alcohol	Avoid heavy alcohol consumption		Avoid any alcohol consumption
Physical activity	≥ 150 minutes of moderate-intensity training per week		
Resistance training	Resistance training is effective and the choice of training should be tailored to patients' preferences	No statement	Resistance training is effective and might be more feasible in patients with poor condition
Pharmaceutical intervention	Restrict pharmaceutical treatment to patients with steatohepatitis or fibrosis		
Pioglitazone	Pioglitazone may be used in patients with biopsy-proven NASH on an individual basis after discussion of risks and benefits		No statement
Statin	Statins can be used safely to prevent cardiovascular disease Insufficient evidence on preventative effect of statins on NAFLD		
GLP-1	No statement	Insufficient evidence	No statement

A recent publication from the Rotterdam Study by Alferink et al. showed that adherence to a plant-based, high-fibre dietary pattern was associated with regression of fatty liver disease in elderly participants.[316] This was one of the first times dietary patterns were investigated longitudinally with proper adjusting for important confounders such as total caloric intake and BMI. Similarly, in the Framingham Heart Study, the Mediterranean Diet Score was positively associated with less liver fat accumulation and reduced risk of incident fatty liver disease.[317] These results warrant confirmation in younger populations but form solid evidence for recommending a (predominantly) plant-based diet with high fibre intake, which seem to align with the Mediterranean diet.

Pooling cross-sectional data did not result in new insights. A meta-analysis published in 2021 that pooled 60 studies could not identify any specific macro-nutrient that could affect fatty liver disease.[318] However, individuals with NAFLD had increased total caloric intake. Although no causality could be demonstrated in this study, this supports the current recommendations for a hypocaloric diet.

In the end, each diet that effectively reduces weight should be able to improve fatty liver disease because 5-10% weight loss is a reliable indicator for disease regression.[74, 75, 319] Hence, summarizing the evidence for specific diets, it has been concluded that not only the Mediterranean diet but also intermittent fasting, ketogenic diet and the dietary approach to stop hypertension (DASH) diet seem to be effective, as long as weight loss is achieved.[320]

Recommendations and future perspectives

Based on this information, future guidelines should continue recommending a hypocaloric diet aiming at substantial weight loss. A plant-based and high-fibre diet is effective for steatosis regression, but long-term adherence to caloric restrictions and a diverse diet may be more important. Personalised diets (based on genetics, microbiota and environmental factors), may even be more effective since they had already favourable outcomes compared to conventional diets in diabetes and obesity care, but warrants investigation in individuals with steatosis.[321] Dietary counselling should be readily available for overweight individuals to prevent comorbidities, including liver disease. This counselling might be best included in combined lifestyle intervention programs, where expertise is concentrated and the intervention is intensified.

Physical activity

The other pillar of disease management is physical activity. Guidelines agree upon the synergistic effect of simultaneous targeting dietary patterns and physical exercise. Although they recommend exercising at least 150 minutes of moderate intensity each week, this duration and intensity of activity are based on general recommendations from the WHO and not on NAFLD-specific research and warrant further investigation.

Within the Rotterdam Study, we have shown that not only moderate or vigorous physical activity was beneficial but also light physical activity in reducing the odds of NAFLD as described in **chapter 4.1**. However, the benefit increased with higher intensity of physical activity.[322] Nonetheless, light activities, for example, walking, shopping, and household activities, were associated with lower odds of NAFLD. Thus, not being able to reach moderate or vigorous activity is no argument to abstain from any activity. In fact, light physical activity should even be recommended for individuals with poor physical condition, unable to reach higher activity intensity. The benefit of light activity is beyond the lower risk of NAFLD. For example, another study showed that replacing 30 minutes of sitting time with light physical activity in inactive individuals was associated with a 14% mortality risk reduction.[323] Moreover, objectively measured physical activity in patients with NAFLD (based on hepatic steatosis index) resulted in improved survival.[235]

In the same year, Tsunoda et al. also reported on accelerometry-based physical activity and fatty liver disease.[324] In contrast, in their cohort of almost 2.000 middle-aged Japanese, they could not demonstrate a beneficial association between NAFLD and light physical activity, but only for moderate-to-vigorous exercise. We note that the cohorts differ on two key characteristics: our cohort was almost 20 years older (70 vs 51 years), and there were apparent differences in BMI (27 kg/m^2 vs 23 kg/m^2). One can imagine that activities categorised as light in elderly overweight individuals are more energy-consuming than in the lean middle-aged populations. Unfortunately, both studies lacked correlation with heart rate during exercise, which could provide support for this hypothesis.

