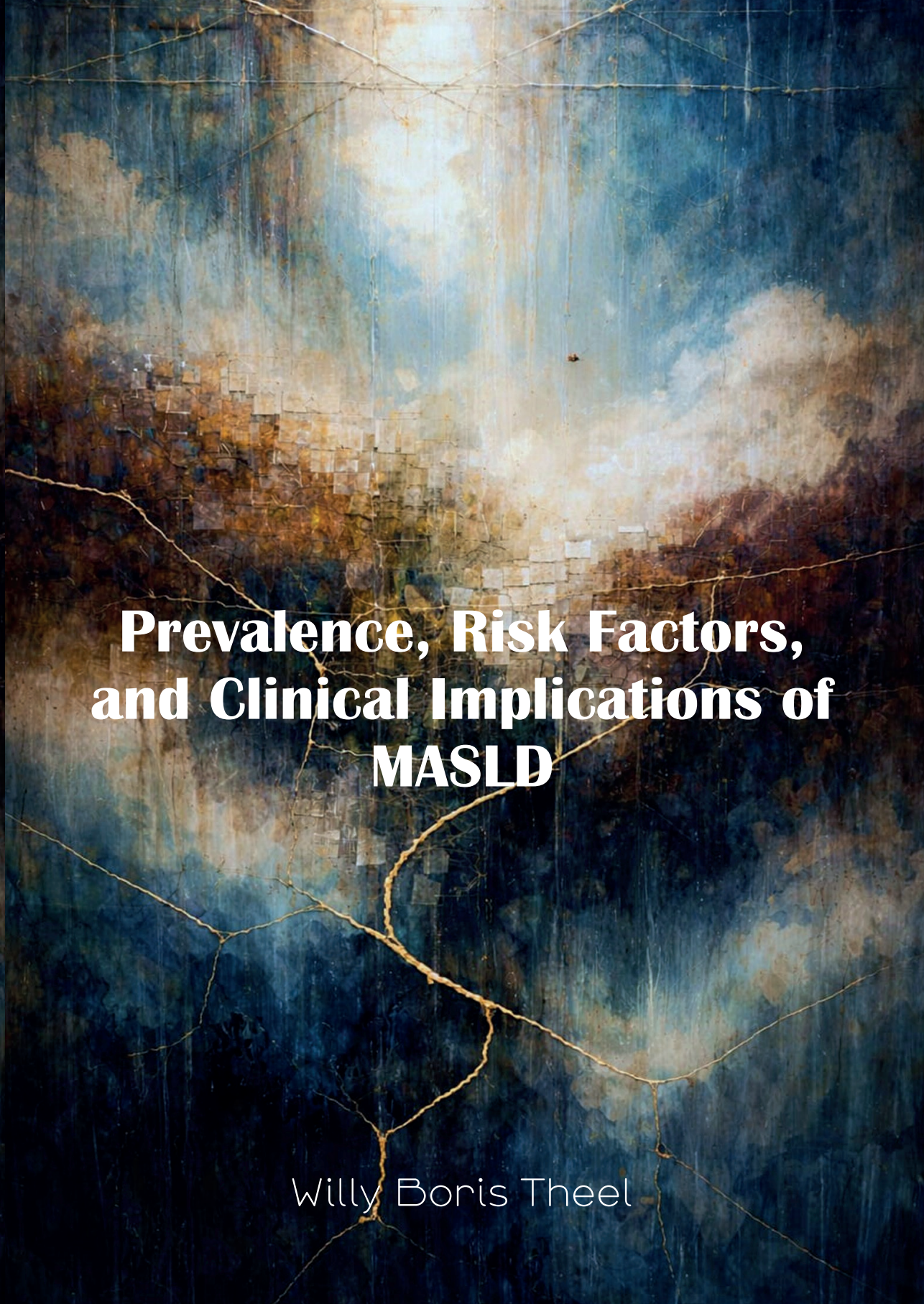




# **Prevalence, Risk Factors, and Clinical Implications of MASLD**

Willy Boris Theel

## Propositions accompanying the thesis:

### **Prevalence, risk factors, and clinical implications of MASLD**

Willy Theel

1. The transition from NAFLD/NASH to MASLD/MASH has improved scientific clarity and reduced patient stigmatization, thereby enhancing clinical communication. (this thesis)
2. In elderly patients with obesity and liver fibrosis, statin therapy should be considered not only for cardiovascular risk reduction but also as a potential modifier of liver-related outcomes. (this thesis)
3. Validated non-invasive tests demonstrate sufficient diagnostic accuracy for the assessment of steatosis and fibrosis in MASLD, rendering liver biopsy unnecessary in the majority of routine clinical settings. (this thesis)
4. Bariatric surgery provides substantial benefits for MASLD regression, but its long-term effect on fibrosis progression remains underexplored and demands structured follow-up. (this thesis)
5. The integration of genetic risk factors into MASLD risk stratification models is essential to personalize management and identify high-risk individuals earlier. (this thesis)
6. Redefining obesity as a disease rather than merely a risk factor represents, a necessary paradigm shift that will fundamentally reshape clinical responsibility, reimbursement structures, and public health accountability.
7. Fragmentation of care between metabolic, cardiovascular, and hepatic disciplines constitutes a greater obstacle to effective MASLD management than the absence of novel pharmacological therapies.
8. Preventive lifestyle interventions initiated during childhood yield a substantially greater long-term societal health impact than therapeutic interventions started in adulthood; this justifies structural investment in early-life prevention programs over reactive healthcare spending.
9. Work-life balance in academia should not be framed as an individual coping skill but as an institutional responsibility; sustainable scientific careers require formal structural safeguards within PhD training programs.
10. Although science excels in organizing empirical and reproducible knowledge, its explanatory framework remains inherently limited in addressing existential dimensions of human experience, which necessitates complementary philosophical or humanistic perspectives.
11. Le hasard ne favorise que les esprits préparés // Chance favors only the prepared mind – Louis Pasteur.

# **Prevalence, Risk Factors, and Clinical Implications of MASLD**

Willy Theel

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# **Prevalence, Risk Factors, and Clinical Implications of MASLD**

Prevalentie, risicofactoren  
en klinische implicaties van MASLD

## **Thesis**

to obtain the degree of Doctor from  
Erasmus University Rotterdam  
by command of the  
rector magnificus  
Prof.dr.ir. A.J. Schuit

and in accordance with the decision of the Doctorate Board.  
The public defence shall be held on  
Tuesday 22 September 2026 at 13.00 hrs

by

Willy Boris Theel  
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# Introduction

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## BACKGROUND AND SIGNIFICANCE

Metabolic dysfunction-associated steatotic liver disease (MASLD), formerly known as nonalcoholic fatty liver disease (NAFLD), is now recognized as the most common chronic liver disease worldwide, driven by the rising prevalence of obesity and type 2 diabetes [1, 2]. Its estimated global prevalence varies considerably, ranging from 15% in parts of sub-Saharan Africa to over 40% in the Middle East and South America, reflecting differences in obesity rates, lifestyle, genetics, and diagnostic practices. These figures likely underestimate the true burden, as MASLD often remains undiagnosed in clinical practice due to its asymptomatic nature and the lack of systematic screening.

Diagnosis requires liver steatosis (detected by ultrasound, transient elastography, or MRI) plus  $\geq 1$  cardiometabolic risk factor: elevated BMI/waist circumference, impaired glucose metabolism or type 2 diabetes, hypertension, hypertriglyceridemia, or low HDL cholesterol (with or without treatment).

The condition encompasses a spectrum of liver abnormalities, ranging from benign steatosis to more severe stages such as metabolic dysfunction-associated steatohepatitis (MASH), formerly known as nonalcoholic steatohepatitis (NASH), and cirrhosis. The severity of the disease is primarily determined by the presence of fibrosis, and without timely intervention, MASLD/MASH can lead to liver cirrhosis and hepatocellular carcinoma (HCC), two life-threatening conditions [3] (see Figure 1).

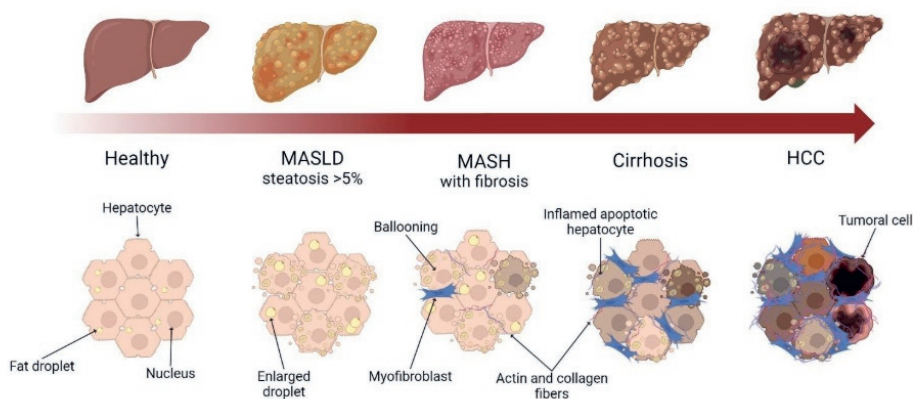


Figure 1 – Stages of MASLD: from health to HCC.

Although environmental and metabolic risk factors dominate, genetic predisposition also plays an important role in MASLD pathogenesis. Variants in genes such as PNPLA3, TM6SF2, and MBOAT7 have been associated with increased susceptibility, faster fibrosis progression, and higher risk of hepatocellular carcinoma. The prevalence of these variants differs substantially between ethnic groups, which partly explains regional differences in disease burden and outcomes.

Liver cirrhosis represents the end stage of chronic liver disease, characterized by extensive scarring (fibrosis) that disrupts liver function. As cirrhosis progresses, patients may develop complications such as liver failure, portal hypertension, and an increased risk of liver cancer. HCC is the most common type of primary liver cancer and often arises in patients with long-standing MASLD/MASH. Once HCC develops, treatment options are limited, and the prognosis worsens significantly. In advanced cases of cirrhosis or liver cancer, the only life-saving intervention may be a liver transplantation [4]

The burden of MASLD has grown so significantly that it is now the leading cause of liver transplantation in women in the United States. This highlights the pressing need for improved disease awareness, early diagnosis, and effective management strategies to prevent disease progression and reduce the need for transplantation.

The recent shift from NAFLD/NASH to MASLD/MASH was motivated by several factors. The term “NAFLD” was considered scientifically inaccurate, as it defined the disease primarily by the absence of alcohol consumption rather than by its true underlying cause, metabolic dysfunction. Additionally, the word “fatty” in the previous terminology was often perceived as stigmatizing and could make patients feel blamed for their condition [5]. By contrast, the updated nomenclature emphasizes the metabolic drivers of the disease, providing a clearer and more respectful medical classification. This change aims to improve disease recognition, enhance communication among healthcare providers, researchers, and patients, and ultimately reduce the trivialization of the condition [6].

Importantly in 2024 and 2025 new definitions of obesity were introduced, redefining it as a disease rather than merely a risk factor for other conditions (Figure 2). These new frameworks acknowledge that excess adiposity itself can cause organ dysfunction and directly contributes to chronic disease, rather than being solely a precursor to metabolic dis-

orders [7]. In the Netherlands, the multidisciplinary obesity guideline already applies a similar approach through the weight-related health risk (*gewichtgerelateerd risico* (GGR)) classification, which combines body mass index (BMI), waist circumference, and obesity-related comorbidities to determine whether an individual qualifies as having obesity and is eligible for treatment. This system closely aligns with the emerging international concepts and provides a practical framework for clinical implementation (Figure 3).

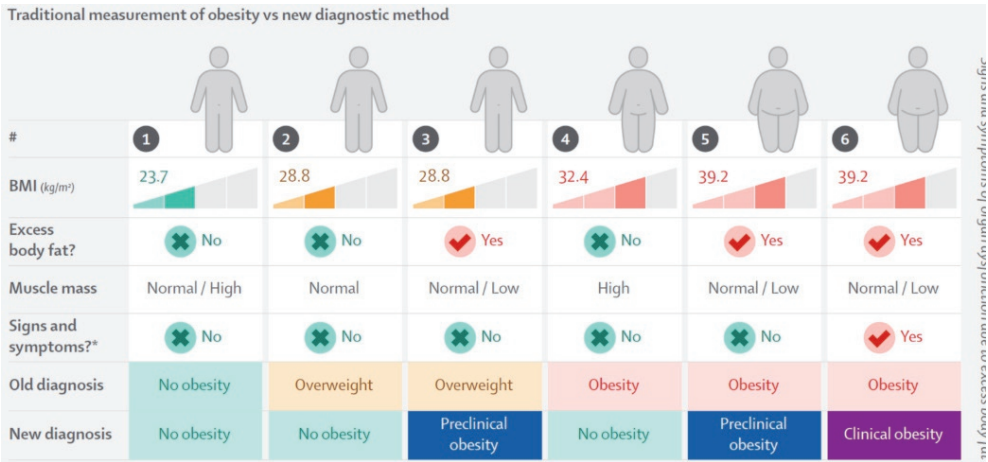


Figure 2 – Definition of obesity. Rubino, Francesco et al. “Definition and diagnostic criteria of clinical obesity.” The lancet. Diabetes & endocrinology vol. 13,3 (2025): 221-262. doi:10.1016/S2213-8587(24)00316-4

| BMI KG/M²                           | GEEN VERGROTE BUIKOMVANG¹ OF COMORBIDITEIT²                                                                                                     | BUIKOMVANG¹<br>Man ≥102 cm   vrouw ≥88 cm                                                                                                                                    | COMORBIDITEIT²                                                                                                             |
|-------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------|
| ≥ 25 BMI < 30<br>Overgewicht        | Adviezen gezonde leefstijl³                                                                                                                     | Basis GLI⁴                                                                                                                                                                   | Basis GLI (met aanvullende zorg)⁴                                                                                          |
|                                     |                                                                                                                                                 | Bij BMI ≥ 27 kg/m²: overweeg gewichtsreducerende medicatie toe te voegen na één jaar behandeling met de geïndiceerde GLI naar oordeel van de zorgprofessional(s)⁴            |                                                                                                                            |
| ≥ 30 BMI < 35<br>Obesitas klasse I  | Adviezen gezonde leefstijl³                                                                                                                     | Basis GLI⁴                                                                                                                                                                   | Basis GLI (met aanvullende zorg)⁴                                                                                          |
|                                     |                                                                                                                                                 | Overweeg gewichtsreducerende medicatie toe te voegen na één jaar behandeling met de geïndiceerde GLI naar oordeel van de zorgprofessional(s)⁴                                |                                                                                                                            |
|                                     |                                                                                                                                                 |                                                                                                                                                                              | Bij DM2: indien GLI en maximale inzet gewichtsreducerende medicatie onvoldoende effect hebben, overweeg metabole chirurgie |
| ≥ 35 BMI < 40<br>Obesitas klasse II | Basis GLI                                                                                                                                       | Gespecialiseerde GLI⁴,⁵<br>(evt. basis GLI met/zonder aanvullende zorg)                                                                                                      | Gespecialiseerde GLI⁴,⁵<br>(evt. basis GLI met aanvullende zorg)                                                           |
|                                     |                                                                                                                                                 | Overweeg toevoegen van gewichtsreducerende medicatie na één jaar behandeling met de geïndiceerde GLI of vroeger in de behandeling naar oordeel van de zorgprofessional(s)⁴,⁵ |                                                                                                                            |
|                                     |                                                                                                                                                 | Patiënt met een Aziatische of Hindoestaanse achtergrond: Overweeg metabole chirurgie                                                                                         |                                                                                                                            |
|                                     |                                                                                                                                                 |                                                                                                                                                                              | Overweeg metabole chirurgie                                                                                                |
| BMI ≥ 40<br>Obesitas klasse III     | Gespecialiseerde GLI⁴,⁵                                                                                                                         |                                                                                                                                                                              |                                                                                                                            |
|                                     | Overweeg toevoegen van gewichtsreducerende medicatie vroeg in de behandeling met de geïndiceerde GLI naar oordeel van de zorgprofessional(s)⁴,⁵ |                                                                                                                                                                              |                                                                                                                            |
|                                     | Overweeg samen met de patiënt en een multidisciplinair behandelteam de mogelijkheid van metabole chirurgie                                      |                                                                                                                                                                              |                                                                                                                            |

Mate van Gewichtsgelateerd gezondheidsrisico (GGR)      Licht verhoogd      Matig verhoogd      Sterk verhoogd      Extreem verhoogd

Figure 3 – Weight-Related Health Risk (GGR) and indication of general treatment options (adapted from the Partnership Obesity Netherlands guideline)

Given that MASLD is closely linked to obesity and metabolic dysfunction, this updated perspective strengthens the need for an integrated approach to managing MASLD within the broader context of metabolic health. Furthermore, traditional BMI-based measures of obesity have been criticized for their limitations in accurately assessing adiposity and individual health risks. The new definition provides objective diagnostic criteria, aiming to refine clinical decision-making and guide therapeutic interventions, which could have significant implications for MASLD diagnosis and treatment strategies.

Beyond its direct hepatic implications, MASLD is strongly associated with cardiometabolic disorders, significantly increasing the risk of cardiovascular disease (CVD). Even in the absence of liver-related complications, individuals with MASLD face a markedly higher risk of non-fatal CVD, coronary heart disease, and heart failure [8–12]. Given its intricate relationship with metabolic and cardiovascular conditions, the effective management of MASLD has become a critical challenge.

A key aspect of addressing MASLD is the implementation of noninvasive diagnostic techniques to assess liver health, particularly in individuals with severe obesity who are at increased risk. Understanding how imaging modalities and biomarker-based scoring systems can accurately detect and stratify liver disease in these high-risk patients remains an essential area of ongoing research. Additionally, integrating these tools into cardiovascular risk stratification can help identify individuals at greater risk of CVD, enabling more comprehensive disease management.

## RESEARCH CONTEXT

The management of MASLD often necessitates a multifaceted approach, integrating advanced diagnostic methods, lifestyle interventions, targeted medical treatments and metabolic surgery. Weight loss, whether achieved through lifestyle changes or metabolic surgery, has been shown to improve both metabolic health and liver disease outcomes [13]. Yet, the extent to which metabolic surgery directly impacts the progression or resolution of liver diseases such as steatohepatitis and fibrosis is not fully understood. Exploring how these surgical interventions influence liver health over time is crucial for optimizing care in individuals with obesity-related MASLD.

MASLD is not confined to a single population but manifests differently across various clinical cohorts. For instance, bariatric surgery patients and cardiometabolic outpatients often exhibit distinct risk factors and disease patterns. Understanding the prevalence of MASLD in these specialized populations, along with the key determinants of its progression, is essential for refining patient stratification and optimizing treatment strategies.

In addition to its links with obesity and metabolic dysfunction, MASLD is increasingly recognized as a significant risk factor for cardiovascular disease, particularly in older populations [14]. However, the relationship between liver fibrosis, a marker of advanced disease, and cardiovascular risk remains an area of active investigation. Moreover, genetic predispositions, such as risk alleles associated with MASLD/MASH, may further modulate this relationship. Clarifying how these genetic and fibrotic factors contribute to cardiovascular outcomes in elderly populations can shed light on the shared pathophysiology of these diseases.

While pharmacological interventions for MASLD remain limited, statin therapy has shown promise in reducing cardiovascular risks and liver fibrosis progression in high-risk individuals [15]. The potential dual benefit of statins in managing both cardiovascular disease and advanced liver disease warrants further exploration, particularly in elderly patients.

This thesis explores the prevalence, risk factors, and clinical implications of MASLD across diverse populations, including individuals with severe obesity, cardiometabolic disease, and rheumatologic conditions, as well as elderly cohorts with obesity and liver fibrosis. It also examines the effect of therapeutic interventions, such as bariatric surgery and statin therapy, on liver health and cardiovascular outcomes. By integrating insights from non-invasive diagnostic techniques, population studies, and intervention trials, this work aims to provide a comprehensive understanding of MASLD as a multisystem disease.

## RESEARCH QUESTIONS

This thesis addresses several critical questions related to MASLD. It examines how effective noninvasive diagnostic tools and imaging techniques are in evaluating MASLD in individuals with severe obesity. It also explores the prevalence of MASLD, MASH, and liver fibrosis in a Dutch bariatric cohort and investigates how these conditions can progress in this population over time. Additionally, the thesis evaluates the use of Fibroscan™ for assessing hepatic steatosis and fibrosis in Dutch cardiometabolic cohorts and discusses the implications for disease management.

The prevalence of MASLD and liver fibrosis in outpatient populations and their associations with cardiometabolic risk factors, such as carotid intima-media thickness (cIMT), are also analyzed. Furthermore, the research investigates the relationships between MASLD, liver fibrosis, and cardiovascular risk factors in patients with rheumatoid arthritis. It proposes to evaluate the impact of bariatric surgery on NAFLD/NASH and its progression to fibrosis while examining its effects on metabolic and cardiovascular outcomes.

In elderly subjects with obesity, this thesis explores how genetic risk alleles and liver fibrosis influence cardiovascular risk. The role of statin treatment in reducing all-cause mortality and cardiovascular risks in elderly patients with liver fibrosis is critically assessed, with insights drawn from the PROSPER trial. Finally, it analyzes the relationship between cardiovascular disease risk in elderly subjects with obesity and liver fibrosis and evaluates the potential benefits of statin treatment in mitigating these risks.

In summary, this thesis addresses key research questions to bridge gaps in current knowledge and develop strategies for diagnosing, stratifying risk, and managing MASLD, particularly in populations at high risk for metabolic and cardiovascular complications. The findings will deepen our understanding of MASLD as a systemic disease and support the development of improved diagnostic and management approaches. Ultimately, this research aims to advance clinical practice and inform public health strategies.

## STRUCTURE OF THIS THESIS

The thesis is structured into three parts to systematically address the key aspects of MASLD. **The first part** includes chapters on the use of non-invasive diagnostic techniques to evaluate MASLD in individuals with severe obesity, the prevalence and progression of MASLD in Dutch bariatric cohorts, and the application of Fibroscan™ in cardiometabolic populations to assess hepatic steatosis and fibrosis. **The second part** explores the metabolic and cardiovascular implications of MASLD, with chapters on its impact in outpatient populations and its associations with cardiovascular risk factors in individuals with rheumatoid arthritis. **The final part** delves into interventions, with chapters on the effect of bariatric surgery on MASLD and MASH, the role of genetic risk factors in elderly subjects with obesity and liver fibrosis, and the benefits of statin therapy for cardiovascular risk reduction in this population.

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# PART I

MASLD in specific populations



# Chapter 1

---

## **Evaluation of non-alcoholic fatty liver disease (NAFLD) in severe obesity using non-invasive tests and imaging techniques.**

W.B. Theel, B.M. Boxma-de Klerk, F. Dirksmeier-Harinck, E.F.C. van Rossum, D.A. Kanhai, J. Apers, B. M. van Dalen, R. J. de Knecht, A.G. Holleboom, M.E. Tushuizen, D.E. Grobbee, J. Wiebolt, M. Castro Cabezas.

Obes Rev. 2022 Aug;23(8):e13481. doi: 10.1111/obr.13481. Epub 2022 Jun 12. PMID: 35692179.

## ABSTRACT

The prevalence of nonalcoholic fatty liver disease (NAFLD) and the more severe and inflammatory type, nonalcoholic steatohepatitis (NASH), is increasing rapidly. Especially in high-risk patients i.e. those with obesity, metabolic syndrome and type 2 diabetes mellitus, the prevalence of NAFLD can be as high as 80% while NASH may be present in 20% of these subjects. With the worldwide increase of obesity it is most likely that these numbers will rise. Since advanced stages of NAFLD and NASH are strongly associated with morbidity and mortality – in particular cardiovascular disease, liver cirrhosis, hepatocellular carcinoma – it is of great importance to identify subjects at risk. A great variety of non-invasive tests has been published to diagnose NAFLD and NASH, especially using blood- and imaging-based tests. Liver biopsy remains the gold standard for NAFLD/NASH. This review aims to summarize the different mechanisms leading to NASH and liver fibrosis, the different non-invasive liver tests to diagnose and evaluate patients with severe obesity.

## INTRODUCTION: EPIDEMIOLOGY, RISKS AND COMPLICATIONS IN NAFLD

In the last decades, the prevalence of obesity has risen rapidly in most parts of the world, closely associated with comorbidities such as type 2 diabetes mellitus (T2DM), dyslipidemia, hypertension, sleep apnea syndrome, non-alcoholic fatty liver disease (NAFLD) and its more severe form non-alcoholic steatohepatitis (NASH) [1]. The silent obesity pandemic also affects 5% of children and young adults in the world [2] and the increasing prevalence of subjects with obesity closely parallels the increasing NAFLD/NASH occurrence in these same groups [3]. The prevalence of overweight and obesity differs significantly between countries. While in Europe, more than half (52.7%) of the population is suffering from overweight (BMI 25–29,9 kg/m<sup>2</sup> and ) and almost 17% from obesity (BMI ≥30 kg/m<sup>2</sup>) [4], the situation in the USA is far more alarming with 73.6% of the population having overweight and 42.4 % obesity [5]. A similar situation can be described for NAFLD/NASH. Multiple studies estimate that NAFLD is present in 17%–33% of the world population making this number one liver disease in many countries [6, 7][8]. In patients with severe obesity (BMI ≥40 kg/m<sup>2</sup>), NAFLD prevalence may be as high as 90% [9–16]. Most of the patients with NAFLD will maintain a mild form of this condition, but a significant percentage (~20%) may progress to NASH [17–20].

For the diagnosis of NAFLD, alcohol use cannot exceed a daily intake of 30 grams for men and 20 grams for women [21]. NAFLD is a risk factor for and a precursor of metabolic syndrome (MetS) with a bidirectional link with a number of metabolic abnormalities [9–11, 22–24]. Hence, the term “*metabolic dysfunction-associated fatty liver disease*” (MAFLD) has been proposed [25, 26]. Major liver-related complications of NAFLD include the development of NASH, liver cirrhosis and hepatocellular carcinoma (HCC) [6, 7, 22, 23]. NASH has been defined as the inflammatory form of NAFLD with one of the 2 additional characteristic histological features: lobular inflammation (with the presence of neutrophil infiltration) and hepatocyte ballooning (reflecting injury of the hepatocytes) [27]. The severity of NASH is determined by the presence and stage of fibrosis [28], which is a key determinant of the liver-specific disease outcome and overall mortality [29–32]. Patients with NASH often have multiple cardiovascular risk factors which generally determine the main cause of death [7, 33]. An association between NAFLD and increased mortality due to cardiovascular disease (CVD) has been shown [22, 31, 34–37].

However, a debate is still ongoing whether NAFLD can be considered as an independent risk factor for CVD or whether the increased risk can be explained by the sum of the risk factors associated to NAFLD like insulin resistance, dyslipidemia, obesity and hypertension [38, 39].

NASH-associated liver cirrhosis is responsible for an increase of the liver transplant waiting list registration in the USA of 114% in men and 80% in women [40]. The economic burden is considerable since it has been estimated that medical care costs related to NASH are in the billions [20]. While there is no registered pharmacological treatment for NASH yet, bariatric surgery in these subjects could be very cost effective [41]. Liver fibrosis decreases significantly in the majority of patients after bariatric surgery but a recent systematic review and meta-analysis showed that 75% of patients with liver fibrosis before bariatric surgery show persistent fibrosis after the procedure [42]. Weight loss, which is considered as therapeutic goal for patients with NAFLD/NASH, is therefore no guarantee for complete remission of liver fibrosis. Weight loss after surgery or non-pharmacological (i.e. lifestyle) interventions is also often not sustainable [43, 44]. Consequently, progression of liver fibrosis to cirrhosis may occur in these patients. This raises the issue of control and evaluation of these patients in the follow-up phase after bariatric surgery in order to detect complications at an early stage. We will review the mechanisms leading to NASH and liver fibrosis, and the different non-invasive tests available to evaluate this condition in patients with severe obesity.

## CELLULAR MECHANISMS UNDERLYING NAFLD/NASH IN OBESITY

### Molecular basis of steatosis in NAFLD

An accumulation of fat in hepatocytes (i.e. >5%) defines the term steatosis [45]. Hepatic fat accumulation has different origins and can be triggered by factors such as peripheral insulin resistance (IR), obesity, gut barrier dysfunction and unhealthy dietary habits [46]. These factors also interact together since we know that adipose tissue is nowadays considered to be a full-fledged organ with different functions. Adipose tissue is considered to be an endocrine organ producing pro-inflammatory hormones called adipokines (i.a. leptin, resistin,  $\text{TNF}\alpha$ , IL6) but also anti-inflammatory adipokines (i.a. adiponectin) [47] (Figure 1). The enlargement of adipose tissue leads to higher levels of leptin whereas adiponectin is decreased in

people with obesity [48–53]. Leptin has an anti-steatotic activity at the early stages of NAFLD, but it may have adverse effects in the long term by stimulating hepatic inflammation and fibrosis [54, 55].

Fat is usually stored in adipocytes, however an excessive fat storage makes them dysfunctional and resistant for insulin [56]. Therefore, IR plays a central role in the development of NAFLD/NASH in obesity [57–60]. Furthermore, IR induces adipocyte lipolysis which leads to high serum concentrations of free fatty acids (FFAs) and enhances uptake by the liver, thereby converting hepatocytes into fat storing cells [61]. These fatty acids impair the lysosomal permeability of hepatocytes stimulating the expression and release of tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), inhibiting insulin receptor activity and enhancing IR [62]. Therefore, control of IR is one of the major targets in clinical practice in order to prevent disease progression.

### Molecular basis of inflammation and cell damage by hepatic steatosis

Intracellular fat accumulation also generates lipid peroxides and reactive oxygen species resulting in lipotoxicity and mitochondrial dysfunction, pro-inflammatory changes, cell damage and finally apoptosis [63–67]. What initially appears to be simple steatosis, in fact activates inflammatory pathways and causes cellular dysfunction until lipo-apoptosis occurs [56]. The accumulation of fatty acids and their metabolites activates the c-Jun N-terminal kinase (JNK) and the inhibitor of nuclear factor kappa-B kinase subunit beta (I $\kappa$ K $\beta$ ) [68], promoting chronic inflammation and the development of hepatic IR [69]. The JNK pathway activates the inflammatory response and regulates hepatocyte apoptosis by the apoptosis signal-regulating kinase 1 (ASK1). I $\kappa$ K $\beta$  activates the nuclear factor kappa-light-chain-enhancer of activated B-cells (NF- $\kappa$ B) during inflammation [70, 71] which stimulates the production of inflammatory cytokines like TNF $\alpha$  and IL-6 by these cells [72, 73].

Activation of these pathways leads to accumulation of immune cells (macrophages, B-lymphocytes, T-lymphocytes and neutrophils) inducing a pro-inflammatory cascade of interleukins and cytokines (e.g., IL-1, IL-6, TNF- $\alpha$ ) in the liver [74]. Adiponectin [49] and obestatin [75] have a favorable effect protecting the liver integrity [76] whereas TNF $\alpha$ , IL-6, RBP4, chemerin and resistin promote steatosis, inflammation and/or fibrosis [74].

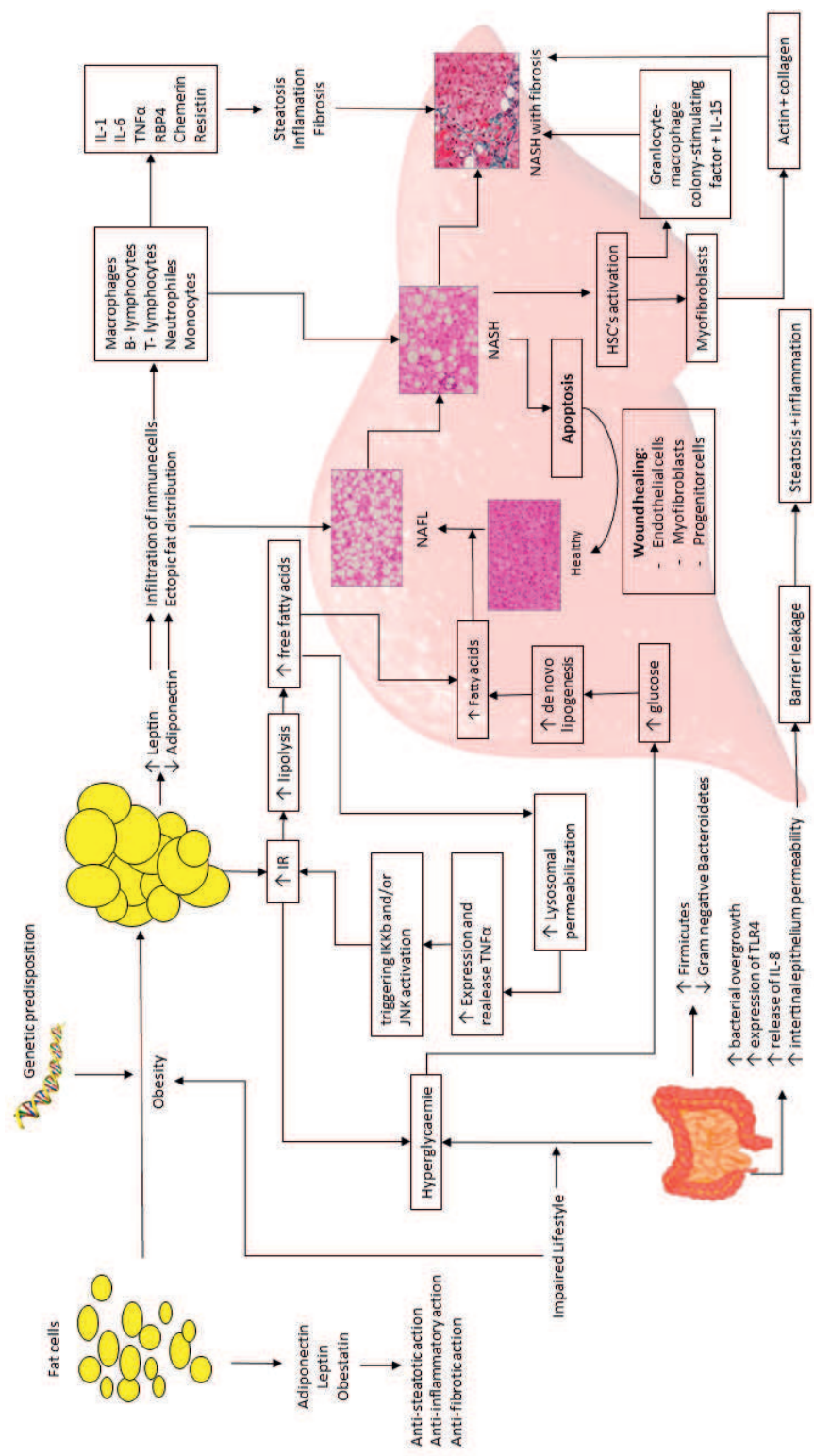


Figure 1 – Physiopathology of NASH in obesity

NAFL: Non-Alcoholic Fatty Liver; NASH: NON-Alcoholic Steato-hepatitis; IL-1: interleukin-1; IL-6: interleukin-6; IL-15: interleukin-15; TNF- $\alpha$ : Tumor Necrosis Factor  $\alpha$ ; RBP4: Retinol binding protein 4;  $\uparrow$ : increased;  $\downarrow$ : decreased; JNK; c-Jun N-terminal kinases; IKK $\beta$ : I $\kappa$ B kinase  $\beta$ ; TLR4: Toll-Like Receptor 4.

The enlargement of adipose tissue leads to higher level of leptin and lower level of adiponectin in people with obesity. Fat is usually stored in adipocytes which become dysfunctional and resistant for insulin contributing to ectopic fat accumulation of TG-derived toxic metabolites. Hypoadiponectemia also induces ectopic fat distribution including the hepatic cells which acquire an adipocyte-like-function. The storage of fat in the form of triglycerides results first in simple steatosis. When hepatocytes cannot dispose the excess of free fatty acids anymore, inflammatory pathways are activated, cellular dysfunction occurs causing lipooapoptosis. The increase of adipose tissue also initiates the infiltration of immune cells. The most common cells are macrophages, B-lymphocytes, T-lymphocytes and neutrophils which produce different cytokines. Adiponectin and obestatin have a favorable effect defending the liver integrity whereas TNF $\alpha$ , IL-6, RBPP4, chemerin or resistin promotes steatosis, inflammation and/or fibrosis.

Leptin, which has an anti-steatotic action at the early stage of NAFLD, becomes an unfavorable factor at a later stage stimulating hepatic inflammation and fibrosis.

The storage of fat in the liver is also emphasized with the insulin resistance of the patient. This situation leads to hyperglycemia and lipolysis resulting in increased fatty acids in the liver. These fatty acids affect the lysosomal permeability which stimulates the expression and release of TNF $\alpha$ . Through the IKK $\beta$  and/or JNK pathways the inhibition of insulin receptor signaling occurs and maintains the insulin resistance.

The hepatocyte injury, also called ballooning, and inflammation, with or without fibrosis, then becomes visible: the initiation of NASH development.

In order to restore the liver integrity, wound-healing cells are engaged. Endothelial cells, myofibroblasts and progenitor cells are recruited after the death or apoptosis of the hepatocytes to recover. The myofibroblasts are hepatocytes stellate cells (HSCs) which have been activated. They contribute to the liver fibrogenesis producing actin and different types of collagens. They also induce the granulocyte-macrophage colony-stimulating factor and IL-15 which may prolong the neutrophils survival and enhance the liver damage and fibrosis.

One of the causes of obesity is an impaired lifestyle. In the situation of a high fat diet a change in the gut microbiota occurs. The ratio firmicutes/bacteroides increase. This type of diet induces a loosening of intestinal tight junctions, increases endotoxin levels and activation of TLR pathways. Eventually, NASH patients show a higher TLR4 expression, an augmentation of IL-8 release and a greater intestinal epithelium permeability. The consequence is a barrier leakage which stimulates the steatosis and inflammation.

The imbalance created between pro- and anti-inflammatory cytokines plays a crucial role in the generation of NASH and fibrosis [77].

Gut microbiota can also induce inflammation. Diet plays a crucial role in the composition of the microbiome and the Western diet offers a good breeding ground for micro-organisms like the Firmicutes species [78]. In the case of obesity, the gut microbiota changes and the ratio Firmicutes/Bacteroides is higher in comparison to normal-weight subjects [79]. This alteration may induce inflammation by increasing endotoxin levels and it may cause disruption of intestinal tight junctions through the induction of Toll-like Receptor 4 (TLR4) pathways [80]. In NASH, a higher prevalence of bacterial overgrowth, a higher TLR4 expression, an augmentation of IL-8 release and a better intestinal epithelium permeability have been reported [81]. Ultimately, barrier leakage may expose the liver to other bacteria and bacterial products that will induce pro-inflammatory signaling pathways in the liver eventually promoting the development of NASH [98].

Altogether, these mechanisms lead to hepatocyte injury resulting in ballooning of these cells which together with inflammatory infiltration and steatosis results in the characteristic histology of NASH [28].

## Molecular basis of fibrosis in NAFLD

In order to restore the liver integrity, wound-healing cells play an important role. Endothelial cells, myofibroblasts and progenitor cells are recruited after apoptosis of the hepatocytes [74]. The myofibroblasts are in fact activated hepatic stellate cells (HSCs). Normally, fenestrated liver sinusoidal endothelial cells (LSECs) actively repress their activation but this is not the case after injury [82, 83]. Moreover, LSECs may promote fibrosis via CXCR4 (an alpha-chemokine receptor) and the fibroblast growth factor receptor 1 (FGFR-1) on their surface [84]. This activation is enhanced when liver macrophages (i.e. Kupffer cells) release cytokines in the case of liver injury and/or inflammation [85]. Kupffer cells may also support HSCs migration by secreting CCL2, CCL3, CCL4, CCL5, CCL7 and CCL8 [86, 87]. Myofibroblasts remodel the liver extra-cellular matrix (ECM) by producing actin and replacing collagens IV and VI in the space of Disse with collagens I, III and fibronectin [88–91] to provide mechanical stability in a damaged liver. They also induce the granulocyte-macrophage colony-stimulating factor (GMCSF) and IL-15 which may prolong the neutrophil survival and enhance liver damage and fibrosis [92]. In the situation of a damaged liver, thrombocytopenia is a common hematological complication due to portal hypertension but also due to a reduced production of hormone thrombopoietin (TPO) [93–95]. Interestingly, thrombocytopenia itself may also stimulate fibrosis. Indeed, platelets and platelet-derived extracts normally hinder the conversion of HSCs into myofibroblasts and consequently prevent fibrosis [96].

## NAFLD in the pediatric population

NAFLD is not only a problem of adulthood but even children show an increasing prevalence of fatty liver disease. In the USA an increase of NAFLD in children between 12 and 19 years has been reported rising from 3.9% between 1988 and 1994 to 10.7% between 2007 and 2010 [97]. Similarly to the situation in adults, this increase parallels closely the rise of obesity seen in children [98]. In children with obesity the prevalence of NAFLD may range from 22.5% to 44% [99].

Different theories have been proposed to explain the development of NASH. The most popular theory is the ‘multiple-hits’ model [69, 100, 101]. This model implies that different factors need to be operative leading to inflammation and fibrosis (Figure 1). An unhealthy maternal lifestyle including obesity and an early exposure to a hyperlipidemic environment during critical developmental periods may represent the “first hit” for the fetal liver and initiate liver histopathogenesis [102, 103]. This per-

spective converges with the developmental origins of disease hypothesis which claims that early exposure to an adverse situation leads to risk of later life disease [104, 105].

The physiopathology of NAFLD in children is similar to adults. The same risk factors (i.e. those of the MetS) are related to the development of NAFLD in children [106]. When compared to non-diabetic subjects, type 2 diabetes raises the risk of NAFLD by twofold, while the presence of NAFLD increases the risk of type 2 diabetes by two to fivefold [107]. Insulin resistance and type 2 diabetes in children have been linked to more advanced forms of NAFLD, such as NASH, or with fibrosis [106, 108]. Insulin resistance is partly responsible of the lipolysis in adipocytes leading to higher levels of circulating fatty acids. The disbalance between hepatic fatty acids acquisition and removal (through hepatic oxidation and VLDL secretion) is responsible for fat accumulation in hepatocytes which causes defects in insulin signaling pathways as explained earlier [109, 110].

## NON-INVASIVE LIVER TESTS (NILTS)

The progression of NAFLD is characterized by three overlapping processes: (a) steatosis (b) inflammation and (c) fibrosis. While all three are based on histological evaluation [111], many non-invasive tests have been described reflecting several of these conditions. Both, serum markers and imaging procedures have been validated for this purpose. Constantinescu et al. recently published an excellent overview of the ultrasound-based techniques to estimate the degree of liver steatosis and fibrosis [112].

### Biomarkers for the diagnosis and evaluation of NAFLD/NASH

Serum alanine aminotransferase (ALT), aspartate transaminase (AST) and gamma glutamyltransferase (GGT) levels have been the most widely used markers to screen for liver pathology [113]. Unfortunately, they are not specific for the diagnosis of NASH and show no correlation with inflammation or fibrosis [114, 115]. Serum transaminases are often elevated in different clinical settings, but they may also be within normal limits in severe stages of NAFLD [116, 117]. Indeed, no relation has been demonstrated between the ALT levels and the possible histological liver changes in adults [118, 119]. However, different algorithms have been described including ALT and AST which can be of clinical use to diagnose

liver steatosis and fibrosis. Laboratory measurements of liver enzymes or inflammatory markers like C-reactive protein (CRP) are neither specific nor sensitive for NASH [117, 120–122]. CRP has been associated with obesity but not with NAFLD/NASH [122]. Some authors have proposed that CRP may be helpful to distinguish severe NASH from NAFLD [123–125] but this has been disputed by others [126–128]. Finally, a low platelet count may reflect hepatic fibrosis [129].

For the initial workup and screening purposes, serum biomarkers have been recommended. The Fatty Liver Index (FLI), the SteatoTest and the NAFLD Liver Fat Score (NAFLD-LFS) have been proposed to identify patients with hepatic steatosis [111] (Table 1). The SteatoTest and NAFLD-LFS have been validated with histology. Validation in obesity was done in a limited number of subjects as shown in Table 1 [130–135]. The best diagnostic accuracy in obesity was shown for the NAFLD-LFS. All three tests are of limited clinical value since a large proportion of subjects will fall in an intermediate range (FLI) or in a gradual score of steatosis (SteatoTest and NAFLD-LFS).

The histological score for fibrosis is based on the 5-point scale proposed by Brunt et al. and modified by Kleiner et al. [136]. This classification uses stage 0 (F0) indicating the absence of fibrosis, stage 1 (F1) for perisinusoidal or portal fibrosis, stage 2 (F2) for perisinusoidal and portal/periportal fibrosis, stage 3 (F3) for septal or bridging fibrosis and stage 4 (F4) for cirrhosis. Clinically significant fibrosis and advanced/severe fibrosis stand for F2 and F3, respectively.

Guidelines recommend the NAFLD Fibrosis Score (NFS) and the fibrosis 4 algorithm (FIB-4) for the identification of cases at high risk for advanced fibrosis/cirrhosis [111]. Recently, other algorithms have been proposed like the Fibrotest, the ADAPT score, the FIB-C3 score, the enhanced liver fibrosis (ELF) test or the AST to platelet ratio index (APRI) for this purpose.

All these algorithms have been validated in subjects with obesity. The best performing was the ELF score followed by the FIB-4 score. However, the former was evaluated in a very limited number of subjects with obesity. All these tests have been validated with histology. Most of these tests use regular clinical parameters, except the ELF test [137–148].

Table 1 – Algorithms for the evaluation of liver steatosis and liver fibrosis

| Components                        | Formula (when available)       | Diagnostic accuracy in the general population / obesity                                                                                                                                   | References                                       | Number of subjects evaluated for validation in the general population/obesity |
|-----------------------------------|--------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------|-------------------------------------------------------------------------------|
| Steatosis                         |                                |                                                                                                                                                                                           |                                                  |                                                                               |
| Fatty Liver Index (FLI)           | $= e^y / (1 + e^y) \times 100$ |                                                                                                                                                                                           |                                                  |                                                                               |
|                                   | Fasting TG                     |                                                                                                                                                                                           |                                                  |                                                                               |
|                                   | GGT                            | Where $y = 0.953 \times \ln[\text{TG (mg/dL)}] + 0.139 \times \text{BMI (kg/m}^2) + 0.718 \times \ln[\text{GGT (U/L)}] + 0.053 \times \text{waist circumference (cm)} - 15.745$           | Bedogni et al. [130]<br>Cuthbertson et al. [131] | 228 / 168                                                                     |
|                                   | BMI                            |                                                                                                                                                                                           |                                                  |                                                                               |
| SteatoTest                        | Waist circumference            |                                                                                                                                                                                           |                                                  |                                                                               |
|                                   | TG                             |                                                                                                                                                                                           |                                                  |                                                                               |
|                                   | GGT                            |                                                                                                                                                                                           |                                                  |                                                                               |
|                                   | BMI                            |                                                                                                                                                                                           |                                                  |                                                                               |
|                                   | Serum cholesterol              |                                                                                                                                                                                           |                                                  |                                                                               |
|                                   | Total bilirubin                | NA                                                                                                                                                                                        |                                                  |                                                                               |
|                                   | Serum glucose                  |                                                                                                                                                                                           | Poynard et al. [132]<br>Lassailly et al. [133]   | 884 / 288                                                                     |
|                                   | $\alpha$ -2-macroglobulin      |                                                                                                                                                                                           |                                                  |                                                                               |
|                                   | apolipoprotein AI              |                                                                                                                                                                                           |                                                  |                                                                               |
|                                   | Haptoglobin                    |                                                                                                                                                                                           |                                                  |                                                                               |
| NAFLD Liver Fat Score (NAFLD-LFS) | ALT                            |                                                                                                                                                                                           |                                                  |                                                                               |
|                                   | AST                            |                                                                                                                                                                                           |                                                  |                                                                               |
|                                   | Fasting serum insulin          |                                                                                                                                                                                           |                                                  |                                                                               |
|                                   | the presence or absence of:    |                                                                                                                                                                                           |                                                  |                                                                               |
|                                   | MetS                           | $= -2.89 + 1.18 \times \text{MetS (yes:1, No:0)} + 0.45 \times \text{T2DM (Yes:2, No:0)} + 0.15 \times \text{insulin (mU/L)} + 0.04 \times \text{AST (U/L)} - 0.94 \times \text{AST/ALT}$ | Fedchuk et al. [134]<br>Kotronen et al. [135]    | 324 / 470                                                                     |
|                                   | T2DM                           |                                                                                                                                                                                           |                                                  |                                                                               |
|                                   | ALT                            |                                                                                                                                                                                           |                                                  |                                                                               |

Table 1 – Algorithms for the evaluation of liver steatosis and liver fibrosis (continued)

| Components                 |                    | Formula (when available)                                                                                                                                                                                                                                                                                                                                                          | Diagnostic accuracy in the general population / obesity | References                                  | Number of subjects evaluated for validation In the general population/obesity |
|----------------------------|--------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------|---------------------------------------------|-------------------------------------------------------------------------------|
| Fibrosis                   |                    |                                                                                                                                                                                                                                                                                                                                                                                   |                                                         |                                             |                                                                               |
| NAFLD Fibrosis Score (NFS) | Age                | $= -1.675 + 0.037 \times \text{Age (years)} + 0.094 \times \text{BMI (kg/m}^2\text{)} + 1.13 \times \text{IFG (yes=1, no=0)} + 0.99 \times \text{AST/ALT ratio} - \text{platelet (x10}^9\text{/L)} - 0.66 \times \text{albumin (g/dL)}$                                                                                                                                           | 82% / 87%                                               | Angulo et al. [137]<br>Drolz et al. [138]   | 733 / 368                                                                     |
|                            | IFG                |                                                                                                                                                                                                                                                                                                                                                                                   |                                                         |                                             |                                                                               |
|                            | BMI                |                                                                                                                                                                                                                                                                                                                                                                                   |                                                         |                                             |                                                                               |
|                            | Platelet count     |                                                                                                                                                                                                                                                                                                                                                                                   |                                                         |                                             |                                                                               |
|                            | Albumin            |                                                                                                                                                                                                                                                                                                                                                                                   |                                                         |                                             |                                                                               |
| FIB-4                      | AST/ALT ratio      | $= (\text{Age} \times \text{AST}) / (\text{Platelet count} \times \sqrt{\text{ALT}})$                                                                                                                                                                                                                                                                                             | NA/89%                                                  | Sterling et al. [139]<br>Drolz et al. [138] | 832 / 368                                                                     |
|                            | Age                |                                                                                                                                                                                                                                                                                                                                                                                   |                                                         |                                             |                                                                               |
|                            | AST                |                                                                                                                                                                                                                                                                                                                                                                                   |                                                         |                                             |                                                                               |
|                            | ALT                |                                                                                                                                                                                                                                                                                                                                                                                   |                                                         |                                             |                                                                               |
| FibroTest                  | Platelet count     | $= 1 / (1 + e^{-z})$<br><br>Where $z = 4.467 \times \log_{10} [\alpha 2\text{M (g/L)}] - 1.357 \times \log_{10} [\text{haptoglobin (g/L)}] + 1.017 \times \log_{10} [\text{GGT (IU/L)}] + 0.0281 \times \text{Age} + 1.737 \times \log_{10} [\text{bilirubin (}\mu\text{mol/L)}] - 1.1184 \times \text{apolipoprotein (g/L)} + 0.301 \times \text{Sex (male=1, female=0)} - 5.54$ | NA/80%                                                  | Rossi et al. [140]<br>Vali et al. [141]     | 125 / 3385                                                                    |
|                            | Haptoglobin        |                                                                                                                                                                                                                                                                                                                                                                                   |                                                         |                                             |                                                                               |
|                            | $\alpha 2\text{M}$ |                                                                                                                                                                                                                                                                                                                                                                                   |                                                         |                                             |                                                                               |
|                            | Apolipoprotein AI  |                                                                                                                                                                                                                                                                                                                                                                                   |                                                         |                                             |                                                                               |
|                            | Bilirubin          |                                                                                                                                                                                                                                                                                                                                                                                   |                                                         |                                             |                                                                               |
| ADAPT                      | GGT                | $= e^t + \text{Diabetes (yes:1, No:0)}$<br><br>Where $t = \log_{10}[(\text{age} \times \text{Pro-C3}) / \sqrt{\text{Platelets}}]$                                                                                                                                                                                                                                                 | 81% / 81%                                               | Daniels et al. [142]<br>Mak et al. [143]    | 431 / 1833                                                                    |
|                            | Age                |                                                                                                                                                                                                                                                                                                                                                                                   |                                                         |                                             |                                                                               |
|                            | Pro-C3             |                                                                                                                                                                                                                                                                                                                                                                                   |                                                         |                                             |                                                                               |
|                            | Platelets          |                                                                                                                                                                                                                                                                                                                                                                                   |                                                         |                                             |                                                                               |
|                            | Diabetes           |                                                                                                                                                                                                                                                                                                                                                                                   |                                                         |                                             |                                                                               |

Table 1 – Algorithms for the evaluation of liver steatosis and liver fibrosis (continued)

| Components                    |                           | Formula (when available)                                                                                                                                         | Diagnostic accuracy in the general population / obesity | References                                | Number of subjects evaluated for validation In the general population/obesity |
|-------------------------------|---------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------|-------------------------------------------|-------------------------------------------------------------------------------|
| FIBC3                         | Age                       | $= -5.939 + (0.053 \times \text{Age}) + (0.076 \times \text{BMI}) + (1.614 \times \text{T2DM}) - (0.009 \times \text{Platelets}) + (0.071 \times \text{Pro-C3})$ | NA / 83%                                                | Boyle et al. [144]                        | NA / 449                                                                      |
|                               | BMI                       |                                                                                                                                                                  |                                                         |                                           |                                                                               |
|                               | T2DM                      |                                                                                                                                                                  |                                                         |                                           |                                                                               |
|                               | Platelet count            |                                                                                                                                                                  |                                                         |                                           |                                                                               |
|                               | Pro-C3                    |                                                                                                                                                                  |                                                         |                                           |                                                                               |
| Enhanced liver fibrosis (ELF) | Hyaluronic acid           | $= -7.412 + 0.681 \ln[\text{HA} (\mu\text{g/L})] + 0.775 \ln[\text{Pro-C3} (\mu\text{g/L})] + 0.494 \ln[\text{TIMP-1} (\mu\text{g/L})]$                          | 93% / 95%                                               | Guha et al. [145]<br>Karlas et al. [146]  | 192 / 41                                                                      |
|                               | Pro-C3                    |                                                                                                                                                                  |                                                         |                                           |                                                                               |
| AST to platelet ratio (APRI)  | TIMP-1                    |                                                                                                                                                                  |                                                         |                                           |                                                                               |
|                               | AST                       | $= \text{AST (IU/L)} / \text{AST upper limit of normal (IU/L)} / \text{Platelets (x10}^9\text{/L)}$                                                              | 83 % / 82%                                              | Wai et al. [147]<br>Siddiqui et al. [148] | 270 / 1904                                                                    |
|                               | AST upper limit of normal |                                                                                                                                                                  |                                                         |                                           |                                                                               |
| Fibroscan-AST (FAST) score    | Platelet count            |                                                                                                                                                                  |                                                         |                                           |                                                                               |
|                               | LSM                       | $= e^w/(1+e^w)$<br><br>Where $w = -1.65 + 1.07 \times \ln[\text{LSM (kPa)}] + 2.66 \times 10^{-8} \times \text{CAP}^3 - 63.3 \times \text{AST}^{-1}$             | NA / 80%                                                | Newsome et al. [170]                      | NA/350                                                                        |
|                               | CAP                       |                                                                                                                                                                  |                                                         |                                           |                                                                               |
|                               | AST                       |                                                                                                                                                                  |                                                         |                                           |                                                                               |

α2M: Alpha(2)-macroglobin; ALT: serum alanine aminotransferase; AST: serum aspartate transaminase; CAP: Controlled Attenuation Parameter; GGT: Gamma glutamyltransferase; IFG: impaired fasting glucose; LSM: Liver Stiffness Measurement; MetS: metabolic syndrome; Pro-C3: Procollagen III amino terminal peptide; T2DM: Type 2Diabetes Mellitus; TG: triglycerides; TIMP-1: Tissue inhibitor of matrix metalloproteinase

## Imaging modalities for the detection and evaluation of NAFLD/NASH

**Conventional ultrasound imaging** (US) is frequently used to establish liver steatosis. This technique has been included in the guidelines [149] and is widely available at relatively low cost. One of the main disadvantages is the fact that US only provides qualitative information on liver steatosis, but it is not informative in relation to inflammation and fibrosis. This accounts for both adults and children [150]. Moreover, ultrasound is not able to detect mild hepatic steatosis ( $< 30\%$ ) [151] and it has a lower sensitivity (39%) for patients with obesity compared to lean subjects (49.1%) [152–154]. Furthermore, US has a significant interobserver variability [155].

