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Non-alcoholic fatty liver disease. Mind the gaps

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NON-ALCOHOLIC FATTY LIVER DISEASE: MIND THE GAPS

PATHOPHYSIOLOGY AND NON-INVASIVE DIAGNOSIS

Pauline Verhaegh

Non-Alcoholic Fatty Liver Disease:

Mind the gaps

Pathophysiology and non-invasive diagnosis

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Non-Alcoholic Fatty Liver Disease:

Mind the gaps

Pathophysiology and non-invasive diagnosis

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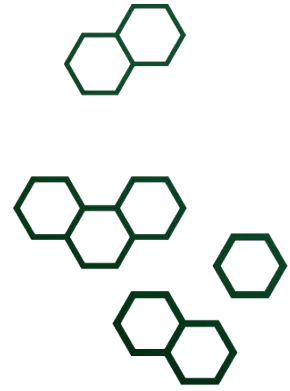
Table of contents

Chapter 1	General introduction	9
Chapter 2	Electron microscopic observations in perfusion-fixed human non-alcoholic fatty liver disease biopsies *Pathology 2021*	29
Chapter 3	Hyperferritinaemia in non-alcoholic fatty liver disease: iron accumulation or inflammation? *Seminars in Liver Disease 2019*	55
Chapter 4	Serum ferritin is associated with non-alcoholic steatohepatitis with significant fibrosis *To be submitted*	83
Chapter 5	Myosteatosis in non-alcoholic fatty liver disease: An exploratory study *Clinics and Research in Hepatology and Gastroenterology 2020*	101
Chapter 6	Non-invasive tests do not accurately differentiate non-alcoholic steatohepatitis from simple steatosis: a systematic review and meta-analysis *Clinical Gastroenterology and Hepatology 2018*	119
Chapter 7	Crashing NASH in patients listed for bariatric surgery *Obesity Surgery 2018*	167
Chapter 8	The increasing burden of non-alcoholic fatty liver disease in the general population: time to bridge the gap between hepatologists and primary care Appendix 1: Survey general practitioners – Liver disease *Hepatology 2017*	175
Chapter 9	General discussion	193

Addendum	Impact paragraph	211
	Summary	219
	Samenvatting	225
	List of publications	233
	Dankwoord	239
	Curriculum Vitae	245



Chapter 1

General introduction

General introduction

Globesity

Obesity is one of today's biggest public health problems. Overnutrition associated with a sedentary lifestyle is leading to an escalating global epidemic of overweight and obesity called "globesity". Obesity is a complex condition, due to associated comorbidity and challenging social and psychological dimensions, affecting all age and socioeconomic groups in both developed and developing countries. In 1995, an estimated 200 million adults worldwide were classified as obese. As of 2000, the number of obese adults has increased to over 300 million,[1] and is anticipated to increase even further.[2]
In 2018, 50.2% of the Dutch adult population had overweight (BMI ≥25 kg/m^2) and 15% was classified as obese (BMI ≥30 kg/m^2). Men have higher rates of overweight (53.3% versus 47.2% in women), whereas women have higher rates of obesity (16.9% versus 13.0% in men)[3]. Obesity poses a major health risk especially for serious non-communicable diseases, including diabetes mellitus, cardiovascular disease, hypertension and stroke, specific types of cancer [1] and non-alcoholic fatty liver disease (NAFLD).[4,5]

Non-alcoholic fatty liver disease

NAFLD is the most widespread liver disease in Western society, with a prevalence up to 25% among the population.[6] The term NAFLD comprises both simple steatosis, a more benign form of fat accumulation, present in the majority of patients, and non-alcoholic steatohepatitis (NASH).[7] NASH is a chronic inflammatory state that develops in about 30% of NAFLD patients and leads to an overall increase in morbidity and mortality due to the progression to fibrosis, cirrhosis and hepatocellular carcinoma (HCC).[8,9]

Pathophysiology

NAFLD is a lifestyle related disease with both hepatic and extra-hepatic complications. The complex pathophysiology is multifactorial and not yet fully elucidated. Factors reported to be involved include amongst others diet, host genetics, metabolic factors like insulin resistance, oxidative stress and lipotoxicity, and intestinal microbiome perturbations.[10,11]

Diet

Excess calorie consumption leading to obesity and related comorbidities,[12] results in lipid accumulation in the liver, mainly in the form of triglycerides, and is the major risk factor for NAFLD.[6] With regard to macronutrient composition, especially high intake of saturated fats, animal proteins, and also simple sugars like fructose, have been associated with NAFLD. A Western diet is characterised by a high consumption of these components,[13, 14] and therefore considered a clear risk factor. In addition, snacking, a common feature of the Western diet, has also been shown to increase liver fat content independent of caloric load.[15]
Once nutrients have been taken up by the enterocytes they are transported to the liver via the portal circulation. Chylomicrons present in the circulation have to be transported through the vascular endothelium to be metabolized and/or stored by tissues. Under physiological conditions, liver sinusoidal endothelial cells (LSECs) are major regulators of the bidirectional lipid exchange between blood and the liver parenchyma. LSEC fenestrae allow efficient transfer of lipoproteins, chylomicron remnants and other macromolecules, from the sinusoidal blood to the space of Disse, and vice versa. Animal models and in vitro studies using primary LSECs have shown that LSECs undergo morphological and functional changes during the process of liver steatosis, characterised by loss of fenestrae (*i.e.* defenestration). It has been shown that this can occur early in the course of NAFLD and can promote steatosis.[16] For example, a study in mice deficient of Plasmalemma Vesicle Associated Protein (PLVAP), a protein required for the formation of fenestrae, showed development of extensive multivesicular steatosis, steatohepatitis and fibrosis around three weeks of life.[17] This indicates that fenestrae may have a role in NAFLD pathophysiology. Up to now, studies in human liver samples are lacking, mainly as detailed evaluation of the LSECs and their fenestrae is not possible in immersion fixed tissue samples, due to collapse of the sinusoids. 18 However, recently techniques have been developed that allow perfusion-type fixation of needle[18] and wedge liver biopsies.[19]

Genetics

Genetic factors were found to regulate susceptibility to liver damage as based on results of a study in twins showing that hepatic steatosis and hepatic fibrosis are heritable traits.[20] At present, several genetic associations have been identified in patients with NAFLD, but only a minority has been validated. Polymorphisms in patatin-like phospholipase domain-containing 3 (PNPLA3) and transmembrane

6 superfamily, member 2 (TM6SF2) are among the best characterised polymorphisms and have been shown to promote the development of NASH and related liver damage.[21] They have been reported in 14[22] and 12%[23] of NAFLD patients, respectively. PNPLA3 encodes adiponutrin, a lipase that regulates both triglyceride and retinoid metabolism. Exact mechanisms through which PNPLA3 acts are however unknown and it should be noted that the PNPLA3 polymorphism (I148M) is neither sufficient nor required to cause NASH, cirrhosis, or liver cancer.[21] The TM6SF2 polymorphism reduces hepatocyte very low-density lipoprotein secretion and is associated with NASH and liver fibrosis. This suggests that proper trafficking of lipids is necessary to prevent lipotoxicity.[21] Furthermore, a lower risk of developing carotid plaques and a lower incidence of cardiovascular events was seen in patients with this polymorphism.[24] More recently, the rs641738 variant in the gene membrane bound O-acyltransferase domain containing 1 (MBOAT1) and the rs72613567 variant in the hydroxysteroid 17-beta dehydrogenase 13 (HSD17B13) gene have also been linked to NAFLD.[25, 26] Overall, there is evidence that about half of the steatosis variability is inherited.[27] However, despite various associations being identified, host genetics cannot fully explain which patients are at risk of developing progressive disease, other factors are also involved. Therefore, at present, genotyping is not yet recommended as routine procedure, but may be considered in selected patients in case of lacking features of insulin resistance and in clinical studies.[28]

Metabolic factors: Insulin resistance, oxidative stress and lipotoxicity

NAFLD is characterised by an excess of hepatic free fatty acids (FFA), resulting from an imbalance between an increased availability and a reduced removal of FFA. Apart from a high dietary intake, major sources of FFA in NAFLD are dysregulated lipolysis in adipose tissue and *de novo* lipogenesis,[29] which both are induced by insulin resistance (IR).[27,30] Furthermore, in case of IR-induced impairment of insulin uptake in extrahepatic tissue, the liver will receive an overload of glucose and insulin. This will induce liver synthesis of FFAs through activation of SREBP-1c [sterol regulatory binding protein-1c] and ChREBP [carbohydrate response element binding protein].[27] The two major ways to process this excess FFA in hepatocytes are mitochondrial beta-oxidation and re-esterification to form triglycerides.[30] In simple steatosis, it is thought that the excess FFA leads to triglyceride accumulation in the form of lipid droplets. This is considered a non-toxic, safe form of storage for lipids in the liver. However in

NASH, enhanced FFA metabolism takes place via mitochondrial β-oxidation, leading to the formation of toxic lipid species (such as ceramides, diacylglycerols, lysophosphatidylcholine). These can induce endoplasmic reticulum stress, hepatocellular injury and cell death contributing to fibrogenesis and genomic instability predisposing to cirrhosis and hepatocellular carcinoma.[30]

NASH patients do have higher oxidative stress levels as compared to patients with simple steatosis.[27] Enhanced free fatty acid metabolism via mitochondrial β-oxidation is considered a crucial pro-oxidizing factor in NASH. Two percent of the oxygen consumed by hepatocytes is transformed into radical oxygen species (ROS) by mitochondria under basal conditions. Enhanced oxidative stress in mitochondria by excess free fatty acids may further damage organelle function. This contributes to a vicious circle, since ROS generation by the damaged respiratory chain is increased, which further damages the respiratory chain. In this way oxidative stress can play a crucial role in NAFLD progression, since oxidative damage to lipid, protein and DNA molecules can trigger several inflammatory and fibrogenic signalling pathways (NF-κB and TGF-β/SMAD3-signalling pathway).[27] Iron is also a well-known pro-oxidant and can thereby play a role in generating reactive oxygen species.[27] An association between NAFLD and iron overload has been shown, with increased hepatic iron found in NAFLD patients. Hepatic iron can be present in three histologic patterns; in the hepatocytes (hepatocellular (HC) iron), in the reticuloendothelial system (RES) or as a mixed pattern (stainable iron in both HC and RES). NAFLD patients with RES iron were found to show more severe NAFLD, according to the NAFLD Activity Score (NAS).[31] It is hypothesised that increased iron depositions can increase oxidative stress by catalysing the production of ROS through the Fenton reaction. In addition, iron is suggested to exacerbate IR through alteration of insulin signalling in adipocytes and hepatocytes. Iron as well as blood iron-transporter transferrin have been shown to induce insulin resistance of glucose transport in adipocytes through a mechanism independent of fatty acids in an *in vitro* model of isolated rat adipocytes.[32] Furthermore, hyperinsulinaemia and hence insulin resistance without impaired glucose tolerance has been demonstrated in patients with idiopathic haemochromatosis in the non-cirrhotic stage.[33] Additionally, ferritin, the iron storage protein, is known to inhibit the secretion of apolipoprotein B post-translationally in rat liver microsomes.[34] Serum ferritin is used as a measure of iron overload, but also reflect inflammation. Ferritin levels are increased in about 30% of patients with

NAFLD,[35,36] but the exact cause of hyperferritinaemia in NAFLD, being iron overload or inflammation, still remains unclear.

Skeletal muscles

Excess FFA do not only affect the liver, but also impact several other organs. Ectopic FFA accumulation in skeletal muscle has been recognized as an early and important source of peripheral IR in NAFLD.[27] In muscle tissue, fat can accumulate within the cytoplasm of myofibres (intra-myocellular lipids; IMCL) or in adipocytes located between muscle fibres (extra-myocellular lipids).[37] High IMCL levels have been associated with IR, but the exact underlying mechanism is unclear.[38] Interestingly, exercise has also been shown to increase IMCL levels, as insulin-sensitive muscles of highly trained athletes have similar IMCL content compared to old type 2 diabetes mellitus patients. This is known as the 'athlete paradox', which suggests that IMCL accumulation per se does not lead to insulin resistance, but specific lipid species or lipid turnover dynamics may play a role.[37,38]

Intestinal involvement

Evidence is increasing that also intestinal factors may contribute to the onset and/or progression of NAFLD. Intestinal epithelial integrity is essential to separate luminal contents from the interstitium and the systemic compartment. This is amongst others determined by the integrity of the epithelial barrier through tight junctions that seal the paracellular space. Intestinal permeability may increase in response to several factors, such as luminal nutrients, mucosal immune mediators and pathogens.[39] An abnormally increased permeability has been associated with several gastrointestinal and systemic diseases.[40-42]
Increased intestinal permeability leads to translocation of bacteria or parts of the bacterial membrane, subsequently leading to increased concentration of lipopolysaccharide (LPS), called endotoxaemia, which can dysregulate the inflammatory tone and insulin sensitivity.[43,44] Animal studies have suggested that a high-fat diet or a high-fructose diet can induce increased intestinal permeability and metabolic endotoxaemia by altering the intestinal tight junctions proteins, mainly zonula occludens-1 and occludin. A high-fat diet can affect the intestinal barrier by the secretion of bile acids, induction of pro-inflammatory mediators and by influencing the intestinal microbiota.[45] A high-fat diet has been shown to increase the proportion of LPS-containing microbes in the gut.[43] Furthermore, in adolescents with NAFLD, postprandial endotoxin

levels were increased compared to healthy subjects in response to fructose, but not glucose, beverages (consumed with meals) in a 24-h feeding challenge.[46] Fructose also directly affects hepatic lipid metabolism, leading to hepatic steatosis.[47]

Human data on intestinal permeability in NAFLD patients are limited. In NAFLD patients, intestinal permeability has been studied using urinary extraction of (51)Cr-ethylene diamine tetraacetate ((51)Cr-EDTA),[48] lactose/mannitol ratio,[49] and by measuring serum zonulin levels.[50] Using these methods, an increased intestinal permeability in NAFLD patients compared with healthy control subjects was observed in some studies.[48-50] However, this increased intestinal permeability was not confirmed by other investigators.[51,52] Some studies showed an increased intestinal permeability only in NASH and not in simple steatosis,[50,53] however another study did not confirm this.[48] Further studies are warranted directly comparing well defined simple steatosis with NASH and with control subjects.

In addition to dietary factors and inflammatory mediators, the intestinal barrier might be directly affected by intestinal bacteria and their products. The intestinal microbiome further provides colonisation resistance, affects epithelial cell and immune functioning and comprises a large metabolic activity. Several studies have observed changes in microbiota composition in NAFLD. It has been shown that concentrations of Gram-negative bacteria such as Enterobacteria are increased, whereas protective bacteria such as *Faecalibacterium prausnitzii* are decreased in NAFLD patients.[54] Although the exact role of the intestinal microbiome in the pathogenesis of NAFLD is not fully elucidated, the observed perturbations may further contribute to the pro-inflammatory phenotype of NAFLD.[54]

Diagnosis

NAFLD is mostly asymptomatic, generally resulting in silent disease progression. To date, a liver biopsy is the most sensitive test for detecting and staging NAFLD and so far is the only reliable method to diagnose NASH.[28] Due to its invasiveness, high costs and risk of complications, liver biopsy is however not suitable to diagnose large populations or for (repeated) disease monitoring.[55] In clinical practice, only subjects at high risk of NASH, usually based on elevated aminotransferase levels, are generally considered to undergo a liver biopsy. This may lead to underdiagnosis of NASH.[56] Unfortunately, a

non-invasive tool to diagnose NASH is lacking, however, at present, non-invasive tools are available for the assessment of steatosis and fibrosis.

Non-invasive assessment of steatosis

Several imaging techniques have been evaluated for the detection of steatosis. Ultrasound is a widely available and rather inexpensive diagnostic tool, with a high sensitivity when liver steatosis is more than 33%. However, sensitivity is poor when the degree of liver steatosis is less than 30%.[57,58] A promising new technique is the addition of controlled attenuation parameter (CAP), that measures the degree of ultrasound attenuation by hepatic fat simultaneously with liver stiffness measurements.[59] This ultrasonic attenuation coefficient is an estimate of the total ultrasonic attenuation (go-and-return path) at 3.5 MHz and is expressed in dB/m.[60] CAP has shown to also reliably detect low grades of steatosis.[61] A technique with a very high sensitivity in case of low degrees of steatosis is magnetic resonance imaging (MRI), including MR spectroscopy. This technique is able to detect hepatic fat at a percentage greater than 5.56% very accurately. The down side of this technique are the high costs and limited availability.[62] Computed tomography does not substantially improve sensitivity in case steatosis is mild and it has the disadvantages of radiation exposure and increased costs.[11] Table 1.1 provides an overview of sensitivity and specific values of the different techniques for moderate-severe and all degrees of steatosis.[63,64]

Table 1.1 Overview of sensitivity and specificity for steatosis

		Moderate-severe steatosis (≥30% OR 33%)	All degrees of steatosis (≥3% OR 5%*/≥5%)
US*	Sensitivity	81.8% - 100.0%	53.3% - 66.6%
	Specificity	98%	77.0% - 93.1%
CT*	Sensitivity	72.7% - 82%	
	Specificity	91.3% - 100%	
CAP#	Sensitivity	67%	91%
	Specificity	97%	79%
MRI*	Sensitivity		76.7% - 90.0%
	Specificity		87.1% - 91%
MRS*	Sensitivity		80.0% - 91.0%
	Specificity		80.2% - 87.0%

*Data from Lee SS, Park SH. World J Gastroenterol 2014;20:7392-7402. #Data from Francque S, Lanthier N, *et al.* Acta Gastroenterol Belg 2018;81:55-81.
Abbreviations: ultrasound (US); computed tomography (CT); controlled attenuation parameter (CAP); magnetic resonance imaging (MRI); magnetic resonance spectroscopy (MRS).

Non-invasive assessment of fibrosis

Fibrosis can be assessed by transient elastography (TE) and magnetic resonance elastography (MRE). TE is fairly accurate in the detection of hepatic fibrosis but more reliable for diagnosing advanced fibrosis[65] (see Table 1.2 for cut-off values and accuracy). While MRE may be even more reliable than TE, it is a costly technique that is not widely available.[66]

Table 1.2 Cut-off values of transient elastography[64]

Type of probe	Stage of fibrosis	Reported cut offs (kPa)	Sens (%)	NPV (%)	Spec (%)	Cut off (kPa)
M Probe	≥2	6.7-7.8	67-88	84	61-84	7,9
	≥3	>8-10.4	65-100	95	75-93	8.7
	4	>8-10.4	78-100	99	87-98	10.3
XL Probe	≥2			89		7.2
	4			98		7.9

Abbreviations: sensitivity (Sens), negative predictive value (NPV), specificity (Spec). Table adapted from Francque *et al.* [64]

In addition to imaging techniques, some predictive models for fibrosis have been developed based on clinical parameters and measures. The NAFLD fibrosis score (NFS) is the best validated score and can help to identify patients with more severe disease, who may need a liver biopsy. The NFS is calculated using various parameters, including age, body mass index, presence/absence hyperglycaemia, platelet count, albumin level, and ratio of aspartate aminotransferase (AST) to alanine aminotransferase (ALT).[67] Other non-invasive scoring systems for fibrosis include FIB-4 index, AST/platelet ratio (APRI) index and BARD score. It should be noted that all these scoring systems, including the NFS, yield high negative predictive values (NPV) but poor positive predictive values (PPV). Therefore, they are best applied to exclude subjects without advanced fibrosis, thus avoiding unnecessary liver biopsies.[68]

Up to now, accurate non-invasive tools to diagnose NASH are largely lacking. Although some parameters and scoring systems have been evaluated, none are available for use in daily clinical practice. There is an urgent need for non-invasive methods to detect NASH among the general population. In the near future, general practitioners (GPs), as gatekeepers of our healthcare system, are going to play a major role in adequately identifying and managing these patients at population level.

Treatment

Lifestyle interventions

Diet and physical exercise are considered the mainstay treatment for the majority of patients with NAFLD.[11,28] Weight loss is widely acknowledged to be beneficial and the degree of liver histological improvement is directly proportional to the amount of weight loss.[11] A weight loss of 10% of body weight was shown to be associated with histological benefit in NASH patients, such as improvement of liver fat, degree of hepatic inflammation and fibrosis and also improvement of serum liver enzymes.[69] However, it is common knowledge that sustained weight loss is very difficult to achieve. Therefore, other treatment options should also be considered.

Pharmacological treatment of NASH

Drug therapy should be considered for progressive NASH, those with significant fibrosis (≥F2) but also for early-stage NASH with increased risk of fibrosis progression, i.e. diabetes mellitus, metabolic syndrome, persistently increased ALT, high necro-inflammation and age >50 years. However, the optimal type and duration of therapy is still unknown.[28] Among promising pharmacological agents for progressive NASH, insulin sensitizers, anti-oxidants and bile acids have been studied most widely. With the insulin sensitizers, thiazolidinediones, also called glitazones (peroxisome proliferator-activated receptor (PPAR) gamma agonists), improvement of all histological features, except for fibrosis has been demonstrated and resolution of NASH was achieved more often (85%) than with placebo (58%).[70-72] However, the side-effects of thiazolidinediones are a concern, including weight gain, increased risk of bone fractures in women and rarely also congestive heart failure.[28] Liraglutide (an incretin mimetic) showed histologic remission of NASH without worsening fibrosis in a small pilot trial,[73] but further research is necessary.

Vitamin E, an anti-oxidant, was found to improve steatosis, inflammation and ballooning and induced remission of NASH in 36% of patients (versus 21% in placebo arm).[72] There are, however, concerns with regard to the long-term safety of vitamin E, relating to increased risks for overall mortality,[74] haemorrhagic stroke[75] and prostate cancer in males older than 50 years.[76]

Ursodeoxycholic acid, a bile acid, has been studied in several randomised controlled trials and showed biochemical but not histological improvements of NAFLD.[77-79] Obeticholic acid, a synthetic farnesoid X receptor agonist, has been

shown to improve all NASH lesions and improve fibrosis in the FLINT trial.[80] However, there is a concern with regard to safety and tolerability of obeticholic acid related to increased low-density lipoprotein (LDL)-cholesterol levels and to pruritus.[28]
Data on lipid-lowering drugs, such as statins, are limited. Statins reduce LDL-cholesterol and prevent cardiovascular risk, with no benefits or harm on liver disease. Currently, there are no data to support their use specifically for NASH.[28]

Although several drugs have been studied, up to now, no drug has been approved for NASH by regulatory agencies, and any drug treatment would be off-label. Due to the multifactorial origin of the NASH it is unlikely that one specific drug will cure or prevent this disease.

Probiotics and prebiotics

In addition to lifestyle interventions and pharmacological therapy, the potential of functional foods receives increasing attention. Probiotics, prebiotics and synbiotics have been studied, but most of the available data have been obtained in animal or *in vitro* studies. While the results of these studies are promising, data from human intervention studies are scarce.[81] In one small-sized human proof-of-concept study, it was shown that compared with placebo, a probiotic mixture resulted in a significant reduction in intrahepatic triglyceride content in patients with hepatic steatosis.[82]

Bariatric surgery

Bariatric surgery is an option for reducing weight and metabolic complications in patients unresponsive to lifestyle changes and pharmacotherapy. Several types of bariatric surgeries are known, such as the gastric sleeve and the Roux-and-Y gastric bypass, though a preference for a certain type of bariatric procedure cannot be given.[83] A recent cohort study with 1-year follow-up showed that bariatric surgery associated weight loss cleared NASH in 85% of patients and improved fibrosis in 34%.[84] The possible benefits of bariatric surgery should be balanced against peri- and postoperative complications.[28]

Liver transplantation

In case lifestyle and other interventions failed and end-stage liver disease has developed, a liver transplantation can be considered as the only curative option. Remarkably, during the past 10 years, the prevalence of NAFLD as an indication

for liver transplantation has increased by 170%. In the near future, it is expected that NAFLD will probably become the leading indication for liver transplantation due to the still rising prevalence of NAFLD.[12] Liver transplantation is an accepted procedure in NASH patients with end-stage liver disease, with comparable overall survival to other indications, despite its invasiveness and the higher cardiovascular mortality.[28] It should be noted that there will not only be an increased need for liver transplantations, with the rapidly growing prevalence of NAFLD, also the pool of potential liver donors is impacted, as it is expected that a higher proportion of potential liver donors will have steatosis or steatohepatitis and might be declined for liver transplantation use.[85]

Aims and outline of this thesis

With the growing epidemic of overweight and obesity, the prevalence of NAFLD is expecting to rise enormously over the coming years. Due to the progressive nature of NASH, it is important to identify patients at risk of progressive disease in an early stage, to prevent progression, promote quality of life and reduce healthcare costs. The studies presented in this thesis were undertaken to gain more insight in underlying pathophysiological mechanisms of NASH, focussing on ultrastructural changes, the role of ferritin and myosteatosis. Furthermore, this thesis addresses the non-invasive diagnosis of NASH and the awareness and knowledge of general practitioners on NAFLD to investigate possibilities for early diagnosis.

First, we aim to study the liver ultrastructure, especially fenestration, in human liver biopsy samples of NAFLD patients, comparing patients with and without NASH, using transmission electron microscopy (**chapter 2**). We hypothesise that fenestrae size and number decrease as the severity of NAFLD increases. Serum ferritin levels can be used as a measure of iron overload, but can also reflect inflammation. Both inflammation and iron overload have been associated with NAFLD. We hypothesise that hyperferritinaemia in NAFLD predominantly is an expression of inflammation rather than iron overload. We performed an extensive review of the available literature (**chapter 3**) and evaluated the relation between serum ferritin and histologic NAFLD severity in a large cohort of patients with biopsy proven NAFLD (**chapter 4**) to test our hypothesis.

Furthermore, NAFLD is considered to be a multisystem disease and can affect extra-hepatic organ systems leading to cardio-vascular disease, type 2 diabetes mellitus, chronic kidney disease and colorectal cancer. One of the potential mechanisms is based on myosteatosis, which is thought to be an early sign of peripheral insulin resistance, which plays a role in NAFLD. In **chapter 5** we aim to investigate whether myosteatosis is associated with the amount of hepatic fat and the presence of liver fibrosis.

Accurate non-invasive diagnosis of NASH is needed to facilitate future research and patient care. In **chapter 6**, we report on an extensive systematic literature search and meta-analyses with available non-invasive diagnostic biomarkers and scores for NASH, with liver biopsy as a reference standard.

Even when the gold standard of liver biopsy is used, a large variation in NASH prevalence is shown, especially within bariatric studies. In **chapter 7** we search for a possible explanation for the variation in NASH prevalence within bariatric studies. In this chapter, we focus on the impact of direct preoperative interventions.

The rising prevalence of NAFLD and its related comorbidities confront us with the challenge to adequately detect and manage these patients at the level of the general population in order to prevent end-stage liver disease. Therefore, we performed a survey among general practitioners with the aim to investigate the current knowledge and common practices concerning NAFLD among Dutch general practitioners (**chapter 8**).

In **chapter 9**, the general discussion, the findings of the various chapters are summarised and new insights are discussed, allowing an overview with future perspectives and new leads, focussing on pathophysiology, diagnosis, pitfalls in NAFLD research and the bridge to primary care.

References

1. World Health Organisation (WHO) - Controlling the global obesity epidemic. https://www.who.int/nutrition/topics/obesity/en/
2. Pineda E, Sanchez-Romero LM, Brown M, Jaccard A, Jewell J, Galea G, et al. Forecasting Future Trends in Obesity across Europe: The Value of Improving Surveillance. Obes Facts 2018;11:360-371.
3. RIVM. Overgewicht, cijfers en context. In; 2019.
4. Bellentani S, Scaglioni F, Marino M, Bedogni G. Epidemiology of non-alcoholic fatty liver disease. Dig Dis 2010;28:155-161.
5. Chalasani N, Younossi Z, Lavine JE, Diehl AM, Brunt EM, Cusi K, et al. The diagnosis and management of non-alcoholic fatty liver disease: practice guideline by the American Gastroenterological Association, American Association for the Study of Liver Diseases, and American College of Gastroenterology. Gastroenterology 2012;142:1592-1609.
6. Younossi ZM, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M. Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. Hepatology 2016;64:73-84.
7. Hashimoto E, Taniai M, Tokushige K. Characteristics and diagnosis of NAFLD/NASH. J Gastroenterol Hepatol 2013;28 Suppl 4:64-70.
8. McCullough AJ. Pathophysiology of nonalcoholic steatohepatitis. J Clin Gastroenterol 2006;40 Suppl 1:S17-29.
9. Ong JP, Pitts A, Younossi ZM. Increased overall mortality and liver-related mortality in non-alcoholic fatty liver disease. J Hepatol 2008;49:608-612.
10. Petta S, Gastaldelli A, Rebelos E, Bugianesi E, Messa P, Miele L, et al. Pathophysiology of Non Alcoholic Fatty Liver Disease. Int J Mol Sci 2016;17.
11. Rinella ME. Nonalcoholic fatty liver disease: a systematic review. JAMA 2015;313:2263-2273.
12. Samji NS, Verma R, Satapathy SK. Magnitude of Nonalcoholic Fatty Liver Disease: Western Perspective. J Clin Exp Hepatol 2019;9:497-505.
13. Perdomo CM, Fruhbeck G, Escalada J. Impact of Nutritional Changes on Nonalcoholic Fatty Liver Disease. Nutrients 2019;11.
14. Papandreou D, Andreou E. Role of diet on non-alcoholic fatty liver disease: An updated narrative review. World J Hepatol 2015;7:575-582.
15. Koopman KE, Caan MW, Nederveen AJ, Pels A, Ackermans MT, Fliers E, et al. Hypercaloric diets with increased meal frequency, but not meal size, increase intrahepatic triglycerides: a randomized controlled trial. Hepatology 2014;60:545-553.
16. Hammoutene A, Rautou PE. Role of liver sinusoidal endothelial cells in non-alcoholic fatty liver disease. J Hepatol 2019;70:1278-1291.
17. Herrnberger L, Hennig R, Kremer W, Hellerbrand C, Goepferich A, Kalbitzer HR, et al. Formation of fenestrae in murine liver sinusoids depends on plasmalemma vesicle-associated protein and is required for lipoprotein passage. PLoS One 2014;9:e115005.
18. Vreuls C, Wisse E, Duimel H, Stevens K, Verheyen F, Braet F, et al. Jet-fixation: a novel method to improve microscopy of human liver needle biopsies. Hepatology 2014;59:737-739.
19. Wisse E, Braet F, Duimel H, Vreuls C, Koek G, Olde Damink SW, et al. Fixation methods for electron microscopy of human and other liver. World J Gastroenterol 2010;16:2851-2866.
20. Loomba R, Schork N, Chen CH, Bettencourt R, Bhatt A, Ang B, et al. Heritability of Hepatic Fibrosis and Steatosis Based on a Prospective Twin Study. Gastroenterology 2015;149:1784-1793.
21. Diehl AM, Day C. Cause, Pathogenesis, and Treatment of Nonalcoholic Steatohepatitis. N Engl J Med 2017;377:2063-2072.

22. Valenti L, Al-Serri A, Daly AK, Galmozzi E, Rametta R, Dongiovanni P, et al. Homozygosity for the patatin-like phospholipase-3/adiponutrin I148M polymorphism influences liver fibrosis in patients with nonalcoholic fatty liver disease. Hepatology 2010;51:1209-1217.
23. Liu YL, Reeves HL, Burt AD, Tiniakos D, McPherson S, Leathart JB, et al. TM6SF2 rs58542926 influences hepatic fibrosis progression in patients with non-alcoholic fatty liver disease. Nat Commun 2014;5:4309.
24. Dongiovanni P, Petta S, Maglio C, Fracanzani AL, Pipitone R, Mozzi E, et al. Transmembrane 6 superfamily member 2 gene variant disentangles nonalcoholic steatohepatitis from cardiovascular disease. Hepatology 2015;61: 506-514.
25. Abul-Husn NS, Cheng X, Li AH, Xin Y, Schurmann C, Stevis P, et al. A Protein-Truncating HSD17B13 Variant and Protection from Chronic Liver Disease. N Engl J Med 2018;378:1096-1106.
26. Mancina RM, Dongiovanni P, Petta S, Pingitore P, Meroni M, Rametta R, et al. The MBOAT7-TMC4 Variant rs641738 Increases Risk of Nonalcoholic Fatty Liver Disease in Individuals of European Descent. Gastroenterology 2016;150:1219-1230 e1216.
27. Bessone F, Razori MV, Roma MG. Molecular pathways of nonalcoholic fatty liver disease development and progression. Cell Mol Life Sci 2019;76:99-128.
28. European Association for the Study of the L, European Association for the Study of D, European Association for the Study of O. EASL-EASD-EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease. J Hepatol 2016;64:1388-1402.
29. Donnelly KL, Smith CI, Schwarzenberg SJ, Jessurun J, Boldt MD, Parks EJ. Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. J Clin Invest 2005;115:1343-1351.
30. Friedman SL, Neuschwander-Tetri BA, Rinella M, Sanyal AJ. Mechanisms of NAFLD development and therapeutic strategies. Nat Med 2018;24:908-922.
31. Nelson JE, Wilson L, Brunt EM, Yeh MM, Kleiner DE, Unalp-Arida A, et al. Relationship between the pattern of hepatic iron deposition and histological severity in nonalcoholic fatty liver disease. Hepatology 2011;53:448-457.
32. Green A, Basile R, Rumberger JM. Transferrin and iron induce insulin resistance of glucose transport in adipocytes. Metabolism 2006;55: 1042-1045.
33. Niederau C, Berger M, Stremmel W, Starke A, Strohmeyer G, Ebert R, et al. Hyperinsulinaemia in non-cirrhotic haemochromatosis: impaired hepatic insulin degradation? Diabetologia 1984;26:441-444.
34. Hevi S, Chuck SL. Ferritins can regulate the secretion of apolipoprotein B. J Biol Chem 2003;278:31924-31929.
35. Moirand R, Mendler MH, Guillygomarc'h A, Brissot P, Deugnier Y. Non-alcoholic steatohepatitis with iron: Part of insulin resistance-associated hepatic iron overload? [2] (multiple letters). J Hepatol 2000;33:1024-1026.
36. Trombini P, Piperno A. Ferritin, metabolic syndrome and NAFLD: Elective attractions and dangerous liaisons. J Hepatol 2007;46:549-552.
37. Nachit M, Leclercq IA. Emerging awareness on the importance of skeletal muscle in liver diseases: time to dig deeper into mechanisms! Clin Sci (Lond) 2019;133:465-481.
38. Bosma M, Kersten S, Hesselink MK, Schrauwen P. Re-evaluating lipotoxic triggers in skeletal muscle: relating intramyocellular lipid metabolism to insulin sensitivity. Prog Lipid Res 2012;51:36-49.
39. Odenwald MA, Turner JR. Intestinal permeability defects: is it time to treat? Clin Gastroenterol Hepatol 2013;11:1075-1083.
40. Cox AJ, Zhang P, Bowden DW, Devereaux B, Davoren PM, Cripps AW, et al. Increased intestinal permeability as a risk factor for type 2 diabetes. Diabetes Metab 2017;43:163-166.

41. Mujagic Z, Ludidi S, Keszthelyi D, Hesselink MA, Kruimel JW, Lenaerts K, et al. Small intestinal permeability is increased in diarrhoea predominant IBS, while alterations in gastroduodenal permeability in all IBS subtypes are largely attributable to confounders. Aliment Pharmacol Ther 2014; 40: 288-297.
42. Wilbrink J, Bernards N, Mujagic Z, van Avesaat M, Pijls K, Klaassen T, et al. Intestinal barrier function in morbid obesity: results of a prospective study on the effect of sleeve gastrectomy. Int J Obes (Lond) 2020; 44: 368-376.
43. Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, et al. Metabolic endotoxemia initiates obesity and insulin resistance. Diabetes 2007; 56: 1761-1772.
44. Cani PD, Possemiers S, Van de Wiele T, Guiot Y, Everard A, Rottier O, et al. Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability. Gut 2009; 58: 1091-1103.
45. Rohrig S, Farecki ML, Boden KT, Haus A, Gutfleisch M, Jung S, et al. Negative Effects of Vital Dyes after Uneventful Vitreomacular Surgery. Retina 2019; 39: 1772-1778.
46. Nicoletti A, Ponziani FR, Biolato M, Valenza V, Marrone G, Sganga G, et al. Intestinal permeability in the pathogenesis of liver damage: From non-alcoholic fatty liver disease to liver transplantation. World J Gastroenterol 2019; 25: 4814-4834.
47. Lim JS, Mietus-Snyder M, Valente A, Schwarz JM, Lustig RH. The role of fructose in the pathogenesis of NAFLD and the metabolic syndrome. Nat Rev Gastroenterol Hepatol 2010; 7: 251-264.
48. Miele L, Valenza V, La Torre G, Montalto M, Cammarota G, Ricci R, et al. Increased intestinal permeability and tight junction alterations in nonalcoholic fatty liver disease. Hepatology 2009; 49: 1877-1887.
49. Volynets V, Kuper MA, Strahl S, Maier IB, Spruss A, Wagnerberger S, et al. Nutrition, intestinal permeability, and blood ethanol levels are altered in patients with nonalcoholic fatty liver disease (NAFLD). Dig Dis Sci 2012; 57: 1932-1941.
50. Hendy OM, Elsabaawy MM, Aref MM, Khalaf FM, Oda AMA, El Shazly HM. Evaluation of circulating zonulin as a potential marker in the pathogenesis of nonalcoholic fatty liver disease. APMIS 2017; 125: 607-613.
51. Farhadi A, Gundlapalli S, Shaikh M, Frantzides C, Harrell L, Kwasny MM, et al. Susceptibility to gut leakiness: a possible mechanism for endotoxaemia in non-alcoholic steatohepatitis. Liver Int 2008; 28: 1026-1033.
52. Wigg AJ, Roberts-Thomson IC, Dymock RB, McCarthy PJ, Grose RH, Cummins AG. The role of small intestinal bacterial overgrowth, intestinal permeability, endotoxaemia, and tumour necrosis factor alpha in the pathogenesis of non-alcoholic steatohepatitis. Gut 2001; 48: 206-211.
53. Chwist A, Hartleb M, Lekstan A, Kukla M, Gutkowski K, Kajor M. A composite model including visfatin, tissue polypeptide-specific antigen, hyaluronic acid, and hematological variables for the diagnosis of moderate-to-severe fibrosis in nonalcoholic fatty liver disease: a preliminary study. Pol Arch Med Wewn 2014; 124: 704-712.
54. Grabherr F, Grander C, Effenberger M, Adolph TE, Tilg H. Gut Dysfunction and Non-alcoholic Fatty Liver Disease. Front Endocrinol (Lausanne) 2019; 10: 611.
55. Rockey DC, Caldwell SH, Goodman ZD, Nelson RC, Smith AD, American Association for the Study of Liver D. Liver biopsy. Hepatology 2009; 49: 1017-1044.
56. Verdam FJ, Dallinga JW, Driessen A, de Jonge C, Moonen EJ, van Berkel JB, et al. Non-alcoholic steatohepatitis: a non-invasive diagnosis by analysis of exhaled breath. J Hepatol 2013; 58: 543-548.
57. Dasarathy S, Dasarathy J, Khiyami A, Joseph R, Lopez R, McCullough AJ. Validity of real time ultrasound in the diagnosis of hepatic steatosis: a prospective study. J Hepatol 2009; 51: 1061-1067.
58. Saadeh S, Younossi ZM, Remer EM, Gramlich T, Ong JP, Hurley M, et al. The utility of radiological imaging in nonalcoholic fatty liver disease. Gastroenterology 2002; 123: 745-750.

59. Sasso M, Miette V, Sandrin L, Beaugrand M. The controlled attenuation parameter (CAP): a novel tool for the non-invasive evaluation of steatosis using Fibroscan. Clin Res Hepatol Gastroenterol 2012;36:13-20.
60. Sasso M, Beaugrand M, de Ledinghen V, Douvin C, Marcellin P, Poupon R, et al. Controlled attenuation parameter (CAP): a novel VCTE guided ultrasonic attenuation measurement for the evaluation of hepatic steatosis: preliminary study and validation in a cohort of patients with chronic liver disease from various causes. Ultrasound Med Biol 2010;36:1825-1835.
61. de Ledinghen V, Vergniol J, Foucher J, Merrouche W, le Bail B. Non-invasive diagnosis of liver steatosis using controlled attenuation parameter (CAP) and transient elastography. Liver Int 2012;32:911-918.
62. Reeder SB, Cruite I, Hamilton G, Sirlin CB. Quantitative Assessment of Liver Fat with Magnetic Resonance Imaging and Spectroscopy. J Magn Reson Imaging 2011;34:729-749.
63. Lee SS, Park SH. Radiologic evaluation of nonalcoholic fatty liver disease. World J Gastroenterol 2014;20:7392-7402.
64. Francque S, Lanthier N, Verbeke L, Reynaert H, Van Steenkiste C, Vonghia L, et al. The Belgian Association for Study of the Liver Guidance Document on the Management of Adult and Paediatric Non-Alcoholic Fatty Liver Disease. Acta Gastroenterol Belg 2018;81:55-81.
65. Myers RP, Elkashab M, Ma M, Crotty P, Pomier-Layrargues G. Transient elastography for the noninvasive assessment of liver fibrosis: a multicentre Canadian study. Can J Gastroenterol 2010;24:661-670.
66. Loomba R, Wolfson T, Ang B, Hooker J, Behling C, Peterson M, et al. Magnetic resonance elastography predicts advanced fibrosis in patients with nonalcoholic fatty liver disease: a prospective study. Hepatology 2014;60:1920-1928.
67. Angulo P, Hui JM, Marchesini G, Bugianesi E, George J, Farrell GC, et al. The NAFLD fibrosis score: a noninvasive system that identifies liver fibrosis in patients with NAFLD. Hepatology 2007;45:846-854.
68. Vilar-Gomez E, Chalasani N. Non-invasive assessment of non-alcoholic fatty liver disease: Clinical prediction rules and blood-based biomarkers. J Hepatol 2018;68:305-315.
69. Vilar-Gomez E, Martinez-Perez Y, Calzadilla-Bertot L, Torres-Gonzalez A, Gra-Oramas B, Gonzalez-Fabian L, et al. Weight Loss Through Lifestyle Modification Significantly Reduces Features of Nonalcoholic Steatohepatitis. Gastroenterology 2015;149:367-378 e365; quiz e314-365.
70. Aithal GP, Thomas JA, Kaye PV, Lawson A, Ryder SD, Spendlove I, et al. Randomized, placebo-controlled trial of pioglitazone in nondiabetic subjects with nonalcoholic steatohepatitis. Gastroenterology 2008;135: 1176-1184.
71. Belfort R, Harrison SA, Brown K, Darland C, Finch J, Hardies J, et al. A placebo-controlled trial of pioglitazone in subjects with nonalcoholic steatohepatitis. N Engl J Med 2006;355:2297-2307.
72. Sanyal AJ, Chalasani N, Kowdley KV, McCullough A, Diehl AM, Bass NM, et al. Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis. N Engl J Med 2010;362:1675-1685.
73. Armstrong MJ, Gaunt P, Aithal GP, Barton D, Hull D, Parker R, et al. Liraglutide safety and efficacy in patients with non-alcoholic steatohepatitis (LEAN): a multicentre, double-blind, randomised, placebo-controlled phase 2 study. Lancet 2016;387:679-690.
74. Bjelakovic G, Nikolova D, Gluud LL, Simonetti RG, Gluud C. Mortality in randomized trials of antioxidant supplements for primary and secondary prevention: systematic review and meta-analysis. JAMA 2007;297:842-857
75. Schurks M, Glynn RJ, Rist PM, Tzourio C, Kurth T. Effects of vitamin E on stroke subtypes: meta-analysis of randomised controlled trials. BMJ 2010;341:c5702.
76. Klein EA, Thompson IM, Jr., Tangen CM, Crowley JJ, Lucia MS, Goodman PJ, et al. Vitamin E and the risk of prostate cancer: the Selenium and Vitamin E Cancer Prevention Trial (SELECT). JAMA 2011;306:1549-1556.

77. Dufour JF, Oneta CM, Gonvers JJ, Bihl F, Cerny A, Cereda JM, et al. Randomized placebo-controlled trial of ursodeoxycholic acid with vitamin e in nonalcoholic steatohepatitis. Clin Gastroenterol Hepatol 2006;4:1537-1543.
78. Leuschner UF, Lindenthal B, Herrmann G, Arnold JC, Rossle M, Cordes HJ, et al. High-dose ursodeoxycholic acid therapy for nonalcoholic steatohepatitis: a double-blind, randomized, placebo-controlled trial. Hepatology 2010;52:472-479.
79. Lindor KD, Kowdley KV, Heathcote EJ, Harrison ME, Jorgensen R, Angulo P, et al. Ursodeoxycholic acid for treatment of nonalcoholic steatohepatitis: results of a randomized trial. Hepatology 2004;39:770-778.
80. Neuschwander-Tetri BA, Loomba R, Sanyal AJ, Lavine JE, Van Natta ML, Abdelmalek MF, et al. Farnesoid X nuclear receptor ligand obeticholic acid for non-cirrhotic, non-alcoholic steatohepatitis (FLINT): a multicentre, randomised, placebo-controlled trial. Lancet 2015;385:956-965.
81. Aron-Wisnewsky J, Warmbrunn M, Nieuwdorp M, Clement K. Nonalcoholic fatty liver disease: modulating gut microbiota to improve severity? Gastroenterology 2020.
82. Wong VW, Won GL, Chim AM, Chu WC, Yeung DK, Li KC, et al. Treatment of nonalcoholic steatohepatitis with probiotics. A proof-of-concept study. Ann Hepatol 2013; 12:256-262.
83. Baldwin D, Chennakesavalu M, Gangemi A. Systematic review and meta-analysis of Roux-en-Y gastric bypass against laparoscopic sleeve gastrectomy for amelioration of NAFLD using four criteria. Surg Obes Relat Dis 2019;15:2123-2130.
84. Lassailly G, Caiazzo R, Buob D, Pigeyre M, Verkindt H, Labreuche J, et al. Bariatric Surgery Reduces Features of Nonalcoholic Steatohepatitis in Morbidly Obese Patients. Gastroenterology 2015;149:379-388; quiz e315-376.
85. Pais R, Barritt ASt, Calmus Y, Scatton O, Runge T, Lebray P, et al. NAFLD and liver transplantation: Current burden and expected challenges. J Hepatol 2016;65:1245-1257.

Chapter 2

Electron microscopic observations in perfusion-fixed human non-alcoholic fatty liver disease biopsies

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is a widespread liver disease in Western society, but its multifactorial pathogenesis is not yet fully understood. Ultrastructural analysis of liver sinusoidal endothelial cells (LSECs) in animal models and *in vitro* studies, shows defenestration early in the course of NAFLD, promoting steatosis. LSECs and fenestrae are important in the transport of lipids across the sinusoids. However, human ultrastructural data, especially on LSECs and fenestrae, are scarce.

This study aimed to explore the ultrastructural changes in perfusion-type fixed liver biopsies of NAFLD patients with and without non-alcoholic steatohepatitis (NASH), with a special focus on LSECs and their fenestration.

Liver biopsies from patients with NAFLD were fixed using two perfusion techniques, jet and injection fixation, for needle and wedge biopsies respectively. Ultrastructural changes were studied using transmission electron microscopy. NASH was diagnosed by bright-field microscopy using the SAF score (Steatosis, Activity, Fibrosis).

Thirty-seven patients were included, of which twelve patients (32.4%) had NASH. Significantly less defenestration was found in NASH compared to noNASH samples (p=0.002). Other features, *i.e.* giant mitochondria and fenestrae size did not differ between groups. Furthermore, we described new structures, *i.e.* single cell steatonecrosis and inflammatory fat follicles that were observed in both groups.

Concluding, defenestration was more common in noNASH compared to NASH in human liver samples. Defenestration was not related to the degree of steatosis or fibrosis. We speculate that defenestration can be a protective mechanism in simple steatosis which is lacking in NASH.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most widespread liver disease in Western society, with a prevalence up to 25%.[1] It is strongly associated with overweight and obesity.[2,3] The term NAFLD comprises both steatosis and non-alcoholic steatohepatitis (NASH).[4] NASH is an inflammatory state that develops in about 30% of NAFLD patients with significantly increased morbidity and mortality due to progression to fibrosis, cirrhosis and hepatocellular carcinoma.[5,6]

The pathophysiology of NASH is still poorly understood and is considered to have a multifactorial aetiology, involving dietary factors, host genetics, insulin resistance, lipotoxicity and microbiome perturbations.[7] Studying ultrastructural changes in human liver biopsies may help to provide leads to further elucidate the pathophysiology of NAFLD.