Furthermore, we aimed to identify an optimum physical activity duration using natural cubic splines. Within our data, we could not demonstrate non-linear associations for the association between NAFLD and physical activity, indicating consistent increasing benefits for each added time-unit of activity. However, we note that even the most active elderly participants were relatively inactive and a potential plateau phase of the maximum benefit of physical activity in our study was therefore unlikely to be reached. Interestingly, the aforementioned Japanese study showed that the benefit of moderate to vigorous physical activity plateaued at 1800 (metabolic equivalent of task) MET-min per week.[324] Since moderate activity is defined as 4-6 MET per minute, this would indicate that moderate activity is no longer beneficial after 300-450 minutes of moderate activity per week and sooner when individuals have additional vigorous activity.

Studies prior to ours have been comprehensively summarised by Machado et al. in their review, and although different interventions were performed, most of them showed beneficial effects of physical activity on fatty liver disease.[325] They conclude that exercise should be proposed to all patients with fatty liver disease, and hepatology clinics should implement a multidisciplinary team that would assist patients in planning an individualised exercise program according to their age and health status.

Recommendations and future perspectives

In light of the accumulated evidence on physical activity and fatty liver disease, we recommend an individualised approach that matches the patient's capabilities. Given the extensive research that is performed on types of exercise which yielded inconsistent results,[326, 327] it is unlikely that a specific type of exercise is exceptionally effective. Therefore, it seems more important that individuals maintain their increased level of physical activity than recommending a specific type or intensity that does not match the condition or preferences of the individual. Future studies should investigate how physical activity can be incorporated into daily routine successfully. The chance of performing physical activity beyond the duration where it is no longer increasingly effective seems to be low, however, this 'plateau-phase' need to be further investigated. Finally, fatty liver disease management cannot build upon one pillar, and dietary improvements are required alongside an increase in physical activity.

Pharmaceutical Intervention

This thesis cannot ignore the important progression in seeking a cure for fatty liver disease. Several drugs have been shown to be efficient in reducing steatohepatitis activity without fibrosis progression in phase IIb trials, the accepted endpoint in clinical trials.[328, 329] These promising novel treatments (**table 3**) yet await further validation in Phase III trials and final approval in both Europe and America.

Table 3: Overview of ongoing or scheduled phase III trials

Agent	Target
Obetecholic acid	FXR agonist
Semaglutide	GLP-1 receptor agonist
Resmetirom	THR-β agonist
Aramchol	SCD1 inhibitor
Lanifibranor	Pan-PPAR agonist
Elafibranor	PPAR-α/δ agonist
Saroglitazar	PPAR-α/γ Agonist
Belapectin	Galectin-3 inhibitor
Dapagliflozin	SGLT2 inhibitor
Oltipraz	AMPK activator
Pentoxifylline	methylxanthine derivative
Cotadutide	Dual GLP-1 and glucagon receptor agonist

Abbreviations: AMPK adenosine monophosphate-activated protein kinase; FXR, farnesoid X receptor, GLP-1 glucagon-like peptide-1; PPAR, peroxisome proliferative activated receptor; SCD1, stearoyl CoA desaturase; SGLT2, Sodium-Glucose Cotransporter-2; THR-β, thyroid hormone receptor beta.

The European Medicines Agency recently accepted Wegovy (semaglutide), a glucagon-like peptide (GLP)-1 agonist, as a treatment to obtain weight reduction in obese or overweight patients with weight-related health issues, regardless of fatty liver disease.[330] Previously, another GLP-1 agonist, liraglutide, has also been accepted in treating overweight. Interestingly, GLP-1 agonists have been shown to improve diabetes but also NAFLD and NASH effectively among patients with type II diabetes.[72] However, among patients without diabetes, reimbursement by insurance companies in the Netherlands is only granted in case a “combined

lifestyle intervention" failed while having a BMI ≥35. Nonetheless, it is fascinating to see whether treatment of GLP-1 agonists in non-diabetics with substantial overweight yields similar benefits for NAFLD in a real-world setting. However, given the strict criteria, the current use of GLP-1 agonists in patients without diabetes is low.