**Magnetic resonance imaging–proton density fat fraction** (MRI–PDFF) is at present considered the best imaging technique providing both qualitative and quantitative information without sampling error as may occur with liver biopsy and transient elastography [154]. MRI–PDFF determines the concentration of triglycerides in organs such as the liver [156]. MRI–PDFF combines two methodologies: the complex-based fitting and the magnitude-based fitting techniques. The fat and water signal fractions are updated after a multi-step nonlinear fitting procedure. The update is based on magnitude signal equations with a multi-peak fat spectral model [157]. Earlier, a comparable method called proton **magnetic resonance spectrometry** (MRS) was developed to determine the lipid volume fractions which seems to be a reliable procedure consistent with computed tomography [158]. However, MRI–PDFF has the best accuracy (i.e. 99%) outperforming all other NILTs [159]. Major drawbacks are the significant costs and limited availability.

**Magnetic resonance elastography** (MRE) uses low frequency mechanical waves in organs. A phase contrast MRI technique models the propagating wave and process the information to create qualitative results defining the mechanical properties. In this way the liver stiffness, and therefore elasticity, is evaluated [160]. In children, MRE showed a good correlation with histology for the evaluation of liver steatosis [161]. In subjects with obesity, MRE may be of added value to MRI–PDFF, for the detection of fibrosis with 93% accuracy [162].

**Transient elastography** (TE) measures the elastic shear wave propagation through the liver parenchyma thereby quantifying the liver fibrosis. TE was developed in the 1990's from the field of seismology

for the food industry and it was adapted in the early 2000s for medical use [163]. There are two types of TE: Shear Wave Elastography (SWE) and Vibration-Controlled Transient Elastography (VCTE). SWE analyses the reflection of a produced shear wave, generated by a focused acoustic beam, to determine the degree of liver fibrosis. The VCTE probe administers shockwaves through a mechanical vibrating part to evaluate liver stiffness which is closely associated with liver fibrosis. VCTE also has an ultrasound transducer to measure the attenuation of the mechanical vibration which is associated with the degree of steatosis. In this way, two measurements are performed to obtain the following results: the degree of liver steatosis using Controlled Attenuation Parameter (CAP expressed in dB/m) and the degree of liver fibrosis (also referred to as elasticity (E) expressed in kPa). According to the European Association for the Study of the Liver (EASL), the European Association for the Study of Diabetes (EASD) and the European Association for the Study of Obesity (EASO), TE is an acceptable non-invasive procedure for the identification of cases at low risk of advanced fibrosis/cirrhosis [111, 149]. In addition, TE can exclude advanced fibrosis and it has been proposed for initial assessment [164]. VCTE correlates well with hepatic histology in both adults and children [165, 166] and it performs better than US for the evaluation of liver steatosis in children [167]. Cut-off values have been determined using matching liver biopsies in 246 patients to classify the degree of fibrosis: 7.2 kPa for significant fibrosis (F2), 8.7 kPa for advanced fibrosis (F3) and 10.3 kPa for cirrhosis (F4) [168]. These values are specific for the M probe but for patients with an increased skin-to-liver capsular distance, e.g. patients with obesity, the XL probe has been developed with its own cut-off values: 7.2 kPa for F3 and 7.9 kPa for F4 [169]. Recently, a combination of TE and AST measurement has been proposed, the Fibroscan-AST (FAST) score [170], to capture better active fibrotic NASH instead of simple steatosis. TE is a non-invasive technique becoming increasingly available in clinical practice [168, 171, 172].

### NILTs in children

The diagnosis and evaluation of NAFLD in children is based on histological findings and can be supported by laboratory measurements, scores and imaging techniques.

Biopsy remains the most accurate method to evaluate NAFLD and to grade its inflammatory and fibrosis component [173]. The main pitfall of liver biopsy is that a small sample (1/50.000 of the liver) is analyzed which can result in misclassification due to the inconsistent character of

NAFLD. Pediatric biopsies, especially those from pre-pubertal boys, may differ from those from adults. The latter are characterized by macrovascular steatosis, hepatocyte ballooning, lobular inflammation and perisinusoidal fibrosis [174]. Histological findings in young patients reveal less ballooning and more portal-based inflammation and fibrosis [175–178].

As started earlier, laboratory findings based on ALT levels do not permit to make any conclusion on the presence and degree of NASH or NAFLD with fibrosis [175, 179–181]. Liver inflammation and NASH may be identified with markers of hepatocyte injury (i.e. cytokeratin 18, leptin and TNF $\alpha$ ) but their accuracy is variable and they are not always available in clinical situations [182–185]. Concerning fibrosis, the number of studies in the young population is very limited. Hyaluronic acid has a fair to good accuracy in detecting minimal-mild and significant fibrosis in children but more validation studies are needed [183, 186]. Plasminogen activator inhibitor-1 (PAI-1) and monocyte chemoattractant protein 1 (MCP-1) may predict advanced fibrosis but this findings have not been validated sufficiently [187].

Scores have also been developed for the pediatric population in order to evaluate NAFLD. Only the Pediatric NAFLD score has been developed to evaluate steatosis [188] but it may be of limited value in clinical practice [189]. The pediatric predictive NASH model has an accuracy of 74% to evaluate NASH in children with NAFLD but it has not been externally validated [190]. Significant liver fibrosis in children cannot be detected accurately using the APRI, NFS or FIB4 score [176, 191]. Only the ELF test may be clinically useful as shown in two pediatric studies evaluating fibrosis compared to liver biopsy [192, 193].

Imaging modalities have been developed and tested to evaluate NAFLD in pediatric patients. Ultrasound is inaccurate to detect fibrosis and unable to detect inflammation in children with NAFLD [150] but it evaluates steatosis with a sensitivity and a specificity from 84% to 94% and from 32% to 70%, respectively in 4 pediatric studies [154, 194–196]. The CAP also showed good performance compared to liver biopsy with an accuracy of 93% in a pediatric study [197]. Magnetic resonance techniques (i.e. MRI, MRS, 1HMRS, MRI-PDFF) are the most accurate imaging methods in adults to detect steatosis and comparable results have been found in pediatric studies [161, 198–201]. A major drawback is the cost of these techniques which makes them less suitable for daily clinical practice.

Finally, 2 pediatric studies showed excellent accuracy to evaluate the presence of any, significant and advanced fibrosis using transient elastography in children with histologically proven NAFLD [166, 202]. The XL probe has not been validated in a pediatric population. Magnetic resonance elastography outperforms TE in adults but a pediatric two-center study showed a fair and good accuracy for detecting any and significant fibrosis respectively in children with biopsy-proven NAFLD [203]. Finally, acoustic radiation force impulse imaging (ARFI) showed a sensitivity and specificity of 79%–82% and of 45%–77%, respectively to establish fibrosis in 2 pediatric studies with children with liver disease other than NAFLD [204, 205].

### Future developments for serum based markers of NAFLD–NASH

Omics-based biomarkers including proteomics, metabolomics and lipidomics are emerging for patients with NAFLD. Different proteins have been identified but none is specific for NASH [206, 207]. Altered levels of bile acids and glutathione have been reported during NAFLD onset [208]. Lipidomic-based markers have also been published [209–211]. These studies are often limited by their size and their ability to distinguish different grades of steatosis. One example is the oxNASH panel which includes different parameters like linoleic acid and 13-HODE (hydroxyoctadecadienoic acid) and has a sensitivity and specificity of 81% and 97%, respectively to detect NASH [212].

Cytokeratin-18 (CK-18) is a promising biomarkers for hepatic apoptosis and NASH. It has a sensitivity and specificity of 66% and 82%, respectively [164]. The combination with fibroblast growth factor 12 (FGF12) improves both, sensitivity and specificity above 90% [213]. In subjects with obesity, CK-18 combined with adiponectin and resistin showed an accuracy between 73% and 91% to detect NASH [214]. The derived-form of CK-18, M30, combined with the serum apoptosis-mediating surface antigen sFas had an accuracy of 79–93% [215].

Several other markers for hepatic fibrosis have also emerged: hyaluronic acid (HA), serum procollagen III amino-terminal peptide (P3NP) and its neo-epitope Pro-C3, and the tissue inhibitor of metalloprotease-1 (TIMP1). HA, represented in the accumulation of extra cellular matrix, has an accuracy of 87% for F2 and of 92% for F4 [216]. P3NP is a reliable biomarker for fibrosis [217] and elevated Pro-C levels correlate with NASH and fibrosis [218]. TIMP1 may be the best biomarker for patients

with obesity with an accuracy of 97% to detect NASH-related fibrosis [219].

Novel developments using non-coding messenger RNAs (mRNAs), long-noncoding RNAs (lncRNAs), circular RNAs (circRNAs) and microRNAs (miRNAs) in patients with NAFLD–NASH are promising [220]. Subjects with severe obesity and NASH show upregulated miR-122 and miR-192 compared to those with simple steatosis [221].

Altogether, so far the best performing marker for the diagnosis of liver fibrosis at an early stage in subjects with obesity seems to be FIB-4. While the search for useful serum markers is still ongoing, promising imaging techniques are being developed like transient elastography which is the most accurate tool for the lowest costs.

## TRANSIENT ELASTOGRAPHY IN THE BARIATRIC WORKUP

The most common problem reported for the measurements with TE is the occurrence of false positive results. This can be partly caused by liver steatosis [222] or, in 20% of the measurements, by generalized obesity with high subcutaneous fat accumulation [223]. A measurement with VCTE can be considered as correctly done, and thus reliable for interpretation, if the interquartile range/median ratio (IQR/M)  $\leq 0.30$  or IQR/M  $> 0.30$  with a liver stiffness evaluation  $< 7.1$  kPa [224]. Different factors also play a role in the determination of the correct value. For example, extrahepatic cholestasis, a limited operator experience and the presence of ascites have been cited as confounding parameters for the measurement [168, 223]. Also in the presence of congestive heart failure, right-sided heart failure, acute inflammation or edema, the results may be less accurate [225–228]. In any event, TE measurement has a high intra- and interobserver level of agreement [229], making it very useful in clinical practice.

An XL probe for the Fibroscan™ has been developed to address the misclassification problem caused by obesity. This new probe aims to lower the failure rate and to improve the accuracy of the measurement. This development brought new problems about the possible cut-off values that physicians need to use. Several studies have shown that the XL probe can be used for a correct evaluation of the liver fibrosis of patients

with obesity, with cut-off values being generally 1.5 to 2.0 kPa lower than with the M probe [223, 230, 231]. There is some literature aiming to define the position of TE for the diagnosis of fibrosis or steatosis stage but, to the best of our knowledge, little is known about the XL probe for patients enrolled for bariatric surgery [232].

European guidelines do not provide any cut-off values for different stages of steatosis using transient elastography. For fibrosis the following cut off values have been recommended: 7.2 kPa for F3 and 7.9 kPa for F4 when using a XL probe. However, these recommendations are based on the study of individuals without obesity [111, 149].

We identified 12 recent studies which evaluated NASH with an XL probe in patients referred for bariatric surgery [233–244] (Table 2 and Table 3). Ten provided cut-off values for steatosis stages and 9 for fibrosis stages. Based on these studies, the best defined stages for steatosis were S1, S2 and S3 with cut-off values from 214 to 330.5 dB/m, 251 to 393 dB/m and 296 to 360 dB/m, respectively. The fibrosis stages identified as F2, F3 and F4 were best defined with cut-off values from 6.3 to 9.0 kPa, 7.6 to 12.6 kPa and 12.5 to 14.25 kPa, respectively. Ten out of the 12 studies confirmed their findings with liver biopsies.

Several reports used references from previous studies [168, 233, 244–246], others included a relatively low number of histological validations [234] or they defined different cutoff values for the same stage of liver steatosis or fibrosis [235]. Different factors may also affect the TE measurement and its accuracy: the presence of diabetes mellitus, high triglyceride levels, a great probe-to-capsule distance and a higher BMI have been mentioned.

The cut-off values proposed in these studies differ from those mentioned by the European guidelines. While the cut off values identified are supported by several studies, the numbers of subjects included is still relatively low, especially when considering the fact that validation with liver biopsies is necessary. Therefore, larger studies including liver biopsy are still necessary.

Table 2 – Cut-off values for steatosis

| Study                         | Cutoff (dB/m) | Sensitivity | Specificity | AUROC |
|-------------------------------|---------------|-------------|-------------|-------|
| Steatosis stage $\geq$ S1     |               |             |             |       |
| Wan et al. (2021)[234]        | 326           | nc          | nc          | nc    |
| Agarwal et al. (2020)1 [235]  | 330,5         | 0,672       | 0,667       | 0,73  |
| Agarwal et al. (2020)2 [235]  | 276           | 0,909       | 0,717       | 0,87  |
| Barsamian et al. (2020) [237] | 294           | 0,77        | 0,68        | 0,77  |
| Shalimar et al. (2020) [236]  | 285           | 0,928       | 0,875       | 0,96  |
| Somda et al (2019) [239]      | 255           | 0,88        | 0,76        | 0,83  |
| Eddowes et al (2019) [241]    | 302           | 0,80        | 0,83        | 0,87  |
| Naveau et al (2017) [238]     | 298           | 0,78        | 0,83        | 0,81  |
| de Barros et al (2016) [233]  | 214           | nc          | nc          | nc    |
| Steatosis stage $\geq$ S2     |               |             |             |       |
| Eilenberg et al. (2021) [242] | 350           | nc          | nc          | 0,703 |
| Wan et al. (2021) [234]       | 393           | 1           | 0,43        | nc    |
| Agarwal et al. (2020)1 [235]  | 350           | 0,647       | 0,638       | 0,67  |
| Agarwal et al. (2020) 2 [235] | 321           | 0,667       | 0,815       | 0,71  |
| Barsamian et al. (2020) [237] | 326           | 0,74        | 0,75        | 0,78  |
| Shalimar et al. (2020) [236]  | 340           | 0,652       | 0,644       | 0,667 |
| Somda et al. (2019) [239]     | 288           | 0,81        | 0,73        | 0,86  |
| Eddowes et al. (2019) [241]   | 331           | 0,70        | 0,76        | 0,77  |
| Garg et al. (2018) [240]      | 336           | 0,739       | 0,755       | 0,74  |
| Naveau et al. (2017) [238]    | 303           | 0,90        | 0,69        | 0,83  |
| de Barros et al. (2016) [233] | 251           | nc          | nc          | nc    |
| Steatosis stage $\geq$ S3     |               |             |             |       |
| Eilenberg et al. (2021) [242] | 353,5         | nc          | nc          | 0,738 |
| Wan et al. (2021) [234]       | 360           | nc          | nc          | nc    |
| Agarwal et al. (2020) 1 [235] | 358,5         | 0,889       | 0,709       | 0,734 |
| Naveau et al. (2017) [238]    | 326           | 0,83        | 0,71        | 0,84  |
| Shalimar et al. (2020) [236]  | 355           | 0,778       | 0,673       | 0,724 |
| Somda et al. (2019) [239]     | 297           | 0,66        | 0,59        | 0,79  |
| Eddowes et al. (2019) [241]   | 337           | 0,72        | 0,63        | 0,70  |
| Garg et al. (2018) [240]      | 357           | 1           | 0,778       | 0,82  |
| de Barros et al. (2016) [233] | 296           | nc          | nc          | nc    |

1: pre-operative measurement; 2: post-operative measurement; nc: not communicated; AUROC: Area Under the Receiver Operating Characteristic

Table 3 – Cut-off values for elasticity

| Study                                               | Cutoff (kPa) | Sensitivity | Specificity | AUROC |
|-----------------------------------------------------|--------------|-------------|-------------|-------|
| <b>Fibrosis stage <math>\geq</math> F2</b>          |              |             |             |       |
| Eilenberg et al. (2021) <sup>[242]</sup>            | 6,3          | 0,788       | 0,537       | 0,687 |
| Agarwal et al. (2020) <sup>1</sup> <sup>[235]</sup> | 7,95         | 0,738       | 0,689       | 0,747 |
| Agarwal et al. (2020) <sup>2</sup> <sup>[235]</sup> | 7,5          | 0,667       | 0,75        | 0,834 |
| Eddowes et al. (2019) <sup>[241]</sup>              | 8,2          | 0,71        | 0,70        | nc    |
| Garg et al. (2018) <sup>[240]</sup>                 | 7,25         | 0,7         | 0,587       | 0,65  |
| Naveau et al. (2014) <sup>[243]</sup>               | 7,6          | 0,73        | 0,78        | 0,81  |
| Wan et al. (2021) <sup>[234]</sup>                  | 9,0          | nc          | nc          | nc    |
| Barsamian et al. (2020) <sup>[237]</sup>            | 8,1          | nc          | nc          | 0,83  |
| Weiss et al. (2016) <sup>[244]</sup>                | 7,1          | nc          | nc          | nc    |
| de Barros et al. (2016) <sup>[233]</sup>            | 7,9          | nc          | nc          | nc    |
| <b>Fibrosis stage <math>\geq</math> F3</b>          |              |             |             |       |
| Eilenberg et al. (2021) <sup>[242]</sup>            | 12,6         | 0,538       | 0,915       | 0,786 |
| Agarwal et al. (2020) <sup>1</sup> <sup>[235]</sup> | 8,95         | 0,895       | 0,759       | 0,854 |
| Agarwal et al. (2020) <sup>2</sup> <sup>[235]</sup> | 12           | 0,6         | 0,616       | 0,835 |
| Eddowes et al. (2019) <sup>[241]</sup>              | 9,7          | 0,71        | 0,75        | 0,80  |
| Garg et al. (2018) <sup>[240]</sup>                 | 12,5         | 0,636       | 0,877       | 0,83  |
| Naveau et al. (2014) <sup>[243]</sup>               | 7,6          | 1           | 0,74        | 0,85  |
| Barsamian et al. (2020) <sup>[237]</sup>            | 8,7          | nc          | nc          | 0,90  |
| Weiss et al. (2016) <sup>[244]</sup>                | 9,5          | nc          | nc          | nc    |
| de Barros et al. (2016) <sup>[233]</sup>            | 9,6          | nc          | nc          | nc    |
| <b>Fibrosis stage <math>\geq</math> F4</b>          |              |             |             |       |
| Agarwal et al. (2020) <sup>1</sup> <sup>[235]</sup> | 14,25        | 0,889       | 0,873       | 0,916 |
| Eddowes et al. (2019) <sup>[241]</sup>              | 13,6         | 0,85        | 0,79        | 0,89  |
| Weiss et al. (2016) <sup>[244]</sup>                | 12,5         | nc          | nc          | nc    |

1: pre-operative measurement; 2: post-operative measurement; nc: not communicated; AUROC: Area Under the Receiver Operating Characteristic

## CONCLUSION

Obesity as a chronic disease is a worldwide growing problem which is associated with comorbidities. The occurrence of NAFLD/NASH is clearly related to obesity and almost ubiquitous in severe obesity. NAFLD/NASH can lead to severe complications such as cirrhosis or HCC. An early diagnosis of NAFLD/NASH is warranted to prevent progression and severe complications. Several diagnostic tools are available to detect and quantify fibrosis. Biomarkers are the first choice to distinguish the major part of patients with a mild form of NAFLD from those at risk for liver fibrosis. For this purpose, algorithms are simple, applicable and also inexpensive tests that can be used to recognize serious cases of fibrosis. In addition, technological development currently allows to help establish the diagnosis, especially for patients with an indeterminate score. Different techniques are available to monitor steatosis and fibrosis in patients with severe obesity before and after bariatric surgery. Nevertheless, some questions remain regarding the availability, the ease of use, the cost and the correct use of cut-off values for each method.

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# Chapter 2

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## Evaluation of the prevalence of MASLD, MASH and liver fibrosis in a Dutch bariatric cohort

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## ABSTRACT

### Background & Aims

The prevalence of metabolic dysfunction-associated steatotic liver disease (MASLD) and metabolic dysfunction-associated steatohepatitis (MASH) in bariatric populations has been widely studied but may vary geographically. This study evaluates MASLD/MASH prevalence and the utility of non-invasive tests (NITs) for liver fibrosis in a Dutch bariatric surgery cohort.

### Methods

This single-center cross-sectional diagnostic accuracy study included 220 patients undergoing bariatric surgery. At baseline, 10 NITs were performed. Patients with liver stiffness measurements  $\geq 8$  kPa using vibration-controlled transient elastography underwent a liver biopsy during surgery. Histology was assessed using the nonalcoholic fatty liver disease Activity Score. Diagnostic accuracy of NITs was evaluated against histology using sensitivity, specificity, and area under the receiver operating characteristic (AUROC). MASH was defined as steatosis with lobular inflammation and ballooning, with or without fibrosis. At-risk MASH included fibrosis  $\geq$  F2.

### Results

Out of 77 patients (35%) eligible for histological analysis, the findings revealed a MASLD prevalence of 50.6%, MASH prevalence of 5.3%, and at-risk MASH prevalence of 2.6%. Most patients had no fibrosis (67.1%), while others exhibited mild fibrosis (F1: 23.7%, F2: 9.2%). Capped MAF-5 effectively identified fibrosis stage  $\geq 2$  (AUROC: 0.809), surpassing FIB-4 (AUROC: 0.645). Both the FAST score and capped MAF-5 demonstrated strong performance in detecting at-risk MASH.

### Conclusion

MASLD/MASH prevalence and advanced fibrosis were lower than expected in this Dutch cohort. Capped MAF-5 demonstrated superior performance for fibrosis detection, while transient elastography and FIB-4 were less reliable. Further studies are needed to optimize NIT selection in bariatric populations.

## INTRODUCTION

Obesity is a worldwide epidemic with an increasing prevalence. Recent data predict a rise of individuals with obesity up to 2 billion in 2035 [1]. This rise is closely linked to the development of obesity-related comorbidities [2]. A partial list includes conditions such as type 2 diabetes mellitus (T2D), cardiovascular disease (CVD) and metabolic dysfunction-associated steatotic liver disease (MASLD). The latter is the most common chronic liver disease and is also associated with T2D and other cardiometabolic risk factors [3]. MASLD encompasses a spectrum of liver conditions, ranging from simple steatosis to metabolic dysfunction-associated steatohepatitis (MASH), which can progress to liver fibrosis and cirrhosis [4]. It is associated with hepatic events such as chronic liver failure and hepatocellular carcinoma (HCC) but also with extra hepatic outcomes like chronic kidney disease, cardiovascular disease, heart failure or colorectal carcinoma [5–9]. At least, 90% of the patients with severe obesity undergoing bariatric surgery may have MASLD [10,11] but data on European subjects are scarce [12,13].

The global burden of MASLD is high. High-risk groups, such as patients with obesity should undergo active screening for liver fibrosis associated with MASLD. Consequently, three scientific societies, the European Association for the Study of Liver, the European Association for the Study of Diabetes, and the European Association for the Study of Obesity, have proposed a strategy for non-invasive assessment of the risk for advanced fibrosis [14]. Although liver biopsies provide valuable information, they are not frequently performed due to associated risks and sampling error. Vibration controlled transient elastography (VCTE) is a good alternative for early diagnosis of liver fibrosis and has shown high accuracy in people with obesity [15]. The severity of liver fibrosis determines the risk of liver-related and cardiovascular outcomes [16].

At present, resmetirom is the only approved drug for treating adults with MASLD in the US. Despite the availability of medication, the primary treatment for MASLD remains weight loss and bariatric surgery is the most effective intervention for reducing body weight [17, 18]. Multi-disciplinary experts agree that bariatric surgery should be recommended for individuals with MASH and severe obesity, while they also acknowledge that MASH or advanced liver fibrosis increase the risk of complications [19].

In this study, we aim to investigate the prevalence of MASLD and MASH in a Dutch cohort scheduled for bariatric surgery and assess the diagnostic value of noninvasive tests (NITs) to determine fibrosis risk in this population. These objectives will be addressed through the Franciscus Obesity NASH Study, as previously described [20].

## METHODS AND ANALYSIS

### Participants

Participants were recruited from the regular bariatric surgery program from the Obesity Center of the Franciscus Gasthuis Hospital in Rotterdam, the Netherlands, between 1 August 2022 and 15 November 2023. Participants fulfilled the criteria of the International Federation for the Surgery of Obesity and Metabolic Disorders at screening [21]. To participate in this study, subjects had to speak and read Dutch in order to provide written informed consent. This study was conducted according to the principles of the Declaration of Helsinki (version 2013), the Guideline for Good Clinical Practice (E6(R2)) and in accordance with the Medical Research Involving Human Subjects Act (WMO) and General Data Protection Regulation. The study was approved by the regional Medical Ethics Committee (MEC-U, Utrecht, The Netherlands) and the Institutional Review Board (IRB) of Stichting Sint Franciscus Vlietland Groep. The study is registered in the International Clinical Trial Registry Platform (ICTRP) (NL79423.100.21).

Patients who met any of the following criteria at screening could not take part to the study: (1) participants younger than 18 years or older than 65 years, (2) known with an established diagnosis of another primary liver disease, (3) alcohol consumption (>20g alcohol/day for woman and >30 g alcohol/day for men as defined in the local protocol), (4) histologically documented liver cirrhosis at screening or in a historical biopsy, (5) known with active HIV infection and/or treatment and (6) known with any type of diagnosed malignancies with or without active treatment.

### Bariatric surgery

The type of surgery—laparoscopic sleeve gastrectomy (LSG), Roux-en-Y gastric bypass (RYGB), one-anastomosis gastric bypass (OAGB), or Nissen sleeve gastrectomy (NSG)—was determined by the surgeon in consultation with the patient. There was no randomization of the procedure allocation.

## Outcome measures

Clinical data such as anthropometric measurements, blood tests and medical history were recorded.

The main outcome is to evaluate the prevalence of MASLD and the severity of liver fibrosis in a bariatric cohort. According to the available data, we evaluated the degree of liver fibrosis using TE, the Fibrosis-4 (FIB-4) index [22, 23], the baseline metabolic dysfunction-associated fibrosis 5 (MAF-5) score [24], the capped MAF-5 score at BMI >35 and over [25], aspartate aminotransferase (AST) to platelet ratio (APRI) [26], the non-alcoholic fatty liver disease (NAFLD) fibrosis score (NFS) [27], and the Fibroscan-based Agile scores [28]. These scores were defined as follows:

$$\text{FIB-4 index} = (\text{Age} * \text{AST}) / (\text{platelets} * \sqrt{\text{ALT}})$$

$$\begin{aligned} \text{Baseline MAF-5} = & -11,3674 + \text{waist circumference} * 0,0282 - \text{BMI} * \\ & 0,1761 + \text{waist circumference} * \text{BMI} * 0,0019 + 2,0762 * \text{diabetes} + \\ & \ln(\text{AST}) * 2,9207 - \text{platelets} * 0,0059 \end{aligned}$$

$$\begin{aligned} \text{Capped MAF-5} = & -11,3674 + \text{waist circumference} * 0,0282 - 35 * 0,1761 \\ & + \text{waist circumference} * 35 * 0,0019 + 2,0762 * \text{diabetes} + \ln(\text{AST}) * \\ & 2,9207 - \text{platelets} * 0,0059 \end{aligned}$$

$$\text{APRI} = (\text{AST}/(\text{AST}(\text{ULN}))) / \text{platelets}$$

$$\begin{aligned} \text{NFS} = & -1,675 + 0,037 * \text{Age} + 0,094 * \text{BMI} + 1,13 * \text{diabetes} + 0,99 * \\ & \text{AST}/\text{ALT} - 0,013 * \text{platelets} - 0,66 * \text{albumin} \end{aligned}$$

$$\begin{aligned} \text{Agile 3+} = & e^{(-.3,92368 + 2,29714 * \ln(\text{LSM}) - 0,00902 * \text{platelets} - \\ & 0,98633 * \text{AST}/\text{ALT} + 1,08636 * \text{diabetes} - 0,38581 * \text{male} + 0,03018 \\ & * \text{age})} / (1 + e^{(-.3,92368 + 2,29714 * \ln(\text{LSM}) - 0,00902 * \text{platelets} \\ & - 0,98633 * \text{AST}/\text{ALT} + 1,08636 * \text{diabetes} - 0,38581 * \text{male} + 0,03018 * \\ & \text{age})) \end{aligned}$$

$$\begin{aligned} \text{Agile 4} = & e^{(7,50139 - 15,42498 * 1/\sqrt{\text{LSM}} - 0,01378 * \text{platelets} - 1,41149 \\ & * \text{ALT}/\text{AST} - 0,53281 * \text{male} + 0,41741 * \text{diabetes})} / (1 + e^{(7,50139 - \\ & 15,42498 * 1/\sqrt{\text{LSM}} - 0,01378 * \text{platelets} - 1,41149 * \text{ALT}/\text{AST} - 0,53281 \\ & * \text{male} + 0,41741 * \text{diabetes})) \end{aligned}$$

The capped MAF-5 is identical to the baseline MAF-5, but it applies a BMI cap of  $35 \text{ kg.m}^{-2}$  to reduce false positive cases resulting from overestimation caused by a high BMI. An elevated FIB-4 value of  $\geq 1.3$ , a baseline and capped MAF-5 value of  $\geq 0$ , an APRI  $\geq 0.918$  or a NFS  $> 0.676$  were used as a surrogate marker for significant liver fibrosis (F2) as defined by Kleiner et al. [29]. A cutoff value of 0.679 was applied to rule in advanced fibrosis (F3) using the Agile 3+ score, while a cutoff value of 0.565 was used to rule in cirrhosis (F4) with the Agile 4 score, as proposed by Sanyal et al. [28].

### Fibroscan measurements and MASLD definition

VCTE (Fibroscan<sup>®</sup>, Echosens SA, Paris, France) with liver-controlled attenuation parameter (CAP) was used to quantify liver steatosis, while liver stiffness measurement (LSM) was used to assess the degree of liver fibrosis. A CAP  $\geq 275 \text{ dB/m}$  was chosen to define MASLD and a LSM  $\geq 8 \text{ kPa}$  defined significant fibrosis.

According to manufacturer's instructions, VCTE was performed on the lying participants, placing the probe in the intercostal space, close to the right lobe of the liver [30, 31]. At least ten valid measurements were done by an operator trained and certified by Echosens. As published elsewhere, the interquartile range – median ratio had to be  $< 30\%$  to consider the measurements as successful [32]. Outside these conditions, the measurements were deemed failures and were not used for analysis. VCTE was performed no more than two weeks prior to surgery.

According to the literature available at the time of protocol submission, a preoperative liver elasticity  $\geq 7.95 \text{ kPa}$  was chosen as the threshold for performing a perioperative liver biopsy. The biopsy was obtained from the diaphragmatic surface of segment three or five of the liver and the size varied between 1 and 3 cm in length and between 1.2 and 2 mm in diameter. Biopsies were assessed for histology (paraffin embedded) and two pathologists determined and individually scored the MASLD/MASH status using the NAFLD Activity Score (NAS) [29]. In case of disagreement on the classification, a third pathologist was consulted and the score set by 2 out of 3 pathologists was recorded.

Participants with liver steatosis  $> 5\%$  automatically met the definition of MASLD due to the presence of obesity as a cardiometabolic risk factor. Both a clinical approach using LSM and a histological approach with liver biopsies were employed to analyze MASLD in our cohort.

MASH was histologically defined by the presence of steatosis, lobular inflammation, and ballooning, with a NAS score of  $\geq 4$ , with or without fibrosis. Cases with significant or advanced fibrosis ( $F \geq 2$ ) on histological evaluation were classified as at-risk MASH.

At-risk MASH was predicted with a Fibroscan AST (FAST) score  $\geq 0,35$ , as described by Newsome et al. [33], or a Fibrotic NASH index (FNI) of  $\geq 0,33$  [34]. Therefore we used the following formulas:

$$\text{FAST} = e^{(-1,65 + 1,07 * \ln(\text{LSM}) + 2,66 * 10^{-8} * \text{CAP3} - 63,3 * \text{AST}^{-1})} / (1 + e^{(-1,65 + 1,07 * \ln(\text{LSM}) + 2,66 * 10^{-8} * \text{CAP3} - 63,3 * \text{AST}^{-1})})$$

$$\text{FNI} = e^{(-10,33 + 2,54 * \ln(\text{AST}) + 3,86 * \ln(\text{HbA1c}) - 1,66 * (\text{HDL}))} / (1 + e^{(-10,33 + 2,54 * \ln(\text{AST}) + 3,86 * \ln(\text{HbA1c}) - 1,66 * (\text{HDL}))})$$

At last, baseline and capped MAF-5 scores were also used in the evaluation of the detection of at-risk MASH cases.

## Sample size

A sample size calculation was performed based on previous studies including TE measurements, epidemiological data, and dropout rates during bariatric follow-up. The sample size calculation for the entire study has been previously conducted and extensively detailed in a prior publication [20].

## Statistical analysis

Continuous variables were evaluated using the two-tailed independent t-test when normally distributed and were expressed as means with standard deviations. Categorical variables were assessed using the Chi-square test and expressed in terms of frequency and percentage. Outliers were not removed. The diagnostic value of NITs was evaluated by calculating sensitivity, specificity, positive predictive value, negative predictive value, and the area under the receiver operating characteristic curve (AUROC), compared to the NAS score from biopsies. We used the highest Youden's index to determine the best cut-off value from the AUROC. Statistical analysis was performed using IBM SPSS version 29 (IBM Corporation, Armonk, New York, USA). P values  $< 0,05$  (two-tailed) were considered statistically significant.

## RESULTS

### Baseline characteristics

A total of 220 patients were recruited between August 2022 and November 2023, all of whom underwent bariatric surgery with a valid preoperative VCTE assessment. The mean age was 43,2 years ( $\pm 11,5$ ), and the mean BMI was 42,9 kg/m<sup>2</sup> ( $\pm 5,7$ ). The majority were women (80,5%). Most patients were of Caucasian ethnicity (74,5%). At baseline, 36 patients (16,3%) had diabetes mellitus (1,8% with type 1 and 14,5% with type 2), 64 (29,1%) had hypertension, and 82 (37,3%) had dyslipidemia (see Table 1).

The mean CAP was 283 dB/m ( $\pm 50,5$ ) and the mean LSM was 8.25 kPa ( $\pm 4,49$ ). A total of 42,3% of participants had an LSM  $\geq 8$  kPa. Table 2 shows the mean NIT scores.

### Risk factors for MASLD

Based on VCTE, individuals with MASLD were significantly older, with the majority being male, and exhibited greater waist circumference despite having a comparable BMI, indicating central adiposity. They also had higher levels of ALT, triglycerides, and HbA1c, despite a comparable proportion of individuals with diabetes or dyslipidemia.

Based on histology, individuals with MASLD were predominantly male, but no differences were found in other demographic characteristics. The laboratory measurements were characterized by higher levels of transaminases, cholesterol/HDL ratio, triglycerides, ferritin, and iron, suggesting possible inflammation.

Baseline differences are shown in Table 1.

In both the VCTE and histology groups, several parameters (baseline MAF-5, Capped MAF-5, CAP, FAST score, and FNI) demonstrated significant differences between individuals with and without MASLD. The APRI was higher in the histology-based MASLD group, with a greater percentage of patients with fibrosis stage  $F \geq 2$ . The Agile 3+ score was also elevated in the TE-based MASLD group; however, the proportion of participants with  $F \geq 3$  did not differ significantly. Other parameters, such as FIB-4, NFS, LSM, and Agile 4, did not show significant differences between the groups. Results of the non-invasive tests (NITs) are presented in Table 2.

Table 1 – Baseline characteristics of the FONS population based on VCTE and liver histology.

| Demographics            | Based on VCTE |              |              |         | Based on histology |              |              |
|-------------------------|---------------|--------------|--------------|---------|--------------------|--------------|--------------|
|                         | Total         | No MASLD     | MASLD        | P-value | Total              | No MASLD     | MASLD        |
| n (%)                   | 220 (100%)    | 105 (47,7%)  | 115 (52,3%)  |         | 77 (100%)          | 38 (49,3%)   | 39 (50,6%)   |
| Age, years ±SD          | 43,2 ± 11,5   | 40,7 ± 11,1  | 45,5 ± 11,4  | 0,002   | 44,7 ± 11,0        | 43,4 ± 12,2  | 45,7 ± 9,7   |
| Women, n(%)             | 177 (80,5%)   | 92 (87,6%)   | 85 (73,9%)   | 0,010   | 58 (75,3%)         | 33 (86,8%)   | 25 (64,1%)   |
| Height, cm              | 169,3 ± 8,2   | 168,5 ± 7,6  | 170,1 ± 8,6  | 0,158   | 170,3 ± 8,8        | 168,4 ± 9,3  | 172,3 ± 8,0  |
| Weight, kg              | 123,3 ± 20,9  | 121,5 ± 19,0 | 124,8 ± 22,5 | 0,238   | 125,8 ± 21,5       | 122,0 ± 17,4 | 129,5 ± 24,5 |
| Waist circumference, cm | 130,5 ± 13,5  | 127,9 ± 13,4 | 132,7 ± 13,3 | 0,008   | 132,5 ± 13,2       | 131,7 ± 12,2 | 133,4 ± 14,1 |
| BMI, kg.m <sup>-2</sup> | 42,9 ± 5,7    | 42,7 ± 5,3   | 43,0 ± 6,00  | 0,674   | 43,2 ± 5,9         | 43,0 ± 4,5   | 43,5 ± 7,1   |
| Etnicity, n (%)         |               |              |              | 0,486   |                    |              | 0,212        |
| Hindu                   | 8 (3,6%)      | 5 (4,8%)     | 3 (2,6%)     |         | 2 (2,6%)           | 1 (2,6%)     | 1 (2,6%)     |
| Asian                   | 1 ( 0,5%)     | 1 (1,0%)     | 0 (0%)       |         | 1 (1,3%)           | 1 (2,6%)     | 0 (0%)       |
| Caucasian               | 164 (74,5%)   | 73 (69,5%)   | 91 (79,1%)   |         | 57 (74,0%)         | 27 (71,1%)   | 30 (76,9%)   |
| Latino-american         | 2 (0,9%)      | 0 (0%)       | 2 (1,7%)     |         | 0 (0%)             | 0 (0%)       | 0 (0%)       |
| North African           | 9 (4,1%)      | 5 (4,8%)     | 4 (3,5%)     |         | 0 (0%)             | 0 (0%)       | 0 (0%)       |
| Other African           | 14 (6,4%)     | 9 (8,6%)     | 5 (4,3%)     |         | 9 (11,7%)          | 7 (18,4%)    | 2 (5,1%)     |
| Turkish                 | 11 (5%)       | 6 (5,7%)     | 5 (4,3%)     |         | 3 (3,9%)           | 0 (0%)       | 3 (7,7%)     |
| Other                   | 11 (5%)       | 6 (5,7%)     | 5 (4,3%)     |         | 5 (6,5%)           | 2 (5,3%)     | 3 (7,7%)     |
| Diabetes, n(%)          | 36 (16,3%)    | 15 (14,3%)   | 21 (18,3%)   | 0,426   | 21 (27,3%)         | 8 (21,1%)    | 13 (33,3%)   |
| No diabetes             | 184 (83,6%)   | 90 (85,7%)   | 94 (81,7%)   |         | 56 (72,7%)         | 30 (78,9%)   | 26 (66,7%)   |
| Type 1                  | 4 (1,8%)      | 3 (20,0%)    | 1 (4,8%)     |         | 1 (1,3%)           | 1 (2,6%)     | 0 (0%)       |
| Type 2                  | 32 (14,5%)    | 12 (80,0%)   | 20 (95,2%)   |         | 20 (26,0%)         | 7 (18,4%)    | 13 (33,3%)   |

Table 1 – Baseline characteristics of the FONS population based on VCTE and liver histology. (continued)

|                                        |               | Based on VCTE |               |        | Based on histology |              |        |
|----------------------------------------|---------------|---------------|---------------|--------|--------------------|--------------|--------|
| Hypertension, n(%)                     | 6,4 (29,1%)   | 25 (23,8%)    | 39 (33,9%)    | 0,099  | 30 (39,0%)         | 13 (34,2%)   | 0,399  |
| Dyslipidemia, n(%)                     | 8,2 (37,3%)   | 4,0 (38,1%)   | 4,2 (36,5%)   | 0,809  | 30 (39,0%)         | 10 (26,3%)   | 0,025  |
| OSAS, n(%)                             | 25 (11,4%)    | 14 (13,3%)    | 11 (9,6%)     | 0,379  | 5 (6,5%)           | 1 (2,6%)     | 0,175  |
| <b>Laboratory measurements</b>         |               |               |               |        |                    |              |        |
| Creatinine, µmol/L                     | 71,4 ± 28,8   | 68,8 ± 12,7   | 73,8 ± 37,8   | 0,197  | 75,9 ± 45,1        | 67,8 ± 8,7   | 0,119  |
| ALT, U/L                               | 32,2 ± 21,5   | 29,0 ± 20,9   | 35,2 ± 21,6   | 0,032  | 35,8 ± 28,4        | 24,2 ± 9,4   | <0,001 |
| AST, U/L                               | 27,4 ± 11,6   | 26,1 ± 12,2   | 28,5 ± 11,0   | 0,133  | 30,0 ± 16,2        | 23,4 ± 4,4   | <0,001 |
| Cholesterol, mmol/L                    | 5,19 ± 1,03   | 5,06 ± 0,96   | 5,31 ± 1,09   | 0,068  | 5,1 ± 1,2          | 4,9 ± 1,1    | 0,105  |
| LDL-cho, mmol/L                        | 3,18 ± 0,87   | 3,13 ± 0,83   | 3,23 ± 0,91   | 0,435  | 3,10 ± 0,92        | 3,01 ± 0,88  | 0,349  |
| HDL-cho, mmol/L                        | 1,21 ± 0,30   | 1,24 ± 0,33   | 1,19 ± 0,26   | 0,217  | 1,16 ± 0,26        | 1,22 ± 0,27  | 0,046  |
| Chol/HDL                               | 4,48 ± 1,25   | 4,32 ± 1,31   | 4,63 ± 1,17   | 0,069  | 4,50 ± 1,16        | 4,06 ± 0,87  | <0,001 |
| Triglycerides, mmol/L                  | 1,84 ± 1,15   | 1,57 ± 1,01   | 2,09 ± 1,21   | <0,001 | 1,84 ± 1,14        | 1,40 ± 0,64  | <0,001 |
| Transferrine, g/L                      | 2,81 ± 0,39   | 2,75 ± 0,40   | 2,87 ± 0,39   | 0,022  | 2,83 ± 0,40        | 2,84 ± 0,47  | 0,771  |
| Ferritine, µg/L                        | 110,3 ± 103,5 | 100,9 ± 106,1 | 119,0 ± 100,8 | 0,194  | 126,7 ± 111,9      | 89,2 ± 70,3  | 0,003  |
| Iron, umol/L                           | 15,1 ± 5,6    | 14,8 ± 5,6    | 15,4 ± 5,6    | 0,381  | 14,9 ± 4,7         | 13,8 ± 4,4   | 0,041  |
| HbA1c, mmol/mol                        | 41,5 ± 10,5   | 39,4 ± 9,4    | 43,4 ± 11,1   | 0,005  | 45,1 ± 13,9        | 42,6 ± 11,2  | 0,132  |
| Albumin, g/L                           | 45,6 ± 2,6    | 45,4 ± 2,7    | 45,8 ± 2,5    | 0,260  | 45,9 ± 2,5         | 45,3 ± 2,5   | 0,040  |
| Hemoglobin, mmol/L                     | 8,7 ± 0,9     | 8,6 ± 0,9     | 8,8 ± 0,8     | 0,030  | 8,8 ± 0,8          | 8,6 ± 0,8    | 0,115  |
| Hematocrite, L/L                       | 0,04          | 0,42 ± 0,04   | 0,43 ± 0,04   | 0,013  | 0,43 ± 0,04        | 0,42 ± 0,04  | 0,119  |
| Platelets, x10 <sup>9</sup> /L         | 301,2 ± 66,9  | 302,9 ± 61,4  | 299,6 ± 71,8  | 0,723  | 296,2 ± 74,5       | 299,1 ± 74,6 | 0,742  |
| White blood cells, x10 <sup>9</sup> /L | 8,2 ± 2,2     | 8,4 ± 2,3     | 8,1 ± 2,1     | 0,366  | 8,3 ± 2,2          | 8,7 ± 2,1    | 0,144  |

Data are given as mean (SD). OSAS: obstructive sleep apnea syndrome; ALT: alanine transaminase; AST: aspartate transaminase; LDL: low density lipoprotein; HDL: high-density lipoprotein. Results are presented as numbers and percentages, or mean and standard deviation. When based on VCTE, MASLD was established based on a CAP value ≥275 dB/m.

Table 2 – Results of noninvasive tests of the FONS cohort based on VCTE and liver histology.

| Non invasive tests            | Based on VCTE     |                   |                   |         | Based on histology |                |                |         |
|-------------------------------|-------------------|-------------------|-------------------|---------|--------------------|----------------|----------------|---------|
|                               | Total             | No MASLD          | MASLD             | P-value | Total              | No MASLD       | MASLD          | P-value |
| N (%)                         | 220 (100%)        | 105 (47,7%)       | 115 (52,3%)       |         | 77 (100%)          | 38 (49,3%)     | 39 (50,6%)     |         |
| FIB-4 index ± SD<br>(=211)    | 0,768 ±<br>0,337  | 0,723 ±<br>0,344  | 0,801 ± 0,326     | 0,063   | 0,854 ±<br>0,396   | 0,812 ± 0,432  | 0,894 ± 0,361  | 0,382   |
| <1,3, n (%)                   | 193 (91,5%)       | 94 (92,2%)        | 99 (90,8%)        | 0,197   | 63 (86,3%)         | 30 (85,7%)     | 33 (86,8%)     | 0,889   |
| ≥ 1,3 and <2,67, n<br>(%)     | 18 (8,5%)         | 8 (7,8%)          | 10 (9,2%)         |         | 10 (13,7%)         | 5 (14,3%)      | 5 (13,2%)      |         |
| ≥ 2,67, n (%)                 | 0 (0%)            | 0 (0%)            | 0 (0%)            |         | 0 (0%)             | 0 (0%)         | 0 (0%)         |         |
| Baseline MAF-5 ± SD<br>(=211) | 3,284 ±<br>2,365  | 2,758 ±<br>2,440  | 3,777 ± 2,191     | 0,002   | 3,990 ±<br>2,505   | 3,125 ± 2,348  | 4,787 ± 2,405  | 0,004   |
| <0, n (%)                     | 10 (4,7%)         | 9 (8,8%)          | 1 (0,9%)          | <0,001  | 1 (1,4%)           | 1 (2,9%)       | 0 (0%)         | 0,172   |
| ≥ 0 and <1, n (%)             | 27 (12,8%)        | 19 (18,6%)        | 8 (7,3%)          |         | 10 (13,9%)         | 7 (20,0%)      | 3 (7,9%)       |         |
| ≥ 1, n (%)                    | 174 (82,5%)       | 74 (72,5%)        | 100 (91,7%)       |         | 61 (84,7%)         | 27 (77,1%)     | 35 (92,1%)     |         |
| Capped MAF-5 ± SD<br>(=211)   | -0,546 ±<br>1,742 | -0,919 ±<br>1,811 | -0,197 ±<br>1,605 | 0,002   | 0,042 ±<br>1,988   | -0,686 ± 1,845 | 0,712 ± 1,897  | 0,002   |
| <0, n (%)                     | 135 (64,0%)       | 72 (70,6%)        | 63 (57,8%)        | 0,153   | 38 (53,4%)         | 24 (68,6%)     | 15 (39,5%)     | 0,031   |
| ≥ 0 and <1, n (%)             | 36 (17,1%)        | 14 (13,7%)        | 22 (20,2%)        |         | 9 (12,3%)          | 4 (11,4%)      | 5 (13,2%)      |         |
| ≥ 1, n (%)                    | 40 (18,9%)        | 16 (15,7%)        | 24 (22,0%)        |         | 25 (34,2%)         | 7 (20,0%)      | 18 (47,4%)     |         |
| APRI ± SD (n=211)             | 0,311 ± 0,205     | 0,285 ± 0,152     | 0,323 ± 0,191     | 0,116   | 0,349 ±<br>0,242   | 0,263 ± 0,093  | 0,428 ± 0,304  | 0,003   |
| ≥ 0,918, n (%)                | 5 (2,4%)          | 2 (2,0%)          | 3 (2,8%)          | 0,706   | 4 (5,5%)           | 0 (0%)         | 4 (10,5%)      | 0,048   |
| NFS ± SD (n=211)              | -1,884 ±<br>1,238 | -1,977<br>±1,262  | -1,801 ±<br>1,233 | 0,307   | -1,624<br>±1,328   | -1,672 ± 1,488 | -1,580 ± 1,180 | 0,769   |

Table 2 – Results of noninvasive tests of the FONS cohort based on VCTE and liver histology. (continued)

|                             | Based on VCTE |               |               | Based on histology |               |               |
|-----------------------------|---------------|---------------|---------------|--------------------|---------------|---------------|
|                             |               |               |               |                    |               |               |
| >0,676, n(%)                | 2 (0,9%)      | 1 (1,0%)      | 1 (0,9%)      | 0,962              | 1 (1,4%)      | 1 (2,9%)      |
| CAP, dB/m (=220)            | 283,00 ± 50,5 | 240,1 ± 27,1  | 322,2 ± 31,5  | <0,001             | 291,7 ± 54,0  | 272,97 ± 49,5 |
| LSM, kPa ± SD (=220)        | 8,25 ± 4,49   | 7,74 ± 4,58   | 8,72 ± 4,37   | 0,109              | 11,77 ± 4,94  | 11,70 ± 4,87  |
| ≥ 8 kPa, n(%)               | 93 (42,3%)    | 38 (36,2%)    | 55 (47,8%)    | 0,081              | 42 (56,8%)    | 19 (50,0%)    |
| IQR/median preoperative (%) | 18,7 ± 6,6    | 18,6 ± 6,5    | 18,9 ± 6,6    | 0,692              | 18,9 ± 6,2    | 19,5 ± 5,7    |
| FAST score ± SD (n=212)     | 0,24 ± 0,17   | 0,16 ± 0,13   | 0,31 ± 0,18   | <0,001             | 0,34 ± 0,20   | 0,25 ± 0,13   |
| ≥ 0,35, n(%)                | 45 (21,2%)    | 10 (9,7%)     | 35 (32,1%)    | <0,001             | 31 (41,9%)    | 7 (19,4%)     |
| FNI ± SD                    | 0,23 ± 0,22   | 0,18 ± 0,19   | 0,25 ± 0,19   | 0,013              | 0,29 ± 0,25   | 0,18 ± 0,17   |
| ≥ 0,33, n(%)                | 39 (18,6%)    | 13 (12,9%)    | 26 (23,9%)    | 0,041              | 21 (28,8%)    | 5 (14,3%)     |
| Agile3+ ± SD (n=211)        | 0,237 ± 0,219 | 0,188 ± 0,192 | 0,283 ± 0,233 | 0,001              | 0,415 ± 0,229 | 0,382 ± 0,223 |
| ≥ 0,679, n(%)               | 13 (6,2%)     | 3 (2,9%)      | 10 (9,2%)     | 0,060              | 11 (15,1%)    | 3 (8,6%)      |
| Agile 4 ± SD (n=211)        | 0,041 ± 0,066 | 0,036 ± 0,064 | 0,046 ± 0,068 | 0,310              | 0,087 ± 0,093 | 0,100 ± 0,109 |
| ≥ 0,565, n(%)               | 0 (0,0%)      | 0 (0,0%)      | 0 (0,0%)      | NA                 | 0 (0,0%)      | 0 (0,0%)      |

Data are given as mean (SD). APRI: AST to platelet ratio; CAP: controlled attenuation parameters; FAST score: Fibroscan-AST score; FNI: fibrotic NASH index; IQR: interquartile range; LSM: liver stiffness measurement; MAF-5: metabolic dysfunction-associated fibrosis 5; NFS: NAFLD fibrosis score; SD: standard deviation; Results are presented as numbers and percentages, or mean and standard deviation. When based on VCTE, MASLD was established based on a CAP value ≥275 dB/m.