Only a limited number of human studies have been performed that focused on ultrastructural changes in NAFLD. These studies showed autophagic vesicles[8] and lipolysosomes in liver biopsies of NAFLD patients,[9] with giant lipolysosomes in case of severe steatosis.[9] Furthermore, Mallory-Denk body formation, 'cellular enlargement' and dilatation of the endoplasmic reticulum have been observed in NASH patients.[10] Mitochondria have also been studied because of their role in beta-oxidation, showing crystalline inclusions and giant mitochondria in some, but not all cases of NASH.[11,12] Ahishali et al. reported larger mitochondrial diameter in NASH livers compared to fatty livers, but did not report a difference in the presence of giant mitochondria comparing NASH and fatty liver samples.[13] However, a shortcoming of studies on ultrastructural changes is the rather small numbers of NAFLD and/or NASH patients included, ranging from 4 to 23, and only one study made a direct comparison of adult NAFLD patients with versus without NASH.[13] Animal models and in vitro studies have also revealed interesting ultrastructural findings in NAFLD, especially with regard to liver sinusoidal endothelial cells (LSECs). LSECs are highly specialised endothelial cells at the interface between blood derived from the visceral adipose tissue and the gut on one side and hepatocytes and hepatic stellate cells on the other side. They have a unique phenotype, they lack a basement membrane and have fenestrae organised into sieves that regulate the transport of macromolecules, including lipids and lipoproteins across the sinusoid.[14] Studies in animal models and *in vitro* studies have shown that the diameter and porosity of fenestrae show a negative correlation with excessive lipid[15,16] and endotoxin[17] exposure. A study in mice, deficient of Plasmalemma

Vesicle Associated Protein (PLVAP), a protein coding gene required for the formation of fenestrae in LSECs, showed development of extensive multivesicular steatosis, steatohepatitis and fibrosis at around three weeks of life.[18] This suggests that loss of fenestrae (defenestration) of LSECs may be an early step in the course of NAFLD.[14] Due to the limited preservation of LSECs and their fenestrae by immersion fixation, as used in previous human electron microscopy studies, data on LSECs and their fenestrae in human samples is scarce.[14] During immersion fixation, the fixative penetrates the tissue by diffusion, on its way fixating the tissues from the periphery to the centre. This leaves the centre of the tissue unfixed for almost an hour[19] and only allows studying the well-fixed peripheral parenchymal cells. Sinusoidal cells and sinusoids collapse and lose details due to this improper fixation.[20]
To overcome this problem, we developed perfusion fixation methods for needle and wedge biopsies,[19,20] which significantly improved the preservation of the sinusoids, sinusoidal cells and endothelial fenestrae, enabling high resolution transmission electron microscopy (TEM) of all hepatic cell types in human liver biopsies. Depending on the size of the tissue sample, wedge versus needle liver biopsy, different perfusion fixation techniques need to be used, namely injection[19] and jet[20] fixation respectively. Our aim was to explore the ultrastructural changes in human liver biopsies of NAFLD patients with and without NASH, with a special focus on LSECs and their fenestration. We hypothesised that fenestrae size and number decrease as the severity of NAFLD increases.

Methods

Patients and tissue sampling

For the current explorative study, samples were available from patients with NAFLD undergoing a liver biopsy either for clinical reasons or as part of a research project. From all participants paraffin embedded needle biopsies were available for standard bright-field microscopy evaluation. Needle liver biopsies were obtained using a 16G needle and a biopsy gun (Bard Magnum Reusable Core Biopsy System). Additionally, biopsy samples were collected for transmission electron microscopy (TEM) analysis. Liver wedge biopsy specimen were taken from 19 patients with NAFLD, between October 2016 and October 2017 in the Zuyderland Medical Centre. Before inclusion, NAFLD had been

diagnosed using imaging. The samples were collected during surgery (cholecystectomy [n=1] and bariatric surgery [n=18]) as part of a study identifying non-invasive markers for NASH. This study was approved by the Medical Ethics Committee Zuyd and registered at clinicaltrials.gov (NCT02717000). All participants gave written informed consent prior to participation. Another 20 needle liver biopsies for TEM analysis were available as residual material from routine liver biopsies obtained from patients with a diagnosis of NAFLD based upon routine histopathological evaluation for clinical reasons at MUMC+ between 2011 and 2016. The Medical Ethics Committee AzM/UM had approved the use of the samples for the present study. The current study was performed according the declaration of Helsinki (latest amendment of 2013, Fortaleza, Brazil).

Clinical evaluation

The medical history, body mass index (BMI) and routine biochemical tests including bilirubin, alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), alanine aminotransferase (ALT), aspartate aminotransferase (AST), fasting glucose, ferritin, total cholesterol, triglycerides, high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C), were collected prospectively for the patients from the Zuyderland Medical Centre using standardised registration forms and by retrospective review of the patients' medical files for the clinical MUMC+ patients.

Bright-field microscopy

Liver needle biopsies for bright-field microscopy analysis were fixed in 4% formalin and embedded in paraffin. Hematoxylin-eosin stained sections were used for NAFLD scoring according to the SAF score (Steatosis, Activity and Fibrosis)[21] by an experienced liver pathologist blinded to the clinical context and laboratory parameters. NASH was defined by score A≥2 including at least 1 point for ballooning and 1 point for inflammation. A score of A<2 or the absence of ballooning was defined as 'noNASH'.[21]

Transmission electron microscopy (TEM)

TEM analysis was performed on needle and wedge liver biopsies. From 19 patients, wedge biopsies were obtained during surgery at Zuyderland Medical Centre. A portion of approximately 5 by 5 mm was subjected to injection

fixation for TEM analysis, according to the method described by Wisse *et al.*[19] In short, a needle is used to inject the fixative into the wedge biopsy at a rate of approximately 1 ml/min. As injection of the fixative is not possible in needle biopsies, due to the smaller size, the technique of jet fixation as described by Vreuls *et al.* was used in 20 needle biopsy samples. This technique sprays the fixative with a constant pressure onto the liver tissue.[20]

All perfusion-fixed biopsies were studied by TEM (FEI Tecnai G2 Spirit BioTWIN iCorr microscope, FEI company, USA) at the Nanoscopy Unit of Maastricht University (M4I). The magnification was calibrated with a 463 nm line grid, before representative images of each biopsy were taken. Measurements of fenestrae diameter were done with Image J software (Rasband, National Institutes of Health, Bethesda, Maryland, USA). Defenestration was defined by no or hardly any fenestrae found in well-fixed livers with preserved fine structured details, after scrutinising the whole samples.

Statistical analysis

Patient characteristics and ultrastructural findings were reported as absolute numbers and percentages for categorical variables and as mean with standard deviation (SD) or median and range, according to the normality of the distribution, for continuous variables. Findings were compared between NAFLD patients with and without NASH according to the SAF score using the independent t-test for normally and the Mann-Whitney U test for non-normally distributed data. Differences in dichotomous variables between groups were tested using the Chi-square test with Fisher exact when necessary. A two-sided p-value of <0.05 was considered statistically significant. All calculations were performed using SPSS version 24 statistical software (IBM Statistics for Macintosh, Chicago, IL, USA).

Results

Baseline characteristics

In total, 19 wedge biopsy samples and 20 needle biopsy samples were available for TEM evaluation and 39 paraffin embedded needle biopsies for standard bright-field microscopic evaluation.

One needle biopsy sample was of insufficient quality to perform a reliable bright-field microscopic scoring and was therefore excluded. All other biopsies contained a sufficient number of portal tracts to allow for correct evaluation of the hepatic architecture. With regard to the TEM samples, one needle biopsy was of insufficient quality for evaluation by TEM and was therefore excluded.
Finally, biopsies of 37 patients were included in the analysis (see Supplementary Figure S2.1: flowchart of patient routing). Patient characteristics, BMI, routine biochemical test results at time of inclusion and bright-field microscopic results for the total group and subgroups (NASH/noNASH) are presented in Table 2.1.
All patients had NAFLD on initial histologic evaluation or imaging, which was confirmed in 30 of them upon revision of the biopsy specimen for the current study. Twelve patients were classified as having NASH according to the SAF score. None of the patients had cirrhosis (F4) or overt pathology other than NAFLD on bright-field microscopic analysis. Patients with NASH showed higher ALP, ALT and AST levels as compared to noNASH patients (all $p<0.05$).

TEM analysis of Liver Sinusoids

An overview of the TEM findings comparing NASH with noNASH samples is given in Table 2.2 and findings of individual patients are presented in Supplementary Table S2.1. The results of both jet and injection fixation are comparable and meet the criteria for good fixation as given by Wisse *et al.*,[19] being open sinusoids, intact topography and fine structure for all cell types. Examples of representative images, for both jet and injection perfusion fixation are given in Supplementary Figure 2.2A/B. Thirty-three of the 37 samples showed successful perfusion fixation, the other four samples showed results comparable to immersion fixation.

Table 2.1 Baseline characteristics of included participants

	Participants (n=37)	NASH (n=12)	noNASH (n=25)	P-value
Gender (male/female)	17/20 (46%/54%)	6/6 (50/50%)	11/14 (44/56%)	0.732
Age (years)	48.4 (11.9)	49.3 (14.5)	48.0 (10.7)	0.769
BMI (kg/m^2)	34.6 (6.0)	31.9 (5.9)	35.9 (5.7)	0.063
Bilirubin (µmol/L)	11.0 (6.7)	14.0 (7.8)	9.6 (5.8)	0.106
ALP (U/L)	119.9 (81.9)	170.7 (114.8)	95.6 (46.1)	0.048
GGT (U/L)	121.4 (180.6)	228.7 (268.6)	69.8 (85.9)	0.068
AST (U/L)	45.4 (34.1)	74.0 (41.6)	31.6 (18.5)	0.005
ALT (U/L)	69.9 (63.9)	118.3 (68.9)	46.6 (47.1)	0.001
Fasting glucose* (mmol/L)	6.4 (3.5)	8.6 (6.1)	5.2 (0.0)	0.171
Ferritin* (µg/L)	238.7 (226.1)	335.8 (158.1)	202.3 (239.6)	0.133
HDL-C* (mmol/L)	1.18 (0.32)	1.3 (0.4)	1.1 (0.3)	0.258
LDL-C* (mmol/L)	2.97 (1.37)	3.4 (1.9)	2.7 (1.0)	0.272
Total cholesterol* (mmol/L)	5.03 (1.59)	5.7 (2.0)	4.7 (1.3)	0.075
Triglycerides* (mmol/L)	2.03 (1.41)	2.9 (2.0)	1.6 (0.8)	0.051
SAF-Steatosis				
S0	7 (19%)	0 (0%)	7 (28%)	
S1	21 (57%)	6 (50%)	15 (60%)	
S2	6 (16%)	3 (25%)	3 (12%)	
S3	3 (8%)	3 (25%)	0 (0%)	
SAF-Activity				
A0	5 (13%)	0 (0%)	5 (20%)	
A1	18 (49%)	0 (0%)	18 (72%)	
A2	5 (13%)	3 (25%)	2 (8%)	
A3	8 (22%)	8 (67%)	0 (0%)	
A4	1 (3%)	1 (8%)	0 (0%)	
SAF-Fibrosis				
F0	7 (19%)	0 (0%)	7 (28%)	
F1	16 (43%)	2 (17%)	14 (56%)	
F2	13 (35%)	9 (75%)	4 (16%)	
F3	1 (3%)	1 (8%)	0 (0%)	
F4	0 (0%)	0 (0%)	0 (0%)	

Table 2.1 shows an overview of the baseline characteristics of the included participants, showing demographic characteristics, blood tests and histology results based on bright-field microscopy. Continuous variables are expressed as mean ±standard deviation (SD), and tested using the independent sample T-test. Categorical variables are expressed as absolute figures and percentages and tested using the Chi-square test with Fisher exact when necessary. A two-sided p-value of <0.05 was considered statistically significant. Abbreviations: Body mass index (BMI), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), alanine aminotransferase (ALT), aspartate aminotransferase (AST), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), non-alcoholic steatohepatitis (NASH). *For these parameters 1-3 subjects are missing per subgroup NASH or noNASH.

Table 2.2. Ultrastructural findings in perfusion fixed human NAFLD samples studied using Transmission Electron Microscopy

	Participants (YES/Total)	NASH (YES/Total)	noNASH (YES/Total)	p-value
Fenestrae observed*	17/33	10/11	7/22	0.002
(Yes/total [%])	(46%)	(91%)	(32%)	
Steatosis present	32/37	12/12	20/25	
(Yes/total [%])	(87%)	(100%)	(80%)	
Sinusoidal embolism	10/32	1/12	9/20	0.050
(Yes/total [%])	(31%)	(8%)	(45%)	
Kupffer cells	32/37	10/12	22/25	
(Yes/total [%])	(87%)	(83%)	(87%)	
Kupffer cell lipofuscin	16/32	5/10	11/22	>0.999
(Yes/total [%])	(50%)	(50%)	(50%)	
Fibrosis	24/37	9/12	15/25	0.476
(Yes/total [%])	(65%)	(75%)	(60%)	
Parenchymal lysosomes	35/37	10/12	25/25	
(Yes/total [%])	(95%)	(83%)	(100%)	
Parenchymal lipofuscin	31/35	8/10	23/25	0.561
(Y/total [%])	(89%)	(80%)	(92%)	
Mitochondria	35/37	11/12	24/25	
(Y/total [%])	(95%)	(92%)	(96%)	
Megamitochondria	18/35	4/11	14/24	0.289
(Yes/total [%])	(51%)	(36%)	(58%)	
SCSN	9/37	5/12	4/25	0.116
(Yes/total [%])	(24%)	(42%)	(16%)	

*4 samples are missing due to insufficient perfusion fixation. Abbreviations: non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), single cell steatonecrosis (SCSN).

Sinusoidal endothelial cells

Perfusion fixation (Figure 2.1A) preserved the fine structure of the liver sinusoids (Figure 2.1B) and sinusoidal cells including the fenestrae (Figure 2.1C), and was successful in 33 out of 37 samples. Fenestrae were observed in seventeen cases, of which in eleven cases the fenestrae size could be measured. Of these eleven cases, six had NASH and five did not. The median fenestrae size did not differ in samples of patients with NASH versus noNASH (121.0nm (116.0-138.0) versus 120.0nm (93.0-128.0) respectively, p=0.792, Supplementary Table S2.2).

Defenestration was identified in 16 cases, for a representative image see Figure 2.1D. Significantly less liver biopsies samples of patients with NASH (9%) showed defenestration as compared to the noNASH group (68%) (p=0.002) (Table 2.2 and Supplementary Table S2.3). Defenestration was not associated with the severity of steatosis or fibrosis (data not shown, p>0.3 for both).

Sinusoidal lipid embolisms, defined as single large fat droplets blocking the sinusoid, were observed in nine of the noNASH and one of the NASH samples (45% versus 8% respectively, p=0.050) (Figure 2.1E/1F).

Kupffer cells and Stellate cells

Kupffer cells were observed in 32 cases (Figure 2.2A). In these cells, lipofuscin was seen in five of ten NASH (50%) and 11 of 22 noNASH samples (50%) (p>0.999) (Figure 2.2B). No apparent signs of storage disease of any substance were found.
In 32 cases, stellate cells were visualised using TEM (Figure 2.2C), which showed a normal appearance. Intracellular lipid droplets were observed in 30 of these cases.
Using TEM, fibrosis was found in 24 cases (75% versus 60%, NASH versus noNASH, respectively, p=0.476), of which the majority was seen perisinusoidal. In the bright-field microscopic analysis, 16 cases showed F1, 13 F2, one F3 and seven cases showed no fibrosis.

TEM analysis of Parenchymal cells

TEM analysis of parenchymal cells could be performed in all 37 perfusion-fixed samples. Perfusion fixation showed intact fat droplets within the parenchymal cells (Figure 2.3A).
Giant mitochondria in the parenchymal cells, as defined by a large size, abnormal shape and crystalline inclusions, were seen in 18 cases (36% in NASH versus 58% in noNASH, p=0.289, Figure 2.3B). Parenchymal lipofuscin was observed in 31 cases (83% in NASH and 91% in noNASH, p=0.561, Figure 2.3C, Table 2.2).

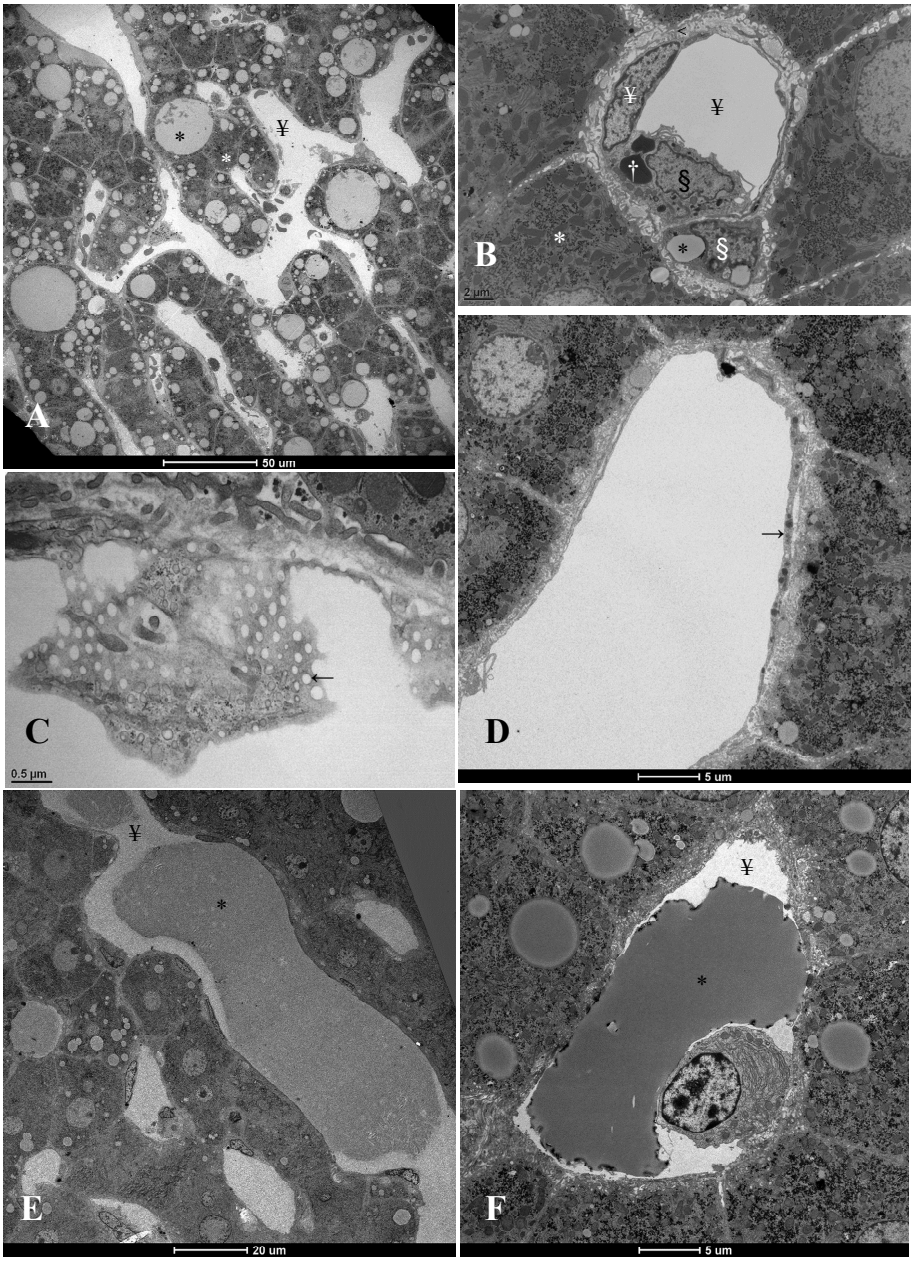

Figure 2.1 **A** Low magnification TEM of a NASH liver needle biopsy sample, fixed by jet fixation. The picture shows parenchymal cells (white *) with different sizes of fat droplets (*) and open sinusoids (¥). Note the openness of the sinusoids and the preserved topography and fine structure of all cells present. **B** Four cell types are united in this figure of a jet-

fixed noNASH liver needle biopsy, showing parenchymal cells (white *) together with one liver sinusoidal endothelial cell (white ¥), a Kupffer cell (§) and a stellate cell (white §) (respectively from top to bottom), together surrounding an open sinusoidal lumen (¥) free of plasma and blood cells. This picture shows the normal topographical arrangement of these cell types that is lost in immersion fixation. A normal space of Disse (<) separates the endothelial lining from the parenchymal cells. The Kupffer cell shows endocytosed electron dense material (white †) and the stellate cell (previously named fat-storing cells or Ito-cells) contains two fat droplets (*). **C** TEM picture at higher magnification from a jet-fixed needle biopsy of a NASH patient. Fenestrae within the endothelial lining are observed when the lining is obliquely cut during sectioning for TEM. Because of the isotonicity of the fixative and the inclusion of the cells in epoxy resin, the size of the fenestrae is presumed to be preserved to represent the size in vivo. Fenestrae are grouped in thin, slightly electron dense cytoplasmic sieve plates (pointed out by the black arrow). **D** TEM figure of a defenestrated sinusoid of a noNASH patient. There are no or hardly any fenestrae in well-fixed livers with preserved fine structured details (black arrow). This condition is called pseudocapillarisation, capillarisation or defenestration by some authors (wedge biopsy, injection fixation). **E and F** Fat droplets (*) in the liver sinusoids (¥) of noNASH patients (sinusoidal lipid embolism) (injection fixation). Phagocytoses of whole fat droplets or parts of it, by e.g. Kupffer cells or other cells was not observed. Furthermore, it is unclear how long fat droplets remain in the sinusoids before being degraded.

In addition to 'known' ultrastructural findings, a new observation was seen which occurred in a minority of samples (24%). First, gigantic fat droplets were observed in the middle of the parenchymal cell showing disintegration of the cytoplasm and disruption of the usual arrangement of organelles (42% in NASH versus 16% in noNASH, p=0.116, Figure 2.4A). In addition, a more complex, but probably further developed situation was observed, in which inflammatory cells, mainly polymorphonuclear granulocytes, and red blood cells, formed a circle around the large fat droplet (Figure 2.4B). Cells with these features did not colocalise, but were spread out in the steatotic region. In none of the samples apoptosis was observed.

Second, a substance was found in several fat droplets across our study samples; namely a filamentous precipitate, that resembles the filamentous structure of Mallory-Denk-Bodies (Supplementary Figure S2.3A/S2.3B).

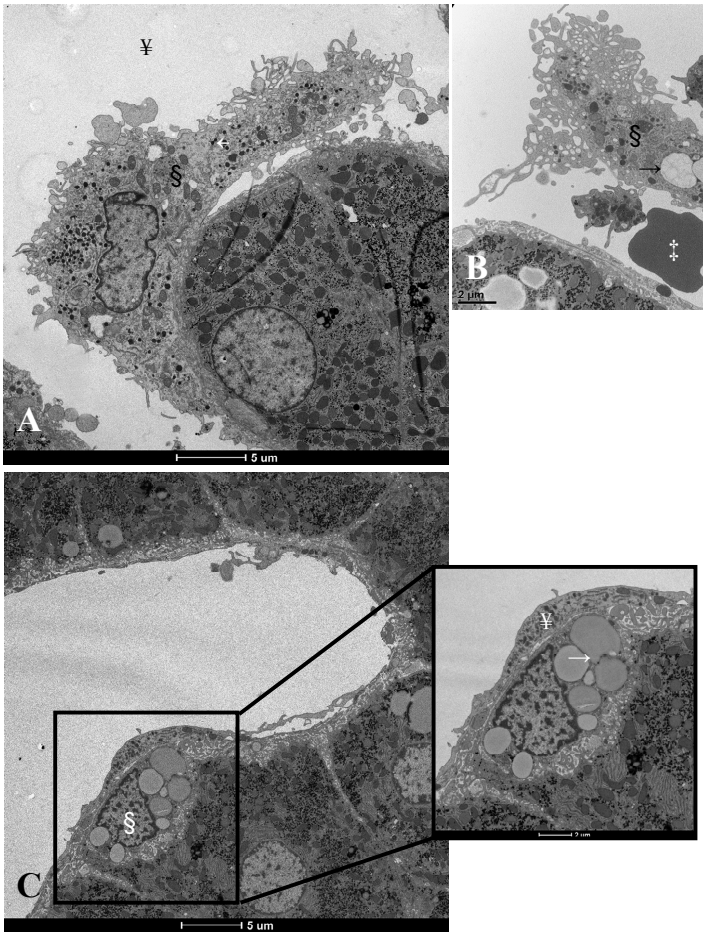

Figure 2.2 **A** TEM picture of a Kupffer cell (§) on top of a parenchymal cell. Kupffer cells have an irregular shape and a rough surface. These cells are exposed to the sinusoidal (¥) blood flow and phagocytose foreign cells and materials present in the blood. This particular Kupffer cell shows the absence of phagocytosed material, but displays the presence of numerous small lysosomes which are the electron dense granules in the cytoplasm (pointed out by the white arrow). Injection fixation of a wedge biopsy of a noNASH patient. **B** TEM picture of a Kupffer cell (§) in a jet-fixed needle biopsy of a noNASH patient, showing the presence of phagocytosed material of different density (lipofuscin) (black arrow). Other cell types, such as a red blood cell (at the bottom) (white ‡) and several white blood cells (white +), probably lymphocytes, are present. **C** TEM picture (needle biopsy, noNASH patient, jet-fixation) of a stellate cell (white §) which is an inhabitant of the space of Disse. In other words, these cells are always covered by the endothelial lining (white ¥ in inset). Therefore, the cell is shielded from the sinusoidal blood flow. Within the cytoplasm of the cell we observe fat droplets (white arrow in inset) that can vary in number and size. Inset: enlargement of the stellate cell.

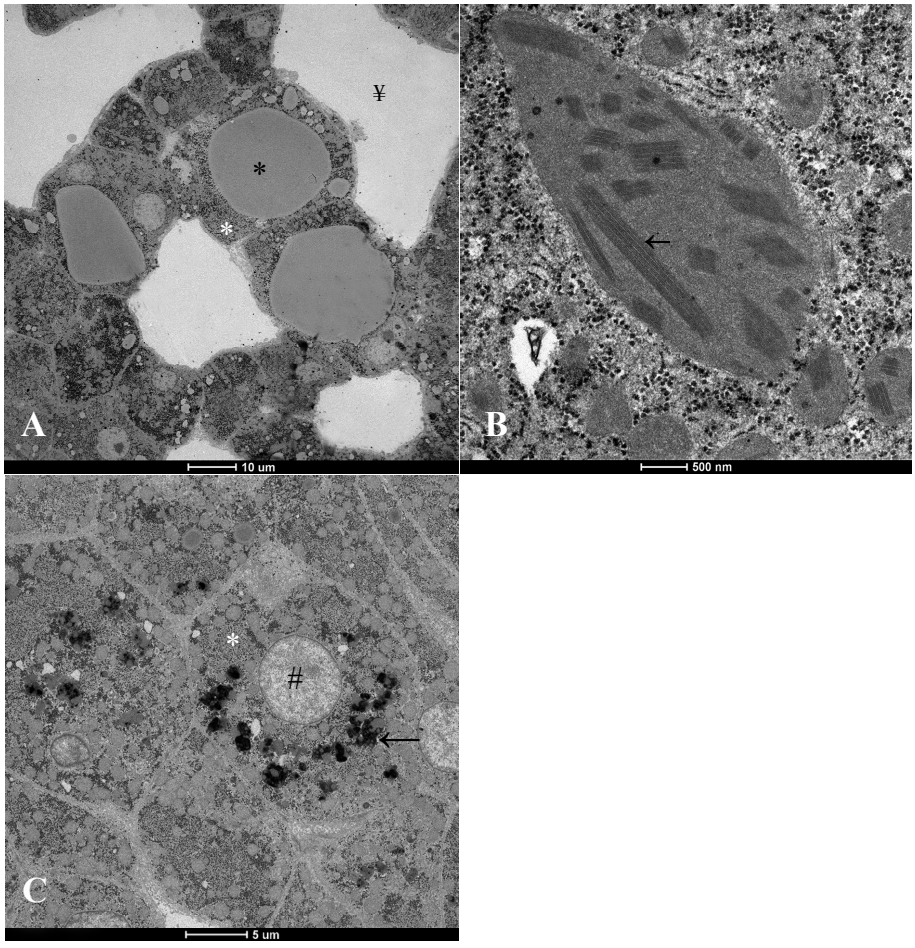

Figure 2.3 **A** In steatosis, the fat droplets (*) in parenchymal cells (white *) can grow to proportions that largely exceed the size of a normal parenchymal cell. In most cases we only find a single large fat droplet per cell. Open sinusoids (¥) are present at several sides of the parenchymal cells. (wedge biopsy, injection fixation, noNASH patient). **B** A giant mitochondrion is shown (wedge biopsy, injection fixation, noNASH patient). The size of giant mitochondria varies between normal and gigantic. Always, crystals (black arrow) can be recognized in the matrix of these intriguing organelles. Apart from the variety in size, the giants also show different shapes and content. They can be round, oval, cylindric, or branched. Their occurrence is regional, but it is not yet clear whether their presence is zonal (portal or central). They never populate all parenchymal cells. They are absent in the livers of experimental animals like rats, mice, rabbits, guinea pig. **C** TEM picture of a parenchymal cell in which the nucleus (#) is surrounded by lipofuscin containing residual bodies (black arrow), which are in fact old (retired) lysosomes with reduced or even absent digesting capacity. Normally lysosomes are oriented to the bile canaliculi, but in the case of larger numbers, they are more dispersed in the cell. (wedge biopsy, injection fixation, noNASH patient).

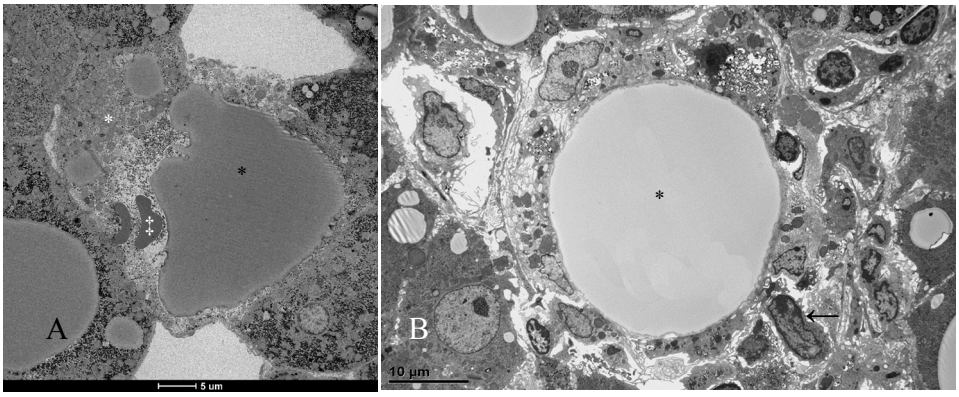

Figure 2.4 **A** TEM figure with a clear example of single cell steatonecrosis (SCSN). The cell shows disorganisation of the cytoplasm resulting in a free suspension of organelles (white *), probably in Brownian movement. A large fat droplet (*) is supposed to have introduced necrosis in this parenchymal cell. The organelles are more or less confined within the boundaries of the cell or the still intact endothelial lining. Two red blood cells (white ‡) are present. It is not yet clear to what extend this situation contributes to developing the inflammatory aspect of steatohepatitis (wedge biopsy, injection fixation, noNASH patient). **B** TEM figure of an inflammatory fat follicle (IFF). Remarkably, in IFF a monolayer of polymorphonuclear neutrophils (PMNs) (black arrow) is surrounding the fat droplet (*). The presence of PMN's suggest that these cells take care of digesting the large fat droplet and at the same time might contribute to the inflammatory process (needle biopsy, jet fixation, NASH patient)

Discussion

The current study used perfusion-type fixation to explore the ultrastructural changes in liver biopsy samples of NAFLD patients, comparing patients with and without NASH Successful perfusion was obtained in 89.2% (33/37) as judged by the following criteria: open sinusoid, intact topography and fine structure for all cell types, including endothelial fenestrae.[19,22] Significantly less defenestration was found in NASH compared to noNASH samples, while fenestrae size and the presence of other features, *i.e.* giant mitochondria, did not differ between groups. Furthermore, new structures were identified, *i.e.* single cell steatonecrosis and inflammatory fat follicles, in a subset of both groups.

This study used perfusion fixation as it allows for proper fixation of all cell types, including the sinusoids and sinusoidal cells, whereas in immersion fixation only the well-fixed peripheral parenchymal cells can be studied.[19] TEM liver biopsy samples in this study consisted of needle and wedge liver biopsies, therefore two

different perfusion techniques were used. Injection fixation was used in wedge biopsies. With this technique the fixative is injected into the wedge biopsy.[19] It is not possible to use this technique in needle biopsies, due to the smaller tissue sample. Therefore, the technique of jet fixation, spraying the fixative onto the biopsy sample, was used in the needle biopsies.[20] Vice versa, it is not possible to use jet fixation in wedge biopsies as the size of the wedge biopsies is too big and perfusion would not take place. The results of both jet and injection fixation are comparable to perfusion through the portal vein in animal studies.[23]

In the current study, special attention was paid to the LSECs and fenestrae since they serve as a sieve and play an important role in the transport of lipids across the sinusoid. The hypothesis of the study was that fenestrae size and number decrease as the severity of NAFLD increases. Unexpectedly, we showed that defenestration was more prevalent in the noNASH samples compared to NASH samples. In addition, defenestration was not related to the degree of steatosis or fibrosis.

Based on the current findings and the fact that defenestration is seen more in simple steatosis, instead of in NASH, one could argue that defenestration is a protective mechanism to handle the excess in lipids and perhaps other toxic substances, such as bacterial products. If defenestration plays a role in inhibiting the entry of chylomicron remnants into the space of Disse[18]; it could protect the liver from receiving more lipids. On the other hand, defenestration also impairs the passage of VLDL excretion towards the sinusoidal lumen, thereby causing hepatic lipid accumulation.[14] Furthermore, inhibition of lipid uptake by defenestration has been proposed to lead to stimulation of *de novo* lipid (DNL) synthesis and thereby induces steatosis.[18] This is due to the fact that hydroxymethylglutaryl-CoA reductase (HMGCR), the rate limiting enzyme for hepatocyte cholesterol synthesis, is no longer inhibited when chylomicron remnants and dietary cholesterol do not cross the fenestrae, thereby activating endogenous cholesterol synthesis in hepatocytes.[24] However, one could argue that in case of defenestration, DNL could be performed at a pace that does not overwhelm beta-oxidation and re-esterification within the liver. This would enable the liver to store the fat as triglycerides in lipid droplets, instead of metabolising them. This fits in the current view on the pathophysiology of NAFLD, which proposes that NASH and simple steatosis are independent twin conditions. Steatosis in NASH is being seen as a benign epiphenomenon rather than a causal factor of inflammation and fibrosis, which are the two main hallmarks of NASH.[25] In NASH, the metabolization of free fatty acids and cholesterol leads to formation of toxic lipid species. However, the underlying

mechanisms of the formation of lipotoxic intermediates at molecular levels remain unknown.[26] Whereas triglyceride accumulation in the form of lipid droplets in simple steatosis may serve as a non-toxic, safer form of storage for lipids in the liver.[27]
When comparing fenestrae size between NASH and noNASH samples, we found no significant difference (121.0 (116.0-138.0) nm in NASH and 120.0 (93.0-128.0) nm in noNASH). Fenestrae size in humans without liver pathology show a diameter of 107 (±1.5) nm.[28]
Our finding in human samples are not in line with the animal model findings of Herrnberger *et al.*,[18] studying PLVAP deficient mice. PLVAP is known to be critical for the development of fenestrae. PLVAP deficient mice developed extensive multivesicular steatosis, but also steatohepatitis and fibrosis at around three weeks of life.[18] This stresses the added value of human studies as animal models do not recapitulate all pathological features of human NAFLD or NASH. Furthermore, they do not capture the large inter-individual variation with regard to host genetics and environmental factors.[29]

The presence of giant mitochondria in this study showed no difference between patients with and without NASH, these findings are in line with Ahishali *et al.*[13] Giant mitochondria have been considered to represent an intermediate phase in evolution of steatohepatitis, regardless of the source of hepatic fat deposition.[12] However, their exact role in the pathophysiology in NAFLD is unclear. Based on our findings, they might play a role in NAFLD, but that role is not limited to steatohepatitis.

In the current study, new structures were identified and described, namely single cell steatonecrosis (SCSN) and inflammatory fat follicles (IFF). SCSN shows an enormous fat droplet in a single parenchymal cell in a well advanced stage of necrosis. In addition, this condition may further develop into a situation where the large central single fat droplet is surrounded with a monolayer of inflammatory cells, such as polymorphonuclear granulocytes, but also by red blood cells. We propose the term 'inflammatory fat follicle (IFF)' for this feature.
It has been shown that accumulating lipid droplets can mechanically distort the intracellular environment, as lipid droplets are stiffer than the surrounding cytosol, thereby leading to cellular damage. Baldini *et al.*[30] have studied the biomechanics of cultured steatotic hepatocytes and showed that hepatocyte cell stiffness and cell viability are directly influenced by the extent of triglyceride accumulation and the size of lipid droplets. Furthermore, the swelling of

parenchymal cells may lead to the narrowing of local sinusoids, and contribute to vascular resistance. The size of the fat droplets in severe steatosis exceeds the diameter of parenchymal cells and sinusoids considerably.[31] Previous research in rats showed an increase in intrahepatic resistance with hemodynamic impact in relation to severe steatosis.[32] This study observed sinusoidal lipid embolisms in both NASH and noNASH patients. One could argue that these can also be a contributing factor to vascular resistance.

When interpreting the results of this study, it is important to note several limitations. Liver biopsy samples were obtained via two different routes. Needle biopsy samples were collected via percutaneous liver biopsies in outpatients with liver enzyme abnormalities as part of clinical care. Wedge biopsy samples were collected during bariatric surgery in overweight patients. Consequently, the liver biopsies originate from different parts of the liver, more peripherally for the wedge biopsies versus more centrally for the needle biopsies. Furthermore, it should be noted that the patients who underwent bariatric surgery followed a calorie restricted diet before surgery.[33] Defenestration has been linked to diets varying in content of macronutrients in animal models,[15,34] suggesting that a diet based on caloric restriction reduces defenestration. However, a larger part of the noNASH group underwent bariatric surgery and a calorie restricted diet, still showing more defenestration compared to the NASH group. Therefore, we think that this calorie restricted diet did not have a major impact on our results.
It should also be noted that only small parts of the liver are evaluated by electron microscopy. It is known that histologic lesions in NAFLD are unevenly distributed throughout the liver and a sampling error can occur when studying lesions using bright-field microscopy.[35] Therefore, it is difficult to quantify parameters for the total organ. In seven patients with steatosis on imaging, steatosis could not be confirmed, when revising the liver biopsy with bright-field microscopy for the current study. Further supporting that sampling errors cannot be excluded. However, when excluding these samples from the analysis for defenestration, the difference between groups remained significant ($p=0.005$, data not shown).
To our knowledge, this is the first study on fenestrae in perfusion fixed human NAFLD liver samples using TEM. Previous studies used immersion fixation,[8-13,36,37] leaving the centre of the biopsy improperly fixed, which does not allow for accurate analysis of sinusoids and sinusoidal cells.[20] Second, a relatively large sample size was included, directly comparing NASH versus

noNASH samples, whereas other studies included smaller sample sizes,[8-12,37] or studied animal models.[18,38]

In conclusion, we demonstrated that perfusion type-fixation techniques are feasible, for both needle and wedge liver biopsies, in human NAFLD liver samples. By using these techniques, new ultrastructural details, such as SCSN and IFF, were revealed. An almost complete lack of fenestrae (defenestration) in NAFLD patients without NASH was found. Defenestration may be a protective mechanism to handle the excess in lipids and perhaps other toxic substances and may serve as a possible explanation why some patients develop NASH and others do not. Future studies are needed to further investigate the role of these findings within the multifactorial aetiology of NASH.

References

1. Younossi ZM, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M. Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. Hepatology 2016;64:73-84.
2. Bellentani S, Scaglioni F, Marino M, Bedogni G. Epidemiology of non-alcoholic fatty liver disease. Dig Dis 2010;28:155-161.
3. Chalasani N, Younossi Z, Lavine JE, Diehl AM, Brunt EM, Cusi K, et al. The diagnosis and management of non-alcoholic fatty liver disease: practice guideline by the American Gastroenterological Association, American Association for the Study of Liver Diseases, and American College of Gastroenterology. Gastroenterology 2012; 142:1592-1609.
4. Hashimoto E, Taniai M, Tokushige K. Characteristics and diagnosis of NAFLD/NASH. J Gastroenterol Hepatol 2013;28 Suppl 4:64-70.
5. McCullough AJ. Pathophysiology of nonalcoholic steatohepatitis. J Clin Gastroenterol 2006;40 Suppl 1:S17-29.
6. Ong JP, Pitts A, Younossi ZM. Increased overall mortality and liver-related mortality in non-alcoholic fatty liver disease. J Hepatol 2008;49:608-612.
7. Friedman SL, Neuschwander-Tetri BA, Rinella M, Sanyal AJ. Mechanisms of NAFLD development and therapeutic strategies. Nat Med 2018;24:908-922.
8. Fukuo Y, Yamashina S, Sonoue H, Arakawa A, Nakadera E, Aoyama T, et al. Abnormality of autophagic function and cathepsin expression in the liver from patients with non-alcoholic fatty liver disease. Hepatol Res 2014;44:1026-1036.
9. Iancu TC, Manov I, Shaoul R, Haimi M, Lerner A. What's in a name?-"Lipolysosome": ultrastructural features of a lipid-containing organelle. Ultrastruct Pathol 2013;37:293-303.
10. Caldwell S, Ikura Y, Dias D, Isomoto K, Yabu A, Moskaluk C, et al. Hepatocellular ballooning in NASH. J Hepatol 2010;53:719-723.
11. Caldwell SH, de Freitas LA, Park SH, Moreno ML, Redick JA, Davis CA, et al. Intramitochondrial crystalline inclusions in nonalcoholic steatohepatitis. Hepatology 2009;49:1888-1895.
12. Caldwell SH, Swerdlow RH, Khan EM, Iezzoni JC, Hespenheide EE, Parks JK, et al. Mitochondrial abnormalities in non-alcoholic steatohepatitis. J Hepatol 1999;31:430-434.
13. Ahishali E, Demir K, Ahishali B, Akyuz F, Pinarbasi B, Poturoglu S, et al. Electron microscopic findings in non-alcoholic fatty liver disease: is there a difference between hepatosteatosis and steatohepatitis? J Gastroenterol Hepatol 2010;25:619-626.
14. Hammoutene A, Rautou PE. Role of liver sinusoidal endothelial cells in non-alcoholic fatty liver disease. J Hepatol 2019;70:1278-1291.
15. Cogger VC, Mohamad M, Solon-Biet SM, Senior AM, Warren A, O'Reilly JN, et al. Dietary macronutrients and the aging liver sinusoidal endothelial cell. Am J Physiol Heart Circ Physiol 2016;310:H1064-1070.
16. Zhang Q, Liu J, Liu J, Huang W, Tian L, Quan J, et al. oxLDL induces injury and defenestration of human liver sinusoidal endothelial cells via LOX1. J Mol Endocrinol 2014;53:281-293.
17. Dobbs BR, Rogers GW, Xing HY, Fraser R. Endotoxin-induced defenestration of the hepatic sinusoidal endothelium: a factor in the pathogenesis of cirrhosis? Liver 1994;14:230-233.
18. Herrnberger L, Hennig R, Kremer W, Hellerbrand C, Goepferich A, Kalbitzer HR, et al. Formation of fenestrae in murine liver sinusoids depends on plasmalemma vesicle-associated protein and is required for lipoprotein passage. PLoS One 2014;9: e115005.
19. Wisse E, Braet F, Duimel H, Vreuls C, Koek G, Olde Damink SW, et al. Fixation methods for electron microscopy of human and other liver. World J Gastroenterol 2010;16:2851-2866.
20. Vreuls C, Wisse E, Duimel H, Stevens K, Verheyen F, Braet F, et al. Jet-fixation: a novel method to improve microscopy of human liver needle biopsies. Hepatology 2014;59:737-739.

21. Bedossa P, Poitou C, Veyrie N, Bouillot JL, Basdevant A, Paradis V, et al. Histopathological algorithm and scoring system for evaluation of liver lesions in morbidly obese patients. Hepatology 2012;56: 1751-1759.
22. Wisse E, Braet F, Luo D, De Zanger R, Jans D, Crabbe E, et al. Structure and function of sinusoidal lining cells in the liver. Toxicol Pathol 1996;24:100-111.
23. Wisse E. An electron microscopic study of the fenestrated endothelial lining of rat liver sinusoids. J Ultrastruct Res 1970;31:125-150.
24. Fraser R, Cogger VC, Dobbs B, Jamieson H, Warren A, Hilmer SN, et al. The liver sieve and atherosclerosis. Pathology 2012;44:181-186.
25. Yilmaz Y. Review article: is non-alcoholic fatty liver disease a spectrum, or are steatosis and non-alcoholic steatohepatitis distinct conditions? Aliment Pharmacol Ther 2012;36: 815-823.
26. Neuschwander-Tetri BA. Hepatic lipotoxicity and the pathogenesis of nonalcoholic steatohepatitis: the central role of nontriglyceride fatty acid metabolites. Hepatology 2010;52:774-788.
27. Bessone F, Razori MV, Roma MG. Molecular pathways of nonalcoholic fatty liver disease development and progression. Cell Mol Life Sci 2019;76:99-128.
28. Wisse E, Jacobs F, Topal B, Frederik P, De Geest B. The size of endothelial fenestrae in human liver sinusoids: implications for hepatocyte-directed gene transfer. Gene Ther 2008;15:1193-1199.
29. Castro RE, Diehl AM. Mistakes in mouse models of nonalcoholic steatophepatitis and how to avoid them. In; 2018.
30. Baldini F, Bartolozzi A, Ardito M, Voci A, Portincasa P, Vassalli M, et al. Biomechanics of cultured hepatic cells during different steatogenic hits. J Mech Behav Biomed Mater 2019;97:296-305.
31. Wisse E, De Zanger RB, Charels K, Van Der Smissen P, McCuskey RS. The liver sieve: considerations concerning the structure and function of endothelial fenestrae, the sinusoidal wall and the space of Disse. Hepatology 1985;5:683-692.
32. Francque S, Laleman W, Verbeke L, Van Steenkiste C, Casteleyn C, Kwanten W, et al. Increased intrahepatic resistance in severe steatosis: endothelial dysfunction, vasoconstrictor overproduction and altered microvascular architecture. Lab Invest 2012; 92:1428-1439.
33. De Munck TJI, Verhaegh PLM, Verbeek J, Verheij J, Greve JW, Jonkers D, et al. Crashing NASH in Patients Listed for Bariatric Surgery. Obes Surg 2019;29:1012-1014.
34. Jamieson HA, Hilmer SN, Cogger VC, Warren A, Cheluvappa R, Abernethy DR, et al. Caloric restriction reduces age-related pseudo-capillarization of the hepatic sinusoid. Exp Gerontol 2007;42:374-378.
35. Ratziu V, Charlotte F, Heurtier A, Gombert S, Giral P, Bruckert E, et al. Sampling variability of liver biopsy in nonalcoholic fatty liver disease. Gastroenterology 2005;128:1898-1906.
36. Le TH, Caldwell SH, Redick JA, Sheppard BL, Davis CA, Arseneau KO, et al. The zonal distribution of megamitochondria with crystalline inclusions in nonalcoholic steatohepatitis. Hepatology 2004;39:1423-1429.
37. Lotowska JM, Sobaniec-Lotowska ME, Bockowska SB, Lebensztejn DM. Pediatric non-alcoholic steatohepatitis: the first report on the ultrastructure of hepatocyte mitochondria. World J Gastroenterol 2014;20: 4335-4340.
38. Kanai M. Ultrastructural and biochemical studies of lipolysis by lipolysosomes in chick hepatocytes. Cell Tissue Res 1989;255:559-565.