Although promising developments regarding pharmaceutical solutions for fatty liver disease are highly relevant and exciting, most patients with fatty liver disease will not qualify for treatment. Because, although not based on actually available treatment, current guidelines recommend only considering future treatment in patients with NASH or fibrosis.[1, 24, 25] However, even with these selection criteria, only up to 20% of NAFLD patients will be eligible for treatment. A recent economic evaluation of future pharmaceutical agents for fatty liver disease indicated that it was unlikely to be cost-effective in case of treatment costs over $12.000 a year.[331] The current novel drugs are increasingly pricy, with average developing costs of 1.3 billion dollars.[332] Hence, it is a challenge for pharmaceutical industries to price their newly developed agent within the range of cost-effectiveness.

Given the coherence between metabolic dysfunction and fatty liver disease, conventional agents designed to improve metabolic health might also be beneficial in disease management. Therefore, we investigated the potential benefits of statins across the fatty liver disease spectrum. Statins are one of the cheapest drugs available, with a year of treatment costing only €7.40 for the actual medication.[333] Although guidelines indicate that statins are safe in patients with NAFLD and effective in preventing cardiovascular events, there is insufficient evidence to recommend them beyond their original indication.[1, 24, 25]

We demonstrated in **chapter 4.2** the potential of statins to prevent fatty liver disease and fibrosis in the Rotterdam Study. Moreover, statins also reduced NASH odds in biopsy-proven NAFLD patients, whereas statins were not associated with fibrosis in this population. In order to overcome these contrasting results, we performed a meta-analysis. We demonstrated that statin use among individuals with metabolic dysfunction might reduce the odds of NAFLD (-30%), but further studies are warranted as this was not significant. The potential benefit of statins became more explicit in patients that already had fatty liver disease, as pooling all available data indicated a 40% lower odds of NASH and a 50% lower odds of fibrosis.

Our experimental studies in **chapter 4.2** strengthened these findings by demonstrating the anti-inflammatory traits of statins in human liver organoids.

In investigating the potential benefits of statins in population studies, one should consider that individuals using statins are likely to have more metabolic dysfunction. Hence this group should be compared to individuals with metabolic health. Indeed, in the Rotterdam Study, we demonstrated that statin users were at a lower risk of fatty liver disease than those with untreated dyslipidemia, but those without dyslipidemia (and no statins) were at the lowest risk of fatty liver disease. This is a strong argument for prescribing statins to individuals with an indication.

Recommendations and future perspectives
Adequate prescription of statins may limit the disease burden of fatty liver disease. These claims are supported by our study and another meta-analysis demonstrating statin use was associated with lower liver enzyme levels, lower risk of NAFLD and improvements in histological characteristics.[73] Future studies should validate these findings preferably in a trial setting and unravel whether statin use is also hepatoprotective among those without dyslipidemia. Further validation of statins yield benefits for liver health in individuals that already have another indication for statin treatment is complicated as one cannot withhold effective treatment.

FINAL REMARKS

Fatty liver disease has undergone an important transition from a neglected disease to a well-recognised global health threat. Policymakers should act accordingly and stimulate a healthy lifestyle to contain the ongoing fatty liver disease pandemic together with deteriorating metabolic health. The proposed name change towards metabolic-dysfunction associated fatty liver disease (MAFLD) better acknowledges the pathophysiology and improves the identification of patients at risk of advanced liver disease. However, a more overarching term with subgroups including all types of fatty liver disease might be preferred. In the years to come, we need to substantially refine risk stratification algorithms to discriminate accurately between those who could benefit from specialists' attention and future pharmaceutical treatment and who could not.

CHAPTER 6.3

Nederlandse samenvatting

Chapter 2 van dit proefschrift bespreekt de gevolgen van het hernoemen en herdefiniëren van niet alcoholische leververvetting (NAFLD) naar metabole dysfunctie geassocieerde leververvetting (MAFLD). NAFLD is gedefinieerd als leververvetting zonder secundaire oorzaken zoals excessief alcoholgebruik en virale hepatitis. MAFLD is gedefinieerd als leververvetting samen met metabole dysfunctie zoals overgewicht of diabetes.