## Liver biopsy

A total of 77 liver biopsies were of sufficient quality for evaluation. Based on the histological analysis, 39 (50,6%) of them exhibit steatosis and therefore met the definition of MASLD. Only 4 of them (5,3%) met the definition of MASH of whom 2 (2,6%) were at-risk MASH. No fibrosis (F0) was found in 51 biopsies (67,1%), perisinusoidal or portal fibrosis (F1) in 18 biopsies (23,7%), and perisinusoidal and periportal fibrosis (F2) in 7 biopsies (9,2%). Neither septal or bridging fibrosis (F3), nor cirrhosis (F4), was found. Based on the TE distinction, only the proportion of individuals with steatosis was significantly higher in the MASLD group. However, 27 individuals (35,1%) were misclassified: 11 biopsies from the no-MASLD group exhibited steatosis, while 16 biopsies from the MASLD group did not. When using the histology for the MASLD classification, the MASLD group exhibited a higher proportion of participants with a NAS score  $\geq 4$  and MASH. These findings are shown in Table 3.

## Evaluation of non-invasive tests

In order to evaluate the diagnostic value of the NITs, we compared them with the set of liver biopsies.

The difference in mean values of NITs between F0-1 and  $\geq F2$  was significant different for, the baseline and capped MAF-5 scores and NFS but not for the FIB-4, APRI or TE. However, only the capped MAF-5 and NFS made a significant distinction between these fibrosis stages. The capped MAF-5 showed a sensitivity of 100% while the NFS a specificity of 100%. In practice, the capped MAF-5 score could be used to screen and rule out F2 and NFS could confirm the diagnosis in positive cases.

Due to the absence of F3 and F4 cases in our biopsy set, we were unable to calculate the test characteristics for Agile 3+ and Agile 4. The Agile 3+ still identified 10 individuals as possibly having F3 fibrosis.

Finally, both MAF-5 scores, the FAST score and FNI showed different mean values between individuals with and without at-risk MASH. These tests are useful for ruling out at-risk MASH; however, further investigation is required in cases of positive results. The tests characteristics are presented in Table 4.

Table 3 – Bariatric surgery and liver biopsies.

| Surgery and liver biopsies   | Based on histology |            |            |         |
|------------------------------|--------------------|------------|------------|---------|
|                              | Total              | No MASLD   | MASLD      | P-value |
| N (%)                        | 77 (100%)          | 38 (49,3%) | 39 (50,6%) |         |
| Type of surgery              |                    |            |            | 0,408   |
| LSG                          | 39 (50,6%)         | 21 (55,3%) | 18 (46,2%) |         |
| RYGB                         | 36 (46,8%)         | 16 (42,1%) | 20 (51,3%) |         |
| OAGB                         | 1 (1,3%)           | 1 (2,6%)   | 0 (0%)     |         |
| Nissen sleeve                | 1 (1,3%)           | 0 (0%)     | 1 (2,6%)   |         |
| NAS score $\geq 4$ , n(%)    | 6 (7,9%)           | 0 (0%)     | 6 (15,4%)  | <0,001  |
| MASH                         | 4 (5,3%)           | 0 (0%)     | 4 (10,3%)  | 0,045   |
| At-risk MASH                 | 2 (2,6%)           | 0 (0%)     | 2 (5,1%)   | 0,163   |
| Steatosis $\geq 1$ , n(%)    | 39 (50,6%)         | 0 (0%)     | 39 (100%)  |         |
| Ballooning $\geq 1$ , n(%)   | 12 (15,6%)         | 3 (7,9%)   | 9 (23,1%)  | 0,074   |
| Inflammation $\geq 1$ , n(%) | 42 (54,5%)         | 19 (50,0%) | 23 (59,0%) | 0,504   |
| Fibrosis                     |                    |            |            | 0,237   |
| 0, n(%)                      | 51 (67,1%)         | 29 (76,3%) | 22 (57,9%) |         |
| 1, n(%)                      | 18 (23,7%)         | 7 (18,4%)  | 11 (28,9%) |         |
| 2, n(%)                      | 7 (9,2%)           | 2 (5,3%)   | 5 (13,2%)  |         |
| 3, n(%)                      | 0 (0%)             | 0 (0%)     | 0 (0%)     |         |
| 4, n(%)                      | 0 (0%)             | 0 (0%)     | 0 (0%)     |         |

LSG: laparoscopic sleeve gastrectomy; RYGB: Roux-en-Y gastric bypass; OAGB: one-anastomosis gastric bypass; NSG: Nissen sleeve gastrectomy. Results are presented as numbers and percentages. When based on TE, MASLD was established based on a CAP value  $\geq 275$  dB/m.

| NIT's              | F0-1                 | $\geq F2$            | p-value | Sensitivity | Specificity | PPV   | NPV   |
|--------------------|----------------------|----------------------|---------|-------------|-------------|-------|-------|
| FIB-4 index        | 0,827 $\pm$<br>0,364 | 1,141 $\pm$<br>0,596 | 0,217   | 28,6%       | 87,7%       | 0.2   | 0,919 |
| <1.3, n (%)        | 57<br>(87,7%)        | 5<br>(71,4%)         |         |             |             |       |       |
| $\geq 1.3$ , n (%) | 8<br>(12,3%)         | 2<br>(28,6%)         | 0,237   |             |             |       |       |
| Baseline MAF-5     | 3,681 $\pm$<br>2,390 | 6,423 $\pm$<br>2,203 | 0,005   | 100%        | 1,5%        | 0,099 | 1     |
| <0, n (%)          | 1<br>(1,5%)          | 0<br>(0,0%)          |         |             |             |       |       |
| $\geq 0$ , n(%)    | 64<br>(98,5%)        | 7<br>(100,0%)        | 0,741   |             |             |       |       |

| NIT's          | F0-1           | ≥F2           | p-value | Sensitivity | Specificity | PPV   | NPV   |
|----------------|----------------|---------------|---------|-------------|-------------|-------|-------|
| Capped MAF-5   | -0,192 ± 1,958 | 1,808 ± 0,994 | 0,010   | 100%        | 60%         | 0,212 | 1     |
| <0, n (%)      | 39 (60,0%)     | 0 (0,0%)      |         |             |             |       |       |
| ≥0, n(%)       | 23 (40,0%)     | 7 (100,0%)    | 0,002   |             |             |       |       |
| APRI ± SD      | 0,325 ± 0,212  | 0,578 ± 0,391 | 0,140   | 28,6%       | 96,9%       | 0,5   | 0,926 |
| < 0,918, n (%) | 63 (96,9%)     | 5 (71,4%)     |         |             |             |       |       |
| ≥ 0,918, n (%) | 2 (3,1%)       | 2 (28,6%)     | 0,005   |             |             |       |       |
| NFS ± SD       | -1,724 ± 1,324 | -0.594 ±1,021 | 0,032   | 14,3%       | 100%        | 1     | 0,915 |
| ≤0,676, n(%)   | 65 (100,0%)    | 6 (85,7%)     |         |             |             |       |       |
| >0,676, n(%)   | 0 (0,0%)       | 1 (14,3%)     | 0.002   |             |             |       |       |
| LSM            | 11,45 ± 4,27   | 13,51 ± 8,95  | 0,567   | 100%        | 1,5%        | 0,093 | 1     |
| <8 kPa, n(%)   | 1 (1,4%)       | 0 (0,0%)      |         |             |             |       |       |
| ≥8 kPa, n(%)   | 68 (98,6%)     | 7 (100,0%)    | 0,748   |             |             |       |       |

| NIT's        | <F3           | ≥F3      | p-value | Sensitivity | Specificity | PPV | NPV |
|--------------|---------------|----------|---------|-------------|-------------|-----|-----|
| Agile 3+     | 0.411 ± 0.228 | NA       | NA      | NA          | NA          | NA  | NA  |
| <0,679, n(%) | 62 (86,1%)    | 0 (0,0%) |         |             |             |     |     |
| ≥0,679, n(%) | 10 (13,9%)    | 0 (0,0%) | NA      |             |             |     |     |

| NIT's        | <F4            | F4       | p-value | Sensitivity | Specificity | PPV | NPV |
|--------------|----------------|----------|---------|-------------|-------------|-----|-----|
| Agile 4      | 0,0874 ± 0,093 | NA       | NA      | NA          | NA          | NA  | NA  |
| <0,565, n(%) | 72 (100,0%)    | 0 (0,0%) |         |             |             |     |     |
| ≥0,565, n(%) | 0 (0.0%)       | 0 (0,0%) | NA      |             |             |     |     |

Table 4 – Evaluation of the NITs: tests characteristics.

| NIT's             | Not At-risk<br>MASH | At-risk<br>MASH  | p-value | Sensitivity | Specificity | PPV   | NPV |
|-------------------|---------------------|------------------|---------|-------------|-------------|-------|-----|
| Baseline<br>MAF-5 | 3,929 ±<br>2,507    | 6,785 ±<br>0,864 | 0,114   | 100%        | 1,4%        | 0,028 | 1   |
| <0, n (%)         | 1 (1,4%)            | 0 (0,0%)         |         |             |             |       |     |
| ≥0, n(%)          | 69 (98,6%)          | 2 (100,0%)       | 0,865   |             |             |       |     |
| Capped<br>MAF-5   | -0,024 ±<br>1,966   | 2,884 ±<br>0,256 | 0,041   | 100%        | 54,2%       | 0,06  | 1   |
| <0, n (%)         | 38 (54,3%)          | 0 (0,0%)         |         |             |             |       |     |
| ≥0, n(%)          | 32 (45,7%)          | 2 (100,0%)       | 0,129   |             |             |       |     |
| FAST score        | 0,33 ± 0,19         | 0,73 ± 0,13      | 0,005   | 100%        | 60,6%       | 0,067 | 1   |
| <0,35, n(%)       | 43 (60,6%)          | 0 (0,0%)         |         |             |             |       |     |
| ≥0,35, n(%)       | 28 (39,4%)          | 2 (100,0%)       | 0,086   |             |             |       |     |
| FNI               | 0,28 ± 0,24         | 0,76 ± 0,17      | 0,003   | 100%        | 72,9%       | 0,095 | 1   |
| <0,33, n(%)       | 51 (72,9%)          | 0 (0,0%)         |         |             |             |       |     |
| ≥0,33, n(%)       | 19 (27,1%)          | 2 (100,0%)       | 0,025   |             |             |       |     |

APRI: AST to platelet ratio; CAP: controlled attenuation parameters; FAST score: Fibroscan-AST score; FNI: fibrotic NASH index; IQR: interquartile range; LSM: liver stiffness measurement; MAF-5: metabolic dysfunction-associated fibrosis 5; NFS: NAFLD fibrosis score; NPV: negative predictive value ; PPV: positive predictive value; SD: standard deviation;. Results are presented as numbers and percentages, or mean and standard deviation.

The AUROCs of the tests (excluding the Agile tests) are shown in Table 5 and visualized in Figure 1 and 2. Both baseline and capped MAF-5 share the highest AUROC of 0.809, making them the top performers for detecting  $F \geq 2$ . The best two test for detecting at-risk MASH were the FAST score and the capped MAF-5 scores, demonstrating AUROCs of 0.957 and 0.943, respectively. The optimal cutoff values for each test were defined using the highest Youden's index.

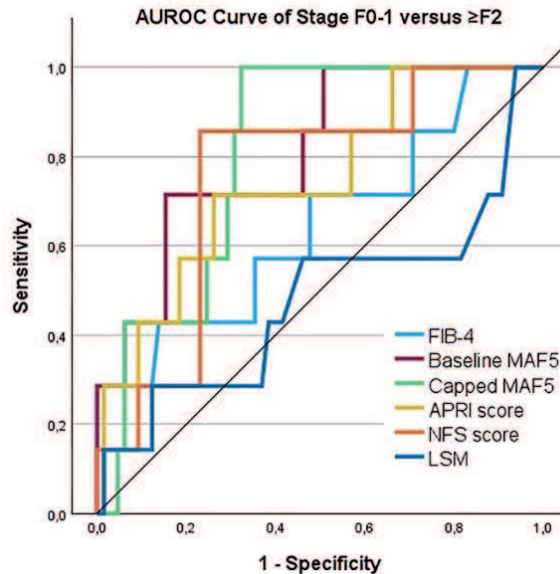


Figure 1 - AUROC curves of NIT's. AUROC curves from FIB-4 index, baseline MAF-5, capped MAF-5, APRI score, NFS score and Fibroscan®(LSM) for the detection of stage  $F \geq 2$  and. The performance of these tests was evaluated against histology. The diagonal line corresponds to chance alone (AUROC curve = 0.50).

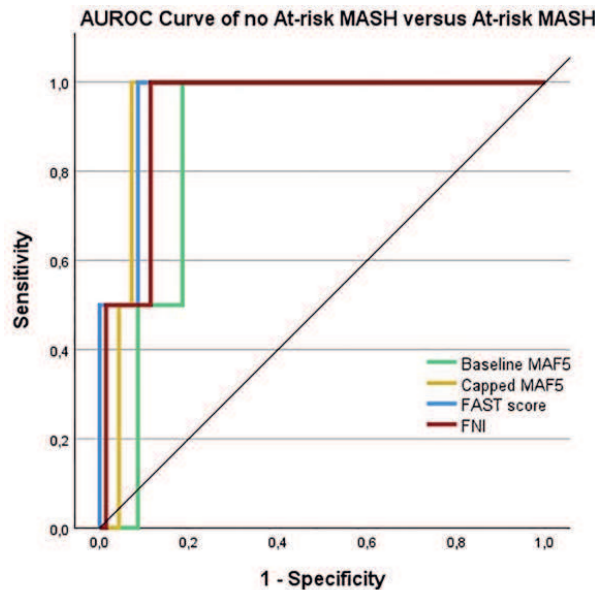


Figure 2 - AUROC curves of NIT's. AUROC curves from Baseline MAF-5, Capped MAF-5, FAST score and FNI for the detection of at-risk MASH. The performance of these tests was evaluated against histology. The diagonal line corresponds to chance alone (AUROC curve = 0.50).

Table 5 – AUROCs and optimal cutoff values for each test in detecting F $\geq$ 2 and At-risk MASH.

| Aera Under the ROC Curve of Stage F0-1 versus $\geq$ F2         |              |             |             |
|-----------------------------------------------------------------|--------------|-------------|-------------|
| FIB-4 index                                                     | 0,645        |             |             |
| Baseline MAF-5                                                  | 0,809        |             |             |
| Capped MAF-5                                                    | 0,809        |             |             |
| APRI score                                                      | 0,743        |             |             |
| NFS score                                                       | 0,754        |             |             |
| LSM                                                             | 0,479        |             |             |
| Aera Under the ROC Curve of no At-risk MASH versus At-risk MASH |              |             |             |
| Baseline MAF-5                                                  | 0,864        |             |             |
| Capped MAF-5                                                    | 0,943        |             |             |
| FAST score                                                      | 0,957        |             |             |
| FNI                                                             | 0,936        |             |             |
| Optimal cutoff values for $\geq$ F2                             | cutoff value | sensitivity | specificity |
| FIB-4 index                                                     | 1,26         | 42,90%      | 13,80%      |
| Baseline MAF-5                                                  | 6,043        | 71,40%      | 15,40%      |
| Capped MAF-5                                                    | 0,475        | 100%        | 32,30%      |
| APRI score                                                      | 0,351        | 71,40%      | 26,20%      |
| NFS score                                                       | -0,708       | 85,70%      | 23,10%      |
| LSM                                                             | 14,3         | 28,60%      | 12,30%      |
| Optimal cutoff values for At-risk MASH                          | cutoff value | sensitivity | specificity |
| Baseline MAF-5                                                  | 6,15         | 100%        | 18,60%      |
| Capped MAF-5                                                    | 2,69         | 100%        | 7,10%       |
| FAST score                                                      | 0,63         | 100%        | 8,60%       |
| FNI                                                             | 0,62         | 100%        | 11,40%      |

## DISCUSSION

In this study investigating the prevalence of MASLD and MASH in a Dutch bariatric cohort, 52.3% of patients had MASLD based on VCTE. Based on histology in a subset of the patients, 50.6% had MASLD, while 5.3% had MASH. Among the patients who underwent biopsy, the majority (67.1%) had no fibrosis, while a smaller percentage exhibited mild fibrosis (F1: 23.7%, F2: 9.2%). No patients were diagnosed with F3 fibrosis or cirrhosis. Additionally, 2.6% of patients had at-risk MASH.

The capped MAF-5 and NFS successfully ruled out F2 and confirmed the diagnosis, where other NITs failed to reliably determine liver fibrosis. In our study, the capped MAF-5 was the most effective NIT for predicting fibrosis stage  $\geq 2$ , with an AUROC of 0.809. The FAST score and capped MAF-5 showed a great AUROC of 0.957 and 0.943 respectively.

These findings differ from previous studies. Recent results from similar populations have been published in various countries, including Germany, China, France, Italy, and Brazil [12, 13, 35–37]. Although the pre-operative mean LSM of 8.25 kPa in our study is consistent with previous reports ranging from 7.01 to 12.9 kPa, there is a substantial difference in the distribution of fibrosis categories. In a 2023 multicenter, open-label, randomized trial in Italy including 431 liver biopsies, 98.7% of patients had liver fibrosis  $\geq F1$ , with 42.4% having  $\geq F2$  [37]. Higher LSM values were also found by others including 101 patients in China, where 64.9% of patients had liver fibrosis [13]. Similarly, a French group reported 62% of patients with liver fibrosis in their cohort including 100 liver biopsies [36]. In our study no patients were diagnosed with F3 fibrosis or cirrhosis, in contrast to a German study where 48% of the patients had bridging fibrosis or cirrhosis prior to bariatric surgery [35]. Finally, our results align more closely with those of Raverdy et al. [38], who reported that 72% of patients had no liver fibrosis prior to bariatric surgery in a French cohort of 2,436 individuals. While they diagnosed patients with F3 and F4 fibrosis, the proportion of these stages was also low, at 4.4% and 0.7%, respectively.

The evaluation of MASLD in our cohort revealed no significant differences in terms of liver fibrosis or other histological characteristics. However, a difference in hepatic fibrosis could have been anticipated, as the group with a CAP  $\geq 275$  dB/m exhibited abdominal obesity, one of the most significant factors in the development of advanced fibrosis, cirrhosis, and HCC [14]. In this group, HbA1c levels were significantly higher, which may support the hypothesis that liver steatosis often precedes the development of T2D. Additional studies should be conducted to further investigate the role of impaired glucose metabolism as an early contributing factor to steatotic liver disease. .

There is a clear discrepancy between previous reports and our study. One possible explanation could be the duration of obesity. The longer individuals have been living with obesity, the higher their risk of developing comorbidities such as MASLD. Unfortunately, no data were available to

assess this factor. Secondly, the proportion of patients with diabetes may also influence the results. In our study, only 16.3% of patients had diabetes at baseline, whereas the studies mentioned earlier reported that up to 44% of their patients had diabetes. Finally, another possible explanation could be the effectiveness of the Dutch healthcare system, which offers free procedures and maintains short waiting times. In contrast, other countries may depend on private healthcare with high costs or have overburdened public systems, leading to delays in patient care and potentially contributing to the progression of MASLD [39].

The use of NITs is helpful for detecting at-risk patients, but our study indicates that there might be an overestimation of the obtained measurements or calculation. The FIB-4 in our study performed significantly worse than in previous validation studies. The AUROC of 0.645 for detecting  $F \geq 2$  is notably lower than the AUROC of 0.81 reported by Siddiqui et al [40]. In contrast to that study, our patients had a much higher BMI and lower transaminases. Therefore the two cohorts are not comparable and it may be the case that FIB-4 is not a valid test in subjects as included in the present study. TE misclassified one-third of our histology-based MASLD cases. To our knowledge, no validation study has yet demonstrated the accuracy of the capped MAF-5, which was the best performer in our study detecting fibrosis stage  $\geq 2$ , with AUROCs of 0.809. Only the AUROCs for the APRI and NFS scores were comparable to those reported by Raverdy et al., at 0.75 and 0.68, respectively.

Therefore, in a Dutch bariatric population with low prevalence of advanced fibrosis or cirrhosis, applying these tests to the entire group may be unnecessary. The evaluation of the liver should target individuals who are at high risk for advanced fibrosis or liver cirrhosis and the choice of test should be carefully considered. Future studies will aim to define the profile of these high-risk individuals to ensure accurate detection and diagnosis.

A significant limitation of this study is the small cohort size, with only 77 liver biopsies performed among 220 individuals. The rationale for this approach was the need to follow guidelines to perform liver biopsy based on current diagnostic algorithms [20]. In comparison, Raverdy et al. analyzed 2,346 liver biopsies, providing a much larger dataset. This small sample size resulted in limited diversity across fibrosis stages, with no diagnoses of F3 or F4 and only a few F2 cases. Consequently, questions may arise regarding the accurate assessment of the diagnostic

value of NITs. In the current study, the FibroScan assessment suggests an overestimation of the risk of hepatic fibrosis.

## CONCLUSION

In the current Dutch cohort, the prevalence of MASLD and MASH in patients prior to bariatric surgery was lower than expected. Histological evaluations revealed that the majority had no liver fibrosis, and none were diagnosed with F3 fibrosis or liver cirrhosis. These results suggest that the current population may exhibit characteristics that differ from those reported in previous epidemiological studies. Furthermore, NITs may have limitations in this population. Among them, the capped MAF-5, which demonstrated the best performance, appears to be the preferred test for ruling out significant fibrosis in patients with severe obesity but a relatively low prevalence of MASLD and MASH. However, traditionally reliable tests such as LSM with TE and FIB-4 index performed worse than expected.

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# Chapter 3

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## **Prevalence of Liver Steatosis and Fibrosis by Transient Elastography in a High Cardiovascular-Risk Outpatient Cohort Including T1DM and T2DM**

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## ABSTRACT

### Background

Metabolic dysfunction-associated steatotic liver disease (MASLD) is common in T2DM, likely due to insulin resistance and obesity. Although screening is recommended in high-risk patients, its prevalence in outpatient cardiovascular clinical settings remains unclear.

### Methods

We analyzed data from 475 patients attending a cardiovascular outpatient clinic: 142 with T2DM, 78 with T1DM, and 255 non-diabetic individuals at elevated cardiovascular risk. Liver steatosis and fibrosis were assessed using vibration-controlled transient elastography (Fibroscan®): steatosis by controlled attenuation parameter (CAP  $\geq 275$  dB/m), and fibrosis risk by liver stiffness measurement (LSM  $\geq 8.1$  kPa). Carotid intima-media thickness (cIMT) was also measured.

### Results

The cohort (47% women, mean age 53 years, BMI 29.8 kg/m<sup>2</sup>) showed MASLD in 39.2% and fibrosis risk in 18.3%. MASLD was most prevalent in T2DM (57.0%), followed by non-diabetics (35.3%) and T1DM (19.2%) ( $p < 0.001$ ). Fibrosis risk was also highest in T2DM (22.5%) vs. T1DM (7.7%) and non-diabetics (19.2%) ( $p = 0.02$ ). CAP values were higher in those with fibrosis risk. T2DM patients with MASLD had higher LSM ( $7.0 \pm 3.0$  kPa) compared to those without MASLD ( $5.1 \pm 2.2$  kPa;  $p < 0.001$ ). cIMT was highest in T2DM ( $0.73 \pm 0.12$  mm;  $p = 0.04$ ), but not associated with MASLD or fibrosis. BMI and triglycerides were the strongest predictors of both MASLD and fibrosis.

### Conclusions

MASLD and risk of significant fibrosis were highest among T2DM patients. Within T2DM, those with MASLD had higher LSM, indicating increased risk of fibrosis. The presence of MASLD and risk of significant fibrosis was not associated with cIMT in this cardiometabolic cohort. BMI and plasma TG were consistent predictors across groups urging for more strict control by body weight reduction and lifestyle interventions.

## INTRODUCTION

Metabolic dysfunction-associated steatotic liver disease (MASLD) encompasses a range of liver disease stages, from isolated steatosis, where lipid droplets accumulate in hepatocytes, to metabolic dysfunction-associated steatohepatitis (MASH), characterized by hepatocyte damage and hepatic inflammation (1). This can further progress to fibrosis, cirrhosis, and, in some cases, hepatocellular carcinoma (HCC), all in the absence of secondary causes such as excessive alcohol consumption or other liver diseases (1–3).

The global prevalence of MASLD is estimated at 30%, with higher rates observed in individuals with obesity and type 2 diabetes (T2DM) (4, 5). As global rates of obesity and T2DM rise, MASLD prevalence is expected to increase, likely becoming the leading indication for liver transplantation in the near future (6). Despite its growing prevalence, MASLD remains underdiagnosed due to limited awareness among both healthcare providers and patients (7–9).

In addition to its hepatic implications, MASLD is closely linked to cardiovascular disease (CVD). Epidemiological studies show that MASLD is associated with increased carotid intima-media thickness (cIMT), a surrogate marker of arterial injury (10, 11). The underlying inflammatory and metabolic mechanisms, such as insulin resistance (IR) and systemic inflammation, contribute to vascular damage thereby increasing CVD risk (10). Moreover, MASLD's severity correlates with a higher incidence of atherosclerotic cardiovascular disease (asCVD) risk factors, such as hypertension and diabetes, making early detection and management essential (12, 13). Recent evidence suggests that MASLD contributes to asCVD beyond shared risk factors. In a cohort of 7507 adults in Eastern China, MASLD severity was independently associated with subclinical coronary atherosclerosis, particularly in individuals with severe MASLD or hypertension (14). Mechanistic and clinical data further highlight links through systemic inflammation, IR, and endothelial dysfunction (11), reinforcing its role as an independent cardiovascular (CV) risk factor. Different scientific organizations, including the American Heart Association (AHA) and the European Association for the Study of the Liver (EASL), have recommended MASLD screening in individuals with cardiometabolic disorders (13, 15). Although liver biopsy is considered the diagnostic gold standard, its invasive nature, cost, and associated risks limit its utility in routine clinical care. To improve accessibility, non-invasive tests (NITs),

such as vibration-controlled transient elastography (VCTE), have been developed (1, 16). VCTE is a non-invasive method that simultaneously assesses liver fibrosis and steatosis. Liver stiffness measurement (LSM), obtained from VCTE, stages fibrosis, while the controlled attenuation parameter (CAP) measures hepatic fat content.

While MASLD has been widely studied in T2DM due to its strong association with IR, relatively little is known about MASLD in patients with type 1 diabetes mellitus (T1DM) (17, 18). Furthermore, few studies have explored MASLD and liver fibrosis in high CV risk populations attending cardiometabolic outpatient clinics; groups that are often overlooked despite sharing many metabolic risk factors.

Therefore, this study aimed to evaluate the prevalence of MASLD and risk of significant fibrosis in patients with T1DM and T2DM, compared to a high CV-risk non-diabetic group from a cardiometabolic outpatient clinic in the Netherlands. Secondary objectives include assessing differences in arterial injury between groups defined by MASLD and fibrosis status, and determining independent predictors of MASLD and significant fibrosis using backward stepwise logistic regression.

## MATERIALS AND METHODS

This study was conducted at the Endocrinology and Diabetes Vascular Center of Franciscus Gasthuis Hospital in Rotterdam, the Netherlands. Patients attending the out-patient clinic for CV risk management were included. Data were collected from electronic health records (EHRs) of patients enrolled between January 2021 and July 2022, all of whom underwent a standard, protocolized CV assessment, including VCTE. The study received ethical approval from the local Medical Ethical Committee of the Franciscus Gasthuis & Vlietland Hospital and was conducted in accordance with the Declaration of Helsinki.

### Study Population

The study involved 475 adult patients: 142 with T2DM, 78 with T1DM, and 255 non-diabetic individuals, all of whom had cardiometabolic disorders. Patients were eligible if they visited the Endocrinology and Vascular Medicine department of Franciscus Gasthuis Hospital and underwent a FibroScan measurement between 1 January 2021, and 1 October 2022. Patients with previously diagnosed chronic liver disease were excluded, as

well as those with conditions associated with secondary liver steatosis or fibrosis, such as HIV, viral hepatitis, hemochromatosis, Wilson's disease, sarcoidosis, or excessive alcohol consumption ( $\geq 3$  units per day for men and  $\geq 2$  units per day for women).

### Data Collection

MASLD was defined as the presence of at least one cardiometabolic disorder and a controlled attenuation parameter (CAP)  $\geq 275$  dB/m (S1), in accordance with the 2021 European Association for the Study of the Liver (EASL) Clinical Practice Guidelines, indicating hepatic steatosis (19). The cardiometabolic disorders considered were: overweight/obesity (BMI  $\geq 25$  kg/m<sup>2</sup>), hypertension (blood pressure  $\geq 130/85$  mmHg or use of antihypertensive medication), hypercholesterolemia (total cholesterol  $\geq 5.0$  mmol/L or use of lipid-lowering medication), dyslipidemia (LDL-C  $\geq 3.0$  mmol/L or HDL-C  $< 1.0$  mmol/L), and diabetes (fasting glucose  $\geq 7.0$  mmol/L, HbA1c  $> 48$  mmol/mol, or use of glucose-lowering medication). All variables were abstracted from the electronic health records. Risk of significant fibrosis was defined as a liver stiffness measurement (LSM)  $\geq 8.1$  kPa ( $\geq F2$ ) (19). CVD was defined as the presence of any of the following conditions: transient ischemic attack (TIA), cerebrovascular accident (CVA), peripheral arterial disease (PAD), or coronary artery disease (CAD). All data were encoded with a unique, non-identifiable code to maintain patient confidentiality.

### Laboratory Examination

Blood samples were analyzed for glucose, HbA1c, total cholesterol, HDL cholesterol (HDL-C), LDL cholesterol (LDL-C), triglycerides (TGs), apolipoproteins (apo) B and AI, C-reactive protein (CRP), hemoglobin, thrombocytes, gamma-glutamyl transferase (GGT), creatinine, albumin, estimated glomerular filtration rate (eGFR), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) at the laboratory of Franciscus Gasthuis using standard procedures. All blood tests were conducted in a non-fasting state.

### Vibration-Controlled Transient Elastography

VCTE was performed using Fibroscan™ (Echosens, Paris, France) by trained personnel at the outpatient clinic. At this cardiometabolic clinic, the procedure is routinely applied to screen for hepatic steatosis and fibrosis. Patients were positioned supine with their right arm under their head. The probe (either M- or XL-probe) was placed in the intercostal

space between the 10th and 12th ribs along the mid-axillary line. A minimum

of 10 valid measurements were required for the VCTE to be considered successful. Reliability was assessed by ensuring the interquartile range (IQR) from the median LSM value was  $\leq 30\%$  (20).

### Carotid Intima Media Thickness

Carotid ultrasound scans were performed using the ART-LAB (Esaote, Genoa, Italy) by trained personnel as described previously in detail (21). Imaging was conducted for each common carotid artery in three distinct projections. The cIMT was determined as the mean of six measurements, with at least two measurements per carotid artery required for inclusion.

### Data Analysis

Data are presented as mean  $\pm$  standard deviation (SD) for normally distributed variables, or median and IQR for non-normally distributed variables. Comparisons of normally distributed variables were performed using Student's T-test, while non-normally distributed variables were analyzed using the Mann-Whitney U test or Kruskal-Wallis. ANOVA with post hoc analyses was used to explore differences between subgroups. Chi-square tests were employed to assess prevalence differences. To identify predictors of MASLD and risk of significant fibrosis, a backward stepwise logistic regression analysis was performed. Predictors included BMI, age, TGs, HDL-C, LDL-C, HbA1c, and sex. Collinearity was assessed using Variance Inflation Factor (VIF) values, with a threshold of VIF  $< 5$  indicating acceptable levels of collinearity. As this study is exploratory in nature, analyses were primarily descriptive, and formal multivariable adjustment was not performed. All statistical analyses were conducted using IBM SPSS Statistics version 28.0.0.0 (IBM SPSS Statistics, New York, NY, USA). A p-value of  $< 0.05$  was considered statistically significant.

## RESULTS

### Baseline Characteristics

The study population consisted of 475 patients (223 women, mean age  $53 \pm 14$  years, mean BMI  $29.8 \pm 6.7$  kg/m<sup>2</sup>) (Table 1). Of the total cohort, 39.2% (N = 186) had MASLD, and 18.3% (N = 87) were at risk of significant fibrosis. The prevalence of MASLD was 57.0%, 19.2%, and 35.3% in patients with T2DM, T1DM, and in those without diabetes, respectively ( $p < 0.001$ ). 22.5%, 7.7%, and 19.2% of the T2DM, T1DM, and non-diabetic patients, respectively, were at risk of significant fibrosis ( $p = 0.02$ ) (Figure 1). The mean cIMT in the overall cohort was 0.71 mm ( $\pm 0.14$ ), with T2DM patients showing the highest cIMT (Figure 2). T2DM patients also had the highest glucose levels, HbA<sub>1c</sub>, and TGs among the three subgroups. Additionally, T2DM patients exhibited significantly higher creatinine levels and reduced eGFR compared to non-diabetic patients. In contrast, T1DM patients had significantly lower BMI and higher HDL-C levels than non-diabetic patients. Non-diabetic patients had the highest LDL-C levels among all subgroups (Table 1). The majority of the cohort was Caucasian (67.7%), with smaller groups of Turkish (6.5%), North African (5.5%), and Hindustani (4.9%) patients. Further details on the ethnicity distribution of the cohort can be found in Supplementary Table S1. Baseline medication use is summarized in Supplementary Table S2.

Table 1. Baseline demographic and clinical characteristics for the overall group and subgroups.

| Characteristics                   | Total<br>(N = 475) | T2DM<br>(N = 142) | T1DM<br>(N = 78) | Non-DM<br>(N = 255) | p-Value |
|-----------------------------------|--------------------|-------------------|------------------|---------------------|---------|
| Age (years)                       | 53 ± 14            | 59 ± 12 ***       | 52 ± 12          | 50 ± 14             | <0.001  |
| M/F                               | 252/223            | 86/56             | 39/39            | 127/128             | 0.101   |
| BMI (kg/m <sup>2</sup> )          | 29.8 ± 6.7         | 30.4 ± 6.4        | 27.1 ± 5.0 ***   | 30.6 ± 7.3          | <0.001  |
| cIMT (mm)                         | 0.71 ± 0.14        | 0.73 ± 0.12       | 0.69 ± 0.14      | 0.68 ± 0.16         | 0.036   |
| Total cholesterol (mmol/L)        | 4.6 (3.8–5.5)      | 4.1 (3.7–5.3)     | 4.3 (3.7–5.0)    | 5.1 (4.5–5.7)       | 0.573   |
| HDL-C (mmol/L)                    | 1.3 ± 0.39         | 1.2 ± 0.32        | 1.5 ± 0.42 ***   | 1.3 ± 0.37          | <0.001  |
| LDL-C (mmol/L)                    | 2.5 ± 0.92         | 2.3 ± 0.93 ***    | 2.3 ± 0.75 ***   | 3.0 ± 0.80          | <0.001  |
| Triglycerides (mmol/L)            | 1.6 (1.0–2.4)      | 2.1 (1.3–3.1) *   | 1.1 (0.8–1.7) ** | 1.5 (1.1–2.3)       | <0.001  |
| Glucose (mmol/L)                  | 6.2 (5.4–7.9)      | 10.0 (7.4–14) *** | 8.5 (5.6–11) **  | 5.8 (5.1–6.4)       | <0.001  |
| HbA1c (mmol/mol)                  | 50.9 ± 15          | 59.0 ± 13 ***     | 57.3 ± 12 ***    | 36.4 ± 5.0          | <0.001  |
| Apo B (g/L)                       | 1.05 ± 0.27        | 1.05 ± 0.38       | 0.82 ± 0.27      | 1.07 ± 0.20         | 0.330   |
| Apo AI (g/L)                      | 1.54 ± 0.26        | 1.49 ± 0.24       | 1.39 ± NA        | 1.59 ± 0.27         | 0.490   |
| CRP (mg/L)                        | 4.0 (2.0–7.0)      | 3.0 (2.0–6.0)     | 8.0 (4.0–25)     | 4.0 (2.0–7.0)       | 0.176   |
| Hemoglobin (mmol/L)               | 8.7 ± 1.1          | 8.7 ± 1.1         | 8.2 ± 1.0 *      | 8.8 ± 1.1           | 0.057   |
| Thrombocytes (10 <sup>9</sup> /L) | 258 ± 87           | 253 ± 93          | 305 ± 114        | 255 ± 82            | 0.099   |
| GGT (U/L)                         | 58 (31–106)        | 71 (38–177)       | 23 (14–140)      | 58 (29–97)          | 0.082   |
| ALT (U/L)                         | 38 (27–52)         | 38 (28–60)        | 22 (18–39)       | 39 (28–51)          | 0.158   |
| AST (U/L)                         | 39 (24–67)         | 38 (25–72)        | 29 (14–36)       | 41 (23–67)          | 0.131   |
| Creatinine (μmol/L)               | 74 (65–87)         | 77 (67–96) **     | 76 (66–92)       | 72 (65–81)          | 0.006   |
| Albumin (g/L)                     | 44 (41–46)         | 44 (42–47)        | 46 (40–46)       | 44 (41–46)          | 0.850   |

Table 1. Baseline demographic and clinical characteristics for the overall group and subgroups. (continued)

| Characteristics                   | Total<br>(N = 475) | T2DM<br>(N = 142) | T1DM<br>(N = 78) | Non-DM<br>(N = 255) | p-Value |
|-----------------------------------|--------------------|-------------------|------------------|---------------------|---------|
| eGFR (mL/min/1.73m <sup>2</sup> ) | 80 (68–88)         | 77 (59–86) ***    | 78 (70–87)       | 82 (75–91)          | <0.001  |
| FIB-4                             | 1.86 ± 1.50        | 1.94 ± 1.46       | 1.73 ± 1.12      | 1.85 ± 1.62         | 0.789   |
| CAP (dB/m)                        | 263 ± 59           | 282 ± 59 ***      | 238 ± 52 *       | 259 ± 58            | <0.001  |
| LSM (kPa)                         | 6.1 ± 3.0          | 6.2 ± 2.8         | 5.4 ± 1.8 *      | 6.3 ± 3.3           | 0.039   |

Data are expressed as mean ± standard deviation, median (IQR), or n (%), as appropriate. Statistical significance is indicated by asterisks \*, \*\*, and \*\*\*, corresponding to probability values of <0.05, <0.01, and <0.001, respectively. These significance levels are in relation to the high CV-risk non-diabetic subgroup. P-values reflect ANOVA differences between groups. Abbreviations: BMI, body mass index; cIMT, carotid intima media thickness; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; HbA1c, hemoglobin A1c; CRP, C-reactive protein; GGT, gamma-glutamyl transferase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; eGFR, estimated glomerular filtration rate; FIB-4, fibrosis-4 index; CAP, controlled attenuation parameter; LSM, liver stiffness measurement.

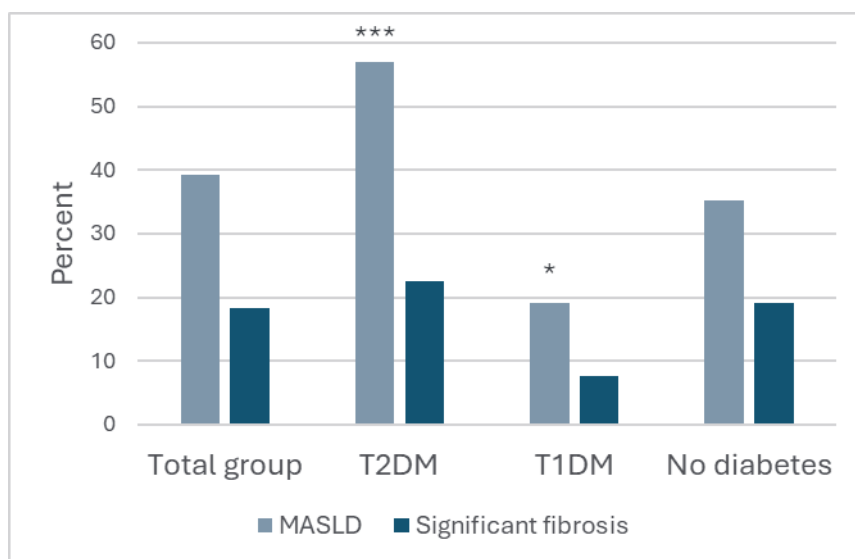


Figure 1. Prevalence of MASLD and risk of significant fibrosis in the total cohort and the among different subgroups. Statistical significance is indicated by asterisks \*, \*\*, and \*\*\*, corresponding to probability values of  $<0.05$ ,  $<0.01$ , and  $<0.001$ , respectively, in relation to the high CV-risk non-diabetic subgroup.

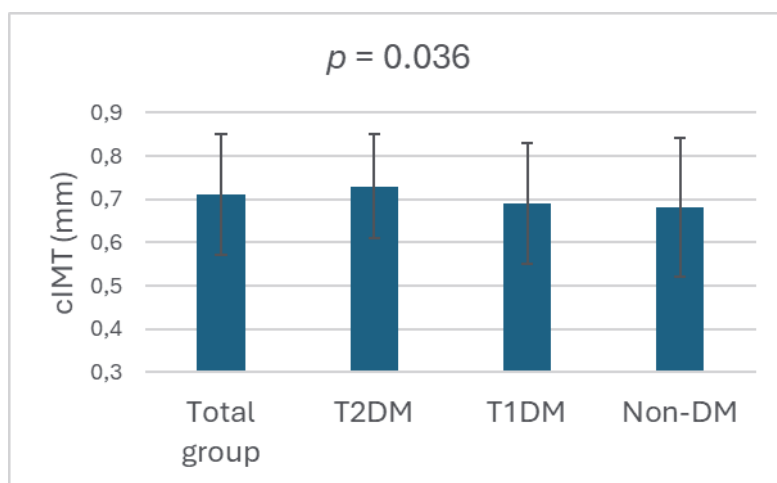


Figure 2. Mean carotid intima-media thickness across the total cohort and subgroups.  $p$ -value indicates ANOVA differences between groups. Abbreviations: T2DM, type 2 diabetes mellitus; T1DM, type 1 diabetes mellitus; non-DM, high CV-risk non-diabetics.

## Characteristics by MASLD and Fibrosis Status

Across the entire cohort and within each subgroup, BMI was significantly greater in patients with MASLD compared to those without. Additionally, in the overall population, individuals with MASLD showed significantly higher plasma TGs, as well as higher liver enzyme levels relative to those without MASLD (Table 2). Within the T2DM group, patients with MASLD showed significantly higher LSM compared to both T1DM patients and non-diabetic individuals. Although T2DM patients had higher cIMT than T1DM patients and high CV-risk non-diabetic patients, neither MASLD nor risk of significant liver fibrosis appeared to significantly affect cIMT within this group. Additionally, T2DM patients with MASLD showed higher LDL-C levels compared to those without MASLD (Table 2).

In the overall cohort, multivariable regression analysis, adjusted for age, sex, and LDL-C, revealed that MASLD was not significantly associated with cIMT ( $\beta = 0.012$ , 95% CI:  $-0.025$  to  $0.049$ ,  $p = 0.516$ ). Similarly, significant fibrosis did not show a significant association with cIMT ( $\beta = 0.044$ , 95% CI:  $-0.025$  to  $0.113$ ,  $p = 0.211$ ). However, age remained significantly positively associated with cIMT ( $\beta = 0.006$ ,  $p < 0.001$ ).

Subjects at higher risk of significant fibrosis had a higher BMI in the overall cohort as well as in the T2DM and T1DM groups (Table 3). Patients who were at risk of fibrosis also showed increased liver enzyme levels in the total population. Moreover, CAP values were significantly elevated in those with risk of fibrosis across both the entire cohort and the diabetic subgroups (Table 3).

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Table 2. Demographic and clinical characteristics by MASLD status in the total population and subgroups.

| Characteristics            | Total<br>(N = 475) |                          |             | T2DM<br>(N = 142) |                         |             | T1DM<br>(N = 78)  |                         |             | Non-DM<br>(N = 255) |                          |             |
|----------------------------|--------------------|--------------------------|-------------|-------------------|-------------------------|-------------|-------------------|-------------------------|-------------|---------------------|--------------------------|-------------|
|                            | MASLD<br>(N = 186) | No<br>MASLD<br>(N = 289) | p-<br>Value | MASLD<br>(N = 81) | No<br>MASLD<br>(N = 61) | p-<br>Value | MASLD<br>(N = 15) | No<br>MASLD<br>(N = 63) | p-<br>Value | MASLD<br>(N = 90)   | No<br>MASLD<br>(N = 165) | p-<br>Value |
| Age (years)                | 52 ± 14            | 54 ± 13                  | 0.068       | 58 ± 12           | 61 ± 11                 | 0.092       | 56 ± 11           | 51 ± 12                 | 0.197       | 45 ± 14             | 52 ± 13                  | <0.001      |
| M/F                        | 100/86             | 152/137                  | 0.803       | 49/32             | 37/24                   | 0.984       | 4/11              | 35/28                   | 0.044       | 47/43               | 80/50                    | 0.568       |
| BMI (kg/m <sup>2</sup> )   | 33.1 ± 6.4         | 27.6 ± 6.0               | <0.001      | 32.3 ± 6.2        | 27.8 ± 5.9              | <0.001      | 31.9 ± 4.2        | 25.9 ± 4.4              | <0.001      | 34.3 ± 6.8          | 28.3 ± 6.7               | <0.001      |
| cIMT (mm)                  | 0.72 ± 0.12        | 0.70 ± 0.14              | 0.409       | 0.73 ± 0.12       | 0.74 ± 0.13             | 0.643       | 0.71 ± 0.12       | 0.68 ± 0.14             | 0.459       | 0.69 ± 0.15         | 0.68 ± 0.17              | 0.975       |
| Total cholesterol (mmol/L) | 5.5 ± 7.0          | 4.6 ± 1.2                | 0.129       | 6.0 ± 9.7         | 4.1 ± 1.2               | 0.207       | 4.5 ± 1.3         | 4.4 ± 0.91              | 0.696       | 5.1 ± 0.95          | 5.1 ± 1.2                | 0.958       |
| HDL-C (mmol/L)             | 1.2 ± 0.36         | 1.3 ± 0.42               | 0.231       | 1.2 ± 0.31        | 1.1 ± 0.33              | 0.942       | 1.6 ± 0.39        | 1.5 ± 0.44              | 0.579       | 1.3 ± 0.35          | 1.3 ± 0.39               | 0.960       |
| LDL-C (mmol/L)             | 2.6 ± 0.90         | 2.5 ± 0.93               | 0.155       | 2.4 ± 1.0         | 2.0 ± 0.87              | 0.042       | 2.4 ± 0.87        | 2.3 ± 0.72              | 0.634       | 3.0 ± 0.70          | 3.1 ± 0.87               | 0.850       |
| Triglycerides (mmol/L)     | 2.2 ± 1.3          | 1.8 ± 1.3                | 0.035       | 2.5 ± 1.5         | 2.2 ± 1.6               | 0.324       | 1.1 ± 0.52        | 1.3 ± 0.74              | 0.455       | 2.0 ± 1.1           | 1.9 ± 1.4                | 0.611       |
| Glucose (mmol/L)           | 7.1 ± 2.7          | 7.6 ± 3.6                | 0.441       | 9.8 ± 3.4         | 11.5 ± 4.6              | 0.222       | 8.1 ± 2.7         | 8.8 ± 3.7               | 0.806       | 5.9 ± 0.93          | 5.9 ± 1.1                | 0.911       |
| HbA1c (mmol/mol)           | 51 ± 14            | 51 ± 15                  | 0.900       | 59 ± 13           | 60 ± 12                 | 0.680       | 56 ± 7.8          | 58 ± 13                 | 0.612       | 37 ± 4.7            | 36 ± 5.3                 | 0.365       |
| ApoB (g/L)                 | 1.1 ± 0.30         | 1.0 ± 0.23               | 0.209       | 1.1 ± 0.39        | 0.90 ± 0.31             | 0.207       | 0.87 ± 0.36       | 0.74 ± NA               | NA          | 1.09 ± 0.21         | 1.05 ± 0.20              | 0.543       |

Table 2. Demographic and clinical characteristics by MASLD status in the total population and subgroups. (continued)

| Characteristics                       | Total<br>(N = 475) |                          |             | T2DM<br>(N = 142) |                         |             | T1DM<br>(N = 78)  |                         |             | Non-DM<br>(N = 255) |                          |             |
|---------------------------------------|--------------------|--------------------------|-------------|-------------------|-------------------------|-------------|-------------------|-------------------------|-------------|---------------------|--------------------------|-------------|
|                                       | MASLD<br>(N = 186) | No<br>MASLD<br>(N = 289) | p-<br>Value | MASLD<br>(N = 81) | No<br>MASLD<br>(N = 61) | p-<br>Value | MASLD<br>(N = 15) | No<br>MASLD<br>(N = 63) | p-<br>Value | MASLD<br>(N = 90)   | No<br>MASLD<br>(N = 165) | p-<br>Value |
| Apo AI (g/L)                          | 1.6 ±<br>0.23      | 1.5 ±<br>0.27            | 0.726       | 1.6 ±<br>0.28     | 1.4 ±<br>0.17           | 0.169       | NA                | 1.4 ±<br>NA             | NA          | 1.5 ±<br>0.12       | 1.6 ±<br>0.30            | 0.680       |
| CRP (mg/L)                            | 7.4 ±<br>9.3       | 8.0 ±<br>16              | 0.781       | 4.2 ±<br>4.1      | 4.7 ±<br>3.5            | 0.686       | 25.0 ±<br>NA      | 10.2 ±<br>10.1          | NA          | 8.6 ±<br>11         | 8.7 ±<br>19              | 0.970       |
| Hemoglobin<br>(mmol/L)                | 8.8 ±<br>1.2       | 8.7 ±<br>1.1             | 0.480       | 8.7 ±<br>1.1      | 8.8 ±<br>1.0            | 0.537       | 8.5 ±<br>0.57     | 8.1 ±<br>1.1            | 0.633       | 8.9 ±<br>1.2        | 8.7 ±<br>1.1             | 0.436       |
| Thrombocytes<br>(10 <sup>9</sup> /L)  | 266 ±<br>83        | 252 ±<br>90              | 0.230       | 264 ±<br>79       | 238 ±<br>110            | 0.283       | 296 ±<br>216      | 306 ±<br>106            | 0.910       | 266 ±<br>83         | 248 ±<br>81              | 0.194       |
| GGT (U/L)                             | 82.4 ±<br>76       | 106 ±<br>141             | 0.190       | 102 ±<br>98       | 173 ±<br>188            | 0.159       | NA                | 66.0 ±<br>73.9          | NA          | 73.9 ±<br>62.5      | 94.7 ±<br>130            | 0.202       |
| ALT (U/L)                             | 55 ±<br>37         | 40 ±<br>26               | 0.003       | 56 ±<br>37        | 45 ±<br>35              | 0.240       | NA                | 31 ±<br>21              | NA          | 53 ±<br>38          | 40 ±<br>24               | 0.010       |
| AST (U/L)                             | 65 ±<br>51         | 42 ±<br>30               | <0.001      | 68 ±<br>63        | 44 ±<br>40              | 0.077       | 26 ±<br>13        | 38 ±<br>34              | 0.624       | 65 ±<br>43          | 41 ±<br>26               | <0.001      |
| Creatinine<br>(μmol/L)                | 79.9 ±<br>21       | 81.3 ±<br>42             | 0.693       | 85.0 ±<br>24      | 91.7 ±<br>77            | 0.486       | 81.5 ±<br>20      | 81.5 ±<br>23            | 0.995       | 73.9 ±<br>15        | 76.0 ±<br>16             | 0.371       |
| Albumin (g/L)                         | 44.1 ±<br>3.3      | 46.7 ±<br>41             | 0.557       | 44.7 ±<br>3.2     | 42.3 ±<br>6.3           | 0.126       | 41.0 ±<br>1.4     | 43.8 ±<br>3.7           | 0.343       | 44.0 ±<br>3.3       | 48.1 ±<br>47             | 0.505       |
| eGFR (mL/<br>min/1.73m <sup>2</sup> ) | 76.1 ±<br>15.3     | 75.6 ±<br>15.3           | 0.802       | 70.7 ±<br>16.7    | 71.8 ±<br>18.0          | 0.758       | 74.2 ±<br>13.9    | 74.7 ±<br>15.6          | 0.917       | 83.3 ±<br>10.5      | 78.4 ±<br>12.6           | 0.064       |

Table 2. Demographic and clinical characteristics by MASLD status in the total population and subgroups. (continued)

| Characteristics | Total<br>(N = 475) |                          | T2DM<br>(N = 142) |                   | T1DM<br>(N = 78)        |             | Non-DM<br>(N = 255) |                          |
|-----------------|--------------------|--------------------------|-------------------|-------------------|-------------------------|-------------|---------------------|--------------------------|
|                 | MASLD<br>(N = 186) | No<br>MASLD<br>(N = 289) | p-<br>Value       | MASLD<br>(N = 81) | No<br>MASLD<br>(N = 61) | p-<br>Value | MASLD<br>(N = 90)   | No<br>MASLD<br>(N = 165) |
| FIB-4           | 1.68 ±<br>1.3      | 1.98 ±<br>1.6            | 0.139             | 1.82 ±<br>1.2     | 2.10 ±<br>1.7           | 0.444       | 1.40 ±<br>0.56      | 1.79 ±<br>1.2            |
| CAP (dB/m)      | 323 ±<br>30        | 223 ±<br>36              | <0.001            | 325 ±<br>32       | 227 ±<br>37             | <0.001      | 318 ±<br>31         | 219 ±<br>35              |
| LSM (kPa)       | 6.7 ± 3.0          | 5.8 ± 2.9                | <0.001            | 7.0 ± 3.0         | 5.1 ± 2.2               | <0.001      | 5.8 ±<br>2.4        | 5.3 ± 1.6                |
|                 |                    |                          |                   |                   |                         |             | 6.6 ±<br>3.1        | 6.2 ± 3.4                |
|                 |                    |                          |                   |                   |                         |             |                     | 2.01 ±<br>1.7            |
|                 |                    |                          |                   |                   |                         |             |                     | 224 ±<br>36              |
|                 |                    |                          |                   |                   |                         |             |                     | <0.001                   |
|                 |                    |                          |                   |                   |                         |             |                     | 0.182                    |

\* Data are given as mean (standard deviation), median (IQR), or numbers (%) where appropriate. \*\* Values shown as mean ± SD. NA = data not available due to small sample size or missing measurements. Abbreviations: T2DM, type 2 diabetes mellitus; T1DM, type 1 diabetes mellitus; non-DM, high CV-risk non-diabetics; BMI, body mass index; cIMT, carotid intima media thickness, HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; HbA1c, hemoglobin A1c; CRP, C-reactive protein; GGT, gamma-glutamyl transferase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; eGFR, estimated glomerular filtration rate; FIB-4, fibrosis-4 index; CAP, controlled attenuation parameter; LSM, liver stiffness measurement.