Supplementary data

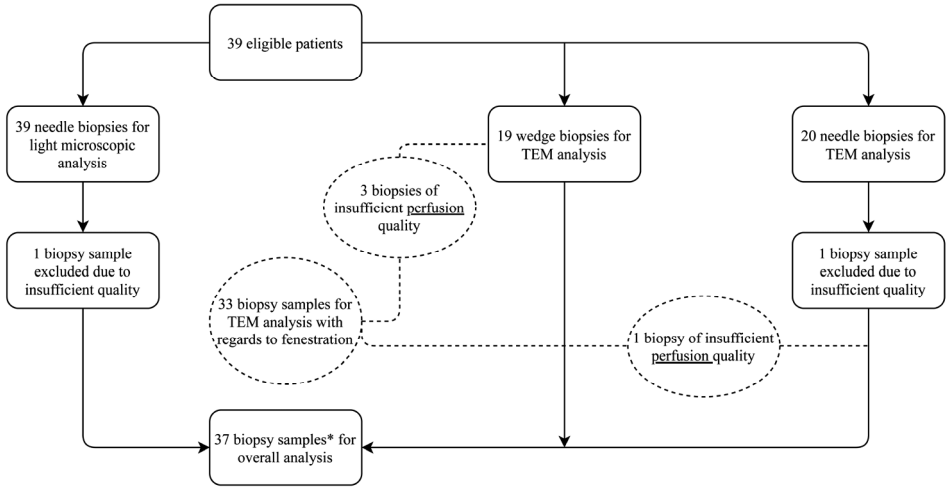

Figure S2.1 Flowchart of patient routing

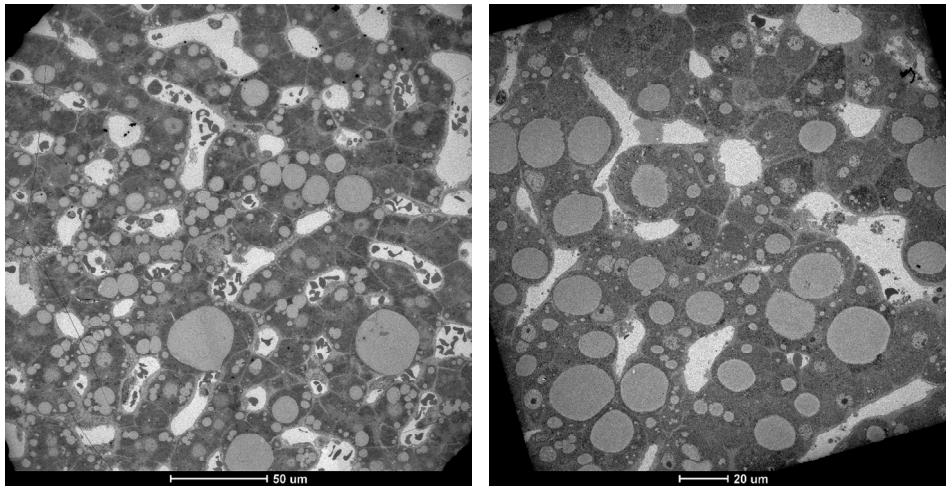

Figure S2.2 Figures **A** (left) and **B** (right) show parenchymal cells with different size of fat droplets and open sinusoids. Figure S2.2A is an image of a needle biopsy samples fixed by jet fixation, figure S2.2B is an image of a wedge biopsy sample fixed by injection fixation. Both figures show the openness of the sinusoids and the preserved topography and fine structure of cells.

Table S2.1 Ultrastructural findings in perfusion-fixed human NAFLD samples studied using TEM per participant

Participant	NASH	SAF score	Perfusion technique	TEM sample quality	Steatosis	Sinusoidal embolism	Parenchymal lysosomes	Parenchymal lipofuscin	Mitochondria	Giant-mitochondria	SCSN	Fenestrae observed	Kupffer cell	Kupffer cell lipofuscin	Stellate cells	Fibrosis
1	No	S1A1F1	Jet	+	+	-	+	+	+	-	+	+	+	-	+	+ (ps)
2	No	S1A1F1	Jet	+	+	+	+	+	+	+	+	+	+	+	+	+ (ps)
3	No	S0A0F0	Jet	+	+	-	+	+	+	+	-	+	+	+	+	+ portal
4	No	S1A0F1	Jet	+	+	-	+	+	-	-	-	+	+	+	+	+ (ps)
5	No	S0A1F1	Jet	+	+	+	+	+	+	+	-	+	+	-	+	+ (ps)
6	No	S2A1F1	Jet	+	+	+	+	+	+	-	-	+	+	+	+	+ (ps)
7	No	S1A1F0	Jet	+	+	-	+	+	+	-	-	-	+	+	+	-
8	No	S1A1F0	Injection	-	+	-	+	+	+	-	-	Insufficient perfusion	-	-	-	+ (ps)
9	No	S1A1F2	Injection	-	+	+	+	+	+	+	+	Insufficient perfusion	+	+	+	-
10	No	S1A1F1	Injection	+	+	-	+	+	+	-	-	-	+	+	+	+ (ps)
11	No	S0A1F0	Injection	+	+	-	+	+	+	+	-	-	+	-	+	+
12	No	S1A0F1	Injection	+	+	+	+	+	+	+	+	-	+	+	+	-
13	No	S2A1F1	Injection	+	+	+	+	+	+	+	-	-	+	+	+	-
14	No	S1A1F1	Injection	+	-	-	+	+	+	+	-	-	-	-	-	-
15	No	S1A1F2	Injection	+	+	+	+	+	+	+	-	-	+	+	+	+ (ps)
16	No	S1A1F1	Injection	+	+	-	+	+	+	+	-	+	+	-	+	+ (ps)
17	No	S1A1F0	Injection	+	-	-	+	+	+	+	-	-	+	-	-	-
18	No	S0A0F0	Injection	-	-	-	+	-	+	-	-	Insufficient perfusion	+	-	+	-
19	No	S1A1F1	Injection	+	-	-	+	+	+	-	-	-	+	-	-	+ (ps)
20	No	S1A1F2	Injection	+	+	-	+	+	+	-	-	-	+	-	+	+ (ps)
21	No	S0A0F1	Injection	+	+	-	+	+	+	+	-	-	+	+	+	+
22	No	S2A1F1	Injection	+	+	+	+	-	+	-	-	-	+	-	+	-
23	No	S0A1F0	Injection	+	+	-	+	+	+	-	-	-	-	-	+	-
24	No	S0A2F1	Injection	+	-	-	+	+	+	+	-	-	+	-	+	-
25	No	S1A2F2	Injection	+	+	+	+	+	+	+	-	-	+	-	+	+

Table S2.1 (continued)

Participant	NASH	SAF score	Perfusion technique	TEM sample quality	Steatosis	Sinusoidal embolism	Parenchymal lysosomes	Parenchymal lipofuscin	Mitochondria	Giant-mitochondria	SCSN	Fenestrae observed	Kupffer cell	Kupffer cell lipofuscin	Stellate cells	Fibrosis
26	Yes	S1A3F2	Jet	+	+	-	+	+	+	-	+	+	+	-	+	+
27	Yes	S2A2F2	Jet	+	+	-	+	-	+	+	-	+	+	-	+	+ (ps)
28	Yes	S3A3F1	Jet	+	+	-	+	+	+	-	+	+	+	+	+	+ (ps)
29	Yes	S1A3F3	Jet	+	+	+	+	+	+	+	-	+	+	-	+	+ (ps)
30	Yes	S2A3F2	Jet	+	+	-	+	+	+	-	+	+	+	+	+	+ (ps)
31	Yes	S3A3F2	Jet	-	+	-	+	+	-	-	+	Insufficient perfusion	-	-	-	+ (ps)
32	Yes	S2A4F1	Jet	+	+	-	+	+	+	-	+	+	+	+	+	-
33	Yes	S1A3F2	Jet	+	+	-	-	-	+	-	-	+	+	+	+	-
34	Yes	S1A2F2	Jet	+	+	-	+	+	+	+	-	+	-	-	+	+ (ps)
35	Yes	S3A3F2	Jet	+	+	-	-	-	+	-	-	+	+	-	+	+
36	Yes	S1A2F2	Jet	+	+	-	+	+	+	+	-	-	+	-	+	+ (ps)
37	Yes	S1A3F2	Jet	+	+	-	+	-	+	-	-	+	+	+	+	-

Abbreviations: non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), steatosis-activity-fibrosis score (SAF score), transmission electron microscopy (TEM), single cell steatotonecrosis (SCSN), Maastricht University Medical Centre (MUMC), Zuyderland medical centre (Zuyd), perisinusoidal (ps).

Table S2.2 Fenestrae size in NASH vs noNASH

	NASH (n=6)	noNASH (n=5)
Fenestrae size (nm)[§]	121.0 (116.0-138.0)	120.0 (93.0-128.0)

[§] Fenestrae size could be measured in eleven samples. No statistically significant difference based on Mann-Whitney U test (p=0.792). Continuous variables are expressed as median (minimum-maximum), and categorical variables as absolute figures and percentages. Abbreviations: non-alcoholic steatohepatitis (NASH), nanometre (nm).

Table S2.3 Defenestration in NASH vs noNASH

	noNASH	NASH	Total
Fenestrae not present (defenestration)[§]	15	1	16
Fenestrae present	7	10	17
	22	11	33[*]

[*]4 biopsies are missing due to insufficient perfusion fixation for fenestrae scoring using transmission electron microscopy (TEM). [§]A statistical significant difference was found using Chi-square test, p=0.002 (two sided). Categorical variables are expressed as absolute figures. Abbreviations: non-alcoholic steatohepatitis (NASH).

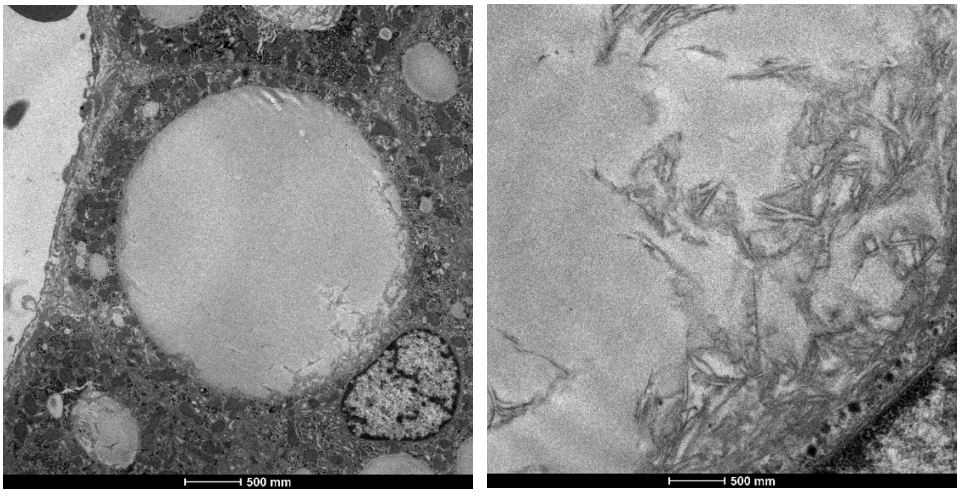

Figure S2.3 **A** (left) Large fat droplets sometimes contain precipitates of fibrillar and membranous materials. The amount can vary between a small quantity and sometimes abundant masses**.** This filamentous precipitate could be incompletely digested cytoskeletal material that due to its lipophilic nature is collected by the fat droplet. Importantly, no compartmenting intracellular membrane is to be seen between the fat droplet and the cytoplasm, giving free access to lipophilic substances. Perhaps, this filamentous material may be similar to the material of the Mallory Denk bodies (needle biopsy, jet fixation, noNASH patient). **B** (right) Transmission electron microscopy (TEM) picture at higher magnification showing a more detailed image of the membranous component of the precipitate. In our study fat is preserved due to type of fixation, resulting in this image in which we observe that lipophilic material is mixed well with the present fat content (needle biopsy, jet fixation, noNASH patient).

Chapter 3

Hyperferritinaemia in non-alcoholic fatty liver disease: iron accumulation or inflammation?

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*both authors contributed equally to this work

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Abstract

Hyperferritinaemia, observed in inflammation, in iron overload as well as in the combination of both, is found in about 30% of non-alcoholic fatty liver disease (NAFLD) patients. We summarised the evidence regarding the potential cause of hyperferritinaemia in NAFLD, as this may affect the indicated therapy.

A systematic literature search was conducted in EMBASE, PubMed, MEDLINE and the Cochrane library.

In the majority of NAFLD patients, hyperferritinaemia is due to inflammation without hepatic iron overload. In a smaller group, a dysmetabolic iron overload syndrome (DIOS) is found, showing hyperferritinaemia in combination with mild iron accumulation in the reticuloendothelial cells. The smallest group consists of NAFLD patients with haemochromatosis. Phlebotomy is only effective with hepatocellular iron overload and should not be the treatment when hyperferritinaemia is related to inflammation, whether or not combined with DIOS. Treatment with lifestyle changes is to date probably the more effective way until new medication is becoming available.

Introduction

Non-alcoholic fatty liver disease (NAFLD), strongly associated with overweight and obesity, is the most common liver disorder in Western society, with a global prevalence of around 25%.[1] NAFLD ranges from hepatic steatosis[2] to non-alcoholic steatohepatitis (NASH), progressive liver fibrosis, cirrhosis and an increased risk to develop hepatocellular carcinoma.[3] It is considered the hepatic manifestation of the metabolic syndrome in which a generalised systemic inflammation plays an important role in the initiation and progression of diabetes mellitus and cardiovascular diseases.[4,5] The incidence of NASH is expected to increase even further over the next decades, which stresses the need to elucidate factors involved in disease progression. Diagnostic markers are urgently needed to identify NAFLD patients that progress to advanced liver- and systemic diseases. Serum ferritin is increased in about 30% of patients with NAFLD.[6,7] Ferritin is an acute phase protein but also a storage protein for iron within cells, being most pronounced in hepatocytes and macrophages.[8,9] Small amounts of ferritin are present in the circulation reflecting the body iron stores in healthy control subjects. Elevated serum ferritin levels can also occur in patients with mutations in genes involved in iron homeostasis, such as hereditary haemochromatosis (HH) (*e.g.* *HFE* mutations),[10,11] or in the case of beta-thalassaemia trait.[12] Transferrin saturation levels are usually increased (>45%) in hyperferritinaemia caused by HH.[13]

In 34.5% - 51.5% of NAFLD patients dysmetabolic iron overload syndrome (DIOS) is present.[14,15] DIOS is characterised by hyperferritinaemia with only a mild increase of both liver and body iron stores. It is associated with various compounds of the metabolic syndrome in the absence of any identifiable genetic mutation or other causes of iron excess.[16] As iron plays a role via the Fenton reaction in the generation of oxidative stress and thereby causes disease progression, it is important to distinguish if hyperferritinaemia is due to classical hepatic iron overload or is the expression of inflammation. If hepatic iron overload in the classical pattern is the primary cause of hyperferritinaemia, therapeutic bloodletting is the only therapeutic option. While in inflammation-related hyperferritinaemia or in patients with DIOS, lifestyle changes such as a hypocaloric diet and physical activity are recommended instead of phlebotomies.

Therefore, the aim of this systematic literature search was to evaluate the role and value of hyperferritinaemia as a marker of iron overload or inflammation in patients with NAFLD.

Methods

Literature search

A systematic literature search was performed in PubMed, Cochrane Library, MEDLINE and EMBASE up to 31st of October 2017 using the following (truncated) keywords: `Non*alcoholic fatty liver disease', `NAFLD', `Fatty liver`, `Hepatic steatosis` or `Liver steatosis` in any combination with `Hyperferritin*' and `Ferritin` and `Dysmetabolic iron overload syndrome`, `Dysmetabolic iron overload`, `Dysmetabolic hepatic iron overload`, `Dysmetabolic hepatic overload syndrome`, `DIOS', `DHIOS` or `iron overload`. This resulted in a total number of 530 hits (117 PubMed, 9 hits Cochrane Library, 252 EMBASE and 152 MEDLINE).

We excluded studies in which the population primarily consisted of patients with hyperferritinaemia, obesity, diabetes and/or metabolic syndrome when they did not report on the role of hyperferritinaemia in NAFLD. We also excluded studies performed *in vitro,* in animals, in minors, in languages other than English, case reports, conference abstracts, reviews, and duplicates.

Titles and abstracts of all 530 hits were screened, leaving 152 articles, of which the full text was checked. The initial screening was done by a single author (WM) and in case of doubt the reference was checked by a second author (PV). In case of disagreement on eligibility, the two reviewers came to consensus after discussing the article with a third reviewer (DJ or GK). Furthermore, eligible papers were cross-checked for references, which resulted in six additional papers. Finally, 42 papers were included in the current review (Figure 3.1). We assessed the quality of the included studies with regard to the risk of bias (Supplementary Table S3.1). Not all articles were described in detail also due to limited description of results applicable to the subject of the review.[17-19]

Results

HFE mutations and iron regulators in NAFLD

The frequency of mutations in genes involved in iron homeostasis has been studied in NAFLD patients with hyperferritinaemia[12,15,20-34] (Supplementary Table S3.2). The overall prevalence of HFE mutations for p.C282Y ranged from 0-7.9% and 5-23.5% for homozygosity and heterozygosity, respectively. For p.H63D the prevalence was between 1-5.6% and 13-44.4% for homozygosity

and heterozygosity, respectively. Some studies found a significantly higher prevalence of HFE mutations (*i.e.* for homozygosity[20] or heterozygosity[20,28,29,30] of p.C282Y and/or heterozygosity of p.H63D[28]) in NAFLD patients versus control subjects. The majority of studies however, did not show significant differences in the presence of HFE mutations between NAFLD patients and control subjects.[12,15,24-27,31,33] Furthermore, no relation was observed between HFE mutations in NAFLD patients with high versus low serum ferritin[22] or with the presence or absence of iron overload.[23,32]

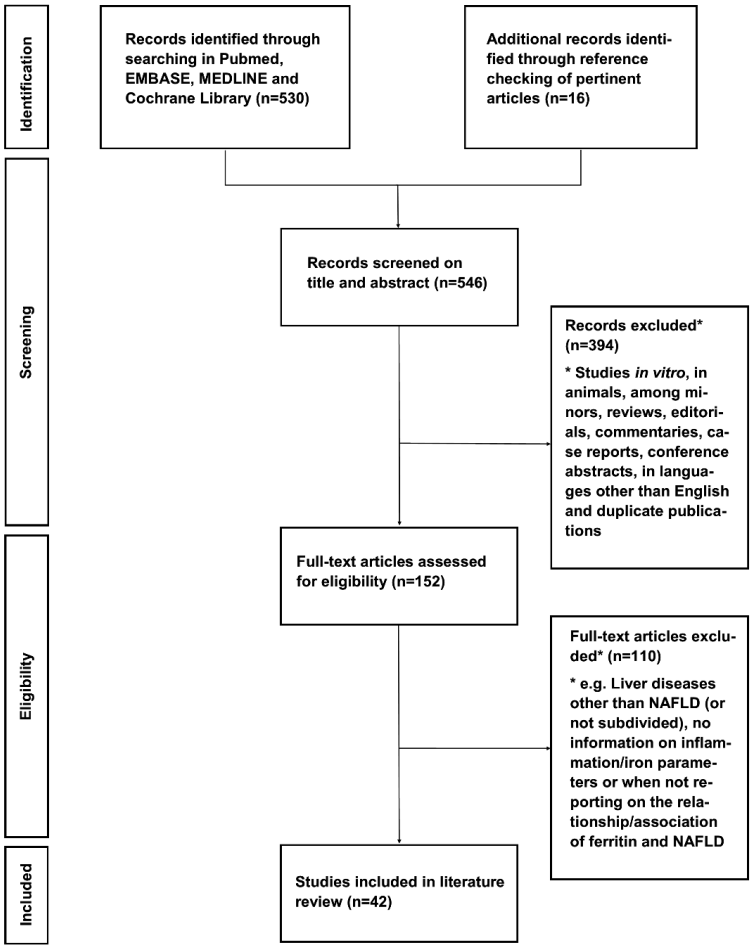

Figure 3.1. **Flowchart study selection process.** Abbreviation: Non-alcoholic fatty liver disease (NAFLD).

In addition to the HFE mutations, the beta-thalassaemia trait, alpha-1-antitripsin mutation (AAT) and the p.Ala736Val variant of transmembrane protease serine 6 (TMPRSS6) were investigated in NAFLD. A significantly higher prevalence of the AAT mutation was found in NAFLD patients (±10%) compared to healthy control subjects (±3.5%).[12,21] The prevalence of beta-thalassaemia was 9.1% in patients with NAFLD, there was no prevalence available in the control group.[12] One study assessing the prevalence of the p.Ala736Val variant of transmembrane protease serine 6, did not find a significant difference between NAFLD patients and healthy control subjects.[25]

The regulators of iron homeostasis focussing on serum transferrin receptor-1 (TfR1), TfR2, divalent metal transporter-1 (DMT-1), ferroportin, hepcidin and hemojuvelin were also studied in NAFLD patients, but results were inconsistent.[12,21,23-25,34-41]

No significant correlations were found between NAFLD patients (NASH and hepatic steatosis (HS)) compared to control subjects in duodenal or serum DMT-1 mRNA levels,[23,39,41] TfR1 and 2 mRNA levels,[23,35,36,41] and serum, duodenal and hepatic ferroportin concentration.[23,24,35,38,39,41] Increased hepcidin levels were found in three studies[23,35,39] measuring hepcidin in NAFLD patients (NASH and HS) compared to control subjects. Furthermore, increased hepcidin levels were found to be associated with hepatic lobular inflammation and NAFLD activity scores (NAS)[40] and iron in reticuloendothelial cells[12] (Supplementary Table S3.2).

An important up-stream regulator of hepcidin expression is hemojuvelin. Two studies[23, 37] investigating hemojuvelin found lower levels in NAFLD patients versus control subjects. Lower copper levels were associated with higher ferritin levels and increased prevalence of siderosis and hepatic iron in NAFLD patients.[24] Hepatic 8-oxodG levels, a product generated by hydroxyl radicals, was found to be positively correlated with body and hepatic iron deposition markers in NAFLD patients.[42]

Different patterns of hepatic iron deposition

The pattern of hepatic iron deposition can vary between iron deposition in the hepatocytes (classical iron overload), iron deposition in the reticuloendothelial cells or a combination of both cell types also called the mixed pattern. The histological NAS score,[43] assessing histological injury of NAFLD patients, is found to be significantly higher in patients with iron depositions in the reticuloendothelial cells,[14,22,44] when compared to iron depositions in

hepatocytes (classical iron overload) or to iron deposition in the mixed patter.[44] Patients with depositions in reticuloendothelial cells or mixed pattern were also found to have significantly higher serum ferritin than those with no iron overload or the hepatocyte pattern.[44] Furthermore, also a correlation was found between elevated serum ferritin levels and increased NAS.[22]

Hyperferritinaemia related to inflammation

In addition to the standard inflammatory serum biomarkers measured in NAFLD patients, ferritin is often found to be significantly higher in NASH patients when compared to controls or patients with HS.[27,36,37,42,45-47] In several of these studies,[36,37,45] serum iron parameters were also investigated, however no significant difference in iron status was found between the groups.

A wide range of indirect and direct inflammatory parameters were found to be significantly higher in NAFLD or NASH patients when compared to control subjects (*e.g.* aspartate aminotransferase, thioredoxin, C-reactive protein, alpha-2-macroglobulin, ceruloplasmin, malondialdehyde, hepatic 8-oxodG, nitric oxide, interleukin-6, interleukin-8 and tumour necrosis factor alpha). Most studies also found ferritin levels to be significantly higher in NASH patients compared to controls and/or patients with HS and NAFLD patients compared to control subjects[27,36,37,42,45-49] (Supplementary Table 3.2). Most of the studies showed no signs of biochemical or histological iron overload to explain the higher ferritin levels.[2,27,36,37,45,48,49] Ferritin showed a significant positive correlation with lobular inflammation,[2,22,44] portal inflammation,[2,50] higher NAS,[22,44] severity of steatosis,[22,26,44,51] presence of fibrosis,[2,22,26,44,51] presence of NASH[48] and ballooning.[22,44]

Effect of interventions on hyperferritinaemia in NAFLD

The proposed treatment of hyperferritinaemia depends on its aetiology. Moderate to vigorous physical exercise showed to decrease steatosis accompanied with significantly improved levels of serum ferritin as well as lipid peroxidation and adiponectine.[52] Eicosapentaenoic acid, an antilipidemic agent also showed to improve hepatic steatosis, fibrosis hepatic ballooning and lobular inflammation as well as ferritin levels in NASH patients.[53]

Phlebotomy therapy in NAFLD patients showed a significant decrease in ferritin and iron status[23,42,54,55] but also in parameters related to inflammation, *e.g.* alanine aminotransferase,[23,42,54] tumour necrosis factor-alfa[23] and hepatic 8-oxodG levels (a DNA base-modified product generated by hydroxy radicals).[42]

In contrast, Adams *et al.* did not find improvement in liver enzymes, hepatic fat or insulin resistance during ferritin reduction by phlebotomy in patients with NAFLD in a randomised, controlled trial comparing phlebotomy and life style advices with life style advices only.[55]

Discussion

This literature review aimed to summarise the evidence for iron overload and/or inflammation as a possible cause of hyperferritinaemia observed in patients with NAFLD. In more severe forms of NAFLD like NASH and advanced fibrosis, systemic inflammation is present in the majority of patients.[22] This low grade systemic inflammation has generally no characteristic laboratory abnormalities, but hyperferritinaemia is frequently observed and this raises the question whether this is an expression of this low grade systemic inflammation in NAFLD.

With this extended literature review, three groups could be distinguished. First, the majority of NAFLD patients that have hyperferritinaemia without evidence of hepatic iron overload or HFE mutations. These NAFLD patients are most likely to have hyperferritinaemia related to ferritin release from damaged hepatocytes[56] and/or related to the systemic inflammatory status (Figure 3.2). Secondly, hyperferritinaemia in NAFLD can also be explained as part of DIOS. In which inflammation up-regulates the hepcidin levels resulting in impaired iron export from the cells causing diminished iron availability[23] which can lead to mild hepatic iron overload in reticuloendothelial system cells whether or not combined with iron in hepatocytes. About 30% of NAFLD patients have DIOS, which consists of hyperferritinaemia in combination with only a mild increase of both liver and body iron stores associated with various compounds of the metabolic syndrome in the absence of any identifiable genetic mutation or other causes of iron excess.[16] The finding of a normal or moderately increased transferrin saturation supports DIOS. Iron overload in DIOS probably plays a prominent role in the pathophysiology of NAFLD, as it can generate reactive oxygen species and thereby lead to oxidative stress[57] causing severe cellular dysfunction, organ damage and promotes the development of insulin resistance and hepatocellular inflammation attributing to NAFLD progression.[58,59]

The third group is relatively small, consisting of NAFLD patients with identifiable p.C282Y homozygote HFE mutations (up to 7.9%). In the majority of these patients the increased serum ferritin levels are found in combination with

elevated transferrin saturation (>45%). Therefore, screening for haemochromatosis mutations in NAFLD with hyperferritinaemia should be considered in case transferrin saturation is also elevated.[60] The influence of the iron regulators (*i.e.* TfR1/2, ferroportin) was the subject of many studies however, no consistent results were found.

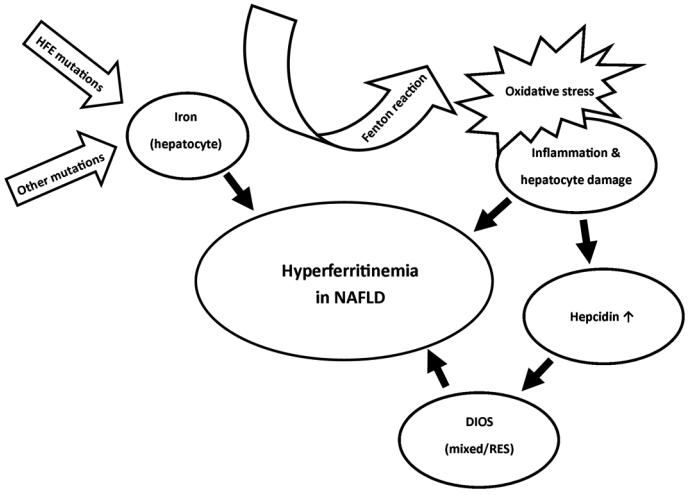

Figure 3.2 Hyperferritinaemia is observed in case of inflammation or in case of iron overload. In non-alcoholic fatty liver disease (NAFLD) a minority of patients has significant hepatic iron overload, caused by mutations in the haemochromatosis gene or other mutations. However, in the majority of patients, there is no evidence of hepatic iron overload, in this case inflammation and hepatocyte damage can contribute to elevated serum ferritin levels. An up-regulation of hepcidin levels in inflammation can result in impaired iron export from the cells, which can lead to mild hepatic iron overload in hepatocytes, and/or reticuloendothelial system cells (RES). Excess iron will contribute to the disease progression, since it will generate reactive oxygen species and lead to oxidative stress through the Fenton reaction.

Liver biopsy is still the gold standard as diagnostic tool in NAFLD staging and is also useful to measure iron status. However, many patients are not willing to undergo a biopsy. MRI is a good alternative to measure the liver iron concentration (LIC) to determine iron accumulation in patients with hyperferritinaemia. The reference value for the LIC is below 36 μmol/g dry weight but the LIC can also be elevated without liver iron excess (*e.g.* in patients with obesity or the metabolic syndrome). For this reason it is suggested to only diagnose iron overload in case the LIC exceeds 150 μmol /g dry weight.[16,61] It

should be noted that others also use three times the upper limit of normal.[62] When increased iron accumulation is suspected, patients should be screened for haemochromatosis and therapeutic phlebotomies should be considered. In case of a hyperferritinaemia accompanied by a normal transferrin saturation there is no need for HFE screening or therapeutic phlebotomy since the hyperferritinaemia is most likely related to inflammation whether or not combined with DIOS. In these patients phlebotomy has not been proven more effective than lifestyle changes.[59] In case of a clear inflammatory state, anti-inflammatory treatment and lifestyle changes should be considered.[16]
Based on the above, we suggest a diagnostic and therapeutic algorithm for the approach of NAFLD patients with hyperferritinaemia (Figure 3.3).

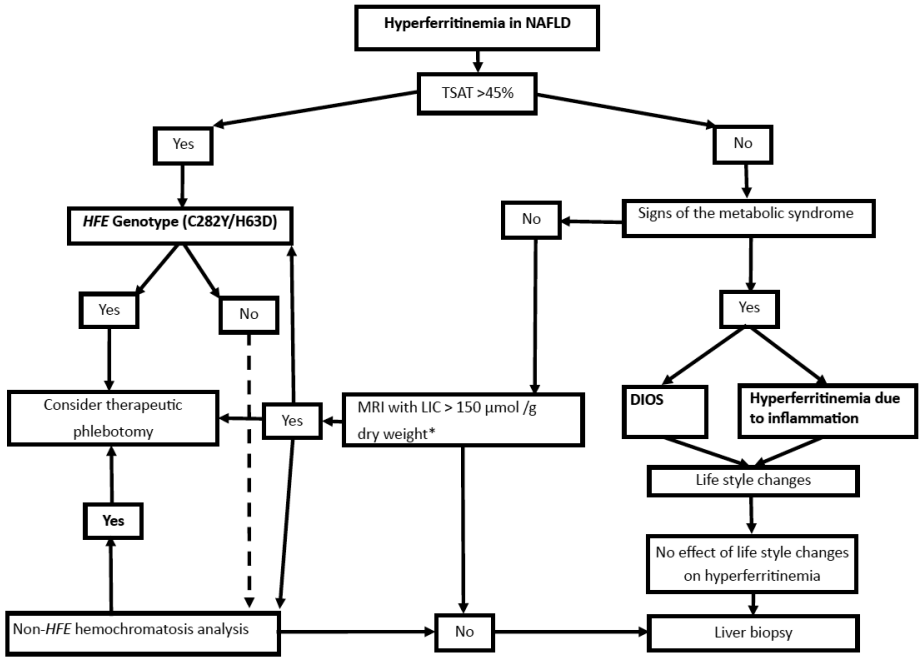

Figure 3.3 Diagnostic and therapeutic algorithm for the approach of hyperferritinaemia in patients with NAFLD. *Advice based on Deugnier et al. 2017. Abbreviations: Non-alcoholic fatty liver disease (NAFLD), Transferrin saturation (TSAT), dysmetabolic iron overload syndrome (DIOS), liver iron concentration (LIC).

This review has several strengths, its focused question with appropriate inclusion criteria, an extended search over a widespread period of years in four databases. We also assessed the quality of the included articles with regard to

the risk of bias (Supplementary Table S3.1). A major limitation is the heterogeneity of the included studies with regard to their objectives, study population and methods. Furthermore, the studies are often based on relatively small retrospective and/or selective cohorts, which makes the results difficult to interpret. Besides, different methods were used to diagnose NAFLD and NASH, and liver biopsy was not always used as golden standard.[33,34,36] Another limitation is the possibility of selection bias in the studies, as some included NAFLD/NASH patients were referred for HFE mutation screening because of their hyperferritinaemia enlarging the a priori chance for positive mutations.[20,30] Also, multiple studies by the same authors are included and it cannot be ruled out that overlapping populations were used.

In conclusion, in the majority of cases, hyperferritinaemia in NAFLD seems to be mainly related to inflammation. In some cases this can lead to mild hepatic iron accumulation in reticuloendothelial- or mixed pattern as an expression of DIOS, that is strongly related to the metabolic syndrome. Only in case of elevated transferrin levels, typically seen in classical iron overload (hepatocyte pattern) screening for HFE mutations should be performed. Phlebotomy is only effective when classical iron overload is proven. Lowering iron content probably decreases the generation of oxidative stress that plays a role in liver- and systemic inflammatory reactions. Large population studies will be necessary to evaluate the role of hyperferritinaemia in the different stages of NAFLD.

References

1. Younossi ZM, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M. Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. Hepatology 2016;64:73-84.
2. Manousou P, Kalambokis G, Grillo F, Watkins J, Xirouchakis E, Pleguezuelo M, et al. Serum ferritin is a discriminant marker for both fibrosis and inflammation in histologically proven non-alcoholic fatty liver disease patients. Liver Int 2011;31:730-739.
3. Neuschwander-Tetri BA. Fatty liver and the metabolic syndrome. Curr Opin Gastroenterol 2007;23:193-198.
4. Lindsay RS, Howard BV. Cardiovascular risk associated with the metabolic syndrome. Curr Diab Rep 2004;4:63-68.
5. Koh KK, Han SH, Quon MJ. Inflammatory markers and the metabolic syndrome: insights from therapeutic interventions. J Am Coll Cardiol 2005;46:1978-1985.
6. Moirand R, Mendler MH, Guillygomarc'h A, Brissot P, Deugnier Y. Non-alcoholic steatohepatitis with iron: Part of insulin resistance-associated hepatic iron overload? [2] (multiple letters). J Hepatol 2000;33:1024-1026.
7. Trombini P, Piperno A. Ferritin, metabolic syndrome and NAFLD: Elective attractions and dangerous liaisons. J Hepatol 2007;46:549-552.
8. Kernan KF, Carcillo JA. Hyperferritinemia and inflammation. Int Immunol 2017;29:401-409.
9. Worwood M: Ferritin. In. Volume 4: Longman Group UK Ltd, 1990; 259-269.
10. Adams PC, Reboussin DM, Barton JC, McLaren CE, Eckfeldt JH, McLaren GD, et al. Hemochromatosis and iron-overload screening in a racially diverse population. N Engl J Med 2005;352:1769-1778.
11. Brissot P, Pietrangelo A, Adams PC, de Graaff B, McLaren CE, Loreal O. Haemochromatosis. Nat Rev Dis Primers 2018;4:18016.
12. Valenti L, Canavesi E, Galmozzi E, Dongiovanni P, Rametta R, Maggioni P, et al. Beta-globin mutations are associated with parenchymal siderosis and fibrosis in patients with non-alcoholic fatty liver disease. J Hepatol 2010;53:927-933.
13. Qaseem A, Aronson M, Fitterman N, Snow V, Weiss KB, Owens DK. Screening for hereditary hemochromatosis: a clinical practice guideline from the American College of Physicians. Ann Intern Med 2005;143: 517-521.
14. Nelson JE, Wilson L, Brunt EM, Yeh MM, Kleiner DE, Unalp-Arida A, et al. Relationship between the pattern of hepatic iron deposition and histological severity in nonalcoholic fatty liver disease. Hepatology 2011;53:448-457.
15. Valenti L, Fracanzani AL, Bugianesi E, Dongiovanni P, Galmozzi E, Vanni E, et al. HFE genotype, parenchymal iron accumulation, and liver fibrosis in patients with nonalcoholic fatty liver disease. Gastroenterology 2010;138:905-912.
16. Deugnier Y, Bardou-Jacquet E, Laine F. Dysmetabolic iron overload syndrome (DIOS). Presse Med 2017;46:e306-e311.
17. Akin K, Beyler AR, Kaya M, Erden E. The importance of iron and copper accumulation in the pathogenesis of non-alcoholic steatohepatitis. Turk J Gastroenterol 2003;14: 228-233.
18. Duseja A, Das R, Nanda M, Das A, Garewal G, Chawla Y. Nonalcoholic steatohepatitis in Asian Indians is neither associated with iron overload nor with HFE gene mutations. World J Gastroenterol 2005;11:393-395.
19. Uraz S, Aygun C, Sonsuz A, Ozbay G. Serum iron levels and hepatic iron overload in nonalcoholic steatohepatitis and chronic viral hepatitis. Dig Dis Sci 2005;50:964-969.
20. George DK, Goldwurm S, MacDonald GA, Cowley LL, Walker NI, Ward PJ, et al. Increased hepatic iron concentration in nonalcoholic steatohepatitis is associated with increased fibrosis. Gastroenterology 1998;114: 311-318.
21. Valenti L, Dongiovanni P, Piperno A, Fracanzani AL, Maggioni M, Rametta R, et al. Alpha 1-antitrypsin mutations in NAFLD: high prevalence and association with altered iron metabolism but not with liver damage. Hepatology 2006;44:857-864.

22. Kowdley KV, Belt P, Wilson LA, Yeh MM, Neuschwander-Tetri BA, Chalasani N, et al. Serum ferritin is an independent predictor of histologic severity and advanced fibrosis in patients with nonalcoholic fatty liver disease. Hepatology 2012;55:77-85.
23. Aigner E, Theurl I, Theurl M, Lederer D, Haufe H, Dietze O, et al. Pathways underlying iron accumulation in human nonalcoholic fatty liver disease. Am J Clin Nutr 2008;87:1374-1383.
24. Aigner E, Theurl I, Haufe H, Seifert M, Hohla F, Scharinger L, et al. Copper availability contributes to iron perturbations in human nonalcoholic fatty liver disease. Gastroenterology 2008;135:680-688.
25. Valenti L, Rametta R, Dongiovanni P, Motta BM, Canavesi E, Pelusi S, et al. The A736V TMPRSS6 polymorphism influences hepatic iron overload in nonalcoholic fatty liver disease. PLoS One 2012;7:e48804.
26. Bugianesi E, Manzini P, D'Antico S, Vanni E, Longo F, Leone N, et al. Relative Contribution of Iron Burden, HFE Mutations, and Insulin Resistance to Fibrosis in Nonalcoholic Fatty Liver. Hepatology 2004;39:179-187.
27. Yoneda M, Nozaki Y, Endo H, Mawatari H, Iida H, Fujita K, et al. Serum ferritin is a clinical biomarker in Japanese patients with nonalcoholic steatohepatitis (NASH) independent of HFE gene mutation. Dig Dis Sci 2010;55:808-814.
28. Bonkovsky HL, Jawaid Q, Tortorelli K, LeClair P, Cobb J, Lambrecht RW, et al. Non-alcoholic steatohepatitis and iron: increased prevalence of mutations of the HFE gene in non-alcoholic steatohepatitis. J Hepatol 1999;31:421-429.
29. Chitturi S, Weltman M, Farrell GC, McDonald D, Liddle C, Samarasinghe D, et al. HFE mutations, hepatic iron, and fibrosis: Ethnic-specific association of NASH with C282Y but not with fibrotic severity. Hepatology 2002; 36:142-149.
30. Valenti L, Dongiovanni P, Fracanzani AL, Santorelli G, Fatta E, Bertelli C, et al. Increased susceptibility to nonalcoholic fatty liver disease in heterozygotes for the mutation responsible for hereditary hemochromatosis. Dig Liver Dis 2003;35:172-178.
31. Zamin I, Jr., Mattos AA, Mattos AZ, Migon E, Bica C, Alexandre CO. Prevalence of the hemochromatosis gene mutation in patients with nonalcoholic steatohepatitis and correlation with degree of liver fibrosis. Arq Gastroenterologia 2006;43:224-228.
32. Deguti MM, Sipahi AM, Gayotto LC, Palacios SA, Bittencourt PL, Goldberg AC, et al. Lack of evidence for the pathogenic role of iron and HFE gene mutations in Brazilian patients with nonalcoholic steatohepatitis. Braz J Med Biol Res 2003;36:739-745.
33. Lin TJ, Lin CL, Wang CS, Liu SO, Liao LY. Prevalence of HFE mutations and relation to serum iron status in patients with chronic hepatitis C and patients with nonalcoholic fatty liver disease in Taiwan. World J Gastroenterol 2005;11:3905-3908.
34. Rametta R, Dongiovanni P, Pelusi S, Francione P, Iuculano F, Borroni V, et al. Hepcidin resistance in dysmetabolic iron overload. Liver Int 2016;36:1540-1548.
35. Tsuchiya H, Ashla AA, Hoshikawa Y, Matsumi Y, Kanki K, Enjoji M, et al. Iron state in association with retinoid metabolism in non-alcoholic fatty liver disease. Hepatol Res 2010;40:1227-1238.
36. Uysal S, Armutcu F, Aydogan T, Akin K, Ikizek M, Yigitoglu MR. Some inflammatory cytokine levels, iron metabolism and oxidan stress markers in subjects with nonalcoholic steatohepatitis. Clin Biochem 2011;44:1375-1379.
37. Boga S, Alkim H, Alkim C, Koksal AR, Bayram M, Yilmaz Ozguven MB, et al. The Relationship of Serum Hemojuvelin and Hepcidin Levels with Iron Overload in Nonalcoholic Fatty Liver Disease. J Gastrointestin Liver Dis 2015;24:293-300.
38. Dongiovanni P, Lanti C, Gatti S, Rametta R, Recalcati S, Maggioni M, et al. High fat diet subverts hepatocellular iron uptake determining dysmetabolic iron overload. PLoS One 2015; 10:e0116855.
39. Hoki T, Miyanishi K, Tanaka S, Takada K, Kawano Y, Sakurada A, et al. Increased duodenal iron absorption through up-regulation of divalent metal transporter 1

from enhancement of iron regulatory protein 1 activity in patients with nonalcoholic steatohepatitis. Hepatology 2015;62:751-761.
40. Zimmermann A, Zimmermann T, Schattenberg J, Pottgen S, Lotz J, Rossmann H, et al. Alterations in lipid, carbohydrate and iron metabolism in patients with non-alcoholic steatohepatitis (NASH) and metabolic syndrome. Eur J Intern Med 2011;22:305-310.
41. Barisani D, Pelucchi S, Mariani R, Galimberti S, Trombini P, Fumagalli D, et al. Hepcidin and iron-related gene expression in subjects with Dysmetabolic Hepatic Iron Overload. J Hepatol 2008;49:123-133.
42. Fujita N, Miyachi H, Tanaka H, Takeo M, Nakagawa N, Kobayashi Y, et al. Iron overload is associated with hepatic oxidative damage to DNA in nonalcoholic steatohepatitis. Cancer Epidemiol Biomarkers Prev 2009;18:424-432.
43. Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology 2005;41:1313-1321.
44. Hagstrom H, Nasr P, Bottai M, Ekstedt M, Kechagias S, Hultcrantz R, et al. Elevated serum ferritin is associated with increased mortality in non-alcoholic fatty liver disease after 16 years of follow-up. Liver Int 2016;36:1688-1695.
45. Canbakan B, Senturk H, Tahan V, Hatemi I, Balci H, Toptas T, et al. Clinical, biochemical and histological correlations in a group of non-drinker subjects with non-alcoholic fatty liver disease. Acta Gastroenterol Belg 2007;70:277-284.
46. Koruk M, Taysi S, Savas MC, Yilmaz O, Akcay F, Karakok M. Serum levels of acute phase proteins in patients with nonalcoholic steatohepatitis. Turk J Gastroenterol 2003;14: 12-17.
47. Sumida Y, Nakashima T, Yoh T, Furutani M, Hirohama A, Kakisaka Y, et al. Serum thioredoxin levels as a predictor of steatohepatitis in patients with nonalcoholic fatty liver disease. J Hepatol 2003;38:32-38.
48. Parikh P, Patel J, Ingle M, Sawant P. Serum ferritin levels predict histological severity in patients with nonalcoholic fatty liver disease in India. Indian J Gastroenterol 2015;34:200-208.
49. Utzschneider KM, Nelson JE, Yeh MY, Kowdley KV, Kahn SE. Serum ferritin is associated with increased liver fat and decreased beta-cell function but is not associated with insulin sensitivity in non-diabetic men and women. Diabetes 2013;62:A474.
50. Rakha EA, Adamson L, Bell E, Neal K, Ryder SD, Kaye PV, et al. Portal inflammation is associated with advanced histological changes in alcoholic and non-alcoholic fatty liver disease. J Clin Pathol 2010;63:790-795.
51. Loguercio C, De Simone T, D' Auria MV, de Sio I, Federico A, Tuccillo C, et al. Non-alcoholic fatty liver disease: A multicentre clinical study by the Italian Association for the Study of the Liver. Dig Liver Dis 2004;36:398-405.
52. Oh S, Shida T, Yamagishi K, Tanaka K, So R, Tsujimoto T, et al. Moderate to vigorous physical activity volume is an important factor for managing nonalcoholic fatty liver disease: a retrospective study. Hepatology 2015;61:1205-1215.
53. Tanaka N, Sano K, Horiuchi A, Tanaka E, Kiyosawa K, Aoyama T. Highly purified eicosapentaenoic acid treatment improves nonalcoholic steatohepatitis. J Clin Gastroenterol 2008;42:413-418.
54. Beaton MD, Chakrabarti S, Adams PC. Inflammation is not the cause of an elevated serum ferritin in non-alcoholic fatty liver disease. Ann Hepatol 2014;13:353-356.
55. Adams LA, Crawford DH, Stuart K, House MJ, St Pierre TG, Webb M, et al. The impact of phlebotomy in nonalcoholic fatty liver disease: A prospective, randomized, controlled trial. Hepatology 2015;61:1555-1564.
56. Adams PC, Barton JC. A diagnostic approach to hyperferritinemia with a non-elevated transferrin saturation. J Hepatol 2011;55:453-458.
57. Pietrangelo A. Iron and the liver. Liver Int 2016;36 Suppl 1:116-123.
58. Maliken BD, Nelson JE, Klintworth HM, Beauchamp M, Yeh MM, Kowdley KV. Hepatic reticuloendothelial system cell

iron deposition is associated with increased apoptosis in nonalcoholic fatty liver disease. Hepatology 2013;57:1806-1813.
59. Murali AR, Gupta A, Brown K. Systematic review and meta-analysis to determine the impact of iron depletion in dysmetabolic iron overload syndrome and non-alcoholic fatty liver disease. Hepatol Res 2018;48:E30-E41.
60. Chalasani N, Younossi Z, Lavine JE, Charlton M, Cusi K, Rinella M, et al. The diagnosis and management of nonalcoholic fatty liver disease: Practice guidance from the American Association for the Study of Liver Diseases. Hepatology 2018;67:328-357.
61. Powell LW, Seckington RC, Deugnier Y. Haemochromatosis. Lancet 2016;388:706-716.
62. Bassett ML, Halliday JW, Powell LW. Value of hepatic iron measurements in early hemochromatosis and determination of the critical iron level associated with fibrosis. Hepatology 1986;6:24-29.