In **Chapter 2.1**, hebben wij de nieuwe MAFLD-criteria toegepast op 5.445 deelnemers van de Rotterdam Studie. Wij toonden dat MAFLD vaker voorkwam dan NAFLD (34.3% versus 29.5%) en dat MAFLD in staat was 96.4% van de deelnemers met NAFLD te identificeren. Een belangrijke bevinding was dat deelnemers gemist met de NAFLD-criteria maar geïdentificeerd met de MAFLD-criteria (MAFLD-only 5.9%) een hogere leverstijfheid hadden en vaker fibrose (OR 5.3) in volledig gecorrigeerde modellen. Dit is in tegenstelling tot deelnemers met NAFLD zonder te voldoen aan MAFLD (NAFLD-only 1.0%), in deze groep was niemand met fibrose en kon er geen associatie met leverstijfheid worden gevonden.

Een directe vergelijking van de NAFLD-only groep met de MAFLD-only groep werd bemoeilijkt door kleine groepen, wat de aanleiding was voor de meta-analyse in **Chapter 2.2.** Hierin hebben we de epidemiologische en klinische karakteristieken NAFLD en MAFLD vergeleken. In totaal hebben we 17 studies geïncludeerd met 9.808.677 personen. Hierin toonden wij aan dat MAFLD vaker voorkomt dan NAFLD (33.0% vs. 29.1%). Bovendien, waren significant meer mensen extra geïdentificeerd met MAFLD (MAFLD-only) dan er werden gemist (NAFLD-only). De MAFLD-only groep had een significant verhoogd risico op fibrose (RR 4.2), hoger ALT (+ 8.0 U/L) en hoger AST (+ 6.4 U/L) in vergelijking met NAFLD-only. Dit duidt op dat de nieuwe MAFLD-definitie superieur is aan NAFLD in de algemene populatie en steunt dus de transitie van NAFLD naar MAFLD.

In **Chapter 2.3** hebben we de klinische relevantie van de nieuwe MAFLD-criteria onderzocht in patiënten met chronische hepatitis B. Deze multicenter cohort studie bestond uit 1.076 chronische hepatitis B patiënten met een mediane follow-up van 9.8 jaar. Wij toonden aan dat MAFLD was geassocieerd met verminderde event-vrije (HR 2.00, 95%CI 1.26–3.19), leverkanker-vrije (HR 1.93, 95%CI 1.17 – 3.21) en transplantatie-vrije overleving (HR 1.80, 95%CI 0.98 – 3.29) in volledig

gecorrigeerde modellen. In patiënten zonder metabole dysfunctie verhoogde steatose niet het risico op een ernstig beloop. Onze bevindingen illustreren de relevantie van metabole dysfunctie in patiënten met chronische hepatitis B.

Chapter 2.4 focust op potentiële interacties tussen MAFLD en excessief alcoholgebruik ten aanzien van sterfte. Onze bevindingen in 12.656 deelnemers (22.9 mediane follow-up, 3.804 overlijdens) van een prospectieve algemene populatie studie toonden dat zowel MAFLD als excessief alcoholgebruik onafhankelijk van elkaar een risicofactor zijn voor sterfte. Er was geen effect modificatie tussen MAFLD en excessief alcoholgebruik ten aanzien van sterfte. MAFLD was geassocieerd met een vergelijkbaar verhoogd risico op sterfte in mensen met en zonder excessief alcohol gebruik. Onze bevindingen waren consistent voor 10-jaars sterfte, andere afkapwaardes voor excessief alcohol gebruik en 'propensity score weighting'. Deze bevindingen onderstrepen de relevantie van MAFLD, juist ook in patiënten met excessief alcoholgebruik.

Chapter 3 gaat over de overwegingen in vroegtijdige detectie van leverziekte. Richtlijnen adviseren screening voor leverziekte in populaties met metabole dysfunctie, wat bij ouderen vaak voorkomt. Daarom hebben wij in **Chapter 3.1** de associaties tussen leververvetting en sterfte onderzocht bij ouderen. Onze bevindingen in 4.093 65-plussers (6.9 jaar mediane follow-up, 793 overlijdens) van een prospectieve algemene populatie studie duiden erop dat leververvetting niet is geassocieerd met sterfte (aHR 0.87, 95%CI 0.73 – 1.03). Deze bevindingen waren consistent voor verschillende klinische relevante subgroepen, verschillende follow-up duur en kanker-gerelateerde en cerebro-cardiovasculaire sterfte. Bovendien was hoge leverstijfheid niet geassocieerd met sterfte in mensen met steatose. Op basis van deze studie lijkt het onwaarschijnlijk dat vroegtijdige detectie van leververvetting bij 65-plussers de uitkomsten verbetert en moet daarom niet worden aanbevolen.