Table 3. Demographic and clinical characteristics by risk of significant fibrosis status in the total population and subgroups.

| Characteristics               | Total<br>(N = 475)   |                             |             | T2DM<br>(N = 142)    |                             |             | T1DM<br>(N = 78)    |                            |             | Non-DM<br>(N = 255)  |                             |             |
|-------------------------------|----------------------|-----------------------------|-------------|----------------------|-----------------------------|-------------|---------------------|----------------------------|-------------|----------------------|-----------------------------|-------------|
|                               | Fibrosis<br>(N = 87) | No<br>Fibrosis<br>(N = 388) | p-<br>Value | Fibrosis<br>(N = 32) | No<br>Fibrosis<br>(N = 110) | p-<br>Value | Fibrosis<br>(N = 6) | No<br>Fibrosis<br>(N = 72) | p-<br>Value | Fibrosis<br>(N = 49) | No<br>Fibrosis<br>(N = 206) | p-<br>Value |
| Age (years)                   | 54 ±<br>13           | 53 ±<br>14                  | 0.453       | 56 ±<br>13           | 60 ± 11                     | 0.124       | 56 ±<br>6.1         | 52 ±<br>12                 | 0.144       | 52 ±<br>14           | 49 ±<br>14                  | 0.187       |
| M/F                           | 54/33                | 198/190                     | 0.062       | 21/11                | 65/45                       | 0.506       | 3/3                 | 36/36                      | 1.000       | 30/19                | 97/109                      | 0.075       |
| BMI (kg/m²)                   | 31.9 ±<br>7.6        | 29.5 ±<br>6.5               | 0.023       | 34.2 ±<br>7.5        | 29.4 ±<br>5.8               | 0.004       | 31.7 ±<br>6.0       | 26.7 ±<br>4.7              | 0.017       | 29.7 ±<br>7.5        | 30.7 ±<br>7.3               | 0.523       |
| cIMT (mm)                     | 0.76 ±<br>0.11       | 0.70 ±<br>0.14              | 0.120       | 0.76 ±<br>0.10       | 0.73 ±<br>0.13              | 0.565       | 0.77 ±<br>0.16      | 0.68 ±<br>0.13             | 0.178       | 0.76 ±<br>NA         | 0.68 ±<br>0.16              | NA          |
| Total cholesterol<br>(mmol/L) | 5.0 ±<br>1.6         | 5.0 ±<br>5.0                | 0.989       | 5.0 ±<br>8.1         | 5.2 ±<br>1.7                | 0.907       | 4.2 ±<br>0.81       | 4.4 ±<br>1.0               | 0.728       | 5.2 ±<br>1.6         | 5.1 ±<br>1.0                | 0.891       |
| HDL-C (mmol/L)                | 1.2 ±<br>0.48        | 1.3 ±<br>0.38               | 0.134       | 1.1 ±<br>0.37        | 1.2 ±<br>0.31               | 0.748       | 1.5 ±<br>0.18       | 1.5 ±<br>0.44              | 0.989       | 1.1 ±<br>0.66        | 1.3 ±<br>0.31               | 0.506       |
| LDL-C (mmol/L)                | 2.7 ±<br>1.2         | 2.5 ±<br>0.87               | 0.575       | 2.6 ±<br>1.3         | 2.2 ±<br>0.84               | 0.257       | 2.0 ±<br>0.55       | 2.3 ±<br>0.76              | 0.382       | 3.1 ±<br>1.2         | 3.0 ±<br>0.74               | 0.762       |
| Triglycerides<br>(mmol/L)     | 2.3 ±<br>1.2         | 1.9 ±<br>1.4                | 0.168       | 2.6 ±<br>1.2         | 2.3 ±<br>1.6                | 0.425       | 1.6 ±<br>0.78       | 1.3 ±<br>0.70              | 0.360       | 2.0 ±<br>1.1         | 1.9 ±<br>1.3                | 0.905       |
| Glucose (mmol/L)              | 8.0 ±<br>3.0         | 7.3 ±<br>3.3                | 0.317       | 10.3 ±<br>3.4        | 10.9 ±<br>4.3               | 0.691       | 9.3 ±<br>1.1        | 8.5 ±<br>3.8               | 0.800       | 6.0 ±<br>0.76        | 5.9 ±<br>1.1                | 0.787       |
| HbA1c (mmol/mol)              | 52 ±<br>15           | 51 ±<br>15                  | 0.656       | 60 ±<br>16           | 59 ±<br>12                  | 0.690       | 57 ±<br>6.5         | 57 ±<br>13                 | 0.997       | 38 ±<br>4.2          | 36 ±<br>5.1                 | 0.079       |
| Apo B (g/L)                   | 1.1 ±<br>0.32        | 1.0 ±<br>0.27               | 0.454       | 1.2 ±<br>0.38        | 1.0 ±<br>0.38               | 0.399       | 0.61 ±<br>NA        | 0.93 ±<br>0.27             | NA          | 1.2 ±<br>0.17        | 1.1 ±<br>0.20               | 0.351       |

Table 3. Demographic and clinical characteristics by risk of significant fibrosis status in the total population and subgroups.  
(continued)

| Characteristics                       | Total<br>(N = 475)   |                             | T2DM<br>(N = 142) |                      | T1DM<br>(N = 78)            |             | Non-DM<br>(N = 255)  |                             |             |
|---------------------------------------|----------------------|-----------------------------|-------------------|----------------------|-----------------------------|-------------|----------------------|-----------------------------|-------------|
|                                       | Fibrosis<br>(N = 87) | No<br>Fibrosis<br>(N = 388) | p-<br>Value       | Fibrosis<br>(N = 32) | No<br>Fibrosis<br>(N = 110) | p-<br>Value | Fibrosis<br>(N = 49) | No<br>Fibrosis<br>(N = 206) | p-<br>Value |
| Apo AI (g/L)                          | 1.4 ±<br>NA          | 1.5 ±<br>0.26               | NA                | 1.4 ±<br>NA          | 1.5 ±<br>0.25               | NA          | NA                   | 1.6 ±<br>0.27               | NA          |
| CRP (mg/L)                            | 6.8 ±<br>8.6         | 8.0 ±<br>15                 | 0.639             | 3.9 ±<br>2.2         | 4.6 ±<br>4.3                | 0.623       | 8.0 ±<br>NA          | 8.9 ±<br>17                 | 0.786       |
| Hemoglobin<br>(mmol/L)                | 8.8 ±<br>1.1         | 8.7 ±<br>1.1                | 0.866             | 8.7 ±<br>0.98        | 8.7 ±<br>1.1                | 0.987       | 8.5 ±<br>0.57        | 8.8 ±<br>1.1                | 0.925       |
| Thrombocytes<br>(10 <sup>9</sup> /L)  | 240 ±<br>87          | 263 ±<br>87                 | 0.085             | 240 ±<br>90          | 259 ±<br>95                 | 0.469       | 193 ±<br>70.7        | 243 ±<br>88                 | 0.308       |
| GGT (U/L)                             | 96.8 ±<br>80.2       | 96.4 ±<br>131               | 0.983             | 117 ±<br>89          | 143 ±<br>175                | 0.537       | 98 ±<br>NA           | 87.3 ±<br>118               | 0.967       |
| ALT (U/L)                             | 57 ±<br>45           | 42 ±<br>24                  | 0.021             | 67 ±<br>47           | 43 ±<br>26                  | 0.052       | 82 ±<br>NA           | 51 ±<br>45                  | 0.277       |
| AST (U/L)                             | 65 ±<br>56           | 48 ±<br>35                  | 0.025             | 91 ±<br>80           | 45 ±<br>37                  | 0.018       | 66 ±<br>43           | 51 ±<br>36                  | 0.793       |
| Creatinine (μmol/L)                   | 76.6 ±<br>17         | 81.6 ±<br>38                | 0.283             | 81.5 ±<br>19         | 89.7 ±<br>60                | 0.490       | 81.0 ±<br>27         | 72.2 ±<br>11                | 0.087       |
| Albumin (g/L)                         | 52.4 ±<br>61         | 43.4 ±<br>4.1               | 0.315             | 44.3 ±<br>2.9        | 43.4 ±<br>5.5               | 0.552       | 43.0 ±<br>4.2        | 43.3 ±<br>3.7               | 0.334       |
| eGFR (mL/<br>min/1.73m <sup>2</sup> ) | 75.5 ±<br>12.5       | 75.8 ±<br>15.7              | 0.901             | 71.7 ±<br>14.7       | 71.2 ±<br>17.8              | 0.973       | 67.3 ±<br>11.9       | 80.5 ±<br>8.7               | 0.916       |

Table 3. Demographic and clinical characteristics by risk of significant fibrosis status in the total population and subgroups.  
(continued)

| Characteristics | Total<br>(N = 475)   |                             | T2DM<br>(N = 142) |                      | T1DM<br>(N = 78)            |             | Non-DM<br>(N = 255) |                            |             |
|-----------------|----------------------|-----------------------------|-------------------|----------------------|-----------------------------|-------------|---------------------|----------------------------|-------------|
|                 | Fibrosis<br>(N = 87) | No<br>Fibrosis<br>(N = 388) | p-<br>Value       | Fibrosis<br>(N = 32) | No<br>Fibrosis<br>(N = 110) | p-<br>Value | Fibrosis<br>(N = 6) | No<br>Fibrosis<br>(N = 49) | p-<br>Value |
| FIB-4           | 1.79 ±<br>1.3        | 1.88 ±<br>1.5               | 0.738             | 1.62 ±<br>1.1        | 2.08 ±<br>1.6               | 0.240       | 2.34 ±<br>NA        | 1.90 ±<br>1.5              | 0.840       |
| CAP (dB/m)      | 283 ±<br>62          | 258 ±<br>58                 | <0.001            | 310 ±<br>46          | 275 ±<br>60                 | <0.001      | 286 ±<br>62         | 266 ±<br>66                | 0.308       |
| LSM (kPa)       | 10.8 ±<br>3.6        | 5.1 ±<br>1.4                | <0.001            | 10.5 ±<br>2.1        | 5.0 ±<br>1.5                | <0.001      | 9.8 ±<br>1.3        | 11.1 ±<br>4.5              | <0.001      |

\* Data are given as mean (standard deviation), median (IQR), or numbers (%) where appropriate. \*\* Values shown as mean ± SD. NA = data not available due to small sample size or missing measurements. Abbreviations: T2DM, type 2 diabetes mellitus; T1DM, type 1 diabetes mellitus; non-DM, high CV-risk non-diabetics; BMI, body mass index; cIMT, carotid intima media thickness, HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; HbA1c, hemoglobin A1C; CRP, C-reactive protein; GGT, gamma-glutamyl transferase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; eGFR, estimated glomerular filtration rate; FIB-4, fibrosis-4 index; CAP, controlled attenuation parameter; LSM, liver stiffness measurement.

## Predictors of MASLD and Fibrosis

Logistic regression analysis identified BMI as a significant predictor of MASLD across all patient groups (Table 4). In the total cohort, each 1 kg/m<sup>2</sup> increase in BMI was associated with a 13.3% increase in the odds of MASLD. Similarly, TGs were significantly associated with MASLD, with a 49.6% increase in the odds for each 1 mmol/L increase. Linearity of continuous predictors was assessed visually, and BMI and TGs showed clear linear trends, supporting their inclusion as continuous variables in the models. In subgroup analyses, BMI remained a crucial predictor of MASLD. In T2DM patients, each 1 kg/m<sup>2</sup> increase in BMI raised the odds of MASLD by 13.2%, while in T1DM patients, the odds increased by 22.3%, and by 11.7% in non-diabetic individuals with high CV-risk (Table 4). BMI was also identified as a predictor of risk for significant fibrosis in both T2DM and T1DM patients (Table 5). While BMI showed a positive association with risk of significant fibrosis in the overall cohort, the association did not reach statistical significance ( $p = 0.08$ ). Additionally, TGs were found to be a strong predictor of risk of significant fibrosis in the overall cohort (Table 5). VIF values between independent variables were all below 5, indicating no significant collinearity.

Table 4. Independent predictors of MASLD from the final backward stepwise logistic regression model in the overall cohort and subgroups.

| Group         | Variable                 | B (SE)         | Exp(B) | 95% CI      | p-Value |
|---------------|--------------------------|----------------|--------|-------------|---------|
| Total cohort  | Constant                 | -4.745 (0.849) | 0.009  |             | <0.001  |
|               | BMI (kg/m <sup>2</sup> ) | 0.125 (0.026)  | 1.133  | 1.077–1.192 | <0.001  |
|               | Triglycerides (mmol/L)   | 0.403 (0.174)  | 1.496  | 1.064–2.102 | 0.020   |
| T2DM          | Constant                 | -3.491 (1.403) | 0.030  |             | 0.013   |
|               | BMI (kg/m <sup>2</sup> ) | 0.125 (0.026)  | 1.132  | 1.033–1.240 | 0.008   |
| T1DM          | Constant                 | -7.001 (2.143) | 0.001  |             | 0.001   |
|               | BMI (kg/m <sup>2</sup> ) | 0.202 (0.071)  | 1.223  | 1.064–1.406 | 0.005   |
| Non-diabetics | Constant                 | -5.152 (1.513) | 0.006  |             | <0.001  |
|               | BMI (kg/m <sup>2</sup> ) | 0.111 (0.038)  | 1.117  | 1.037–1.203 | 0.004   |

a Variables entered on step 1: Age, BMI, HbA1c, triglycerides, HDL-C, LDL-C, sex.  
b Abbreviations: T2DM, type 2 diabetes mellitus; T1DM, type 1 diabetes mellitus; non-diabetics, high CV-risk non-diabetics.

Table 5. Independent predictors of significant fibrosis from the final backward stepwise logistic regression model in the overall cohort and subgroups.

| Group         | Variable                 | B (SE)         | Exp(B) | 95% CI      | p-Value |
|---------------|--------------------------|----------------|--------|-------------|---------|
| Total cohort  | Constant                 | -4.337 (1.047) | 0.013  |             | <0.001  |
|               | Triglycerides (mmol/L)   | 0.422 (0.208)  | 1.524  | 1.015–2.289 | 0.042   |
| T2DM          | Constant                 | -4.746 (1.544) | 0.009  |             | 0.002   |
|               | BMI (kg/m <sup>2</sup> ) | 0.104 (0.047)  | 1.110  | 1.012–1.217 | 0.027   |
| T1DM          | Constant                 | -8.904 (3.238) | 0.000  |             | 0.006   |
|               | BMI (kg/m <sup>2</sup> ) | 0.212 (0.097)  | 1.236  | 1.022–1.494 | 0.029   |
| Non-diabetics | Constant                 | -3.020 (0.724) | 0.049  |             | <0.001  |

a Variables entered on step 1: Age, BMI, HbA1c, triglycerides, HDL-C, LDL-C, sex.  
b Abbreviations: T2DM, type 2 diabetes mellitus; T1DM, type 1 diabetes mellitus; non-diabetics, high CV-risk non-diabetics.

MASLD and Fibrosis in CVD Patients

In the entire cohort, 86 patients (18.1%) had a history of CVD. Among these, 35 patients (40.7%) had MASLD, and 18 patients (20.9%) were at risk of significant fibrosis. Chi-square tests revealed no statistically significant differences in the prevalence of MASLD or risk of significant fibrosis between patients with and without CVD ( $p = 0.75$  and  $p = 0.49$ , respectively).

DISCUSSION

In this cross-sectional study of patients attending a Dutch cardiometabolic outpatient clinic, we observed a high prevalence of MASLD (39.2%) and risk of significant fibrosis (18.3%), indicating a considerable burden of undiagnosed liver disease in this high-risk population. The prevalence of MASLD was highest among patients with T2DM followed by high CV-risk non-diabetic individuals and patients with T1DM. A similar pattern was observed for the risk of significant fibrosis. Notably, T2DM patients with MASLD had a higher LSM, suggesting that MASLD may more strongly predispose this group to fibrosis development. Interestingly, neither MASLD nor risk of significant fibrosis were associated with cIMT. This may be explained that other CV risk factors may be operative in this high-risk population and therefore, MASLD does not differentiate in this respect. Of course, the number of subjects was limited which may have

influenced these results. Our diagnostic model identified BMI and TGs as the strongest predictors of MASLD and risk of significant fibrosis, reinforcing their role as key metabolic risk factors. These data clearly point at the relevance of weight control and lifestyle as main interventions in MASLD.

In line with existing literature, MASLD prevalence was higher in T2DM patients compared to the general population (17, 22, 23). Interestingly, MASLD was least prevalent in T1DM patients. This lower prevalence may reflect the unique metabolic profile of T1DM, including absent endogenous insulin, reduced portal insulin levels, and increased GH and reduced IGF-1, fostering a catabolic, fasting-like state that limits hepatic fat accumulation (24). These distinct metabolic characteristics may confer protection against MASLD development and progression in T1DM. While the small sample size warrants cautious interpretation, similar prevalence rates were observed in another Dutch T1DM cohort (25).

Relative to global data, the MASLD prevalence observed in our cohort was lower than the 65.3% pooled prevalence reported in a recent meta-analysis of T2DM patients worldwide, which included over 2.2 million individuals. The meta-analysis also noted regional variations, with the highest prevalence in Eastern Europe (80.6%) and the Middle East (71.2%) (26). The lower prevalence in our study may partly reflect differences in diagnostic approaches, as the meta-analysis used estimated rates, whereas we used VCTE, providing more direct assessment of liver steatosis and fibrosis.

Few studies have assessed the prevalence of clinically significant liver fibrosis (defined as  $\geq 8.1$  kPa) in the general population, with reported rates ranging from 2% to 13.8% (27–31). In our cohort, the risk of significant fibrosis was substantially higher than these general population estimates, though still lower than rates reported in T2DM patients from Eastern European studies. For instance, a Romanian study of 424 T2DM patients using VCTE reported a significant fibrosis prevalence of 57.1%, while a Croatian study, applying a lower cutoff value of 7.0 kPa, found a prevalence of 46.6% in T2DM patients (32, 33). These differences likely reflect variations in study populations, diagnostic criteria, VCTE cutoff thresholds, as well as potential influences of healthcare practices, genetic predispositions, and lifestyle factors specific to the Netherlands. Notably, the prevalence of significant fibrosis observed among T1DM patients in

our study (7.7%) is consistent with earlier Dutch findings, which reported rates around 6.7% in similar cohorts assessed with VCTE (25).

Previous Dutch MASLD research has primarily focused on general population cohorts, for example, the Lifelines study in Northern Holland, which used the Fatty Liver Index (FLI) for diagnosis (34), and the Rotterdam Study, which assessed an elderly population using ultrasound (35). De Vries et al. further compared MASLD prevalence between patients with T1DM and T2DM (25). Our study builds on this work by including non-diabetic patients with high CV risk, thereby offering a more comprehensive and representative overview of MASLD prevalence in routine cardiometabolic clinical practice.

In our cohort, VCTE provided quantitative LSM and CAP values to identify patients at risk of significant fibrosis. Although many studies focus primarily on advanced fibrosis (F3–F4), detecting significant fibrosis is crucial, as this population represents the main target for clinical trials and early intervention. VCTE enables reliable risk stratification in outpatient settings, and while traditional scores such as FIB-4 and NAFLD Fibrosis Score (NFS) can complement it, they may underestimate F2–F3 prevalence. Recent studies have explored combined scoring systems, blood-based biomarkers, and machine learning approaches to improve identification of patients with significant fibrosis, supporting the integration of these tools into routine outpatient screening strategies (36–38).

Our findings reveal a substantial burden of undiagnosed liver disease in this cohort, particularly among T2DM patients, reinforcing the importance of incorporating NITs into routine screening. Early detection using these methods could help identify patients at risk of progression, leading to earlier interventions and potentially improving long-term outcomes. These results support current recommendations from several organizations that support non-invasive MASLD assessment in individuals with cardiometabolic risk (13, 15, 39).

Nonetheless, some limitations should be noted. The cross-sectional design of the current study restricts our ability to establish causality or to evaluate the risk of long-term liver-related morbidity within the cohort. However, the presence of fibrosis has been consistently associated with adverse liver-related outcomes in longitudinal studies (40), underscoring the importance of its identification. Moreover, statistical

power was limited by the small T1DM subgroup and the low number of fibrosis events, restricting the ability to draw definitive conclusions for these patients. Additionally, residual confounding could have influenced the observed null associations between cIMT, MASLD, and fibrosis, given the exploratory study design, incomplete covariate data, and small T1DM and T2DM subgroups that restricted fully adjusted analyses.

While VCTE is a reliable and non-invasive method for assessing steatosis and fibrosis, it cannot fully substitute for liver biopsy, the diagnostic gold standard. Some serum markers, such as FLI and routine abdominal ultrasound, were not assessed, and FIB-4 was available for only a subset of participants, possibly limiting detection of differences. Additionally, all blood samples were collected in the non-fasting state, which may particularly influence triglyceride values, although diabetes classification was primarily based on HbA1c and EHR diagnoses. The single-center, high CV-risk nature of the cohort may limit generalizability of our findings to broader populations. Furthermore, baseline medication use was recorded but not adjusted for, which may affect liver and cardiometabolic outcomes.

Future studies with larger cohorts, fasting laboratory assessments, and additional non-invasive markers alongside VCTE are needed to better evaluate MASLD and significant fibrosis.

## CONCLUSIONS

In conclusion, this study highlights a substantial burden of undiagnosed MASLD and risk of significant fibrosis in cardiometabolic outpatient populations, particularly among patients with T2DM. Elevated LSM values observed in T2DM patients with MASLD suggest an increased risk for fibrosis progression. The absence of an association between MASLD or fibrosis and cIMT may reflect distinct mechanisms driving hepatic and vascular risk in this cohort. BMI and plasma TG were consistent predictors across groups urging for more strict control by body weight reduction and lifestyle interventions.

## SUPPLEMENTARY MATERIAL

Table S1. Ethnicity distribution in the total cohort presented as n (%).

| Ethnicity      | Total cohort (N=470) |
|----------------|----------------------|
| Caucasian      | 318 (67.7)           |
| North African  | 26 (5.5)             |
| Other African  | 11 (2.3)             |
| Turkish        | 31 (6.6)             |
| Hindustani     | 23 (4.9)             |
| Asian          | 20 (4.3)             |
| Latin-American | 13 (2.8)             |
| Other          | 28 (6.0)             |

\*Ethnicity data were available for 470 of 475 participants; 5 participants had missing data.

Table S2. Baseline medication use in the total cohort and subgroups presented as n (%).

| Characteristics | Total<br>(N = 475) | T2DM<br>(N = 142) | T1DM<br>(N = 78) | Non-DM<br>(N = 255) |
|-----------------|--------------------|-------------------|------------------|---------------------|
| Metformin       | 102 (21.5)         | 90 (63.4) ***     | 11 (14.1) **     | 1 (0.4)             |
| Statin          | 190 (40)           | 99 (69.7) ***     | 45 (57.7) ***    | 46 (18.0)           |
| SU-derivative   | 27 (5.7)           | 26 (18.3) ***     | 1 (1.3)          | 0 (0)               |
| GLP-1           | 19 (4.0)           | 16 (11.3) ***     | 1 (1.3)          | 2 (0.8)             |
| SGLT2i          | 25 (5.3)           | 24 (16.9) ***     | 1 (1.3)          | 0 (0)               |
| ACEi            | 53 (11.2)          | 30 (21.1) ***     | 10 (12.8)        | 13 (5.1)            |
| ARB             | 109 (22.9)         | 59 (41.5) ***     | 20 (25.6) *      | 30 (11.8)           |
| CCB             | 72 (15.2)          | 30 (21.1) *       | 14 (17.9)        | 28 (11.0)           |
| Beta blocker    | 58 (12.2)          | 32 (22.5) ***     | 6 (7.7)          | 20 (7.8)            |
| Diuretics       | 66 (13.9)          | 30 (21.1) **      | 9 (11.5)         | 27 (10.6)           |
| DPP4-inhibitor  | 14 (2.9)           | 14 (9.9) ***      | 0 (0)            | 0 (0)               |
| Alpha blocker   | 6 (1.3)            | 4 (2.8)           | 0 (0)            | 2 (0.8)             |

Statistical significance is indicated by asterisks \*, \*\*, and \*\*\*, corresponding to probability values of <0.05, <0.01, and <0.001, respectively. These significance levels are in relation to the non-diabetic subgroup. Abbreviations: T2DM, type 2 diabetes mellitus; T1DM, type 1 diabetes mellitus; non-diabetics, high CV-risk non-diabetics; SU-derivative, sulfonylurea derivative; GLP-1, glucagon-like peptide-1; SGLT2i, sodium-glucose cotransporter 2 inhibitor; ACEi, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker; CCB, calcium channel blocker; DPP4-inhibitor, dipeptidyl peptidase-4 inhibitor.

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# PART II

MASLD, cardiovascular risk and  
metabolic associations



# Chapter 4

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**Prevalence of MASLD and liver fibrosis  
in an outpatient population:  
limited impact on cIMT but strong  
metabolic association**

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Submitted

## ABSTRACT

### Introduction

Metabolic dysfunction-associated steatotic liver disease (MASLD) affects up to 30% of the global adult population, with a higher prevalence in high-risk groups, such as individuals with type 2 diabetes mellitus (T2D) or obesity, which contributes to an increased risk of cardiovascular disease. This study aims to evaluate the prevalence of MASLD and liver fibrosis across various BMI among outpatients and examine their association with carotid intima-media thickness (cIMT).

### Materials and methods

This retrospective study analyzed data from the outpatient clinic at Franciscus Hospital, Rotterdam. Liver steatosis and stiffness were measured via transient elastography and cIMT with carotid ultrasound. The cohort was stratified by BMI.

### Results

A total of 475 patients were included, with a mean BMI of 29.8 kg/m<sup>2</sup> ( $\pm 6.7$ ). The cohort comprised 35.7% overweight and 41.6% obese individuals. MASLD was present in 40.2% of patients, and significant fibrosis in 18.9%, both showing higher prevalence in groups with elevated BMI. Multiple regression analysis revealed that BMI (OR = 1.147, 95% CI: 1.083–1.215,  $p < 0.001$ ) and triglycerides (OR = 1.536, 95% CI: 1.016–2.322,  $p = 0.042$ ) were positively associated with MASLD, with BMI alone (OR = 1.074, 95% CI: 1.002–1.151,  $p = 0.045$ ) as a significant predictor of liver fibrosis. Additionally, BMI ( $B = 0.004$ ,  $\beta = 0.165$ ,  $p = 0.032$ ), age ( $B = 0.007$ ,  $\beta = 0.610$ ,  $p < 0.001$ ), and LDL cholesterol ( $B = 0.024$ ,  $\beta = 0.160$ ,  $p = 0.044$ ) were positively associated with cIMT. Conversely, HDL cholesterol was negatively associated with cIMT ( $B = -0.070$ ,  $\beta = -0.219$ ,  $p = 0.022$ ).

### Conclusion

MASLD and liver fibrosis were more prevalent in the outpatient cohort compared to the general population, driven by metabolic risk factors such as BMI and triglycerides. While cIMT was modestly associated with age, BMI, and LDL cholesterol, it showed no significant relationship with liver steatosis or fibrosis, limiting its utility as a marker for liver-related conditions.

## INTRODUCTION

Metabolic dysfunction-associated steatotic liver disease (MASLD) may be present in 30 % of the adult world population with higher prevalence in risk groups such as patients with type 2 diabetes mellitus (T2D), obesity, metabolic syndrome or arterial hypertension [1]. Patients with MASLD can develop metabolic dysfunction associated steatohepatitis (MASH), the inflammatory form of MASLD, and liver fibrosis which determines the severity of the disease [2]. At term, MASLD/MASH can lead to liver cirrhosis and hepatocellular carcinoma (HCC) if no intervention is proposed on time [3–5]. If individuals do not develop liver-related complications, the risk of non-fatal cardiovascular disease (CVD), coronary heart disease or heart failure is significantly higher for people with MASLD [6–8]. Each individual may experience unique progression and complications, highlighting the need for personalized treatment [9–11].

The risk of liver fibrosis is increased for patients with T2D [10, 12, 13] and the outcomes are poor for those with cirrhosis or HCC [14, 15]. The risk of HCC development and HCC-related mortality is also increased for people with obesity [16]. Data on MASLD/MASH in the Netherlands is very limited compared to other countries where more detailed information has been published [17].

MASLD/MASH often remains underdiagnosed due to a lack of awareness among healthcare professionals [18] and the absence of symptoms in most of the cases. Liver biopsy is currently the gold standard for diagnosis but this invasive and expensive method carries risks such as bleeding, infection or pain [19]. In order to avoid unnecessary biopsies, non-invasive diagnostic tools have been developed. In clinical practice, guidelines recommend first serum-based non-invasive methods, such as the FIB-4 index, and then the use of a non-invasive test like vibration-controlled transient elastography (TE) [20]. Early detection of CVD and risk assessment can be achieved through the measurement of carotid intima-media thickness (cIMT), a marker of subclinical atherosclerosis. [21–23].

To enhance clinical outcomes, this study aims to map the prevalence of MASLD and liver fibrosis across various BMI among outpatients in Rotterdam, the Netherlands. Additionally, the study will assess the relationship between liver steatosis, liver fibrosis, and cIMT. These findings will provide valuable insights into the extent of this issue within the patient population.

## METHODS

### Study design

This retrospective database study was conducted using data obtained from the outpatient clinic of Endocrinology, Diabetes and Vascular Medicine of the Franciscus Gasthuis hospital, Rotterdam, the Netherlands. Data were collected between January the 1<sup>st</sup> of 2021 and October the 1<sup>st</sup> of 2022.

### Study population

Patients were eligible for the study if they were 18 years or older at the time of inclusion. Diabetes was defined by one of the following criteria: fasting glucose levels  $\geq 7.0$  mmol/L, HbA1c  $\geq 48$  mmol/mol, or the use of antidiabetic medication. The type of diabetes was obtained from the patients' medical records.

Patients were excluded if they consumed alcohol in excess of 20 g/day for women and 30 g/day for men, or if they had any other form of chronic liver disease that could potentially affect transient elastography results, including viral hepatitis, hemochromatosis, or Wilson's disease.

### Transient elastography

The primary objective is to evaluate the prevalence of MASLD and the severity of liver fibrosis in an at-risk cohort. We used TE (Fibroscan®, Echosens, Paris, France) with the controlled attenuation parameter (CAP) to quantify liver steatosis, and liver stiffness measurement (LSM) to assess the degree of liver fibrosis. Following the manufacturer's instructions, TE was performed on participants lying down, with the probe placed in the intercostal space near the right lobe of the liver. [24][25, 26]. At least ten valid measurements were performed by an operator trained and certified by Echosens. As reported elsewhere, the interquartile range-to-median ratio had to be  $<30\%$  for the measurements to be considered successful [27]. Measurements that did not meet these criteria were deemed unsuccessful and excluded from the analysis.

### FIB-4 score

We calculated the FIB-4 index as described in recent guidelines to evaluate liver fibrosis [20]. The index was calculated when possible using the patient's age, aspartate transaminase (AST), alanine transaminase (ALT), and blood platelet count [28]. The index was defined as follows:  $\text{FIB-4 index} = (\text{Age} * \text{AST}) / (\text{platelets} * \sqrt{\text{ALT}})$

## Carotid intima media thickness

Carotid ultrasound scans were conducted using the ART-LAB system (Esaote, Italy) by experienced sonographers. Patients were positioned supine with their heads comfortably resting and their necks slightly extended and rotated away from the probe. B-mode ultrasound was used to capture images of the common carotid arteries (CCA), creating two echogenic lines representing the combined thickness of the artery's intima and media layers, measured 1 cm from each CCA. Three distinct projections were obtained for each CCA: 210°, 240°, and 270° on the left, and 90°, 120°, and 150° on the right. Measurements were taken in triplicate for each segment using semi-automated methods. In cases where technological issues limited data availability, mean cIMT was calculated using measurements from at least two angles per side.

## Data analysis

The study group was divided into three subgroups according to their body mass index (BMI): lean (BMI <25 kg/m<sup>2</sup>), overweight (BMI 25–29,9 kg/m<sup>2</sup>) and obese (BMI ≥30 kg/m<sup>2</sup>).

TE data were analyzed to evaluate both liver steatosis and fibrosis. As defined in the recent guidelines, a CAP value of ≥275 dB/m was chosen to define steatosis, and a LSM ≥8 kPa for significant fibrosis (F2) as defined by Kleiner et al [29].

The FIB-4 score determines the risk for liver fibrosis as follows: low risk (<1.30), intermediate risk (≥1.30 and ≤2.67), and high risk (>2.67) [28, 30].

The study assessed the occurrence of liver steatosis and fibrosis in the entire study population, categorizing by BMI subgroups.

Logistic regression analysis was performed to evaluate the effects of age, gender, BMI, HbA1c triglycerides, HDL-C, LDL-C and sex on liver steatosis and fibrosis. Finally, a linear regression analysis was conducted to determine the influence of these factors, liver steatosis, and liver fibrosis on cIMT.

All analyses were conducted using IBM SPSS version 29 (IBM Corporation, Armonk, New York, USA). Associations with a p-value <0.05 were considered statistically significant.

# RESULTS

## General baseline characteristics

For this study, a cohort of 475 patients with a valid TE measurement was included. In this cohort, 46.9% of the participants were women. The overall cohort had a mean BMI of 29.8 ( $\pm 6.7$ ), indicating overweight. The proportion of individuals with obesity was higher than those classified as overweight or lean, with rates of 41.6%, 35.7%, and 22.6%, respectively. Hypertension was present in 37.6% of the patients, while hypercholesterolemia was found in 20.7%. Diabetes was present in 46.3% of the cohort, with 29.9% diagnosed with T2D.

The non-invasive tests revealed a mean FIB-4 index of 1.86, indicating an intermediate risk for liver fibrosis. Additionally, TE reported a mean LSM of 6.1 kPa, and liver steatosis was present in 40.4% of the patients. This results are presented in table 1.

Table 1 – Baseline characteristics of the FENDATA population.

|                                | Total (n=475)    |
|--------------------------------|------------------|
| Age, years                     | 53.01 $\pm$ 13.7 |
| Female, n (%)                  | 223 (46.9%)      |
| BMI, kg/m <sup>2</sup>         | 29.8 $\pm$ 6.7   |
| Lean, n (%)                    | 88 (22.6%)       |
| Overweight, n (%)              | 139 (35.7%)      |
| Obese, n (%)                   | 162 (41.6%)      |
| Hypertension, n(%)             | 178 (37.6%)      |
| Hypercholesterolemia, n(%)     | 98 (20.7%)       |
| Creatinine, $\mu$ mol/L        | 80.7 $\pm$ 35.4  |
| ALT, U/L                       | 45.7 $\pm$ 31.6  |
| AST, U/L                       | 51.7 $\pm$ 41.6  |
| Cholesterol, mmol/L            | 5.00 $\pm$ 4.71  |
| LDL-chol, mmol/L               | 5.55 $\pm$ 0.92  |
| HDL-chol, mmol/L               | 1.28 $\pm$ 0.39  |
| Triglycerides, mmol/L          | 1.97 $\pm$ 1.35  |
| Albumin, g/L                   | 45.6 $\pm$ 30.3  |
| Hemoglobin, mmol/L             | 8.7 $\pm$ 1.1    |
| Platelets, x10 <sup>9</sup> /L | 257.6 $\pm$ 87.4 |

Table 1 – Baseline characteristics of the FENDATA population. (*continued*)

|                          | Total (n=475)  |
|--------------------------|----------------|
| HbA1c, mmol/mol (n=475)  | 50.9 ± 14.8    |
| None, n (%)              | 255 (53.7%)    |
| DM1, n (%)               | 78 (16.4%)     |
| DM2, n (%)               | 142 (29.9%)    |
| FIB4 score (n=218)       | 1.86 ± 1.50    |
| low risk, n (%)          | 100 (45.7%)    |
| intermediate risk, n (%) | 79 (36.1%)     |
| high risk, n (%)         | 40 (18.3%)     |
| CAP (dB/m) (n=475)       | 262.5 (± 59.2) |
| ≥ 275 dB/m, n (%)        | 193 (40.4%)    |
| LSM (kPa) (n=475)        | 6.1 (± 3.0)    |
| ≥ 8.0 kPa, n (%)         | 90 (18.9%)     |
| cIMT, mm (=203)          | 0.708 (±0.138) |

Data are given as mean standard ± deviation or numbers (%) where appropriate.

## MASLD characteristics in BMI groups

In this cohort, 40.2% of the outpatients have MASLD. These individuals exhibited significantly higher BMI (24.5 vs. 33.0 kg/m<sup>2</sup>,  $p < 0.001$ ), transaminase levels, and LSM measurements (5.7 vs. 6.8 kPa,  $p < 0.001$ ) compared to those without MASLD. No significant differences were observed in other clinical characteristics between the two groups.

Upon stratification by BMI, additional differences emerged. MASLD patients in the lean group had higher triglyceride levels, those in the overweight group had elevated GGT levels, and those in the obese group had higher AST levels. Finally, LSM measurements in MASLD patients were significantly higher in both the overweight and obese groups, although they did not reach the threshold of 8 kPa, which is indicative of significant fibrosis. No significant differences in cIMT were found between the MASLD group and the group without MASLD, both in the overall cohort and within each BMI group. The results are presented in table 2.

## Liver fibrosis characteristics in BMI groups

In this cohort, 18.9% of the outpatients have significant fibrosis. These individuals with TE-diagnosed liver fibrosis exhibited similar characteristics to those with MASLD, including higher BMI (29.5 vs. 31.8 kg/m<sup>2</sup>,  $p=0.026$ ), elevated transaminase levels, and increased CAP measurements (257.6 vs. 283.9 dB/m,  $p<0.001$ ). No significant differences were observed in other clinical characteristics between the two groups.

Table 2 – Clinical and biochemical characteristics of participants with and without MASLD, stratified by BMI categories

| Characteristics           | Total (n=475) |              |         |  | Lean (n=88)  |               |         |  | Overweight (n=139) |              |         |  | Obese (n=162) |              |         |  |
|---------------------------|---------------|--------------|---------|--|--------------|---------------|---------|--|--------------------|--------------|---------|--|---------------|--------------|---------|--|
| TE                        | No MASLD      | MASLD        | P-value |  | No MASLD     | MASLD         | P-value |  | No MASLD           | MASLD        | P-value |  | No MASLD      | MASLD        | P-value |  |
| N (%)                     | 283 (59.6%)   | 192 (40.4%)  |         |  | 78 (88.6%)   | 10 (11.4%)    |         |  | 86 (61.9%)         | 53 (38.1%)   |         |  | 61 (37.7%)    | 101 (62.3%)  |         |  |
| Age, years                | 53,9 ± 13,3   | 51,7 ± 14,3  | 0,091   |  | 55,1 ± 13,3  | 62,2 ± 8,7    | 0,104   |  | 53,5 ± 12,0        | 54,3 ± 13,0  | 0,711   |  | 50,6 ± 13,4   | 49,6 ± 15,4  | 0,678   |  |
| Woman, n (%)              | 134 (47,3)    | 89 (46,4)    | 0,839   |  | 40 (51,3)    | 4 (40,0)      | 0,502   |  | 33 (38,4)          | 23 (43,4)    | 0,557   |  | 37 (60,7)     | 53 (52,5)    | 0,31    |  |
| BMI, kg/m2                | 24,5 ± 6,0    | 33,0 ± 6,4   | <0,001  |  | 22,2 ± 3,0   | 23,5 ± 1,3    | 0,165   |  | 27,2 ± 1,5         | 27,6 ± 1,4   | 0,145   |  | 35,0 ± 5,3    | 36,8 ± 5,2   | 0,034   |  |
| Total cholesterol, mmol/l | 4,58 ± 1,19   | 5,50 ± 6,90  | 0,127   |  | 4,41 ± 1,16  | 5,24 ± 1,16   | 0,071   |  | 4,46 ± 1,01        | 4,63 ± 1,20  | 0,482   |  | 4,75 ± 1,25   | 6,03 ± 9,01  | 0,361   |  |
| HDL-C, mmol/l             | 1,3 ± 0,40    | 1,25 ± 0,39  | 0,317   |  | 1,46 ± 0,46  | 1,08 ± 0,63   | 0,061   |  | 1,30 ± 0,34        | 1,34 ± 0,40  | 0,613   |  | 1,18 ± 0,31   | 1,21 ± 0,31  | 0,674   |  |
| LDL-C, mmol/l             | 2,46 ± 0,94   | 2,65 ± 0,89  | 0,123   |  | 2,33 ± 0,97  | 2,85 ± 0,98   | 0,2     |  | 2,36 ± 0,77        | 2,47 ± 0,87  | 0,551   |  | 2,65 ± 0,99   | 2,74 ± 0,86  | 0,671   |  |
| Triglycerides, mmol/l     | 1,84 ± 1,36   | 2,14 ± 1,33  | 0,079   |  | 1,34 ± 0,81  | 2,32 ± 1,15   | 0,006   |  | 1,83 ± 1,47        | 1,71 ± 0,83  | 0,662   |  | 2,19 ± 1,56   | 2,39 ± 1,53  | 0,537   |  |
| HbA1c, mmol/mol           | 50,9 ± 15,2   | 50,9 ± 14,4  | 0,987   |  | 52,3 ± 14,8  | 51,8 ± 11,4   | 0,936   |  | 52,4 ± 16,3        | 50,6 ± 13,8  | 0,609   |  | 49,5 ± 14,7   | 51,1 ± 15,1  | 0,576   |  |
| CRP, mg/l                 | 8,1 ± 16,4    | 7,2 ± 9,2    | 0,686   |  | 6,3 ± 7,5    | 2,5 ± 1,3     | 0,34    |  | 10,3 ± 25,2        | 9,2 ± 13,1   | 0,881   |  | 9,3 ± 11,1    | 7,4 ± 8,1    | 0,482   |  |
| Hemoglobin, mmol/l        | 8,67 ± 1,05   | 8,81 ± 1,16  | 0,282   |  | 8,40 ± 1,01  | 8,39 ± 1,74   | 0,98    |  | 8,66 ± 1,01        | 8,62 ± 1,00  | 0,883   |  | 8,84 ± 1,04   | 8,87 ± 1,06  | 0,891   |  |
| Thrombocytes, 109/l       | 251,7 ± 89,8  | 265,4 ± 83,9 | 0,228   |  | 257,5 ± 86,6 | 263,7 ± 114,1 | 0,881   |  | 250,3 ± 94,7       | 261,3 ± 88,6 | 0,66    |  | 267,9 ± 88,2  | 279,1 ± 79,2 | 0,542   |  |

Table 2 – Clinical and biochemical characteristics of participants with and without MASLD, stratified by BMI categories (continued)

| Characteristics     | Total (n=475) | Lean (n=88)   | Overweight (n=139) | Obese (n=162) |
|---------------------|---------------|---------------|--------------------|---------------|
| GGT, U/l            | 106,6 ± 142,5 | 76,6 ± 99,4   | 135,4 ± 203,4      | 123,5 ± 142,5 |
|                     | 82,2 ± 74,8   | 172,6 ± 147,8 | 55,5 ± 26,9        | 78,8 ± 67,7   |
| ALT, U/l            | 40,3 ± 26,6   | 37,7 ± 25,5   | 40,1 ± 33,2        | 43,0 ± 28,3   |
|                     | 54,0 ± 36,8   | 40,4 ± 8,0    | 47,3 ± 24,4        | 56,3 ± 33,2   |
| AST, U/l            | 41,7 ± 29,9   | 43,4 ± 38,4   | 43,3 ± 27,3        | 42,4 ± 31,4   |
|                     | 64,6 ± 50,3   | 48,5 ± 24,8   | 59,5 ± 39,3        | 66,6 ± 59,3   |
| Creatinine, umol/l  | 81,5 ± 43,0   | 86,8 ± 76,3   | 83,4 ± 23,2        | 78,4 ± 19,5   |
|                     | 79,6 ± 20,3   | 86,6 ± 26,8   | 81,0 ± 17,6        | 78,8 ± 21,4   |
| Albumine, g/l       | 46,7 ± 41,0   | 40,4 ± 5,7    | 42,9 ± 5,2         | 41,1 ± 3,5    |
|                     | 44,1 ± 3,3    | 44,3 ± 3,1    | 44,4 ± 5,1         | 44,2 ± 2,9    |
| eGFR, ml/min/1,73m2 | 75,4 ± 15,4   | 73,0 ± 16,9   | 73,6 ± 16,9        | 78,8 ± 13,4   |
|                     | 76,3 ± 15,2   | 66,5 ± 20,9   | 74,0 ± 14,5        | 78,7 ± 14,9   |
| FIB-4               | 2,00 ± 1,59   | 2,03 ± 1,44   | 1,86 ± 1,73        | 2,07 ± 1,79   |
|                     | 1,67 ± 1,33   | 1,78 ± 0,67   | 1,57 ± 1,17        | 1,63 ± 1,47   |
| CAP, dB/m           | 222,4 ± 35,3  | 208,5 ± 34,8  | 224,5 ± 32,5       | 236,0 ± 30,0  |
|                     | 321,7 ± 30,8  | 315,8 ± 42,4  | 318,0 ± 28,5       | 326,5 ± 32,0  |
| LSM, kPa            | 5,7 ± 2,6     | 5,5 ± 2,4     | 5,1 ± 2,1          | 5,6 ± 2,4     |
|                     | 6,8 ± 3,3     | 8,4 ± 7,0     | 6,1 ± 2,5          | 7,0 ± 3,2     |
| cIMT, mm            | 0,701 ± 0,143 | 0,682 ± 0,120 | 0,716 ± 0,169      | 0,711 ± 0,137 |
|                     | 0,719 ± 0,127 | 0,732 ± 0,101 | 0,700 ± 0,119      | 0,740 ± 0,138 |

Data are given as mean standard ± deviation or numbers (%) where appropriate. Abbreviations: BMI, body mass index; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; HbA1c, hemoglobin A1c; CRP, C-reactive protein; GGT, gamma-glutamyl transferase; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; eGFR, estimated glomerular filtration rate; FIB-4, fibrosis-4 index; CAP, controlled attenuation parameter; LSM, liver stiffness measurement; cIMT, carotid intima media thickness.

Table 3 – Clinical and biochemical characteristics of participants with and without liver fibrosis, stratified by BMI categories

| Characteristics              | Total (n=475)   |                 |         | Lean (=88)      |                 |         | Overweight (n=139) |                  |         | Obese (n=162)   |                 |         |
|------------------------------|-----------------|-----------------|---------|-----------------|-----------------|---------|--------------------|------------------|---------|-----------------|-----------------|---------|
|                              | No              | Fibrosis        | p-value | No              | Fibrosis        | p-value | No                 | Fibrosis         | p-value | No              | Fibrosis        | p-value |
| TE                           |                 |                 |         |                 |                 |         |                    |                  |         |                 |                 |         |
| N (%)                        | 385<br>(81.1%)  | 90<br>(18.9%)   |         | 74<br>(84.1%)   | 14<br>(15.9%)   |         | 126<br>(90.6%)     | 13<br>(9.4%)     |         | 127<br>(78.4%)  | 35<br>(21.6%)   |         |
| Age, years                   | 52,8 ±<br>13,9  | 54,2 ±<br>13,3  | 0,376   | 55,8 ±<br>13,4  | 56,6 ±<br>11,4  | 0,832   | 53,5 ±<br>12,7     | 56,9 ±<br>7,7    | 0,187   | 49,7 ±<br>14,9  | 50,8 ±<br>13,7  | 0,701   |
| Woman, n (%)                 | 188<br>(48,8)   | 35 (38,9)       | 0,089   | 41<br>(55,4)    | 3<br>(21,4)     | 0,02    | 49<br>(38,9)       | 7<br>(53,8)      | 0,295   | 74<br>(58,3)    | 16<br>(45,7)    | 0,186   |
| BMI, kg/m2                   | 29,5 ±<br>6,5   | 31,8 ±<br>7,5   | 0,026   | 22,3 ±<br>3,1   | 22,2 ±<br>2,0   | 0,848   | 27,4 ±<br>1,4      | 27,5 ±<br>1,4    | 0,757   | 35,8 ±<br>5,4   | 37,2 ±<br>4,9   | 0,161   |
| Total cholesterol,<br>mmol/l | 4,998 ±<br>5,07 | 4,99 ±<br>1,58  | 0,99    | 4,56 ±<br>1,20  | 4,43 ±<br>1,24  | 0,785   | 4,56 ±<br>1,09     | 4,22 ±<br>1,20   | 0,46    | 5,56 ±<br>7,86  | 5,40 ±<br>1,60  | 0,93    |
| HDL-C, mmol/l                | 1,29 ±<br>0,38  | 1,21 ±<br>0,49  | 0,266   | 1,43 ±<br>0,44  | 1,27 ±<br>0,81  | 0,629   | 1,34 ±<br>0,36     | 0,97 ±<br>0,24   | 0,049   | 1,19 ±<br>0,32  | 1,22 ±<br>0,26  | 0,756   |
| LDL-C, mmol/l                | 2,53 ±<br>0,87  | 2,64 ±<br>1,18  | 0,634   | 2,42 ±<br>0,98  | 2,38 ±<br>1,03  | 0,93    | 2,41 ±<br>0,80     | 2,36 ±<br>1,13   | 0,904   | 2,68 ±<br>0,83  | 2,83 ±<br>1,25  | 0,634   |
| Triglycerides,<br>mmol/l     | 1,92 ±<br>1,38  | 2,27 ±<br>1,20  | 0,142   | 1,47 ±<br>0,88  | 1,73 ±<br>1,28  | 0,497   | 1,78 ±<br>1,25     | 1,74 ±<br>0,83   | 0,936   | 2,26 ±<br>1,62  | 2,54 ±<br>1,18  | 0,439   |
| HbA1c, mmol/mol              | 50,7 ±<br>14,8  | 52,2 ±<br>15,1  | 0,529   | 53,1 ±<br>14,6  | 48,1 ±<br>12,7  | 0,346   | 51,5 ±<br>15,4     | 55,0 ±<br>14,3   | 0,66    | 49,3 ±<br>14,5  | 55,2 ±<br>16,0  | 0,071   |
| CRP, mg/l                    | 8,1 ±<br>15,0   | 6,8 ±<br>8,6    | 0,639   | 4,3 ±<br>4,2    | 9,0 ±<br>11,8   | 0,433   | 9,8 ±<br>22,7      | 10,3 ±<br>13,8   | 0,957   | 8,7 ±<br>10,0   | 5,2 ±<br>2,2    | 0,041   |
| Hemoglobin,<br>mmol/l        | 8,73 ±<br>1,10  | 8,71 ±<br>1,12  | 0,901   | 8,31 ±<br>1,14  | 8,70 ±<br>1,18  | 0,377   | 8,68 ±<br>0,97     | 8,41 ±<br>1,21   | 0,459   | 8,85 ±<br>1,08  | 8,90 ±<br>0,96  | 0,832   |
| Thrombocytes,<br>109/l       | 262,0 ±<br>87,3 | 244,1 ±<br>87,1 | 0,168   | 277,7 ±<br>83,0 | 196,4 ±<br>88,7 | 0,023   | 250,5 ±<br>87,4    | 274,8 ±<br>119,1 | 0,47    | 277,9 ±<br>82,5 | 266,1 ±<br>82,7 | 0,57    |

Table 3 – Clinical and biochemical characteristics of participants with and without liver fibrosis, stratified by BMI categories (continued)

| Characteristics     | Total (n=475) |               | Lean (n=88)   |               | Overweight (n=139) |               | Obese (n=162) |               |
|---------------------|---------------|---------------|---------------|---------------|--------------------|---------------|---------------|---------------|
| GGT, U/l            | 94,9 ± 129,8  | 101,0 ± 84,6  | 98,1 ± 132,9  | 90,8 ± 64,5   | 104,3 ± 169,1      | 71,1 ± 28,9   | 97,7 ± 116,0  | 100,1 ± 84,6  |
| ALT, U/l            | 42,0 ± 23,8   | 55,5 ± 45,2   | 38,3 ± 24,0   | 37,8 ± 23,8   | 42,3 ± 32,1        | 46,4 ± 18,2   | 42,4 ± 17,3   | 75,1 ± 49,5   |
| AST, U/l            | 48,2 ± 35,5   | 62,9 ± 55,7   | 44,8 ± 39,2   | 42,8 ± 30,1   | 47,3 ± 33,2        | 65,2 ± 32,5   | 49,1 ± 37,5   | 88,9 ± 80,2   |
| Creatinine, umol/l  | 81,7 ± 38,4   | 76,7 ± 16,8   | 87,5 ± 77,6   | 83,0 ± 23,0   | 83,1 ± 21,8        | 76,1 ± 9,2    | 79,2 ± 21,3   | 76,7 ± 18,0   |
| Albumine, g/l       | 43,4 ± 4,1    | 52,4 ± 60,9   | 41,8 ± 5,6    | 37,4 ± 4,3    | 43,4 ± 4,8         | 43,6 ± 8,4    | 44,1 ± 3,2    | 44,4 ± 2,8    |
| eGFR, ml/min/1,73m2 | 75,8 ± 15,8   | 75,5 ± 12,2   | 71,6 ± 18,1   | 73,6 ± 15,2   | 73,6 ± 16,6        | 74,9 ± 8,4    | 79,3 ± 14,2   | 75,4 ± 15,1   |
| FIB-4               | 1,88 ± 1,55   | 1,79 ± 1,31   | 1,91 ± 1,07   | 2,45 ± 2,54   | 1,82 ± 1,67        | 1,42 ± 0,40   | 1,94 ± 1,77   | 1,33 ± 0,77   |
| CAP, dB/m           | 257,6 ± 57,6  | 283,9 ± 61,5  | 215,4 ± 44,9  | 248,5 ± 62,9  | 256,6 ± 53,0       | 294,1 ± 65,8  | 287,2 ± 54,9  | 311,2 ± 46,2  |
| LSM, kPa            | 5,1 ± 1,4     | 10,7 ± 3,6    | 4,7 ± 1,4     | 11,5 ± 4,8    | 4,9 ± 1,4          | 10,7 ± 2,9    | 5,2 ± 1,4     | 10,8 ± 3,1    |
| cIMT, mm            | 0,704 ± 0,140 | 0,750 ± 0,109 | 0,686 ± 0,120 | 0,697 ± 0,116 | 0,706 ± 0,153      | 0,796 ± 0,048 | 0,722 ± 0,139 | 0,763 ± 0,126 |

Data are given as mean standard ± deviation or numbers (%) where appropriate. Abbreviations: BMI, body mass index; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; HbA1c, hemoglobin A1c; CRP, C-reactive protein; GGT, gamma-glutamyl transferase; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; eGFR, estimated glomerular filtration rate; FIB-4, fibrosis-4 index; CAP, controlled attenuation parameter; LSM, liver stiffness measurement; cIMT, carotid intima media thickness.