Table S3.1 The adjusted Quality Assessment scale with regards to risk of bias based on the NewCastle-Ottawa Quality Assessment scale

Author	Year	Selection				Comparability	Outcome		
		Is the study population defined adequately?	Is the comparative group defined adequately?	Representativeness of the study population?	Representativeness of the controls comparative group?	Comparability of groups on the basis of design or analysis	Assessment of the outcome iron	Assessment of outcome inflammation	In case of ≥2 groups that are compared: same method of ascertainment for groups
Adams *et al.*	2015	B*	B*	B	B	B*	B*	D	A*
Aigner *et al.* (Am J Clin Nutr.)	2008	A*	A*[1]	B	B	C	A* + B*	A* + B*	A*
Aigner *et al.* (gastroenterology)	2008	A*	A*	A*	B	C	C	C	A*
Akin *et al.*	2003	A*	NA	B	NA	C	D	D	NA
Barisani *et al.*	2008	A*	A*[2]	B	B	C	A*	D	A*
Beaton *et al.*	2014	A*	NA	B	NA	NA	A* + B*	A* + B*	NA
Boga *et al.*	2015	A*	B	A*	B	C	A* + B*	A* + B*	B
Bonkovsky *et al.*	1999	A*	D[4]	B	B	C	C	C	A [5]
Bugianesi *et al.*	2004	A*/B*[3]	NA	A*	NA	B*	A* + B*	A*	A*
Canbakan *et al.*	2007	A*	NA	B	NA	C	A* + B*	A* + B*	A*
Chitturi *et al.*	2002	A*	D[4]	A*	B	A*	A* + B*	A*	A*[5]
Deguti *et al.*	2003	A*	NA	A*	NA	C	A* + B*	A*	A*
Dongiovanni *et al.*	2015	A*	A*	B	B	C	A* + B*	A*	A*
Duseja *et al.*	2005	D[7]	NA	B	NA	NA	A* + B*	A*	NA
Fujita *et al.*	2009	A*	A*	A*	B	B*	C	C	A*
George *et al.*	1998	A*	D[4]	B	A	C	A* + B*	A*	A*[5]
Hagström *et al.*	2016	A*	NA	A*	NA	B*	A*	A* + B*	A*
Hoki *et al.*	2015	A*	D	A*	B	C	A* + B*	A*	B
Koruk *et al.*	2003	A*	B*	B	B	C	D	A* + B*	B
Kowdley *et al.*	2014	A*	NA	A*	NA	B*	A* + B*	A*	A*
Lin *et al.*	2005	B*	C	A*	B	C	B	D	B
Loguercio *et al.*	2004	A*	NA	A*	NA	B*	A*	A* + B*	A*
Manousou *et al.*	2011	A*	NA	A*	NA	B*	C	A*	A*
Nelson *et al.*	2011	A*	NA	A*	NA	B*	C	C	A*
Oh *et al.*	2015	A*	NA	B	NA	C	B*	B*	A*
Parikh *et al.*	2015	A*	B*	A*	B	A*	B*	A*	B
Rahka *et al.*	2010	A*	NA	A*	NA	NA	A*	A*	NA
Rametta *et al.*	2016	A*/B*[3]	A* (DIOS), B* (C), B* (HH)[5]	B	B[1]	B*	B*	D	A*

Table S3.1 (continued)

Author	Year	Selection				Comparability	Outcome		
		Is the study population defined adequately?	Is the comparative group defined adequately?	Representativeness of the study population?	Representativeness of the controls comparative group?	Comparability of groups on the basis of design or analysis	Assessment of the outcome iron	Assessment of outcome inflammation	In case of ≥2 groups that are compared: same method of ascertainment for groups
Sumida *et al.*	2003	A*	D[6]	B	B[6]	B*	B*	B*	A*
Tanaka *et al.*	2008	A*	NA	B	NA	NA	B*	A* + B*	NA
Tsuchiya *et al.*	2010	A*	A*	B	B	C	A* + B*	C	A*
Uraz *et al.*	2005	A*	NA	B	NA	D	A* + B*	A*	NA
Utzschneider *et al.*	2014	A*/B*[3]	C	B	B	A	B*	A* + B*	A*
Uysal *et al.*	2011	D[4]	B	B	B	C	B*	B*	A*
Valenti *et al.*	2003	A*/B*[3]	D[2]	A*	B	B*	A*	A*	A*[2]
Valenti *et al.*	2012	A*	C	B	B	B*	A*	A*	B
Valenti *et al.*	2006	A*	C	A*	B	B*	A*	A* + B*	B
Valenti *et al.* (J. Hepatology)	2010	A*	C	A*	A*	A*	A*	C	B
Valenti *et al.* (Gastroenterology)	2010	A*	C	A*	A*	B*	A*	A*	B
Yoneda *et al.*	2010	A*	C	B	B	C	B*	A* + B*	B
Zamin *et al.*	2006	A*	C[6]	B	B[6]	C	A* + B*	A*	A*[2]
Zimmerman *et al.*	2011	A*	C	A*	B	A*	A* + B*	A* + B*	A*

[1] Answer applicable for all groups (since more than one comparative group) used in the study [2] Diagnosis NAFLD is not excluded (by biopsy or imaging) nevertheless a star was scored since this was not relevant for aim of the study and our review [3] Not all patients underwent a liver biopsy, some were diagnosed by imaging [4]Diagnosis of NASH by ultrasound examination [5]More than one comparative group with different answers [6]Not all comparative groups used since they were not relevant for the aim of the review

Quality assessment with regards to bias: Questions 1-4 relate to the selection process. Per question one star (*) can be scored. In case of 4, 3, 2-1 stars the risk of bias is ranked as low , intermediate or high , respectively. The comparative group could include non-NAFLD patients or subjects with NAFLD/NASH. Due to the heterogeneity of the studies, question 2 was interpreted as follows: not applicable (in case of no comparative group or the comparative group being not relevant in context of this review (e.g. hepatitis C or alcoholic fatty liver disease patients); which was scored as high risk of bias. When studies lacked a control group and/or when the diagnosis NASH was made by imaging, they were also scored as high risk of bias.

For question 6 and 7, more than one star could be scored. In case of 5, 4-3, 2-1 stars the risk of bias is ranked as low , intermediate or high , respectively. Some studies subdivide their patient group during the study, in this case only comparability and the method of ascertainment will be scored.

Table S3.2 All included studies and their results about inflammatory and iron parameters

Author, year	Population (biopsy proven diagnosis: yes/no)	Mean serum ferritin levels in µmol/L (prevalence of hyperferritinaemia)	Genetic mutations or regulators iron homeostasis (group comparison)	Significant changes in (surrogate) inflammatory parameters (group comparison)	Significant changes in iron parameters (group comparison)
George *et al.* 1998	51 NASH (yes) 2465 Controls	504 (62%)	homozygosity p.C282Y 8% vs. 1% * heterozygosity p.C282Y 24% vs. 12% * homozygosity p.H63D 4% vs. 2% ¥ heterozygosity p.H63D 28% vs. 22% ¥ (NASH vs. controls)	NP	NASH: TS (Elevated in 22%), HIC (Elevated in 23%) Sig. association between ferritin levels and HIC
Bonkovsky *et al.* 1999	57 NASH (yes) 348 Controls	447	homozygosity p.C282Y 3% vs. 0% * heterozygosity p.C282Y 17% vs. 11% ¥ homozygosity p.H63D 6% vs. 3% ¥ heterozygosity p.H63D 44% vs. 26% * (NASH vs. controls)	NP	NASH: Significant association between ferritin levels and the presence of iron deposits
Chitturi *et al.* 2002	93 NASH (yes) 206 Controls	387 (40%)	homozygosity p.C282Y 0% vs. 1% ¥ heterozygosity p.C282Y 22% vs. 9% * homozygosity p.H63D 2% vs. 1% ¥ heterozygosity p.H63D 17% vs. 25% ¥ (NASH vs. controls)	NP	NASH: TS (elevated in 5%)
Deguti *et al.* 2003	3 NASH with hepatic siderosis 29 NASH without hepatic siderosis (yes)	♂ 764 *, ♀ NP ♂ 349*, ♀ 220	heterozygosity p.C282Y 0% vs. 4% ¥ homozygosity p.H63D 0% vs. 4% ¥ heterozygosity p.H63D 33% vs. 21% ¥ (NASH with hepatic siderosis vs. without hepatic siderosis)	No significant difference in histological inflammation between groups	NP

Table S3.2 (continued)

Author, year	Population (biopsy proven diagnosis: yes/no)	Mean serum ferritin levels in µmol/L (prevalence of hyperferritinaemia)	Genetic mutations or regulators iron homeostasis (group comparison)	Significant changes in (surrogate) inflammatory parameters (group comparison)	Significant changes in iron parameters (group comparison)
Lin *et al.* 2004	33 NAFLD (no) 125 Controls	331	homozygosity p.C282Y 0% vs. 0% ¥ heterozygosity p.C282Y 0% vs. 0% ¥ homozygosity p.H63D 3% vs. 3% ¥ heterozygosity p.H63D 3% vs. 3% ¥ (NAFLD vs. controls)	NP	NP
Zamin *et al.* 2006[a]	29 NASH (yes) 20 controls	258 (69%)	homozygosity p.C282Y 0% vs. 0% ¥ heterozygosity p.C282Y 34% vs. 25% ¥ homozygosity p.H63D 3% vs. 0% ¥ heterozygosity p.H63D 14% vs. 5% ¥ (NASH vs. controls)	NP	NASH: No association between ferritin levels and the presence of iron deposits
Valenti *et al.* 2002	134 NAFLD (yes in 67 patients) 291 controls	45%	(homozygosity p.C282Y 1% vs. 0% ¥ heterozygosity p.C282Y 17% vs. 2% * homozygosity p.H63D 2% vs. 3% ¥ heterozygosity p.H63D 27% vs. 23% ¥ (NAFLD vs. controls)	NP	NAFLD: TS (elevated in 12%) Sig. association between ferritin levels and the presence of iron deposits
Valenti *et al.* 2010 (Gastro-enterology)	587 NAFLD (yes) 179 controls	NP	heterozygosity p.C282Y 6% vs. 4% ¥ homozygosity p.H63D 3% vs. 4% ¥ heterozygosity p.H63D 26% vs. 29% ¥ (NAFLD vs. controls)	NP	NAFLD: Significant association between ferritin levels and the presence of iron deposits

Table S3.2 (continued)

Author, year	Population (biopsy proven diagnosis: yes/no)	Mean serum ferritin levels in µmol/L (prevalence of hyperferritinaemia)	Genetic mutations or regulators iron homeostasis (group comparison)	Significant changes in (surrogate) inflammatory parameters (group comparison)	Significant changes in iron parameters (group comparison)
Nelson *et al.* 2011	556 NAFLD with negative iron stain (Fe -) 293 NAFLD with positive iron stain (Fe +) (yes)	106* 328 *	NP	NAFLD Fe-: NAS significantly higher among those with iron in RES	NP
Kowdley *et al.* 2012	500 NAFLD with SF >1.5 x ULN 128 NAFLD with SF <1.5 x ULN (yes)	547 * 121 *	heterozygosity p.C282Y 8% vs. 9% ¥ homozygosity p.H63D 1% vs. 2% ¥ heterozygosity p.H63D 20% vs. 24% ¥ (NAFLD with SF 1.5x ULN vs. SF <1.5 ULN)	NAFLD with SF >1.5 x ULN : NAS*↑ vs. NAFLD with SF <1.5 x ULN	NAFLD with SF >1.5 x ULN : Iron in RES and hepatocytes* ↑ vs. NAFLD with SF <1.5 x ULN
Hagstrom *et al.* 2016	89 NAFLD with hyperferritinemia (♂ >350 µg/L, ♀ >150 µg/L) 133 NAFLD without hyperferritinemia (yes)	455 * (40%) 132*	NP	NAFLD with hyperferritinemia: sign. higher NAS and more fibrosis vs. NAFLD without hyperferritinemia No differences in CRP and WBC between groups	Total 204/222 patients with iron staining In NAFLD with hyperferritinemia sig. more positive iron staining 48% vs. 21% (NAFLD without hyperferritinemia)

Table S3.2 (continued)

Author, year	Population (biopsy proven diagnosis: yes/no)	Mean serum ferritin levels in µmol/L (prevalence of hyperferritinaemia)	Genetic mutations or regulators iron homeostasis (group comparison)	Significant changes in (surrogate) inflammatory parameters (group comparison)	Significant changes in iron parameters (group comparison)
Aigner *et al.* 2008 (Am J Clin Nutr.)	32 NAFLD biopsy proven Fe+	796	heterozygosity p.C282Y 12.5% vs. 10.3 ¥ heterozygosity p.H63D 18.7% vs. 20.7 ¥ (NAFLD Fe + vs NAFLD Fe -) Liver FP-1 mRNA/protein expression, HJV mRNA: sign. Lower in NAFLD Fe+/Fe- vs. 20 controls	NAFLD Fe +: Serum TNFα sign. higher vs NAFLD Fe- No differences in NAS between groups	Siderosis grade sign. correlated with hepcidin in both groups
	29 NAFLD biopsy proven Fe- (yes)	196	Hepatic TfR-1 mRNA, duodenal FP-1 protein expression sign. Lower in NAFLD Fe+ compared to controls and NAFLD Fe-) Hepcidin mRNA sign. higher in NAFLD Fe+ compared to controls/NAFLD Fe- and correlates with ferritin in NAFLD Fe+/Fe- No differences in DMT-1 expression between groups		
Tsuchiya *et al.* 2010	11 NASH	225 ¥	HJV mRNA , TfR2 mRNA/protein, TfR1 mRNA, FP-1 Mrna/ protein sig. higher in NASH/HS compared to controls NASH: Hepcidin mRNA*↑ vs. HS No differences in hepcidin protein between groups	NP	NASH: Hepatic iron sign. elevated vs. controls No differences in serum iron and TS between groups
	17 HS (Yes)	179 ¥			
	8 Controls	180 ¥			

Table S3.2 (continued)

Author, year	Population (biopsy proven diagnosis: yes/no)	Mean serum ferritin levels in µmol/L (prevalence of hyperferritinaemia)	Genetic mutations or regulators iron homeostasis (group comparison)	Significant changes in (surrogate) inflammatory parameters (group comparison)	Significant changes in iron parameters (group comparison)
Valenti *et al.* 2010 (J. Hepatology)	274 NAFLD (yes)	46%	Heterozygosity p.C282Y 10% vs. 6% ¥, Homozygosity p.H63D 5% vs. 3% ¥ (NAFLD vs. controls)	NP	Liver siderosis in: Hepatocytes: 11.7% In non-parenchymal cells or Mixed pattern: 30.6% No hepatic iron: 57.7%
	179 Controls	NP	NAFLD: Beta-thalassemic trait *↑ vs controls and a sign. higher risk of hepatocellular siderosis No differences in prevalence of polymorphism FP-1 between groups NAFLD: (n57) Serum hepcidin sign. more frequently >3 Nm in patients with non-parenchymal siderosis		
Valenti *et al.* 2012	216 NAFLD (yes)	286	Heterozygosity p.C282Y 7% vs. 1% ¥ Homozygosity p.H63D 4% vs. 3% ¥ Heterozygosity p.H63D 25% vs. 28% ¥ Homozygosity TMPRSS6 p.Ala736Val ¥ (NAFLD vs controls)	NP	NAFLD: Liver siderosis correlated with heterozygosity p.C282Y and homozygosity p.H63D
	271 Controls	37			
Hoki *et al.* 2015	25 NASH	182*	NASH: Duodenal DMT-1 mRNA *↑ vs controls	NASH: NAS*↑ vs HS	NASH: Iron absorption: TS, serum iron (After OIAT test) sig. higher vs. HS/controls
	15 HS (Yes)	145	Duodenal FP- 1 mRNA ¥ not sig. different between groups Serum hepcidin-25 sig. Higher in NASH/HS compared to controls		
	9 Controls	75*			

Table S3.2 (continued)

Author, year	Population (biopsy proven diagnosis: yes/no)	Mean serum ferritin levels in µmol/L (prevalence of hyperferritinaemia)	Genetic mutations or regulators iron homeostasis (group comparison)	Significant changes in (surrogate) inflammatory parameters (group comparison)	Significant changes in iron parameters (group comparison)
Oh *et al.* 2015	87 NAFLD with MPVA ≥250 min/week Before/after/ change 82 NAFLD with MPVA <250 min/week Before/after/ change (Yes)	198/171/-27 *↓ NP*	NP	NAFLD with MPVA ≥250 min/week: TBARS *↓, Steatosis*↓ vs. NAFLD with MPVA <250 min/week (more improvement) No significant differences in loghsCRP, logTNFα and IL-6 between groups	NP
Rametta *et al.* 2016	18 NAFLD (yes) 23 controls	140 63	Homozygosity p. C282Y 0% vs. 0% ¥ Heterozygosity p. H63D 26% vs. 26% ¥ Serum hepcidin ¥ (NAFLD vs controls)	NP	No differences in TS between groups
Aigner *et al.* 2008 (gastroenterology)	140 NAFLD (yes) 25 controls	NP	Heterozygosity p. C282Y 8% vs 12% ¥ Heterozygosity p. H63D 14% vs 16% ¥ (NAFLD vs controls)	NP	NP
Barisani *et al.* 2008	8 NAFLD Fe – 25 DIOS (yes)	266 1032	NAFLD Fe-: TfR1 mRNA *↑, DMT1 mRNA *↑, urinary hepcidin *↓vs. DIOS No differences in TfR2 mRNA, FP-1 mRNA and hepcidin mRNA between groups	NP	NP

Table S3.2 (continued)

Author, year	Population (biopsy proven diagnosis: yes/no)	Mean serum ferritin levels in µmol/L (prevalence of hyperferritinaemia)	Genetic mutations or regulators iron homeostasis (group comparison)	Significant changes in (surrogate) inflammatory parameters (group comparison)	Significant changes in iron parameters (group comparison)
Boga *et al.* 2015	66 NAFLD (Yes) 35 Controls	140* 98*	NAFLD: HJV *↓ vs. controls No differences in serum hepcidin between groups	NAFLD: hsCRP *↑ vs controls	Positive iron staining in 18% in NAFLD patients no differences in TS between groups
Beaton *et al.* 2014	28 NAFLD (yes) pre phlebotomy (Baseline) 28 NAFLD Post phlebotomy	384 (61%) 88	NP	No differences in presence of NASH between groups No association found between ESR, CRP and SF	HIC elevated in 11% with sig. decrease post phlebotomy Sig. correlation between HIC and SF
Dongiovanni *et al.* 2015	23 NASH + 23 HS (yes) 10 Controls	689 240	Sig. higher FP-1 mRNA in NASH compared to HS and controls	NP	NP
Rahka *et al.* 2015	206 NAFLD (yes)	237	NP	Portal inflammation correlated with serum ferritin levels	NP
Manousou *et al.* 2011	65 NASH 47 HS (yes)	309 * 146 *	NP	No differences in CRP between groups In NASH + HS: Portal inflammation correlated with serum ferritin levels	No differences in serum iron and TIBC between groups
Yoneda *et al.* 2008	62 NASH 24 HS (yes)	279 * 165*	NP	NASH: CRP*↑ vs. HS	NP

Table S3.2 (continued)

Author, year	Population (biopsy proven diagnosis: yes/no)	Mean serum ferritin levels in µmol/L (prevalence of hyperferritinaemia)	Genetic mutations or regulators iron homeostasis (group comparison)	Significant changes in (surrogate) inflammatory parameters (group comparison)	Significant changes in iron parameters (group comparison)
Zimmerman *et al.* 2011	25 NASH (yes) 37 Controls	180 (sig. correlated with serum hepcidin)	Serum hepcidin not sig. different vs controls	NASH: CRP not sig. correlated with serum hepcidin NAS sig. correlated with serum hepcidin	NASH: No iron deposition in liver biopsy
Utzschneider *et al.* 2013	15 NAFLD (yes) 15 Controls	362* 207*	NP	No differences in TNFα, hsCRP and IL-6 between groups	NP
Parikh *et al.* 2015	55 NAFLD (yes)	51	NP	Presence of NASH correlated with SF	NP
Uysal *et al.* 2011	60 NASH (no) 28 Controls	118*↑ 27*	NASH: sTfR*↑ vs. controls No difference in hepcidin level between groups	NASH: TNFα, IL-6, IL-8 and MDA*↑ vs. controls	No sig. differences in serum iron and UIBC between groups
Bugianesi *et al.* 2004	263 NAFLD (yes) 200 controls	239 (21%)	Homozygosity p.C282Y 0% vs. 0% Heterozygosity p.C282Y 5% vs. 3% Homozygosity p.H63D 1% Heterozygosity p.H63D 24% vs. 19% (NAFLD vs controls)	In NAFLD: correlation between steatosis, fibrosis and serum ferritin levels	In NAFLD: TS (elevated in: 7.4%), no association found between hepatic iron and *HFE* mutations
Loguercio *et al.* 2004	305 NAFLD (yes)	NP (35%)	NP	Sign. correlation between: steatosis, fibrosis, lobular, portal inflammation and serum ferritin levels	Histological hepatic hemosiderosis (elevated in: 19%) (sign. correlated with SF)

Table S3.2 (continued)

Author, year	Population (biopsy proven diagnosis: yes/no)	Mean serum ferritin levels in µmol/L (prevalence of hyperferritinaemia)	Genetic mutations or regulators iron homeostasis (group comparison)	Significant changes in (surrogate) inflammatory parameters (group comparison)	Significant changes in iron parameters (group comparison)
Koruk *et al.* 2002	18 NASH (yes) 16 Controls	173 * 82*	NP	NASH: CRP*↑ vs. controls And hepatic inflammation, fibrosis, fatty infiltration not sig. correlated with serum ferritin levels	NP
Canbakan *et al.* 2007	38 HS 67 NASH (yes)	71* 110 *	NP	NASH: MDA and NO*↑ vs. HS	No hepatic iron deposition found in any group. No difference in serum iron, TS and TIBC between groups.
Tanaka *et al.* 2008	23 NASH pre EPA treatment 23 NASH post EPA treatment	228 * 124 *	NP	Pre EPA treatment TRX*↑ vs. post EPA treatment and no difference in TNFα	No difference in serum iron and TS between groups
Sumida *et al.* 2001	31 NAFLD (yes) 17 controls	170	NP	NAFLD: TRX*↑ vs. controls and sig. correlation between TRX and serum ferritin levels	NP
Adams *et al.* 2015	33 NAFLD with phlebotomy 41 NAFLD without phlebotomy (lifestyle advice)	Only in NAFLD with phlebotomy sign. decrease ferritin after intervention compared with before	NP	NP	NP

Table S3.2 (continued)

Author, year	Population (biopsy proven diagnosis: yes/no)	Mean serum ferritin levels in µmol/L (prevalence of hyperferritinaemia)	Genetic mutations or regulators iron homeostasis (group comparison)	Significant changes in (surrogate) inflammatory parameters (group comparison)	Significant changes in iron parameters (group comparison)
Fujita *et al.* 2009	38 NASH 24 HS (yes)	283 * 139 *	NP	NASH: Hepatic 8-oxodG vs. HS No difference in steatosis between groups HS: correlation between Hepatic 8-oxodG and serum ferritin levels	NASH: Sig. higher serum iron, TS and total iron score vs. HS
Akin *et al.* 2003	53 NASH (yes)	136	NP	NP	NP
Duseja *et al.* 2005	31 NASH (Yes)	NP	NP	NP	No association found between the presence of iron deposition and inflammation or fibrosis
Uraz *et al.* 2005	25 NASH (yes)	140	NP	NP	NP

Abbreviations: not provided (NP), non-alcoholic steatohepatitis (NASH), hepatic iron concentration (HIC), transferrin saturation (TS), total iron-binding capacity (TIBC), NAFLD activity score (NAS), reticuloendothelial system (RES), iron in both hepatocytes and the reticuloendothelial system (mixed), serum ferritin (SF), upper limit of normal (ULN), moderate to vigorous intensity physical activity (MVPA), C-reactive protein (CRP), white blood count (WBC), patients with iron overload (Fe+), patients without iron overload (Fe-), tumour necrosis factor α (TNFα), ferroportin (FP-1), hemojuvelin (HJV), transferrin receptor (TfR 1), divalent metal transporter (DMT1), homozygosity for the alfa-1-antitrypsine mutation (non-MM genotypes AAT), Transmembrane protease serine 6 (TMPRSS6), oral iron absorption test (OIAT), Thiobarbituric acid reactive substances (TBARS), interleukin (IL), erythrocyte sedimentation rate (ESR), soluble transferrin receptor (sTfR), unsaturated iron binding capacity (UIBC), malondialdehyde (MDA), hepatic steatosis HS), purified eicosapentaenoic acid (EPA), nitric oxide (NO), thioredoxin (TXR). *significant difference between marked values in horizontal columns ($p<0.05$), ¥ no significant difference between marked values in horizontal columns.

Chapter 4

Serum ferritin is associated with non-alcoholic steatohepatitis with significant fibrosis

EMBARGOED

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To be submitted

Chapter 5

Myosteatosis in non-alcoholic fatty liver disease:

An exploratory study

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Abstract

Background and Aim
Insulin resistance (IR) plays a central role in the complex pathophysiology of non-alcoholic fatty liver disease (NAFLD). IR is linked to fat infiltration in skeletal muscle (myosteatosis) and loss of skeletal muscle mass and function (sarcopenia). The clinical significance of myosteatosis in NAFLD is not well investigated. In this exploratory study we aimed to investigate the association between myosteatosis and NAFLD related hepatic and systemic variables in a well characterised NAFLD cohort.

Methods
We cross-sectionally studied forty-five NAFLD patients. The muscle fat fraction (MFF) was measured with chemical shift Magnetic Resonance Imaging (MRI). In addition, the hepatic fat fraction (MRI), liver stiffness (FibroScan®) and appendicular skeletal muscle mass (Dual-energy X-ray absorptiometry) were analysed.

Results
The median hepatic fat fraction was 15.64% (IQR 12.05-25.13) and significant (F2-F3) liver fibrosis (liver stiffness ≥7kPa) was diagnosed in 18 NAFLD patients (40%). MFF was not correlated with hepatic fat fraction (r=-0.035, p=0.823) and did not differ between subjects with or without significant fibrosis (p=0.980). No patient was diagnosed with sarcopenia based on the skeletal muscle mass index. In a linear regression model, anthropometric parameters, including body mass index (BMI) (p=0.018) and total body fat percentage (p=0.005), were positively associated with MFF while no association with insulin resistance (HOMA-IR) was observed.

Conclusion
Myosteatosis did not correlate with the degree of hepatic steatosis or fibrosis in this well characterised NAFLD cohort, but was positively correlated with total body fat percentage and BMI.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease in the Western society, with an estimated prevalence of 20%-30%.[1] Its progressive form, non-alcoholic steatohepatitis (NASH) is characterised by a chronic progressive liver and systemic inflammation. In particular, NASH can progress to liver fibrosis, liver cirrhosis and hepatocellular carcinoma (HCC).[2] In addition, patients with NAFLD are at increased risk to the development or progression of diabetes mellitus and cardiovascular diseases.[3]

The pathogenesis of NAFLD is not completely elucidated. Factors that are thought to be involved are dietary habits, gut microbiota perturbations and metabolic-toxic factors, such as insulin resistance (IR).[4,5] It is thought that IR is a key driver in the pathogenesis of NAFLD and worsens with progression to NASH and liver fibrosis.[6] IR contributes to NAFLD through dysregulated lipolysis in adipose tissue resulting in excessive delivery of fatty acids to the liver.[6] Skeletal muscle is quantitatively the major contributor to whole body insulin-mediated glucose disposal. Fat accumulation in skeletal muscle (myosteatosis) negatively affects insulin sensitivity.[7,8]

Myosteatosis is believed to be a precursor of IR and sarcopenia, and might be an early manifestation of NAFLD disease progression.[9,10] Myosteatosis comprises fat infiltration within myocytes (intramyocellular lipids [IMCL]) and between myofibres within the fascia of the muscles (intermyocellular-intrafascial adipose tissue). Recent data suggests that both, IMCL and intermyocellular-intrafascial adipose tissue are associated with IR.[7,11,12] The exact mechanism remains unclear but lipid metabolites and muscle inflammation are thought to play a role.[10,12]

The degree of myosteatosis, determined as the muscle fat fraction (MFF) can be measured reliably with chemical shift Magnetic Resonance Imaging (MRI) showing excellent agreement with histopathologic findings.[13] Although MRI is not able to distinguish between IMCL and intermyocellular-intrafascial adipose tissue, good correlations with IMCL measurements have been observed.[14-16] Chemical shift MRI is also widely used to measure hepatic fat fraction in NAFLD patients making it possible to accurately measure hepatic and muscle fat fraction with a single examination.

The association between myosteatosis and chronic liver disease has been the subject of several studies. Most studies investigate myosteatosis in an advanced disease setting including liver cirrhosis, HCC and liver transplant patients. The degree of myosteatosis is observed to be a prognostic factor of long-term

mortality in liver cirrhosis and HCC.[17-20] In NAFLD, data concerning myosteatosis is scare and contradictory results were found with respect to the association between the degree of myosteatosis and the hepatic fat fraction or liver fibrosis.[21-23]

We here hypothesise that myosteatosis is associated with the amount of hepatic fat and the presence of liver fibrosis before sarcopenia is present. For this reason, we analysed myosteatosis in well-characterised NAFLD cohort.

Methods

Study population

All data were obtained from a prospective cohort of 63 subjects with NAFLD recruited from the Maastricht University Medical Centre (MUMC+) and CO-EUR (a second line eating disorder clinic) between September 2015 and November 2018. Forty-five subjects had 3D fast gradient echo mDixon MRI data available for research purpose and were included in this study. All subjects were between 18 and 65 years old, had a BMI of 25 kg/m^2 or higher and proven NAFLD on MRI (hepatic fat fraction >5%). Participants with excessive ethanol consumption (>7 and >14 beverages per week for females and males, respectively) were excluded from this cohort. Other exclusion criteria were: causes for secondary hepatic fat accumulation (medication, Wilson's disease, viral infections, starvation or parenteral nutrition and microvesicular steatosis on liver biopsy), pregnancy and breastfeeding, a history of bariatric surgery, liver cirrhosis and/or hepatocellular carcinoma, extrahepatic malignancy within the last 5 years and individuals about to undergo or recovering from a surgical or otherwise medical procedure. The Medical Ethics Committee of the Maastricht University Medical Centre approved the protocol (ClinicalTrials.gov Identifier: NCT02422238) and all participants had given written informed consent prior to participation. The study was performed according the declaration of Helsinki (latest amendment of 2013, Fortaleza, Brazil).

Data collection

Bodyweight, height, waist and hip circumference were measured in all participants. Venous blood was collected after an overnight fast in all participants. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma - glutamyl transferase (GGT), alkaline phosphatase (ALP), total

cholesterol, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglyceride, albumin, ferritin, C-reactive protein (CRP), fasting plasma glucose, glycosylated haemoglobin type A1c (HbA1C) and fasting plasma insulin concentrations were determined. IR was defined by means of the homeostasis model of assessment for insulin resistance (HOMA-IR). In subjects on insulin therapy (n=2), HOMA-IR was not calculated. Metabolic syndrome was diagnosed based on the updated International Diabetes Federation (IDF) definition with abdominal obesity (WC ≥94 cm in male and ≥80 cm in female) not being prerequisite for metabolic syndrome diagnosis.[24] The definition of diabetes at baseline included any patient with a current treatment for type 2 diabetes or any patient with an HbA1c ≥6.5% or a fasting plasma glucose of ≥7 mmol/l. [25]

Body composition

Fat mass index (FMI, fat mass/height^2), body fat percentage and skeletal muscle mass index (SMI) (appendicular skeletal mass/height^2) were calculated with dual-energy X-ray absorptiometry (DXA). Low SMI was defined as ≤5.67 kg/m^2 in women and ≤7.25 in men based on the cut-off values recommended by the European working group on Sarcopenia in Older People (EWGSOP).[26] Hand grip strength was assessed three times for each hand using a Saehan hand dynamometer; the highest measurement of the dominant hand was used for analysis. Low grip strength was defined as ≤30 kg for BMI 24.1 to 28 kg/m^2 or ≤32 for BMI > 28 kg/m^2 in men; and ≤17.3 kg for BMI 23.1 to 26 kg/m^2, ≤18 kg for BMI 26.1 to 29 kg/m^2 or ≤21 kg for BMI >29 kg/m^2 in women.[26]

Transient elastography

In all participants liver stiffness measurements (LSM) were performed using the FibroScan® (touch 502, Echosens, Paris, France). Both the M (3.5 MHz) and the XL probe (2.5 MHz) were available for this study. The LSM is expressed in kilo Pascal (kPa) with a range of 2.5–75 kPa. All participants were in fasting condition for at least 3 hours before the examination. Only procedures with at least 10 valid measurements, a minimal success rate of 60% and interquartile range divided by median for liver stiffness estimation ≤ 30% were accepted for further analysis. The final result of LSM was the median of 10 valid individual measurements. Based on previous research the FibroScan® cut-off value for histological grade 2 fibrosis (F2) or grade 3 fibrosis (F3) were 7.0 kPa and 8.7 kPa, respectively.[27]

Magnetic Resonance Imaging

For research purpose all participant received an MRI analysis after a minimal fasting period of 3 hours within one month of the other measurements. 3D fast gradient echo mDixon sequence were obtained with a 1.5T or 3T Achieva MRI system (Philips, Best, The Netherlands) in all subjects. The parameters employed for 1.5T and 3T were respectively: flip angle =15° and 10°, TR =5.8 and 3.4 ms, TE1/TE2 =2.37/4.75 and 1.19/2.37ms, field-of-view (FOV) =320 x 320 and 384 x 384 mm, acquisition matrix = 288 x 227 and 252 x 209, slice thickness 5 and 3 mm.

Image analysis

We followed the region of interest (ROI) segmentation method to determine muscle fat as described previously by Crawford *et al.*[28]. The multifidus and erector spinae muscle were segmented bilaterally at the lumbar 1 (L1) level using Weasis software (Figure 5.1). The large fat-filled "tent" which can be observed between the longissimus and iliocostalis muscle was not included in the ROI. The ROI borders were manually traced on the in-phase image (IP image) and copied to the corresponding opposed-phase image (OP image). The average ROI signal intensity (SI) for the IP and OP image were calculated. The relative signal intensity loss, a measure for muscle fat fraction (MFF) was calculated with the following formula: (SI IP – SI OP)/ SI IP*100%.[13] Representative images of subjects with different MFF percentages are shown in Figure 5.1.
Hepatic steatosis was assessed similar to muscle fat fraction.[29] On three MRI sections, four circular ROIs of 5 cm^2 were drawn in the liver. Artefact, vascular and biliary structures were avoided and the ROI was copied from the IP image to the OP image. The mean signal intensity loss of all 12 ROIs was calculated with the following formula: (SI IP- SI OP)/ 2*SI IP*100%.[30]

Statistical analysis

Data were analysed with IBM SPSS statistics version 25 (IBM Statistics for Macintosh, Chicago, IL, USA). Results were expressed as median and interquartile range for continuous variables and as absolute number and percentages for categorical variables. Mann - Whitney U tests was used to determine statistically significant differences between two groups. Partial correlation controlling for gender were evaluated by partial spearman's rho correlation coefficients. Multiple linear regression analysis in which sex and age

were considered as covariate was performed to analyse the associations between MFF as a dependent variable and liver function test, IR or body composition as independent variables. Due to skewed data, liver transaminases AST and ALT were LN transformed prior to analysis. A two-sided P-value of <0.05 was considered statistically significant. Bonferroni-Holm correction was used to correct for multiple testing.[31]

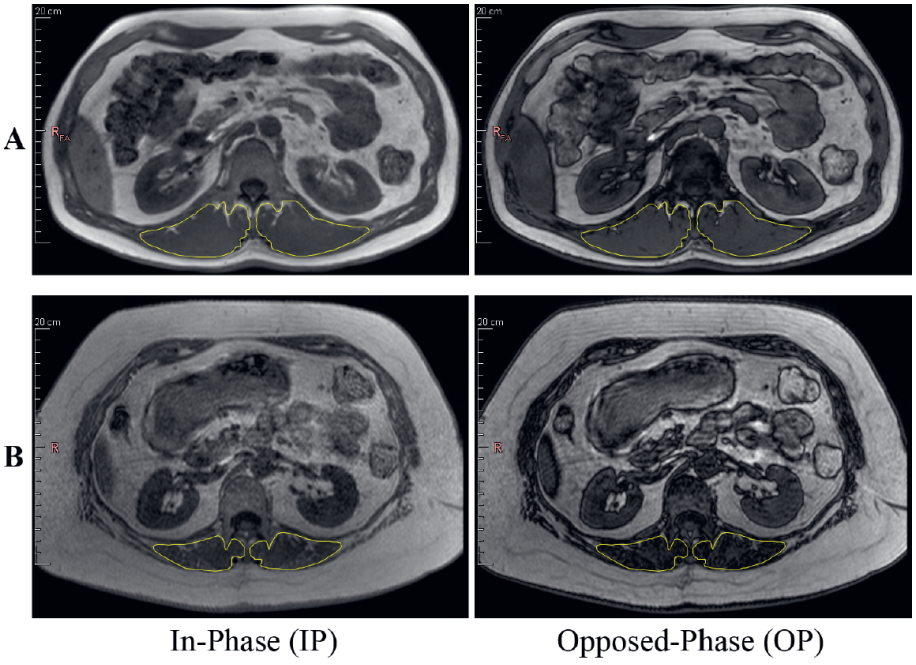

Figure 5.1 **Muscle fat fraction calculation**. **A** represents a subject with a relative low muscle fat fraction (MFF) of the multifidus and erector spinae muscle (right 7.41%, left 8.75%, average 8.08%) and **B** represents a subject with a relative high MFF of the multifidus and erector spinae muscle (right 47.13%, left 44.67%, average 45.90%).

Results

Baseline characteristics

In total, 45 subjects with proven NAFLD were included of whom 21 were female. Median age was 53 years and median BMI was 33.3 kg/m^2. Furthermore, median MRI hepatic fat fraction was 15.64% (IQR 12.05-25.13) and significant (F2-F3) liver fibrosis (liver stiffness ≥7kPa) was diagnosed in 18 NAFLD patients

(40%). None of the NAFLD subjects had a low SMI, consequently none of the participant were diagnosed with sarcopenia.[26] Only two subjects had a low grip strength. Median MFF (p=0.003) and SMI (p=0.003) differed significantly between male and female subjects (see table 1). In five subjects HOMA-IR results were not available, due to missing fasting insulin concentrations. In two male participants FibroScan® results were not obtained as the FibroScan® was unavailable because of regular maintenance of the device. Anthropometric and biochemical parameters of all participants are presented in Table 5.1.

Table 5.1 Baseline characteristics of the study cohort

	Female (n=21)	Male (n=24)	Total (n=45)
Age (years)	54 (50-61)	52 (39-60)	53 (43-61)
BMI (kg/m^2)	33.7 (31.3-36.7)	31.5 (27.4-35.7)	33.3 (28.4-35.9)
Waist Circumference (cm)	106.3 (100.5-114.9)	109.8 (99.3-118.7)	106.7 (100.5-117.0)
FMI (kg/m^2)	13.75 (12.43-6.20)**	9.85 (7.45-10.65)**	10.80 (9.07-14.58)
Body Fat (%)	41.54 (37.53-45.91)**	29.47 (27.21-34.67)**	36.31 (28.62- 41.79)
HOMA-IR (n = 38)	2.65 (1.48-4.15)	3.25 (2.72-4.15)	3.05 (2.18-4.15)
Diabetic Mellitus Type 2 (Yes)	5 (24%)	4 (17%)	9 (20%)
Total cholesterol (mmol/L)	5.4 (4.5-6.1)	5.1 (4.3-5.7)	5.1 (4.5-5.9)
LDL-C (mmol/L)	2.8 (2.6-4.0)	3.2 (2.5-3.9)	3.0 (2.4-3.9)
HDL-C (mmol/L)	1.5 (1.1-1.7)*	1.1 (0.9-1.3)*	1.2 (1.0-1.6)
Triglycerides (mmol/L)	1.50 (1.27-2.43)	1.51 (1.19-2.38)	1.51 (1.22-2.36)
ALT (U/L)	31.0 (19.5-39.5)*	41.0 (29.3-62.5)*	37.0 (22.5-50.5)
AST (U/L)	24.0 (20.0-30.0)	27.0 (24.0-80.0)	27.0 (22.5-33.0)
GGT (U/L)	30.0 (20.5-57.0)	41.0 (27.8-80.0)	33 (25.0-66.5)
ALP (U/L)	89.0 (83.0-119.5)	84.0 (69.5-105.3)	89.0 (74.5-106.0)
Ferritin (ug/L)	118.0 (45.5-145.5)*	196.0 (63.3-274.2)*	122.0 (61.0-224.0)
Albumin (g/L)	39.0 (36.7-41.0)	41.1 (39.5-43.3)	40.5 (37.8-42.2)
CRP (mg/L)	3 (1-5)	2 (1-4)	3 (1-5)
Hepatic fat fraction (%)	13.55 (11.12-24.95)	17.04 (12.96-23.52)	15.64 (12.05-25.13)
LSM (kPa) (n=43)	6.2 (5.2-9.1)	6.0 (5.0-8.7)	6.0 (5.1-8.7)
F2 (7- 8.7 kPa) (yes/total)	3/21 (14%)	3/22 (13%)	6/43 (14%)
≥ F3 (≥8.7 kPa)(yes/total)	6/21 (29%)	6/22 (25%)	12/43 (28%)
SMI (kg/m2)	8.10 (7.19-8.89)**	9.42 (8.25-9.83)**	8.57 (7.90-9.56)
Hand Grip Strength (cm)	28.0 (22.5-33.5)**	41.5 (38.0-50.25)**	36 (28-42)
Low Grip Strength	2 (10%)	0 (0%)	2 (4%)
MFF	40.75 (31.04-47.59)**	31.04 (23.51-38.99)**	35.57 (29.98-43.46)

Continuous variables are expressed as median (Q1-Q3). Categorical variables are expressed as number (percentage of the total group). Mann-Whitney test was used to determine statistically significant differences ($p<0.05$) between male and female subjects. * $p<0.05$, ** $p<0.01$. Abbreviations: body mass index (BMI), fat mass index (FMI), Homeostatic model assessment for insulin resistance (HOMA-IR), low-density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP), c-reactive protein (CRP), liver stiffness measurement (LSM), skeletal muscle mass index (SMI), muscle fat fraction (MFF).

Muscle fat infiltration and skeletal muscle mass index are not correlated with hepatic fat fraction or the presence of significant fibrosis

To investigate possible correlation between the hepatic fat fraction and MFF and SMI partial correlation controlling for gender was used. No significant correlations were found between MFF or SMI and hepatic fat fraction (Figure 5.2A-C). Furthermore, no difference in MFF (p=0.980) or SMI (p=0.291) between NAFLD patients with significant fibrosis (F2-F3) and NAFLD patients without fibrosis was observed (Figure 5.2B-D). Results were not adjusted for gender because gender was evenly divided across both groups (F0-F1 vs. F2-F3).

Insulin sensitivity is correlated with the degree of hepatic steatosis and the presence of significant fibrosis.

Insulin sensitivity was defined by means of HOMA-IR. We observed a moderate positive correlation (r=0.528, p=0.001) between HOMA-IR and hepatic fat fraction in our cohort (Figure 5.2E). Furthermore HOMA-IR was significantly elevated in NAFLD patients with significant fibrosis (p=0.039) (Figure 5.2F).

MFF is not associated with the metabolic syndrome, liver transaminases and insulin sensitivity but is associated with body composition

A multiple linear regression analysis in which sex and age were considered as covariates was performed to investigate the association between body composition and MFF. BMI, fat mass index (FMI, fat mass/heigt^2) and body fat percentage were found to be positively associated with MFF (see Table 5.2). The metabolic syndrome, HOMA-IR, AST and ALT levels, SMI and hand grip strength were not associated with MFF. None of the associations remained significant after Bonferroni-Holm correction for multiple testing.

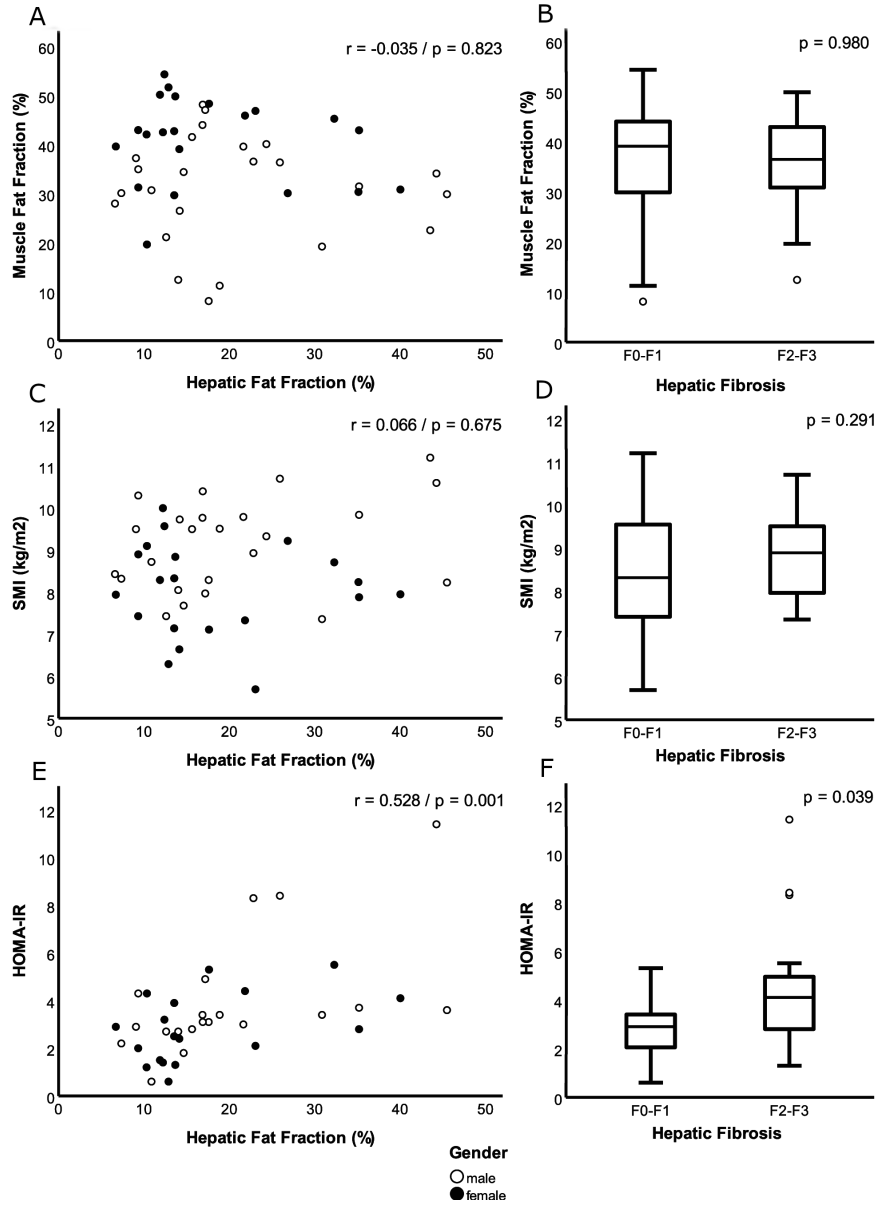

Figure 5.2 Hepatic Fat Fraction and liver fibrosis were not associated with MFF or SMI but with HOMA-IR. **A/C/E** Partial Spearman correlation corrected for gender of muscle fat fraction (MFF), skeletal muscle mass index (SMI) and Homeostatic model assessment for insulin resistance (HOMA-IR) with hepatic fat fraction. Black dots represent female subjects and white dots male subjects. **B/D/F** Boxplot showing the MFF, SMI and HOMA-IR in non-alcoholic fatty liver disease patients with (F2-3) and without significant fibrosis (F0-F1). Mann-Whitney U tests was used to determine statistically significant differences ($p<0.05$).

Table 5.2 Association between MFF and IR or body composition variables in patients with NAFLD

	Unstandardised B	95% CI	p-value
MS (no/yes)	0.082	-6.215; 6.379	0.979
MS (number of components)	-0.337	-2.824; 2.151	0.786
ALT (U/L)	-0.305	-12.214; 11.603	0.959
AST (U/L)	1.898	-14.424; 18.220	0.815
HOMA-IR	0.277	-1.407; 1.961	0.740
HbA1C (%)	-1.556	-4.810; 1.701	0.340
BMI (kg/m^2)	0.714	0.130; 1.299	0.018
FMI (kg/m^2)	1.221	0.357; 2.085	0.007
Body Fat (%)	0.879	0.275; 1.484	0.005
SMI (kg/m2)	1.525	-1.577; 4.626	0.326
Hand Grip (cm)	-0.049	-0.498; 0.399	0.826

The multiple linear model included sex and age as covariates, muscle fat fraction (MFF) as dependent variable and insulin resistance (IR) and body composition variables as independent variables. Abbreviations: metabolic syndrome (MS), alanine aminotransferase (ALT), aspartate aminotransferase (AST), Homeostatic model assessment for insulin resistance (HOMA-IR), glycosylated haemoglobin type A1c (HbA1C), body mass index (BMI), fat mass index (FMI), skeletal muscle mass index (SMI), confidence interval (CI). Skewed data (ALT and AST) were LN (natural logarithm) transformed prior to analysis.

Discussion

Myosteatosis is suggested to be a precursor of sarcopenia and may be an early sign of the progression from simple steatosis to NASH.[32] In this exploratory study, the degree of myosteatosis was not associated with the amount of hepatic fat, liver transaminases or the presence of significant liver fibrosis. Our findings are in line with those of Machado et al. who found no correlation between biopsy proven myosteatosis and the degree of hepatic steatosis and HOMA-IR in 51 biopsy proven morbidly obese NAFLD patients.[22] In contradiction to our results they observed a progressive increase in the prevalence of intramyocellular lipids and the severity of hepatic fibrosis. Kitajima et al. investigated CT muscle attenuation in 208 biopsy proven Japanese NAFLD patients of which 179 had NASH.[23] The degree of myosteatosis increased significantly with the severity of NASH, however the relationship with liver fibrosis was not investigated.[23] An explanation for the difference in results between studies is at least partially explained by the characteristics of the various study populations. In our cohort of 45 patients, 18 subjects had elevated liver stiffness measurements, nine had type 2 diabetes mellitus (DM2) and in five subjects ALT levels were elevated more than 1.5 times upper limit of normal (male >67 U/L; female >51 U/L). In addition, only subjects younger than 65 years were included without liver

cirrhosis. In the study of Machado et al. morbidly obese patients were studied and of them 51% had DM2 and 43% had NASH with significant fibrosis.[22] In the study of Kitajima et al. 82% of the study population had NASH, mean ALT value was 77 IU/L and significant fibrosis was present in 64% of the study population.[23] Our cohort could be characterised as NAFLD in a less advanced phase as NASH prevalence is expected to be lower in our cohort compared to previous NAFLD myosteatosis studies. Chronic low grade inflammation and ageing are well known risk factors for loss of muscle strength, mass and functionality. A correlation between myosteatosis and the expression of systemic inflammatory cytokines IL-6 and TNF-a has been observed.[33] NASH is associated with more pronounced systemic inflammation and may enhance myosteatosis. In the presented study, we were not able to investigate the link between hepatic inflammation and myosteatosis because liver biopsies were not available. While myosteatosis may possibly be an important hallmark for hepatic inflammation and NASH, based on our data the relevance of myosteatosis for the degree of hepatic steatosis or fibrosis is minimal.