Chapter 3.2 van dit proefschrift gaat over de associaties tussen leverstijfheid en klinische uitkomsten in relatie tot de aanwezigheid van hartfalen. In 4.266 deelnemers van de Rotterdam Studie toonden wij aan dat verhoogde leverstijfheid (≥8.0 kPa) was geassocieerd met sterfte (aHR 1.38, 95%CI 1.00 – 1.89). Opmerkelijk was dat deze associatie werd gedreven door deelnemers met hartfalen (aHR 2.69, 95%CI 1.28 – 5.64). Er werd geen associatie gevonden tussen leverstijfheid en

sterfte in deelnemers zonder hartfalen (aHR 1.09, 95%CI 0.72 – 1.65). Verschillende cardiovasculaire karakteristieken en hartfalen waren geassocieerd met een hogere leverstijfheid. Deze bevindingen duiden op de beperkingen van screening op gevorderde leverziekte met elastografie in populaties met een lage fibrose prevalentie.

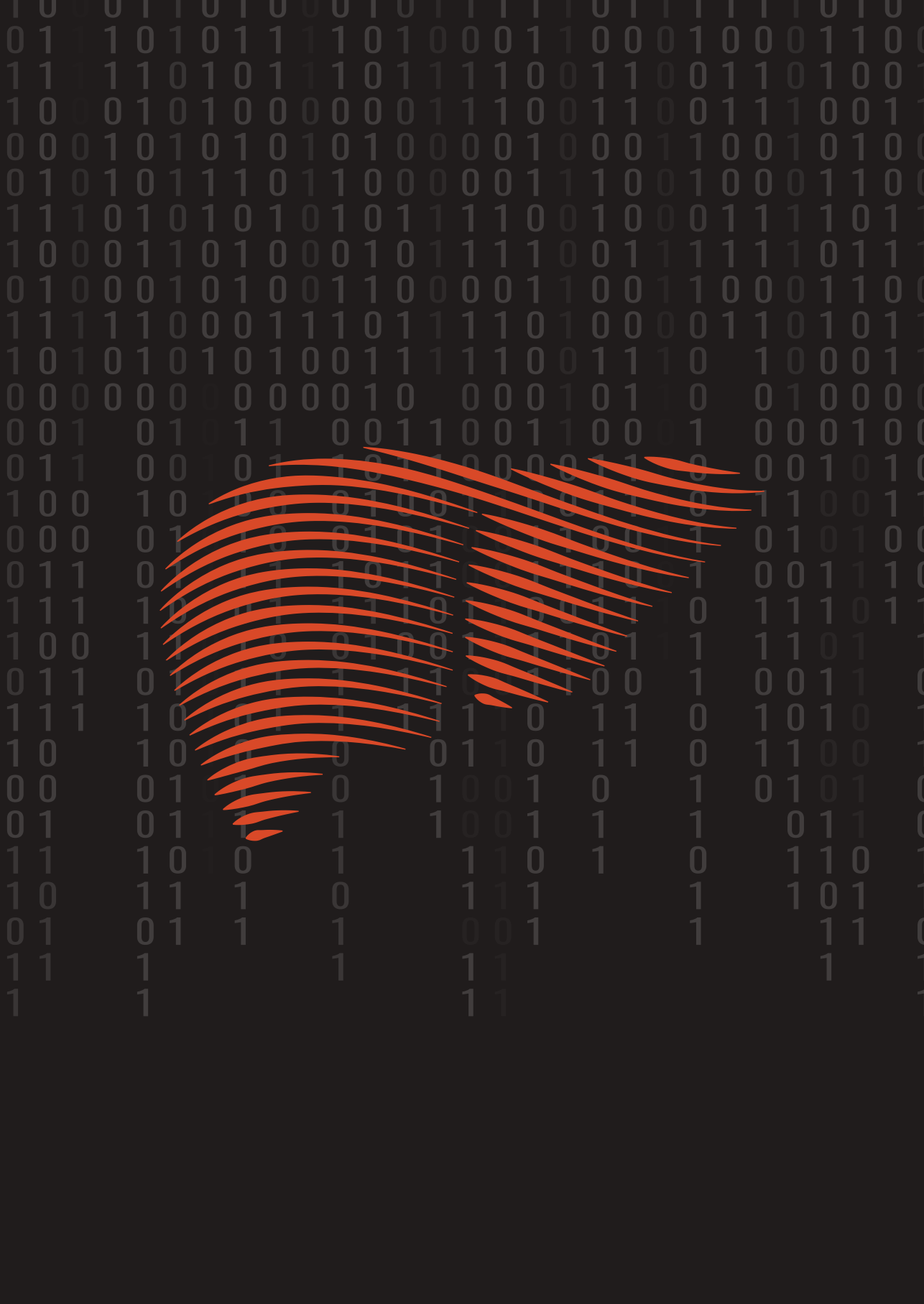
In 5.825 deelnemers van de Rotterdam Studie toonden wij in **Chapter 3.3** aan dat niet leververvetting (OR 0.80, 95%CI 0.62 – 1.03), maar leverstijfheid (OR 1.09 per kPa, 95%CI 1.03 – 1.16) is geassocieerd met atriumfibrilleren. Opmerkelijk was dat dit alleen gold voor deelnemers zonder steatose (OR 1.18 per kPa, 95%CI 1.08 – 1.29). Een belangrijke bevinding was dat zowel atriumfibrilleren en leverstijfheid geassocieerd zijn met subklinische tekenen van veneuze congestie, wat er op duidt dat de associatie van atriumfibrilleren met leverstijfheid verklaard kan worden door veneuze congestie en mogelijk niet puur door fibrose. Op basis van deze studie, adviseren wij daarom het controleren van de cardiovasculaire gezondheid bij mensen met een hoge leverstijfheid, zeker in de afwezigheid van evidente leverziekte.