When analyzing BMI groups, higher CAP values were observed in individuals with liver fibrosis, regardless of BMI. Those with a lean BMI and liver fibrosis were more likely to be women with lower platelet levels. Interestingly, individuals with liver fibrosis and obesity exhibited higher transaminase levels but lower C-reactive protein levels, suggesting a reduced inflammatory status. Furthermore, the FIB-4 index was lower in the fibrosis group with obesity, challenging the assumption that higher FIB-4 scores are consistently associated with liver fibrosis. The results are presented in table 3.

Logistic regression analysis for liver steatosis

A logistic regression analysis was conducted to assess the impact of several variables on the likelihood of liver steatosis. The model included age, gender, BMI, HbA1c triglycerides, HDL-C, LDL-C and sex as predictors.

The results indicated that BMI and triglycerides were significant predictors. BMI was significantly associated with liver steatosis ( $B = 0.137$ ,  $p < 0.001$ ), with an odds ratio (OR) of 1.147 (95% CI: 1.083–1.215). This suggests that for every one-unit increase in BMI, the odds of liver steatosis increased by 14.7%. Triglycerides were also a significant predictor ( $B = 0.429$ ,  $p = 0.042$ ), with an odds ratio of 1.536 (95% CI: 1.016–2.322), indicating a 53.6% increase in odds for each unit increase in triglycerides. The analysis is presented in Table 4.

Table 4 – Logistic Regression Analysis of predictors for MASLD

| Total         | B      | S.E.  | Wald   | df | Sig.   | Exp(B) | 95% C.I. for EXP(B) |       |
|---------------|--------|-------|--------|----|--------|--------|---------------------|-------|
|               |        |       |        |    |        |        | Lower               | Upper |
| Age           | 0,003  | 0,013 | 0,058  | 1  | 0,810  | 1,003  | 0,978               | 1,029 |
| BMI           | 0,137  | 0,029 | 21,876 | 1  | <0,001 | 1,147  | 1,083               | 1,215 |
| HbA1c         | -0,003 | 0,011 | 0,063  | 1  | 0,802  | 0,997  | 0,976               | 1,019 |
| Triglycerides | 0,429  | 0,211 | 4,150  | 1  | 0,042  | 1,536  | 1,016               | 2,322 |
| HDL-C         | 0,477  | 0,511 | 0,872  | 1  | 0,350  | 1,612  | 0,592               | 4,388 |
| LDL-C         | 0,008  | 0,192 | 0,002  | 1  | 0,965  | 1,008  | 0,692               | 1,469 |
| Sex           | 0,387  | 0,337 | 1,323  | 1  | 0,250  | 1,473  | 0,761               | 2,849 |
| Constant      | -5,973 | 1,746 | 11,705 | 1  | <0,001 | 0,003  |                     |       |

Logistic regression analysis for liver fibrosis

A logistic regression analysis was performed to identify predictors of liver fibrosis. The results showed that BMI was the only statistically signifi-

cant predictor, while other variables, including age, HbA1c, triglycerides, HDL-C, LDL-C, and sex, were not significant. BMI was significantly associated with liver fibrosis ( $B = 0.071$ ,  $p = 0.045$ ), with an odds ratio of 1.074 (95% CI: 1.002–1.151). This indicates that for each one-unit increase in BMI, the odds of liver fibrosis increased by 7.4%. The analysis is presented in table 5.

Table 5 – Logistic Regression Analysis of predictors for liver fibrosis

|               | B      | S.E.  | Wald  | df | Sig.  | Exp(B) | 95% C.I. for EXP(B) |       |
|---------------|--------|-------|-------|----|-------|--------|---------------------|-------|
|               |        |       |       |    |       |        | Lower               | Upper |
| Age           | 0,013  | 0,018 | 0,550 | 1  | 0,458 | 1,013  | 0,979               | 1,049 |
| BMI           | 0,071  | 0,036 | 4,017 | 1  | 0,045 | 1,074  | 1,002               | 1,151 |
| HbA1c         | 0,022  | 0,014 | 2,528 | 1  | 0,112 | 1,022  | 0,995               | 1,049 |
| Triglycerides | 0,304  | 0,244 | 1,553 | 1  | 0,213 | 1,356  | 0,840               | 2,189 |
| HDL-C         | 0,216  | 0,703 | 0,094 | 1  | 0,759 | 1,241  | 0,313               | 4,925 |
| LDL-C         | 0,134  | 0,252 | 0,283 | 1  | 0,595 | 1,143  | 0,698               | 1,874 |
| Sex           | 0,589  | 0,463 | 1,614 | 1  | 0,204 | 1,802  | 0,726               | 4,469 |
| Constant      | -7,468 | 2,418 | 9,541 | 1  | 0,002 | 0,001  |                     |       |

Linear regression analysis for cIMT

A multiple linear regression analysis was conducted to identify predictors of carotid intima-media thickness (cIMT). The following variables were included in the model: age, BMI, sex, HbA1c, triglycerides, HDL-C, LDL-C, CAP, and LSM.

Age was the strongest predictor of cIMT ( $B = 0.007$ ,  $\beta = 0.610$ ,  $p < 0.001$ ), indicating that for each year increase in age, the cIMT value increases by 0.007 mm.

BMI was positively associated with cIMT ( $B = 0.004$ ,  $\beta = 0.165$ ,  $p = 0.032$ ), with a 0.004 mm increase in cIMT for each one-unit increase in BMI.

HDL-C had a significant negative association with cIMT ( $B = -0.070$ ,  $\beta = -0.219$ ,  $p = 0.022$ ), suggesting that higher HDL-C levels are associated with lower cIMT. LDL-C also showed a positive association with cIMT ( $B = 0.024$ ,  $\beta = 0.160$ ,  $p = 0.044$ ), meaning that higher LDL-C levels are associated with an increase in cIMT. The results are presented in table 6.

Table 6 – Logistic Regression Analysis of predictors for cIMT

|             | Unstandardized Coefficients |            | Standardized Coefficients | t      | Sig.   |
|-------------|-----------------------------|------------|---------------------------|--------|--------|
|             | B                           | Std. Error | Beta                      |        |        |
| (Constant)  | 0,297                       | 0,094      |                           | 3,175  | 0,002  |
| Leeftijd    | 0,007                       | 0,001      | 0,610                     | 8,167  | <0,001 |
| BMI         | 0,004                       | 0,002      | 0,165                     | 2,176  | 0,032  |
| Geslacht    | 0,008                       | 0,020      | 0,033                     | 0,421  | 0,674  |
| HbA1c       | -0,001                      | 0,001      | -0,069                    | -0,916 | 0,361  |
| Tot_triglyc | -0,007                      | 0,012      | -0,060                    | -0,641 | 0,523  |
| HDL         | -0,070                      | 0,030      | -0,219                    | -2,317 | 0,022  |
| LDL         | 0,024                       | 0,012      | 0,160                     | 2,036  | 0,044  |
| CAP         | 0,000                       | 0,000      | -0,054                    | -0,633 | 0,528  |
| LSM         | 0,007                       | 0,005      | 0,116                     | 1,464  | 0,146  |

## DISCUSSION

This study investigated the prevalence of liver steatosis and liver fibrosis, and the association with cIMT in an outpatient cohort. The analysis revealed that 40.4% of these patients have liver steatosis, and 18.9% have significant fibrosis. All participants had at least one cardiometabolic risk factor, meeting the definition of MASLD when steatosis was present. The prevalence of MASLD in the general population exceeds 30% and is rising [1, 31, 32]. Younossi et al. [33] reported a prevalence of 55% for MASLD among individuals with type 2 diabetes in a systematic review and meta-analysis that included 49,149 participants. In this cohort, the proportion of MASLD was even higher among patients with obesity, reaching 62.3%. These findings, along with the logistic regression analysis, confirm that BMI and triglycerides are a key determinant in the development of liver steatosis. This aligns with previous studies describing an obesity-driven pathophysiology [34, 35], highlighting the importance of weight management and the treatment of overweight and obesity to prevent MASLD.

Outpatients with liver fibrosis were more likely to also suffer from obesity. The logistic regression analysis confirmed that BMI is a predictor for liver fibrosis, reinforcing its role in the development of liver pathology. However, in contrast to Stefan et al., who assert that T2D is associated with a high risk of liver fibrosis [10], our analysis did not demonstrate a significant association between HbA1c and liver fibrosis.

Previous studies showed a positive association between BMI, age and an increased cIMT [36–38] and obesity has been defined as an independent risk factor for CVD [39]. Our findings suggest that BMI may contribute to increased cIMT, albeit with a small effect. A recent study involving 451,355 participants found cIMT increased with BMI and showed an adverse association with central adiposity [40]. A cross-sectional study has demonstrated a significant correlation between MASLD and subclinical atherosclerosis [41]. However, our analysis did not support this association, as only age, BMI and LDL cholesterol were identified as predictors of increased cIMT. Conversely, a recent meta-analysis of 57 studies including 29,502 patients reported a decrease in HDL cholesterol levels with increasing cIMT. Our analysis align with this observation, confirming that HDL cholesterol is negatively associated with cIMT measurements.

The primary limitation of our study is its cross-sectional design, which limits the ability to assess the impact of changes in clinical and chemical values over time on liver steatosis, liver fibrosis, and cIMT. Another limitation is the absence of data on waist circumference (WC). Abdominal obesity, independent of BMI, has long been linked to chronic diseases and increased mortality [42]. Investigating the role of WC in liver steatosis and fibrosis would be valuable. An ongoing study will analyze the relationship between abdominal obesity, MASLD, and fibrosis [43]. Overall, BMI has demonstrated limitations as a reference measure, as it does not distinguish between adipose and lean body mass, nor does it account for sex- and ethnicity-related differences. [44]. Additionally, we did not adjust the BMI categories as recommended by the World Health Organization for the Asian population [45]. Another limitation of our study is the use of transient elastography, a non-invasive method, for the identification of liver steatosis and fibrosis. While this technique has been validated and is endorsed in the latest guidelines [20, 46, 47], the possibility of misclassification cannot be excluded. Ideally, a liver biopsy would have been preferred as it remains the gold standard for accurately diagnosing steatosis and fibrosis. Moreover, recent research has identified two distinct subtypes of MASH, each with unique risks and outcomes [48]. In our study, we were unable to distinguish between the liver-specific MASH and cardiometabolic MASH subtypes. The liver-specific subtype is genetically driven and associated with a lower risk of cardiovascular complications, while the cardiometabolic subtype is closely linked to diabetes and cardiovascular disease. Future studies should focus on distinguishing these subtypes to better determine individual risks. Finally, our analysis lacked data on potential confounders,

such as smoking status, medication use and the duration of diabetes or obesity, which could have influenced the outcomes.

## CONCLUSION

In this outpatient cohort, MASLD and liver fibrosis were notably more prevalent than in the general population, reflecting an elevated risk profile. Liver steatosis was significantly associated with BMI and triglyceride levels, while liver fibrosis demonstrated a direct correlation with BMI. Although cIMT was positively associated with age, BMI, and LDL cholesterol, its effect was modest and showed no significant relationship with liver steatosis or fibrosis. These findings highlight the critical role of metabolic risk factors in the progression of liver disease and emphasize the need for targeted public health interventions. Strategies focusing on obesity management, lipid control, and metabolic health optimization could mitigate the burden of liver disease and its associated complications in at-risk populations.

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# Chapter 5

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## **Metabolic dysfunction–associated steatotic liver disease and cardiovascular risk factors in rheumatoid arthritis–associated steatotic liver disease and cardiovascular risk factors in rheumatoid arthritis**

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## ABSTRACT

### Objectives

Rheumatoid arthritis (RA) is a chronic autoimmune disease linked with metabolic dysfunction-associated steatotic liver disease (MASLD), which may increase cardiovascular (CV) risk. This study explores the association between liver fibrosis, assessed by the Fibrosis-4 (FIB-4) index, and CV risk factors in RA patients.

### Methods

Cross-sectional data from the Franciscus Rheumatoid Arthritis and Cardiovascular Intervention Study (FRANCIS), a randomized, cardiovascular single center, intervention study involving RA patients without cardiovascular disease (CVD) or type 2 diabetes (T2DM), were analyzed. Liver fibrosis was assessed using FIB-4, with a cut-off point of  $\geq 1.3$  to define high fibrosis risk, and its relationship with CV risk factors, medication use, and subclinical atherosclerosis, measured by carotid intima-media thickness (cIMT), was evaluated.

### Results

Among 326 patients (68.4% female, age  $53 \pm 11$  years, BMI  $26.5 \pm 4.5$  kg/m<sup>2</sup>), those with high FIB-4 ( $n = 49$ ) had higher cIMT ( $p = 0.002$ ), apolipoprotein B48 ( $p = 0.04$ ), systolic blood pressure ( $p = 0.007$ ), alkaline phosphatase ( $p = 0.002$ ), and anti-CCP levels ( $p = 0.02$ ). High FIB-4 was associated with lower leukocyte count and complement component 3. Statin use was linked to higher FIB-4 (OR = 4.49,  $p = 0.014$ ), while hydroxychloroquine use was associated with lower FIB-4 (OR = 0.11,  $p = 0.004$ ). Disease activity scores did not differ between low and high FIB-4 groups.

### Conclusions

Elevated FIB-4 in RA patients is associated with increased cIMT, higher blood pressure, and elevated atherogenic remnants. Incorporating FIB-4 measurements into routine clinical care for RA populations could effectively identify individuals at the highest CV risk, enabling the implementation of more intensive CV risk management strategies.

## INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune and inflammatory disease which can lead to significant disability and reduced quality of life [1]. Beyond joint involvement, RA has notable extra-articular manifestations, impacting the lungs, skin, eyes, heart, kidneys, nervous system, and gastrointestinal tract [2, 3]. One potential clinical outcome of RA is liver involvement, which can manifest as autoimmune biliary disease, abnormal liver test results, or metabolic dysfunction–associated steatotic liver disease (MASLD) [4–6]. MASLD is characterized by excess fat accumulation in the liver along with at least one cardiometabolic risk factor, in the absence of secondary causes of hepatic steatosis or excessive alcohol consumption [7]. Approximately one-third of RA patients are affected by MASLD [8, 9], which can range from simple steatosis to more severe forms like metabolic dysfunction–associated steatohepatitis (MASH), potentially advancing to cirrhosis and hepatocellular carcinoma (HCC) [10]. Furthermore, MASLD is associated with an increased risk for CVD [11].

### Treatment with disease-modifying antirheumatic drugs

(DMARDs) may also contribute to the risk of MASLD in RA. Methotrexate (MTX), the most frequently used DMARD for RA, has been linked to various hepatic side effects, including elevated liver enzymes and an increased risk of liver fibrosis, especially with long-term use or higher doses [12, 13]. Other RA medications, such as biologics, have also been associated with hepatotoxicity, although the extent of their contribution to liver fibrosis remains unclear [14]. Given the widespread use of these medications in RA management, it is crucial to evaluate their potential impact on liver health, particularly on the progression of liver fibrosis.

RA is closely linked to cardiovascular disease (CVD), primarily due to the combined effects of systemic inflammation, potentially causing harm to the arterial wall, and the presence of traditional CV risk factors, which are often undertreated in this population [15]. Patients with RA have a 1.5-fold increased risk of CVD and experience higher rates of CVD-related morbidity and mortality compared to the general population [16, 17]. The increased risk of premature mortality in RA patients is attributed to both persistent inflammation and inadequate management of traditional CV risk factors [18–22].

In addition to traditional CV risk factors, the carotid intima-media thickness (cIMT), a surrogate marker for sub-clinical atherosclerosis, has also been shown to be increased in RA patients [23, 24]. Recent studies also suggested that the increased CVD risk in RA may be associated with accumulation of atherogenic intestinal remnants carrying apolipoprotein (apo) B48 [25, 26]. Although CVD and MASLD are closely interconnected and inflammation plays a central role in both, this has not been studied in detail in a typical systemic inflammatory condition like RA. We hypothesized that the presence of MASLD, particularly liver fibrosis, a key determinant of MASLD prognosis, has been associated with increased CV risk [25].

While liver biopsy is considered the gold standard for diagnosing liver fibrosis, it is an invasive and costly procedure which is not routinely performed unless there is a clear clinical indication [27]. Therefore, we used the Fibrosis-4 (FIB-4) index, a non-invasive blood test recommended by the European Association for the Study of the Liver (EASL) guidelines, for the assessment of liver fibrosis [7, 28]. The aims of this study were to investigate the association between liver fibrosis, as estimated by the FIB-4, and CV risk factors, including hypertension, lipid levels, and cIMT in patients with RA. Additionally, it aimed to investigate the association between liver fibrosis and medication use in this patient population.

## MATERIALS AND METHODS

### Study design and study population

This is a post-hoc analysis of the Franciscus Rheumatoid Arthritis and Cardiovascular Intervention Study (FRAN-CIS), an open-label randomized controlled trial that aimed to investigate the effectiveness of a strict treatment of CV risk factors in RA [26, 29, 30]. The study population ( $n = 326$ ) consisted of patients attending the outpatient clinic of the Department of Rheumatology at the Franciscus Gasthuis Hospital in Rotterdam, the Netherlands. Participants were included if they were less than 70 years of age and if they had been diagnosed with rheumatoid arthritis, defined according to the criteria of the American College of Rheumatology [31]. Patients were excluded if they had type 1 diabetes mellitus (T1DM) or T2DM at baseline, defined as a fasting glucose of  $> 7.0$  mmol or HbA1c  $> 48$  mmol/mol, or being treated for these conditions or if they had a history of CVD. The latter of which was defined as a prior

myocardial infarction, cerebrovascular event, intermittent claudication, amputation due to peripheral artery disease (PAD), coronary artery bypass graft (CABG), or percutaneous transluminal coronary angioplasty (PTCA). Additionally, patients were excluded if they had kidney disease, defined as an estimated glomerular filtration rate (eGFR) of  $< 40 \text{ ml/min/1.73m}^2$ . The study was approved by the medical ethical committee of the Maasstad hospital in Rotterdam, the Netherlands, and was conducted in accordance with the Declaration of Helsinki (The Dutch Trial register, NTR3873; ABR no. NL32669.101.10) [32].

### Data collection

Baseline data was collected during outpatient clinic visits between 2009 and 2012. Blood samples were obtained after an overnight fast. In addition, anthropomorphic measurements (e.g., weight, height, blood pressure) were recorded. Variables included for medication use were methotrexate (MTX), NSAIDs, biologicals, statins, antihypertensives, leflunomide, hydroxychloroquine (HCQ), prednisone, and anti-TNF therapy. Furthermore, RA disease activity was evaluated using the Disease Activity Score (DAS28) wherein 28 joints are evaluated [33].

### Fibrosis-4 index

The FIB-4 was calculated according to the following formula:  $\text{age (years)} \times \text{AST(U/L)}/\text{platelet (PLT)} (10^9/\text{L}) \times \sqrt{\text{ALT(U/L)}}$ . Low hepatic fibrosis risk was defined as a FIB-4  $< 1.3$  and high fibrosis risk as a FIB-4  $\geq 1.3$ . The FIB-4 has been extensively validated in a broad spectrum of MASLD subjects and in RA patients [7, 28].

### Carotid intima media thickness

cIMT was measured by trained and experienced sonographers using the ART-LAB (Esaote, Italy) as described earlier [29]. Using B-mode ultrasounds which produced two echogenic lines, ultrasound images were made of the far wall of each common carotid artery (CCA). These lines represented the overall thickness of the media and intima layers of the arterial wall. Atherosclerotic plaque was defined as a lesion with a focal cIMT  $\geq 1.0 \text{ mm}$ .

### Laboratory measurements

In each patient, a standard set of laboratory measurements was performed at the Department of Clinical Chemistry of the Franciscus Gasthuis hospital. Blood samples were drawn following the standard procedures. Liver enzymes and renal tests as well as total cholesterol,

HDL-C, glucose, C-reactive protein (CRP), and triglycerides (TGs) were measured using DxC analyzers (Beckman Coulter, United States) or Synchron LX20 (Beckman Coulter, United States) [34, 35]. LDL-C was measured using the Friedewald formula if TGs were below 4.00 mmol/L. Complement C3 levels were measured using the Atellica® Solution (Siemens Healthineers, Germany). Furthermore, using an ELISA, apoB48 serum levels were quantified as previously described [36].

### Statistical analysis

Data are presented as mean  $\pm$  standard deviation (SD), unless otherwise specified. Normally distributed variables were analyzed using the independent *T*-test, while non-normally distributed parameters were assessed with the Mann-Whitney *U* test or Kruskal-Wallis test. Correlation analyses were conducted using Pearson's or Spearman's correlation coefficients, where appropriate. Multivariate binary logistic regression was performed with FIB-4 categories (low vs. high) as the dependent variable and medication use as the independent variables. Chi-square tests were also conducted. A multiple regression analysis was performed to investigate the association between FIB-4 (low vs. high) and cIMT adjusting for hypertension and lipid levels. All statistical analyses were performed using IBM SPSS Statistics version 28.0.0.0 (IBM SPSS Statistics, New York, United States), with statistical significance set at a *p*-value  $< 0.05$ .

## RESULTS

The study population included 223 women and 103 men, with an average age of 53 years ( $\pm 11$ ) and an average BMI of 26.5 kg/m<sup>2</sup> ( $\pm 4.5$ ). The majority of the cohort had a low FIB-4 score ( $n = 277$ , 85.0%). Patients with an elevated FIB-4 score were significantly older and had higher systolic blood pressure, higher apoB48 concentrations, higher cIMT values, higher alkaline phosphatase, and higher anti-cyclic citrullinated peptide (anti-CCP) concentrations (Table 1; Fig. 1). In addition, they had statistically significant lower BMI, as well as lower levels of thrombocytes and a lower leukocyte count, particularly neutrophils and lymphocytes, and lower levels of complement C3. There was no difference in disease activity score between groups (Table 1).

Table 1 – Total group (n = 326).

Baseline characteristics in the low FIB-4 versus high FIB-4 groups. p-value is for comparison between FIB-4 groups. Data are expressed as mean  $\pm$  standard deviation, median (IQR), or n (%), as appropriate

|                                       | Low FIB-4        | High FIB-4       | p-value |
|---------------------------------------|------------------|------------------|---------|
|                                       | N=277            | N=49             |         |
| <b>Demographic characteristics</b>    |                  |                  |         |
| Age (years)                           | 52 $\pm$ 11      | 62 $\pm$ 7.2     | < 0.001 |
| Sex (m/f)                             | 83/194 (30/70)   | 20/29 (41/59)    | 0.132   |
| Smoking (no/yes)                      | 207/55 (79/21)   | 39/9 (81/19)     | 0.724   |
| <b>Clinical characteristics</b>       |                  |                  |         |
| Body mass index (kg/m <sup>2</sup> )  | 26.7 $\pm$ 4.6   | 25.2 $\pm$ 3.6   | 0.031   |
| Systolic blood pressure (mmHg)        | 131 $\pm$ 18     | 139 $\pm$ 22     | 0.007   |
| Diastolic blood pressure (mmHg)       | 78.9 $\pm$ 10    | 81 $\pm$ 12      | 0.142   |
| Total cholesterol (mmol/L)            | 5.4 $\pm$ 1.1    | 5.6 $\pm$ 1.1    | 0.224   |
| HDL-C (mmol/L)                        | 1.5 $\pm$ 0.41   | 1.6 $\pm$ 0.43   | 0.069   |
| LDL-C (mmol/L)                        | 3.4 $\pm$ 0.94   | 3.4 $\pm$ 0.90   | 0.537   |
| Triglycerides (mmol/L)                | 1.04 (0.72–1.55) | 1.05 (0.76–1.49) | 0.757   |
| ApoAI (g/L)                           | 1.68 $\pm$ 0.37  | 1.73 $\pm$ 0.37  | 0.441   |
| Apo B (g/L)                           | 1.00 $\pm$ 0.26  | 1.00 $\pm$ 0.25  | 1.000   |
| Apo B48 (mg/L)                        | 8.2 (5.0–12.1)   | 9.6 (6.8–15.5)   | 0.036   |
| Fasting glucose (mmol/L)              | 5.5 $\pm$ 0.54   | 5.5 $\pm$ 0.63   | 0.350   |
| HbA1c (mmol/mol)                      | 35.3 $\pm$ 4.5   | 35.1 $\pm$ 3.9   | 0.839   |
| CRP (mg/L)                            | 3.0 (1.0–6.0)    | 2.0 (1.0–5.0)    | 0.181   |
| Hemoglobin (g/dL)                     | 8.5 $\pm$ 0.8    | 8.7 $\pm$ 0.5    | 0.002   |
| Thrombocytes (10 <sup>9</sup> /L)     | 255 $\pm$ 63.2   | 172 $\pm$ 30.9   | < 0.001 |
| Leukocyte counts (10 <sup>9</sup> /L) | 6.7 $\pm$ 2.0    | 5.2 $\pm$ 1.5    | < 0.001 |
| Neutrophils (10 <sup>9</sup> /L)      | 4.1 $\pm$ 1.7    | 3.1 $\pm$ 1.2    | < 0.001 |
| Lymphocytes (10 <sup>9</sup> /L)      | 1.8 (1.4–2.28)   | 1.5 (1.1–1.75)   | 0.024   |
| Monocytes (10 <sup>9</sup> /L)        | 0.55 $\pm$ 0.19  | 0.49 $\pm$ 0.18  | 0.057   |
| Eosinophils (10 <sup>9</sup> /L)      | 0.16 $\pm$ 0.11  | 0.16 $\pm$ 0.16  | 0.979   |
| Basophils (10 <sup>9</sup> /L)        | 0.03 $\pm$ 0.05  | 0.02 $\pm$ 0.04  | 0.128   |
| Complement C3 (g/L)                   | 1.19 $\pm$ 0.23  | 1.08 $\pm$ 0.18  | < 0.001 |
| Complement C4 (g/L)                   | 0.21 $\pm$ 0.06  | 0.19 $\pm$ 0.05  | 0.053   |
| Alkaline phosphatase (U/L)            | 70.5 $\pm$ 23.1  | 83.1 $\pm$ 38.2  | 0.002   |

Table 1 – Total group (n = 326).  
Baseline characteristics in the low FIB-4 versus high FIB-4 groups. p-value is for comparison between FIB-4 groups. Data are expressed as mean ± standard deviation, median (IQR), or n (%), as appropriate (*continued*)

|                       | Low FIB-4        | High FIB-4       | p-value |
|-----------------------|------------------|------------------|---------|
| Gamma-GT (U/L)        | 21 (16–29)       | 21 (16–32)       | 0.399   |
| Creatinine (µmol/L)   | 69.5 ± 14.7      | 73.3 ± 13.9      | 0.092   |
| Microalbumin (mg/L)   | 7.0 (5.0–14)     | 7.0 (5.0–15)     | 0.249   |
| Uric acid (mmol/L)    | 0.29 (0.25–0.34) | 0.32 (0.26–0.36) | 0.878   |
| TSH (mU/L)            | 1.9 (1.3–2.7)    | 2.0 (1.3–3.8)    | 0.221   |
| Anti-CCP (U/mL)       | 0.64 ± 0.48      | 0.80 ± 0.41      | 0.027   |
| DAS28CRP              | 2.5 ± 1.1        | 2.2 ± 0.93       | 0.171   |
| DAS28BSE              | 2.6 ± 1.3        | 2.4 ± 0.98       | 0.287   |
| ALAT (U/L)            | 16 (13–20)       | 23 (19.5–27)     | < 0.001 |
| ASAT (U/L)            | 25 (19–32)       | 27 (20–35)       | 0.086   |
| FIB-4                 | 0.73 ± 0.28      | 1.75 ± 0.45      | < 0.001 |
| <b>Medication use</b> |                  |                  |         |
| Antihypertensives     | 43 (15.6)        | 10 (20.4)        | 0.405   |
| Statins               | 9 (3.3)          | 6 (12.2)         | 0.015   |
| Leflunomide           | 3 (1.1)          | 2 (4.1)          | 0.166   |
| Hydroxychloroquine    | 71 (25.8)        | 2 (4.1)          | < 0.001 |
| Anti-TNF              | 97 (35.3)        | 19 (38.8)        | 0.747   |
| Prednisone            | 34 (12.4)        | 5 (10.2)         | 0.814   |
| Methotrexate          | 206 (74.9)       | 38 (77.6)        | 0.589   |
| NSAID                 | 120 (43.6)       | 20 (40.8)        | 0.755   |

HDL-C high-density lipoprotein cholesterol, LDL-C low-density lipoprotein cholesterol, remnant-C remnant cholesterol, apoAI apolipoprotein AI, apoB apolipoprotein B, apoB48 apolipoprotein B48, HbA1c hemoglobin A1C, CRP C-reactive protein, Gamma-GT gamma-glutamyl transferase, TSH thyroid-stimulating hormone, anti-CCP anti-cyclic citrullinated peptide, DAS28CRP Disease Activity Score 28-joint count with C-Reactive Protein, DAS28BSE Disease Activity Score 28-joint count with Erythrocyte Sedimentation Rate, ALT alanine aminotransferase, AST aspartate aminotransferase, FIB-4 Fibrosis-4 index

Table 2 Logistic regression analysis of medication effects on FIB-4 (low versus high)

| Variable           | B       | S.E   | Wald   | df | Sig     | Exp(B) | 95% CI for Exp(B) |
|--------------------|---------|-------|--------|----|---------|--------|-------------------|
| Constant           | - 1.826 | 0.461 | 15.686 | 1  | < 0.001 | 0.161  |                   |
| Antihypertensives  | 0.257   | 0.423 | 0.370  | 1  | 0.543   | 1.293  | 0.565-2.962       |
| Statins            | 1.501   | 0.612 | 6.019  | 1  | 0.014   | 4.488  | 1.352-14.890      |
| Hydroxychloroquine | - 2.176 | 0.749 | 8.436  | 1  | 0.004   | 0.113  | 0.026-0.493       |
| Anti-TNF           | - 0.070 | 0.343 | 0.041  | 1  | 0.839   | 0.933  | 0.476-1.828       |
| Prednisone         | - 0.265 | 0.536 | 0.244  | 1  | 0.621   | 0.767  | 0.269-2.193       |
| Methotrexate       | 0.403   | 0.420 | 0.921  | 1  | 0.337   | 1.497  | 0.657-3.411       |
| NSAID              | - 0.096 | 0.335 | 0.082  | 1  | 0.774   | 0.909  | 0.472-1.750       |

Table 3 Multiple regression analysis of cIMT and FIB-4, adjusted for confounding variables

| Variable | B     | 95% CI      | p-value |
|----------|-------|-------------|---------|
| Model 1  | 0.055 | 0.021-0.089 | 0.002   |
| Model 2  | 0.037 | 0.005-0.068 | 0.024   |

Model 1, crude model; model 2, adjusted for systolic blood pressure and LDL-C

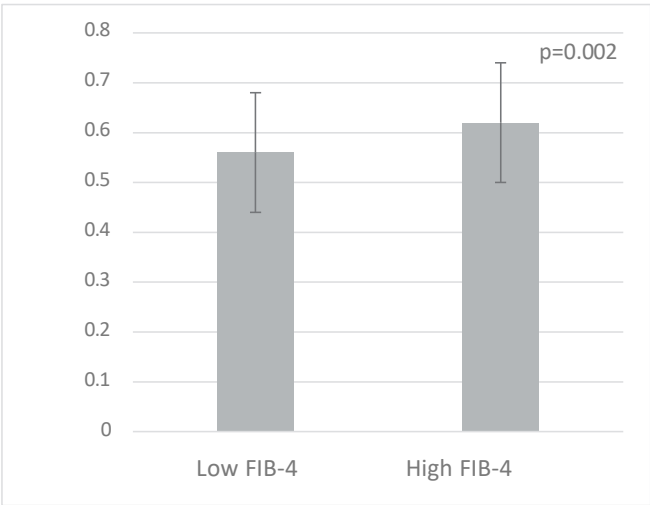


Figure 1 - Mean carotid intima media thickness (cIMT) in the low FIB-4 versus high FIB-4 group; 0.57 mm  $\pm$  0.12 in the low FIB-4 group versus 0.62 mm  $\pm$  0.12 in the high FIB-4 group ( $p = 0.002$ )

Correlation analyses showed significant positive associations between FIB-4 and systolic blood pressure ( $\rho = 0.301$ ,  $p < 0.001$ ), cIMT ( $\rho = 0.409$ ,  $p < 0.001$ ), total cholesterol ( $\rho = 0.212$ ,  $p < 0.001$ ), LDL-C ( $\rho = 0.190$ ,  $p < 0.001$ ), and apoB48 ( $\rho = 0.141$ ,  $p = 0.011$ ). In contrast, negative correlations were found between FIB-4 and complement C3 ( $\rho = -0.161$ ;  $p = 0.004$ ) and leukocyte count ( $\rho = -0.399$ ,  $p < 0.001$ ). There was no statistically significant correlation between FIB-4 and triglycerides ( $\rho = 0.060$ ,  $p = 0.278$ ).

Logistic regression analysis showed that statin use was associated with high FIB-4, while HCQ use was associated with lower FIB-4 (Table 2). To further explore these associations, we conducted univariate logistic regression analyses for each medication separately (Supplementary Table S1). These analyses confirmed that statin use was significantly associated with high FIB-4, while HCQ use was significantly associated with lower FIB-4. No significant associations were observed for antihypertensives, leflunomide, anti-TNF agents, prednisone, MTX, NSAIDs, or other biologics (all  $p > 0.05$ ). In terms of medication use, 12.2% of the patients in the high FIB-4 group used statins, compared to only 3.3% in the low FIB-4 group (Table 1). Similarly, HCQ use showed a statistically significant difference between the two groups, with 4.1% of the patients in the high FIB-4 group using HCQ, compared to 25.8% in the low FIB-4 group. These associations were found independently of the use of other medications included in the analysis. No significant differences were found for other medications, including anti-hypertensives, leflunomide, anti-TNF agents, prednisone, MTX, NSAIDs, and biologicals (all  $p > 0.05$ ) (Table 1).

Higher FIB-4 scores are associated with increased cIMT.

This relationship persists even when adjusting for potential confounders, including systolic blood pressure and LDL-C (Table 3). While adjustments were also made for HDL-C and diastolic blood pressure, the linearity with these variables was weaker than that of those included in the final model.

## DISCUSSION

The results from the current study have extended the findings from our previous observations reporting on the association between RA and CV risk factors, by also showing an association with liver fibrosis as estimated by FIB-4 [25, 26, 37]. The findings of the current study showed

that RA patients with a high FIB-4 have higher cIMT values, even after adjusting for potential confounders, reinforcing the notion that liver fibrosis may independently contribute to CV risk in RA patients. This underscores FIB-4's potential as a marker for both liver health and CV risk stratification in RA. Additionally, high systolic blood pressure was found to be significantly associated with increased cIMT, regardless of FIB-4 levels. This highlights the importance of managing blood pressure in RA patients, particularly in those with liver fibrosis, to reduce CV risk. There was no difference in disease activity score between those with low and high FIB-4, suggesting that liver fibrosis, as measured by FIB-4, may be independent of overall RA disease activity, which highlights the need to consider additional factors when assessing liver health in RA patients.

Previously, we reported elevated apoB48 concentrations in RA patients compared to controls [25, 26]. The present study expands that observation by also demonstrating an association with liver fibrosis. It is well known that chylomicrons and their remnants can directly infiltrate intact endothelium, become trapped, and induce an inflammatory response along with the formation of foam cells. This process plays a crucial role in the initiation and progression of atherosclerosis [37, 38]. This mechanism may explain the increased cIMT observed in RA patients with liver fibrosis. While the relationship between cIMT and liver fibrosis is evident, the pathophysiology of the accumulation of chylomicron remnants remains unclear. A key factor in the clearance of chylomicron remnants is a receptor-mediated pathway in the liver. Liver fibrosis could disrupt this process by reducing the number or function of these receptors. One could speculate that this impairment may lead to higher levels of circulating chylomicron remnants due to reduced hepatic clearance. Another important consideration is that RA patients with high FIB-4 levels also were more frequently on statins. Normally, statin use increases LDL-receptor expression, thereby enhancing the clearance of lipoproteins. This could either mean that these subjects had already been identified as having a higher CV risk and were therefore on appropriate treatment or that liver fibrosis might reduce statin effectiveness by impairing LDL-receptor activity, thus hindering the clearance of atherogenic lipoproteins despite statin treatment. At this stage, there is no clinical or experimental evidence supporting the latter hypothesis.

Another factor that may contribute to liver complications in RA is the presence of anti-CCP antibodies. While these antibodies are well-known for targeting various cell types in RA, their impact on liver cells is less

understood. Recent laboratory studies have revealed that anti-CCP antibodies can influence hepatocytes by triggering the release of inflammatory proteins, impairing fat and glucose metabolism, and ultimately causing liver damage and apoptosis [39]. This mechanism could potentially explain the increased anti-CCP levels observed in patients with high FIB-4 scores.

Our study demonstrated a significant association between HCQ use and lower FIB-4 scores, a finding consistent with recent research suggesting that HCQ may have a protective effect against the development of MASLD [40]. HCQ is known to influence adiponectin levels, which play a key role in regulating metabolic processes. Higher adiponectin levels, linked to HCQ use, are associated with reduced systemic inflammation and improved insulin sensitivity, both of which can help mitigate liver damage [41]. This may explain the lower FIB-4 scores observed in patients using HCQ. However, it is important to consider that this association may be confounded by indication. The protective effect of HCQ against liver fibrosis observed in this study may, in part, be attributed to its use in patients with lower RA disease activity. As low disease activity is inherently associated with reduced systemic inflammation, it is possible that the observed benefit reflects better-controlled RA rather than a direct pharmacological effect of HCQ. Given these considerations, the findings need to be interpreted with caution, and further studies are warranted to explore these potential mechanisms and establish the independent role of HCQ in liver fibrosis prevention.

Moreover, in our study, we did not find any significant associations between MTX use and liver fibrosis. Similarly, a recent multicenter study involving around 1000 patients with RA or psoriasis also found no association between the cumulative dose or duration of MTX exposure and liver fibrosis [42]. Although MTX has been previously linked to hepatotoxicity and an increased risk of liver fibrosis [13, 43], these newer findings suggest that the actual risk may be lower than previously believed. This discrepancy underscores the need for further research to clarify MTX's impact on liver health and to better understand the factors that may influence these outcomes.

The lower inflammatory markers observed in patients with high FIB-4 levels could be attributed to the effects of advanced liver fibrosis. Advanced liver fibrosis often leads to portal hypertension and resultant hypersplenism, causing the sequestration of leukocytes and thrombocytes in the spleen, thereby reducing their levels in the bloodstream. Alternatively, the inverse relationship between leukocyte count and FIB-4 may also be attributed to MTX use, which can cause myelosuppression and reduce leukocyte counts, often leading to neutropenia, anemia, and thrombocytopenia [43, 44]. Additionally, in contrast with previous studies, our study did not find significant associations between glucocorticoid use and liver fibrosis [40, 45]. This may be due to differences in glucocorticoid dosage, treatment duration, or comorbidities within our cohort. Further research with larger samples and more detailed data on glucocorticoid use is needed to clarify its potential impact on liver fibrosis in patients with RA.

This study had some limitations. Firstly, the cross-sectional design limits our ability to determine causality and observe changes over time. Longitudinal studies are needed to explore these aspects further. Secondly, while FIB-4 provides valuable insights, it is not the gold standard for liver fibrosis assessment; liver biopsy remains the preferred method. Additionally, only a small portion of the study population had a high FIB-4. Another limitation is the lack of data on cumulative steroid use. As this information was not collected prospectively, it was not feasible to include it in our analysis. However, given the primary focus of this study on the associations between liver fibrosis and CVD risk factors, as well as medication use in patients with RA, we believe the absence of this data is unlikely to have impacted the validity of our key findings though it is an important consideration for future research. Moreover, some patients in this study were prescribed medications such as HCQ, prednisolone, and NSAIDs, which are not currently recommended as first-line treatments for rheumatoid arthritis. These prescriptions likely reflect individual clinical decisions, including intolerance or contraindications to recommended therapies like methotrexate, or historical prescribing practices. Such variations highlight the complexity of managing rheumatoid arthritis in real-world settings and should be considered when interpreting the study's findings.

In conclusion, RA patients with a high FIB-4 showed increased cIMT, indicating higher CV risk. This relationship appears to be influenced by hypertension and the accumulation of intestinal atherogenic chylomicron remnants. The observed inverse relationship between FIB-4 with systemic inflammatory markers suggests a complex interaction that warrants further research. Hydroxychloroquine may have the potential to offer some protective effect against liver fibrosis in RA patients, though further research is needed to confirm this finding.

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# PART III

## Interventions and prognosis



# Chapter 6

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## The effect of bariatric surgery on NAFLD/NASH: a single centre observational prospective cohort study

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## ABSTRACT

### Introduction

The prevalence of nonalcoholic fatty liver disease (NAFLD) ranges from 25% in the general population to 90% in patients with obesity scheduled for bariatric surgery. NAFLD can progress towards nonalcoholic steatohepatitis (NASH) associated with complications like cirrhosis, hepatocellular carcinoma and cardiovascular disease. To date, losing weight and lifestyle modifications are the best known treatments for NASH. Bariatric surgery significantly improves NAFLD/NASH in the short term. However, the extent of this improvement is not yet clear and long term data on the natural course of NAFLD/NASH after bariatric surgery are lacking. The factors involved in NAFLD/NASH regression after bariatric surgery have not been elucidated.

### Methods and analysis

This is an observational prospective cohort study including patients scheduled for bariatric surgery. Extensive metabolic and cardiovascular analyses will be carried out including measurements of carotid intima media thickness and pulse wave velocity. Genomic, proteomic, lipidomic and metabolomic studies will be done. Microbioma analyses before and 1 year after surgery will be done. Transient elastography measurements will be performed before and at 1, 3 and 5 years after surgery. For those with an elevated preoperative transient elastography measurement by Fibroscan, a laparoscopic liver biopsy will be performed during surgery. Primary outcome measures are the change of steatosis and liver fibrosis 5 years after surgery. Secondary outcome measure is the comparison of the transient elastography measurements with the NAFLD Activity Score from the biopsies.

## INTRODUCTION

Nonalcoholic steatohepatitis (NASH), usually preceded by nonalcoholic fatty liver disease (NAFLD), is an inflammatory condition with fat accumulation and sometimes development of fibrosis in the liver [1]. Recent studies have shown that NASH is the most frequent cause of liver transplantation in females in the USA and it will soon reach a similar first position in men [2]. At least, 90% of the patients with morbid obesity undergoing bariatric surgery may have NAFLD [3, 4]. NAFLD is the most frequent liver disease worldwide, with an estimate of 25% of the population having this disorder [5–7]. At least 20% of patients with NAFLD will progress to NASH [1]. Some ethnicities may also be at higher risk to develop NAFLD and NASH [6, 7]. Data in the Netherlands on the prevalence of this condition are scarce [8, 9].

The cause of NAFLD and NASH development remains unclear although it is generally accepted that the presence of insulin resistance is a prerequisite and that a genetic predisposition may play a role in the pathogenesis [10, 11]. Gut microbiota composition may also be involved in the pathogenesis of NAFLD/NASH. The intestine barrier permeability may be altered exposing the liver to other bacteria or metabolites that will induce pro-inflammatory signaling pathways in the liver. In general, alterations in the gut microbial composition have been frequently observed in individuals with NAFLD and an alteration in plasma metabolites due to microbiota and/or diet has been linked to the development of NAFLD [12–14].

NAFLD is an independent risk factor for cirrhosis, liver failure, hepatocellular carcinoma, coronary artery disease, chronic kidney disease and colorectal carcinomas [5, 7, 15–17]. Patients with NAFLD are also more likely to develop type 2 diabetes mellitus (T2DM) [18] and they are at risk for developing cardiovascular disease (CVD) and heart failure (HF) [19, 20].

The global disease burden of NAFLD/NASH is high. European guidelines advocate active screening of NAFLD and NASH in high risk groups like patients with diabetes, obesity, hypertension and subjects with the metabolic syndrome [21, 22]. For this purpose, a diagnostic flow-chart and active screening have been proposed by three different scientific societies, namely the European Association for the Study of Liver (EASL), the

European Association for the Study of Diabetes (EASD) and the European Association for the Study of Obesity (EASO).

In addition, it is of great importance to determine the severity of the liver fibrosis (according to the Metavir classification) since this largely determines the prognosis [23]. Subjects with stage F3 or F4 of NASH have a significantly worse prognosis than those at stages F2 or lower. Of note, Fibroscan™ measurements are very sensitive and specific for F1 and F4 fibrosis stages but Fibroscan™ cannot reliably differentiate between F2 and F3 [24, 25]. Therefore, confirmation with histology in patients with intermediate to severe fibrosis classified as F2 and F3 by Fibroscan is warranted.

Although a great number of pharmacological interventions are being investigated, so far none has been approved for clinical use [26, 27]. The cornerstone of the treatment at present is to lose weight by lifestyle management and improved nutritional interventions. However, the evidence for this approach is based on small studies including a limited number of subjects and with limited follow up [28]. Different lifestyle interventions have been described with the Mediterranean diet being the most efficient [29]. Bariatric surgery is the most effective surgical intervention to decrease body weight [30–34] and it may also lead to NAFLD/NASH regression. Bariatric surgery is usually part of an extensive protocol with a long term follow-up where attention is given to lifestyle changes. Data on the possible remission of NASH after bariatric surgery are limited [1]. In one systematic review, 9940 subjects were included in 45 studies of which 2 studies reported follow-up with liver biopsies. Only 7 studies were comparative, evaluating Roux-Y-Gastric Bypass (RYGB) with sleeve gastrectomy (SG). All other studies only evaluated biochemical variables. So far, no study has included measurements of liver fibrosis with transient elastography in the follow up [35].

In the present study we aim to investigate the prevalence and the severity of NAFLD/NASH in patients enrolled for bariatric surgery in the Netherlands, the association with other comorbidities and the effect of bariatric surgery on the resolution of these disorders. We also aim to establish a correlation between histology (liver biopsy) and transient elastography measurements in these patients. By publishing this protocol we aim to create awareness of this condition and to encourage research within this topic.

## METHODS AND ANALYSIS

### Study design and participants

This is an observational prospective cohort study. It will take place in a single Obesity Center in the Netherlands in a large teaching semi-academic municipal hospital: the Franciscus Gasthuis & Vlietland Hospital. We aim to include 300 patients from the regular morbid obesity bariatric program from September 1<sup>st</sup>, 2022, with a 5 years postoperative follow-up. Participants must fulfil the criteria of the International Federation for the Surgery of Obesity (IFSO) at screening. In order to participate in this study, subjects have to speak and read Dutch in order to provide written informed consent. Patients who meet any of the following criteria at screening will be excluded from the study: participants younger than 18 years or older than 65 years, participants with an established diagnosis of another primary liver disease, histologically documented liver cirrhosis (fibrosis stage F4) at screening or in a historical biopsy, participants with active HIV infection and/or treatment and participants with diagnosed malignancies with or without active treatment. Moreover, participants with a history or evidence of any other clinically significant condition or planned or expected procedure that may compromise the patient's safety or ability to complete the study will be excluded.

The measurements will be performed at the specified time points, at the Department of Endocrinology, Vascular Medicine and Diabetes of our hospital (Table 1).

Table 1 – Timetable and intervention planning. FU: follow-up; IMT: intima media thickness; PWV: pulse wave velocity.

|                        | Subject<br>n= | Enrollment | Visit 1 | Surgery  | Follow-up: moment after surgery |                             |                             |
|------------------------|---------------|------------|---------|----------|---------------------------------|-----------------------------|-----------------------------|
| Time                   |               | -2 months  | 0 month | 3 months | 15 months<br>(1 year<br>FU)     | 39 months<br>(3 year<br>FU) | 63 months<br>(5 year<br>FU) |
| Weighing               | 300           | x          | x       | x        | x                               | x                           | x                           |
| Genetic analysis       | 300           |            | x       |          |                                 |                             |                             |
| Metabolomics analysis  | 300           |            |         | x        | x                               |                             |                             |
| Transient Elastography | 300           |            | x       | x        | x                               | x                           | x                           |

Table 1 – Timetable and intervention planning. FU: follow-up; IMT: intima media thickness; PWV: pulse wave velocity. (continued)

|                       | Subject<br>n= | Enrollment | Visit 1 | Surgery | Follow-up: moment after surgery |   |   |
|-----------------------|---------------|------------|---------|---------|---------------------------------|---|---|
| Lifestyle coaching    | 300           |            | x       |         |                                 |   |   |
| IMT measurement       | 300           |            | x       |         | x                               | x | x |
| PWV measurement       | 300           |            | x       |         | x                               | x | x |
| Feces Analysis        | 300           |            | x       |         | x                               |   |   |
| Heart Echography      | 60            |            | x       |         | x                               |   |   |
| Liver Biopsy (if ≥F2) | 60            |            |         | x       |                                 |   |   |

After the 5 years follow-up, patients will be referred back to the general practitioner following international guidelines and our local protocol. After bariatric surgery, patients will be referred to the department of Gastroenterology and Hepatology for further follow up in case of persistent abnormal elastography measurements (F2 with elevated liver enzymes or ≥F3) (see figure 1).

The type of surgery will be determined by the surgeon together with the patient. The type of operation offered, depends on several clinical characteristics (co-morbidities, medication use, etc.) and this decision will not be influenced by participation in this study. In our hospital we use 3 types of surgery: gastric sleeve resection, Roux-en-Y gastric bypass and one-anastomosis gastric bypass.

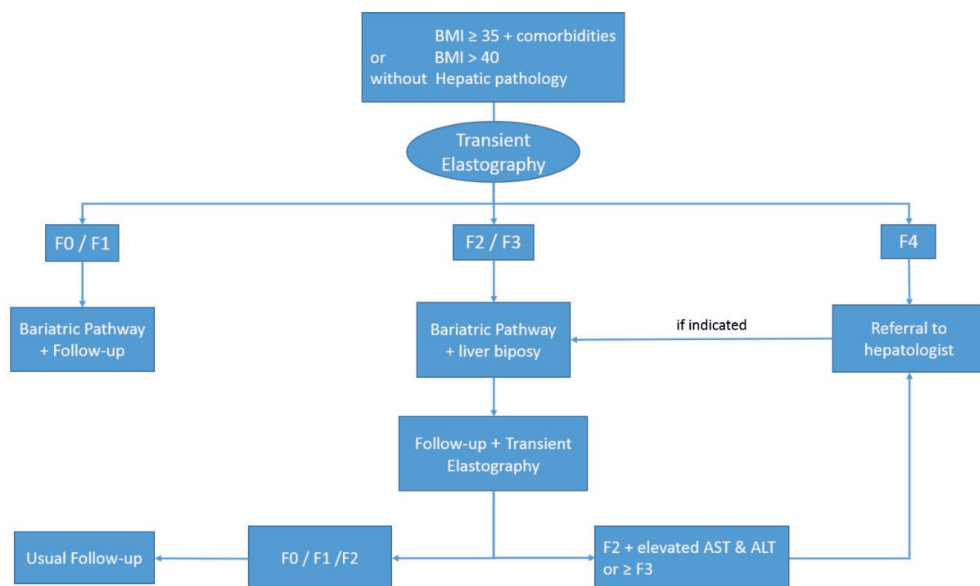


Figure 1 – Decision tree for liver biopsy and referral to hepatologist.

## Patient an public involvement

Patients were not involved in the design and conduct of this research. During the feasibility stage, priority of the research question, choice of outcome measures, and methods of recruitment were informed by discussions with the medical team. During the trial, no patient will join the research team. Once the study is published, participants will be informed of the results on their demand.