Furthermore, we showed that insulin resistance, the possible link between liver- and myosteatosis, was not correlated with the amount of myosteatosis. On the other hand, IR was associated with the hepatic fat fraction and the presence of significant fibrosis. Our results are in line with previous NAFLD myosteatosis studies.[21,22] In DM2 populations however, a positive association between degree of myosteatosis and IR or DM2 has been observed by several authors.[7,34,35] Our results are not in line with these findings. A possible explanation for this difference in results might be that only nine DM2 patients were included in our study. To date, it is still uncertain if myosteatosis is a causal mechanism in IR development.

IMCL and intermyocellular-intrafascial adipose tissue have both been associated with IR development in numerous studies.[7,11,12,36] However, the accumulation of IMCL (stored as lipid droplet inside myocytes) is believed to have a more crucial role in this process. Among other, morphological characteristics, protein coating and composition (diacylglycerol and cermides) of these lipid droplets have been observed to influence insulin sensitivity via unknown mechanisms.[36] IR increases free fatty acid (FFA) delivery to the liver and stimulates de novo lipogenesis both resulting in the development of hepatic steatosis. In this study, however, we were not able to discriminate between IMCL and intermyocellular-intrafascial adipose. This may explain why the hypothesised positive association between the degree of hepatic steatosis and myosteatosis was not observed. IMCL might be associated with the degree of hepatic steatosis and IR, while

myosteatosis in general is not. To further understand the role of myosteatosis in IR and NAFLD development future studies using advanced imaging techniques (e.g. magnetic resonance spectroscopy) and muscle biopsies with detailed analysis are needed.

In addition, we found associations between body composition and the amount of myosteatosis. BMI, DXA measured FMI and body fat percentage were positively associated with MFF. This positive association between body fatness and MFF is in line with previous research data.[21,37]

Finally, none of the NAFLD subjects in our cohort were diagnosed with sarcopenia and similar to the MFF no association between SMI and hepatic fat fraction or significant fibrosis was observed. The association between NAFLD and sarcopenia has been more widely investigated than the association between NAFLD and myosteatosis. Large Korean population studies focussing on sarcopenia in the NAFLD population showed that sarcopenia is more prevalent in NAFLD compared to healthy controls and is related to NAFLD severity including liver fibrosis.[5,38] In contrast to these studies, our population is relatively young with a median age of 53 years which may explain why sarcopenia was not observed. In a recent Dutch population based study, an independent association between SMI and overweight NAFLD was not found. In line with our results a low SMI was only present in 3% of the 1462 overweight NAFLD subjects.[39] Myosteatosis on the other hand is believed to be a precursor of sarcopenia and it may be an early sign of the progression from simple liver steatosis to NASH.[32]

With regards to our cohort, several factors need to be taken into account. First, the number of patients with hepatic fibrosis was probably overestimated with the FibroScan® because the positive predictive value of the FibroScan® for F2 is only 70%.[27] Second, the MRI images were targeted to the liver and because of this MFF was calculated at L1 level instead of the more commonly used L3 level. However, in previous research MFF of L1 correlated very well with MFF of the complete lumbar spine (L1-L5).[40] Third, both T1.5 and T3 MRI images were used. In previous research, no significant difference between MFF measurements across field strengths (1.5T and 3.0T) was observed.[41] Fourth, our cohort was relatively small in numbers and no control group was available which limits the generalisability of the study. However, in this exploratory study we aimed to investigate the association between the degree of myosteatosis and different prognostic factors within the NAFLD population for which a control group is not needed. Furthermore, a control group without

hepatic steatosis and BMI matched to the NAFLD population is difficult to obtain.
A strength of our study is that all NAFLD subjects are well characterised with biochemical parameters, imaging (MRI and FibroScan®), and tests for muscle mass (DXA) and function (hand grip strength). In this way, we were able to evaluate muscle fat fraction, muscle mass and muscle function. Second, chemical shift MRI is known to have a very good diagnostic accuracy for hepatic steatosis and is widely available in the clinical setting.[29,30] Third, hepatic steatosis, insulin sensitivity and MFF were all measured as a continuous variable, which is ideal for correlation analysis.

Conclusion

Myosteatosis was not associated with the degree of hepatic steatosis or fibrosis in this NAFLD cohort but was associated with factors related to body composition. Further research with longitudinal studies focussing on the complete spectrum of muscle wasting including myosteatosis, sarcopenia and muscle functionality are necessary to investigate in more depth the role of myosteatosis in NAFLD pathophysiology.

References

1. Sayiner M, Koenig A, Henry L, Younossi ZM. Epidemiology of Nonalcoholic Fatty Liver Disease and Nonalcoholic Steatohepatitis in the United States and the Rest of the World. Clin Liver Dis 2016;20:205-214.
2. Calzadilla Bertot L, Adams LA. The Natural Course of Non-Alcoholic Fatty Liver Disease. Int J Mol Sci 2016;17.
3. Anstee QM, Targher G, Day CP. Progression of NAFLD to diabetes mellitus, cardiovascular disease or cirrhosis. Nature Reviews Gastroenterology &Amp; Hepatology 2013;10:330.
4. Asrih M, Jornayvaz FR. Metabolic syndrome and nonalcoholic fatty liver disease: Is insulin resistance the link? Mol Cell Endocrinol 2015;418 Pt 1:55-65.
5. Hong HC, Hwang SY, Choi HY, Yoo HJ, Seo JA, Kim SG, et al. Relationship between sarcopenia and nonalcoholic fatty liver disease: the Korean Sarcopenic Obesity Study. Hepatology 2014;59:1772-1778.
6. Friedman SL, Neuschwander-Tetri BA, Rinella M, Sanyal AJ. Mechanisms of NAFLD development and therapeutic strategies. Nature medicine 2018;24:908-922.
7. Boettcher M, Machann J, Stefan N, Thamer C, Haring HU, Claussen CD, et al. Intermuscular adipose tissue (IMAT): association with other adipose tissue compartments and insulin sensitivity. J Magn Reson Imaging 2009;29:1340-1345.
8. Miljkovic I, Zmuda JM. Epidemiology of myosteatosis. Curr Opin Clin Nutr Metab Care 2010;13:260-264.
9. Bosma M, Kersten S, Hesselink MK, Schrauwen P. Re-evaluating lipotoxic triggers in skeletal muscle: relating intramyocellular lipid metabolism to insulin sensitivity. Prog Lipid Res 2012;51:36-49.
10. Wu H, Ballantyne CM. Skeletal muscle inflammation and insulin resistance in obesity. J Clin Invest 2017;127:43-54.
11. Goodpaster BH, He J, Watkins S, Kelley DE. Skeletal muscle lipid content and insulin resistance: evidence for a paradox in endurance-trained athletes. J Clin Endocrinol Metab 2001;86:5755-5761.
12. Eckardt K, Taube A, Eckel J. Obesity-associated insulin resistance in skeletal muscle: Role of lipid accumulation and physical inactivity. Rev Endocr Metab Disord 2011;12:163-172.
13. Gaeta M, Scribano E, Mileto A, Mazziotti S, Rodolico C, Toscano A, et al. Muscle fat fraction in neuromuscular disorders: dual-echo dual-flip-angle spoiled gradient-recalled MR imaging technique for quantification--a feasibility study. Radiology 2011;259:487-494.
14. Larson-Meyer DE, Smith SR, Heilbronn LK, Kelley DE, Ravussin E, Newcomer BR. Muscle-associated triglyceride measured by computed tomography and magnetic resonance spectroscopy. Obesity (Silver Spring) 2006;14:73-87.
15. Fischer MA, Nanz D, Shimakawa A, Schirmer T, Guggenberger R, Chhabra A, et al. Quantification of muscle fat in patients with low back pain: comparison of multi-echo MR imaging with single-voxel MR spectroscopy. Radiology 2013;266:555-563.
16. Goodpaster BH, Kelley DE, Thaete FL, He J, Ross R. Skeletal muscle attenuation determined by computed tomography is associated with skeletal muscle lipid content. J Appl Physiol (1985) 2000;89:104-110.
17. Tachi Y, Kozuka A, Hirai T, Ishizu Y, Honda T, Kuzuya T, et al. Impact of myosteatosis on skeletal muscle volume loss in patients with chronic liver disease. J Gastroenterol Hepatol 2018.
18. Fujiwara N, Nakagawa H, Kudo Y, Tateishi R, Taguri M, Watadani T, et al. Sarcopenia, intramuscular fat deposition, and visceral adiposity independently predict the outcomes of hepatocellular carcinoma. J Hepatol 2015;63:131-140.
19. Praktiknjo M, Book M, Luetkens J, Pohlmann A, Meyer C, Thomas D, et al. Fat-free muscle mass in magnetic resonance imaging predicts acute-on-chronic liver failure and survival in

decompensated cirrhosis. Hepatology 2018;67:1014-1026.
20. Montano-Loza AJ, Angulo P, Meza-Junco J, Prado CMM, Sawyer MB, Beaumont C, et al. Sarcopenic obesity and myosteatosis are associated with higher mortality in patients with cirrhosis. Journal of cachexia, sarcopenia and muscle 2016;7:126-135.
21. Kitajima Y, Eguchi Y, Ishibashi E, Nakashita S, Aoki S, Toda S, et al. Age-related fat deposition in multifidus muscle could be a marker for nonalcoholic fatty liver disease. Journal of Gastroenterology 2010;45:218-224.
22. Machado MV, Ferreira DM, Castro RE, Silvestre AR, Evangelista T, Coutinho J, et al. Liver and muscle in morbid obesity: the interplay of fatty liver and insulin resistance. PLoS One 2012;7:e31738.
23. Kitajima Y, Hyogo H, Sumida Y, Eguchi Y, Ono N, Kuwashiro T, et al. Severity of non-alcoholic steatohepatitis is associated with substitution of adipose tissue in skeletal muscle. J Gastroenterol Hepatol 2013;28:1507-1514.
24. Alberti KGMM, Eckel RH, Grundy SM, Zimmet PZ, Cleeman JI, Donato KA, et al. Harmonizing the Metabolic Syndrome. Circulation 2009;120:1640-1645.
25. American Diabetes A. Diagnosis and classification of diabetes mellitus. Diabetes Care 2010;33 Suppl 1:S62-S69.
26. Cruz-Jentoft AJ, Baeyens JP, Bauer JM, Boirie Y, Cederholm T, Landi F, et al. Sarcopenia: European consensus on definition and diagnosis: Report of the European Working Group on Sarcopenia in Older People. Age Ageing 2010;39:412-423.
27. Wong VW, Vergniol J, Wong GL, Foucher J, Chan HL, Le Bail B, et al. Diagnosis of fibrosis and cirrhosis using liver stiffness measurement in nonalcoholic fatty liver disease. Hepatology 2010;51:454-462.
28. Crawford RJ, Cornwall J, Abbott R, Elliott JM. Manually defining regions of interest when quantifying paravertebral muscles fatty infiltration from axial magnetic resonance imaging: a proposed method for the lumbar spine with anatomical cross-reference. BMC Musculoskelet Disord 2017;18:25.
29. van Werven JR, Marsman HA, Nederveen AJ, Smits NJ, ten Kate FJ, van Gulik TM, et al. Assessment of hepatic steatosis in patients undergoing liver resection: comparison of US, CT, T1-weighted dual-echo MR imaging, and point-resolved 1H MR spectroscopy. Radiology 2010;256:159-168.
30. Cassidy FH, Yokoo T, Aganovic L, Hanna RF, Bydder M, Middleton MS, et al. Fatty liver disease: MR imaging techniques for the detection and quantification of liver steatosis. Radiographics 2009;29:231-260.
31. Holm S. A Simple Sequentially Rejective Multiple Test Procedure. Scandinavian Journal of Statistics 1979;6:65-70.
32. Nachit M, Leclercq IA. Emerging awareness on the importance of skeletal muscle in liver diseases: time to dig deeper into mechanisms! Clin Sci (Lond) 2019;133:465-481.
33. Beasley LE, Koster A, Newman AB, Javaid MK, Ferrucci L, Kritchevsky SB, et al. Inflammation and Race and Gender Differences in Computerized Tomography-measured Adipose Depots. Obesity 2009;17:1062-1069.
34. Goodpaster BH, Krishnaswami S, Resnick H, Kelley DE, Haggerty C, Harris TB, et al. Association between regional adipose tissue distribution and both type 2 diabetes and impaired glucose tolerance in elderly men and women. Diabetes Care 2003;26:372-379.
35. Kiefer LS, Fabian J, Rospleszcz S, Lorbeer R, Machann J, Storz C, et al. Assessment of the degree of abdominal myosteatosis by magnetic resonance imaging in subjects with diabetes, prediabetes and healthy controls from the general population. Eur J Radiol 2018;105:261-268.
36. Gemmink A, Goodpaster BH, Schrauwen P, Hesselink MKC. Intramyocellular lipid droplets and insulin sensitivity, the human perspective. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids 2017;1862:1242-1249.
37. Ryan AS, Nicklas BJ. Age-related changes in fat deposition in mid-thigh muscle in women: relationships with metabolic cardiovascular disease risk factors. Int J

Obes Relat Metab Disord 1999;23:126-132.
38. Lee YH, Kim SU, Song K, Park JY, Kim DY, Ahn SH, et al. Sarcopenia is associated with significant liver fibrosis independently of obesity and insulin resistance in nonalcoholic fatty liver disease: Nationwide surveys (KNHANES 2008-2011). Hepatology 2016;63:776-786.
39. Alferink LJM, Trajanoska K, Erler NS, Schoufour JD, de Knegt RJ, Ikram MA, et al. Nonalcoholic Fatty Liver Disease in The Rotterdam Study: About Muscle Mass, Sarcopenia, Fat Mass, and Fat Distribution. Journal of Bone and Mineral Research 2019;34:1254-1263.
40. Crawford RJ, Filli L, Elliott JM, Nanz D, Fischer MA, Marcon M, et al. Age- and Level-Dependence of Fatty Infiltration in Lumbar Paravertebral Muscles of Healthy Volunteers. AJNR Am J Neuroradiol 2016;37:742-748.
41. Smith AC, Parrish TB, Abbott R, Hoggarth MA, Mendoza K, Chen YF, et al. Muscle-fat MRI: 1.5 Tesla and 3.0 Tesla versus histology. Muscle Nerve 2014;50:170-176.

Chapter 6

Non-invasive tests do not accurately differentiate non-alcoholic steatohepatitis from simple steatosis: a systematic review and meta-analysis

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Abstract

Background

Non-alcoholic fatty liver disease (NAFLD) is a rapidly increasing health problem. Liver biopsy analysis is the most sensitive test to differentiate between non-alcoholic steatohepatitis (NASH) and simple steatosis (SS), but non-invasive diagnostics are needed. We performed a systematic review on non-invasive tests for differentiating NASH from SS, focusing on blood markers.

Methods

We performed a systematic search in PubMed, Medline and Embase (1990-2016) using defined keywords, limited to full-text papers in English and human adults, resulted in 2608 hits. Two independent reviewers screened the articles an identified 122 eligible articles that used liver biopsy as gold standard. If at least 2 studies were available, pooled sensitivity ($sens_p$) and specificity ($spec_p$) were determined using the Meta-Analysis Package for R (metafor).

Results

In the 122 studies analysed, 219 different blood markers (107 single markers and 112 scoring systems) were identified to differentiate NASH from SS. Markers identified related to several pathophysiological mechanisms. The markers analysed in the largest proportion of studies were alanine aminotransferase ($sens_p$ 63.5%, $spec_p$ 74.4%) within routine biochemical tests, adiponectin ($sens_p$ 72.0%, $spec_p$ 75.7%) within inflammatory markers, CK18-M30 ($sens_p$ 68.4%, $spec_p$ 74.2%) within markers of cell death/proliferation and homeostatic model assessment of insulin resistance ($sens_p$ 69.0%, $spec_p$ 72.7%) within the metabolic markers. Two scoring systems could also be pooled: the NASH test (NASH vs borderline NASH plus SS with $sens_p$ 22.9%, $spec_p$ 95.3%) and the GlycoNASH test ($sens_p$ 67.1%, $spec_p$ 63.8%).

Conclusion

In the meta-analysis, we found no test to differentiate NASH from SS with a high level of pooled sensitivity and specificity (≥80%). However, some blood markers, when included in scoring systems in single studies, identified patients with NASH with ≥80% sensitivity and specificity. Replication studies and more standardised study designs are urgently needed. At present, no marker or scoring system can be recommended for use in clinical practice to differentiate NASH from SS.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is one of the most common chronic liver disorders in the world with a prevalence of 25%,[1] and is expected to increase further in line with the worldwide overweight and obesity epidemic.[2-5] NAFLD encompasses simple steatosis and non-alcoholic steatohepatitis (NASH).[6] NASH is the early stage of progression that develops to cirrhosis in 15-25% of patients and can lead to the development of hepatocellular carcinoma.[7-9] In the near future NASH accounts for the majority of liver transplantations.[10] Of the NASH-related cirrhosis patients, 30-40% die of a liver-related cause over a 10-year period[7]. To date, liver-related mortality is the third most common cause of death after cardiovascular diseases and malignancies.[11]

NAFLD is mostly asymptomatic, generally resulting in 'silent' disease progression. Liver biopsy is the gold standard to diagnose NASH but has several limitations. Its invasiveness, high costs, and risk of complications make it unsuitable for diagnosis in large populations or disease monitoring.[12] In clinical practice, only subjects at high risk of NASH, usually based upon elevated aminotransferase levels, undergo a liver biopsy. This may lead to under-diagnosis of NASH.[13] Screening large numbers of subjects with NAFLD will provide more detailed insight in pathophysiological factors and early identification of patients at risk for developing NASH. Therefore, an accurate non-invasive test to diagnose NASH is highly warranted.

Imaging techniques have potential to assess and quantify hepatic fat but cannot accurately diagnose hepatic inflammation.[14] Several biomarkers have been studied, ranging from liver enzymes to markers for specific pathophysiologic processes associated with NASH, such as glucose and lipid metabolism, oxidative stress and inflammation. In addition, attention has been given to scoring systems based on combinations of markers and/or omics approaches. We conducted a systematic review with meta-analyses to identify the best performing non-invasive diagnostic test for NASH in (suspected) NAFLD patients with liver biopsy as a reference test. We specifically focused on blood markers, either for single use or as part of scoring systems.

Methodology

Literature search

A systematic literature search was conducted in PubMed, Embase and Medline (1990 until July 2016), using the following (truncated) keywords and MeSH terms: 'non*alcoholic fatty liver disease, NAFL, fatty liver, NASH or non*alcoholic steatohepatitis', in any combination with 'routine diagnostic tests, diagnosi*, diagnosti*, diagnostic imaging, radiography, radiograph*, radiol*, *marker*, biological marker or genetic markers' (see supplementary file Search). The search was limited to full-text papers in English language and human adults (3398 hits) (see supplementary data, search strategy). Double hits were excluded leaving 2608 single hits.

Inclusion and exclusion criteria

Studies on non-invasive markers and/or tests to diagnose NASH in (suspected) NAFLD patients with liver biopsy as a reference test were eligible for inclusion. In case of inclusion of a population of suspected NAFLD patients, studies were eligible if at least the majority had biopsy proven NAFLD. Studies had to present one of the following values for diagnosing NASH: sensitivity, specificity, likelihood ratio (LR), Area Under the Receiver Operating Characteristic curve (AUROC) or diagnostic accuracy (DA). Studies performed *in vitro*, in animals, in children (≤18 years), reviews, case reports/series, studies on alcoholic or other chronic liver diseases and studies in patients with liver diseases of mixed aetiologies not reporting on NAFLD separately, were excluded. Studies not differentiating NASH from simple steatosis, but focusing only on steatosis or fibrosis in NAFLD were also excluded. Studies on the diagnosis of NAFLD or NASH versus other causes of chronic liver diseases were not eligible for inclusion.

Selection process

This systematic review and meta-analyses were performed according to the PRISMA statement.[15]

Titles and abstracts were screened for eligibility, leaving 438 articles, of which full texts were checked by two independent reviewers. For 18 abstracts, a request for the full text was sent to the corresponding authors to a maximum of three attempts; only one author replied. In case of disagreement on eligibility,

the two reviewers came to consensus after discussing the article with a third reviewer. Scrutinizing full texts resulted in exclusion of 324 articles. Eight additional papers were found by crosschecking references of eligible articles and reviews. Finally, 122 articles were included. Details on the selection process can be found in the flowchart (Figure 6.1).

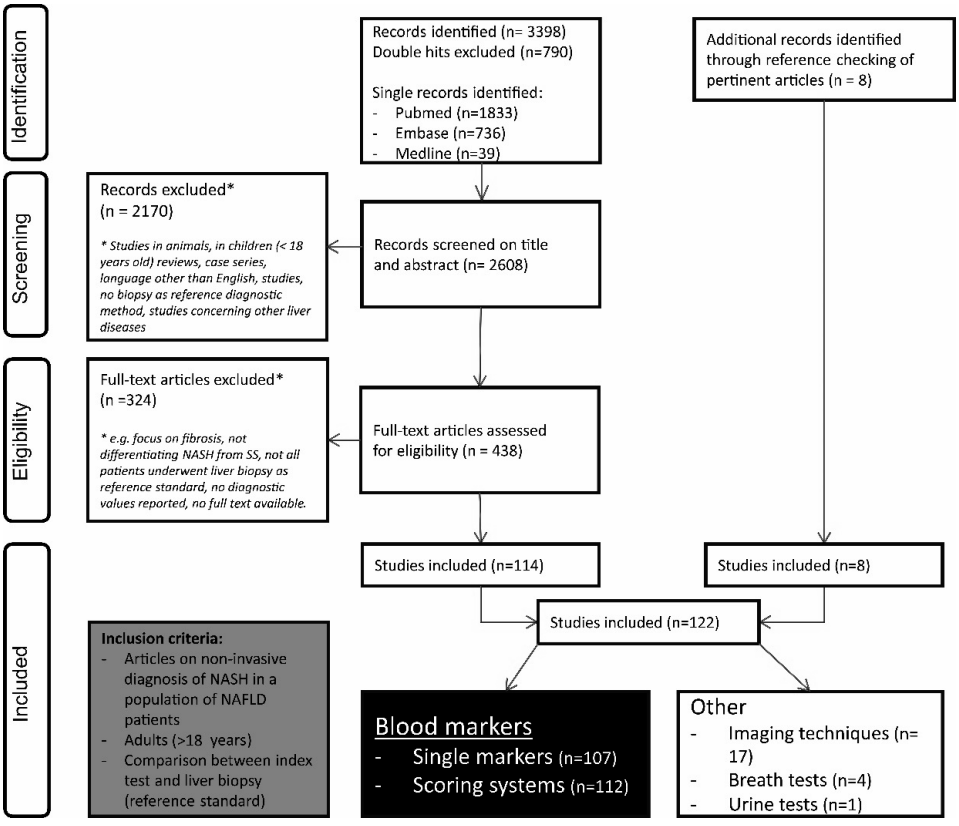

Figure 6.1 **Flow chart of the selection process**; from identification of all the possible eligible articles to the articles included in the review. NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; SS, simple steatosis.

Data extraction

Two reviewers carried out the data extraction using a standardised data extraction form. Seven authors were contacted to clarify their data, one replied. As blood analyses are implemented in all clinical practices, the present review

focuses on serum or plasma markers, either as single marker or as part of scoring systems. An overview of additional biochemical markers (*i.e.* in urine and breath) or imaging techniques is provided in the supplementary tables only.

Quality assessment

The methodological quality for each of the included studies was assessed using phase 3 of the Quality Assessment of Diagnostic Accuracy Studies-2 (QUADAS-2) scoring system. QUADAS-2, assessing the quality of primary diagnostic accuracy studies, consists of four domains covering patient selection, index test, reference standard, and flow of patients through the study and timing of the index test(s) and reference standard. The QUADAS-2 assesses the risk of bias in each domain (low, unclear, high), including selection-, work-up-, and expectation bias. The first three domains are also assessed in terms of concerns regarding applicability (low, unclear and high) by making use of signalling question.[16]

Statistical analyses

A meta-analysis was performed if at least two studies evaluated the same blood marker. Sensitivity and specificity, as statistical measures of the performance of a binary classification test (NASH vs. simple steatosis), were used for pooling, as these data were available or could be calculated in most studies. Furthermore, only studies in which all subjects underwent liver biopsy as reference, were included in the meta-analysis. The histological criteria used to diagnose NASH and the stages of fibrosis in the individual studies are indicated in Supplementary Tables S6.1-S6.3.

When different cut-offs were given in one study, those resulting in the best overall sensitivity and specificity (smallest Euclidean distance) were used for pooling. Different cut-offs between studies had to cover largely the same range to be eligible for pooling. In case of mutually exclusive cut-offs, no meta-analysis was performed. When available, sub-analyses for highest sensitivity and highest specificity were performed using respective cut-offs. In case of largely overlapping cohorts analysing the same marker, those showing the best diagnostic values (smallest Euclidean distance) were included in the meta-analysis.

Meta-analyses were performed using a random effect model by the metafor package in R (version 3.2.2).[17] When the sensitivity or specificity for all markers included in the meta-analysis was between 30-70%, proportion for raw data (PR)

was used for calculation of pooled sensitivity and specificity. When either sensitivities or specificities of one of the markers included in the meta-analysis was <30% or >70%, Freeman-Tukey double Arcsine transformed proportion (PFT) was used, to overcome confidence limits outside the 0-100% range and variance instability.[18]

Analyses of sensitivity and specificity were performed univariate, as less than 6 studies is a contra-indication for a bivariate random effects model.[19] However, sensitivity analyses, using this bivariate random effects model (mada package R, version 3.2.2.), were performed for those markers evaluated in at least 6 studies to check the robustness of the univariate analyses.[20]

All calculations were rounded off to one decimal. The estimates of pooled sensitivities and specificities with 95% confidence intervals (CIs) were presented in forest plots. Heterogeneity was indicated by the I-squared test.

Results

Study characteristics

In total, 122 studies were identified (see supplementary data, list of included articles), in which 219 different blood markers, either as single marker (n=107) or as part of scoring systems (n=112), and 22 other diagnostic tests (*i.e.*, imaging, breath and urine tests) were evaluated to diagnose biopsy proven NASH versus simple steatosis. Meta-analyses could be performed, for 19 blood markers (and 41 studies), including 1 glycomics approach and 1 scoring system. Studies including (healthy) subjects without liver biopsy in the control group used to calculate the diagnostic values were included in the review and supplementary tables (16 studies[21-36]), but not in the meta-analysis, as for a substantial part no appropriate gold standard was available.

The studies on blood markers were categorised as follows: routine blood markers (*e.g.,* liver enzymes); markers related to inflammation, immune system and oxidative stress; markers of cell death, proliferation and cell structure; metabolic parameters; and -omics approaches. An overview of all diagnostic tests is given in Supplementary Table S6.1 for blood markers as single markers, Supplementary Table S6.2 for blood markers as part of scoring systems and in Supplementary Table S6.3 for other tests. The supplementary tables provide information on histologic scoring methods, fibrosis stages, definition and number of patients included and the study design.

Individual studies on blood markers showing both sensitivity and specificity ≥80% and results of the meta-analyses will be discussed in the sections below. Data on other biochemical markers and imaging tests can be found in the supplementary tables only.

Quality assessment

The results of the QUADAS-2 assessment for each domain (*i.e.,* patient selection, index test, reference standard, and patient flow and timing of the tests) are summarised in Table 6.1 and Supplementary Figure S6.1. Sixty-two studies scoring "high" for risk of bias on the domain 'Patient Selection' as well as on 'Flow and Timing', also included (healthy) controls without biopsy. However, most of these studies (46/62 studies) did not include the controls in the analyses to calculate the diagnostic values.
Studies scoring "unclear" on the domain 'Patient Selection', did not state whether consecutive or random patients were used (25 studies). In the domain 'Flow and Timing', "unclear" scores were mainly due to not describing the time interval between liver biopsy and index test (17 studies).

Routine blood markers

Liver enzymes are widely used to diagnose and monitor liver diseases. Alanine aminotransferase (ALT),[13,21,22,37-58] aspartate aminotransferase (AST),[21,38,39,41,42,44,46-48,51,53-55,57,59-65] gamma-glutamyl transferase (GGT),[41,44,47,49,53,57] and alkaline phosphatase (ALP)[44,53] have been studied to diagnose NASH. Pirvulescu *et al.*[44] showed both sensitivities and specificities over 80% for ALT and AST, as did Hendrikx *et al.*[21] for ALT (Supplementary Table S6.1).
Eight studies on ALT,[13,38,41,44,53,54,56,58] 5 on AST,[41,44,47,53,54] 4 on GGT,[41,44,47,53] and 2 on ALP,[44,53] could be pooled. ALT, AST, GGT and ALP showed pooled sensitivity ($sens_p$) and specificity ($spec_p$) of 63.5% and 74.4%, 76.9% and 61.9% (Figure 6.2), 63.7% and 64.2%, and 68.5% and 64.6% (Supplementary Figure S6.2), respectively, for the diagnosis of NASH versus SS (Table 6.2).

Table 6.1 An overview of the methods of patient selection and summary of the QUADAS-2 scoring for all included studies

			Patient Selection		Index Test		Reference Test		Flow and Timing
Author	Year	Methods of patient selection	Could the selection of patients have introduced bias?	Is there concern that the included patients do not match the review question?	Could the conduct or interpretation of the index test have introduced bias?	Is there concern that the index test, its conduct or interpretation differ from review question?	Could the reference test, its conduct or its interpretation have introduced bias?	Is there a concern that the target condition as defined by the reference test does not match the review question?	Could the patient flow have introduced bias?
Abdelaziz	2015	Patients presenting with obesity, fatigue and accidental discovery of elevated liver enzymes during routine check-up at Kasr El Aini University Hospital over a period of 1 year.	**high**	low	low	low	low	low	**high**
Abdel-Razik	2016	Patients with elevated liver enzymes, hepatosteatosis on US and patients admitted for cholecystectomy were included from the Mansoura University, Zagazig University, Aswan University between May 2012-Jul 2015	low	low	low	low	low	low	low
Abrigo	2014	Biopsy proven NAFLD patients (with persistently deranged liver function tests) recruited from a university hospital in Hong Kong	**high**	low	low	low	low	low	**high**
Aida	2014	Biopsy proven NAFLD patients from the Jikei University Katsushika Medical Centre, not further specified	*unclear*	low	low	low	low	low	low
Alaaeddine	2012	Newly diagnosed NASH patients at St George Hospital University Medical Centre and the Montreal University Hospital Centre	**high**	low	low	low	low	low	**high**
Alkhouri	2012	Patients who underwent LB for suspicion of NAFLD by their treating physician. All had elevated liver enzymes, in the absence of alternative causes of elevated aminotransferases.	low	low	low	low	low	low	low
Alkhouri	2014	Patients referred for liver biopsy to the Cleveland Clinic with clinical suspicion of NAFLD	low	low	low	low	low	low	*unclear*
Angulo	2014	Database of well-characterised and liver biopsy confirmed NAFLD patients from Newcastle University, University of Sydney, University of Torino, University of Kentucky (retrospective).	low	low	low	low	low	low	low

Table 6.1 (continued)

Anty	2010	Morbidly obese patients referred for bariatric surgery between Jan 2003-Apr 2009	*unclear*	low	low	low	low	low	low
Ballestri	2012	Biopsy proven NAFLD patients. Biopsies performed (2005-2009) based on clinical indications for suspected fatty liver disease, not further specified.	**high**	low	low	low	low	low	low
Banasch	2011	Patients with suspected NAFLD who underwent histological examination	**high**	low	low	low	low	low	**high**
Barr	2012	Biopsy proven NAFLD patients, reason for biopsy was due to suspected NAFLD by the treating physician (usually based on persistently abnormal liver enzymes or radiological evidence of fatty liver) or liver biopsy was performed during surgery (cholecystectomy or bariatric surgery)	*unclear*	low	low	low	low	low	*unclear*
Bastati	2014	Retrospective study including biopsy proven NAFLD patients between Jul 2007 and Jul 2013, not further specified.	low	low	low	low	low	low	low
Becker	2015	Biopsy proven NAFLD patients from 2 cohorts; moderate obese patients from Hannover Medical school and Severely obese patients undergoing bariatric surgery from Wurzburg University Hospital.	**high**	low	low	low	low	low	**high**
Bell	2010	Patients suspected of NAFLD who underwent liver biopsy were enrolled.	**high**	low	low	low	low	low	**high**
Blomme	2012	Retrospective study of biopsy proven NAFLD patients from three Belgian academic centres (Ghent, Antwerp and Erasmus). Patients underwent liver biopsy during bariatric surgery or percutaneous liver biopsy	low	low	low	low	low	low	low
Bookman	2006	Patients who were referred to the liver clinic due to abnormal liver biochemical tests.	**high**	low	low	low	low	low	**high**
Campos	2008	Patients undergoing elective bariatric surgery at the University of California San Francisco between Aug 2011-Jan 2006	low	low	low	low	low	low	low
Canbakan	2010	Biopsy proven NAFLD patients not further specified	*unclear*	low	low	low	low	low	*unclear*
Cengiz	2013	Patients with persistently elevated liver enzymes and hepatosteatosis on ultrasound were retrospectively studied	**high**	low	low	low	low	low	**high**
Cengiz	2014	Biopsy proven NASH patients and patients with persistently elevated liver enzymes and hepatosteatosis on US.	**high**	low	low	low	low	low	**high**
Chan	2014	Patients with NAFLD, scheduled for liver biopsy. NAFLD based on US finding and exclusion of secondary causes of fatty liver	**high**	low	low	low	low	low	**high**
Chen	2008	Biopsy proven NAFLD patients, not further specified	**high**	low	low	low	low	low	**high**
Chen	2011	Biopsy proven NAFLD patients who underwent MR elastography between Jan 2007-Mar 2010, retrospectively studied, not further specified.	low	low	low	low	low	low	**high**
Chrisholm	2012	Patients who underwent liver biopsy during bariatric surgery were enrolled.	low	low	low	low	low	low	low
Cusi	2014	Overweight/obese patients referred from general medicine or hepatology clinics	*unclear*	low	low	low	low	low	*unclear*

Table 6.1 (continued)

Diab	2008	Patients who underwent liver biopsy at the time of bariatric surgery	*unclear*	low	low	low	low	low	low
Dixon	2001	Patients with severe obesity (>35 kg/m2), having an adjustable gastric band inserted for weight reduction were biopsied during surgery.	*unclear*	low	low	low	low	low	*unclear*
Fedchuk	2014	Biopsy proven NAFLD patients between 2000-2010 for clinical and/or ultrasonographic suspicion of NAFLD were retrospectively studied.	low	low	low	low	low	low	low
Feldstein	2009	Biopsy proven NAFLD patients seen at 8 centres across US who participate in NASH CRN, not further specified.	**high**	low	low	low	low	low	**high**
Feldstein	2010	Biopsy proven NAFLD patients, referred by the treating physician for a baseline liver biopsy in the context of diagnostic evaluation for suspected NAFLD	low	low	low	low	low	low	low
Fierbinteanu (J Gastrointestin Liver Dis)	2013	Biopsy proven NAFLD patients, biopsy was performed for abnormal liver function test or suspected NAFLD on US, diagnosed at University Hospital Bucharest between 2007 and 2010	**high**	low	low	low	low	low	**high**
Fierbinteanu (Ultrasound in Med. & Biol)	2013	Biopsy proven NAFLD patients, diagnosed at University Hospital Bucharest between 2007 and 2010, not further specified.	**high**	low	low	low	low	low	**high**
Fotiadu	2010	Patients who presented at outpatient clinic of Hippokrateion Hospital of Thessaloniki with possible NAFLD.	*unclear*	low	low	low	low	low	low
Friedrich	2012	Biopsy proven NAFLD patients enrolled between May 2009-Dec 2010, not further specified.	low	low	low	low	low	low	**high**
Garcia-Galiano	2007	Morbidly obese patients undergoing bariatric surgery	**high**	low	low	low	low	low	**high**
Gholam	2007	Severely obese subjects (BMI≥40 kg/m2) undergoing Roux-en-Y gastric bypass surgery	low	low	low	low	low	low	low
Grigorescu	2012	Patients with biopsy proven NAFLD, who underwent liver biopsy for abnormal liver function tests (>6 months) and suspected NAFLD at US.	low	low	low	low	low	low	*unclear*
Guajardo	2010	Patients who underwent a Roux-en-Y gastric bypass between 2005 and 2006 were retrospectively reviewed	low	low	*unclear*	*unclear*	low	low	**high**
Gulsen	2005	Biopsy proven NAFLD patients with chronic hypertransaminasemia (> 6 months) not further specified.	**high**	low	low	low	low	low	**high**
Hendrikx	2015	Biopsy proven NAFLD patients, who underwent biopsy due to abnormal high ALT levels.	**high**	low	low	low	low	low	**high**
Hui	2004	Biopsy proven NAFLD patients, who were referred for the assessment of abnormal liver function tests or hepatic steatosis detected on US	**high**	low	low	low	low	low	**high**
Huyogo	2007	Patients with biopsy proven NAFLD, referred to Hiroshima University Hospital for abnormal liver function tests or hepatic steatosis on US/CT	**high**	low	low	low	low	low	**high**

Table 6.1 (continued)

Imajo	2012	Biopsy proven NAFLD patients from 4 academic medical centres in Japan, not further specified.	**high**	low	low	low	low	low	**high**
Imajo	2016	Biopsy proven NAFLD patients, from the Yokohama City University Hospital, enrolled between Jul 2013-Apr 2015, not further specified.	**high**	low	low	low	low	low	**high**
Jamali	2016	Subjects with persistently elevated serum aminotransferases and fatty liver in US admitted to the hepatology clinic of a general university hospital	**high**	low	low	low	low	low	**high**
Joka	2012	Biopsy proven NAFLD patients, not further specified.	**high**	low	low	low	low	low	**high**
Kamada	2015	Biopsy proven NAFLD patients recruited in five hepatology centres in Japan between 2002-2013, not further specified.	*unclear*	low	low	low	low	low	*unclear*
Kamada (Plos One)	2013	Sera of biopsy proven NAFLD patients remaining after medical check-up were used after permission of the subject, not further specified	**high**	low	low	low	low	low	**high**
Kamada (Proteomics Clin Appl)	2013	Sera of biopsy proven NAFLD patients remaining after medical check-up were used after permission of the subject, not further specified	**high**	low	low	low	low	low	**high**
Kashyap	2009	Patients undergoing laparoscopic obesity surgery (liver biopsy obtained during surgery)	low	low	low	low	low	low	low
Kawanaka	2015	Biopsy proven NAFLD patients who underwent liver biopsy at Kawasaki Hospital between 2007-2012, not further specified.	*unclear*	low	low	low	low	low	low
Kazankov	2015	Patients from a gastric surgery program, prospectively recruited between 2003-2012 at the Hotel-Dieu and Pitie-Salpetriere Hospitals	*unclear*	low	low	low	low	low	low
Kikuchi	2009	Biopsy proven NAFLD patients, who underwent liver biopsy for clinical suspicion of NAFLD by their treating hepatologist.	*unclear*	low	low	low	low	low	*unclear*
Kim	2013	Patients who underwent liver biopsy for suspected NAFLD between Jan 2009-Jul 2011 at ten Korean University hospitals (elevated transaminases for 3 months and fatty liver on US were main reason for liver biopsy)	low	low	low	low	low	low	*unclear*
Koehler	2012	patients with class III obesity who underwent bariatric surgery	low	low	low	low	low	low	low
Kunde	2005	Women with class II/III obesity undergoing bariatric surgery were retrospectively studied	low	low	low	low	low	low	*unclear*
Lassaily	2011	Severely obese patients referred to the unit for evaluation in view of bariatric surgery were considered for inclusion.	low	low	low	low	low	low	low
Le Corvec	2016	A biobank of patients with a suspicion of steatosis, not further specified.	*unclear*	low	low	low	low	low	*unclear*
Lemoine	2009	Biopsy proven NAFLD patients hospitalised between 2002 and 2004 at Saint-Antoine and Pitie Saletriere hospitals. Liver biopsy was performed for abnormal liver tests.	**high**	low	low	low	low	low	**high**

Table 6.1 (continued)

Leon-Mimila	2015	Severely obese subjects who underwent bariatric surgery, recruited from the Hospital General Ruben Lenero in Mexico City.	*unclear*	low	low	low	low	low	low
Liang	2007	Biopsy proven NAFLD patients who were admitted for laparoscopic bariatric surgery were enrolled.	low	low	low	low	low	low	low
Loomba	2015	Biopsy proven NAFLD patients from the University of California at San Diego NAFLD research clinic (Jan 2011-Nov 2012), not further specified	**high**	low	low	low	low	low	**high**
Maher	2015	Biopsy proven NAFLD patients from the internal medicine and hepatology outpatient clinics and inpatient wards of Ain Shams University Hospital (Dec 2012-Jul 213)	**high**	low	low	low	low	low	**high**
Malik	2008	Patients attending Beth Israel Deaconess Medical Centre liver clinic	low	low	low	low	low	low	low
Manousou	2011	Clinical records of patients with a pathological diagnosis of steatosis and/or steatohepatitis were reviewed retrospectively.	**high**	low	low	low	low	low	low
Miele	2009	Biopsy proven NAFLD patients who were observed in the outpatient liver unit of the catholic University of the Sacred Heart Medical Centre in Rome.	low	low	low	low	low	low	low
Miller	2014	Sera of biopsy proven NAFLD patients, not further specified	**high**	low	low	low	low	low	**high**
Morita	2015	Morbidly obese patients admitted at Base Hospital of the Sao Jose do Rio Preto Medical School between August 2010 and January 2014 for Roux-en-Y gastric bypass surgery were evaluated.	*unclear*	low	low	low	low	low	*unclear*
Murakami	2012	Biopsy proven NASH patients, not further specified	**high**	low	low	low	low	low	**high**
Ogawa	2013	Biopsy proven NAFLD patients from the Yokohama City University Hospital, not further specified	**high**	low	low	low	low	low	**high**
Ozenirler	2014	Biopsy proven NASH patients with abnormal ALT persisting for more than 6 months and US diagnosed fatty liver were retrospectively reviewed	**high**	low	low	low	low	low	**high**
Ozturk	2015	Patients admitted to the hepatology outpatient clinic within the last year and had been diagnosed with NAFLD based on liver biopsy.	**high**	low	low	low	low	low	**high**
Page	2013	Sera of biopsy proven NAFLD patients, not further specified.	*unclear*	low	low	low	low	low	*unclear*
Palekar	2006	Patients were identified from the liver biopsy database at Brooke Army Medical Centre, patients were considered for inclusion if they had histopathologic evidence of fatty liver disease, either simple steatosis or NASH	**high**	low	low	low	low	low	**high**
Papatheodoridis	2010	NAFLD patients admitted for liver biopsy, who were followed at the outpatient clinic for the previous 6 months.	**high**	low	low	low	low	low	**high**
Parente	2015	Type 2 diabetic subjects from the endocrinology department with a clinical indication for liver biopsy for the evaluation of NAFLD (included between Jun 2010-Feb 2012	low	low	low	low	low	low	*unclear*

Table 6.1 (continued)

Pimentel	2016	Subjects were enrolled in the NAFLD patient registry at Beth Israel Deaconess Medical Centre from 2009-2014	*unclear*	low	low	low	low	low	low
Pirola	2015	Sera of biopsy proven NAFLD patients, not further specified	**high**	low	low	low	low	low	**high**
Pirvulescu	2012	Overweight, obese and morbidly obese patients with significant weight-related comorbidities admitted in Fundeni Clinical Institute or Delta Clinical Hospital between 2009-2010	*unclear*	low	low	low	low	low	low
Polyzos (Annals of Hepatology)	2013	Biopsy proven NAFLD patients were recruited on an outpatient basis between Jun 2008-Nov 2010.	**high**	low	low	low	low	low	**high**
Polyzos (Biomarkers)	2013	Adult Greek NAFLD patients were recruited on an outpatient basis between Jun 2008-Nov 2010.	*unclear*	low	low	low	low	low	low
Portincasa	2006	Biopsy proven NAFLD patients with hypertransaminasemia, referred to the department of internal medicine at the university of Bari.	**high**	low	low	low	low	low	**high**
Poynard	2006	Patients with suspicion of NAFLD hospitalised in our department having undergone liver biopsy (for abnormal serum transaminases or GGT, or steatosis at US or features of the metabolic syndrome). Another part of patients was recruited via the CYTOL study, which recruits patients with chronic abnormal ALT or GGT.	**high**	low	low	low	low	low	low
Poynard	2012	Severely obese patients, not further specified	*unclear*	low	low	low	low	low	low
Pulzi	2011	Medical records of patients submitted to gastroplasty at the hospital da Irmandade de Santa Casa de misericordia de Sao Paulo (Mar 2004-Apr 2006), who had liver biopsy during Roux-en-Y distal gastric bypass were reviewed	low	low	low	low	low	low	**high**
Rahman	2012	Biopsy proven NAFLD patients, recruited from the liver clinics at Royal Prince Alfred and Westmead hospitals, not further specified.	**high**	low	low	low	low	low	**high**
Ratziu	2006	Patients with suspicion of NAFLD hospitalised in our department having undergone liver biopsy (for abnormal serum transaminases or GGT, or steatosis at US or features of the metabolic syndrome). Another part of patients was recruited via the CYTOL study, which recruits patients with chronic abnormal ALT or GGT.	**high**	low	low	low	low	low	**high**
Shen (Ap&T)	2012	Patients who underwent liver biopsy for suspected NAFLD at Prince of Wales Hospital	**high**	low	low	low	low	low	**high**
Shen (Journal of Hepatology)	2012	Patients who underwent liver biopsy for suspected NAFLD at Prince of Wales Hospital and patients from a prospective study with paired liver biopsies. (main reason for liver biopsy were abnormal liver function tests and/or abnormal imaging)	**high**	low	low	low	low	low	**high**

Table 6.1 (continued)

Shigefuku	2012	Patients with NAFLD who had had a liver biopsy and had undergone Xe-CT at St. Marianna University School of Medicine Hospital between Dec 2003-Jan 2011	*unclear*	low	low	low	low	low	low
Shimada	2007	Biopsy proven NAFLD patients, not further specified	*unclear*	low	low	low	low	low	*unclear*
Smits	2016	Biopsy proven NAFLD patients, not further specified.	**high**	low	low	low	low	low	**high**
Sookoian	2009	Biopsy proven NAFLD patients from a county hospital in Buenos Aires, not further specified.	*unclear*	low	low	low	low	low	**high**
Sumida	2011	Biopsy proven NAFLD patients who were seen at the Centre for Digestive and liver diseases, Nara City Hospital from 2002-2008, not further specified. A validation cohort consisted of biopsy proven NAFLD patients from eight hepatology centres in Japan (2002-2008)	low	low	low	low	low	low	*unclear*
Suzuki	2010	Biopsy proven NAFLD patients from the Yokohama City University Hospital, not further specified.	**high**	low	low	low	low	low	low
Tamimi	2011	Initial cohort: Patients undergoing liver biopsy for clinical suspicion of NASH by their treating hepatologists (mostly due to abnormal liver enzymes) Validation cohort: underwent liver biopsy during bariatric surgery.	low	low	low	low	low	low	low
Tanwar	2013	Patients visiting the outpatient clinic from the Queen's Medical Centre, Nottingham and the Freeman Hospital, Newcastle-Upon-Tyne.	low	low	low	low	low	low	low
Tarantino	2007	Patients with overweight or obesity, who were referred during a 3-year period to the metabolic unit of the University Medical Centre were included and a liver biopsy was performed.	**high**	low	low	low	low	low	**high**
Tarantino	2009	Patients referred to obesity outpatient clinic of a university hospital	**high**	low	low	low	low	low	**high**
Tomita	2008	Patients undergoing a liver biopsy due to clinical suspicion of NAFLD by the treating hepatologist (steatosis on US, elevated liver enzymes > 6 months, evidence of clinical components of metabolic syndrome).	low	low	low	low	low	low	low
Tomita	2011	Biopsy proven NAFLD patients, with elevated liver enzymes for more than 6 months	low	low	low	low	low	low	low
Trak	2009	Candidates for bariatric surgery	**high**	low	low	low	low	low	**high**
Ulukaya	2009	Biopsy proven NAFLD patients, not further specified	**high**	low	low	low	low	low	**high**
Verdam	2013	Overweight and obese patients undergoing cholecystectomy or bariatric surgery (intraoperative liver biopsy)	low	low	low	low	low	low	low
Vongsuvahn	2012	Biopsy proven NAFLD patients recruited from a tertiary liver clinic, patients were referred for abnormal liver tests or hepatic steatosis on US.	**high**	low	low	low	low	low	**high**

Table 6.1 (continued)

Wieckowska	2006	Patients undergoing liver biopsy for clinical suspicion of NAFLD by their treating hepatologist. All patients had persistently elevated liver enzymes for more than 3 months and evidence of clinical components of the metabolic syndrome.	**high**	low	low	low	low	low	**high**
Yaman	2005	Biopsy proven NAFLD patients with chronic hypertransaminasemia, not further specified.	**high**	low	low	low	low	low	**high**
Yamasaki	2015	Biopsy proven NAFLD patients admitted to Okayama University Hospital, not further specified (retrospectively studied).	low	low	low	low	low	low	low
Yang	2015	Biopsy proven NAFLD patients who underwent liver biopsy for suspected NAFLD at Beijing 302 hospital were retrospectively and prospectively screened.	**high**	low	low	low	low	low	**high**
Yilmaz	2007	Patients with suspected NAFLD, not further specified.	**high**	low	low	low	low	low	**high**
Yilmaz	2012	Patients were identified from a larger cohort of patients who underwent liver biopsy for suspected NAFLD.	**high**	low	low	low	low	low	**high**
Yilmaz	2009	Patients with suspected NAFLD from the department of Gastroenterology, Uludag University Medical School.	**high**	low	low	low	low	low	**high**
Yilmaz	2010	Biopsy proven NAFLD patients seen at the specialised outpatient clinic in the past 12 months, with US steatosis.	**high**	low	low	low	low	low	**high**
Yilmaz	2014	Biopsy proven NAFLD patients enrolled in a tertiary referral hepatology clinic, not further specified.	low	low	low	low	low	low	low
Yoneda	2007	Biopsy proven NAFLD patients, who underwent liver biopsy at Yokohama city University Hospital between apr 2004-Sept 2006, not further specified.	*unclear*	low	low	low	low	low	*unclear*
Yoneda	2008	Biopsy proven NAFLD patients, not further specified	**high**	low	low	low	low	low	**high**
Yoneda	2010	Biopsy proven NAFLD patients, not further specified	**high**	low	low	low	low	low	**high**
Yoshimura	2016	Biopsy proven NAFLD patients, diagnosed between Jul 2011-Jun 2013 in the Saiseikai Suita Hospital (Japan). The validation cohort was recruited in the same centre from Jun 2013-Apr 2014.	*unclear*	low	low	low	low	low	low
Younossi	2008	Serum samples were obtained from the epidemiology of NAFLD database and specimen repository (EPI-NAFLD), which includes biopsy proven NAFLD patients from Inova Fairfax Hospital (from Oct 2001 onwards)	**high**	low	low	low	low	low	**high**
Younossi	2010	Samples from biopsy proven NAFLD patients from the Inova Fairfax Hospital.	*unclear*	low	low	low	low	low	*unclear*
Younossi	2011	Serum samples were obtained from the epidemiology of NAFLD database and specimen repository (EPI-NAFLD), which includes biopsy proven NAFLD patients from Inova Fairfax Hospital (from Oct 2001 onwards)	*unclear*	low	low	low	low	low	low

Quality Assessment of Diagnostic Accuracy Studies-2 (QUADAS-2) is designed to assess the quality of primary diagnostic accuracy studies. It consists of four key domains: patient selection, index test, reference standard, and flow of patients through the study and timing of the index test(s) and reference standard ("flow and timing"). Each domain is assessed in terms of the risk of bias and the first three are also assessed in terms of concerns regarding applicability; the scores (low, unclear, high) and methods of patient selection are shown in the table. Abbreviations: non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), clinical research network (CRN), body mass index (BMI), ultrasound (US), xenon-computed tomography (Xe-CT), gamma-glutamyl transferase (GGT), alanine aminotransferase (ALT).