In **Chapter 3.4** hebben we een evaluatie gedaan van de nieuwe screening strategie voor leververvetting zoals beschreven door de EASL non-invasieve test werkgroep. Bijna 50% van de 3.891 deelnemers opteerde voor een FIB-4 beoordeling gebaseerd op de aanwezigheid van het metabool syndroom of excessief alcoholgebruik. Wij toonden dat het toepassen van de FIB-4 in deze populatie met hoog risico op chronische leverziekte een slechte onderscheidende waarde had (sensitiviteit 75%, specificiteit 42%, diagnostic odds ratio 2.1). Vergelijkbare slechte prestaties werden gezien in geselecteerde groepen met hoger risico op fibrose. Ondanks het verwijspercentage van 60% werd nog steeds 25% van de deelnemers met hoge leverstijfheid gemist. Deze bevindingen duiden op de behoefte voor alternatieve niet-invasieve handvatten om mensen met verhoogd risico op gevorderde leverziekte te identificeren en betere beschikbaarheid van leverstijfheidsmetingen buiten gespecialiseerde klinieken.

Chapter 4 heeft als doel de huidige behandelopties en preventie doelen verder te onderzoeken. In **Chapter 4.1** hebben we gefocust op de impact van objectief gemeten beweging op NAFLD in 667 deelnemers van de Rotterdam Studie. Hierin toonden wij dat beweging significant was geassocieerd met lagere prevalente van

NAFLD. Een belangrijke bevinding was dat elke intensiteit van beweging nuttig was, met uiteraard wel grotere effecten per tijdseenheid voor intensievere beweging. Het nut van beweging was totaal gemedieerd door verbeteringen in metabole functie, in het bijzonder afname van buikomvang en verbetering in insulineresistentie. Op basis van deze studie adviseren wij lichamelijke beweging zoveel mogelijk te incorporeren in NAFLD-management en nationale preventie strategie, juist ook voor degenen die niet intensief kunnen bewegen.

In **Chapter 4.2** toonden wij dat statinegebruik in de algemene populatie was geassocieerd met lagere kans op NAFLD in vergelijking met deelnemers die geen statine gebruiken ondanks dyslipidemie. Mensen met NAFLD op statine therapie hadden minder vaak verhoogde leverstijfheid. In een biopsie bewezen NAFLD-patiënten cohort was statinegebruik geassocieerd met lager risico op NASH, maar niet op fibrose. Onze meta-analyse bevestigde deze resultaten: statine gebruik resulteerde in een lager risico op NAFLD (OR 0.69, 95%CI 0.46 – 1.01), NASH (OR 0.59, 95%CI 0.44 – 0.79) en fibrose (OR 0.48, 95%CI 0.33 – 0.70) bij mensen met metabole dysfunctie. Tot slot, onze lab-studie toonde dat statines de expressie van pro-inflammatoire cytokines remden wat de anti-NASH en anti-fibrotische eigenschappen van statines kunnen verklaren. Nu er voldoende bewijs is voor de beschermende effecten van statines op de levergezondheid moeten ze worden overwogen in patiënten met NAFLD, zeker in de aanwezigheid van een bestaande indicatie.