## Outcome measures

The primary outcome measure is to evaluate NAFLD/NASH changes in terms of steatosis and liver elasticity in patients with morbid obesity at 5 years after bariatric surgery. Therefore we will use the FIB4 score [36] and then proceed with evaluation using transient elastography (Fibroscan™, Paris, France) before surgery and at 1, 3 and 5 years after surgery. The secondary outcome measure is to establish the correlation between histology (liver biopsy) and transient elastography measurement in a population with morbid obesity. For this purpose, a laparoscopic liver biopsy will be performed during the bariatric procedure in patients with an elevated measurement with transient elastography ( $\geq 7.95$  kPa, see Table 2) estimated preoperatively. Two pathologists will score independently the liver biopsies (NAFLD/NASH status using the NAFLD Activity Score (NAS)) [37]. In case of disagreement on the classification, a third

pathologist will be consulted and the score set by 2 out of 3 pathologists will be recorded.

Table 2 – Cut-off values for the evaluation of liver fibrosis with Fibroscan™ according to Theel et al. [38]. E: elasticity

| Fibrosis stage | F0   | F1         | F2          | F3           | F4      |
|----------------|------|------------|-------------|--------------|---------|
| E (kPa)        | <6,9 | 6,9 – 7,94 | 7,95 – 8,94 | 8,94 – 14,24 | ≥ 14,25 |

In case of perioperative complications, all (serious) adverse events will be registered to evaluate the safety of the surgery procedure for the participants. During follow up, we will also record all cases of liver failure, decompensation, hepatocarcinoma or need for transplantation in order to gain insight in the pathophysiology of NAFLD and weight loss as well as other cardiovascular and renal complications and death. Besides, participants needing a (laparoscopic) cholecystectomy after the bariatric procedure will be registered.

The Franciscus Obesity NASH Study also aims to evaluate determinants of liver steatosis and elasticity. Therefore, subanalyses will be carried out in relation to the type of bariatric surgery (gastric sleeve resection, Roux-en-Y gastric bypass, one-anastomosis gastric bypass), the presence of diabetes and if indicated the use and type of medication (insulin, oral medication, GLP1 analogues), the polymorphism of genetic markers (among others: PNPLA3, TM6SF2, MBOAT7, GCKR and HSD17B13), the effect of lifestyle intervention and the Firmicutes/Bacteroides ratio in the microbioma.

Metabolomics, lipidomics and proteomics analyses will be performed in order to identify potential biomarkers of NASH.

Full panels of metabolites from EDTA-plasma samples will be analysed using mass-spectrometry (MS) based metabolomics and lipidomics methods. A global metabolomics high- resolution platform will be used allowing the targeted and untargeted/global analyses of a wide range of small molecules including amino acids, central energy and carbon metabolism regulators, together with exogenous molecules such as dietary chemicals, microbiome-derived metabolites, environmental chemicals, commercial products and drugs. A lipidomics platform will allow the analysis and classification of more than 27 lipid classes including diglycerides, triglycerides, phospholipids, ceramides, sphingolipids and

many more. A targeted MS/MS platform will be used to cover more than 300 modified free fatty acids that act as bioactive lipid mediators, both locally and systemically, and that are involved in innate immunity, inflammation, chemotaxis, cell survival, cell proliferation, differentiation and apoptosis. Targeted and untargeted proteomics (SomaScan, O-link panels) will be used to identify serological protein biomarkers, which will then be validated through ELISAs or Multiplex assays.

A cardiovascular analysis will also be performed including measurements of carotid intima media thickness, pulse wave velocity and echocardiography to determine if changes in liver fibrosis stage are associated with change in cardiovascular function. Moreover, any cardiovascular event will be registered in order to evaluate the risk of cardiovascular complications in participants with different stadia of NAFLD/NASH.

### Data management

Patient data and material will be coded and handled confidentially. For each subject a unique identification code (neither based on patient initials nor birth-date) will be used to link the study data to the subject's personal characteristics. The key to this code is safeguarded by the principal investigators, who also have access to the source data. Data will be stored using Castor EDC (Amsterdam, the Netherlands), a web-based electronic data capture software program for clinical research. The database will be accessible by the study team only and according to study team-role if applicable.

### Sample size

Based on a recent study [39] we expect to find a mean elastography of 9.7 kPa (SD 4.4) before surgery and a mean elastography of 7.2 kPa (SD 4.0) 5 years after bariatric surgery. Using a two-sided t-test of equal means with 5% significance and at least 80% power, at least 25 subjects (paired observations) will be required for the analysis of bariatric surgery on NASH changes based on elastography.

We expect to have a considerable drop out during follow up. The study of Van de Laar et al. provided information on follow up rate for Dutch patients 5 years after bariatric surgery (783 patients after 5 years from 9393 before surgery or 8.33% from the total) [40]. Thus, given this low follow-up rate and the fact that calculations indicate that we need at least 25 patients with 5 years follow-up to answer our primary objec-

tive, we will need to include at least 300 patients ( $300 \times 8.33\% = 25$ ) before surgery.

According to the literature [6, 41, 42] about 20% of our subjects scheduled for bariatric surgery are expected to demonstrate histological abnormalities reflecting NASH ( $\geq F2$ ). Therefore, from the 300 subjects enrolled in our study, about 60 of these may be eligible for a laparoscopic liver biopsy during the bariatric procedure to determine their actual degree of fibrosis.

### Statistical analysis

This study is designed as an observational, prospective, non-randomized study. For statistical analysis IBM SPSS version 28.0 (IBM Corporation, Armonk, New York, USA) will be used. Efforts will be made to prevent missing data by checking the completeness of the collected data. Missing data may be expected due to the low follow up rate of this patient population or failure of transient elastography measurements. Difference in steatosis and liver elasticity will be analyzed comparing the measurements before and 5 years after bariatric surgery using a paired t-tests (if normally distributed otherwise the Wilcoxon signed rank test). We aim to compare the classification of the liver fibrosis (Metavir F1–4) according to the TE measurement with the NAS score from the biopsies. For this, we will use a Chi-square test for trend to determine if there is any significant difference of classification between the two methods. P values  $< 0.05$  (2-tailed) will be considered statistically significant.

## DISCUSSION

In the last decades, the prevalence of obesity has risen rapidly in most parts of the world, closely associated with comorbidities such as NAFLD and its more severe form NASH. Multiple studies estimate that NAFLD may be present in 17% to 33% of the world population [5][43] but little is known about the prevalence in patients enrolled for bariatric surgery and the evolution after such a procedure. In this study, a consistent analysis of these patients will give insight into the preoperative prevalence of patients with NAFLD, those who are more at risk for NASH and the metabolic changes induced by bariatric surgery. A weakness of our study is that we will not perform a liver biopsy in all participants but only in those with a preoperative elevated liver elasticity measured with transient elastography.

Bariatric surgery has proven its long term effect on weight loss and it currently remains the best therapeutic option to maintain weight loss [32]. The causal relationship with obesity is reflected by the fact that after weight loss, NAFLD decreases significantly, although not in every patient and not to the same extent [30, 44]. This study aims to determine through different measurements which bariatric procedure and which mechanism improves liver steatosis and fibrosis at 5 years. The high drop-out rate may constitute an important limitation. This point will be analyzed and discussed. Special attention will be given in the correction of possible disparities in age and gender.

NAFLD is often underdiagnosed in patients with obesity. The reason for that is often a lack of knowledge and equipment to evaluate the risk of developing serious events. To our knowledge, the literature does not provide specific cut-off values for the bariatric population to distinguish patients with a higher risk [38]. The study will use transient elastography measurements and will compare these with histological findings in order to establish cut-off values.

This study will expand the current knowledge on NAFLD/NASH in severe obesity and we will be able to investigate in detail changes in pathophysiological and cardiovascular mechanisms associated with severe obesity in these subjects. The aim is to identify markers and metabolic pathways that will help to identify subjects at high risk of developing complications due to NAFLD.

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# Chapter 7

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## Genetic risk factors for MASLD/MASH and cardiovascular disease in elderly individuals

Short title: Genetic Risk in MASLD and Cardiovascular Disease

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## ABSTRACT

### Introduction and objectives

Metabolic dysfunction-associated steatotic liver disease (MASLD) and its progressive form, MASH, are closely related to obesity and lifestyle. From previous studies, MASLD is also known for its strong genetic background. This study aimed to investigate the association between previously reported genetic polymorphisms of MASLD/MASH and clinical outcomes in an elderly Western European population.

### Patients and Methods

We performed an analysis of 5,244 participants (mean age 75.3 years) from the PROSPER cohort study who were followed for 3.2 years. FIB-4 scores were calculated using age, AST, ALT, and platelet count, applying age-specific cutoffs. Thirty MASLD/MASH-associated SNPs, previously identified in GWAS analyses, were evaluated for their association with FIB4 scores. For SNPs showing significant differences in FIB-4 score across genotypes, associations with clinical outcomes and all-cause mortality were assessed using Cox proportional hazards regression models, adjusted for relevant clinical covariates.

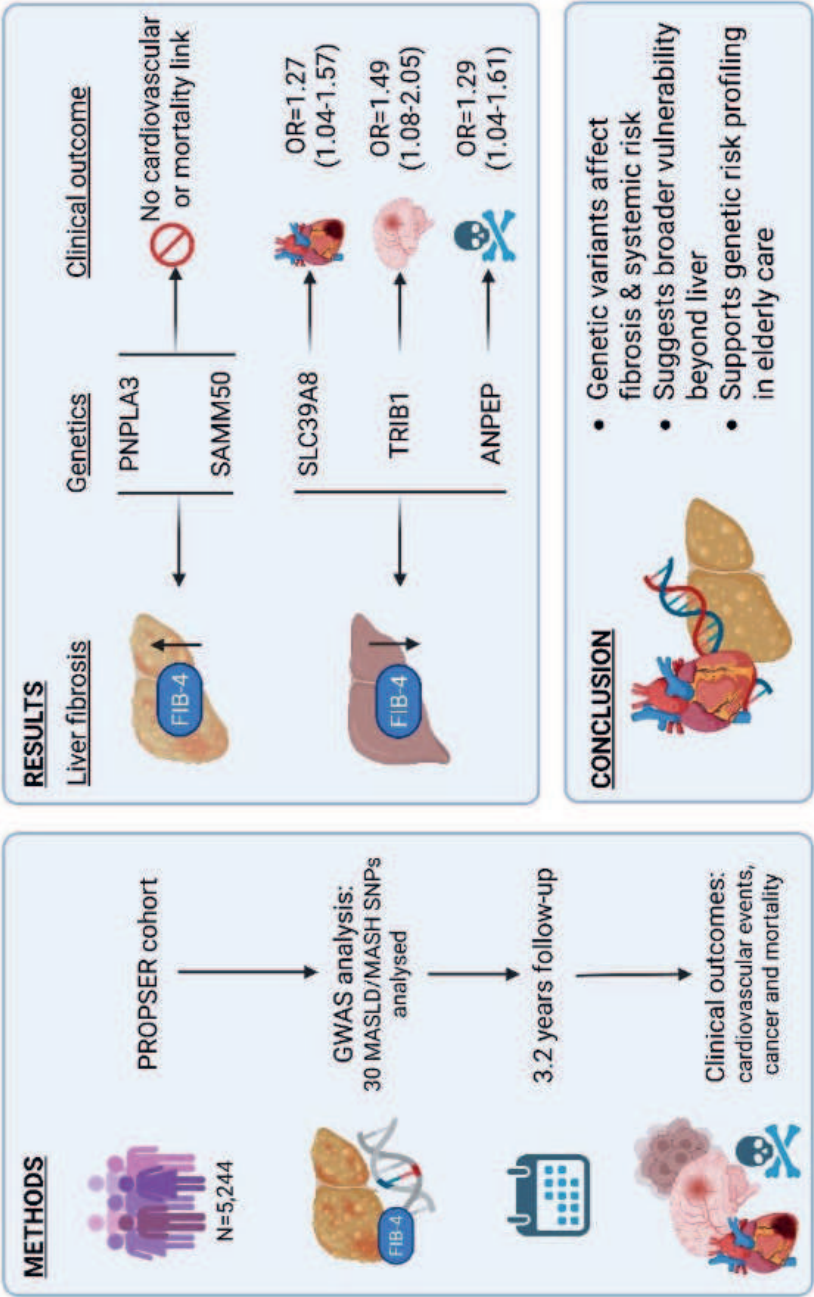
### Results

Five SNPs were significantly associated with FIB-4 scores. PNPLA3\_rs738409 and SAMM50\_rs3761472 showed higher FIB-4 scores in mutant homozygotes, while SLC39A8\_rs13107325, TRIB1\_rs2954021, and ANPEP\_rs7168849 showed the opposite trend. Despite statistical significance, absolute FIB-4 scores remained within ranges typically associated with low fibrosis risk. TRIB1\_rs2954021 was linked to stroke, SLC39A8\_rs13107325 to myocardial infarction and ANPEP\_rs7168849 to all-cause and cancer-related mortality. No significant associations were observed for PNPLA3\_rs738409 or SAMM50\_rs3761472 with cardiovascular and mortality outcomes.

### Conclusions

Our findings reveal that specific MASLD/MASH-associated genetic variants influence FIB-4 scores and are linked to cardiovascular and mortality outcomes in an elderly population. These results suggest that liver fibrosis may reflect not only hepatic injury, but also broader systemic vulnerability driven by genetic predisposition. Incorporating genetic risk profiles may improve stratification for both liver and cardiometabolic disease.

Genetic risk factors for MASLD/MASH and cardiovascular disease in elderly individuals



Graphical abstract

## INTRODUCTION

Metabolic dysfunction–associated steatotic liver disease (MASLD), previously known as nonalcoholic fatty liver disease (NAFLD), represents a spectrum of liver conditions characterized by hepatic steatosis in combination with at least one cardiometabolic risk factor such as type 2 diabetes, obesity, or dyslipidemia (1). MASLD may progress to metabolic dysfunction–associated steatohepatitis (MASH), with or without liver fibrosis, and in advanced stages, can lead to cirrhosis and hepatocellular carcinoma (2).

Fibrosis is a key prognostic factor in MASLD, as its severity strongly predicts liver-related and overall mortality (3–5). Beyond hepatic complications, MASLD is also associated with an increased risk of cardiovascular disease (CVD), which remains the leading cause of death in these patients (6–8). In clinical practice, non-invasive tools such as the Fibrosis-4 Index (FIB-4) are recommended to assess fibrosis risk, especially in settings where liver biopsy is not feasible (9). Recent data suggest that a high FIB-4 score ( $\geq 2.67$ ) is not only linked to liver-related outcomes, but also to increased all-cause mortality and a higher incidence of stroke in patients with obesity (10, 11).

The pathogenesis of MASLD is multifactorial and includes insulin resistance, lipotoxicity, gut dysbiosis, and genetic predisposition. Genome-wide association studies (GWAS) have identified several genetic variants that influence susceptibility to MASLD and its progression to fibrosis, including polymorphisms in *PNPLA3*, *TM6SF2*, *HSD17B13*, *MBOAT7*, and *GCKR* (12–15). Notably, the rs738409 variant in *PNPLA3* and the rs58542926 variant in *TM6SF2* are strongly associated with increased liver fat content and fibrosis severity (16).

Recent evidence suggests the existence of two distinct subtypes of MASH: a genetically driven, liver-predominant form with relatively lower cardiovascular risk, and a cardiometabolic form that is closely tied to features like diabetes and cardiovascular complications (17). However, despite the well-established genetic contributions to MASLD, the link between these polymorphisms and cardiovascular disease remains insufficiently understood. Some studies have even reported that certain MASLD-related single nucleotide polymorphisms (SNPs) may not increase cardiovascular mortality and may potentially offer a protective effect (18–20).

Our group previously demonstrated that a high FIB-4 score predicts all-cause mortality in an elderly cohort and that treatment with pravastatin mitigates this risk (11). In the present study, we aim to investigate whether well-known MASLD-associated genetic variants contribute to cardiovascular complications in this elderly population.

## METHODS

### Study population and genotyping

The PROspective Study of Pravastatin in the Elderly at Risk (PROSPER) provided all the data. The PROSPER study was a clinical trial investigating the effect of the cholesterol-lowering drug pravastatin in an elderly population with an increased risk of cardiovascular disease (i.e., due to smoking, hypertension, or diabetes). The detailed study set-up has been published in great detail elsewhere (21, 22). Between 1997 and 1999, 5,804 individuals aged 70–82 years from Scotland, Ireland and the Netherlands were included and randomized in a 1:1 ratio to receive either 40 mg pravastatin or placebo. The study was completed in 2002. The primary composite endpoint was the combined outcome of definite or suspected death from coronary heart disease, non-fatal myocardial infarction (MI), and fatal or non-fatal stroke, assessed across the entire cohort.

Secondary endpoints included the separate evaluation of coronary and cerebrovascular events. Additionally, subgroup analyses were planned to assess the primary endpoint by sex and by presence or absence of pre-existing cardiovascular disease.

Tertiary endpoints comprised the incidence of transient ischemic attack (TIA), heart failure, cancer, and all-cause mortality. Furthermore, exploratory analyses were intended to examine the potential magnitude of benefit in relation to baseline risk factors such as smoking status, hypertension history, diabetes, and LDL/HDL cholesterol levels.

### Genotyping and SNPs selection

A genome-wide association screening (GWAS) was performed in the PROSPER/PHASE study using Illumina 660-Quad beadchips, as described previously (23). The current study focused on single nucleotide polymorphisms (SNPs) associated with MASLD (13–15, 24) which have been extracted from this PROSPER/PHASE GWAS analysis with PLINK software.

Four recent GWAS studies in the field of MASLD and MASH identified SNPs with robust associations to MASLD and its progressive form, MASH (13–15, 24). These studies yielded 13 SNPs associated with MASH and 44 SNPs linked exclusively to MASLD. Cross-referencing this list of SNPs available in PROSPER, we identified 30 SNPs of interest: 7 associated with MASH and 23 with MASLD. We evaluated them by comparing FIB-4 scores across genotype categories. For SNPs showing significant differences in FIB-4 across genotypes, associations with cardiovascular outcomes and all-cause mortality were assessed using Cox proportional hazards regression models, adjusted for relevant clinical covariates.

### FIB-4 index

We calculated the FIB-4 index as described in recent guidelines to evaluate liver fibrosis (9). The index was calculated using the patient's age, aspartate transaminase (AST), alanine transaminase (ALT), and blood platelet count (25). The FIB-4 index was defined as follows:

$$\text{FIB-4 index} = (\text{Age} * \text{AST}) / (\text{platelets} * \sqrt{\text{ALT}})$$

Since age is a factor in the algorithm used to calculate FIB-4, fibrosis may be overestimated in elderly individuals, resulting in lower sensitivity of FIB-4 in this population. To assess this, we applied age-specific cut-offs as described by McPherson et al. (26): low risk of liver fibrosis (<2.0), intermediate risk ( $\geq 2.0$  and  $\leq 2.66$ ), and high risk ( $\geq 2.67$ ).

### Statistical Analysis

Continuous variables are reported as mean  $\pm$  standard deviation (SD), while categorical variables are presented as counts and proportions. Descriptive characteristics of the population were provided for each FIB-4 subgroup. Comparisons between subgroups were conducted using a one-way ANOVA. The time-to-event data were analyzed with Cox proportional hazards models for identified SNPs. For each endpoint, either time to first occurrence of the event or study closure (censored observation) was taken, depending on which came first. The models were adjusted for gender, alcohol use, LDL cholesterol levels, and the use of antihypertensives, lipid lowering medication, and diabetic medications. Hazard ratios (HR) with 95% confidence intervals (CI) were calculated for each endpoint. Statistical significance was defined as  $p < 0.05$  assessed with SPSS software version 27.

## Ethical Considerations

The medical ethics committees of each participating institution gave their approval to the study protocol. Every study participant provided written, informed consent. The study was compliant with the 1975 Declaration of Helsinki's ethical principles

## RESULTS

### Population demographics

A total of 5,244 subjects of Caucasian descent with available DNA samples were included in this analysis, of which 51.9% were female, with a mean age of 75.3 years and a mean BMI of 26.8 kg/m<sup>2</sup>. These subjects were followed for a mean of 3.2 years.

Higher FIB-4 scores were associated with older age, male sex, a history of vascular disease, lower BMI, and a more favorable lipid profile (table 1). LDL cholesterol, total cholesterol, and triglycerides were lower in individuals with high FIB-4, while HDL cholesterol was slightly higher.

Although no significant differences were observed in the prevalence of hypertension or diabetes, current smoking and alcohol consumption were more frequent in the high FIB-4 group.

### FIB-4 and MASLD/MASH associated SNPs

Table 2 presents the association between MASLD/MASH-associated SNPs and FIB-4 classification across genotypes. Only the five SNPs that showed a statistically significant relationship with FIB-4 scores are highlighted in the main text and table 2 for clarity. The associations for the remaining 25 SNPs, which did not demonstrate significant differences, are included in the supplementary material (Supplementary Table 1) to provide a comprehensive overview.

Individuals with the homozygous mutant (M/M) genotype of *PNPLA3\_rs738409* had significantly higher FIB-4 scores compared to those with the wild-type (WT/WT) genotype. *SAMM50\_rs3761472* followed the same trend, with the highest FIB-4 score observed in the M/M genotype and the lowest score in the WT/WT genotype.

Table 1 – Baseline general characteristics of the subjects of the PROSPER study in relation to FIB-4

|                                                | All (n=5244) | Low FIB-4<br>(n=3549) | Intermediate FIB-4<br>(n=1132) | High FIB-4<br>(n=518) | p-value <sup>b</sup><br>intermediate vs low | p-value<br>vs low |
|------------------------------------------------|--------------|-----------------------|--------------------------------|-----------------------|---------------------------------------------|-------------------|
| Age (years)                                    | 75.3 ± 3.4   | 75.0 ± 3.3            | 76.0 ± 3.3                     | 76.3 ± 3.5            | <0.001                                      | <0.001            |
| Females, n (%)                                 | 2720 (51.9)  | 2003 (56.4)           | 497 (43.9)                     | 195 (37.6)            | <0.001                                      | <0.001            |
| FIB-4                                          | 1.81 ± 0.66  | 1.45 ± 3.20           | 2.27 ± 1.87                    | 3.21 ± 0.59           | NA                                          | NA                |
| BMI (kg/m <sup>2</sup> )                       | 26.8 ± 4.18  | 26.9 ± 4.2)           | 26.6 ± 4.0                     | 26.2 ± 4.3            | <0.001                                      | <0.001            |
| Current Smokers n (%)                          | 1392 (26.5)  | 1006 (28.3)           | 257 (22.7)                     | 119 (23.0)            | <0.001                                      | 0.011             |
| Alcohol (units per week)                       | 5.2 ± 9.2    | 4.6 ± 8.5             | 6.0 ± 9.8                      | 7.8 ± 11.7            | <0.001                                      | <0.001            |
| Diabetes n (%)                                 | 544 (10.4)   | 375 (10.6)            | 105 (9.3)                      | 56 (10.8)             | 0.665                                       | 0.866             |
| Hypertension n (%)                             | 3257 (62.1)  | 2199 (62.0)           | 708 (62.5)                     | 318 (61.4)            | 0.971                                       | 0.803             |
| History of vascular disease <sup>a</sup> n (%) | 2336 (44.5)  | 1502 (42.3)           | 536 (47.3)                     | 277 (53.5)            | <0.001                                      | <0.001            |
| Systolic Blood Pressure (mmHg)                 | 154.6 ± 21.9 | 154.2 ± 21.63         | 155.2 ± 22.3                   | 156.1 ± 22.7          | 0.032                                       | 0.059             |
| Diastolic Blood pressure (mmHg)                | 83.7 ± 11.4  | 83.4 ± 11.4           | 83.8 ± 11.0                    | 85.2 ± 12.4           | 0.002                                       | <0.001            |
| LDL cholesterol (mmol/L)                       | 3.79 ± 0.80  | 3.82 ± 0.80           | 3.74 ± 0.79                    | 3.68 ± 0.80           | <0.001                                      | <0.001            |
| HDL cholesterol (mmol/L)                       | 1.28 ± 0.35  | 1.28 ± 0.34           | 1.28 ± 0.36                    | 1.32 ± 0.37           | 0.021                                       | 0.004             |
| Total Cholesterol (mmol/L)                     | 5.69 ± 0.90  | 5.72 ± 0.91           | 5.63 ± 0.87                    | 5.54 ± 0.89           | <0.001                                      | <0.001            |

Table 1 – Baseline general characteristics of the subjects of the PROSPER study in relation to FIB-4 (continued)

|                                     | All (n=5244) | Low FIB-4<br>(n=3549) | Intermediate FIB-4<br>(n=1132) | High FIB-4<br>(n=518) | p-value <sup>b</sup> | p-value<br>intermediate vs low | p-value high<br>vs low |
|-------------------------------------|--------------|-----------------------|--------------------------------|-----------------------|----------------------|--------------------------------|------------------------|
| Triglycerides (mmol/L)              | 1.55 ± 0.71  | 1.58 ± 0.71           | 1.52 ± 0.72                    | 1.37 ± 0.66           | <0.001               | 0.009                          | <0.001                 |
| Diabetes medication<br>n (%)        | 347 (6.6)    | 243 (6.8)             | 61 (5.4)                       | 38 (7.3)              | 0.637                | 0.083                          | 0.682                  |
| Antihypertensive<br>medication n(%) | 3882 (74.0)  | 2616 (73.7)           | 841 (74.3)                     | 390 (75.3)            | 0.425                | 0.698                          | 0.445                  |

Data are presented as number (percentage), mean ±SD, or median ±IQR.

<sup>a</sup> Any of stable angina, intermittent claudication, stroke, transient ischemic attack, myocardial infarction, peripheral artery disease surgery, or amputation for vascular disease more than 6 months before study entry.

<sup>b</sup> p value represents the difference between the FIB-4 subgroups.

In contrast, *SLC39A8\_rs13107325*, *TRIB1\_rs2954021*, and *ANPEP\_rs7168849* exhibited the opposite pattern, where the WT/WT genotype had the highest FIB-4 score, followed by WT/Mutant, and the lowest score was seen in Mutant/Mutant.

Clinical endpoints

The SNPs that exhibited a significant difference in FIB-4 among the different genotypes were further tested for their associations with cardiovascular and mortality outcomes (Table 3). To improve transparency and interpretability, we also added Supplementary Table 2, which summarizes the number of participants and outcome events per SNP genotype (WT/WT, WT/M, M/M), as well as genotype frequencies.

*TRIB1\_rs2954021* was associated with a higher risk of fatal or nonfatal stroke, while *SLC39A8\_rs13107325* was linked to an increased risk of fatal or nonfatal myocardial infarction. *ANPEP\_rs7168849* showed significant associations with both all-cause and cancer-specific mortality. *PNPLA3\_rs738409* showed no significant associations with cardiovascular or cancer-related outcomes, nor did *SAMM50\_rs3761472*.

Table 2 - Association between the MASLD/MASH-associated SNPs and FIB-4 index across genotypic categories.

| SNP                | WT/WT       | WT/Mutant   | Mutant/Mutant | p-value |
|--------------------|-------------|-------------|---------------|---------|
| MASLD & MASH       |             |             |               |         |
| PNPLA3_rs738409    | 1.79 (0.64) | 1.82 (0.68) | 1.95 (0.76)   | <0.001  |
| SLC39A8_rs13107325 | 1.82 (0.67) | 1.76 (0.64) | 1.57 (0.44)   | 0.004   |
| MASLD only         |             |             |               |         |
| SAMM50_rs3761472   | 1.80 (0.66) | 1.81 (0.68) | 1.96 (0.67)   | 0.042   |
| TRIB1_rs2954021    | 1.83 (0.68) | 1.82 (0.66) | 1.76 (0.65)   | 0.014   |
| ANPEP_rs7168849    | 1.81 (0.67) | 1.76 (0.65) | 1.73 (0.57)   | 0.035   |

All data is presented as Mean (SD) FIB-4 risk. WT: wild type

Table 3 – Association between FIB-4 and related liver fibrosis SNPS with cardiovascular outcomes and cancer.

Analysis was conducted using Cox regression and adjusted for statin treatment, gender, alcohol use, LDL cholesterol, and the use of antihypertensive medication and diabetic medication. Data are presented as hazard ratio (95% confidence interval).

|                       | Combined<br>Endpoint | Fatal/<br>non-fatal<br>Stroke/TIA   | Fatal /<br>nonfatal MI              | Heart<br>failure    | All-cause<br>mortality              | Cancer<br>mortality                  |
|-----------------------|----------------------|-------------------------------------|-------------------------------------|---------------------|-------------------------------------|--------------------------------------|
| <b>FIB-4</b>          |                      |                                     |                                     |                     |                                     |                                      |
| <b>SNPs</b>           |                      |                                     |                                     |                     |                                     |                                      |
| PNPLA3<br>rs738409    | 0.98<br>(0.85–1.14)  | 1.12<br>(0.86–1.45)                 | 0.93<br>(0.78–1.10)                 | 0.96<br>(0.72–1.28) | 0.96<br>(0.81–1.15)                 | 0.88<br>(0.65–1.21)                  |
| SLC39A8<br>rs13107325 | 1.16<br>(0.97–1.39)  | 1.05<br>(0.75–1.48)                 | <b>1.27</b><br><b>(1.04–1.57) *</b> | 1.22<br>(0.86–1.72) | 1.19<br>(0.96–1.48)                 | 1.31<br>(0.91–1.89)                  |
| TRIB1<br>rs2954021    | 1.06<br>(0.91–1.25)  | <b>1.49</b><br><b>(1.08–2.05) ^</b> | 0.95<br>(0.79–1.13)                 | 1.08<br>(0.79–1.48) | 1.03<br>(0.85–1.25)                 | 1.08<br>(0.77–1.51)                  |
| SAMM50<br>rs3761472   | 1.04<br>(0.88–1.21)  | 1.25<br>(0.95–1.65)                 | 0.97<br>(0.80–1.17)                 | 0.89<br>(0.65–1.22) | 0.97<br>(0.80–1.18)                 | 0.95<br>(0.68–1.33)                  |
| ANPEP<br>rs7168849    | 0.99<br>(0.81–1.20)  | 1.16<br>(0.83–1.63)                 | 0.94<br>(0.74–1.19)                 | 0.77<br>(0.51–1.18) | <b>1.29</b><br><b>(1.04–1.61) #</b> | <b>1.62</b><br><b>(1.14–2.32) **</b> |

\*p-value=0.021, ^p-value =0.016, #p-value=0.022, \*\*p-value=0.007

## DISCUSSION

In this analysis of 5,244 subjects of the PROSPER study, we identified several key findings regarding the relationship between demographic factors, MASLD/MASH-associated genetic variants, and FIB-4 scores, as well as their potential links to cardiovascular outcomes. First, higher FIB-4 scores were associated with older age, male sex, lower BMI, a history of vascular disease, and a more favorable lipid profile. Second, five genetic variants (PNPLA3\_rs738409, SAMM50\_rs3761472, SLC39A8\_rs13107325, TRIB1\_rs2954021, and ANPEP\_rs7168849) were significantly associated with FIB-4 classification, with divergent patterns suggesting both risk-enhancing and potentially protective effects. Third, three of these variants (TRIB1, SLC39A8, and ANPEP) were also associated with cardiovascular or mortality endpoints, supporting the notion of shared genetic pathways between liver fibrosis and systemic disease processes.

These findings align well with existing literature and further clarify the interplay between genetic risk and liver fibrosis. The observed association between higher FIB-4 scores and older age, male sex, and

vascular disease is consistent with prior studies highlighting fibrosis as a marker of systemic metabolic burden and cumulative organ damage (27, 28). The inverse relationship between FIB-4 and BMI may initially seem paradoxical, but this likely reflects potential confounders like age, metabolic alterations, and muscle catabolism (29–31). These data support the existence of a “lean fibrosis” phenotype, characterized by metabolic dysfunction despite a lower BMI.

The association of lipid profile changes with FIB-4 scores also mirrors prior findings. Lower LDL, total cholesterol, and triglycerides in individuals with higher FIB-4 scores may reflect impaired hepatic lipid synthesis and transport in advanced liver disease, while slightly elevated HDL cholesterol may indicate compensatory shifts in lipid metabolism (32). Moreover, the higher prevalence of smoking and alcohol use among those with elevated FIB-4 underscores the contribution of lifestyle factors to fibrotic progression and broader metabolic dysregulation.

Our genetic analysis builds upon established knowledge by reinforcing the role of PNPLA3\_rs738409 as a major genetic driver of fibrosis in MASLD and MASH (33, 34). The observed associations with SAMM50\_rs3761472 further support its involvement in hepatic mitochondrial function and fibrogenesis. In contrast, SLC39A8\_rs13107325, TRIB1\_rs2954021, and ANPEP\_rs7168849 were associated with lower FIB-4 scores in their mutant homozygous forms, suggesting a potential protective role. This dichotomy in genetic influence reflects variant-specific effects on hepatic inflammation, oxidative stress responses, lipid homeostasis, or immune function (35, 36).

Importantly, several of these SNPs also demonstrated associations with cardiovascular outcomes, suggesting that genetic risk for liver fibrosis may also extend to vascular disease. TRIB1\_rs2954021 was linked to stroke, consistent with its known effects on lipid metabolism and triglyceride regulation (37). SLC39A8\_rs13107325, associated with myocardial infarction, is known to influence vascular integrity, inflammatory signaling, and oxidative stress response pathways (38, 39). These results support the hypothesis that liver fibrosis and cardiovascular disease share common genetic and pathophysiological mechanisms.

The association between ANPEP\_rs7168849 and both all-cause and cancer-specific mortality suggests that this variant may influence processes beyond hepatic fibrosis, potentially via its known roles in immune

modulation, extracellular matrix remodeling, and angiogenesis (40–42). Although previous studies have highlighted altered ANPEP expression in various cancers (43–45), the specific impact of rs7168849 on cancer progression remains underexplored and warrants further investigation.

Interestingly, PNPLA3\_rs738409, despite being strongly associated with higher FIB-4 scores, was not linked to cardiovascular or oncological outcomes. This suggests that while PNPLA3 is a potent hepatic risk factor, it may exert limited systemic effects outside the liver, consistent with prior reports (46).

However, recent evidence from Mendelian randomization (MR) studies has challenged this view. In a large two-sample, two-step MR analysis, genetically proxied PNPLA3 inhibition was found to significantly increase the risk of several major cardiovascular diseases, including coronary atherosclerosis, coronary heart disease, and myocardial infarction (47). The absence of such associations in our study and given the inclusion of older adults (>70 years), may reflect survival bias since individuals with more deleterious genotypes may have developed complications earlier and were therefore not represented in this cohort. MR studies are typically conducted in larger, population-based cohorts, which offer greater statistical power and broader generalizability. In our cohort, individuals carrying the PNPLA3 risk allele who also had high cardiovascular risk may not have survived older age, potentially leading to an attenuation of the observed associations. As Smit and colleagues have elegantly described, genetic variants with strong effects at younger ages may appear to have diminished effects in older cohorts, not because biological pathways have changed, but because of selective survival [48]. In this context, the lack of association between PNPLA3 and cardiovascular endpoints in our study does not necessarily contradict previous findings but rather highlights the importance of age-related bias when interpreting genetic data in elderly populations.

These findings contribute to a growing body of evidence supporting liver fibrosis as a systemic disease marker, with shared genetic determinants that may drive both hepatic and cardiovascular pathology [49, 50]. From a clinical perspective, this raises the possibility that genetic profiling could help identify individuals at elevated risk for both advanced fibrosis and cardiovascular disease, enabling targeted prevention strategies.

This study has several strengths. It includes a large, well-characterized study, allowing for robust genetic association analyses. The integration of metabolic, genetic, and cardiovascular outcome data provides a comprehensive view of the interrelated nature of liver and vascular health. We acknowledge that a proportion of participants exceeded the recommended limits for alcohol consumption, raising the possibility of mixed metabolic-alcoholic liver disease (MetALD). While this overlap cannot be fully excluded, alcohol intake was included as a covariate in all adjusted analyses. The observed associations remained robust after adjustment, suggesting that moderate alcohol consumption did not substantially influence the reported genetic associations with liver fibrosis or cardiovascular outcomes.

However, several limitations must be acknowledged. First, the cohort may not be fully representative of more diverse populations, which may limit generalizability. Second, we relied on FIB-4 as a non-invasive fibrosis marker rather than histological confirmation. While FIB-4 is widely validated, it has not been specifically validated in elderly populations. Its use in this study was based on its broad applicability and availability within the PROSPER dataset. Biopsy data would have provided more definitive staging. Third, liver-related outcomes were not systematically recorded in the PROSPER trial and were therefore beyond the scope of the present study; cases of liver cancer, when present, were included in the cause-specific mortality analyses. Fourth, mechanistic insights into how specific SNPs influence fibrosis or cardiovascular risk remain speculative and require functional validation. Fifth, although we observed statistically significant differences in FIB-4 scores across several genotypic categories, the absolute FIB-4 values remained within a range typically associated with low risk of advanced liver fibrosis. Therefore, while the findings are relevant for understanding genetic influences on liver fibrosis risk, their immediate clinical implications may be limited.

Finally, it is important to note that our study focused on an elderly population, which may have influenced both the phenotypic expression of genetic variants and the prevalence of comorbid conditions. Age-related changes in liver function, body composition, and vascular health may interact with genetic risk in different ways. Future research should explore these associations in younger cohorts, where genetic predispositions may be more strongly linked to early metabolic changes or subclinical liver fibrosis. Studying younger individuals could help identify at-risk popu-

lations earlier in the disease course, potentially enabling more effective prevention strategies and timely interventions.

## CONCLUSION

In this analysis, we identified key demographic and genetic determinants of liver fibrosis, as reflected by FIB-4 scores, and demonstrated that several MASLD/MASH-associated genetic variants also influence cardiovascular and mortality outcomes. Our findings underscore the complex interplay between liver fibrosis, systemic metabolic health, and genetic predisposition. While variants such as PNPLA3\_rs738409 and SAMM50\_rs3761472 were associated with increased fibrosis risk, other SNPs (TRIB1\_rs2954021, SLC39A8\_rs13107325, and ANPEP\_rs7168849) exhibited potential protective or pleiotropic effects that extended beyond the liver to influence cardiovascular and oncological outcomes.

These results highlight the value of incorporating genetic information into the risk stratification of patients with liver fibrosis and support the hypothesis that fibrosis is not only a hepatic condition but also a marker of broader systemic vulnerability. Future research should aim to validate these associations in diverse cohorts, explore underlying mechanisms through functional studies, and assess the utility of genetic screening in guiding personalized management strategies for both liver and cardiovascular disease.

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## SUPPLEMENTARY MATERIAL

Supplementary table 1 – Association between the MASLD/MASH-associated SNPs and FIB-4 index across genotypic categories.

| SNP                     | WT/WT              | WT/Mutant          | Mutant/Mutant      | p-value          |
|-------------------------|--------------------|--------------------|--------------------|------------------|
| <b>MASLD &amp; MASH</b> |                    |                    |                    |                  |
| PNPLA3_rs738409         | <b>1.79 (0.64)</b> | <b>1.82 (0.68)</b> | <b>1.95 (0.76)</b> | <b>&lt;0.001</b> |
| HSD17B13_rs9992651      | 1.80 (0.66)        | 1.80 (0.69)        | 1.91 (0.71)        | 0.096            |
| HSD17B13_rs13118664     | 1.80 (0.65)        | 1.80 (0.68)        | 1.87 (0.69)        | 0.194            |
| GCKR_rs1260326          | 1.82 (0.69)        | 1.81 (0.66)        | 1.78 (0.62)        | 0.246            |
| LEPR_rs12077210         | 1.80 (0.66)        | 1.83 (0.67)        | 1.68 (0.44)        | 0.620            |
| SLC39A8_rs13107325      | <b>1.82 (0.67)</b> | <b>1.76 (0.64)</b> | <b>1.57 (0.44)</b> | <b>0.004</b>     |
| TMPRSS6_rs4820268       | 1.82 (0.66)        | 1.81 (0.67)        | 1.79 (0.65)        | 0.312            |
| <b>MASLD only</b>       |                    |                    |                    |                  |
| C2ORF16_rs1919127       | 1.81 (0.68)        | 1.81 (0.66)        | 1.74 (0.56)        | 0.214            |
| ZNF512_rs2068834        | 1.81 (0.68)        | 1.80 (0.66)        | 1.76 (0.57)        | 0.158            |
| PBX4_rs10500212         | 1.80 (0.66)        | 1.81 (0.65)        | 2.17 (0.94)        | 0.264            |
| PYGO1_rs11858624        | 1.81 (0.67)        | 1.78 (0.65)        | 2.12 (0.77)        | 0.610            |
| GCKR_rs780094           | 1.82 (0.69)        | 1.80 (0.66)        | 1.77 (0.62)        | 0.116            |
| SAMM50_rs3761472        | <b>1.80 (0.66)</b> | <b>1.81 (0.68)</b> | <b>1.96 (0.67)</b> | <b>0.042</b>     |
| SAMM50_rs2143571        | 1.80 (0.66)        | 1.80 (0.68)        | 1.92 (0.65)        | 0.360            |
| NCAN_rs2228603          | 1.80 (0.66)        | 1.83 (0.69)        | 2.07 (0.83)        | 0.083            |
| TRIB1_rs2954021         | <b>1.83 (0.68)</b> | <b>1.82 (0.66)</b> | <b>1.76 (0.65)</b> | <b>0.014</b>     |
| MBOAT7_rs641738         | 1.82 (0.67)        | 1.80 (0.65)        | 1.80 (0.68)        | 0.505            |
| TRIB1_rs2980888         | 1.82 (0.67)        | 1.82 (0.69)        | 1.79 (0.59)        | 0.638            |
| PPP1R3B_rs4240624       | 1.81 (0.67)        | 1.80 (0.64)        | 1.89 (0.63)        | 0.777            |
| AKNA_rs7041363          | 1.80 (0.64)        | 1.80 (0.67)        | 1.82 (0.68)        | 0.530            |
| ERLIN1_rs10883451       | 1.80 (0.64)        | 1.81 (0.68)        | 1.81 (0.66)        | 0.641            |
| TRIM5_rs11601507        | 1.80 (0.67)        | 1.82 (0.67)        | 2.02 (0.82)        | 0.585            |
| CCDC18_rs6541349        | 1.81 (0.67)        | 1.79 (0.67)        | 1.84 (0.68)        | 0.778            |
| SLC3OA10_rs12137855     | 1.82 (0.66)        | 1.79 (0.67)        | 1.79 (0.67)        | 0.170            |
| MTARC1_rs2642438        | 1.81 (0.68)        | 1.83 (0.68)        | 1.77 (0.64)        | 0.694            |
| MLXIPL_rs799165         | 1.80 (0.67)        | 1.79 (0.64)        | 1.78 (0.69)        | 0.572            |
| ANPEP_rs7168849         | <b>1.81 (0.67)</b> | <b>1.76 (0.65)</b> | <b>1.73 (0.57)</b> | <b>0.035</b>     |
| APOL3_rs132665          | 1.81 (0.68)        | 1.80 (0.64)        | 1.80 (0.61)        | 0.731            |
| MERTK_rs11683409        | 1.80 (0.67)        | 1.80 (0.66)        | 1.83 (0.65)        | 0.558            |
| MERTK_rs4374383         | 1.80 (0.67)        | 1.80 (0.66)        | 1.83 (0.65)        | 0.617            |

All data is presented as Mean (SD) FIB-4 risk.

Supplementary table 2 – Numbers of events per SNP.

| SNPs               | N total | Combined<br>Endpoint<br>N (%) events | Fatal/ non-fatal<br>Stroke/TIA<br>N (%) events | Fatal/nonfatal<br>MI<br>N (%) events | Heart failure<br>N (%) events | All-cause<br>mortality<br>N (%) events | Cancer<br>mortality<br>N (%) events |
|--------------------|---------|--------------------------------------|------------------------------------------------|--------------------------------------|-------------------------------|----------------------------------------|-------------------------------------|
| PNPLA3_rs738409    |         |                                      |                                                |                                      |                               |                                        |                                     |
| WT/WT              | 3332    | 507 (15.2)                           | 145 (4.4)                                      | 383 (11.5)                           | 136 (4.1)                     | 351 (10.5)                             | 121 (3.6)                           |
| WT/M               | 1616    | 245 (15.2)                           | 81 (5.0)                                       | 173 (10.7)                           | 64 (4.0)                      | 159 (9.8)                              | 50 (3.1)                            |
| M/M                | 224     | 29 (12.9)                            | 8 (3.6)                                        | 22 (9.8)                             | 8 (3.6)                       | 29 (12.9)                              | 10 (4.5)                            |
| SLC39A8_rs13107325 |         |                                      |                                                |                                      |                               |                                        |                                     |
| WT/WT              | 4421    | 658 (14.9)                           | 202 (4.6)                                      | 478 (10.8)                           | 172 (3.9)                     | 448 (10.1)                             | 147 (3.3)                           |
| WT/M               | 791     | 135 (17.1)                           | 37 (4.7)                                       | 109 (13.8)                           | 38 (4.8)                      | 97 (12.3)                              | 35 (4.4)                            |
| M/M                | 31      | 5 (16.1)                             | 2 (6.5)                                        | 3 (9.7)                              | 1 (3.2)                       | 3 (9.7)                                | 1 (3.2)                             |
| TRIB1_rs2954021    |         |                                      |                                                |                                      |                               |                                        |                                     |
| WT/WT              | 1348    | 199 (14.8)                           | 46 (3.4)                                       | 160 (11.9)                           | 52 (3.9)                      | 140 (10.4)                             | 45 (3.3)                            |
| WT/M               | 2600    | 393 (15.1)                           | 140 (5.4)                                      | 272 (10.5)                           | 102 (3.9)                     | 273 (10.5)                             | 88 (3.4)                            |
| M/M                | 1294    | 205 (15.8)                           | 54 (4.2)                                       | 158 (12.2)                           | 57 (4.4)                      | 135 (10.4)                             | 50 (3.9)                            |
| SAMD50_rs3761472   |         |                                      |                                                |                                      |                               |                                        |                                     |
| WT/WT              | 3895    | 585 (15.0)                           | 167 (4.3)                                      | 441 (11.3)                           | 161 (4.1)                     | 408 (10.5)                             | 137 (3.5)                           |
| WT/M               | 1219    | 197 (16.2)                           | 68 (5.6)                                       | 139 (11.4)                           | 48 (3.9)                      | 127 (10.4)                             | 42 (3.4)                            |
| M/M                | 125     | 13 (10.4)                            | 4 (3.2)                                        | 9 (7.2)                              | 2 (1.6)                       | 12 (9.6)                               | 4 (3.2)                             |
| ANPEP_rs7168849    |         |                                      |                                                |                                      |                               |                                        |                                     |
| WT/WT              | 4115    | 628 (15.3)                           | 190 (4.6)                                      | 462 (11.2)                           | 172 (4.2)                     | 408 (9.9)                              | 131 (3.2)                           |
| WT/M               | 736     | 110 (14.9)                           | 39 (5.3)                                       | 77 (10.5)                            | 25 (3.4)                      | 96 (13.0)                              | 38 (5.2)                            |
| M/M                | 37      | 5 (13.5)                             | 1 (2.7)                                        | 4 (10.8)                             | 0 (0)                         | 2 (5.4)                                | 2 (5.4)                             |



# Chapter 8

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## **Pravastatin reduces all-cause mortality in elderly at risk of liver fibrosis: post-hoc analysis of the PROSPER trial**

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## ABSTRACT

### Background & Aims

Metabolic dysfunction-associated steatotic liver disease (MASLD), and especially the progressive stages accompanied by liver fibrosis, are associated with liver-related and cardiovascular (CV) complications in middle-aged cohorts. We evaluated whether liver fibrosis is associated with increased mortality and cause-specific endpoints in an elderly population, and whether statin treatment could reduce these risks.

### Methods

PROSPER (PROspective Study of Pravastatin in the Elderly at Risk) was a double-blind randomized clinical trial comparing pravastatin to placebo in an elderly Caucasian population of 5,804 patients (> 70 years) at increased risk of cardiovascular disease. Endpoints were composite and single (CV) endpoints and all-cause mortality. The FIB-4 score was classified as: low risk of liver fibrosis ( $\text{FIB-4} < 2.0$ ), indeterminate risk ( $2.0 \leq \text{FIB-4} \leq 2.66$ ) and high risk ( $\text{FIB-4} \geq 2.67$ ). Time-to-event data was analysed using the Cox proportional hazards model.

### Results

Most participants were classified in the low FIB-4 class ( $n = 3919$ ), followed by the indeterminate ( $n = 1269$ ) and high classes ( $n = 561$ ). In the placebo group, the risk of all-cause mortality increased with a high FIB-4 classification: high class HR of 1.54 (95% CI: 1.10–2.17), compared to the low class (reference group). In the pravastatin group, the HR for all-cause mortality was not associated with FIB-4 classification: high class HR 1.01 (95% CI: 0.69–1.49). Interaction between FIB-4 class and treatment was significant ( $p\text{-value} = 0.049$ ). High FIB-4 classifications were not significantly associated with major adverse cardiovascular events (MACE) or other endpoints in both arms.

### Conclusions

A high FIB-4 classification was associated with increased all-cause mortality in elderly, pravastatin appears to mitigate this increased risk.

## INTRODUCTION

Metabolic dysfunction-associated steatotic liver disease (MASLD) can progress from hepatic steatosis to liver fibrosis and inflammation, and may ultimately result in liver cirrhosis, decompensation, or hepatocellular carcinoma (1). Given MASLD's close association with type 2 diabetes and obesity, its prevalence is also rapidly increasing. MASLD is currently estimated to affect 38% of the global population (2). The pathogenesis of MASLD is multifactorial with insulin resistance as a primary driver. In cohorts of middle-aged patients with biopsy-proven MASLD/MASH, the fibrosis stage is the most important predictor of adverse liver-related outcomes and mortality (3–5). Whether liver fibrosis is also associated with mortality in elderly populations is still unclear (6).

Recently, the cardiovascular implications of MASLD have received increased attention, as reflected in updated clinical guidelines (7,8) and adoption of the new nomenclature (9), wherein MASLD is defined as hepatic steatosis in the presence of cardiometabolic risk factors. MASLD has been linked to atherosclerotic cardiovascular disease (asCVD), heart failure, and an elevated risk of cardiovascular (CV) events in middle-aged patients (10–12). The most common causes of death in MASLD patients seem to be related to asCVD, not to the liver per se (4,7,13,14). This underscores the importance of addressing both hepatic and CV health in the management of MASLD patients.

Recently the first pharmacological treatment for MASLD was approved by the FDA (15). In Europe, the only available therapies are intensive lifestyle intervention and, in selected cases, bariatric surgery (16,17). The long-term success of lifestyle intervention remains low (18). Repurposing available drugs could provide immediate support and be more cost-effective. Hydroxymethylglutaryl-coenzyme A reductase inhibitors (statins) are a class of medication primarily used to lower LDL cholesterol levels and thereby reducing asCVD risk. It has been suggested that statins could have additional anti-inflammatory and anti-fibrotic effects (19–22). The current study utilized the *PROspective Study of Pravastatin in the Elderly at Risk* (PROSPER) trial data (23) to evaluate whether liver fibrosis is associated with increased mortality or cause-specific endpoints in elderly at elevated CV risk. Secondly, we will investigate if pravastatin treatment may reduce any increased risks.

## PATIENTS AND METHODS

### Study population

The PROspective Study of Pravastatin in the Elderly at Risk (PROSPER) was a randomised double-blind clinical trial investigating the effect of pravastatin in an elderly population with a history or increased risk of CVD (i.e., due to smoking, hypertension or diabetes). The detailed study set-up has been published previously (23,24) . Between 1997 and 1999, 5,804 persons aged 70–82 years from Scotland, Ireland and the Netherlands were included and randomized in a 1:1 ratio to receive either 40 mg pravastatin or matching placebo. The mean follow-up duration was 3.2 years, the study was completed in 2002.

### Endpoints

The composite endpoint of the trial was major adverse cardiovascular events (MACE), defined as definite or suspected coronary heart disease death, non-fatal myocardial infarction, and either fatal or non-fatal stroke. The other endpoints included in this analysis are: fatal/non-fatal stroke and transient ischaemic attack (TIA); fatal/non-fatal myocardial infarction (MI); heart failure; all-cause mortality; cancer mortality and CV mortality.

### FIB-4 score

The presence of liver fibrosis was estimated using the Fibrosis-4 Index (FIB-4) (25) . The FIB-4 algorithm is based on age, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and platelet count, and was calculated at baseline using the following formula:  $FIB-4 = [age (years) * AST (U/L)] / [platelet count (10^9/L) * \sqrt{ALT (U/L)}]$ . As the population was above 65 years of age, we used the stricter age-corrected lower cut-off value (2.0 instead of 1.3) as proposed by McPherson et al. (26) . The population was split into three classes based on the following cut-off values: rule-out advanced fibrosis ( $FIB-4 < 2.0$ ), indeterminate results ( $2.0 \leq FIB-4 \leq 2.66$ ) and rule-in advanced fibrosis ( $FIB-4 \geq 2.67$ ). Reports from the LITMUS and LiverScreen consortia indicate that FIB-4 has adequate prognostic abilities at population level (27,28) .

## Statistical analysis

Analyses were based on the intention-to-treat dataset. Population descriptive statistics are provided stratified by FIB-4 classification. Continuous data are presented as mean  $\pm$  SE, categorical data as count and proportion. The time-to-event data were analysed with Cox proportional hazards models according to FIB-4 classification, for the placebo and pravastatin groups separately. For each endpoint, either time to first occurrence of the event or study closure (censored observation) was taken, depending on which came first. The models were adjusted for sex, current smokers, BMI, diabetes and history of vascular disease. For each endpoint the hazard ratio (HR) and its 95% confidence interval were estimated. Data analysis was conducted using SPSS (version 29).