Table 6.2 An overview of the pooled sensitivity and specificity of biomarkers and scoring system pooled in the meta-analyses

Test	Number of studies	Sensitivity % (95% CI)	NASH (n)	Specificity % (95% CI)	SS (n)
Biomarkers					
ALT	8	63.5 (44.1-81.0)	447	74.4 (59.7-86.8)	566
AST	5	76.9 (62.3-88.9)	185	61.9 (35.7-85.0)	323
GGT	4	63.7 (41.1-83.7)	125	64.2 (49.5-77.6)	185
ALP	2	68.5 (57.8-79.2)	73	64.6 (54.0-75.1)	79
Ferritin	2	79.1 (49.2-97.9)	126	71.9 (60.7-82.0)	71
N/L ratio	2	73.0 (66.0-79.5)	170	75.3 (72.3-78.3)	804
Adiponectin	4	72.0 (46.3-92.0)	210	75.7 (64.5-85.6)	172
IL-6	3	60.6 (51.7-69.6)	114	83.9 (44.7-100.0)	94
CK18-M30	15	68.4 (62.4-74.0)	1017	74.2 (68.3-79.7)	839
CK18-M65	5	76.2 (62.8-87.5)	198	73.7 (66.9-80.0)	192
CK18-M65ED	2	91.2 (63.7-100.0)	81	64.1 (44.5-81.7)	88
FGF-21	2	67.0 (40.3-89.0)	150	75.5 (68.8-81.7)	175
HOMA IR	6	69.0 (56.9-80.0)	274	72.7 (63.6-81.1)	230
Fuc-Hpt	2	66.9 (60.4-73.4)	202	79.4 (66.4-90.1)	48
Mac2bp	2	79.4 (68.9-88.2)	204	70.4 (56.2-83.0)	47
Triglycerides	2	73.7 (65.3-81.4)	126	59.9 (47.9-71.8)	110
Total cholesterol	2	72.6 (44.5-94.2)	25	55.7 (45.8-65.6)	97
Omics approach					
GlycoNASH test	2	67.1 (24.7-97.5)	100	63.8 (18.1-98.4)	153
Subanalyses					
CK18-M30 highest sensitivity	7	82.5 (75.1-88.8)	303	61.5 (43.6-78.0)	316
CK18-M30 highest specificity	7	53.2 (35.1-70.9)	303	93.3 (83.4-99.2)	316
Scoring system					
NASH Test	2	22.9 (7.2-43.9)	148	95.3 (93.1-97.0)	603
*NASH vs. borderline NASH + SS**					
NASH Test	2	90.9 (85.6-95.2)	371	41.3 (25.4-57.3)	380
*NASH + borderline NASH vs. SS**					

Non-alcoholic steatohepatitis (NASH) is the number of patients diagnosed with NASH via liver biopsy. Simple steatosis (SS) is the number of patients with biopsy proven simple steatosis (Whether or not this includes SS or the combination of SS and normal (NL) depends on the individual studies). Due to interstudy variation with regards to the classification used to define NASH, whether or not borderline NASH was included in the NASH group or in the SS group depends on the individual studies and classification system used (supplementary tables). *Classification based on NAS score: 0-2 no NASH, 3-4 borderline NASH, ≥5 NASH. Abbreviations: alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP), neutrophil/lymphocyte ratio (N/L ratio), interleukin (IL), cytokeratin (CK), fibroblast growth factor (FGF), Homeostatic model assessment for insulin resistance (HOMA-IR), Fucosylated-haptoglobin (Fuc-Hpt), Mac2 binding protein (Mac2bp), confidence interval (CI).

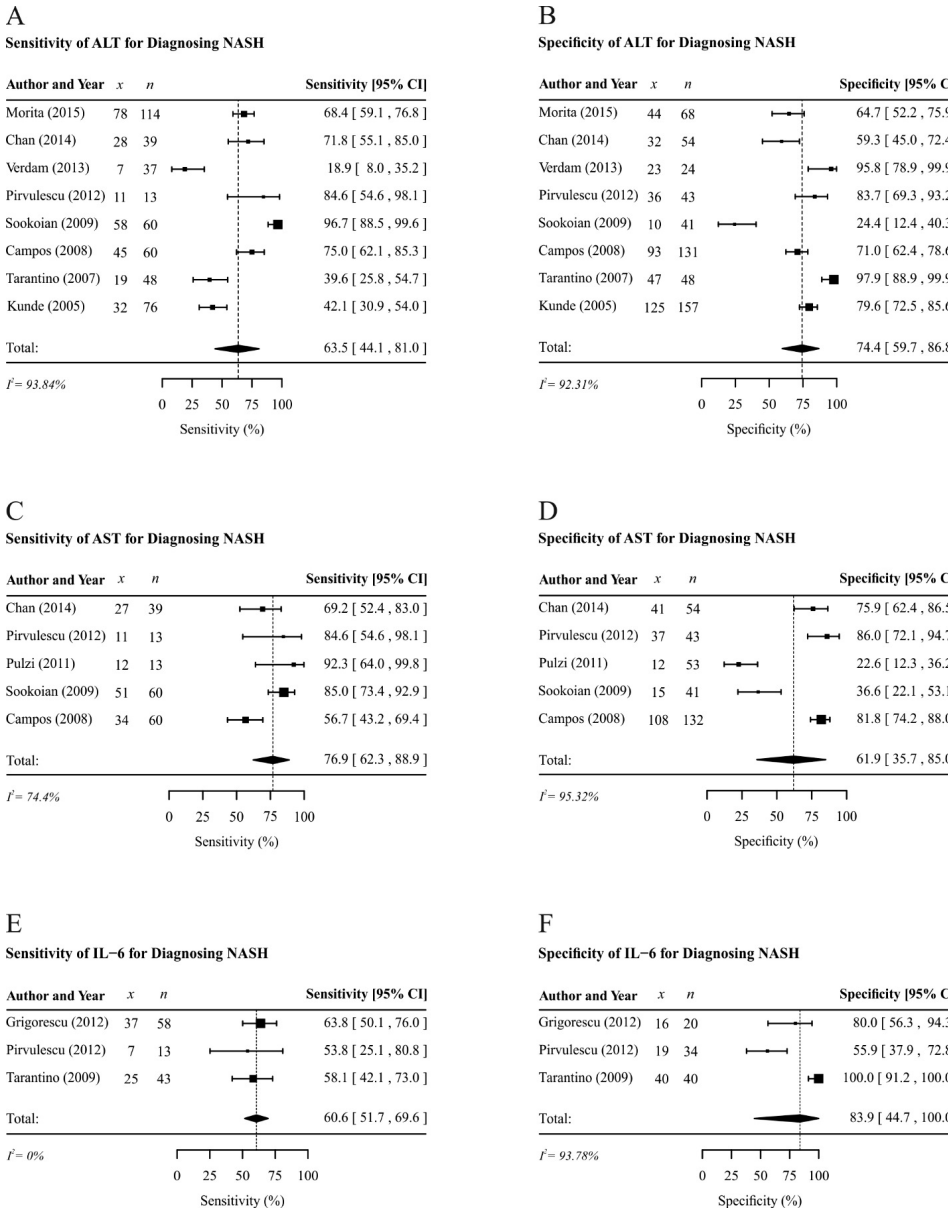

Figure 6.2 **Forest plots** for pooled sensitivity of alanine aminotransferase (ALT) (**A**), pooled specificity of ALT (**B**), pooled sensitivity of aspartate aminotransferase (AST) (**C**), pooled specificity of AST (**D**), pooled sensitivity of interleukin-6 (IL-6) (**E**) and pooled specificity of IL-6 (**F**). Any discrepancy between the sensitivity and specificity for an individual study given in the forest plot and the original article is due to rounding off and/or reported missing data. A difference of ≤2% was accepted. Abbreviations: non-alcoholic steatohepatitis (NASH), confidence interval (CI).

Inflammation, immunologic and oxidative stress parameters

As NASH is considered the inflammatory entity of NAFLD, several inflammatory-, oxidative stress-, and immune parameters have been investigated. Sensitivity and specificity ≥80% was only reported for adiponectin in one study[44], showing a sensitivity of 92.3% and a specificity of 86.7%.
Ferritin,[61,66] Neutrophil/Lymphocyte (N/L) ratio,[60,67] Interleukin(IL)-6,[43,44,68] and adiponectin[43,44,69,70] could be pooled (Table 6.2), resulting in a $sens_p$ of 79.1% and $spec_p$ of 71.9% for ferritin (Supplementary Figure S6.2), $sens_p$ of 73.0% and $spec_p$ of 75.3% for N/L ratio (supplementary figure S6.3), $sens_p$ of 60.6% and $spec_p$ of 83.9% for IL-6 (Figure 6.2), and $sens_p$ of 72.0% and $spec_p$ of 75.7% for adiponectin (Supplementary Figure S6.3).

Markers of cell death, proliferation and cell structure

Among markers of cell death, cytokeratin 18 (CK18) has been studied extensively. This is the major intermediate filament protein of the liver, which is cleaved by caspases during apoptosis of hepatocytes, generating several fragments.[71] Assays measure either the cleaved CK18-M30 or the cleaved and intact CK18-M65.[72]
CK18-M30 was evaluated in 29 studies;[26,27,37,39,41,44,49,73-94] of which 15[41,44,73-75,77,79,82,84,86-88,91,93,94] could be combined, resulting in 68.4% $sens_p$ and 74.2% $spec_p$ (Table 6.2, Figure 6.3). Two studies showed both a sensitivity and specificity of ≥80% for CK18-M30.[26,92] Since various cut-offs for CK18-M30 were investigated within studies, sub-analyses were conducted for cut-offs optimising sensitivity or specificity,[41,84,86-88,90,92] resulting in a highest $sens_p$ of 82.5% (with 61.5% specificity) and highest $spec_p$ of 93.3% (with 53.2% sensitivity; Table 6.2, Supplementary Figure S6.4).
Five[43,44,82,83,91] out of six studies[27,43,44,82,83,91] on CK18-M65 could be pooled, showing 76.2% $sens_p$ and 73.7% $spec_p$ (Table 6.2, Figure 6.3). Two studies[82,83] assessing CK18-M65ED, which differs from CK18-M65 in the way of analysis,[72] showed a $sens_p$ of 91.2% and a $spec_p$ of 64.1% (Table 6.2, Figure 6.3).
Only one study showed a sensitivity of 100% and specificity of 80%, for CK18-M65 and –M65ED (Supplementary Table S6.1).[82]

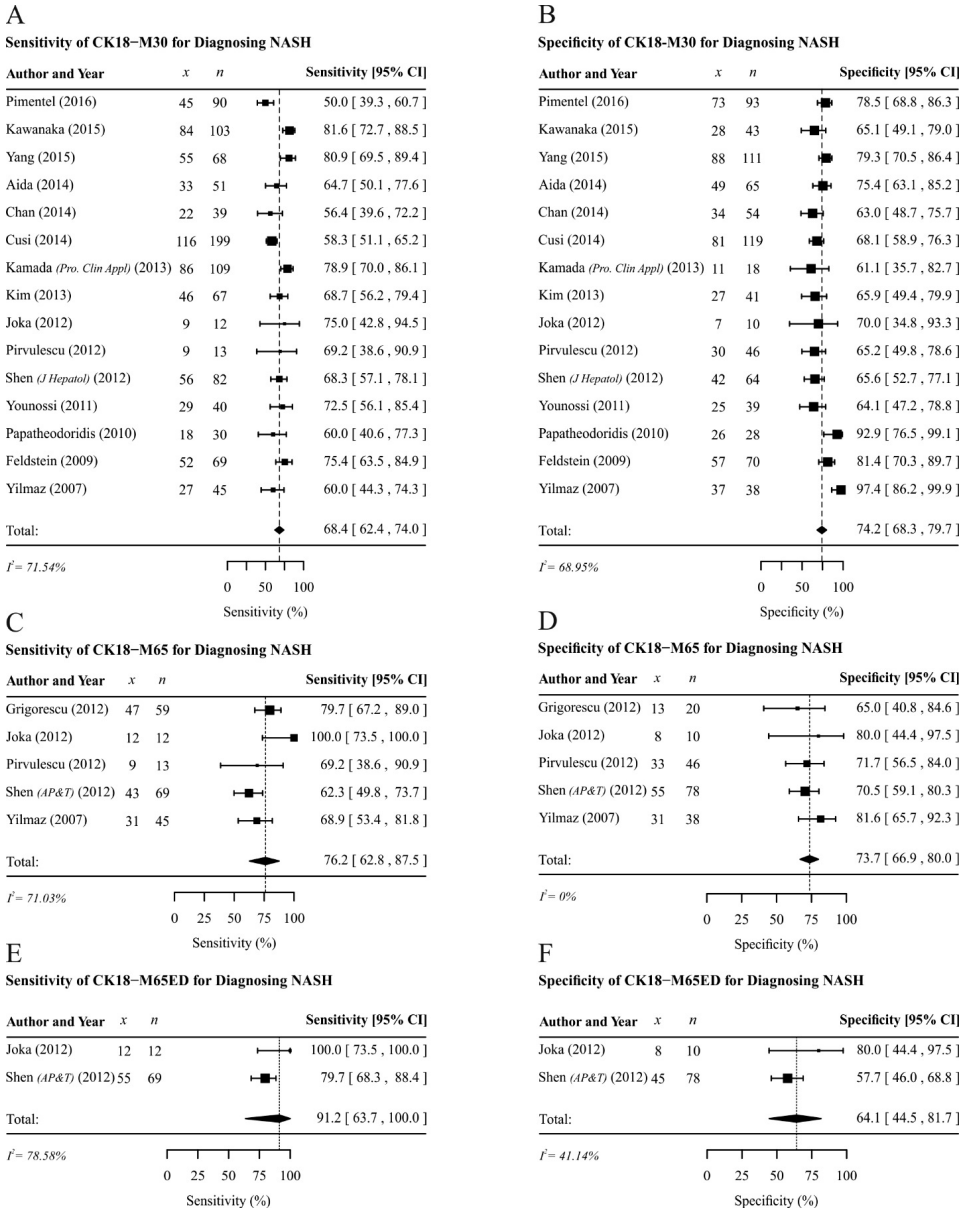

Figure 6.3 **Forest plots** for pooled sensitivity of cytokeratin (CK) 18-M30 (**A**), pooled specificity of CK18-M30 (**B**), pooled sensitivity of CK18-M65 (**C**), pooled specificity of CK18-M65 (**D**), pooled sensitivity of CK18-M65ED (**E**) and pooled specificity of CK18-M65ED (**F**). Any discrepancy between the sensitivity and specificity for an individual study given in the forest plot and the original article is due to rounding off and/or reported missing data. A difference of ≤2% was accepted. Abbreviations: non-alcoholic steatohepatitis (NASH), confidence interval (CI).

Several growth factors (fibroblast growth factor 21 (FGF-21),[73,84] vascular endothelial growth factor,[68] insulin-like growth factor,[95] and pigment epithelium-derived factor (PEDF)[73]) were studied, of which data for FGF-21 could be pooled, showing a $sens_p$ of 67.0% and a $spec_p$ of 75.5% (Table 6.2, Supplementary Figure S6.3).

Additional markers to diagnose NASH can be found in table S6.1. None of these could be pooled. Sensitivities and specificities ≥80% were reported for tissue polypeptide specific antigen (91.7%/95.8%)[56] and tissue inhibitor metalloproteases(TIMP)-1 (96.7%/100%) and TIMP-2 (93.3%/100%).[30]

Metabolic parameters

Of the glycaemic and lipidaemic parameters studied, Gulsen *et al.*[96] showed a sensitivity of 91.67% and specificity of 95.65% for homocysteine to diagnose NASH in a biopsy proven NAFLD population (Supplementary Table S6.1).
A meta-analysis of 6 studies[44,53,69,70,95,97] investigating HOMA-IR could be performed; showing 69.0% $sens_p$ and 72.7% $spec_p$ (Table 6.2). Furthermore, pooling fucosylated-haptoglobin (Fuc-Hpt),[78,98] Mac2 binding protein (Mac2bp),[77,98] triglycerides (TG)[38,44] and total cholesterol (TC)[44,47] resulted in a $sens_p$ of 66.9% and $spec_p$ of 79.4% for Fuc-Hpt, $sens_p$ of 79.4% and $spec_p$ of 70.4% for Mac2bp, $sens_p$ of 73.7% and $spec_p$ of 59.9% for TG, and $sens_p$ of 72.6% and $spec_p$ of 55.7% for TC for diagnosing NASH (Supplementary Figures S6.5-6.6 and table 6.2). For Fuc-Hpt and Mac2bp, it should be noted that potential overlap between cohorts was unclear.[77,78,98]

Omics approaches and scoring systems

The application of –omics approaches to identify discriminating molecules or profiles for disease diagnosis is emerging. The Glycomarker[81]/GlycoNASH test[99] data could be pooled, showing a $sens_p$ of 67.1% and $spec_p$ of 63.8% (Table 6.2, Supplementary Figure S6.6). The clinical and phosphoproteomic model of Younossi *et al.*[100] showed a sensitivity of 81.3% and specificity of 87.0%. The MiRNA profile (MiR-122, -192, -21) combined with CK18-asp396 by Beckers *et al.* also showed good sensitivity and specificity (91% and 83%, resp.).[37] None of the other (combinations of) markers identified by the -omics approaches did result in both sensitivities and specificities of ≥80% (Supplementary Table S6.1).
Additionally, blood markers were often combined in scoring systems. The NASHTest,[45,57] combining 10 markers with age, gender, weight, height, and a

positive result on the SteatoTest, showed a $spec_p$ of 95.3% for differentiating NASH from borderline NASH and SS ($sens_p$ 22.9%), and 90.9% $sens_p$ and 41.3% $spec_p$ for differentiating NASH plus borderline NASH from SS (Table 6.2, Supplementary Figure S6.7).
Another 12 studies reporting on various scoring systems showing both sensitivities and specificities ≥80% (Supplementary Table S6.2),[21,35,43,44,49,67,73,85,91,101-103] however, none of these could be pooled.

Sensitivity analysis

For the markers evaluated in at least six studies (*i.e.*, ALT, CK18-M30, HOMA-IR and CK18-M30), $sens_p$ and $spec_p$ were also calculated by the bivariate random effects model (Supplementary Table S6.4). Results were comparable to univariate analyses, indicating robustness of the findings.

Discussion

NASH is a rapidly increasing health problem, for which non-invasive diagnostics are crucial due to its progressive nature and the fact that liver biopsy is unsuitable for diagnosis in large populations. To provide an overview on the current status, we performed this systematic review with meta-analysis on non-invasive diagnostic tests for NASH versus simple steatosis with biopsy as standard. A wide variety of blood markers, either as single marker or as part of scoring systems have been studied, but only for a limited number sensitivity and specificity were evaluated in at least two studies. None of the pooled markers showed both sensitivity and specificity ≥80%. However, it should be noted that the diagnostic tests that could be pooled, covered largely the same range in cut-off values, with the largest range for AP, CK18, FGF21, Adiponectin and Fuc-Hpt. Furthermore, the majority of studies contained only limited numbers of patients with significant fibrosis (F3-4) or did not provide the stages of fibrosis. The above should be taken into account when interpreting the meta-analysis results and shows the urgent need for more standardised study designs, including histological scoring.

Although the exact pathogenesis is still unclear, it is generally accepted that multiple hits (in parallel) play a role in the progression to NASH.[104] They include *e.g.*, altered metabolic make up, inflammation, oxidative stress, and

fibrogenesis (Figure 6.4). Due to the complexity of the disease, it is unlikely that a single marker will be able to differentiate between NASH and simple steatosis. This is supported by the meta-analysis results showing that none of the pooled markers showed both sensitivity and specificity ≥80%. However, several scoring systems[49,73,91,101] and –omics approaches,[37] that combine markers of at least two domains related to specific pathophysiologic processes associated with NASH (Figure 6.4) in well-designed studies, showed promising findings (sensitivities and specificities ≥80%). Although internal validation was included in some studies, external validation of promising markers and scores (including previously studied cut-off values) are urgently needed. When applying the sensitivity and specificity of these promising scores[49,73,91,101] and omics approaches[37] to hypothetical high (50%) and low (10%) prevalence NASH populations, false positive and false negative rates would vary between 4.4 and 16.7% and between 10 and 20%, respectively, for the low prevalence population, and between 4 and 16% and between 6 and 18%, respectively, for the high prevalence population.

The major strengths of our systematic review with meta-analysis include the comprehensive search, adherence to criteria for conducting a meta-analysis, and the qualitative assessment of the studies. Furthermore, an extensive overview of all studied diagnostic tests is provided.

With regard to the individual studies included in this review, study methods vary widely. Different criteria have been used for patient recruitment; many studies included subjects with suspected NAFLD (based on elevated liver enzymes, steatosis on US or suffering from one or more components of the metabolic syndrome, 40%), being overweight/obese and undergoing a liver biopsy (whether or not during bariatric surgery, 20%) or proven NAFLD without further specification (about 30%). It should be noted that the selection criterion, having elevated liver enzymes and/or steatosis on US, could lead to work-up bias. Furthermore, selection bias should be taken into account, as not all NAFLD patients have elevated liver enzymes and more severely ill patients are more likely to undergo a liver biopsy. However, liver biopsy still is the current gold standard to diagnose NASH. Therefore, studies including (healthy) subjects without liver biopsy in the calculation of the diagnostic values (n=16), were included in the review and supplementary tables, but not in the meta-analysis, as for a substantial part of the subjects in the analysis no appropriate gold standard was available. Although the majority of studies used the criteria by Brunt *et al.*[105] or Kleiner *et al.*[106] to score liver biopsies (see supplementary tables), the criteria applied did differ between studies. Furthermore, not all

studies reported data on the quality of histology (e.g. adequate biopsy length, number of portal tracts) and/or fibrosis stage. Expectation bias is thought to be low, as in 60% of studies the liver biopsy results were interpreted without knowledge of the index test, in the other 40% this was unclear.

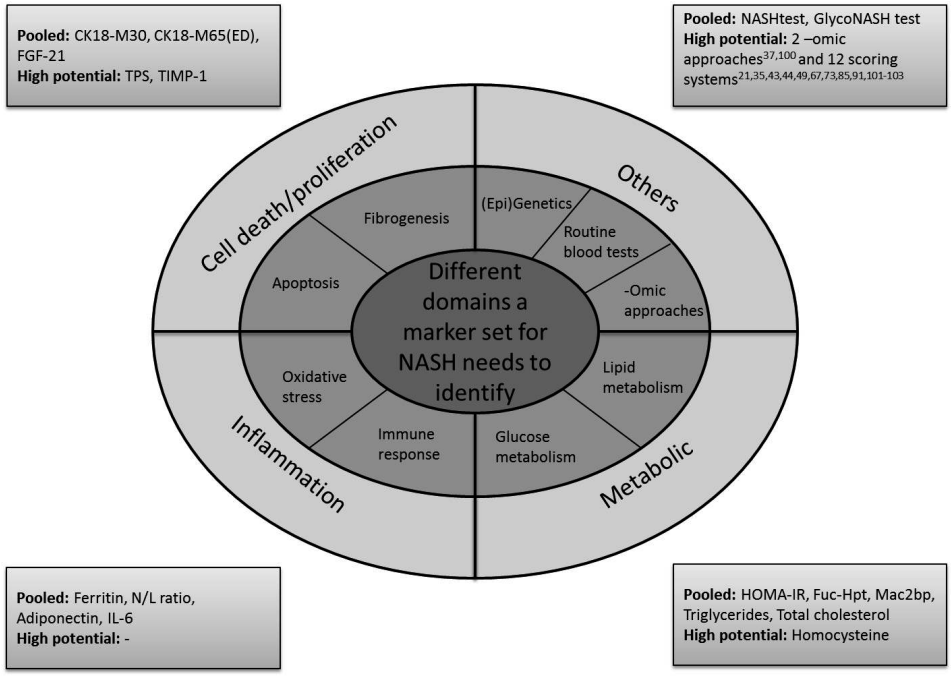

Figure 6.4 **Domains that play an important role in the progression of NAFLD to NASH**, such as inflammation, metabolic make up and fibrogenesis. A marker set combining markers from these processes could help to non-invasively diagnose NASH. Pooled markers (evaluated in at least 2 studies) and high potential markers, showing both sensitivity and specificity ≥80% in single studies, are indicated in the respective domains. Abbreviations: Cytokeratin (CK), Fibroblast growth factor (FGF), Tissue polypeptide specific antigen (TPS), Tissue inhibitor of metalloproteinase (TIMP), Neutrophil/lymphocyte ratio (N/L ratio), Interleukin (IL), Homeostatic model assessment-insulin resistance (HOMA-IR), Fucosylated-haptoglobin (Fuc-Hpt), Mac2 binding protein (Mac2bp).

Furthermore, it should be noted that this review focused on diagnostic tests that could replace liver biopsy (test replacement strategy), and was not aiming to identify subjects at risk for NASH, as these risk scores usually have a large grey area in which patients cannot be classified and liver biopsies are still necessary. When calculating the positive and negative predictive values (PPV and NPV

respectively) of the promising scores[49,73,91,101] and omics approaches[37] in hypothetical high (50%) and low (10%) prevalence NASH populations, PPV and NPV would be 37.5-69.2% and 97.0-98.7%, respectively, for the low prevalence population and 84.3-95.9% and 84.2-94.1%, respectively, for the high prevalence population. This indicates that especially in the low prevalence population the promising tools could serve as screening tools to identify patients with only steatosis and thereby avoid liver biopsy in these patients.

Although promising scoring systems as non-invasive diagnostic tests for NASH were found in single studies, none of the pooled results revealed both good sensitivity and specificity (≥80%). Replication studies and more standardised study designs on non-invasive diagnostics for NASH are urgently needed; as methodological differences and limitations hinder comparisons between studies. Based on the results and the multifactorial pathogenesis, combining markers related to various pathophysiologic processes is anticipated to have the highest potential to non-invasively diagnose NASH. For the time being, no blood marker, either as single marker or as part of a scoring system, can be recommended for use in clinical practice to diagnose NASH.

References

1. Younossi ZM, Koenig AB, Abdelatif D, et al. Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. Hepatology. 2016;64(1):73-84.
2. Bellentani S, Scaglioni F, Marino M, et al. Epidemiology of non-alcoholic fatty liver disease. Dig Dis. 2010;28(1): 155-61.
3. Blachier M, Leleu H, Peck-Radosavljevic M, et al. The burden of liver disease in Europe: a review of available epidemiological data. J Hepatol. 2013; 58(3):593-608.
4. Chalasani N, Younossi Z, Lavine JE, et al. The diagnosis and management of non-alcoholic fatty liver disease: practice guideline by the American Gastroenterological Association, American Association for the Study of Liver Diseases, and American College of Gastroenterology. Gastroenterology. 2012;142(7):1592-609.
5. Marchesini G, Brizi M, Bianchi G, et al. Nonalcoholic fatty liver disease: a feature of the metabolic syndrome. Diabetes. 2001;50(8):1844-50.
6. Hashimoto E, Taniai M, Tokushige K. Characteristics and diagnosis of NAFLD/NASH. J Gastroenterol Hepatol. 2013;28 Suppl 4:64-70.
7. McCullough AJ. Pathophysiology of nonalcoholic steatohepatitis. J Clin Gastroenterol. 2006;40 Suppl 1:S17-29.
8. Ascha MS, Hanouneh IA, Lopez R, et al. The incidence and risk factors of hepatocellular carcinoma in patients with nonalcoholic steatohepatitis. Hepatology. 2010;51(6):1972-8.
9. Wong VW, Wong GL, Choi PC, et al. Disease progression of non-alcoholic fatty liver disease: a prospective study with paired liver biopsies at 3 years. Gut. 2010;59(7):969-74.
10. Shaker M, Tabbaa A, Albeldawi M, et al. Liver transplantation for nonalcoholic fatty liver disease: new challenges and new opportunities. World J Gastroenterol. 2014;20(18):5320-30.
11. Ong JP, Pitts A, Younossi ZM. Increased overall mortality and liver-related mortality in non-alcoholic fatty liver disease. J Hepatol. 2008;49(4):608-12.
12. Rockey DC, Caldwell SH, Goodman ZD, et al. Liver biopsy. Hepatology. 2009;49(3): 1017-44.
13. Verdam FJ, Dallinga JW, Driessen A, et al. Non-alcoholic steatohepatitis: a non-invasive diagnosis by analysis of exhaled breath. J Hepatol. 2013;58(3):543-8.
14. Lee SS, Park SH. Radiologic evaluation of nonalcoholic fatty liver disease. World J Gastroenterol 2014;20(23):7392-402.
15. Moher D, Liberati A, Tetzlaff J, et al. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. J Clin Epidemiol. 2009;62(10): 1006-12.
16. Whiting PF, Rutjes AW, Westwood ME, et al. QUADAS-2: a revised tool for the quality assessment of diagnostic accuracy studies. Ann Intern Med. 2011;155(8): 529-36.
17. Viechtbauer W. Conducting Meta-Analyses in R with the metafor Package. J STAT SOFTW. 2010;36(3).
18. Barendregt JJ, Doi SA, Lee YY, et al. Meta-analysis of prevalence. J Epidemiol Community Health. 2013;67(11):974-8.
19. Takwoingi Y, Guo B, Riley RD, et al. Performance of methods for meta-analysis of diagnostic test accuracy with few studies or sparse data. Stat Methods Med Res. 2015.
20. Doebler P, Holling H. Meta-analysis of Diagnostic Accuracy with mada.
21. Hendrikx T, Walenbergh SM, Jeurissen ML, et al. Plasma IL-1 receptor antagonist levels correlate with the development of non-alcoholic steatohepatitis. Biomark Med. 2015;9(12):1301-9.
22. Bell LN, Theodorakis JL, Vuppalanchi R, et al. Serum proteomics and biomarker discovery across the spectrum of nonalcoholic fatty liver disease. Hepatology. 2010;51(1):111-20.
23. Cengiz M, Candir BA, Yilmaz G, et al. Is increased red cell distribution width an indicating marker of nonalcoholic steatohepatitis and fibrotic stage? World J Gastroenterol. 2013;19(42):7412-8.

24. Alaaeddine N, Sidaoui J, Hilal G, et al. TNF-alpha messenger ribonucleic acid (mRNA) in patients with nonalcoholic steatohepatitis. Eur Cytokine Netw. 2012; 23(3):107-11.
25. Ozenirler S, Erkan G, Konca Degertekin C, et al. The relationship between advanced oxidation protein products (AOPP) and biochemical and histopathological findings in patients with nonalcoholic steatohepatitis. J Dig Dis. 2014;15(3):131-6.
26. Maher M, Ibrahim W, Saleh S, et al. Cytokeratin 19 as a non invasive marker in diagnosis of NASH and its usefulness in correlation with disease seveirty in Egyptian patients. The Egyptian Journal of Medical Human Genetics. 2015;16:41-6.
27. Younossi ZM, Jarrar M, Nugent C, et al. A novel diagnostic biomarker panel for obesity-related nonalcoholic steatohepatitis (NASH). Obes Surg. 2008; 18(11):1430-7.
28. Yilmaz Y, Yonal O, Kurt R, et al. Serum levels of osteoprotegerin in the spectrum of nonalcoholic fatty liver disease. Scand J Clin Lab Invest. 2010;70(8):541-6.
29. Cengiz M, Ozenirler S, Yucel AA, et al. Can serum pin1 level be regarded as an indicative marker of nonalcoholic steatohepatitis and fibrotic stages? Digestion. 2014;90(1):35-41.
30. Abdelaziz R, Elbasel M, Esmat S, et al. Tissue Inhibitors of Metalloproteinase-1 and 2 and Obesity Related Non-Alcoholic Fatty Liver Disease: Is There a Relationship. Digestion. 2015;92(3):130-7.
31. Ozturk O, Colak Y, Senates E, et al. Increased serum soluble lectin-like oxidized low-density lipoprotein receptor-1 levels in patients with biopsy-proven nonalcoholic fatty liver disease. World J Gastroenterol. 2015;21(26):8096-102.
32. Yilmaz Y, Ulukaya E, Atug O, et al. Serum concentrations of human angiopoietin-like protein 3 in patients with nonalcoholic fatty liver disease: association with insulin resistance. Eur J Gastroenterol Hepatol. 2009;21(11):1247-51.
33. Murakami Y, Toyoda H, Tanahashi T, et al. Comprehensive miRNA expression analysis in peripheral blood can diagnose liver disease. PloS One. 2012;7(10): e48366.
34. Portincasa P, Grattagliano I, Lauterburg BH, et al. Liver breath tests non-invasively predict higher stages of non-alcoholic steatohepatitis. Clin Sci. 2006;111(2): 135-43.
35. Yilmaz Y, Eren F. Identification of a support vector machine-based biomarker panel with high sensitivity and specificity for nonalcoholic steatohepatitis. Clin Chim Acta. 2012;414:154-7.
36. Rahman W, Huang P, Belov L, et al. Analysis of human liver disease using a cluster of differentiation (CD) antibody microarray. Liver Int. 2012;32(10):1527-34.
37. Becker PP, Rau M, Schmitt J, et al. Performance of Serum microRNAs -122, -192 and -21 as Biomarkers in Patients with Non-Alcoholic Steatohepatitis. PloS One. 2015;10(11):e0142661.
38. Morita S, Neto Dde S, Morita FH, et al. Prevalence of Non-alcoholic Fatty Liver Disease and Steatohepatitis Risk Factors in Patients Undergoing Bariatric Surgery. Obes Surg. 2015;25(12):2335-43.
39. Pirola CJ, Fernandez Gianotti T, Castano GO, et al. Circulating microRNA signature in non-alcoholic fatty liver disease: from serum non-coding RNAs to liver histology and disease pathogenesis. Gut. 2015;64(5):800-12.
40. Angulo P, George J, Day CP, et al. Serum ferritin levels lack diagnostic accuracy for liver fibrosis in patients with nonalcoholic fatty liver disease. Clin Gastroenterol Hepatol. 2014;12(7):1163-9 e1.
41. Chan WK, Sthaneshwar P, Nik Mustapha NR, et al. Limited utility of plasma M30 in discriminating non-alcoholic steatohepatitis from steatosis--a comparison with routine biochemical markers. PloS One. 2014;9(9):e105903.
42. Polyzos SA, Kountouras J, Slavakis A, et al. A novel noninvasive index for nonalcoholic steatohepatitis: a pilot study. Biomarkers. 2013;18(7):607-13.
43. Grigorescu M, Crisan D, Radu C, et al. A novel pathophysiological-based panel of biomarkers for the diagnosis of

nonalcoholic steatohepatitis. J Physiol Pharmacol. 2012;63(4):347-53.
44. Pirvulescu I, Gheorghe L, Csiki I, et al. Noninvasive clinical model for the diagnosis of nonalcoholic steatohepatitis in overweight and morbidly obese patients undergoing bariatric surgery. Chirurgia. 2012;107(6):772-9.
45. Poynard T, Lassailly G, Diaz E, et al. Performance of biomarkers FibroTest, ActiTest, SteatoTest, and NashTest in patients with severe obesity: meta analysis of individual patient data. PloS One. 2012;7(3):e30325.
46. Tomita K, Teratani T, Yokoyama H, et al. Plasma free myristic acid proportion is a predictor of nonalcoholic steatohepatitis. Dig Dis Sci. 2011;56(10):3045-52.
47. Pulzi FB, Cisternas R, Melo MR, et al. New clinical score to diagnose nonalcoholic steatohepatitis in obese patients. Diabetol Metab Syndr. 2011;3(1):3.
48. Sumida Y, Yoneda M, Hyogo H, et al. A simple clinical scoring system using ferritin, fasting insulin, and type IV collagen 7S for predicting steatohepatitis in nonalcoholic fatty liver disease. J Gastroenterol. 2011;46(2):257-68.
49. Anty R, Iannelli A, Patouraux S, et al. A new composite model including metabolic syndrome, alanine aminotransferase and cytokeratin-18 for the diagnosis of non-alcoholic steatohepatitis in morbidly obese patients. Aliment Pharmacol Ther. 2010;32(11-12):1315-22.
50. Canbakan B, Senturk H, Canbakan M, et al. Is alanine aminotransferase level a surrogate biomarker of hepatic apoptosis in nonalcoholic fatty liver disease? Biomark Med. 2010;4(2):205-14.
51. Feldstein AE, Lopez R, Tamimi TA, et al. Mass spectrometric profiling of oxidized lipid products in human nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. J Lipid Res. 2010;51(10):3046-54.
52. Kashyap SR, Diab DL, Baker AR, et al. Triglyceride levels and not adipokine concentrations are closely related to severity of nonalcoholic fatty liver disease in an obesity surgery cohort. Obesity. 2009;17(9):1696-701.
53. Sookoian S, Castano G, Burgueno AL, et al. A diagnostic model to differentiate simple steatosis from nonalcoholic steatohepatitis based on the likelihood ratio form of Bayes theorem. Clin Biochem. 2009;42(7-8):624-9.
54. Campos GM, Bambha K, Vittinghoff E, et al. A clinical scoring system for predicting nonalcoholic steatohepatitis in morbidly obese patients. Hepatology. 2008;47(6):1916-23.
55. Yoneda M, Uchiyama T, Kato S, et al. Plasma Pentraxin3 is a novel marker for nonalcoholic steatohepatitis (NASH). BMC Gastroenterol. 2008;8:53.
56. Tarantino G, Conca P, Coppola A, et al. Serum concentrations of the tissue polypeptide specific antigen in patients suffering from non-alcoholic steatohepatitis. Eur J Clin Invest. 2007;37(1):48-53.
57. Poynard T, Ratziu V, Charlotte F, et al. Diagnostic value of biochemical markers (NashTest) for the prediction of non alcoholo steato hepatitis in patients with non-alcoholic fatty liver disease. BMC Gastroenterol. 2006;6:34.
58. Kunde SS, Lazenby AJ, Clements RH, et al. Spectrum of NAFLD and diagnostic implications of the proposed new normal range for serum ALT in obese women. Hepatology. 2005;42(3):650-6.
59. Yamasaki Y, Nouso K, Miyahara K, et al. Use of non-invasive serum glycan markers to distinguish non-alcoholic steato-hepatitis from simple steatosis. J Gastroenterol Hepatol. 2015;30(3):528-34.
60. Alkhouri N, Morris-Stiff G, Campbell C, et al. Neutrophil to lymphocyte ratio: a new marker for predicting steatohepatitis and fibrosis in patients with nonalcoholic fatty liver disease. Liver Int. 2012;32(2):297-302.
61. Manousou P, Kalambokis G, Grillo F, et al. Serum ferritin is a discriminant marker for both fibrosis and inflammation in histologically proven non-alcoholic fatty liver disease patients. Liver Int. 2011;31(5):730-9.
62. Fotiadu A, Gagalis A, Akriviadis E, et al. Clinicopathological correlations in a series of adult patients with non-alcoholic fatty

liver disease. Pathol Int. 2010;60(2):87-92.
63. Suzuki K, Kirikoshi H, Yoneda M, et al. Measurement of spleen volume is useful for distinguishing between simple steatosis and early-stage non-alcoholic steatohepatitis. Hepatol Res. 2010;40(7):693-700.
64. Yoneda M, Mawatari H, Fujita K, et al. High-sensitivity C-reactive protein is an independent clinical feature of nonalcoholic steatohepatitis (NASH) and also of the severity of fibrosis in NASH. J Gastroenterol. 2007;42(7):573-82.
65. Palekar NA, Naus R, Larson SP, et al. Clinical model for distinguishing nonalcoholic steatohepatitis from simple steatosis in patients with nonalcoholic fatty liver disease. Liver Int. 2006;26(2):151-6.
66. Yoneda M, Nozaki Y, Endo H, et al. Serum ferritin is a clinical biomarker in Japanese patients with nonalcoholic steatohepatitis (NASH) independent of HFE gene mutation. Dig Dis Sci. 2010;55(3):808-14.
67. Abdel-Razik A, Mousa N, Shabana W, et al. A novel model using mean platelet volume and neutrophil to lymphocyte ratio as a marker of nonalcoholic steatohepatitis in NAFLD patients: multicentric study. Eur J Gastroenterol Hepatol. 2016;28(1):e1-9.
68. Tarantino G, Mazzarella C, Tarantino M, et al. Could high levels of tissue polypeptide specific antigen, a marker of apoptosis detected in nonalcoholic steatohepatitis, improve after weight loss? Dis Markers. 2009;26(2):55-63.
69. Koehler E, Swain J, Sanderson S, et al. Growth hormone, dehydroepiandrosterone and adiponectin levels in non-alcoholic steatohepatitis: an endocrine signature for advanced fibrosis in obese patients. Liver Int. 2012;32(2):279-86.
70. Shimada M, Kawahara H, Ozaki K, et al. Usefulness of a combined evaluation of the serum adiponectin level, HOMA-IR, and serum type IV collagen 7S level to predict the early stage of nonalcoholic steatohepatitis. Am J Gastroenterol. 2007;102(9):1931-8.
71. Kwok R, Tse YK, Wong GL, et al. Systematic review with meta-analysis: non-invasive assessment of non-alcoholic fatty liver disease--the role of transient elastography and plasma cytokeratin-18 fragments. Aliment Pharmacol Ther. 2014;39(3):254-69.
72. Choi S, Diehl AM. After goodbye? Dead hepatocytes as a biomarker for fibrosis and steatohepatitis. Hepatology. 2012;55(2):333-5.
73. Yang M, Xu D, Liu Y, et al. Combined Serum Biomarkers in Non-Invasive Diagnosis of Non-Alcoholic Steatohepatitis. PloS One. 2015;10(6):e0131664.
74. Aida Y, Abe H, Tomita Y, et al. Serum cytokeratin 18 fragment level as a noninvasive biomarker for non-alcoholic fatty liver disease. Int J Clin Exp Med. 2014;7(11):4191-8.
75. Cusi K, Chang Z, Harrison S, et al. Limited value of plasma cytokeratin-18 as a biomarker for NASH and fibrosis in patients with non-alcoholic fatty liver disease. J Hepatol. 2014;60(1):167-74.
76. Yilmaz Y, Yesil A, Gerin F, et al. Detection of hepatic steatosis using the controlled attenuation parameter: a comparative study with liver biopsy. Scand J Gastroenterol. 2014;49(5):611-6.
77. Kamada Y, Fujii H, Fujii H, et al. Serum Mac-2 binding protein levels as a novel diagnostic biomarker for prediction of disease severity and nonalcoholic steatohepatitis. Proteomics Clinical applications. 2013.
78. Kamada Y, Akita M, Takeda Y, et al. Serum Fucosylated Haptoglobin as a Novel Diagnostic Biomarker for Predicting Hepatocyte Ballooning and Nonalcoholic Steatohepatitis. PloS One. 2013;8(6):e66328.
79. Kim YS, Jung ES, Hur W, et al. Noninvasive predictors of nonalcoholic steatohepatitis in Korean patients with histologically proven nonalcoholic fatty liver disease. Clin Mol Hepatol. 2013;19(2):120-30.
80. Tanwar S, Trembling PM, Guha IN, et al. Validation of terminal peptide of procollagen III for the detection and assessment of nonalcoholic steatohepatitis in patients with

nonalcoholic fatty liver disease. Hepatology. 2013;57(1):103-11.
81. Blomme B, Francque S, Trepo E, et al. N-glycan based biomarker distinguishing non-alcoholic steatohepatitis from steatosis independently of fibrosis. Dig Liver Dis. 2012;44(4):315-22.
82. Joka D, Wahl K, Moeller S, et al. Prospective biopsy-controlled evaluation of cell death biomarkers for prediction of liver fibrosis and nonalcoholic steatohepatitis. Hepatology. 2012;55(2):455-64.
83. Shen J, Chan HL, Wong GL, et al. Assessment of non-alcoholic fatty liver disease using serum total cell death and apoptosis markers. Aliment Pharmacol Ther. 2012;36(11-12):1057-66.
84. Shen J, Chan HL, Wong GL, et al. Non-invasive diagnosis of non-alcoholic steatohepatitis by combined serum biomarkers. J Hepatol. 2012;56(6):1363-70.
85. Tamimi TI, Elgouhari HM, Alkhouri N, et al. An apoptosis panel for nonalcoholic steatohepatitis diagnosis. J Hepatol. 2011;54(6):1224-9.
86. Younossi ZM, Page S, Rafiq N, et al. A biomarker panel for non-alcoholic steatohepatitis (NASH) and NASH-related fibrosis. Obes Surg. 2011;21(4):431-9.
87. Papatheodoridis GV, Hadziyannis E, Tsochatzis E, et al. Serum apoptotic caspase activity in chronic hepatitis C and nonalcoholic Fatty liver disease. J Clin Gastroenterol. 2010;44(4):e87-95.
88. Feldstein AE, Wieckowska A, Lopez AR, et al. Cytokeratin-18 fragment levels as noninvasive biomarkers for nonalcoholic steatohepatitis: a multicenter validation study. Hepatology. 2009;50(4):1072-8.
89. Malik R, Chang M, Bhaskar K, et al. The clinical utility of biomarkers and the nonalcoholic steatohepatitis CRN liver biopsy scoring system in patients with nonalcoholic fatty liver disease. J Gastroenterol Hepatol. 2009;24(4):564-8.
90. Diab DL, Yerian L, Schauer P, et al. Cytokeratin 18 fragment levels as a noninvasive biomarker for nonalcoholic steatohepatitis in bariatric surgery patients. Clin Gastroenterol Hepatol. 2008;6(11):1249-54.
91. Yilmaz Y, Dolar E, Ulukaya E, et al. Soluble forms of extracellular cytokeratin 18 may differentiate simple steatosis from nonalcoholic steatohepatitis. World J Gastroenterol. 2007;13(6):837-44.
92. Wieckowska A, Zein NN, Yerian LM, et al. In vivo assessment of liver cell apoptosis as a novel biomarker of disease severity in nonalcoholic fatty liver disease. Hepatology. 2006;44(1):27-33.
93. Pimentel CF, Jiang ZG, Otsubo T, et al. Poor Inter-test Reliability Between CK18 Kits as a Biomarker of NASH. Dig Dis Sci. 2016;61(3):905-12.
94. Kawanaka M, Nishino K, Nakamura J, et al. Correlation between serum cytokeratin-18 and the progression or regression of non-alcoholic fatty liver disease. Ann Hepatol. 2015;14(6):837-44.
95. Garcia-Galiano D, Sanchez-Garrido MA, Espejo I, et al. IL-6 and IGF-1 are independent prognostic factors of liver steatosis and non-alcoholic steatohepatitis in morbidly obese patients. Obes Surg. 2007;17(4):493-503.
96. Gulsen M, Yesilova Z, Bagci S, et al. Elevated plasma homocysteine concentrations as a predictor of steatohepatitis in patients with non-alcoholic fatty liver disease. J Gastroenterol Hepatol. 2005;20(9):1448-55.
97. Lemoine M, Ratziu V, Kim M, et al. Serum adipokine levels predictive of liver injury in non-alcoholic fatty liver disease. Liver Int. 2009;29(9):1431-8.
98. Kamada Y, Ono M, Hyogo H, et al. A novel noninvasive diagnostic method for nonalcoholic steatohepatitis using two glycobiomarkers. Hepatology. 2015;62(5):1433-43.
99. Chen C, Schmilovitz-Weiss H, Liu XE, et al. Serum protein N-glycans profiling for the discovery of potential biomarkers for nonalcoholic steatohepatitis. J Proteome Res. 2009;8(2):463-70.
100. Younossi ZM, Baranova A, Stepanova M, et al. Phosphoproteomic biomarkers predicting histologic nonalcoholic steatohepatitis and fibrosis. J Proteome Res. 2010;9(6):3218-24.
101. Yoshimura K, Okanoue T, Ebise H, et al. Identification of novel noninvasive

markers for diagnosing nonalcoholic steatohepatitis and related fibrosis by data mining. Hepatology. 2016;63(2):462-73.
102. Dixon JB, Bhathal PS, O'Brien PE. Nonalcoholic fatty liver disease: predictors of nonalcoholic steatohepatitis and liver fibrosis in the severely obese. Gastroenterology. 2001;121(1):91-100.
103. Miele L, Forgione A, La Torre G, et al. Serum levels of hyaluronic acid and tissue metalloproteinase inhibitor-1 combined with age predict the presence of nonalcoholic steatohepatitis in a pilot cohort of subjects with nonalcoholic fatty liver disease. Transl research. 2009; 154(4):194-201.
104. Tilg H, Moschen AR. Evolution of inflammation in nonalcoholic fatty liver disease: the multiple parallel hits hypothesis. Hepatology. 2010;52(5): 1836-46.
105. Brunt EM, Janney CG, Di Bisceglie AM, et al. Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. Am J Gastroenterol. 1999;94(9):2467-74.
106. Kleiner DE, Brunt EM, Van Natta M, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology. 2005;41(6):1313-21.