Appendices

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Abbreviations
Contributing authors
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PhD portfolio
Acknowledgements
About the author

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Abbreviations

AIC	Akaike information criterion
ALT	alanine transaminase
aOR	adjusted odds ratio
APRI	AST platelet ratio
AST	aspartate aminotransferase
AUC	area under the curve
AUROC	area under the receiver operating characteristic curve
BMI	body mass index
CAP	controlled attenuation parameter
CHB	chronic hepatitis B
CHD	coronary heart disease
CI	confidence interval
CRP	C-reactive protein
CTR	control
DAG	directed acyclic graph
DASH	dietary approach to stop hypertension
df	degrees of freedom
EASL	European association for the study of the liver
ELF-test	enhanced liver fibrosis test
FAST	Fibroscan-AST
FFQ	food frequency questionnaire
FLD	fatty liver disease
FLI	fatty liver index
FXR	farnesoid X receptor
GGT	gamma-glutamyl transpeptidase
HBeAg	hepatitis B envelope antigen
HBsAg	hepatitis B surface antigen
HCC	hepatocellular carcinoma
HDL-C	high-density lipoprotein cholesterol
HF	heart failure
HOMA-IR	homeostatic model assessment of insulin resistance
HR	hazard rate
ICO	intrahepatic cholangiocyte organoids

ICTRP	international clinical trials registry platform
IQR	interquartile range
IVC	inferior vena cava
JBI	Joanna Briggs institute
kPa	kilopascal
LSM	liver stiffness measurement
LVEDD	left ventricular end-diastolic dimension
LVESD	left ventricular end-systolic dimension
MAFLD	metabolic dysfunction-associated fatty liver disease
MET	metabolic equivalent of task
MRE	magnetic resonance elastography
MRI	magnetic resonance imaging
NAFLD	non-alcoholic fatty liver disease
NAS	NAFLD activity score
NASH	non-alcoholic steatohepatitis
NFS	NAFLD fibrosis score
NHANES	national health and nutrition examination survey
NHCS	national center for health statistics
NTR	national trial register
OR	odds ratio
P25-P75	25th - 75th percentile
PERSONS	prospective epidemic research specifically of NASH
PPAR	peroxisome proliferator-activated receptor
PRISMA	preferred reporting items for systematic reviews and meta-analyses
pSWE	pulse shear wave elastography
RR	relative risk
RS	Rotterdam study
SD	standard deviation
SREBPs	sterol regulatory element-binding proteins
ULN	upper limit of normal

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21. **Authors' response – Letter to the editor: focus on MAFLD, even if patients have a history of excessive alcohol consumption** van Kleef LA, de Knegt RJ, Brouwer WP. *Hepatology*. 2022 Epub ahead of print.

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33. **Spleen stiffness measurement to detect clinically significant portal hypertension in liver disease patients in realworld practice** Lantinga MA, van Kleef LA, den Hoed CM, de Knegt RJ. *Submitted*

34. **Liver stiffness is associated with excess mortality in the general population driven by Heart failure: the Rotterdam Study** van Kleef LA, Sonneveld MJ, Zhu F, Ikram MA, Kavousi M, de Knegt RJ. *Submitted*

35. **Fatty Liver Index and mortality after myocardial infarction: A prospective analysis in the Alpha Omega Cohort** Heerkens L, van Kleef LA, de Knegt RJ, Voortman T, Geleijnse JM. *Submitted*

36. **Presence of metabolic comorbidities is associated with increased risk for liver-related events in patients with chronic hepatitis B** Patmore LA*, Katwaroe W*, van der Spek D, Choi HSJ, Patel K, Brakenhoff S, van der Meer AJ, Brouwer WP, van Kleef LA, de Knegt RJ, Hansen BE, de Man RA, Feld JJ, Janssen HLA, Sonneveled MJ. *Submitted*