## RESULTS

The baseline FIB-4 score was calculated for 5,749 participants of the total intention-to-treat population of 5,804 participants, for 55 participants values to calculate FIB-4 were missing (patient flow visualized in Supplemental Figure 1). The mean ( $\pm$  SE) FIB-4 in the placebo group and treatment group was similar ( $1.80 \pm 0.01$  placebo vs.  $1.81 \pm 0.01$  treatment). Most participants were classified in the low FIB-4 class ( $n = 3,919$ ), followed by the indeterminate class ( $n = 1,269$ ) and fewest in the high FIB-4 class ( $n = 561$ ). In the lowest FIB-4 class, most participants were female, while in the indeterminate and high FIB-4 classes most were male. The mean age ( $\pm$  SE) of the classes ranged from  $74.9 (\pm 0.07)$  to  $76.4 (\pm 0.21)$ . Around 9–13% of the population had diabetes. A slightly increasing prevalence of a history of vascular disease was seen from the low (42%), to the indeterminate (47%) to the high FIB-4 (53%) classes ( $p < 0.001$ ). Regarding medication, use of metformin and insulin was minimal and did not differ between the classes. Use of ACE-/Angiotensin II-inhibitors did not differ significantly between the classes and arms. The baseline characteristics are summarized in Table 1.

Table 1: Baseline characteristics of the PROSPER population stratified by FIB-4 classification and treatment. Continuous data is presented as mean ( $\pm$  SE), categorical data as count (%).

|                                | Placebo      |                     |              | Pravastatin |              |                          |              |         |
|--------------------------------|--------------|---------------------|--------------|-------------|--------------|--------------------------|--------------|---------|
|                                | Low FIB-4    | Indeterminate FIB-4 | High FIB-4   | p-value     | Low FIB-4    | Indetermi-<br>nate FIB-4 | High FIB-4   | p-value |
| Participants (n)               | 1,975        | 636                 | 273          |             | 1,944        | 633                      | 288          |         |
| Characteristics                |              |                     |              |             |              |                          |              |         |
| FIB-4                          | 1.45 (0.01)  | 2.27 (0.01)         | 3.23 (0.04)  | NA          | 1.45 (0.01)  | 2.28 (0.01)              | 3.18 (0.03)  | NA      |
| Age (years)                    | 74.9 (0.07)  | 76.0 (0.13)         | 76.4 (0.21)  | NA          | 75.1 (0.07)  | 75.9 (0.13)              | 76.0 (0.20)  | NA      |
| Females (n, %)                 | 1115 (56.5)  | 271 (42.6)          | 106 (38.8)   | <0.001      | 1088 (56.0)  | 286 (45.2)               | 105 (36.5)   | <0.001  |
| BMI (kg/m²)                    | 26.9 (0.10)  | 26.7 (0.16)         | 26.2 (0.27)  | 0.007       | 27.0 (0.09)  | 26.6 (0.16)              | 26.1 (0.24)  | <0.001  |
| Current Smokers (n, %)         | 581 (29.3)   | 146 (23.0)          | 70 (25.6)    | 0.010       | 540 (27.8)   | 151 (23.9)               | 58 (20.1)    | 0.002   |
| Systolic Blood pressure (mmHg) | 154 (0.5)    | 156 (0.9)           | 156 (1.3)    | 0.081       | 154 (0.5)    | 155 (0.9)                | 156 (1.4)    | 0.203   |
| (Fasting) clinical values      |              |                     |              |             |              |                          |              |         |
| Glucose (mmol/L)               | 5.48 (0.03)  | 5.44 (0.06)         | 5.49 (0.10)  | 0.771       | 5.49 (0.03)  | 5.35 (0.05)              | 5.39 (0.09)  | 0.061   |
| Insulin (mIU/L)                | 10.6 (0.26)  | 9.6 (0.40)          | 9.8 (0.46)   | 0.070       | 10.5 (0.24)  | 10.1 (0.43)              | 10.5 (0.73)  | 0.660   |
| HOMA-IR                        | 2.64 (0.07)  | 2.45 (0.13)         | 2.53 (0.15)  | 0.262       | 2.64 (0.07)  | 2.47 (0.11)              | 2.59 (0.19)  | 0.409   |
| Triglycerides (mmol/L)         | 1.57 (0.02)  | 1.50 (0.03)         | 1.35 (0.04)  | <0.001      | 1.58 (0.02)  | 1.52 (0.03)              | 1.38 (0.04)  | <0.001  |
| HDL cholesterol (mmol/L)       | 1.27 (0.01)  | 1.28 (0.01)         | 1.31 (0.02)  | 0.112       | 1.28 (0.01)  | 1.29 (0.02)              | 1.32 (0.02)  | 0.163   |
| LDL cholesterol (mmol/L)       | 3.82 (0.02)  | 3.73 (0.03)         | 3.63 (0.05)  | <0.001      | 3.82 (0.02)  | 3.76 (0.03)              | 3.73 (0.05)  | 0.029   |
| Creatinine (Umol/L)            | 100.3 (0.50) | 102.8 (0.91)        | 101.3 (1.24) | 0.067       | 101.0 (0.51) | 102.1 (0.89)             | 102.5 (1.23) | 0.165   |

Table 1: Baseline characteristics of the PROSPER population stratified by FIB-4 classification and treatment. Continuous data is presented as mean ( $\pm$  SE), categorical data as count (%). (continued)

|                                                    | Placebo      |              |              | Pravastatin |              |              |              |       |
|----------------------------------------------------|--------------|--------------|--------------|-------------|--------------|--------------|--------------|-------|
| AST (U/L)                                          | 22.9 (0.16)  | 27.9 (0.31)  | 33.4 (0.86)  | NA          | 22.6 (0.16)  | 27.6 (0.33)  | 34.6 (0.80)  | NA    |
| ALT (U/L)                                          | 23.6 (0.24)  | 23.3 (0.47)  | 23.7 (1.03)  | NA          | 23.0 (0.22)  | 22.6 (0.45)  | 26.2 (1.08)  | NA    |
| Platelets (10 <sup>9</sup> /L)                     | 253.8 (1.22) | 199.6 (1.41) | 171.4 (2.02) | NA          | 254.4 (1.33) | 199.1 (1.44) | 170.0 (1.97) | NA    |
| Medical history                                    |              |              |              |             |              |              |              |       |
| Diabetes (n, %)                                    | 217 (11.0)   | 64 (10.1)    | 34 (12.5)    | 0.792       | 210 (10.8)   | 56 (8.8)     | 31 (10.8)    | 0.501 |
| Vascular disease (n, %)                            | 811 (41.1)   | 291 (45.8)   | 146 (53.5)   | <0.001      | 841 (43.3)   | 302 (47.7)   | 150 (52.1)   | 0.002 |
| Medication use                                     |              |              |              |             |              |              |              |       |
| Ace-inhibitors / angio-tensin II inhibitors (n, %) | 334 (16.9)   | 123 (19.3)   | 56 (20.5)    | 0.067       | 357 (18.4)   | 117 (18.5)   | 69 (24.0)    | 0.065 |
| Metformin (n, %)                                   | 125 (6.3)    | 35 (5.5)     | 20 (7.3)     | 0.886       | 125 (6.4)    | 28 (4.4)     | 19 (6.6)     | 0.438 |
| Insulin (n, %)                                     | 22 (1.1)     | 6 (0.9)      | 2 (0.7)      | 0.521       | 14 (0.7)     | 4 (0.6)      | 3 (1.0)      | 0.709 |

FIB-4 classes: low FIB-4 <2.0; indeterminate FIB-4 2.0–2.66; high FIB-4  $\geq$  2.67. Numerical variables compared using ANOVA, categorical using Chi-square test. p-values <0.05 were considered statistically significant. NA = testing not applicable, as these variables are part of FIB-4 algorithm.

A high FIB-4 classification was not significantly associated with the composite endpoint MACE, see Table 2. Regarding the single endpoints, the risk of all-cause mortality was increased with higher FIB-4 classification in the placebo group (see Figure 1). In the placebo group, the high FIB-4 class a HR of 1.54 (95% CI 1.10–2.17) for all-cause mortality, as compared to the low class (reference). However, in the pravastatin group the hazard ratios did not increase with a higher FIB-4 classification. In the pravastatin group, the high class a HR of 1.01 (0.69–1.49) for all-cause mortality, as compared to the low class (reference). The interaction term between FIB-4 class and statin treatment was significant ( $p = 0.049$ ), indicating that the effect of treatment depended on FIB-4 classification (or vice versa). A higher FIB-4 classification was not associated with any of the other single endpoints studied: fatal/non-fatal stroke/TIA; fatal/nonfatal MI; heart failure; cancer mortality (See Table 2).

Table 2: Hazard ratios (HR) and corresponding 95% CI for each endpoint for each FIB-4 class in the placebo arm and the treatment arm. Low FIB-4 is the reference class (HR set to 1). The Cox proportional hazards models were adjusted for sex, smoking, BMI, diabetes and history of vascular disease. \*95% confidence interval corresponding to  $\alpha=0.05$ , does not overlap with 1.

|                                   | Placebo       |                        |                      | Pravastatin   |                        |                     |
|-----------------------------------|---------------|------------------------|----------------------|---------------|------------------------|---------------------|
| FIB-4 class                       | Low<br>N=1975 | Indeterminate<br>N=636 | High<br>N=273        | Low<br>N=1944 | Indeterminate<br>N=633 | High<br>N=288       |
| MACE                              | 1.0           | 0.98<br>(0.79–1.22)    | 0.82<br>(0.59–1.14)  | 1.0           | 1.03<br>(0.81–1.31)    | 0.98<br>(0.70–1.37) |
| <b>Single endpoints</b>           |               |                        |                      |               |                        |                     |
| Fatal/<br>non-fatal<br>Stroke/TIA | 1.0           | 1.20<br>(0.79–1.81)    | 1.15<br>(0.65–2.04)  | 1.0           | 0.98<br>(0.64–1.52)    | 1.34<br>(0.79–2.26) |
| Fatal/<br>nonfatal MI             | 1.0           | 0.92<br>(0.71–1.19)    | 0.72<br>(0.48–1.06)  | 1.0           | 1.04<br>(0.78–1.38)    | 0.90<br>(0.60–1.36) |
| Heart<br>failure                  | 1.0           | 0.99<br>(0.64–1.52)    | 0.77<br>(0.40–1.49)  | 1.0           | 0.89<br>(0.55–1.44)    | 1.26<br>(0.71–2.24) |
| All-cause<br>mortality            | 1.0           | 1.28<br>(0.97–1.68)    | 1.54<br>(1.10–2.17)* | 1.0           | 0.97<br>(0.73–1.29)    | 1.01<br>(0.69–1.49) |
| Cancer<br>Incidence               | 1.0           | 1.12<br>(0.80–1.57)    | 1.14<br>(0.72–1.82)  | 1.0           | 0.87<br>(0.63–1.20)    | 0.93<br>(0.60–1.43) |
| Cancer<br>mortality               | 1.0           | 1.16<br>(0.70–1.91)    | 1.15<br>(0.57–2.33)  | 1.0           | 0.85<br>(0.53–1.36)    | 0.95<br>(0.50–1.79) |
| CV<br>mortality                   | 1.0           | 1.39<br>(0.97–2.00)    | 1.11<br>(0.65–1.89)  | 1.0           | 1.08<br>(0.71–1.64)    | 1.15<br>(0.66–2.00) |

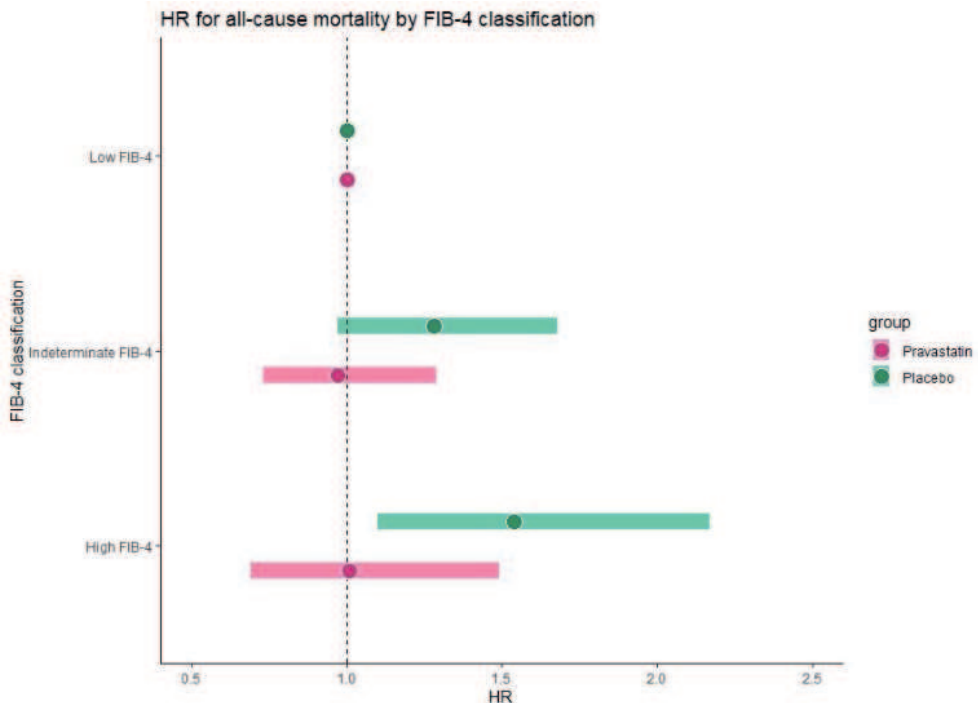


Figure 1: Hazard ratio (dot) and 95% CI (bar) for all-cause mortality for each FIB-4 classification compared to the reference class of low FIB-4 (HR set at 1) for the placebo and treatment group. The Cox proportional hazards model was adjusted for sex, smoking, BMI, diabetes and history of vascular disease. \*95% confidence interval corresponding to  $\alpha=0.05$ , does not overlap with 1.

## DISCUSSION

In the current analysis of the PROSPER trial data a high FIB-4 classification was associated with increased all-cause mortality in elderly in the placebo arm. Pravastatin treatment appears to abolish this risk.

The most studied prognostic outcome in relation to FIB-4 is all-cause mortality. Systematic reviews and meta-analyses indicate that FIB-4 can accurately predict all-cause mortality (27,29). A individual participant data meta-analysis showed that FIB-4 is as good as histologic fibrosis staging to predict a composite endpoint of all-cause mortality and liver-related outcomes (mean age 54 years) (30). In our elderly population, a high FIB-4 classification ( $\geq 2.67$ ) was also associated with an increased risk of all-cause mortality in the placebo arm. In contrast, an elderly general population cohort showed that both liver steatosis and liver fi-

brosis were not associated with increased mortality (mean age 74 years, median follow-up almost 7 years) (31). While age and BMI were relatively similar between both studies, the prevalence of diabetes was higher in the general population cohort, but the presence of CV disease was higher in our study. Also the assessment of fibrosis differed between the studies (FIB-4 vs. transient elastography). A registry-based study of Taiwanese heart failure patients (mean age of 75 years) did again show that FIB-4 was predictive of mortality in elderly (32), although different FIB-4 cut-offs were used than in the current analysis.

Several studies describe the prognostic relation between FIB-4 and CVD, although the results are less clear as for mortality. In a large meta-analysis, FIB-4 was not associated with CV events, although the number of events was relatively low (78 events in  $n=2518$ , 3%) (30). In contrast, when using electronic health record data of patients with obesity and type 2 diabetes (6002 CV events in  $n=44,481$ , 13%) a higher FIB-4 classification was predictive of CV events (mean age 59 years) (33). In the NHANES database a high FIB-4 ( $\geq 2.67$ ) was also predictive of CV events (mean age 53 years) (34). In our elderly population, we did not find any association with MACE or any of the other CV endpoints. The power of our post-hoc analysis might have been too low to detect differences in other single endpoints, although a relatively high number of combined CV events was observed for MACE (870 events in  $n=5749$ , 15%). However, the prospective relation between FIB-4 and all-cause mortality might be stronger than for combined and single CV endpoints.

Statins are well-known for their cholesterol-lowering effects. Lowering LDL-cholesterol might protect patients who are at increased risk of CV complications due to liver fibrosis, regardless of any potential effect on the fibrosis itself. Statins were associated with a decrease in mortality in MASLD patients in the NHANES database (22). Our study confirms these retrospective NHANES findings, as we show that treatment with statins decreased all-cause mortality in PROSPER.

Statins might have additional anti-inflammatory and anti-fibrotic effects (35). Preclinical data has demonstrated a beneficial effect of statins on diet-induced MASH (36,37) and fibrosis (37). Only two small-sized RCTs on statins in MASLD have been performed (38,39) which were both classified as having a high risk of bias in the only Cochrane review to-date (40), and therefore no definite conclusion was drawn. As no large-scale trials with adequate follow-up are available, the clinical evidence

of statins in MASLD is limited to cohort data and post-hoc analyses of RCTs. Statins were associated with protection against MASH and fibrosis in a cross-sectional study (41). Recently, two large meta-analyses reported that statin use was associated with a lower risk of MASH and fibrosis (19,20). Statins are considered safe in MASLD patients (42), however elderly with liver fibrosis might be at a slightly increased risk of liver toxicity. In the current analysis potential adverse effects did not compensate for the survival benefit. Still, in an elderly population close monitoring of those with pre-existing liver conditions, multi-morbidity and polypharmacy remains important.

The prevalence of MASLD is higher in patients with type 2 diabetes as compared to the general population (43), although this might be mainly driven by hepatic steatosis and insulin resistance. In the PROSPER trial the prevalence of type 2 diabetes was around 9–13% and did not differ within the different fibrosis classifications.

To appreciate these findings, some aspects of this study need to be addressed. The strength of the current study is the use of a large dataset from a RCT with an adequate follow-up time (4 years). The elderly study population at risk of CVD is comparable to patients that would be considered for statin treatment in practice. The main limitation of this study was the lack of direct liver assessment by either imaging or biopsy, and FIB-4 was selected as proxy for liver fibrosis. The FIB-4 has been validated as diagnostic marker for advanced fibrosis on a population level, as reflected in available guidelines (44). However, the diagnostic accuracy of FIB-4 in specific subpopulations like type 2 diabetes patients is lower. The accuracy of its use as prognostic marker is less studied, although recent data show that FIB-4 has discriminative power in patients with type 2 diabetes (33). Additionally, FIB-4 is mostly validated (as diagnostic marker) in populations under 65 years old. The FIB-4 algorithm includes age and thereby likely overestimates the presence of fibrosis in elderly participants. Therefore, in this analysis, we used age-specific cut-offs as proposed by McPherson et al (26) and adopted in the 2024 clinical practice guideline (8). The PROSPER trial compared pravastatin to placebo, while other types of statin (i.e., simvastatin and atorvastatin) are more commonly prescribed. As the mechanism of action of statins is fairly similar, we expect the results to be generalizable to other statins as well. A high level of high intra-individual variation was observed in this study, possibly the response to statin treatment could differ based on underlying genotype or metabolic profile (41).

Non-randomized studies have indicated potential benefits of statin use in middle-aged MASLD patients (19,22,41), but no consensus or standard recommendation has been reached. Our analysis of the PROSPER RCT suggests that the beneficial effects of statins also extend to an elderly population with a high risk of liver fibrosis. In conclusion, our findings obtained in a large population of elderly participant at elevated CV risk support the view that increased risks of fibrosis lead to increased risks of all-cause mortality. Moreover, the results of a randomized comparison suggest that pravastatin treatment, or possibly statin treatment in general, may mitigate these risks.

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# Chapter 9

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## **Risk of cardiovascular disease in elderly subjects with obesity and liver fibrosis and the potential benefit of statin treatment**

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## ABSTRACT

### Background

Liver fibrosis progression is influenced by older age and cardiometabolic risk factors such as obesity, and is associated with an increased risk of cardiovascular events. While statins may protect against cardiovascular complications, their effects in elderly individuals with obesity and liver fibrosis are unclear.

### Method

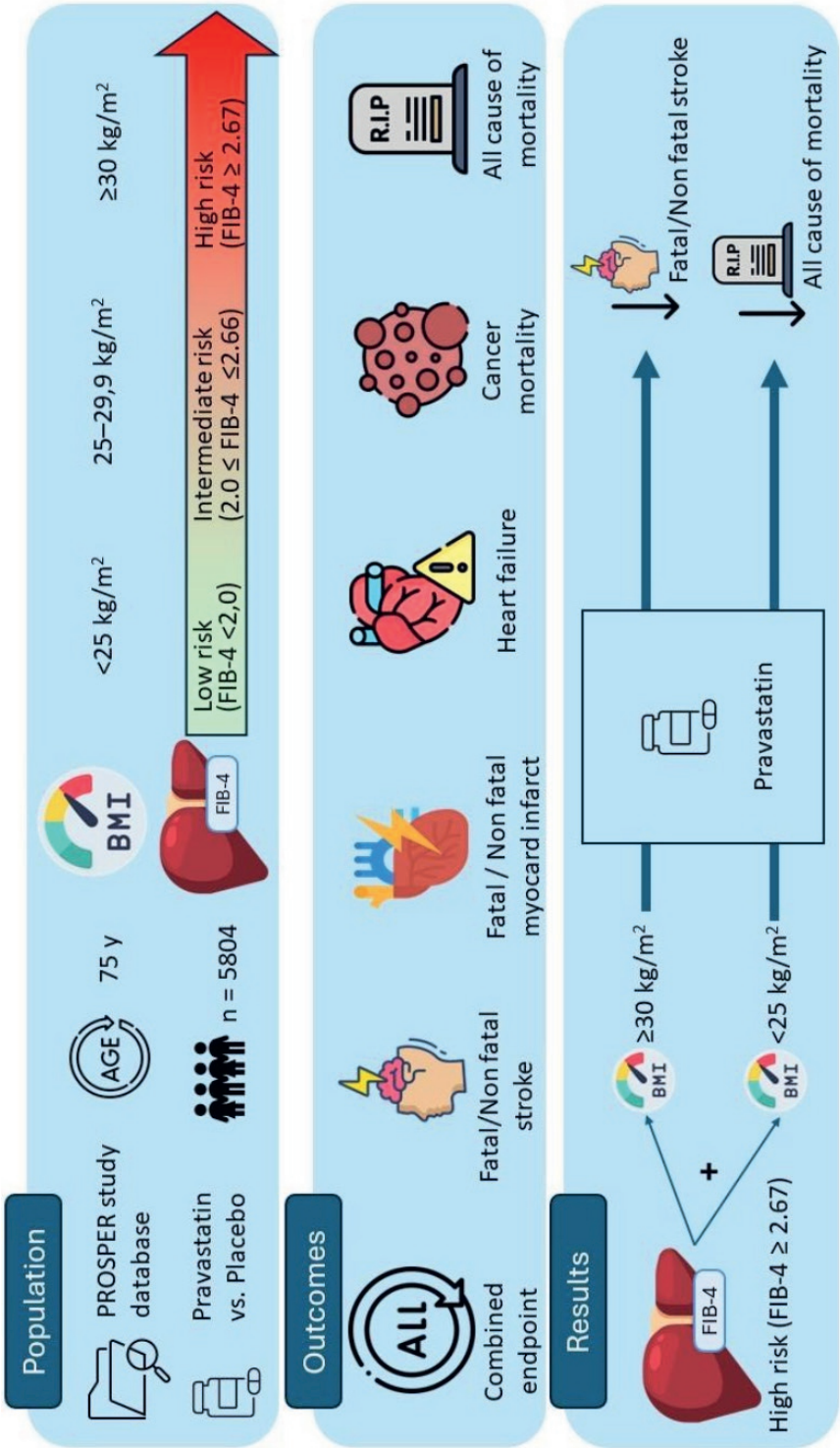
The PROspective Study of Pravastatin in the Elderly at Risk (PROSPER) database was used to evaluate the effect of pravastatin on major adverse cardiovascular events in an elderly population (>70 years). Subjects were categorized by BMI: lean (<25kg/m<sup>2</sup>), overweight (25–29,9kg/m<sup>2</sup>), and obese (≥30kg/m<sup>2</sup>). Liver fibrosis was assessed using the FIB-4 index: low risk (<2,0), intermediate risk (2,0–2,66), and high risk (≥2,67). Time-to-event data were analyzed using the Cox proportional hazards model, adjusted for confounders, and compared the placebo and pravastatin groups.

### Results

A total of 5.804 subjects were included. In the placebo group, the highest risk group (high FIB-4 and obesity) had a significantly higher hazard ratio for (non-)fatal stroke (HR 2.74; 95%CI 1.19–6.29) compared to the low FIB-4, lean BMI group. This risk disappeared in the same pravastatin group. Pravastatin did not affect other cardiovascular endpoints. All-cause mortality was significantly higher in subjects with lean weight and high FIB-4 on placebo (HR 1.88; 95%CI 1.14–3.11), but not on pravastatin (HR 0.58; 95%CI 0.28–1.20).

### Conclusion

Elderly individuals with obesity and liver fibrosis are at higher risk for (non-)fatal stroke, which is reduced with pravastatin. Pravastatin also potentially lowers all-cause mortality in subjects with lean weight and liver fibrosis.



Graphical abstract of the study

## INTRODUCTION

The global prevalence of obesity is on the rise, with predictions estimating up to 2 billion cases by 2035 [1]. Obesity is a key factor in the development of cardiovascular disease and chronic liver conditions. Both obesity and older age accelerate liver fibrosis progression [2], potentially leading to cirrhosis, liver failure, or hepatocellular carcinoma [3]. Individuals with liver fibrosis may also face extrahepatic complications, including kidney disease, cardiovascular disease, heart failure, or colorectal cancer [4–8]. Obesity and liver fibrosis are strongly linked to an increased risk of cardiovascular events, one of the leading causes of death.

The severity of liver fibrosis is a critical determinant of liver-related outcomes and overall mortality [9–14]. To estimate liver fibrosis non-invasively, several tests have been developed, reducing the need for liver biopsies. In line with this, guidelines from the European Association for the Study of the Liver (EASL), the European Association for the Study of Diabetes (EASD), and the European Association for the Study of Obesity (EASO) recommend using simple and cost-effective tools like the Fibrosis-4 index (FIB-4) to identify individuals at high risk for advanced fibrosis or cirrhosis [15]. This can be followed by imaging techniques or more advanced serum-based tests.

Currently, only one specific drug is approved in the U.S. for treating steatotic liver with fibrosis [16]. Some evidence suggests that lipid-lowering drugs, such as statins, may reduce both hepatic and extrahepatic complications [17–19]. Hydroxymethylglutaryl-coenzyme A reductase inhibitors (statins) therapy has been shown to lower cardiovascular risk in individuals with obesity [20], similarly to those without obesity [21, 22]. It may also help reduce inflammation and liver fibrosis [23–26]. While the impact of BMI and liver fibrosis on cardiovascular complications has been studied, there is limited information on their role in elderly populations regarding these risks [27–30].

This study used the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER) trial [31] to analyze the association between cardiovascular outcomes and cancer mortality across different BMI and FIB-4 categories. It will evaluate the effect of pravastatin on clinical outcomes in relation to BMI and FIB-4 combinations. It remains unclear whether statin treatment produces varying results in patients with different stages of liver fibrosis.

## SUBJECTS AND METHODS

### Study population

The PROspective Study of Pravastatin in the Elderly at Risk (PROSPER) database was used for this study. Details on PROSPER have been published in detail elsewhere [31, 32]. The study evaluated the benefits of 40 mg pravastatin treatment in elderly individuals (aged 70 to 82) at risk of vascular disease in Scotland, Ireland, and the Netherlands. Randomization was conducted in a 1:1 ratio with placebo, using a computerized pseudorandom number generator with balanced blocks of size four. The study was completed in 2002, with a mean follow-up of 3.2 years (range: 2.8–4.0). The inclusion and exclusion criteria are provided in Supplementary Material 1.

### Endpoints

The combined effect of death from coronary heart disease, non-fatal myocardial infarction (MI), and fatal and non-fatal stroke was the primary endpoint. The distinct coronary and cerebral vascular components of the primary objective were the secondary endpoints. The medical ethics committees of each participating institution gave their approval to the study protocol. Every study participant provided written, informed consent. The 1975 Declaration of Helsinki's ethical principles were followed by the study protocol.

### Stratification

The study group was divided into three subgroups based on their body mass index (BMI): lean (BMI <25 kg/m<sup>2</sup>), overweight (BMI 25–29.9 kg/m<sup>2</sup>) and obese (BMI ≥30 kg/m<sup>2</sup>).

The FIB-4 was calculated for all patients using aspartate transaminase (AST), alanine transaminase (ALT) and blood platelets count. We used specific cutoff values adapted for elderly subjects as described by McPherson et al. [33]: low risk of advanced fibrosis (FIB-4 < 2.0), intermediate risk (2.0 ≤ FIB-4 ≤ 2.66) and high risk (FIB-4 ≥ 2.67).

### Statistical analysis

IBM® SPSS® Statistics software was used to analyze the data. Continuous data are presented as mean ± standard deviation (SD) when normally distributed, median ± interquartile range (IQR) when not normally distributed and as count and proportion for categorical ones. The descriptive characteristics of the population were defined for each BMI subgroup

first and then according to the FIB-4. One-way ANOVA test was used for comparisons between subgroups.

First, we assessed the effect of either BMI or FIB-4 by calculating the hazard ratio across different groups for the following outcomes: fatal/non-fatal stroke/transient ischemic attack, fatal/non-fatal myocardial infarction, heart failure, all-cause mortality, cancer mortality, and combined endpoint. Next, we repeated the analysis in combined groups of BMI and FIB-4 to search for interaction effects. Finally, stratified analyses were performed to investigate the effect of pravastatin in several BMI-FIB-4 groups. Statistical significance was reached when  $p < 0.05$  (two-sided).

A Cox regression analysis was performed and adjusted for gender, alcohol use, LDL cholesterol, and the use of antihypertensive and diabetic medication across the various BMI and FIB-4 combinations. The cohort characterized by a lean BMI and a low FIB-4 served as the baseline for comparison.

## RESULTS

### Baseline characteristics

In total 5804 subjects were included in this analysis. The baseline characteristics are presented for each BMI group in Table 1. Participants with obesity were mostly female (61.9%) and they had more often diabetes and hypertension. Overall, the FIB-4 was negatively associated to BMI.

### Clinical endpoints in BMI and FIB-4 groups.

In order to analyze the association between cardiovascular outcomes and cancer mortality across different BMI and FIB-4 groups, we computed the hazard ratios for each subgroup. The reference points for comparison were established using the cohorts exhibiting low BMI or low FIB-4. The analysis was adjusted for gender, alcohol use, LDL cholesterol, and the use of antihypertensive medication and diabetic medication. No significant differences concerning the clinical endpoints was found across the different groups as shown in Table 2.

Table 1 – Baseline general characteristics of the subjects of the PROSPER study in relation to BMI

|                                                   | All (n=5804) | Lean<br>(n=1975) | Overweight<br>(n=2640) | Obese<br>(n=1189) | p-value <sup>b</sup> | p-value over –<br>weight vs lean | p-value obese vs lean |
|---------------------------------------------------|--------------|------------------|------------------------|-------------------|----------------------|----------------------------------|-----------------------|
| Age (years)                                       | 75.0 ± 5.5   | 75.4 ± 5.5       | 74.9 ± 5.4             | 74.8 ± 5.4        | 0.017                | 0.008                            | 0.004                 |
| Females, n (%)                                    | 3000 (51.7)  | 1021 (51.7)      | 1243 (47.1)            | 736 (61.9)        | <0.001               | 0.002                            | <0.001                |
| FIB-4                                             | 1.80 ± 0.66  | 1.83 ± 0.68      | 1.81 ± 0.67            | 1.74 ± 0.61       | <0.001               | 0.227                            | <0.001                |
| BMI (kg/m <sup>2</sup> )                          | 26.83 ± 4.20 | 22.60 ± 1.92     | 27.26 ± 2.85           | 32.93 ± 2.85      | NA                   | NA                               | NA                    |
| Current Smokers n (%)                             | 1558 (6.8)   | 776 (39.3)       | 610 (23.1)             | 172 (14.5)        | <0.001               | <0.001                           | <0.001                |
| Alcohol (units per week)                          | 5.21 ± 9.29  | 5.42 ± 9.12      | 5.61 ± 9.97            | 3.99 ± 7.82       | <0.001               | 0.510                            | <0.001                |
| Diabetes n (%)                                    | 623 (10.7)   | 143 (7.2)        | 305 (11.6)             | 175 (14.7)        | <0.001               | <0.001                           | <0.001                |
| Hypertension n (%)                                | 3592 (61.9)  | 1014 (51.3)      | 1679 (63.6)            | 899 (75.6)        | <0.001               | <0.001                           | <0.001                |
| History of vascular disease <sup>a</sup><br>n (%) | 2565 (44.2)  | 864 (43.7)       | 1226 (46.4)            | 475 (39.9)        | 0.129                | 0.069                            | 0.036                 |
| Systolic Blood Pressure<br>(mmHg)                 | 154.7 ± 21.8 | 152.4 ± 22.4     | 155.2 ± 21.6           | 157.3 ± 21.0      | <0.001               | <0.001                           | <0.001                |
| Diastolic Blood pressure<br>(mmHg)                | 83.8 ± 11.5  | 81.8 ± 11.2      | 84.3 ± 11.2            | 85.9 ± 11.9       | <0.001               | <0.001                           | <0.001                |
| LDL cholesterol (mmol/L)                          | 3.79 ± 0.80  | 3.77 ± 0.81      | 3.82 ± 0.80            | 3.77 ± 0.78       | 0.511                | 0.023                            | 0.807                 |
| HDL cholesterol (mmol/L)                          | 1.28 ± 0.35  | 1.40 ± 0.38      | 1.23 ± 0.32            | 1.19 ± 0.29       | <0.001               | <0.001                           | <0.001                |
| Total Cholesterol (mmol/L)                        | 5.68 ± 0.91  | 5.67 ± 0.90      | 5.69 ± 0.92            | 5.68 ± 0.94       | 0.852                | 0.528                            | 0.942                 |
| Triglycerides (mmol/L)                            | 1.54 ± 0.70  | 1.31 ± 0.56      | 1.60 ± 0.72            | 1.79 ± 0.77       | <0.001               | <0.001                           | <0.001                |

Table 1 – Baseline general characteristics of the subjects of the PROSPER study in relation to BMI (continued)

|                                       | All (n=5804) | Lean<br>(n=1975) | Overweight<br>(n=2640) | Obese<br>(n=1189) | p-value <sup>b</sup> | p-value over-<br>weight vs lean | p-value obese vs lean |
|---------------------------------------|--------------|------------------|------------------------|-------------------|----------------------|---------------------------------|-----------------------|
| Diabetes medication n (%)             | 402 (6.9)    | 90 (4.6)         | 203 (7.7)              | 109 (9.2)         | <0.001               | <0.001                          | <0.001                |
| Antihypertensive medica-<br>tion n(%) | 4285 (73.8)  | 1270 (64.3)      | 2008 (76.1)            | 1007 (84.7)       | <0.001               | <0.001                          | <0.001                |

Dara are presented as number (percentage), mean ±SD or median ±IQR.

<sup>a</sup> Any of: stable angina, intermittent claudication, stroke, transient ischemic attack, myocardial infarction, peripheral artery disease surgery, or amputation for vascular disease more than 6 months before study entry.

<sup>b</sup> p value represents difference between the lean, overweight and obese subgroups.

NA: Not applicable

Table 2 - Hazard ratio for each BMI and FIB-4 group for an event to occur.

| Total Group              | Lean<br>(n=1975) | Overweight<br>(n=2640) | Obese<br>(n=1189) | Low FIB-4<br>(n=3919) | Middle FIB-4<br>(n=1269) | High FIB-4<br>(n=560) |
|--------------------------|------------------|------------------------|-------------------|-----------------------|--------------------------|-----------------------|
| Combined Endpoint        | 1.0 (ref)        | 0.94 (0.81-1.09)       | 1.01 (0.84-1.22)  | 1.0 (ref)             | 0.99 (0.84-1.17)         | 0.91 (0.72-1.15)      |
| Fatal / non-fatal stroke | 1.0 (ref)        | 0.81 (0.62-1.06)       | 0.81 (0.57-1.14)  | 1.0 (ref)             | 1.08 (0.80-1.45)         | 1.27 (0.86-1.86)      |
| Fatal / Non-fatal MI     | 1.0 (ref)        | 1.01 (0.84-1.21)       | 1.10 (0.89-1.37)  | 1.0 (ref)             | 0.96 (0.80-1.16)         | 0.81 (0.61-1.08)      |
| Heart failure            | 1.0 (ref)        | 1.05 (0.77-1.42)       | 1.27 (0.89-1.81)  | 1.0 (ref)             | 0.93 (0.68-1.28)         | 1.01 (0.66-1.56)      |
| All-cause mortality      | 1.0 (ref)        | 0.83 (0.69-0.99)       | 0.83 (0.66-1.04)  | 1.0 (ref)             | 1.06 (0.87-1.29)         | 1.22 (0.95-1.58)      |
| Cancer mortality         | 1.0 (ref)        | 1.14 (0.84-1.55)       | 0.93 (0.62-1.41)  | 1.0 (ref)             | 0.91 (0.65-1.28)         | 0.96 (0.60-1.54)      |
| Cancer incidence         | 1.0 (ref)        | 0.94 (0.76-1.16)       | 0.94 (0.72-1.23)  | 1.0 (ref)             | 0.95 (0.75-1.20)         | 0.99 (0.72-1.35)      |

Analysis were conducted using Cox regression and adjusted for gender, alcohol use, LDL cholesterol, and the use of antihypertensive medication and diabetic medication. Data are presented as hazard ratio (95% confidence interval).

## Clinical endpoints in relation to BMI and FIB-4 combinations

Table 3 shows the associations between the BMI and FIB-4 combined categories and the clinical outcomes. The group exhibiting a lean weight and a low FIB-4 was used as reference. The same correction as in Table 2 had been made. There were no significant difference between these groups.

## Effect of pravastatin on clinical endpoints in different BMI and FIB-4 group combinations

When we stratified for the use of placebo or pravastatin, it appears that pravastatin may have a protective role in two situations.

First, the hazard ratio for a (non-) fatal stroke was significantly higher (HR 2.74; 95% CI 1.19–6.29) in elderly subjects with obesity and liver fibrosis on placebo. In the group on pravastatin, no increased risk was present for that group (HR 0.93; 95% CI 0.22–3.91). Pravastatin did not affect other cardiovascular endpoints in elderly subjects with obesity and liver fibrosis.

Secondly, the hazard ratio for all-cause mortality was also significantly higher (HR 1.88; 95% CI 1.14–3.11) in subjects with a lean weight and a high FIB-4 on placebo. This risk disappears in the same group on pravastatin (HR 0.58; 95% CI 0.28–1.20).

Lastly, the hazard ratio for heart failure is significantly increased (HR 3.76; 95% CI 1.26–11.27) in patients with obesity and a high FIB-4 on pravastatin. The confidence interval is abnormally wide, raising questions about the reliability of this result. The results are presented in Table 4.

Table 3 - Hazard ratio for each BMI and FIB-4 group combination for an event to occur.

| BMI                      | Lean          |                     |                     | Overweight          |                     |                     | Obese               |                     |                     |
|--------------------------|---------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|
|                          | Low<br>N=1296 | Medium<br>N=458     | High<br>N=211       | Low<br>N=1765       | Medium<br>N=584     | High<br>N=261       | Low<br>N=858        | Medium<br>N=227     | High<br>N=88        |
| FIB-4 risk               |               |                     |                     |                     |                     |                     |                     |                     |                     |
| Combined Endpoint        | 1.0 (ref)     | 0.94<br>(0.71-1.24) | 0.99<br>(0.68-1.43) | 0.92<br>(0.76-1.11) | 0.99<br>(0.77-1.27) | 0.64<br>(0.43-0.95) | 0.99<br>(0.79-1.24) | 0.88<br>(0.61-1.29) | 1.31<br>(0.81-2.10) |
| Fatal / non-fatal stroke | 1.0 (ref)     | 0.76<br>(0.46-1.27) | 1.13<br>(0.63-2.05) | 0.77<br>(0.55-1.06) | 0.86<br>(0.55-1.34) | 0.63<br>(0.31-1.26) | 0.57<br>(0.37-0.90) | 1.08<br>(0.59-1.96) | 1.88<br>(0.93-3.79) |
| Fatal / Non-fatal MI     | 1.0 (ref)     | 1.07<br>(0.78-1.48) | 1.02<br>(0.65-1.59) | 1.04<br>(0.83-1.30) | 1.08<br>(0.81-1.44) | 0.68<br>(0.43-1.08) | 1.23<br>(0.95-1.59) | 0.81<br>(0.50-1.29) | 1.09<br>(0.59-2.03) |
| Heart failure            | 1.0 (ref)     | 0.82<br>(0.45-1.49) | 1.29<br>(0.65-2.56) | 1.05<br>(0.72-1.52) | 1.10<br>(0.68-1.79) | 0.84<br>(0.41-1.73) | 1.28<br>(0.84-1.95) | 1.14<br>(0.57-2.26) | 1.43<br>(0.56-3.61) |
| All-cause mortality      | 1.0 (ref)     | 1.05<br>(0.77-1.43) | 1.15<br>(0.77-1.72) | 0.82<br>(0.66-1.03) | 0.92<br>(0.69-1.24) | 0.92<br>(0.62-1.38) | 0.82<br>(0.62-1.09) | 0.71<br>(0.44-1.16) | 1.37<br>(0.79-2.39) |
| Cancer mortality         | 1.0 (ref)     | 0.95<br>(0.54-1.67) | 0.89<br>(0.40-1.96) | 1.14<br>(0.78-1.65) | 0.96<br>(0.57-1.63) | 1.31<br>(0.69-2.49) | 0.93<br>(0.57-1.51) | 0.96<br>(0.43-2.14) | 0.35<br>(0.05-2.58) |
| Cancer incidence         | 1.0 (ref)     | 0.97<br>(0.67-1.41) | 0.86<br>(0.51-1.45) | 0.93<br>(0.72-1.20) | 0.84<br>(0.58-1.20) | 1.02<br>(0.65-1.62) | 0.90<br>(0.65-1.25) | 0.93<br>(0.55-1.58) | 0.86<br>(0.37-1.96) |

Analysis were conducted using Cox regression and adjusted for gender, alcohol use, LDL cholesterol, and the use of antihypertensive medication and diabetic medication. Data are presented as hazard ratio (95% confidence interval).

Table 4 – Hazard ratio for each BMI and FIB-4 group for an event to occur.

| BMI<br>FIB-4 index        | Treatment   | Lean      |                     |                                  | Overweight          |                     |                     | Obese               |                     |                                  |
|---------------------------|-------------|-----------|---------------------|----------------------------------|---------------------|---------------------|---------------------|---------------------|---------------------|----------------------------------|
|                           |             | Low       | Medium              | High                             | Low                 | Medium              | High                | Low                 | Medium              | High                             |
| Combined<br>Endpoint      | Placebo     | 1.0 (ref) | 1.03<br>(0.71-1.50) | 0.92<br>(0.54-1.55)              | 1.07<br>(0.83-1.38) | 1.10<br>(0.78-1.55) | 0.69<br>(0.39-1.23) | 0.99<br>(0.72-1.35) | 0.75<br>(0.43-1.31) | 1.39<br>(0.76-2.53)              |
|                           | Pravastatin |           | 0.80<br>(0.53-1.21) | 1.07<br>(0.64-1.79)              | 0.76<br>(0.57-1.00) | 0.91<br>(0.63-1.31) | 0.62<br>(0.36-1.05) | 0.97<br>(0.71-1.34) | 1.03<br>(0.62-1.71) | 1.16<br>(0.53-2.51)              |
| Fatal/Non<br>fatal stroke | Placebo     | 1.0 (ref) | 1.03<br>(0.53-2.01) | 0.73<br>(0.26-2.05)              | 0.78<br>(0.48-1.27) | 1.00<br>(0.54-1.86) | 0.53<br>(0.16-1.73) | 0.69<br>(0.37-1.28) | 0.89<br>(0.35-2.30) | 2.74<br>(1.19-6.29) <sup>a</sup> |
|                           | Pravastatin |           | 0.51<br>(0.23-1.14) | 1.50<br>(0.73-3.12)              | 0.73<br>(0.47-1.15) | 0.73<br>(0.38-1.41) | 0.68<br>(0.28-1.62) | 0.47<br>(0.24-0.90) | 1.22<br>(0.57-2.63) | 0.93<br>(0.22-3.91)              |
| Fatal/Non<br>fatal MI     | Placebo     | 1.0 (ref) | 1.02<br>(0.66-1.59) | 0.96<br>(0.52-1.77)              | 1.17<br>(0.87-1.58) | 1.14<br>(0.77-1.68) | 0.73<br>(0.38-1.43) | 1.08<br>(0.76-1.55) | 0.72<br>(0.37-1.40) | 0.94<br>(0.41-2.16)              |
|                           | Pravastatin |           | 1.08<br>(0.67-1.74) | 1.09<br>(0.57-2.08)              | 0.87<br>(0.62-1.22) | 1.05<br>(0.68-1.62) | 0.66<br>(0.35-1.26) | 1.37<br>(0.94-1.98) | 0.91<br>(0.46-1.78) | 1.30<br>(0.52-3.26)              |
| Heart failure             | Placebo     | 1.0 (ref) | 0.75<br>(0.34-1.65) | 1.70<br>(0.77-3.73)              | 0.82<br>(0.49-1.35) | 0.92<br>(0.47-1.78) | 0.18<br>(0.02-1.32) | 1.08<br>(0.61-1.90) | 1.27<br>(0.55-2.94) | 0.39<br>(0.05-2.88)              |
|                           | Pravastatin |           | 0.90<br>(0.36-2.28) | 0.66<br>(0.15-2.82)              | 1.40<br>(0.80-2.47) | 1.40<br>(0.68-2.88) | 1.69<br>(0.73-3.93) | 1.58<br>(0.83-3.10) | 0.88<br>(0.26-2.98) | 3.76<br>(1.26-11.27)             |
| All cause of<br>mortality | Placebo     | 1.0 (ref) | 1.39<br>(0.91-2.12) | 1.88<br>(1.14-3.11) <sup>b</sup> | 0.96<br>(0.69-1.33) | 1.16<br>(0.77-1.76) | 1.10<br>(0.60-2.01) | 0.84<br>(0.56-1.25) | 0.60<br>(0.28-1.32) | 1.42<br>(0.68-2.99)              |
|                           | Pravastatin |           | 0.78<br>(0.49-1.24) | 0.58<br>(0.28-1.20)              | 0.70<br>(0.51-0.96) | 0.75<br>(0.49-1.15) | 0.78<br>(0.46-1.35) | 0.80<br>(0.55-1.17) | 0.82<br>(0.43-1.54) | 1.29<br>(0.56-2.99)              |
| Cancer<br>Mortality       | Placebo     | 1.0 (ref) | 1.24<br>(0.54-2.84) | 1.65<br>(0.61-4.43)              | 1.20<br>(0.66-2.17) | 1.25<br>(0.58-2.70) | 1.33<br>(0.45-3.97) | 1.28<br>(0.64-2.54) | 1.06<br>(0.31-3.60) | NA                               |
|                           | Pravastatin |           | 0.77<br>(0.35-1.68) | 0.41<br>(0.10-1.72)              | 1.08<br>(0.67-1.72) | 0.78<br>(0.38-1.62) | 1.20<br>(0.54-2.66) | 0.66<br>(0.33-1.34) | 0.93<br>(0.32-2.67) | 0.69<br>(0.09-5.12)              |
| Cancer inci-<br>dence     | Placebo     | 1.0 (ref) | 1.12<br>(0.65-1.93) | 1.14<br>(0.60-2.34)              | 0.96<br>(0.65-1.44) | 1.06<br>(0.63-1.77) | 1.02<br>(0.48-2.18) | 1.08<br>(0.67-1.73) | 1.03<br>(0.46-2.29) | 1.17<br>(0.42-3.28)              |
|                           | Pravastatin |           | 0.88<br>(0.53-1.45) | 0.65<br>(0.30-1.42)              | 0.89<br>(0.64-1.25) | 0.67<br>(0.40-1.11) | 0.97<br>(0.54-1.71) | 0.76<br>(0.49-1.19) | 0.89<br>(0.44-1.80) | 0.54<br>(0.13-2.20)              |

Analysis were conducted using Cox regression and adjusted for gender, alcohol use, LDL cholesterol, and the use of antihyper-tensive medication and diabetic medication. Data are presented as hazard ratio (95% confidence interval).

<sup>a</sup>  $p=0.018$ ; <sup>b</sup>  $p=0.014$

## DISCUSSION

In this study, which investigated the association between cardiovascular outcomes and cancer mortality across various BMI and FIB-4 groups, a significant difference in the risk of (non-) fatal stroke was observed in patients with obesity and a high FIB-4. This risk was mitigated by treatment with pravastatin. These findings underscore the potential contribution of statins in reducing the risk of (non-) fatal stroke within this particular subset of the elderly population. Previous research has highlighted the positive impact of lipid-lowering drugs in patients with obesity [17–22]. Indeed, statin treatment can prevent and improve liver steatosis, inflammation and fibrosis [34–36] and in one study even reduced cardiovascular morbidity [37]. We observed an elevated hazard ratio for mortality in the lean group with a high FIB-4, which does not support the findings of Van Kleef et al., who found no association between liver fibrosis and mortality in an elderly cohort [38]. Moreover, two studies involving younger participants (mean ages 59 and 53 years) demonstrated that a high FIB-4 was associated with cardiovascular events [39, 40]. Similarly, in our study, a high FIB-4 was linked to an increased risk of stroke in individuals with obesity.

No significant differences in other clinical endpoints were observed, particularly in cancer mortality. Unfortunately, the data only pertains to cancer mortality prevalence, as the PROSPER study did not collect information on new cancer diagnoses. This may be due to the relatively short follow-up period, which also does not account for variations in BMI and FIB-4 over time. A longer follow-up could provide more clarity on the role of statin therapy and the influence of factors like BMI and FIB-4 on cardiovascular outcomes and cancer mortality or diagnosis.

Interestingly, an inverse relationship between BMI and FIB-4 was observed, suggesting a lower risk of hepatic fibrosis in elderly patients with higher BMI. These findings contrast with previously published data which showed a positive association between BMI and liver fibrosis [41, 42] and the contribution of obesity and liver fibrosis to cardiovascular diseases [43, 44]. These observations may be explained by “survivorship bias”. Therefore, prospective long term studies are necessary in participants with obesity included at a younger age in order to determine the impact on these clinical outcomes.

Certain aspects of this study need to be commented upon. The analyses primarily rely on the FIB-4, a frequently utilized non-invasive test for assessing the likelihood of liver fibrosis [45]. It is important to note that the FIB-4 was originally developed for patients with HIV/HCV infection and an average age of 40 [46]. To address this issue and improve the accuracy of liver fibrosis estimation, we used age-specific values as recommended in international guidelines for screening [15, 33]. FIB-4 is known to be less accurate in patients with diabetes, but no correction exists to compensate this default. Other non-invasive tests, such as vibration-controlled transient elastography or magnetic resonance elastography may detect more reliably subjects with liver fibrosis [47, 48]. Ideally, a liver biopsy would have been the preferred method to diagnose and stage liver fibrosis.

Another limitation of the study was the lack of information about the presence of sarcopenia or abdominal adiposity. We identified a lean BMI group without the guarantee that individuals were healthy in terms of fat distribution or proportion. Indeed, sarcopenia may be an important determinant of morbidity and mortality in the elderly [49]. Moreover, abdominal obesity, as a marker of visceral fat, mediates the majority of cardiometabolic risk and may have influenced the outcomes in our cohort [50].

While in PROSPER pravastatin was the investigational drug, other statins (simvastatin or pravastatin) are more frequently prescribed. We expect similar findings with other statins given the similar mechanisms of action. It is not yet known whether other lipid-lowering interventions, such as cholesterol absorption inhibitors, bempedoic acid, or PCSK9 inhibitors, will show similar results.

## CONCLUSION

In conclusion, the findings of this study, conducted in a cohort of elderly individuals with a randomized comparison between placebo and pravastatin, support the view that subjects with a combination of increased BMI and liver fibrosis are at an increased risk of (non-)fatal stroke. This risk is reduced by pravastatin. Additionally, statin treatment may reduce all-cause mortality in a subgroup of elderly subjects with a lean body weight and liver fibrosis.

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# PART IV

## Discussion and Summary



# Chapter 10

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## General Discussion



## MASLD IDENTIFICATION

Metabolic dysfunction-associated steatotic liver disease (MASLD), previously known as non-alcoholic fatty liver disease (NAFLD), is the most prevalent liver-associated condition. Liver fat accumulation is the first step in the generation of the disease, and insulin resistance plays a prominent role in its pathophysiology. Therefore, it is not surprising that the highest prevalence is found in people with type 2 diabetes mellitus (T2DM) or overweight/obesity. The current thesis aimed to evaluate metabolic, genetic and cardiovascular aspects of MASLD, especially in people living with overweight or obesity. **Chapter 1** provides a comprehensive overview of its prevalence, risk factors, and clinical importance across different populations, with particular emphasis on patients with severe obesity and cardiometabolic disease. This chapter shows that MASLD has serious long-term consequences beyond the liver. Important in this respect is intrahepatic inflammation. This will eventually lead to the development of metabolic dysfunction-associated steatohepatitis (MASH), liver fibrosis, cirrhosis, and hepatocellular carcinoma. **Chapter 1** also shows that MASLD is independently associated with increased cardiovascular risk, insulin resistance, T2DM, and other components of the metabolic syndrome [1].

To establish the severity of MASLD, liver biopsy has been considered the gold standard. Until recently, a liver biopsy was also mandatory in clinical trials, evaluating therapies to treat MASLD. However, given the limitations of liver biopsy, non-invasive tests have become increasingly important in MASLD evaluation. It is also well recognized that liver biopsy is an invasive procedure with potential complications. In addition, sampling error limits the usefulness of this procedure and this has prompted the widespread use of other techniques such as serum biomarker panels (e.g., FIB-4, NAFLD Fibrosis Score, ELF test) and imaging-based modalities such as transient elastography and magnetic resonance elastography and MRI-PDFF. The use of imaging techniques in complementing biomarker-based scoring systems for improved diagnosis is also addressed in **chapter 1**.