Supplementary data

Search Strategy

Pubmed
((((((((non*alcoholic fatty liver disease) OR "Non-alcoholic Fatty Liver Disease") OR nafl*) OR "Fatty Liver"[Mesh])) OR ((non*alcoholic steatohepatitis) OR NASH))) AND (((((*marker*) OR "Biological Markers"[Mesh]) OR "Genetic Markers"[Mesh])) OR (((((radiolog*) OR "Radiography"[Mesh]) OR "Radiology"[Mesh]) OR radiograph*) OR "Diagnostic Imaging"[Mesh]))) AND ((((("Diagnosis"[Mesh]) OR "Diagnostic Tests, Routine"[Mesh]) OR diagnosi*) OR diagnosti*)
Filters: date from 1990/01/01 to 2015/12/31, Humans, English, Adult

Embase
(((fatty liver/or non-alcoholic fatty liver/ or non*alcoholic fatty liver disease.mp or fatty liver/or non-alcoholic fatty liver/or NAFL*.mp) or (non*alcoholic steatohepatitis.mp or NASH.mp)) and (radiology/ or radiolog*.mp or radiography/ or radiography*.mp or radiology/ or radiography/ or diagnostic imaging/or imaging.mp or imaging/) and (biological marker/ or DNA marker/ or biochemical marker/ or disease merker/ or genetic marker/ or marker*.mp or diagnosis/ or diagnos*.mp))
Filters: adult and human

Medline
(((Fatty Liver/or non*alcoholic fatty liver disease.mp or Fatty Liver/or NAFL*.mp) or (Non*alcoholic steatohepatitis.mp or NASH.mp)) and ((marker*.mp or Biologica Markers/ or Genetic Markers/) or (Radiography/ or Radiology/ or Diagnostic Imaging/ or radiolog*.mp) or radiograph*.mp)) and (Diagnosis/or diagnos*.mp))
Filters: human and adult

Single records identified: PubMed 1833, EMBASE 736 and Medline 39 hits

List of the 122 included articles

1. Verdam FJ, Dallinga JW, Driessen A, et al. Non-alcoholic steatohepatitis: a non-invasive diagnosis by analysis of exhaled breath. Journal of hepatology. 2013;58(3):543-8.
2. Becker PP, Rau M, Schmitt J, et al. Performance of Serum microRNAs -122, -192 and -21 as Biomarkers in Patients with Non-Alcoholic Steatohepatitis. PloS one. 2015;10(11):e0142661.
3. Hendrikx T, Walenbergh SM, Jeurissen ML, et al. Plasma IL-1 receptor antagonist levels correlate with the development of non-alcoholic steatohepatitis. Biomarkers in medicine. 2015;9(12):1301-9.
4. Morita S, Neto Dde S, Morita FH, et al. Prevalence of Non-alcoholic Fatty Liver Disease and Steatohepatitis Risk Factors in Patients Undergoing Bariatric Surgery. Obesity surgery. 2015;25(12):2335-43.
5. Pirola CJ, Fernandez Gianotti T, Castano GO, et al. Circulating microRNA signature in non-alcoholic fatty liver disease: from serum non-coding RNAs to liver histology and disease pathogenesis. Gut. 2015;64(5):800-12.
6. Angulo P, George J, Day CP, et al. Serum ferritin levels lack diagnostic accuracy for liver fibrosis in patients with nonalcoholic fatty liver disease. Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association. 2014;12(7):1163-9 e1.
7. Chan WK, Sthaneshwar P, Nik Mustapha NR, et al. Limited utility of plasma M30 in discriminating non-alcoholic steatohepatitis from steatosis--a comparison with routine biochemical markers. PloS one. 2014;9(9):e105903.
8. Polyzos SA, Kountouras J, Slavakis A, et al. A novel noninvasive index for nonalcoholic steatohepatitis: a pilot study. Biomarkers : biochemical indicators of exposure, response, and susceptibility to chemicals. 2013;18(7):607-13.
9. Grigorescu M, Crisan D, Radu C, et al. A novel pathophysiological-based panel of biomarkers for the diagnosis of nonalcoholic steatohepatitis. Journal of physiology and pharmacology : an official journal of the Polish Physiological Society. 2012;63(4):347-53.
10. Pirvulescu I, Gheorghe L, Csiki I, et al. Noninvasive clinical model for the diagnosis of nonalcoholic steatohepatitis in overweight and morbidly obese patients undergoing bariatric surgery. Chirurgia. 2012;107(6):772-9.
11. Poynard T, Lassailly G, Diaz E, et al. Performance of biomarkers FibroTest, ActiTest, SteatoTest, and NashTest in patients with severe obesity: meta analysis of individual patient data. PloS one. 2012;7(3):e30325.
12. Tomita K, Teratani T, Yokoyama H, et al. Plasma free myristic acid proportion is a predictor of nonalcoholic steatohepatitis. Digestive diseases and sciences. 2011;56(10):3045-52.
13. Pulzi FB, Cisternas R, Melo MR, et al. New clinical score to diagnose nonalcoholic steatohepatitis in obese patients. Diabetology & metabolic syndrome. 2011;3(1):3.
14. Sumida Y, Yoneda M, Hyogo H, et al. A simple clinical scoring system using ferritin, fasting insulin, and type IV collagen 7S for predicting steatohepatitis in nonalcoholic fatty liver disease. Journal of gastroenterology. 2011;46(2):257-68.
15. Anty R, Iannelli A, Patouraux S, et al. A new composite model including metabolic syndrome, alanine aminotransferase and cytokeratin-18 for the diagnosis of non-alcoholic steatohepatitis in morbidly obese patients. Alimentary pharmacology & therapeutics. 2010;32(11-12):1315-22.
16. Bell LN, Theodorakis JL, Vuppalanchi R, et al. Serum proteomics and biomarker discovery across the spectrum of nonalcoholic fatty liver disease. Hepatology. 2010;51(1):111-20.
17. Canbakan B, Senturk H, Canbakan M, et al. Is alanine aminotransferase level a surrogate biomarker of hepatic apoptosis in nonalcoholic fatty liver disease? Biomarkers in medicine. 2010;4(2):205-14.
18. Feldstein AE, Lopez R, Tamimi TA, et al. Mass spectrometric profiling of oxidized lipid products in human nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. Journal of lipid research. 2010;51(10):3046-54.

19. Kashyap SR, Diab DL, Baker AR, et al. Triglyceride levels and not adipokine concentrations are closely related to severity of nonalcoholic fatty liver disease in an obesity surgery cohort. Obesity. 2009;17(9):1696-701.
20. Sookoian S, Castano G, Burgueno AL, et al. A diagnostic model to differentiate simple steatosis from nonalcoholic steatohepatitis based on the likelihood ratio form of Bayes theorem. Clinical biochemistry. 2009;42(7-8):624-9.
21. Campos GM, Bambha K, Vittinghoff E, et al. A clinical scoring system for predicting nonalcoholic steatohepatitis in morbidly obese patients. Hepatology. 2008;47(6):1916-23.
22. Yoneda M, Uchiyama T, Kato S, et al. Plasma Pentraxin3 is a novel marker for nonalcoholic steatohepatitis (NASH). BMC gastroenterology. 2008;8:53.
23. Tarantino G, Conca P, Coppola A, et al. Serum concentrations of the tissue polypeptide specific antigen in patients suffering from non-alcoholic steatohepatitis. European journal of clinical investigation. 2007;37(1):48-53.
24. Poynard T, Ratziu V, Charlotte F, et al. Diagnostic value of biochemical markers (NashTest) for the prediction of non alcoholo steato hepatitis in patients with non-alcoholic fatty liver disease. BMC gastroenterology. 2006;6:34.
25. Kunde SS, Lazenby AJ, Clements RH, et al. Spectrum of NAFLD and diagnostic implications of the proposed new normal range for serum ALT in obese women. Hepatology. 2005;42(3):650-6.
26. Yamasaki Y, Nouso K, Miyahara K, et al. Use of non-invasive serum glycan markers to distinguish non-alcoholic steatohepatitis from simple steatosis. Journal of gastroenterology and hepatology. 2015;30(3):528-34.
27. Alkhouri N, Morris-Stiff G, Campbell C, et al. Neutrophil to lymphocyte ratio: a new marker for predicting steatohepatitis and fibrosis in patients with nonalcoholic fatty liver disease. Liver international : official journal of the International Association for the Study of the Liver. 2012;32(2):297-302.
28. Manousou P, Kalambokis G, Grillo F, et al. Serum ferritin is a discriminant marker for both fibrosis and inflammation in histologically proven non-alcoholic fatty liver disease patients. Liver international : official journal of the International Association for the Study of the Liver. 2011;31(5):730-9.
29. Fotiadu A, Gagalis A, Akriviadis E, et al. Clinicopathological correlations in a series of adult patients with non-alcoholic fatty liver disease. Pathology international. 2010;60(2):87-92.
30. Suzuki K, Kirikoshi H, Yoneda M, et al. Measurement of spleen volume is useful for distinguishing between simple steatosis and early-stage non-alcoholic steatohepatitis. Hepatology research : the official journal of the Japan Society of Hepatology. 2010;40(7):693-700.
31. Yoneda M, Mawatari H, Fujita K, et al. High-sensitivity C-reactive protein is an independent clinical feature of nonalcoholic steatohepatitis (NASH) and also of the severity of fibrosis in NASH. Journal of gastroenterology. 2007;42(7):573-82.
32. Palekar NA, Naus R, Larson SP, et al. Clinical model for distinguishing nonalcoholic steatohepatitis from simple steatosis in patients with nonalcoholic fatty liver disease. Liver international : official journal of the International Association for the Study of the Liver. 2006;26(2):151-6.
33. Kamada Y, Fujii H, Fujii H, et al. Serum Mac-2 binding protein levels as a novel diagnostic biomarker for prediction of disease severity and nonalcoholic steatohepatitis. Proteomics Clinical applications. 2013.
34. Abdel-Razik A, Mousa N, Shabana W, et al. A novel model using mean platelet volume and neutrophil to lymphocyte ratio as a marker of nonalcoholic steatohepatitis in NAFLD patients: multicentric study. European journal of gastroenterology & hepatology. 2016;28(1):e1-9.
35. Shimada M, Kawahara H, Ozaki K, et al. Usefulness of a combined evaluation of the serum adiponectin level, HOMA-IR, and serum type IV collagen 7S level to predict the early stage of nonalcoholic steatohepatitis. The American journal of gastroenterology. 2007;102(9):1931-8.

36. Cengiz M, Candir BA, Yilmaz G, et al. Is increased red cell distribution width an indicating marker of nonalcoholic steatohepatitis and fibrotic stage? World journal of gastroenterology : WJG. 2013;19(42):7412-8.
37. Tanwar S, Trembling PM, Guha IN, et al. Validation of terminal peptide of procollagen III for the detection and assessment of nonalcoholic steatohepatitis in patients with nonalcoholic fatty liver disease. Hepatology. 2013;57(1):103-11.
38. Yoneda M, Nozaki Y, Endo H, et al. Serum ferritin is a clinical biomarker in Japanese patients with nonalcoholic steatohepatitis (NASH) independent of HFE gene mutation. Digestive diseases and sciences. 2010;55(3):808-14.
39. Kazankov K, Tordjman J, Moller HJ, et al. Macrophage activation marker soluble CD163 and non-alcoholic fatty liver disease in morbidly obese patients undergoing bariatric surgery. Journal of gastroenterology and hepatology. 2015;30(8):1293-300.
40. Ogawa Y, Imajo K, Yoneda M, et al. Soluble CD14 levels reflect liver inflammation in patients with nonalcoholic steatohepatitis. PloS one. 2013;8(6):e65211.
41. Alaaeddine N, Sidaoui J, Hilal G, et al. TNF-alpha messenger ribonucleic acid (mRNA) in patients with nonalcoholic steatohepatitis. European cytokine network. 2012;23(3):107-11.
42. Koehler E, Swain J, Sanderson S, et al. Growth hormone, dehydroepiandrosterone and adiponectin levels in non-alcoholic steatohepatitis: an endocrine signature for advanced fibrosis in obese patients. Liver international : official journal of the International Association for the Study of the Liver. 2012;32(2):279-86.
43. Trak-Smayra V, Dargere D, Noun R, et al. Serum proteomic profiling of obese patients: correlation with liver pathology and evolution after bariatric surgery. Gut. 2009;58(6):825-32.
44. Tarantino G, Mazzarella C, Tarantino M, et al. Could high levels of tissue polypeptide specific antigen, a marker of apoptosis detected in nonalcoholic steatohepatitis, improve after weight loss? Disease markers. 2009;26(2):55-63.
45. Yang M, Xu D, Liu Y, et al. Combined Serum Biomarkers in Non-Invasive Diagnosis of Non-Alcoholic Steatohepatitis. PloS one. 2015;10(6):e0131664.
46. Ozenirler S, Erkan G, Konca Degertekin C, et al. The relationship between advanced oxidation protein products (AOPP) and biochemical and histopathological findings in patients with nonalcoholic steatohepatitis. Journal of digestive diseases. 2014;15(3):131-6.
47. Maher M, Ibrahim W, Saleh S, et al. Cytokeratin 19 as a non invasive marker in diagnosis of NASH and its usefulness in correlation with disease seveirty in Egyptian patients. The Egyptian Journal of Medical Human Genetics. 2015;16:41-6.
48. Aida Y, Abe H, Tomita Y, et al. Serum cytokeratin 18 fragment level as a noninvasive biomarker for non-alcoholic fatty liver disease. International journal of clinical and experimental medicine. 2014;7(11):4191-8.
49. Cusi K, Chang Z, Harrison S, et al. Limited value of plasma cytokeratin-18 as a biomarker for NASH and fibrosis in patients with non-alcoholic fatty liver disease. Journal of hepatology. 2014;60(1):167-74.
50. Yilmaz Y, Yesil A, Gerin F, et al. Detection of hepatic steatosis using the controlled attenuation parameter: a comparative study with liver biopsy. Scandinavian journal of gastroenterology. 2014;49(5):611-6.
51. Kamada Y, Akita M, Takeda Y, et al. Serum Fucosylated Haptoglobin as a Novel Diagnostic Biomarker for Predicting Hepatocyte Ballooning and Nonalcoholic Steatohepatitis. PloS one. 2013;8(6):e66328.
52. Kim YS, Jung ES, Hur W, et al. Noninvasive predictors of nonalcoholic steatohepatitis in Korean patients with histologically proven nonalcoholic fatty liver disease. Clinical and molecular hepatology. 2013;19(2):120-30.
53. Blomme B, Francque S, Trepo E, et al. N-glycan based biomarker distinguishing non-alcoholic steatohepatitis from steatosis independently of fibrosis. Digestive and liver disease : official journal of the Italian Society of Gastroenterology and the Italian Association for the Study of the Liver. 2012;44(4):315-22.

54. Joka D, Wahl K, Moeller S, et al. Prospective biopsy-controlled evaluation of cell death biomarkers for prediction of liver fibrosis and nonalcoholic steatohepatitis. Hepatology. 2012;55(2):455-64.
55. Shen J, Chan HL, Wong GL, et al. Assessment of non-alcoholic fatty liver disease using serum total cell death and apoptosis markers. Alimentary pharmacology & therapeutics. 2012;36(11-12):1057-66.
56. Shen J, Chan HL, Wong GL, et al. Non-invasive diagnosis of non-alcoholic steatohepatitis by combined serum biomarkers. Journal of hepatology. 2012;56(6):1363-70.
57. Tamimi TI, Elgouhari HM, Alkhouri N, et al. An apoptosis panel for nonalcoholic steatohepatitis diagnosis. Journal of hepatology. 2011;54(6):1224-9.
58. Younossi ZM, Page S, Rafiq N, et al. A biomarker panel for non-alcoholic steatohepatitis (NASH) and NASH-related fibrosis. Obesity surgery. 2011;21(4):431-9.
59. Papatheodoridis GV, Hadziyannis E, Tsochatzis E, et al. Serum apoptotic caspase activity in chronic hepatitis C and nonalcoholic Fatty liver disease. Journal of clinical gastroenterology. 2010;44(4):e87-95.
60. Feldstein AE, Wieckowska A, Lopez AR, et al. Cytokeratin-18 fragment levels as noninvasive biomarkers for nonalcoholic steatohepatitis: a multicenter validation study. Hepatology. 2009;50(4):1072-8.
61. Malik R, Chang M, Bhaskar K, et al. The clinical utility of biomarkers and the nonalcoholic steatohepatitis CRN liver biopsy scoring system in patients with nonalcoholic fatty liver disease. Journal of gastroenterology and hepatology. 2009;24(4):564-8.
62. Diab DL, Yerian L, Schauer P, et al. Cytokeratin 18 fragment levels as a noninvasive biomarker for nonalcoholic steatohepatitis in bariatric surgery patients. Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association. 2008;6(11):1249-54.
63. Younossi ZM, Jarrar M, Nugent C, et al. A novel diagnostic biomarker panel for obesity-related nonalcoholic steatohepatitis (NASH). Obesity surgery. 2008;18(11):1430-7.
64. Yilmaz Y, Dolar E, Ulukaya E, et al. Soluble forms of extracellular cytokeratin 18 may differentiate simple steatosis from nonalcoholic steatohepatitis. World journal of gastroenterology : WJG. 2007;13(6):837-44.
65. Wieckowska A, Zein NN, Yerian LM, et al. In vivo assessment of liver cell apoptosis as a novel biomarker of disease severity in nonalcoholic fatty liver disease. Hepatology. 2006;44(1):27-33.
66. Pimentel CF, Jiang ZG, Otsubo T, et al. Poor Inter-test Reliability Between CK18 Kits as a Biomarker of NASH. Digestive diseases and sciences. 2016;61(3):905-12.
67. Kawanaka M, Nishino K, Nakamura J, et al. Correlation between serum cytokeratin-18 and the progression or regression of non-alcoholic fatty liver disease. Annals of hepatology. 2015;14(6):837-44.
68. Garcia-Galiano D, Sanchez-Garrido MA, Espejo I, et al. IL-6 and IGF-1 are independent prognostic factors of liver steatosis and non-alcoholic steatohepatitis in morbidly obese patients. Obesity surgery. 2007;17(4):493-503.
69. Yilmaz Y, Yonal O, Kurt R, et al. Serum levels of osteoprotegerin in the spectrum of nonalcoholic fatty liver disease. Scandinavian journal of clinical and laboratory investigation. 2010;70(8):541-6.
70. Cengiz M, Ozenirler S, Yucel AA, et al. Can serum pin1 level be regarded as an indicative marker of nonalcoholic steatohepatitis and fibrotic stages? Digestion. 2014;90(1):35-41.
71. Abdelaziz R, Elbasel M, Esmat S, et al. Tissue Inhibitors of Metalloproteinase-1 and 2 and Obesity Related Non-Alcoholic Fatty Liver Disease: Is There a Relationship. Digestion. 2015;92(3):130-7.
72. Lemoine M, Ratziu V, Kim M, et al. Serum adipokine levels predictive of liver injury in non-alcoholic fatty liver disease. Liver international : official journal of the International Association for the Study of the Liver. 2009;29(9):1431-8.

73. Bookman ID, Pham J, Guindi M, et al. Distinguishing nonalcoholic steatohepatitis from fatty liver: serum-free fatty acids, insulin resistance, and serum lipoproteins. Liver international : official journal of the International Association for the Study of the Liver. 2006;26(5):566-71.
74. Hyogo H, Yamagishi S, Iwamoto K, et al. Elevated levels of serum advanced glycation end products in patients with non-alcoholic steatohepatitis. Journal of gastroenterology and hepatology. 2007;22(7):1112-9.
75. Kamada Y, Ono M, Hyogo H, et al. A novel noninvasive diagnostic method for nonalcoholic steatohepatitis using two glycobiomarkers. Hepatology. 2015;62(5):1433-43.
76. Page S, Birerdinc A, Estep M, et al. Knowledge-based identification of soluble biomarkers: hepatic fibrosis in NAFLD as an example. PloS one. 2013;8(2):e56009.
77. Ozturk O, Colak Y, Senates E, et al. Increased serum soluble lectin-like oxidized low-density lipoprotein receptor-1 levels in patients with biopsy-proven nonalcoholic fatty liver disease. World journal of gastroenterology : WJG. 2015;21(26):8096-102.
78. Imajo K, Fujita K, Yoneda M, et al. Plasma free choline is a novel non-invasive biomarker for early-stage non-alcoholic steatohepatitis: A multi-center validation study. Hepatology research : the official journal of the Japan Society of Hepatology. 2012;42(8):757-66.
79. Yilmaz Y, Ulukaya E, Atug O, et al. Serum concentrations of human angiopoietin-like protein 3 in patients with nonalcoholic fatty liver disease: association with insulin resistance. European journal of gastroenterology & hepatology. 2009;21(11):1247-51.
80. Gulsen M, Yesilova Z, Bagci S, et al. Elevated plasma homocysteine concentrations as a predictor of steatohepatitis in patients with non-alcoholic fatty liver disease. Journal of gastroenterology and hepatology. 2005;20(9):1448-55.
81. Fierbinteanu-Braticevici C, Plesca DA, Tribus L, et al. The role of (1)(3)C-methacetin breath test for the non-invasive evaluation of nonalcoholic fatty liver disease. Journal of gastrointestinal and liver diseases : JGLD. 2013;22(2):149-56.
82. Portincasa P, Grattagliano I, Lauterburg BH, et al. Liver breath tests non-invasively predict higher stages of non-alcoholic steatohepatitis. Clinical science. 2006;111(2):135-43.
83. Banasch M, Ellrichmann M, Tannapfel A, et al. The non-invasive (13)C-methionine breath test detects hepatic mitochondrial dysfunction as a marker of disease activity in non-alcoholic steatohepatitis. European journal of medical research. 2011;16(6):258-64.
84. Yaman H, Cakir E, Ozcan O, et al. Elevated urine neopterin levels in nonalcoholic steatohepatitis. Clinical biochemistry. 2005;38(2):187-90.
85. Chen C, Schmilovitz-Weiss H, Liu XE, et al. Serum protein N-glycans profiling for the discovery of potential biomarkers for nonalcoholic steatohepatitis. Journal of proteome research. 2009;8(2):463-70.
86. Miller MH, Walsh SV, Atrih A, et al. Serum proteome of nonalcoholic fatty liver disease: a multimodal approach to discovery of biomarkers of nonalcoholic steatohepatitis. Journal of gastroenterology and hepatology. 2014;29(10):1839-47.
87. Younossi ZM, Baranova A, Stepanova M, et al. Phosphoproteomic biomarkers predicting histologic nonalcoholic steatohepatitis and fibrosis. Journal of proteome research. 2010;9(6):3218-24.
88. Ulukaya E, Yilmaz Y, Moshkovskii S, et al. Proteomic analysis of serum in patients with non-alcoholic steatohepatitis using matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Scandinavian journal of gastroenterology. 2009;44(12):1471-6.
89. Barr J, Caballeria J, Martinez-Arranz I, et al. Obesity-dependent metabolic signatures associated with nonalcoholic fatty liver disease progression. Journal of proteome research. 2012;11(4):2521-32.
90. Loomba R, Quehenberger O, Armando A, et al. Polyunsaturated fatty acid metabolites as novel lipidomic biomarkers for noninvasive diagnosis of nonalcoholic steatohepatitis. Journal of lipid research. 2015;56(1):185-92.
91. Murakami Y, Toyoda H, Tanahashi T, et al. Comprehensive miRNA expression analysis in peripheral blood can diagnose liver disease. PloS one. 2012;7(10):e48366.

92. Leon-Mimila P, Vega-Badillo J, Gutierrez-Vidal R, et al. A genetic risk score is associated with hepatic triglyceride content and non-alcoholic steatohepatitis in Mexicans with morbid obesity. Exp Mol Pathol. 2015;98(2):178-83.
93. Guajardo-Salinas GE, Hilmy A. Prevalence of nonalcoholic fatty liver disease (NAFLD) and utility of FIBROspect II to detect liver fibrosis in morbidly obese Hispano-American patients undergoing gastric bypass. Obesity surgery. 2010;20(12):1647-53.
94. Liang RJ, Wang HH, Lee WJ, et al. Diagnostic value of ultrasonographic examination for nonalcoholic steatohepatitis in morbidly obese patients undergoing laparoscopic bariatric surgery. Obesity surgery. 2007;17(1):45-56.
95. Ballestri S, Lonardo A, Romagnoli D, et al. Ultrasonographic fatty liver indicator, a novel score which rules out NASH and is correlated with metabolic parameters in NAFLD. Liver international : official journal of the International Association for the Study of the Liver. 2012;32(8):1242-52.
96. Shigefuku R, Takahashi H, Kobayashi M, et al. Pathophysiological analysis of nonalcoholic fatty liver disease by evaluation of fatty liver changes and blood flow using xenon computed tomography: can early-stage nonalcoholic steatohepatitis be distinguished from simple steatosis? Journal of gastroenterology. 2012;47(11):1238-47.
97. Parente DB, Paiva FF, Oliveira Neto JA, et al. Intravoxel Incoherent Motion Diffusion Weighted MR Imaging at 3.0 T: Assessment of Steatohepatitis and Fibrosis Compared with Liver Biopsy in Type 2 Diabetic Patients. PloS one. 2015;10(5):e0125653.
98. Abrigo JM, Shen J, Wong VW, et al. Non-alcoholic fatty liver disease: spectral patterns observed from an in vivo phosphorus magnetic resonance spectroscopy study. Journal of hepatology. 2014;60(4):809-15.
99. Bastati N, Feier D, Wibmer A, et al. Noninvasive differentiation of simple steatosis and steatohepatitis by using gadoxetic acid-enhanced MR imaging in patients with nonalcoholic fatty liver disease: a proof-of-concept study. Radiology. 2014;271(3):739-47.
100. Smits LP, Coolen BF, Panno MD, et al. Noninvasive Differentiation between Hepatic Steatosis and Steatohepatitis with MR Imaging Enhanced with USPIOs in Patients with Nonalcoholic Fatty Liver Disease: A Proof-of-Concept Study. Radiology. 2016;278(3):782-91.
101. Tomita K, Tanimoto A, Irie R, et al. Evaluating the severity of nonalcoholic steatohepatitis with superparamagnetic iron oxide-enhanced magnetic resonance imaging. Journal of magnetic resonance imaging : JMRI. 2008;28(6):1444-50.
102. Chen J, Talwalkar JA, Yin M, et al. Early detection of nonalcoholic steatohepatitis in patients with nonalcoholic fatty liver disease by using MR elastography. Radiology. 2011;259(3):749-56.
103. Friedrich-Rust M, Romen D, Vermehren J, et al. Acoustic radiation force impulse-imaging and transient elastography for non-invasive assessment of liver fibrosis and steatosis in NAFLD. European journal of radiology. 2012;81(3):e325-31.
104. Fierbinteanu Braticevici C, Sporea I, Panaitescu E, et al. Value of acoustic radiation force impulse imaging elastography for non-invasive evaluation of patients with nonalcoholic fatty liver disease. Ultrasound in medicine & biology. 2013;39(11):1942-50.
105. Imajo K, Kessoku T, Honda Y, et al. Magnetic Resonance Imaging More Accurately Classifies Steatosis and Fibrosis in Patients With Nonalcoholic Fatty Liver Disease Than Transient Elastography. Gastroenterology. 2016;150(3):626-37 e7.
106. Kikuchi M, Tomita K, Nakahara T, et al. Utility of quantitative 99mTc-phytate scintigraphy to diagnose early-stage non-alcoholic steatohepatitis. Scandinavian journal of gastroenterology. 2009;44(2):229-36.
107. Lassailly G, Caiazzo R, Hollebecque A, et al. Validation of noninvasive biomarkers (FibroTest, SteatoTest, and NashTest) for prediction of liver injury in patients with morbid obesity. European journal of gastroenterology & hepatology. 2011;23(6):499-506.
108. Ratziu V, Massard J, Charlotte F, et al. Diagnostic value of biochemical markers (FibroTest-FibroSURE) for the prediction of liver fibrosis in patients with non-alcoholic fatty liver disease. BMC gastroenterology. 2006;6:6.

109. Alkhouri N, Berk M, Yerian L, et al. OxNASH score correlates with histologic features and severity of nonalcoholic fatty liver disease. Digestive diseases and sciences. 2014;59(7):1617-24.
110. Fedchuk L, Nascimbeni F, Pais R, et al. Performance and limitations of steatosis biomarkers in patients with nonalcoholic fatty liver disease. Alimentary pharmacology & therapeutics. 2014;40(10):1209-22.
111. Polyzos SA, Kountouras J, Papatheodorou A, et al. Adipocytokines and cytokeratin-18 in patients with nonalcoholic fatty liver disease: Introduction of CHA index. Annals of hepatology. 2013;12(5):749-57.
112. Dixon JB, Bhathal PS, O'Brien PE. Nonalcoholic fatty liver disease: predictors of nonalcoholic steatohepatitis and liver fibrosis in the severely obese. Gastroenterology. 2001;121(1):91-100.
113. Vongsuvanh R, George J, McLeod D, et al. Visceral adiposity index is not a predictor of liver histology in patients with non-alcoholic fatty liver disease. Journal of hepatology. 2012;57(2):392-8.
114. Miele L, Forgione A, La Torre G, et al. Serum levels of hyaluronic acid and tissue metalloproteinase inhibitor-1 combined with age predict the presence of nonalcoholic steatohepatitis in a pilot cohort of subjects with nonalcoholic fatty liver disease. Translational research : the journal of laboratory and clinical medicine. 2009;154(4):194-201.
115. Yilmaz Y, Eren F. Identification of a support vector machine-based biomarker panel with high sensitivity and specificity for nonalcoholic steatohepatitis. Clinica chimica acta; international journal of clinical chemistry. 2012;414:154-7.
116. Hui JM, Hodge A, Farrell GC, et al. Beyond insulin resistance in NASH: TNF-alpha or adiponectin? Hepatology. 2004;40(1):46-54.
117. Chisholm J, Seki Y, Toouli J, et al. Serologic predictors of nonalcoholic steatohepatitis in a population undergoing bariatric surgery. Surgery for obesity and related diseases : official journal of the American Society for Bariatric Surgery. 2012;8(4):416-22.
118. Gholam PM, Flancbaum L, Machan JT, et al. Nonalcoholic fatty liver disease in severely obese subjects. The American journal of gastroenterology. 2007;102(2):399-408.
119. Rahman W, Huang P, Belov L, et al. Analysis of human liver disease using a cluster of differentiation (CD) antibody microarray. Liver international : official journal of the International Association for the Study of the Liver. 2012;32(10):1527-34.
120. Le Corvec M, Charpentier F, Kachenoura A, et al. Fast and non-invasive medical diagnostic using mid infrared sensor The AMNIFIR project. IRBM. 2016;37:116-23.
121. Jamali R, Arj A, Razavizade M, et al. Prediction of Nonalcoholic Fatty Liver Disease Via a Novel Panel of Serum Adipokines. Medicine (Baltimore). 2016;95(5):e2630.
122. Yoshimura K, Okanoue T, Ebise H, et al. Identification of novel noninvasive markers for diagnosing nonalcoholic steatohepatitis and related fibrosis by data mining. Hepatology. 2016;63(2):462-73.

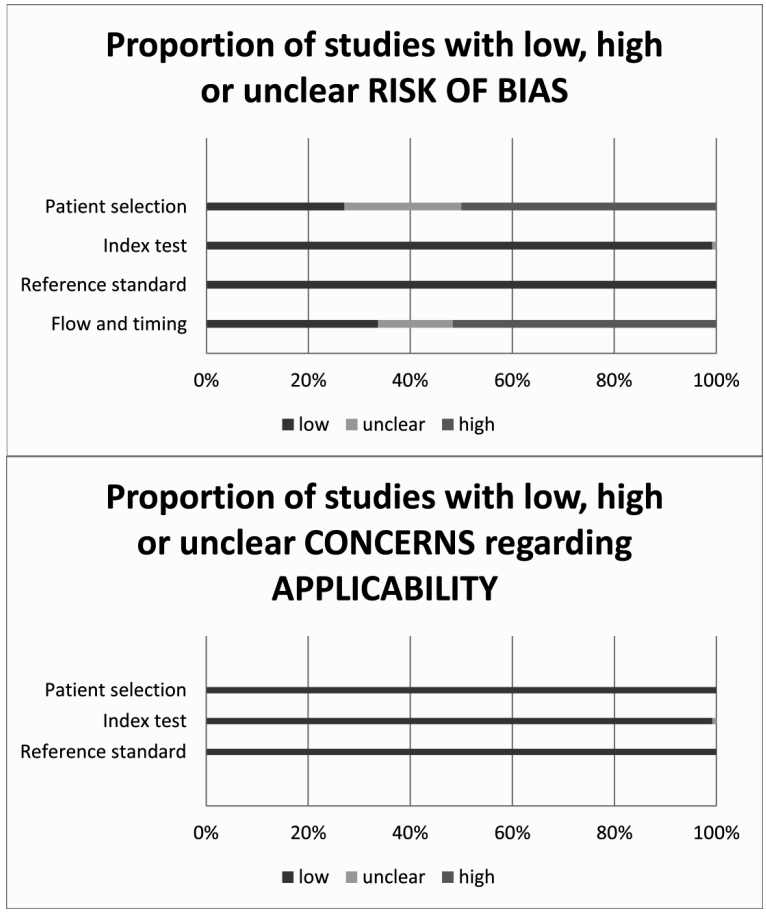

Figure S6.1 QUADAS-2 (Quality Assessment of Diagnostic Accuracy Studies) assessment; an overview with regards to risk of bias (low, unclear, high) and concerns regarding applicability (low, unclear, high) is shown.

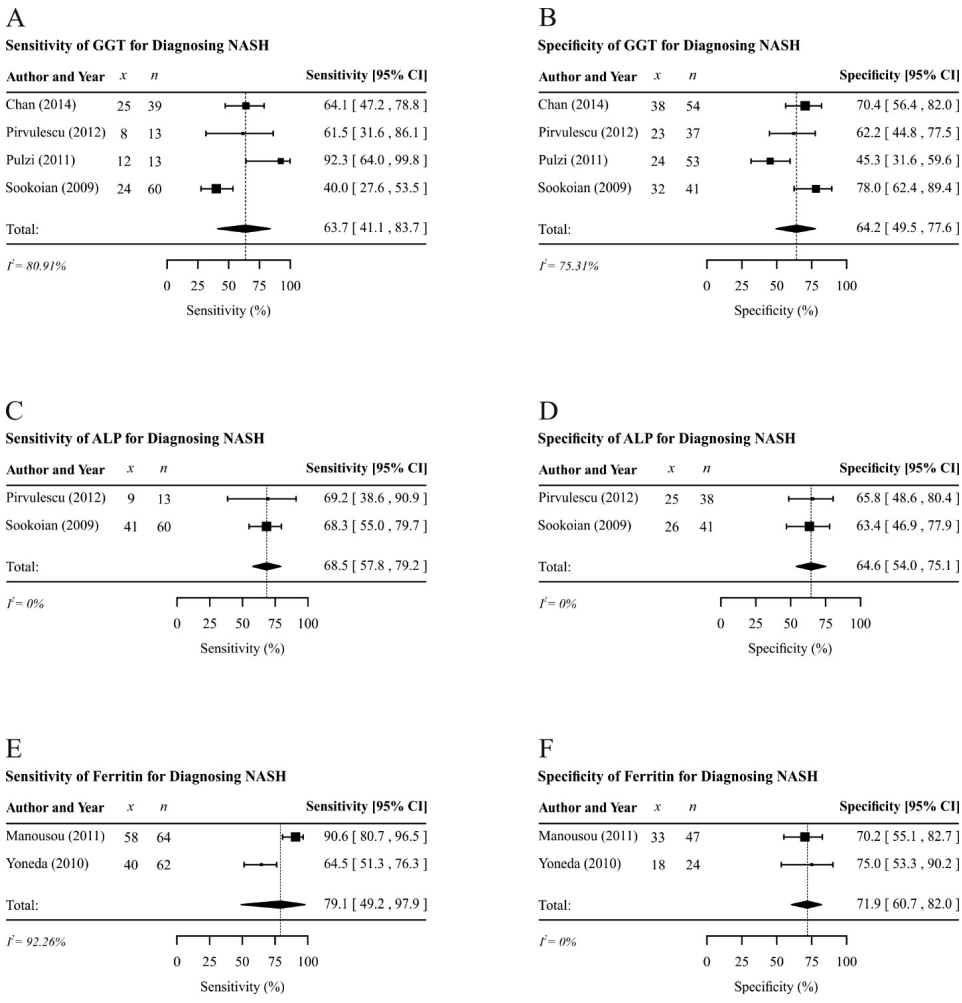

Figure S6.2 **Forest plots** for pooled sensitivity of gamma-glutamyl transferase (GGT) (**A**), pooled specificity of GGT (**B**), pooled sensitivity of alkaline phosphatase (ALP) (**C**), pooled specificity of ALP (**D**), pooled sensitivity of ferritin (**E**), pooled specificity of ferritin (**F**). Any discrepancy between the sensitivity and specificity for an individual study given in the forest plot and the original article is due to rounding off and/or reported missing data. A difference of ≤2% was accepted. Abbreviations: non-alcoholic steatohepatitis (NASH), confidence interval (CI).

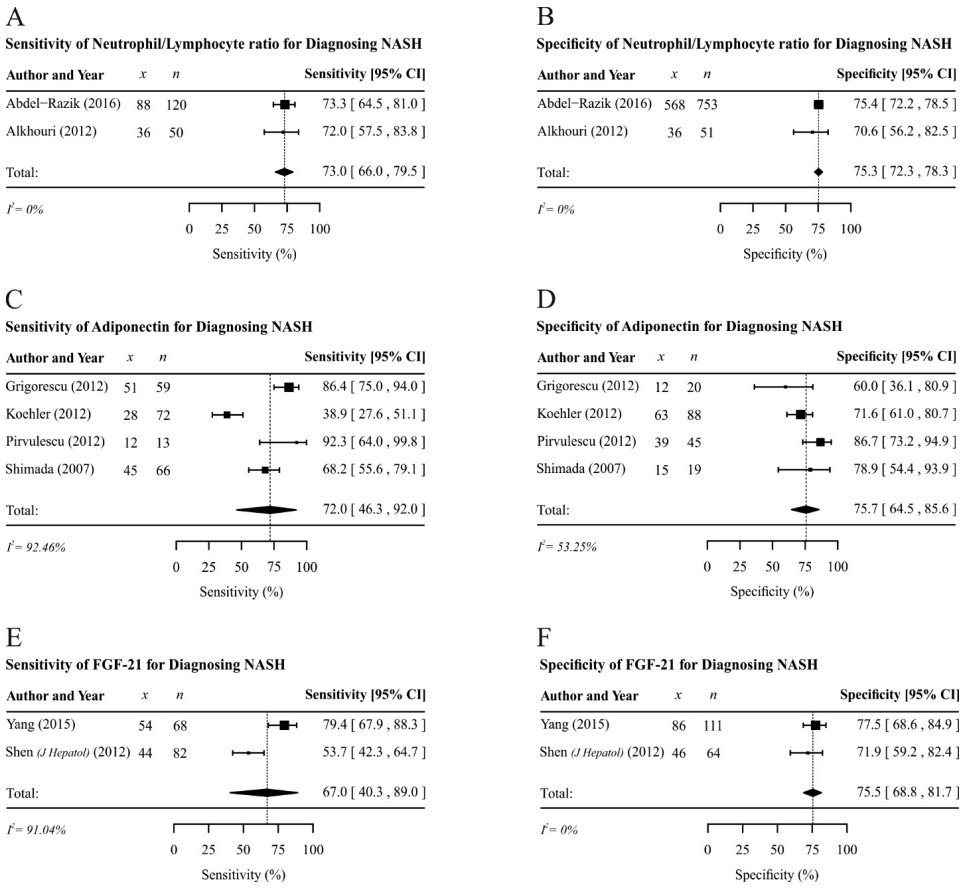

Figure S6.3 **Forest plots** for pooled sensitivity of neutrophil/lymphocyte ratio (N/L ratio) (**A**), pooled specificity of N/L ratio (**B**), pooled sensitivity of adiponectin (**C**), pooled specificity of adiponectin (**D**), pooled sensitivity of fibroblast growth factor (FGF) 21(**E**), pooled specificity of FGF-21(**F**). Any discrepancy between the sensitivity and specificity for an individual study given in the forest plot and the original article is due to rounding off and/or reported missing data. A difference of ≤2% was accepted. Abbreviations: non-alcoholic steatohepatitis (NASH), confidence interval (CI).

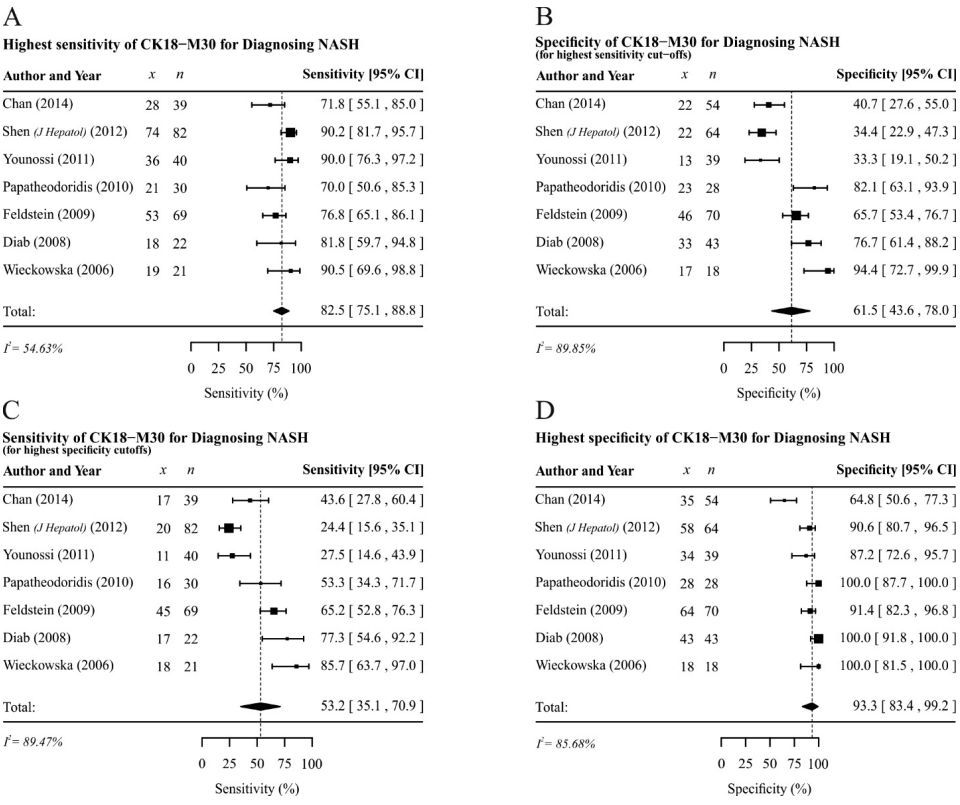

Figure S6.4 **Forest plots** for pooled sensitivity of cytokeratin (CK) 18-M30 (highest sensitivity) (**A**), pooled specificity of CK18-M30 (for highest sensitivity cut-offs) (**B**), pooled sensitivity of CK18-M30 (for highest specificity cut-offs) (**C**) and pooled specificity of CK18-M30 (highest specificity) (**D**). Abbreviations: non-alcoholic steatohepatitis (NASH), confidence interval (CI).