37. **Alterations in gut microbiome and plasma metabolome in metabolic dysfunction-associated fatty liver disease in the Rotterdam Study** Zhang X, Chen J, van Kleef LA, Su J, Vojinovic D, Ikram MA, van Meurs JB, Medina-Gomez C, Kraaij R, de Knegt RJ, Ahmad S, Ghanbari M. *Submitted*

38. **External validation of the steatosis-associated fibrosis estimator (SAFE) score in the general population** van Kleef LA, de Knegt RJ, Ayada I, Pan Q, Brouwer WP. *Submitted*

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(2) *"MAFLD and excessive alcohol consumption are both independent risk factors for mortality"*

(3) *"Simple anthropometrics may improve non-invasive tests for the prediction of fibrosis in an elderly population: The Rotterdam Study"*

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(2) *"Simple anthropometrics may improve non-invasive tests for the prediction of fibrosis in an elderly population: The Rotterdam Study"*

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(1) *"Association of non-alcoholic fatty liver disease and fibrosis with incident dementia and cognitive function: the Rotterdam Study"*

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(2) *"Objectively measured physical activity is inversely associated with NAFLD: The Rotterdam Study"*
(3) *"Liver stiffness, but not NAFLD is associated with atrial fibrillation"*

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nuttige commentaar op mijn eerste stukken. Ik hoop dat ik een waardige opvolger van je ben geweest!

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Een aanzienlijk deel van mijn promotietijd heb ik doorgebracht op het ERGO. Dankjewel **Paulien** voor het wegwijsmaken op het centrum. Bovendien enorm veel dank voor de echo's die je hebt gemaakt tussen 2009 en 2020! Alle **research dames** en **Andy**, dank voor de gezelligheid op de Briandplaats. Zonder jullie was het op *het centrum* niet zo leuk geweest als ik het nu heb ervaren! Mijn dank gaat bovendien uit naar de **deelnemers van het ERGO** hun vrijwillige inzet was bepalend voor de totstandkoming van dit proefschrift!

Moreover, I would like to thank the **LiverScreen consortium**. Working together on such a relevant and important research project was a pleasure! It was challenging to get inclusions during COVID, but we will get to the 30.000 participants soon! I am already so excited to see the outcomes of this unique project and how this will change the views on screening for liver disease in the general population.

Uiteraard wil ik ook **alle PhD collega's** op Na-6 bedanken voor de mooie tijd. Door COVID heeft het even geduurd voordat ik iedereen kende, maar dat is ruimschoots goed gemaakt met de vele koffietjes en borrels die daarna hebben plaatsgevonden. **Sylvia** en **Laurèlle** wat een eer dat jullie mij willen bijstaan als paranimfen tijdens de verdediging! Terecht zitten we in de appgroep *de coffee lover*s, want met weinig mensen heb ik meer koffiegedronken (gelukkig ook vaak met taart!) dan met jullie. Overigens, *coffee lovers* vind ik wel een betere naam voor de gezamenlijke

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Ibrahim, we hebben veel samen opgetrokken in NAFLD research land. Ik blik terug op een zeer prettige samenwerking en heel wat mooie projecten waarbij epidemiologie en lab toch niet zo ver uit elkaar liggen als je initieel zou denken. Ik zie er naar uit om je promotie bij te wonen! **Dr. Q. Pan**, thank you for initiating the collaboration between *the lab* and *the clinic* emphasizing on fatty liver disease. This has now resulted in the promising NAFLD-NASH working group.

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Laurens van Kleef, Januari 2023

About the author

Laurens A. van Kleef was born on August 29th 1994 in Vlissingen, The Netherlands. In 2012 he completed his secondary school at Calvijn College, Goes. That year, he started medical school at the Erasmus University of Rotterdam. During his master, he investigated the recurrence of primary sclerosing cholangitis after liver transplantation. After graduating in 2019, he started as an intern at Albert Schweitzer Hospital in Dordrecht in the internal medicine department. In 2020 he started his PhD program at the hepatology department under the supervision of dr. Robert J. de Knegt and prof. Robert A. de Man. He focused on fatty liver disease in the general population. Specifically, the impact of redefining and renaming NAFLD to MAFLD, considerations in early detection of advanced liver disease and potential targets for disease prevention and management. During his time as a researcher, he became a Delphi panel member for the renaming of NAFLD and mastered abdominal ultrasound and transient elastography. After defending his thesis, he will begin postgraduate training in Gastroenterology and Hepatology by starting as a resident of Internal Medicine at the Ikazia Hospital in Rotterdam.