**Chapter 2** investigates the prevalence of MASLD/MASH in a Dutch cohort scheduled for bariatric surgery (the Franciscus Obesity NASH Study, FONS) with various biomarker-based scoring systems and vibration-controlled transient elastography. The prevalence of MASLD was 50.6% and 9.2% for fibrosis, but the prevalence of MASH and at-

risk MASH were relatively low (5.3% and 2.6%, respectively), illustrating that severe disease may be less common than anticipated based on BMI alone. Capped MAF-5 outperformed both FIB-4 and transient elastography in detecting significant fibrosis, highlighting the value of selecting non-invasive tests (NITs) tailored to bariatric populations. These findings were somewhat unexpected, as previous studies in bariatric cohorts have reported higher rates of steatohepatitis with stage 2 fibrosis or higher, ranging from 22% to 51% [2–4]. One possible explanation may lie in differences in study design, inclusion criteria of the FONS protocol, which could have led to the selection of a relatively healthier subgroup or an underestimation of disease severity. Indeed, the FONS study included patients who met the local selection criteria for bariatric intervention, whereas other groups screened a broader population of patients suffering from severe obesity. Moreover, liver biopsy was only performed based on vibration controlled transient elastography liver stiffness measurements (LSM) as a surrogate for liver fibrosis. A new study should perhaps place greater emphasis on standardizing inclusion criteria and utilizing multiple complementary diagnostic techniques, such as detailed imaging and several serum biomarkers, to provide a more comprehensive view of disease prevalence and severity. This would also align better with the multiple-tier screening approach as recommended in the most recent guidelines.

There is a clear scientific need for further validation of NITs. Moreover, the development of more refined, patient-tailored diagnostic strategies is essential. This would not only improve the accuracy of MASLD/MASH diagnoses but also help identify high-risk patients for developing advanced liver diseases, such as cirrhosis and hepatocellular carcinoma, which is crucial for timely intervention. Newly designed liquid biopsy techniques with for example extracellular exosome determination, may be helpful and lead to a better identification of patients at different stages of MASLD/MASH.

NITs have well known limitations in individuals with severe obesity, due to factors such as increased liver stiffness measurement failure rates and altered aminotransferase kinetics. The capped MAF-5 showed to be the most accurate and appeared to be the preferred test for ruling out significant fibrosis in this setting. However, the modest diagnostic yield and the low prevalence of advanced fibrosis raise the question whether systematic screening for MASLD/MASH in bariatric candidates is warranted. Current results suggest that universal screening may not be cost-

effective, but targeted assessment in patients with metabolic comorbidities or abnormal liver tests could be justified. Given that the diagnostic performance of NITs varies across populations, further studies are needed to refine and optimize risk stratification models. This includes determining widely applicable cut-offs for NITs, integrating additional metabolic and genetic risk factors, and validating combined algorithms that may improve the identification of patients at risk for advanced fibrosis. Such optimization could help balance the need for accurate detection against the burden and cost of unnecessary diagnostic procedures, ultimately guiding more efficient screening strategies in bariatric populations. **Chapter 3** supports this notion by confirming the value of transient elastography for stratifying liver disease severity, particularly in outpatient settings where invasive procedures are not feasible.

The findings from **chapter 3** show that MASLD, when investigated in a cardiovascular outpatient setting, is more common among patients with T2DM compared to both non-diabetic individuals and to those with type 1 diabetes mellitus. If we want to reduce the long-term impact of MASLD, it is essential to integrate MASLD screening into routine cardiometabolic risk assessments and increase awareness of MASLD among healthcare providers. Public health policy and clinical care pathways need to put emphasis on early diagnosis in at-risk populations, including obesity, T2DM, and metabolic syndrome. Preselection of subjects at risk is needed. Given the long-term effects and ongoing research, diagnostic guidelines and algorithms should be updated regularly to ensure timely intervention, prevent liver complications, and improve cardiometabolic health [5].

This thesis evaluates the potential of NITs for MASLD screening, but several methodological constraints should be noted. The diagnostic accuracy of NITs is population-specific and can be influenced by factors such as obesity, systemic inflammation, and age. At the start of this study, we expected NITs to show a promising accuracy for detecting MASLD across various patient populations, based on existing literature [6–11]. However, the findings indicate that while NITs are useful, their performance varies significantly depending on the patient's clinical profile.

A major consideration for future studies is the need for tailored selection criteria, which can better capture the right patients for MASLD screening. One recent development for improving MASLD screening accuracy is the integration of artificial intelligence (AI) tools to refine diagnostic

algorithms. AI can analyze complex, multi-dimensional data, such as patient demographics, clinical history, ethnicity, blood samples, comorbidities (e.g. diabetes, hypertension), and imaging, enabling the identification of at-risk subgroups that may be overlooked using traditional methods. By considering these diverse factors, AI can see the “bigger picture” and detect patterns that might be missed when focusing on one patient group alone.

AI-driven models could enhance personalized screening strategies by prioritizing high-risk patients and recommending the most appropriate non-invasive tests, such as transient elastography or MRI-PDFF. This approach would lead to more accurate risk stratification, improving early detection of advanced liver disease, and optimizing treatment. However, further research and validation are needed to ensure the effectiveness and safety of AI-based tools in clinical practice.

Moreover, future studies should aim to validate a combination of NITs, possibly integrating imaging-based techniques like transient elastography or magnetic resonance elastography with serum biomarkers such as the MAF-5 score.

In summary, while NITs have great potential, further research is needed to optimize patient selection, to establish comprehensive diagnostic algorithms, and to assess their clinical applicability in different risk populations. The ultimate goal should be to develop a robust, scalable screening pathway that integrates MASLD and liver fibrosis risk evaluation into routine cardiometabolic risk assessments, enabling early identification and intervention in high-risk patients. Efforts are currently underway such as the Global Research Initiative for Patient Screening on MASH (GRIPonMASH, IHI no. 101132946) to improve such pathways, with ongoing studies working to refine these strategies and enhance their practical implementation in clinical settings.

## MASLD AND CARDIOVASCULAR RISK

Besides T2DM, MASLD and its primary driver, abdominal obesity, share similar pathophysiological mechanisms, including insulin resistance, chronic inflammation, and dyslipidemia. Insulin resistance not only maintains hepatic steatosis through increased lipogenesis and impaired lipid metabolism but also enhances systemic metabolic dysfunc-

tion, promoting development of both MASLD and cardiovascular disease (CVD) [12]. In addition, atherogenic dyslipidemia, as has been suggested to occur also in MASLD, characterized by elevated triglycerides, reduced high-density lipoprotein (HDL) cholesterol, and raised small dense low-density lipoprotein (LDL) particles, enhances the progression of atherosclerosis, leading to increased risk for cardiovascular events such as myocardial infarction and stroke.

Liver fibrosis is associated with CVD risk independently of the usual determinants such as obesity, hypertension, and hyperlipidemia [13, 14]. The progression from simple steatosis to advanced fibrosis is accompanied by enhanced inflammatory and fibrogenic activity involving mediators such as the transforming growth factor beta (TGF- $\beta$ ) and platelet-derived growth factor (PDGF), which may contribute to vascular injury, cardiac remodeling, and a pro-atherogenic lipid profile [15]. However, the direction of causality remains uncertain, as systemic metabolic dysfunction and inflammation could both drive hepatic fibrosis and promote cardiovascular disease through shared pathophysiological pathways. Hence, assessment of the severity of fibrosis is crucial for the prediction of both liver-related complications as well as long-term cardiovascular risk.

**Chapters 4 and 5** reinforce the strong and bidirectional relationship between MASLD and cardiovascular risk, emphasizing that MASLD is not a singular hepatic disease but a multisystem disorder with serious metabolic and cardiovascular consequences. Individuals with MASLD have an elevated risk of CVD [16, 17], both with or without advanced liver fibrosis, which confirms that isolated hepatic steatosis contributes to systemic metabolic dysfunction.

As the prevalence of obesity increases across the globe, MASLD will increase accordingly, causing further burden to the already strained healthcare systems [18, 19]. The trend specifies an urgent demand for screening, prevention, and treatment measures. Although lifestyle modification, such as dietary improvements and physical activity remain the cornerstone of MASLD management, newer pharmacotherapies like glucagon-like peptide-1 receptor agonists (GLP-1 RAs, e.g., semaglutide), SGLT2 inhibitors, FXR agonists and multireceptor agonists (e.g. GLP-1/GIP receptor agonists) hold promise in the management of both hepatic and cardiovascular complications [20, 21]. Semaglutide has already re-

ceived accelerated approval by the FDA and is currently under evaluation by the EMA for treatment of fibrotic MASH.

Beyond these metabolic and fibrotic determinants, genetic factors also play a role in determining cardiovascular risk in patients with MASLD, as demonstrated in our study of elderly individuals with obesity. **Chapter 7** addresses this genetic predisposition in an elderly population with similar genetic West-European background (PROSPER cohort), highlighting polymorphisms such as PNPLA3, TM6SF2, and HSD17B13, which influence both the severity of MASLD and susceptibility to cardiovascular complications. Notably, variants of TM6SF2 increase hepatic fat accumulation, but paradoxically polymorphisms of this gene have been associated with reduced circulating LDL cholesterol. The identification of such genetic risk markers may enhance screening strategies by allowing earlier detection of individuals predisposed to hepatic fat accumulation despite normal serum lipid profiles.

Moreover, integrating genetic information with metabolic risk factors could support individualized prevention and treatment approaches, for example, by tailoring lifestyle interventions or pharmacotherapy to those genetically at higher risk for both cardiovascular and liver disease. Although the observed associations between MASLD and cardiovascular outcomes are consistent with previous literature, it is important to note the observational design of the included cohorts. Additionally, potential residual confounding factors, such as medication use, diet, and physical activity, cannot be ruled out. Despite MASLD's growing prevalence, healthcare professional awareness remains suboptimal, contributing to delayed diagnosis, underuse of non-invasive liver tests, and missed early intervention opportunities [22]. To address this, it is essential to implement targeted educational initiatives for healthcare professionals, emphasizing the importance of early screening and the use of non-invasive liver tests. These initiatives should start with general practitioners (GPs), as they are often the first point of contact for patients. Primary care physicians play a key role in early case finding, metabolic risk assessment, lifestyle counseling, and follow-up. Therefore, FIB-4 was the first tool recommended for screening [5, 23], but there is still ongoing discussion about its use, particularly for elderly patients and those with obesity and T2DM, where its performance may be suboptimal. Additionally, the high false negative rate of FIB-4 in real-world cohorts highlights the need for caution when using it in broader populations [24]. Attention should also be given to newer screening methods, such as the MAF-5 or

the LiverPRO, which may offer improved diagnostic accuracy and help fill the gaps left by FIB-4 [25, 26]. Additionally, integrating MASLD assessment into routine cardiovascular risk evaluations and developing standardized screening protocols could significantly improve early detection and intervention.

Current practice is limited by the lack of MASLD-specific registries and by inconsistent inclusion in clinical guidelines, thus limiting systematic identification and management of individuals at risk. Including MASLD assessment in current cardiovascular and obesity guidelines may improve early detection, facilitate risk stratification, and support integrated management in clinical practice. Early detection may promote lifestyle changes by increasing awareness and the sense of urgency among patients, as seen in some studies, but the long-term sustainability of these improvements remains uncertain [27]. Additionally, real-world implementation of screening programs depends heavily on local health system structures, with large-scale screening requiring increased capacity, funding, and personnel. While cost-effectiveness has not yet been proven, current models suggest that screening only high-risk individuals for advanced fibrosis may be worthwhile [28–30]. However, these findings may not translate to European healthcare systems, where MASLD-specific treatments are limited to some countries.

## MASLD MANAGEMENT

Change of nomenclature from NAFLD/NASH to MASLD/MASH places the emphasis on the metabolic etiology of the disease, with obesity and metabolic dysfunction as key drivers [5]. The traditional BMI-based criteria do not measure true metabolic risk since patients who have a normal BMI but also have high visceral adiposity progress to MASLD and its complications [31].

Independent of the definition of overweight or obesity, weight loss in patients with obesity, whether achieved through lifestyle modification, drug therapy, or surgery, has been reported to improve metabolic health and mitigate MASLD severity [32]. Improvement of histology is achieved through at least 7–10% reduction in body weight [33]. Currently, MASLD's therapeutic setting is rapidly evolving and GLP-1 RAs and bariatric surgery have shown improvements in metabolic and liver

parameters including improvement of the long-term histologic and cardiovascular endpoints [34–36].

Bariatric surgery remains among the most powerful treatments for long-term weight loss in severe obesity improving insulin sensitivity, reducing systemic inflammation, and resolving MASLD in the majority of patients. However, long-term maintenance of these improvements and risk of recrudescence need to be studied as a recent study showed that the median life expectancy in bariatric patients is still 5.5 years shorter than in the general population [37]. **Chapter 6** describes the study protocol of FONS, aiming to investigate long-term changes in frequency and severity of MASLD/MASH among patients undergoing bariatric surgery.

While bariatric surgery remains a cornerstone of treatment for MASLD and severe obesity, its application will likely alter with increasing availability of effective anti-obesity medications (AOMs) [38]. With pharmacological management such as GLP-1 RAs and dual or triple incretin agonists also showing promise, the future of MASLD and obesity treatment is likely to shift. Interestingly, recent evidence has shown that these drugs not only induce weight loss but also have therapeutic effects on the liver, largely mediated through the reduction in hepatic fat and inflammation maybe as a result of weight loss. This can slow or even reverse fibrosis in MASLD patients [39]. However, it is important to note, as discussed at the AASLD meeting in 2025, that these effects are primarily linked to the weight loss itself, rather than being direct liver-specific actions, in contrast to drugs like resmetirom, which target liver-specific mechanisms [40–42].

As described earlier for the identification and risk stratification of MASLD/MASH, prevention and treatment modalities in the future must adopt an integrated multidisciplinary approach including hepatology, cardiology, and internal medicine/endocrinology. Hepatologist establish fibrosis staging and complex disease management; cardiologists treat existing CVD and optimize cardiovascular risk factor management, while internists/endocrinologists manage complex metabolic disease like diabetes, as well as (complex forms of) obesity.

According to revised (inter)national guidelines, treatment of obesity must start by screening underlying etiologies and comorbidities (e.g. lifestyle, psychosocial causes, endocrine, medication-related and other medical and genetic causes) [43][44, 45]. Recently an innovative online screen-

ing- and monitoring tool has been developed: [www.checkcausesobesity.com](http://www.checkcausesobesity.com) ( in Dutch: [www.checkoorzakenovergewicht.nl](http://www.checkoorzakenovergewicht.nl)). This integrated strategy ensures identification of underlying causes and contributing factors, which facilitates determining the optimal approach for an individual such as referral to lifestyle intervention, psychosocial treatment, optimization of weight-promoting medications, and where appropriate, additional pharmacotherapy with newer AOMs, and metabolic surgery. New evidence has proven that these interventions, in addition to obesity pharmacotherapy and metabolic surgery not only cause clinically relevant weight reduction but also have indirect hepatic and cardiovascular effects by improving insulin sensitivity and reducing adverse cardiovascular event rates[46–49].

Guidelines emphasize the importance of early intervention, better risk assessment, and using advances in lifestyle and medication. This helps clinicians address both MASLD and cardiovascular disease, improving patient outcomes and reducing long-term healthcare costs. AOMs can also be employed as a non-surgical alternative to bariatric surgery in carefully selected patients with reduced risk and shorter recovery periods for therapeutic options. In certain situations, a dual approach consisting of the integration of AOMs and bariatric surgery might enhance patient outcomes, requires further investigation [50]. Upcoming strategies will likely involve a patient-specific approach, integrating lifestyle and psychosocial therapy, surgical and pharmacologic treatment options to tailor treatment based on individual patient profiles, severity of disease, and response to medical treatment. Even as the management of successful MASLD is most important in preventing complications such as CVD, there is mounting evidence that certain therapies used first in cardiovascular health will also play a role in MASLD treatment [51].

This confluence of therapy presents new opportunities for a broader patient population, most particularly those at the interface of liver and metabolic disease.

Indeed, **Chapters 8 and 9**, which are based on data from the PROSPER (PROspective Study of Pravastatin in the Elderly at Risk) trial, further delineate the association between liver fibrosis and cardiovascular disease in an elderly population. **Chapter 8** showed that in elderly patients participating in this double-blind study including 5,804 subjects aged over 70 years at increased cardiovascular risk, elevated FIB-4, a marker for liver fibrosis, was associated with a 54% higher risk of all-cause mor-

tality compared with those in the low FIB-4 category (HR 1.54; 95% CI 1.10–2.17). Notably, this excess risk was abolished in participants treated with pravastatin (HR 1.01; 95% CI 0.69–1.49), suggesting that statin therapy may counteract the adverse prognostic impact of liver fibrosis in the elderly.

**Chapter 9** demonstrates that elderly individuals with both obesity and elevated FIB-4 scores had a markedly increased risk of (non-)fatal stroke, with a hazard ratio of 2.74 (95% CI 1.19–6.29) compared with lean individuals with low FIB-4 values. This excess risk was not observed among participants treated with pravastatin, indicating that statin therapy may mitigate the cardiovascular risk associated with coexisting obesity and liver fibrosis. Moreover, pravastatin appeared to attenuate the increased all-cause mortality observed in lean individuals with advanced fibrosis. Collectively, these findings suggest that statins may exert dual benefits, reducing both hepatic and cardiovascular risk, and highlight their potential role as part of an integrated management strategy for MASLD in elderly populations.

The decision to initiate statin therapy in elderly individuals is often influenced by their baseline cardiovascular risk, which is traditionally determined by tools such as the SCORE2 and SCORE2-OP algorithms. These scoring systems assess the ten-year risk of cardiovascular disease based on factors such as age, gender, smoking status, systolic blood pressure, and cholesterol levels, including non-HDL cholesterol. These risk calculators are pivotal in clinical decision-making for initiation of lipid lowering medication in the general elderly population.

However, while these tools remain essential in identifying individuals at high cardiovascular risk, they do not account for comorbidities such as MASLD, liver fibrosis and obesity, which are increasingly recognized as important contributors to cardiovascular morbidity and mortality. Data derived from studies such as the PROSPER trial (**Chapter 8**) highlight the role of liver fibrosis as an independent risk factor for cardiovascular outcomes in the elderly. Our analysis from the PROSPER trial demonstrated that elevated liver fibrosis markers, such as FIB-4, are associated with significantly higher mortality risk, particularly in elderly patients with cardiovascular risk factors with a beneficial effect by pravastatin in patients with liver fibrosis.

This observation aligns with some theories suggesting that statins may also attenuate hepatic fibrosis and inflammation. Statins have been shown to have pleiotropic effects, including reducing liver inflammation and fibrosis, which are central features of conditions like MASLD. However, historical concerns about hepatotoxicity in patients with liver disease have previously contributed to the underuse of statins in MASLD populations. While these concerns were based on earlier evidence, recent studies have shown that statins are generally safe in MASLD patients, with any increases in ALT being mild and transient. This safety profile, combined with the potential benefits in improving outcomes, supports a broader application of statins in patients with MASLD and hepatic fibrosis. [52].

The PROSPER trial population could be classified as secondary prevention, as the participants in this study were already at high cardiovascular risk due to their age and pre-existing conditions. While the trial demonstrates the benefit of statin therapy in reducing cardiovascular risk in individuals with comorbidities, the findings do not directly address the potential role of statins in the primary prevention of cardiovascular disease in individuals with “only” liver fibrosis and/or obesity. Therefore, future studies should aim to evaluate statin therapy in populations with MASLD, liver fibrosis and/or obesity but without a history of cardiovascular events. Such studies could provide valuable insights into the additional benefit of statins in preventing cardiovascular disease in these individuals, who may not yet show signs of overt atherosclerotic cardiovascular disease but are at increased risk due to their hepatic and metabolic conditions.

Current guidelines, such as those from the Dutch Society of General Practitioners (Nederlands Huisartsen Genootschap), suggest that the benefit of statin therapy in elderly individuals without known cardiovascular disease becomes less convincing with advancing age. This is especially true for vulnerable elderly populations, where the evidence supporting the effectiveness of statins in reducing cardiovascular morbidity and mortality is more uncertain. In light of these limitations, it becomes evident that statin therapy in the elderly should not be based solely on traditional cardiovascular risk calculations but should also consider additional factors like liver fibrosis and obesity.

Given the increasing recognition of liver fibrosis as a significant cardiovascular risk factor, future guidelines and clinical strategies should incorporate a more nuanced approach to statin initiation, especially for older adults. This could include integrating liver fibrosis screening into routine cardiovascular risk assessments, particularly in individuals with metabolic conditions like obesity. The incorporation of NITS for MASLD and liver fibrosis such as FIB-4 in conjunction with traditional cardiovascular risk calculators may help refine treatment decisions, ensuring that statin therapy is tailored to the unique needs of the elderly population.

Moreover, more research is needed to evaluate the long-term effects of statins in elderly individuals with liver fibrosis and obesity, as well as to determine whether statins can mitigate the adverse prognostic impact of these conditions in this age group. In the future, personalized treatment strategies that account for both cardiovascular and hepatic health could offer a more effective approach to managing elderly patients at risk for cardiovascular events.

In conclusion, while statins remain a cornerstone of cardiovascular disease prevention, their use in the elderly should evolve to incorporate factors such as liver fibrosis and obesity. By integrating these elements into risk assessments, clinicians can make more informed decisions regarding statin therapy, ultimately improving outcomes for this vulnerable patient population. Additionally, the growing body of evidence suggesting that statins may benefit hepatic health offers a promising avenue for expanding their use in individuals with liver fibrosis, thereby addressing a broader spectrum of health concerns in elderly patients. Importantly, future studies aimed at primary prevention in individuals with liver fibrosis and/or obesity, without established cardiovascular disease, are necessary to assess the true value of statin treatment in these populations.

## CONCLUSION

MASLD is now the most common cause of chronic liver disease world-wide, closely and inseparably linked to obesity, T2DM, and other features of metabolic dysfunction. This thesis demonstrates that MASLD is not merely extremely common among high-risk individuals but it is also highly linked with cardiovascular morbidity and mortality. Non-invasive screening can detect high-risk individuals, and genetic studies have implicated the involvement of metabolic and lipid pathways in hepatic and cardiovascular disease. Taken together, these findings highlight the significance of early detection and multi-faceted management of MASLD within the broader cardiometabolic spectrum.

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# Chapter 11

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## Summary



## INTRODUCTION

The introduction provides an overview of the epidemiology, pathophysiology, and clinical significance of metabolic dysfunction-associated steatotic liver disease (MASLD), a condition now recognized as the most prevalent chronic liver disease worldwide. It outlines how MASLD arises at the intersection of obesity, type 2 diabetes, and metabolic dysfunction, and explains its reclassification from NAFLD to MASLD/MASH to better reflect its metabolic origins.

This part discusses the global variation in disease burden, the critical role of fibrosis and genetic predisposition in determining outcomes, and the growing recognition of MASLD as both a hepatic and systemic metabolic disorder. It also highlights the strong bidirectional link between MASLD and cardiovascular disease, emphasizing the need for integrated approaches to screening and management.

Finally, it considers recent conceptual shifts in defining obesity and their implications for diagnosing and treating MASLD within the broader context of metabolic health.

## PART I – MASLD IN SPECIFIC POPULATIONS

This body of work focuses on the epidemiology, pathophysiology, and non-invasive assessment of MASLD/MASH across distinct high-risk populations.

**Chapter 1** provides a comprehensive review of the mechanisms leading to steatosis, inflammation, and fibrosis in MASLD/MASH (NAFLD/NASH in the text). It emphasizes the rising global prevalence driven by obesity and type 2 diabetes, the strong association with cardiovascular and liver-related morbidity and mortality, and the urgent need for reliable non-invasive diagnostic tools. Although several blood- and imaging-based tests are available, liver biopsy remains the diagnostic gold standard.

**Chapter 2** examines the prevalence and diagnostic accuracy of non-invasive tests (NITs) in a Dutch bariatric surgery cohort. Among 220 patients, MASLD prevalence was 50.6%, while MASH and at-risk MASH were identified in 5.3% and 2.6%, respectively, lower than expected for this high-risk group. The study found that the capped MAF-5 score

outperformed FIB-4 and transient elastography for detecting significant fibrosis ( $\geq F2$ ), suggesting it may be a more reliable tool in bariatric populations.

**Chapter 3** investigates MASLD and fibrosis in cardiometabolic outpatients, including individuals with and without diabetes. MASLD was present in 39.2% overall and was most common in type 2 diabetes (57%). Fibrosis risk was also highest in T2DM (22.5%), but MASLD and fibrosis were not associated with carotid intima-media thickness, indicating that hepatic and vascular pathology may progress independently. BMI and triglycerides were consistent predictors of MASLD and fibrosis, underscoring the importance of metabolic control and weight reduction.

## PART II – MASLD, CARDIOVASCULAR RISK AND METABOLIC ASSOCIATIONS

In part 2 of this thesis, I demonstrate that MASLD and liver fibrosis are common and metabolically driven in diverse outpatient populations, and that non-invasive markers of fibrosis, such as FIB-4, may also reflect cardiovascular vulnerability.

**Chapter 4** investigated the prevalence of MASLD and liver fibrosis in an outpatient population and examined their relationship with carotid intima-media thickness (cIMT) as a marker of subclinical atherosclerosis. In this cohort of 475 individuals, MASLD and significant fibrosis were highly prevalent, affecting 40.2% and 18.9% of patients, respectively. Both conditions were strongly associated with metabolic risk factors, particularly higher body mass index (BMI) and triglyceride levels. Multiple regression analyses confirmed BMI as an independent predictor of both MASLD and fibrosis. Although cIMT correlated positively with age, BMI, and LDL cholesterol, no significant association was observed between cIMT and liver steatosis or fibrosis, indicating that vascular changes may occur independently of hepatic alterations. These findings highlight that MASLD and fibrosis are common in the outpatient setting but that cIMT has limited utility as a marker of liver-related disease burden.

**Chapter 5** explored the association between liver fibrosis, estimated using the FIB-4 index, and cardiovascular risk factors in patients with rheumatoid arthritis (RA) participating in the FRANCIS study. Among 326 RA patients without type 2 diabetes or established CVD, 15% had

an elevated FIB-4 score ( $\geq 1.3$ ), indicating higher fibrosis risk. Patients with elevated FIB-4 displayed increased carotid intima-media thickness (cIMT), higher systolic blood pressure, and elevated levels of apolipoprotein B48 and alkaline phosphatase, as well as reduced leukocyte counts and complement component 3 levels. Medication analyses revealed that statin use was associated with higher FIB-4, while hydroxychloroquine use correlated with lower FIB-4, suggesting possible medication effects on hepatic or metabolic status. Disease activity was not related to FIB-4 levels. These results indicate that even in the absence of diabetes or overt CVD, RA patients with higher FIB-4 scores exhibit greater cardiovascular vulnerability, underscoring the potential value of incorporating non-invasive fibrosis assessment into CV risk stratification in this population.

## PART III – INTERVENTIONS AND PROGNOSIS

Part 3 addresses the complex interplay between MASLD, obesity, genetic susceptibility, and cardiovascular outcomes across different clinical and demographic contexts.

**Chapter 6** describes the design of a prospective cohort study investigating the long-term hepatic and metabolic effects of bariatric surgery in patients with obesity and MASLD/MASH (NAFLD/NASH in the text). By integrating metabolic, cardiovascular, genomic, proteomic, lipidomic, metabolomic, and microbiome analyses, this study aims to elucidate the mechanisms underlying regression of hepatic steatosis and fibrosis following weight loss surgery. The longitudinal approach, including transient elastography and histological evaluation up to five years postoperatively, seeks to identify predictors of sustained hepatic improvement and metabolic health restoration.

**Chapter 7** shifts the focus to the genetic determinants of MASLD/MASH in an elderly Western European population from the PROSPER cohort. Five single nucleotide polymorphisms (SNPs) previously associated with MASLD/MASH were linked to variation in FIB-4 scores, reflecting differences in fibrosis risk. Furthermore, select variants (TRIB1, SLC39A8, and ANPEP) were associated with cardiovascular and mortality outcomes, suggesting that genetic predisposition to hepatic fibrosis may also confer systemic vulnerability. These findings underscore the potential of incorporating genetic risk profiling to refine both hepatic and cardiometabolic disease stratification in aging populations.

**Chapter 8** further explores the prognostic relevance of hepatic fibrosis, assessed by FIB-4, in relation to mortality and cardiovascular endpoints within the same elderly cohort. Participants with high FIB-4 scores had increased all-cause mortality, independent of cardiovascular events. Notably, this excess risk was attenuated in those receiving pravastatin, suggesting a possible protective role of statin therapy beyond lipid lowering. This observation supports the concept that hepatic fibrosis serves as a systemic marker of vulnerability that may be modifiable through pharmacologic intervention.

**Chapter 9** expands upon these findings by examining the combined impact of obesity and liver fibrosis on cardiovascular risk and the modifying effect of pravastatin. Elderly individuals with both high BMI and high FIB-4 scores demonstrated a markedly increased risk of (non-) fatal stroke in the placebo group, an association that was abolished by pravastatin treatment. In contrast, lean individuals with high FIB-4 exhibited higher all-cause mortality in the absence of statin therapy, a risk that was again mitigated by pravastatin. These results suggest that both metabolic and fibrotic phenotypes influence cardiovascular vulnerability, and that statin therapy may confer differential protective effects depending on body composition and fibrosis status.

Collectively, these chapters reveal that MASLD and liver fibrosis represent systemic expressions of metabolic dysfunction and genetic predisposition that extend beyond the liver. They highlight the potential of integrating omics-based profiling, fibrosis assessment, and cardiometabolic risk evaluation to guide individualized preventive and therapeutic strategies. Moreover, the observed mitigating effect of statin therapy on mortality and stroke risk in high-fibrosis groups underscores its possible role in modulating the hepatic-vascular axis in elderly individuals.

A broad discussion in **Chapter 10** contextualizes our findings and identifies directions for further study.





# Chapter 12

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Nederlandse samenvatting



## INLEIDING

De inleiding geeft een overzicht van de epidemiologie, pathofysiologie en klinische betekenis van metabool disfunctie-geassocieerde steatotische leverziekte (MASLD), een aandoening die nu wordt erkend als de meest voorkomende chronische leverziekte wereldwijd. Er wordt uitgelegd hoe MASLD ontstaat op het kruispunt van obesitas, type 2-diabetes en metabole disfunctie, en waarom de herclassificatie van NAFLD naar MASLD/MASH heeft plaatsgevonden om de metabole oorsprong beter te weer- spiegelen.

Dit deel bespreekt de wereldwijde variatie in ziektelast, de cruciale rol van fibrose en genetische predispositie bij het bepalen van uitkomsten, en de toenemende erkenning van MASLD als zowel een lever- als een systemische metabole aandoening. Tevens wordt de sterke bidirectio- nele relatie tussen MASLD en cardiovasculaire ziekte benadrukt, wat het belang van geïntegreerde benaderingen voor screening en behandeling benadrukt.

Tot slot worden recente conceptuele verschuivingen in de definitie van obesitas besproken en de implicaties daarvan voor het diagnosticeren en behandelen van MASLD binnen de bredere context van metabole gezond- heid.

## DEEL I – MASLD IN SPECIFIEKE POPULATIES

Dit deel richt zich op de epidemiologie, pathofysiologie en niet-invasieve beoordeling van MASLD/MASH in verschillende hoogrisicopopulaties.

**Hoofdstuk 1** geeft een uitgebreid overzicht van de mechanismen die lei- den tot steatose, ontsteking en fibrose bij MASLD/MASH (NAFLD/NASH in de tekst). Er wordt de nadruk gelegd op de toenemende wereldwijde prevalentie, gedreven door obesitas en type 2-diabetes, de sterke associ- atie met cardiovasculaire en levergerelateerde morbiditeit en mortaliteit, en de dringende behoefte aan betrouwbare niet-invasieve diagnostische hulpmiddelen. Hoewel verschillende bloed- en beeldvormende tests be- schikbaar zijn, blijft leverbiopsie de diagnostische gouden standaard.

**Hoofdstuk 2** onderzoekt de prevalentie en diagnostische nauwkeurigheid van niet-invasieve tests (NITs) in een Nederlandse bariatrische chirur-

giecohort. Onder 220 patiënten was de prevalentie van MASLD 50,6%, terwijl MASH en “at-risk” MASH respectievelijk in 5,3% en 2,6% werden geïdentificeerd, lager dan verwacht voor deze hoogrisicogroep. De studie vond dat de gecapte MAF-5 score beter presteerde dan FIB-4 en transient elastografie bij het detecteren van significante fibrose ( $\geq F2$ ), wat suggereert dat dit een betrouwbaarder instrument kan zijn in bariatrische populaties.

**Hoofdstuk 3** onderzoekt MASLD en fibrose bij cardiometabole poliklinische patiënten, inclusief personen met en zonder diabetes. MASLD werd aangetroffen bij 39,2% van de patiënten en was het meest voorkomend bij type 2 diabetes (57%). Het risico op fibrose was ook het hoogst bij T2DM (22,5%), maar MASLD en fibrose waren niet geassocieerd met de dikte van de carotis intima-media, wat suggereert dat lever- en vaatpathologie onafhankelijk van elkaar kunnen voortschrijden. BMI en triglyceriden bleken consistente voorspellers van MASLD en fibrose, wat het belang van metabole controle en gewichtsreductie benadrukt.

## DEEL II – MASLD, CARDIOVASCULAIR RISICO EN METABOLE ASSOCIATIES

In deel 2 van deze thesis wordt aangetoond dat MASLD en leverfibrose vaak voorkomen en metabool gedreven zijn in diverse poliklinische populaties, en dat niet-invasieve markers van fibrose, zoals FIB-4, mogelijk ook cardiovasculaire kwetsbaarheid weerspiegelen.

**Hoofdstuk 4** onderzocht de prevalentie van MASLD en leverfibrose in een poliklinische populatie en onderzocht hun relatie met carotis intima-media dikte (cIMT) als marker van subklinische atherosclerose. In dit cohort van 475 individuen kwamen MASLD en significante fibrose veel voor, respectievelijk bij 40,2% en 18,9% van de patiënten. Beide condities waren sterk geassocieerd met metabole risicofactoren, met name hogere BMI en triglyceriden. Meervoudige regressieanalyses bevestigden BMI als onafhankelijke voorspeller van zowel MASLD als fibrose. Hoewel cIMT positief correleerde met leeftijd, BMI en LDL-cholesterol, werd geen significante associatie waargenomen tussen cIMT en leversteatose of fibrose, wat aangeeft dat vasculaire veranderingen onafhankelijk van leververanderingen kunnen optreden. Deze bevindingen benadrukken dat MASLD en fibrose veel voorkomen in de poliklinische setting, maar

dat cIMT beperkte bruikbaarheid heeft als marker van levergerelateerde ziektelast.

**Hoofdstuk 5** onderzocht de associatie tussen leverfibrose, geschat met de FIB-4 index, en cardiovasculaire risicofactoren bij patiënten met reumatoïde artritis (RA) die deelnamen aan de FRANCIS-studie. Onder 326 RA-patiënten zonder type 2-diabetes of gevestigde CVD, had 15% een verhoogde FIB-4 score ( $\geq 1,3$ ), wat wijst op een hoger fibrose risico. Patiënten met verhoogde FIB-4 vertoonden verhoogde cIMT, hogere systolische bloeddruk, verhoogde niveaus van apolipoproteïne B48 en alkalische fosfatase, en verminderde leukocyten en complementcomponent 3. Medicatieanalyses lieten zien dat statinegebruik geassocieerd was met hogere FIB-4, terwijl hydroxychloroquinegebruik correleerde met lagere FIB-4, wat mogelijke medicamenteuze effecten op lever- of metabole status suggereert. Ziekteactiviteit was niet gerelateerd aan FIB-4 niveaus. Deze resultaten tonen aan dat RA-patiënten met hogere FIB-4 scores een grotere cardiovasculaire kwetsbaarheid vertonen, zelfs zonder diabetes of CVD, wat het potentieel van niet-invasieve fibrosebeoordeling in CV-risicostatificatie benadrukt.

## DEEL III – INTERVENTIES EN PROGNOSE

Deel 3 behandelt de complexe interactie tussen MASLD, obesitas, genetische vatbaarheid en cardiovasculaire uitkomsten in verschillende klinische en demografische contexten.

**Hoofdstuk 6** beschrijft het ontwerp van een prospectieve cohortstudie die de langetermijneffecten van bariatrische chirurgie op lever- en metabole gezondheid onderzoekt bij patiënten met obesitas en MASLD/MASH. Door metabolische, cardiovasculaire, genomische, proteomische, lipidomische, metabolomische en microbiome-analyses te integreren, heeft deze studie tot doel de mechanismen achter regressie van leversteatose en fibrose na gewichtsverlieschirurgie te verduidelijken. De longitudinale aanpak, inclusief transient elastografie en histologische evaluatie tot vijf jaar postoperatief, beoogt voorspellers van blijvende leververbetering en herstel van metabole gezondheid te identificeren.

**Hoofdstuk 7** verschuift de focus naar genetische determinanten van MASLD/MASH in een oudere West-Europese populatie uit de PROSPER-cohort. Vijf single nucleotide polymorfismen (SNPs) die eerder met

MASLD/MASH geassocieerd werden, waren gekoppeld aan variatie in FIB-4 scores, wat verschillen in fibrose risico weerspiegelt. Daarnaast waren selecte varianten (TRIB1, SLC39A8 en ANPEP) geassocieerd met cardiovasculaire en mortaliteitsuitkomsten, wat suggereert dat genetische predispositie voor leverfibrose ook systemische kwetsbaarheid kan veroorzaken. Deze bevindingen benadrukken het potentieel van genetische risicoprofilering om zowel lever- als cardiometabole ziektelast in verouderende populaties te verfijnen.

**Hoofdstuk 8** onderzoekt verder de prognostische relevantie van leverfibrose, beoordeeld met FIB-4, in relatie tot mortaliteit en cardiovasculaire eindpunten binnen hetzelfde oudere cohort. Deelnemers met hoge FIB-4 scores hadden een verhoogde algehele sterfte, onafhankelijk van cardiovasculaire gebeurtenissen. Opvallend was dat dit verhoogde risico werd verminderd bij degenen die pravastatine gebruikten, wat een mogelijk beschermend effect van statinebehandeling suggereert naast cholesterolverlaging. Deze observatie ondersteunt het concept dat leverfibrose een systemische marker van kwetsbaarheid is die mogelijk modificeerbaar is door farmacologische interventie.

**Hoofdstuk 9** breidt deze bevindingen uit door het gecombineerde effect van obesitas en leverfibrose op cardiovasculair risico en het modificerende effect van pravastatine te onderzoeken. Oudere personen met zowel hoge BMI als hoge FIB-4 scores hadden een duidelijk verhoogd risico op (niet-)fatale beroerte in de placebogroep, een associatie die werd opgeheven door pravastatinebehandeling. Daarentegen vertoonden slanke individuen met hoge FIB-4 hogere algehele sterfte zonder statinetherapie, een risico dat opnieuw werd verminderd door pravastatine. Deze resultaten suggereren dat zowel metabole als fibrotische fenotypes de cardiovasculaire kwetsbaarheid beïnvloeden en dat statinetherapie verschillende beschermende effecten kan bieden afhankelijk van lichaamssamenstelling en fibrosestatus.

Samengevat laten deze hoofdstukken zien dat MASLD en leverfibrose systemische uitingen zijn van metabole disfunctie en genetische predispositie die verder reiken dan de lever. Ze benadrukken het potentieel van het integreren van omics-profilering, fibrosebeoordeling en cardiometabole risicobeoordeling om individuele preventieve en therapeutische strategieën te sturen. Bovendien onderstreept het waargenomen dependente effect van statinetherapie op sterfte en beroerterisico bij hoog-

fibrose groepen het mogelijke belang ervan bij het moduleren van de lever-vasculaire as bij ouderen.

**Hoofdstuk 10** behandelt de resultaten van de studies in dit proefschrift. In dit document behandel ik de waarde van onze resultaten en onze aanbevelingen voor de toekomst.



# Appendices

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## LIST OF PUBLICATIONS

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**Theel W**, Boxma-de Klerk BM, Dirksmeier-Harinck F, et al. Evaluation of nonalcoholic fatty liver disease (NAFLD) in severe obesity using noninvasive tests and imaging techniques. *Obes Rev.* 2022;23(8):e13481. doi:10.1111/obr.13481

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**Theel W**, Brouwer WP, van Rossum E, Apers J, Ter Borg S, Noordermeer T, Castro Cabezas M. Evaluation of the prevalence of MASLD, MASH and liver fibrosis in a Dutch bariatric cohort. *PLoS One.* 2025 Jun 24;20(6):e0324813. doi: 10.1371/journal.pone.0324813. PMID: 40554612; PMCID: PMC12186956.

Saidi AN, **Theel WB**, Grobbee DE et al. Prevalence of liver steatosis and fibrosis assessed by transient elastography in a high cardiovascular-risk outpatient cohort including T1DM and T2DM patients. Submitted

**Theel WB**, de Jong VD, Grobbee DE, Jukema JW, Trompet S, Castro Cabezas M. Genetic risk factors for MASLD/MASH and cardiovascular disease in elderly individuals. *Ann Hepatol.* 2026 Jan-Jun;31(1):102170. doi: 10.1016/j.aohp.2025.102170. Epub 2025 Dec 3. PMID: 41349715.

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Türk Y, **Theel W**, Kasteleyn MJ, et al. High intensity training in obesity: a Meta-analysis. *Obes Sci Pract.* 2017;3(3):258–271. Published 2017 May 29. doi:10.1002/osp4.109

Smelt, H. J. M., Pouwels, S., Heusschen, L., Hazebroek, E. J., van Rutte, P. W. J., **Theel, W.**, & Smulders, J. F. (2023). Factors Affecting Patient Adherence to Multivitamin Intake After Bariatric Surgery: Thematic Analysis of Qualitative Data From a Multicenter Study. *Cureus*, 15(8), e42928. <https://doi.org/10.7759/cureus.42928>

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## PORTFOLIO W.B. THEEL

|      | Courses                                                                                                                                                                        | ECTS |
|------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------|
| 2021 | <b>Practical Biostatistics</b>                                                                                                                                                 | 5,7  |
|      | Amsterdam UMC/AMC, the Netherlands                                                                                                                                             |      |
|      | <b>WMO/GCP training</b>                                                                                                                                                        | 1,5  |
|      | Dutch Clinical Research Foundation                                                                                                                                             |      |
|      | <b>Biomedical English Writing</b>                                                                                                                                              | 2    |
|      | Erasmus MC, the Netherlands                                                                                                                                                    |      |
| 2023 | <b>WMO/GCP training</b>                                                                                                                                                        | 1,5  |
|      | Dutch Clinical Research Foundation                                                                                                                                             |      |
| 2025 | <b>Scientific integrity</b>                                                                                                                                                    | 0,3  |
|      | Erasmus MC, the Netherlands                                                                                                                                                    |      |
|      | <b>Presentations</b>                                                                                                                                                           |      |
| 2021 | Vitamins: are they just supplement? 13 <sup>th</sup> Frankfurter Meeting. Frankfurt, Germany                                                                                   | 0,5  |
|      | Obesitas: diagnose en behandeling voor nu en de toekomst. NAPA Invest. Den Bosch, the Netherlands                                                                              | 1    |
| 2022 | Leefstijl en obesitas. Physio global, Rotterdam, the Netherlands                                                                                                               | 0,5  |
|      | Obesitas en bariatrische chirurgie. Nascholing Zorgorganisatie Eerste Lijn. Naaldwijk, the Netherlands                                                                         | 1,5  |
|      | De rol van de internist in de bariatric en medicamenteuze behandeling van obesitas. Internal training at Franciscus Gasthuis, Rotterdam, the Netherlands                       | 1    |
|      | Evaluation of liver fibrosis using the Fibroscan™ in the bariatric workup: recommendations for clinical practice. European Fatty Liver Conference, Maastricht, the Netherlands | 1    |
| 2023 | Obésité et sexualité. Oraal presentatie. Medical Education Fit for Me. Lille, France                                                                                           | 0,5  |
|      | Bariatric in de 1 <sup>e</sup> lijn. Oral presentation. Regionale nascholing voor huisartsen. Medical Education Fit for Me. Rotterdam, the Netherlands                         | 1    |
|      | Liver steatosis and fibrosis in severe obesity in diabetic and non-diabetic subjects: first report of the FONS. Poster presentation. EASL LSD summit, Prague, Czech Republic   | 1    |
|      | Genetic markers of MASLD in an elderly Caucasian population and the association with obesity. Poster presentation. EASL LSD summit, Prague, Czech Republic                     | 1    |
| 2024 | Obesitas: een groot probleem. Regio Overstijgend Evenement voor Medici (ROEM), Rotterdam, the Netherlands                                                                      | 1    |

|           | Courses                                                                                                                                                                                                                                        | ECTS |
|-----------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------|
|           | Effect of pravastatin on cardiovascular diseases in an elderly population with obesity and liver fibrosis. Poster presentation. EAS congress, Lyon, France.                                                                                    | 0,5  |
|           | Liver steatosis and fibrosis in severe obesity in diabetic and non-diabetic subjects: report of the FONS. Poster presentatie. IFSO congress European chapter, Vienna, Austria.                                                                 | 0,5  |
|           | Effect of pravastatin on cardiovascular outcomes in an elderly population with obesity and liver fibrosis. Oraal and poster presentation. Tweede prijs voor beste presentatie. Science day of Franciscus Gasthuis, Rotterdam, the Netherlands. | 1,5  |
|           | Liver fibrosis and obesity associated with stroke in an elderly population: protected effect by pravastatin. Poster tour. EASL congress, Milan, Italy.                                                                                         | 1,5  |
| 2025      | Praktisch aspecten van interventies voor gewichtsverlies, Oral presentation, Capelle aan den IJssel, the Netherlands                                                                                                                           | 1,5  |
|           | NASO spring meeting, Oral Presentation, Utrecht, the Netherlands                                                                                                                                                                               | 1    |
|           | Prevalence of MASLD, MASH and live fibrosis in a Dutch bariatric cohort. Poster presentatie. 32nd ECO. EASO, Malaga, Spain.                                                                                                                    | 0,5  |
| 2012/2024 | Biweekly MASLD research meeting, Franciscus Gasthuis, Rotterdam, the Netherlands                                                                                                                                                               | 3    |
|           | Monthly P3N meeting, online.                                                                                                                                                                                                                   | 1    |
|           | <b>Conferences</b>                                                                                                                                                                                                                             |      |
| 2021      | 23rd European Congress of Endocrinology. ESE. Virtual.                                                                                                                                                                                         | 0,3  |
|           | 28th European Congress on Obesity. EASO. Virtual                                                                                                                                                                                               | 0,3  |
|           | 12th Frankfurter Meeting. Frankfurt, Germany.                                                                                                                                                                                                  | 0,3  |
|           | 9th Congress of the International Federation for the Surgery of Obesity and Metabolic Disorders – European Chapter. IFSO-EC. Prague, Czech Republic                                                                                            | 0,6  |
|           | Physician Assistant Invest congress. Utrecht, the Netherlands                                                                                                                                                                                  | 0,3  |
|           | Science day of Franciscus Gasthuis. Rotterdam, the Netherlands                                                                                                                                                                                 | 0,3  |
|           | AGEM Amsterdam Gastroenterology Endocrinology Metabolism Symposium, Amsterdam, the Netherlands                                                                                                                                                 | 0,3  |
| 2022      | 29th European Congress on Obesity. EASO. Maastricht, the Netherlands                                                                                                                                                                           | 0,6  |
|           | European Fatty Liver Conference, Maastricht, the Netherlands                                                                                                                                                                                   | 0,6  |
|           | Physician Assistant Invest congress. Den Bosch, the Netherlands                                                                                                                                                                                | 0,6  |
|           | Science day of Franciscus Gasthuis. Rotterdam, the Netherlands                                                                                                                                                                                 | 0,3  |
| 2023      | 26th IFSO World Congress of Bariatric Surgery and Metabolic Surgery. Naples, Italy.                                                                                                                                                            | 0,6  |
|           | Steatotic Liver Disease Summit. EASL. Prague, Czech Republic                                                                                                                                                                                   | 0,6  |
|           | EASL Congress 2023. Vienna, Austria.                                                                                                                                                                                                           | 0,6  |

|           | Courses                                                                                                                                            | ECTS        |
|-----------|----------------------------------------------------------------------------------------------------------------------------------------------------|-------------|
|           | 11th Congress of the International Federation for the Surgery of Obesity and Metabolic Disorders – European Chapter. IFSO–EC. Zürich, Switzerland. | 0,6         |
|           | Science day of Franciscus Gasthuis. Rotterdam, the Netherlands                                                                                     | 0,6         |
| 2024      | 92nd European Atherosclerosis Society Congress. EAS. Lyon, France                                                                                  | 0,3         |
|           | Nationaal Obesitas Congres, Amersfoort, the Netherlands                                                                                            | 0,3         |
|           | EASL Congress 2024. Milan, Italy                                                                                                                   | 0,6         |
|           | 31th European Congress on Obesity. EASO. Venice, Italy.                                                                                            | 0,6         |
|           | 12th Congress of the International Federation for the Surgery of Obesity and Metabolic Disorders – European Chapter. IFSO–EC. Vienna, Austria.     | 0,6         |
|           | Science day of Franciscus Gasthuis. Rotterdam, the Netherlands                                                                                     | 0,3         |
|           | Vetleverziekten: en dan?, Rotterdam, the Netherlands                                                                                               | 0,3         |
| 2025      | Gateway to Better Health: The Case for Coordinated EU Action on Obesity, Brussel, Belgium                                                          | 0,3         |
|           | Nationaal Obesitas Congres, Amersfoort, the Netherlands                                                                                            | 0,3         |
|           | NASO spring meeting, Utrecht, the Netherlands                                                                                                      | 0,6         |
|           | EASL Congress 2025. Amsterdam, the Netherlands                                                                                                     | 0,6         |
|           | 32th European Congress on Obesity. EASO. Malaga, Spain.                                                                                            | 0,6         |
|           | 13th Congress of the International Federation for the Surgery of Obesity and Metabolic Disorders – European Chapter. IFSO–EC. Venice, Italy.       | 0,3         |
|           | <b>Teaching activities</b>                                                                                                                         |             |
| 2021–2025 | Transient Elastography workshop residents                                                                                                          | 0,6         |
|           | <b>Other Activities</b>                                                                                                                            |             |
| 2022      | Peer reviewer: Obesity Reviews, Plos One, BMC Gastroenterology                                                                                     | 3           |
| 2024      | Podcast recording for the European Liver Patients' Association: Beyond Weight Loss: The Science of Movement and Liver Health in MASLD and MASH.    | 1           |
|           | Winner of the BOF call for medical scientific research                                                                                             | 1,5         |
|           | Second price for best presentation during the science day of Franciscus Gasthuis                                                                   | 1           |
| 2025      | Top submission for the Netherlands Association for the Study of Obesity (NASO)                                                                     | 1           |
|           | <b>Total</b>                                                                                                                                       | <b>51,3</b> |

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## ABOUT THE AUTHOR

Willy Theel was born on August 29, 1985, in Dijon, France. He grew up in Plombières-les-Dijon with his parents, Philippe and Myriam, his older sister Elina, and his younger brother Florian. In 2003, he obtained his Baccalauréat Européen Scientifique with a specialization in mathematics. He subsequently completed his propaedeutic year in medicine before starting at the Institut de Formation en Masso-Kinésithérapie. In 2007, he graduated as a physiotherapist and specialized in cardiorespiratory rehabilitation.



In 2009, he moved to Rotterdam, the Netherlands, to join the Department of Pulmonology at the Franciscus Gasthuis. Together with his physiotherapy colleagues, he was responsible for both the pulmonary ward and pulmonary rehabilitation. In 2012, he obtained a university diploma in cardiorespiratory physiotherapy from the University of Lyon, France. During this period, he became actively involved in clinical research on respiratory diseases and obesity.

Motivated to contribute more directly to patient care in obesity treatment, he enrolled in the Master's program for Physician Assistants at the University of Applied Sciences in Utrecht, the Netherlands, graduating in 2018. He subsequently worked as a Physician Assistant in internal medicine in close collaboration with the bariatric surgery department.

On January 1st, 2021, alongside his outpatient clinical duties, he began his PhD research under the supervision of Prof. Dr. Liesbeth van Rossum and Dr. Castro Cabezas at Erasmus MC. His research focused on metabolic dysfunction-associated steatotic liver disease (MASLD), a topic that bridges the disciplines he encounters daily in his clinical work.

Over the years, Willy has combined professional growth with personal milestones, including the birth of his son Scott in 2013 and his daughters Olivia and Diana in 2015.

After completing his PhD, he will continue contributing to research and treatment of obesity and fatty liver disease while transitioning into a role as Medical Science Liaison at Eli Lilly. He aims to remain engaged in the broader scientific and clinical field, with the ambition to be both a dedicated professional and a present, happy father.

## INTERPRETATIVE DESCRIPTION OF THE ARTWORK

This abstract expressionist work visually represents a nuanced landscape of mental resilience and emotional depth. The upper section features a delicate geometric framework in cool blues and soft whites, evoking structure, clarity, and intellectual control. This sense of order conveys the capacity to navigate complex responsibilities with focus and composure.

The central area transitions into layered, textured earth tones, symbolizing the accumulation of experience, effort, and sustained engagement. The thick, rich textures suggest strength under pressure and the ability to integrate challenges into a cohesive whole.

The lower section flows into deep blues and subtle darkness, punctuated by fine golden lines reminiscent of kintsugi. These lines highlight the beauty of repair and resilience, emphasizing that even in the presence of strain or vulnerability, there is elegance, strength, and continuity.

Overall, the painting conveys a positive narrative of endurance, self-awareness, and adaptive strength, celebrating the dynamic interplay between structure, depth, and transformation. It serves as a visual metaphor for the balance of cognitive clarity and emotional richness, offering a contemplative insight into the layered nature of mental and emotional well-being.