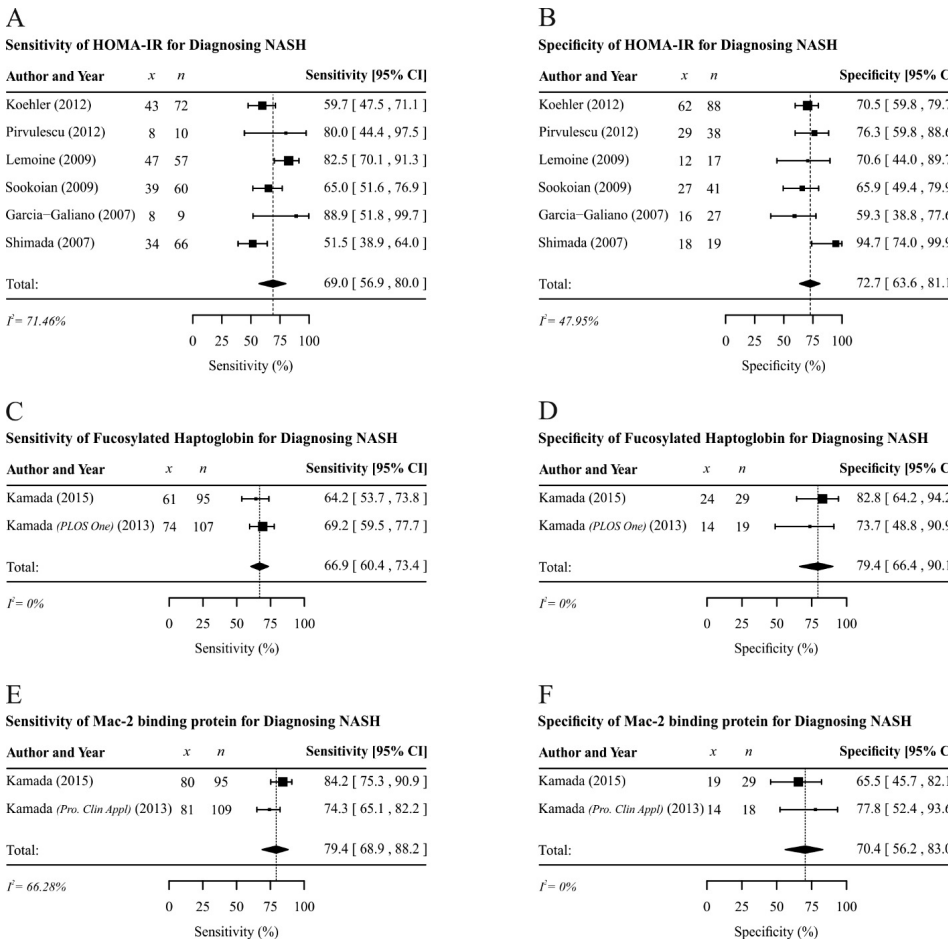

Figure S6.5 Forest plots for pooled sensitivity of homeostatic model assessment for insulin resistance (HOMA-IR) (**A**), pooled specificity of HOMA-IR (**B**), pooled sensitivity of fucosylated-haptoglobin (Fuc-Hpt) (**C**), pooled specificity of Fuc-Hpt (**D**), pooled sensitivity of Mac2 binding protein (Mac2bp) (**E**), pooled specificity of Mac2bp (**F**). Any discrepancy between the sensitivity and specificity for an individual study given in the forest plot and the original article is due to rounding off and/or reported missing data. A difference of ≤2% was accepted. Abbreviations: non-alcoholic steatohepatitis (NASH), confidence interval (CI).

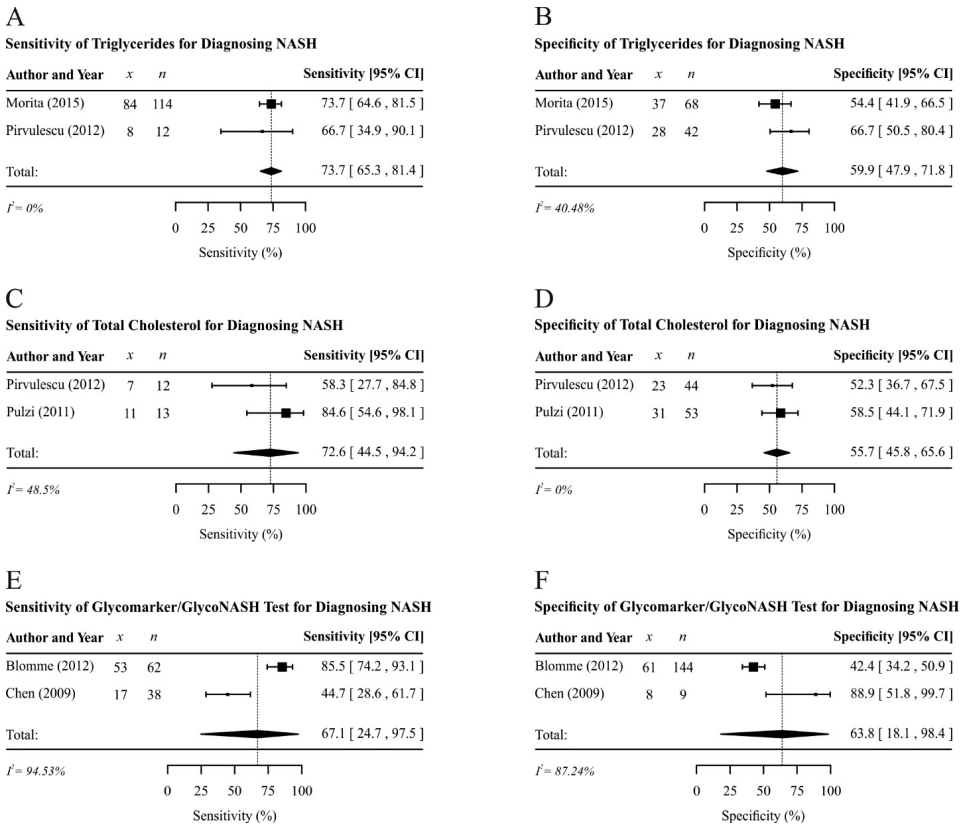

Figure S6.6 Forest plots for pooled sensitivity of triglycerides (TG) (**A**), pooled specificity of TG (**B**), pooled sensitivity of total cholesterol (TC) (**C**), pooled specificity of TC (**D**), pooled sensitivity of GlycoNASH test (**E**), pooled specificity of GlycoNASH test (**F**). Any discrepancy between the sensitivity and specificity for an individual study given in the forest plot and the original article is due to rounding off and/or reported missing data. A difference of ≤2% was accepted. Abbreviations: non-alcoholic steatohepatitis (NASH), confidence interval (CI).

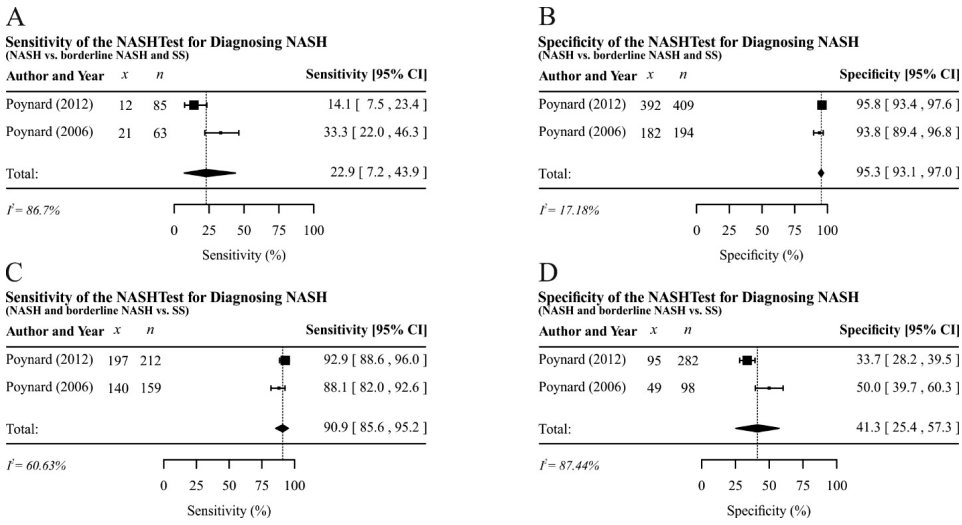

Figure S6.7 Forest plots for pooled sensitivity of NASHTest (NASH vs bNASH+SS) (**A**), pooled specificity of NASHTest (NASH vs bNASH+SS) (**B**), pooled sensitivity of NASHTest (NASH+bNASH vs SS) (**C**) and pooled specificity of NASHTest (NASH+bNASH vs SS) (**D**). Abbreviations: non-alcoholic steatohepatitis (NASH), simple steatosis (SS), borderline (b), confidence interval (CI). Any discrepancy between the sensitivity and specificity for an individual study given in the forest plot and the original article is due to rounding off and/or reported missing data. A difference of ≤2% was accepted.

Due to the extent of Supplementary Tables S6.1-S6.3 (128 pages), they were not printed in this thesis. The tables can be accessed on https://ars.els-cdn.com/content/image/1-s2.0-S1542356517309904-mmc3.pdf (Supplementary Table S6.1), https://ars.els-cdn.com/content/image/1-s2.0-S1542356517309904-mmc4.pdf (Supplementary Table S6.2) and https://ars.els-cdn.com/content/image/1-s2.0-S1542356517309904-mmc5.pdf (Supplementary Table S6.3).

Table S6.4 Sensitivity analysis: an overview of the pooled sensitivity and specificity using the mada package of R

Test	Number of studies	Sensitivity % (95% CI)	NASH (n)	Specificity % (95% CI)	SS (n)
Biomarkers					
ALT	8	63.0 (40.0-81.3)	447	74.4 (55.2-87.3)	566
CK18-M30	15	68.3 (62.5-73.5)	1017	71.8 (67.2-76.0)	839
HOMA-IR	6	68.4 (55.5-79.0)	274	69.6 (62.5-76.0)	230
Subanalyses					
CK18-M30 highest sensitivity	7	80.8 (72.9-86.7)	303	60.5 (41.0-77.2)	316
CK18-M30 highest specificity	7	53.2 (34.9-70.7)	303	91.4 (79.9-96.6)	316

Non-alcoholic steatohepatitis (NASH) is the number of patients diagnosed with NASH via liver biopsy. Simple steatosis (SS) is the number of patients with biopsy proven simple steatosis (Whether or not this includes SS or the combination of SS and normal (NL) depends on the individual studies). Due to interstudy variation with regards to the classification used to define NASH, whether or not borderline NASH was included in the NASH group or in the NoNASH group depends on the individual studies and classification system used (supplementary tables). Abbreviations: alanine aminotransferase (ALT), cytokeratin (CK), homeostatic model assessment for insulin resistance (HOMA-IR), confidence interval (CI).

Chapter 7

Crashing NASH in patients listed for bariatric surgery

Toon De Munck, Pauline Verhaegh, Jef Verbeek, Joanne Verheij, Jan Willem Greve, Daisy Jonkers, Ad Masclee, Ger Koek

Obes Surg. 2019;29(3):1012-1014

We read, with great interest, the publication of Ooi *et al.* describing the prevalence of non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) in a bariatric population.[1] They discussed the fact that there is a large variation in NASH prevalence in bariatric studies ranging from 7.3% to 56%. In their cohort they found a NASH prevalence of 17.1%, which was lower-than-expected based on previous literature. This lower than expected NASH prevalence was in line with our observation. We intra-operatively collected liver needle and wedge biopsies from 20 obese patients undergoing bariatric surgery (Table 7.1).

Table 7.1 Patient characteristics

	Total group n=20
Age (years)	51 (37.8-57.0)
Female gender	13/20 (65%)
BMI before crash (kg/m2)	41.2 (40.1-42.5)
BMI after crash (kg/m2)	38.6 (37.5-40.3)
Weight loss (kg)	8.0 (6.0-8.8)
Weight loss (%)	6.2 (5.3-7.6)
Hypertension (y/n)	9/20 (45%)
Type 2 diabetes mellitus (y/n)	4/20 (25%)
Dyslipidemia (y/n)	6/20 (30%)
Obstructive sleep apnoea (y/n)	8 (40%)
Total Cholesterol (5.0-6.4 mmol/L)	4.3 (4.0-4.6)
LDL-C (3.5-4.4 mmol/L)	2.7 (2.2-2.9)
HDL-C (>0,9 mmol/l)	1.0 (0.8-1.2)
Triglycerides (0.80-1.94 mmol/l)	1.3 (0.9-2.3)
ALT (F <34, M <45U/L)	31.0 (19.0-39.8)
AST (F <31, M <35U/L)	26.5 (19.0-30.8)
GGT (F <38, M <55U/L)	22.5 (17.3-28.0)
ALP (F <98, M <115U/L)	82.5 (73.3-96.8)
Fasting glucose (3.1-6.1mmol/L)	5.4 (5.0-5.9)
HbA1C (25-44 mmol/mol)	35.0 (33.0-42.8)

Continuous variables are expressed as median (Q1-Q3). Categorical variables are expressed as number (percentage of the total group). Abbreviations: Body mass index (BMI), Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Gamma-glutamyl transferase (GGT), Alkaline phosphatase (ALP), Low-density lipoprotein cholesterol (LDL-C), High-density lipoprotein cholesterol (HDL-C), glycosylated haemoglobin, type A1c (HbA1c).

Patients with significant alcohol use (>14 standard beverages a week for men and >7 for women), secondary causes of hepatic fat accumulation and chronic inflammatory diseases other than NASH were excluded. Strikingly, NASH, defined as the combined presence of steatosis, lobular inflammation and

hepatocyte ballooning, was not present in any of our obese patients and the maximal NAFLD activity score was only three (Table 7.2).

Table 7.2 Histological evaluation of liver biopsies

Patient	Steatosis	Lobular Inflammation	Portal Inflammation	Ballooning*	Fibrosis	NAS	SAF
1	0	0	0	0	0	0	S0A0F0
2	0	0	0	0	0	0	S0A0F0
3	0	0	0	0	1	0	S0A0F1
4	0	1	0	0	0	1	S0A1F0
5	0	1	0	0	0	1	S0A1F0
6	0	1	1	0	0	0	S0A1F0
7	0	1	2	0	1	1	S0A2F1
8	1	0	1	0	1	1	S1A0F1
9	1	1	0	0	2	2	S1A1F2
10	1	1	1	0	0	2	S1A1F0
11	1	1	1	0	1	2	S1A1F1
12	1	1	1	0	1	2	S1A1F1
13	1	1	1	0	1	2	S1A1F1
14	1	1	2	0	1	2	S1A1F1
15	1	1	2	0	2	2	S1A1F2
16	1	1	2	0	2	2	S1A1F2
17	1	2	2	0	2	3	S1A2F2
18	2	1	0	0	1	3	S2A1F1
19	2	1	0	0	1	3	S2A1F1
20	2	1	0	0	1	3	S2A1F1

Steatosis: 0: <5%, 1: 5%-33%, 2: 34%-66%, 3: >66%. Lobular Inflammation: 0: no foci, 1: <2 foci/200 x field, 2: 2-4 foci/200 x field, 3: >4 foci/200 x field. Portal inflammation: 0: none, 1: mild, 2: moderate, 3: severe. Fibrosis: 0: none, 1: perisinusoidal or periportal, 2: perisinusoidal and portal/periportal, 3: bridging fibrosis, 4: Cirrhosis. * Ballooning is scored using the SAF (Steatosis, Activity, Fibrosis) and Kleiner scoring system. In both scoring systems all biopsies scored 0. Abbreviations: NAFLD activity score (NAS), Steatosis, Activity and Fibrosis score (SAF).

Questions arise why none of our patients had NASH. We hypothesise that the absence of NASH in our cohort could be related to the pre-operative low-calorie diet (so-called crash diet). All our patients received a 4-week food-based crash diet as part of standard clinical care. Crash diets have been shown to reduce liver volume by 12% and intrahepatic fat by 40%.[2] The median amount of weight loss in our cohort after the 4-week crash diet was 8.0 kg or 6.2% of total body weight. Recently, it has been shown that this amount of weight loss (5-10% of initial body weight) can lead to improvement of lobular inflammation, hepatocellular ballooning and NASH resolution in 84%, 64% and 45% of the cases respectively.[3] Although we cannot prove the presence of NASH *before* initiation of the diet, the presence of fibrosis in 14/20 (70%) patients is suggestive for burned-out NASH with resolution of hepatocyte ballooning after

the dietary intervention.[4] A pre-operative diet is prescribed in numerous bariatric centres in order to decrease liver volume, to diminish operation time, complexity and post-operative complications.[5,6] Although encouraged, pre-operative weight loss is not obligated in all bariatric centres.[7] However, a survey among 99 Australian dieticians revealed that all dieticians provide bariatric patients with some form of nutritional intervention in the pre-operative stage.[8] Therefore, our question to Ooi *et al.* is if a crash diet or other type of pre-operative dietary intervention was prescribed before the operation.

Taken together, we hypothesise that large variations in NAFLD and NASH prevalence between different bariatric studies may be at least partly explained by the use of a pre-operative crash diet. In addition, a crash diet may lead to bias in NASH biomarker and pathophysiology research. Therefore, we advocate that the application of a pre-operative diet or other pre-operative interventions should always be mentioned in bariatric NASH studies.

References

1. Ooi GJ, Burton PR, Bayliss J, Raajendiran A, Earnest A, Laurie C, et al. Effect of Body Mass Index, Metabolic Health and Adipose Tissue Inflammation on the Severity of Non-alcoholic Fatty Liver Disease in Bariatric Surgical Patients: a Prospective Study. Obes Surg. 2019; 29(1): 99-108
2. Edholm D, Kullberg J, Haenni A, Karlsson FA, Ahlstrom A, Hedberg J, et al. Preoperative 4-week low-calorie diet reduces liver volume and intrahepatic fat, and facilitates laparoscopic gastric bypass in morbidly obese. Obes Surg. 2011; 21(3): 345-50.
3. Vilar-Gomez E, Martinez-Perez Y, Calzadilla-Bertot L, Torres-Gonzalez A, Gra-Oramas B, Gonzalez-Fabian L, et al. Weight Loss Through Lifestyle Modification Significantly Reduces Features of Nonalcoholic Steatohepatitis. Gastroenterology. 2015; 149(2): 367-78. e5; quiz e14-5.
4. Brunt EM. Nonalcoholic Fatty Liver Disease: Pros and Cons of Histologic Systems of Evaluation. Int J Mol Sci. 2016; 17(1).
5. Thorell A, MacCormick AD, Awad S, Reynolds N, Roulin D, Demartines N, et al. Guidelines for Perioperative Care in Bariatric Surgery: Enhanced Recovery After Surgery (ERAS) Society Recommendations. World J Surg. 2016; 40(9): 2065-83.
6. Cassie S, Menezes C, Birch DW, Shi X, Karmali S. Effect of preoperative weight loss in bariatric surgical patients: a systematic review. Surg Obes Relat Dis. 2011; 7(6): 760-7.
7. Mechanick JI, Youdim A, Jones DB, Garvey WT, Hurley DL, McMahon M, et al. Clinical Practice Guidelines for the Perioperative Nutritional, Metabolic, and Nonsurgical Support of the Bariatric Surgery Patient—2013 Update: Cosponsored by American Association of Clinical Endocrinologists, The Obesity Society, and American Society for Metabolic & Bariatric Surgery(). Obesity (Silver Spring). 2013; 21(0 1): S1-27.
8. Bourne R, Tweedie J, Pelly F. Preoperative nutritional management of bariatric patients in Australia: The current practice of dietitians. Nutr Diet. 2018; 75(3): 316-23.

Chapter 8

The increasing burden of non-alcoholic fatty liver disease in the general population: time to bridge the gap between hepatologists and primary care

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Abstract

Introduction

Non-alcoholic fatty liver disease (NAFLD) has become the leading cause of chronic liver disease worldwide due to the rising prevalence of obesity, diabetes mellitus, and metabolic syndrome. This confronts us with the challenge to adequately identify and manage these patients at population level. General practitioners (GPs), as the gatekeepers of our health care system, may play a major role in this important health issue. We aimed to investigate the knowledge and practices concerning NAFLD among Dutch GPs.

Methods

A survey, by using a questionnaire, was performed among GPs in the area of Maastricht, Netherlands, from April 2016 to May 2016.

Results

The acronyms NAFLD and NASH (non-alcoholic steatohepatitis) were not known by 34% and 53% of the GPs, respectively. Although 63% of the GPs acknowledged NAFLD as an important health problem, most GPs never (33%) or rarely (63%) screened for its presence. Referral to specialist care was highly dependent on the presence of increased liver enzymes. Lifestyle advice (weight loss) and referral to a dietician were mostly applied as treatment. Referral to an obesity treatment facility or starting medication rarely occurred. Follow-up of patients with NAFLD was usually done on a yearly basis (52%). The need for increased awareness and knowledge on NAFLD in the field of primary care was endorsed by 84% of the GPs.

Conclusion

In dialogue with GPs, we should develop, validate, and communicate, non-invasive tools and algorithms for the detection, referral, and (parallel) follow-up of high-risk individuals of NAFLD progression in the primary care setting in the Netherlands, taking into account cost-effectiveness.

Introduction

Non-alcoholic fatty liver disease (NAFLD) has become the leading cause of chronic liver disease worldwide due to the rising prevalence of obesity, diabetes mellitus, and metabolic syndrome.[1,2] It is estimated that the global prevalence of NAFLD is as high as 1 billion individuals.[1] Non-alcoholic steatohepatitis (NASH) is the most rapidly growing indication for liver transplantation in the United States.[3] In order to counter these alarming trends, a better knowledge and understanding of factors associated with poor outcomes in NAFLD is needed.
Fibrosis stage is the strongest predictor for disease-specific mortality in NAFLD; no other histologic features are associated with long-term outcomes of patients with NAFLD.[4,5] Koehler *et al.*[6] demonstrates the high prevalence of liver fibrosis in the context of NAFLD in a large, population-based cohort in the Netherlands. These results confront us with the challenge to adequately identify and manage these patients at general population level.[7] In this respect, general practitioners (GPs), as gatekeepers of our health care system, may play a major role. With the rapidly growing health problem of NAFLD, we aimed to investigate the knowledge and current medical approach concerning NAFLD among Dutch GPs.

Methods

General practitioners

We performed a survey among GPs in the area of Maastricht, The Netherlands, from April 2016 to May 2016. Questionnaires were sent to 120 GPs in the area of Maastricht.

Questionnaire

The questionnaire consisted of 22 questions of which the first five questions were about baseline characteristics. The remaining 17 questions were included to get more insight in the knowledge and approaches of GPs concerning NAFLD (including e.g. screening and diagnosing NASH/referral/treatment). An example of the questionnaire is provided in appendix 8.1.

Statistics

Data were analysed using SPSS version 23 statistical software (IBM Statistics for Macintosh, Chicago, IL, USA). GPs' baseline characteristics and knowledge and practices regarding NAFLD were reported as mean and standard deviation or median and range, when appropriate, for continuous variables and as absolute numbers and percentages for categorical variables.

Results

In total, 64 GPs completed the questionnaire (response rate 53%). Baseline characteristics of the participating GPs are depicted in Table 8.1.

Table 8.1 Baseline characteristics

	Total group of GPs
Age (years)	44.7 (10.8)
Gender (F/M [%])*	31/32 (49%/51%)
Experience (years)	14.0 (10.9)
Number of patients a week	108 (40.5)

Continuous variables are expressed as mean ±standard deviation (SD) and categorical variables are expressed as absolute numbers and percentages. *one missing value

Screening for NAFLD and differentiating NASH

Strikingly, 34% and 53% of the GPs, respectively, were not familiar with the acronyms NAFLD and NASH. Although 63% of the GPs acknowledged NAFLD to be an important health problem, 33% of the GPs never and 63% only rarely screened for its presence. Methods used for screening for NAFLD are shown in Figure 8.1.

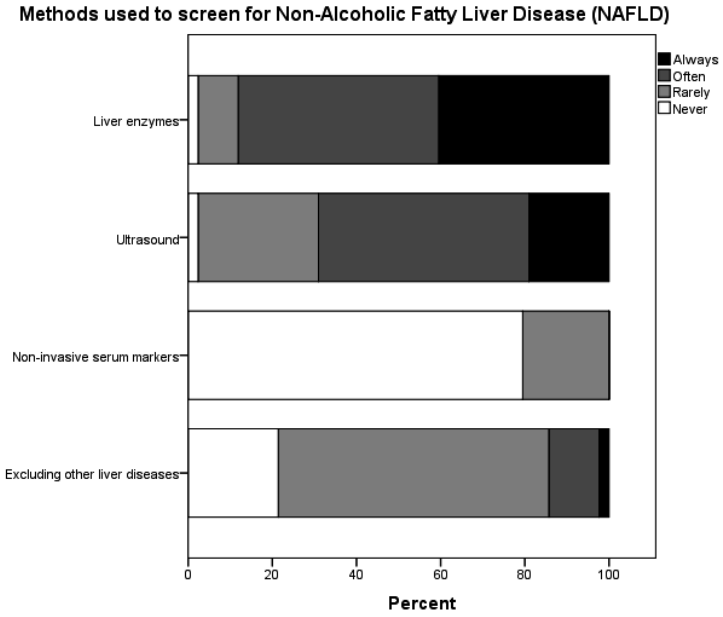


Figure 8.1 **Bar chart** showing how often (always/often/rarely/never) different methods were used to screen for non-alcoholic fatty liver disease (NAFLD). Percentages based on the number of GPs that indicated to screen for NAFLD; 42 out of 64 GPs, except for 'non-invasive serum markers' (N=39 GPs).

GPs were also questioned about the difference between simple steatosis and NASH. Although 80% of the GPs acknowledged the importance of differentiating simple steatosis from NASH, only 47% of the questioned GPs did this in daily practice. 51% of the GPs always or often used liver enzymes and 44% always or often used liver ultrasound to differentiate between simple steatosis and NASH. Non-invasive serum markers/scores other than liver enzymes were never (73%) or rarely (22%) used for this purpose (Figure 8.2).

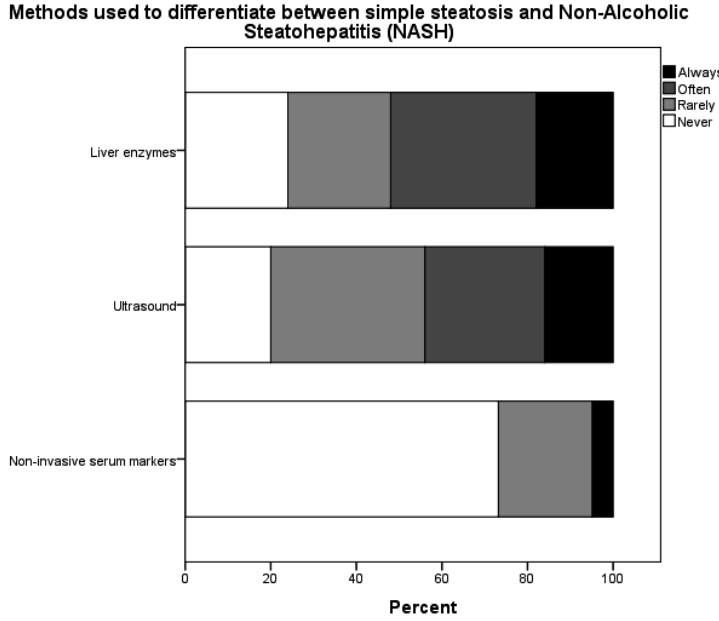


Figure 8.2. **Bar chart** showing how often (always/often/rarely/never) different methods were used to differentiate between simple steatosis and NASH. Percentages based on the number of GPs that indicated to differentiate between simple steatosis and NASH; 50 out of 64 GPs for liver enzymes and ultrasounds and 41 out of 64 GPs for non-invasive serum markers.

Referral

Referral to specialist care turned out to be highly dependent on the presence of increased liver enzymes. Patients with liver steatosis on ultrasound and normal liver enzymes were never or rarely referred (53% and 47% respectively). In contrast, 57% of patients with liver steatosis and abnormal liver enzymes were referred by GPs (being often 47% and always 10%) (Figure 8.3). In case of referral, the majority of GPs (95%) referred their patients to gastroenterologists or hepatologists.

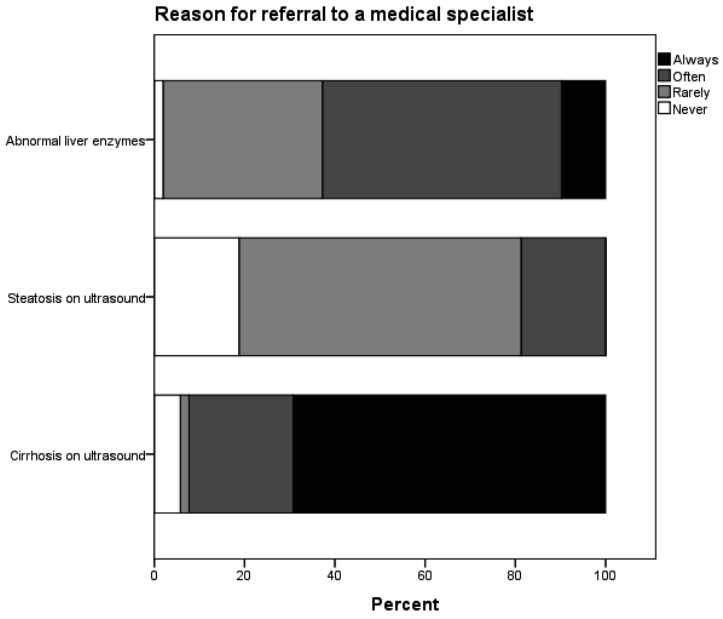


Figure 8.3. **Bar chart** showing how often (always/often/rarely/never) different reasons led to referral of a patient to the medical specialist. Percentages based on the number of GPs that indicated to refer to a medical specialist; 51 out of 64 GPs, 48 out of 64 GPs, 52 out of 64 GPs respectively for 'abnormal liver enzymes', 'steatosis on ultrasound' and 'cirrhosis on ultrasound'.

Treatment

In case GPs would start treatment for NAFLD, lifestyle advice (weight loss) (often in 37% and always in 60%) and referral to a dietician (often in 64% and always in 10%) were mostly applied as treatment. Referring a patient to a physical therapist was less common (never in 32% and rarely in 59%) and referral to an obesity treatment facility (never in 8% and rarely in 78%) or starting medication (never in 80% and rarely in 18%) was seldom done (Figure 8.4). Follow-up of patients with NAFLD was usually done once a year (52%) compared to never (19%), twice a year (24%) or more than twice a year (5%).

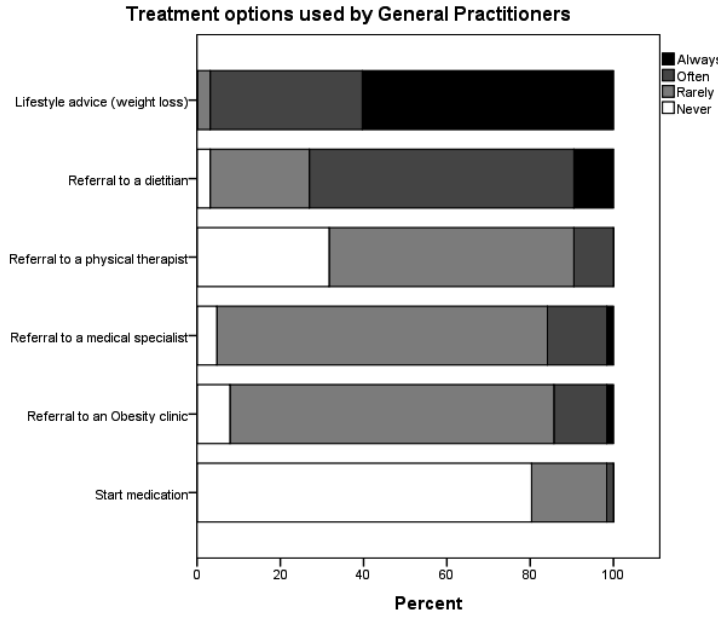


Figure 8.4. **Bar chart** showing how often (always/often/rarely/never) different treatment options were chosen to treat NAFLD patients. Percentages based on the number of GPs that indicated to start treatment; 63 out of 64 GPs, except for 'Start medication' (N=61 GPs).

Awareness and guidelines

GPs are not familiar with current clinical practice guidelines on NAFLD as developed by our national and international hepatology medical societies. Only one of the questioned GPs consulted the clinical practice guidelines of the European Association for the Study of the Liver (EASL) or the American Association for the Study of Liver Diseases. However, 86% of the GPs consulted the Dutch general practitioners guidelines for liver diseases. The need for increased awareness and knowledge on NAFLD in the field of primary care was endorsed by 84% of the GPs.

Discussion

Our aim was to obtain a snapshot of the knowledge and current approaches concerning NAFLD in primary care. The results of our questionnaire indicate the need for increased awareness and knowledge on NAFLD in primary care. Our

findings, together with those of Koehler *et al.*,[6] demonstrate the urgent need for the gastroenterology and hepatology societies to reach out and start training and informing GPs.

The growing burden of obesity has resulted in a global prevalence of NAFLD of more than 25%.[8] The number of patients diagnosed and referred with NAFLD is expected to rise even further in the near future. Although there is substantial evidence that some NAFLD patients have an increased risk to develop progressive liver disease that can lead to cirrhosis and liver cancer or to develop diabetes, specific risk factors to identify these patients are currently lacking.[9] Due to the high prevalence but low severity of NAFLD evidence based guidelines with regard to selection of patients for referral to secondary care and for follow-up of patients in primary care are urgently needed. Only by doing so, we will be able to manage this health issue in the coming years.

The current Dutch general practitioners guideline for liver diseases has also included NAFLD in their guideline for liver disease. It states that NAFLD is the most common cause of asymptomatic, mildly elevated liver enzymes and should be suspected in patients with obesity, diabetes mellitus or dyslipidaemia. However, it is a diagnosis of exclusion. Lifestyle advice is the recommended treatment. Furthermore, a cardiovascular risk profile should be assessed and yearly follow-up of alanine aminotransferase (ALT) levels should be performed. Referral to a gastroenterologist should only be considered in case of persistent elevated ALT levels (1.5-10 times upper limit of normal) or in case of doubt about the diagnosis.[10] While this guideline focusses on elevated liver enzymes, it is known that the entire histologic spectrum of NAFLD can be seen in individuals with normal ALT values.[11]

The EASL guideline (2016) "Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease" provides a flow-chart (Figure 1 in the EASL guideline, p 1393)[12] for the proposed assessment and monitoring in the presence of suspected NAFLD and metabolic risk factors both in the presence and absence of liver enzyme elevation.

The EASL guideline advises screening for NAFLD by use of an abdominal ultrasound in individuals with obesity, type 2 diabetes mellitus, metabolic syndrome, or incidentally discovered abnormal aminotransferase levels. In case significant fibrosis cannot be ruled out non-invasively by using fibrosis scores, e.g. Fibrosis-4 score (FIB-4),[13] a patient should be referred to a specialist.[12] The use of transient elastography (FibroScan®) in secondary care could be useful in this group to rule out or confirm significant fibrosis. Then a liver biopsy is only

indicated in patients with invalid FibroScan® measurements or in case of doubt about the diagnosis.
In addition, the EASL guideline advises for all cases with diabetes or diabetes risk to go to a diabetes clinic for optimal management. Those with diabetes risk and patients with obesity should be included in structured lifestyle modification programs. Finally, all cases should receive cardiovascular disease work-up.[12]
The optimal follow-up regimen of patients with NAFLD still needs to be determined. In this respect, risk of progression and workload for healthcare providers need to be considered. The EASL guideline advises to monitor patients with NAFLD without worsening of metabolic risk factors at 2-3 years intervals. Patients with NASH and/or fibrosis should be monitored more frequently, once a year. This group should most probably be followed up by a hepatologist, and not in primary care. Monitoring should include routine biochemistry, assessment of comorbidities and non-invasive monitoring of liver fibrosis.[12]
It is crucial to get a better insight in patients at risk for disease progression. Until specific risk factors or risk profiles have been identified, the flowchart provided by the EASL guideline should be followed to help route patients to the right healthcare professional.

In conclusion, our survey showed the need for increased awareness and knowledge on NAFLD in primary care. We should validate and communicate algorithms for the detection, referral, and (parallel) follow-up of NAFLD patients in the primary care setting in the Netherlands with GPs. Thereby, taking into account cost-effectiveness. Only close harmony between primary care and hepatologists will decrease the number of patients with undiagnosed NAFLD/NASH and insufficient referral.

References

1. Loomba R, Sanyal AJ. The global NAFLD epidemic. Nat Rev Gastroenterol Hepatol 2013;10:686-690.
2. Rinella ME. Nonalcoholic fatty liver disease: a systematic review. JAMA 2015; 313:2263-2273.
3. Wong RJ, Aguilar M, Cheung R, Perumpail RB, Harrison SA, Younossi ZM, et al. Nonalcoholic steatohepatitis is the second leading etiology of liver disease among adults awaiting liver transplantation in the United States. Gastroenterology 2015;148: 547-555.
4. Angulo P, Kleiner DE, Dam-Larsen S, Adams LA, Bjornsson ES, Charatcharoenwitthaya P, et al. Liver Fibrosis, but No Other Histologic Features, Is Associated With Long-term Outcomes of Patients With Nonalcoholic Fatty Liver Disease. Gastroenterology 2015;149:389-397 e310.
5. Ekstedt M, Hagstrom H, Nasr P, Fredrikson M, Stal P, Kechagias S, et al. Fibrosis stage is the strongest predictor for disease-specific mortality in NAFLD after up to 33 years of follow-up. Hepatology 2015;61:1547-1554.
6. Koehler EM, Plompen EP, Schouten JN, Hansen BE, Darwish Murad S, Taimr P, et al. Presence of diabetes mellitus and steatosis is associated with liver stiffness in a general population: The Rotterdam study. Hepatology 2016;63:138-147.
7. Ahmed A, Perumpail RB, Harrison SA. High prevalence of hepatic fibrosis in the setting of coexisting diabetes and hepatic steatosis: A case for selective screening in the general population? Hepatology 2016; 63:20-22.
8. Younossi ZM, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M. Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. Hepatology 2016;64:73-84.
9. Ekstedt M, Nasr P, Kechagias S. Natural History of NAFLD/NASH. Curr Hepatol Rep 2017;16:391-397.
10. Genootschap NH. Standaard Virushepatitis en andere leveraandoeningen. In; 2016.
11. Mofrad P, Contos MJ, Haque M, Sargeant C, Fisher RA, Luketic VA, et al. Clinical and histologic spectrum of nonalcoholic fatty liver disease associated with normal ALT values. Hepatology 2003;37:1286-1292.
12. European Association for the Study of the L, European Association for the Study of D, European Association for the Study of O. EASL-EASD-EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease. J Hepatol 2016;64:1388-1402.
13. Fibrosis-4 (FIB-4) Calculator. In. p. Source: Sterling RK, Lissen E, Clumeck N, et. al. Development of a simple noninvasive index to predict significant fibrosis patients with HIV/HCV co-infection. Hepatology 2006;2043:1317-1325.

Appendix 8.1

Survey General Practitioners - Liver disease

Dear colleague,
We are researchers of the department gastroenterology and hepatology at the MUMC+. At this moment we are investigating, in cooperation with the department of primary care, diagnosis and treatment of common liver diseases in primary care. Our goal is to improve the collaboration between primary care and secondary/tertiary care. We are looking for general practitioners (GP) that are willing to fill in a short questionnaire on this topic. All records will be handled anonymously. Filling in the questionnaire will take about 5 minutes of your time. After completing the questionnaire you can return it in the attached return envelope.
Thank you in advance for your cooperation!

M. van Asten, final year medical student
Dr. J. Verbeek, Gastroenterologist in training
Dr. G. Koek, Gastroenterologist

DATE: .....
NAME GENERAL PRACTITIONER:
Sir/Madam.....
NAME GP practice:
.....
CITY GP practice:
.....
.....

1. What is your age?
   ..... years old

2. How many years are you working as a trained GP?
   ..... years. In case of GP in training, number of years working as a GP in training:
   ..... years.

3. How many patients do you see on average in a week?
   .....

4. Are you familiar with the abbreviation NAFLD (circle your answer)
   a) no b) yes; what does the abbreviation stand for:
   .....

5. Are you familiar with the abbreviation NASH? (circle your answer)
   a) no b) yes; what does the abbreviation stand for:
   .....

The following questions are on fatty liver/steatosis hepatis in the context of metabolic syndrome.

6. Do you have an idea what percentage of the general adult Dutch population has liver fat in the context of metabolic syndrome?
   Answer: ..... %

7. The following questions are on screening for the presence of steatosis hepatis.
   Do you actively screen for steatosis hepatis? (circle your answer)
   a) never *(continue to question 10)* b) rarely
   c) often d) always

8. In case you do screen, what is your motivation to screen for steatosis hepatis? (circle your answer)

Obesity	never / rarely / often / always
Hypertension	never / rarely / often / always
Hypercholesterolaemia	never / rarely / often / always
Diabetes	never / rarely / often / always

   Other:.....

9. In case you screen, do you never, rarely, often or always using (circle your answer):
   a) Liver enzymes never / rarely / often / always
   b) Ultrasound of the liver never / rarely / often / always
   c) Excluding other causes of liver disease
   (HBV, HCV, EBV, CMV, HEV) never / rarely / often / always
   d) Non-invasive blood markers/scores,
   other than routine liver enzymes never / rarely / often / always
   e) Other, namely .....

10. Do you think it is important to differentiate between simple steatosis and chronic liver inflammation? (circle your answer)
    a) no b) yes

11. Do you differentiate between simple steatosis and chronic liver inflammation? (circle your answer)
    a) no b) yes

12. How do you differentiate between simple steatosis and chronic liver inflammation? Are you never, rarely, often or always using (circle your answer):
    a) Liver enzymes never / rarely / often / always
    b) Imaging (ultrasound) of the liver never / rarely / often / always
    c) Non-invasive serum markers/scores never / rarely / often / always
    d) Other, namely .....

13. In case you suspect steatosis hepatis in the context of metabolic syndrome in a patient, do you never, rarely, often or always refer this patient to a medical specialist? (circle your answer)
    a) never b) rarely c) often d) always

14. In case you refer a patient, what are your motives for referral? (circle your answer)
    a) Elevated liver enzymes never / rarely / often / always
    b) Steatosis on ultrasound never / rarely / often / always
    c) cirrhosis / portal hypertension on ultrasound never / rarely / often / always
    d) Other, namely .....

15. How often do you refer a patients with steatosis hepatis on ultrasound and **normal** liver enzymes to a medical specialis? (circle your answer)
    a) never b) rarely c) often d) always

16. How many patients with steatosis hepatis on ultrasound and **abnormal** liver enzymes do you refer to a specialis? (circle your answer)
    a) never b) rarely c) often d) always

17. To which medical specialist do you refer patients with steatosis hepatis? (circle your answer)
    a) Gastroenterologist/hepatologist
    b) Internist/Endocrinologist
    c) Other, namely.....

18. Do you consult the following guidelines on diagnosis and treatment of steatosis hepatis (circle your answer):
    a) Dutch general practitioner guideline yes / no
    b) AASLD (American Association for the Study of Liver Diseases) guideline on NAFLD yes / no
    c) EASL (European Association for the Study of Liver) guideline on NAFLD yes / no
    d) Other, namely .....

19. The following questions are on the treatment of patients with steatosis hepatis. Do you never, rarely, often or always (circle your answer):
    a) Give the advice to lose weight never / rarely / often / always
    b) Refer to a dietician never / rarely / often / always
    c) Refer to a physical therapist never / rarely / often / always
    d) Refer to a medical specialist never / rarely / often / always
    e) Refer to an obesity clinic (e.g. CO-EUR) never / rarely / often / always
    f) Start medication never / rarely / often / always
    g) In case you start medication, what do you start?.....
    e) Other, namely .....

20. How often per year do you follow-up patients with steatosis hepatis? (circle your answer)
    a) never b) once a year c) twice a year d) more than twice a year

21. Is steatosis hepatis/NAFLD in your opinion an important health problem? (circle your answer)
    a) no b) yes

22. Should something about the awareness and knowledge on steatosis hepatis be improved in primary care, according to you? In case your answer is yes, what and how? (circle your answer)
    a) no b) yes,
    namely.....
    .....

This is the end of the questionnaire. ***Thank you very much for your cooperation!***

Chapter 9

General discussion

General discussion

Main Findings

In this thesis, we aimed to gain further insight in underlying pathophysiological mechanisms and non-invasive diagnostic tools for non-alcoholic steatohepatitis (NASH).

With regard to pathophysiological mechanisms, this thesis addressed hepatic factors, namely liver sinusoidal endothelial cells and the role of ferritin, but also extrahepatic factors, namely the skeletal muscles. Furthermore, an extensive overview of non-invasive biomarkers and scores was presented. Finally, the role of general practitioners (GPs) in identifying and managing NAFLD patients was discussed.

Pathophysiology: How far have we reached?

Getting further insight in factors contributing to the onset and development of NASH is relevant to help identifying NAFLD patients at risk for disease progression.

Although the exact pathophysiology of NAFLD is not yet clear, much progress has been made in the last three decades.

In 1998, the two-hit theory was proposed to explain NAFLD disease progression.[1] According to this theory, the first hit is triggered by lipid accumulation in hepatocytes. This would predispose the liver to a second hit, for example oxidative stress, which would be required for the development of NASH.

Growing interest in NAFLD led to an increase in research and the concept evolved to a 'parallel multiple hit theory',[2] in which it was suggested that not simple steatosis but insulin resistance (IR) may induce inflammation in NAFLD. In this theory, steatosis is only seen as a marker of insulin resistance, since insulin resistance is the most important factor predisposing to hepatic free fatty acid (FFA) accumulation due to lipolysis of peripheral fat and exacerbated hepatic lipogenesis.[3] After initial hepatic fat infiltration, the liver becomes vulnerable for parallel hits such as oxidative damage and gut- and adipose tissue-derived signals, which play a central role in the cascade of inflammation in NASH.[2] This theory conceptualises NASH as a condition which is generally preceded by simple steatosis from a histological point of view and insulin resistance from a pathophysiological point of view.

Thereafter, the 'distinct hit theory' emerged, in which simple steatosis and NASH are regarded as two separate pathological entities caused by IR. Hereby, NASH may present without any or with much steatosis. Steatosis in NASH is considered a benign epiphenomenon rather than a causal factor of inflammation and fibrosis, which are the two main hallmarks of NASH.[3,4] Triglyceride accumulation as lipid droplets, as seen in simple steatosis, seems to be an adaptive, beneficial response in situations where hepatocytes are exposed to potentially toxic cholesterol and FFA metabolites due to IR and increased caloric consumption.[2] Accumulation of triglycerides is not a pre-requisite for the development of NASH. In NASH, it is thought that hepatic metabolism of FFAs and cholesterol leads to the formation of toxic metabolites *i.e.* ceramides, diacylglycerols, lysophosphatidylcholine (lipotoxicity), which are considered to be responsible for oxidative stress, inflammation and liver cell injury.[2,3] However, the exact underlying mechanisms of the formation of lipotoxic intermediates at molecular levels remain to be elucidated.[5] As triglyceride accumulation often is a process parallel to the development of lipotoxic injury, it is however difficult to separate the respective roles of triglycerides and FFA metabolites in causing inflammation and cell injury.[5]

In **chapter 2**, the ultrastructure of the liver in human NAFLD samples was studied, focusing on liver sinusoidal endothelial cells (LSECs) and their fenestrae. LSECs form the liver endothelium and are highly specialised cells, being at the interface between blood derived from the visceral adipose tissue and the gut on the one side and hepatic stellate cells and hepatocytes on the other side. LSECs have a unique phenotype, they lack a basement membrane and have fenestrae organised into sieves that regulate the transport of macromolecules, including lipids and lipoproteins across the sinusoid.[6] We showed that defenestration is more common in patients with simple steatosis compared to patients with NASH. This finding can be supportive for the 'distinct hit theory' as it could be argued that defenestration is a protective mechanism to handle the excess in lipids and perhaps other toxic substances provided to the liver. By inhibiting the entry of chylomicron remnants into the space of Disse,[7] defenestration could protect the liver from receiving more lipids. However, defenestration could also contribute to triglyceride accumulation as it impairs the passage of VLDL excretion towards the sinusoidal lumen, thereby contributing to hepatic lipid accumulation.[6] Additionally, inhibition of lipid uptake due to defenestration has been proposed to lead to stimulation of *de novo* lipid (DNL) synthesis and thereby induction of steatosis.[7] This is due to the fact

that hydroxymethylglutaryl-CoA reductase (HMGCR), the rate limiting enzyme for hepatocyte cholesterol synthesis, is no longer inhibited when chylomicron remnants and dietary cholesterol do not cross the fenestrae into the liver, thereby activating endogenous cholesterol synthesis in hepatocytes.[8] However, one can speculate that in case of defenestration as seen in simple steatosis, DNL could be performed at a pace that does not overwhelm beta-oxidation and re-esterification within the liver. This would enable the liver to store the fat as 'innocent' triglycerides in lipid droplets, instead of metabolising the lipids. Metabolization of FFA and cholesterol is suggested to cause the lipotoxic injuries in NASH.[2,3]

The finding of defenestration in patients without NASH provides support for the distinct hit theory, because a distinct aetiological mechanism, that might contribute to the development of NASH, was shown. However, even if the 'distinct hit theory' applies within NAFLD pathophysiology, multiple hits will probably be needed to lead to NASH.

Another possible hit has been suggested in this thesis, namely ferritins. Ferritin is an iron storage protein, it comprises of 24 units of ferritin heavy and light chains and can store up to 4500 iron atoms bound to oxygen.[9] Serum ferritin was associated with having NASH with significant fibrosis (F2-4) and being p.C2828Y or p.H63D heterozygote, in the presence and absence of hepatocellular iron, in a cross-sectional study in biopsy proven NAFLD patients (**chapter 4**). Whether ferritin levels are a consequence of inflammation or may contribute to inflammation in NAFLD is not completely clear. A possible mechanism via which ferritin might cause inflammation in NAFLD has been proposed by Hevi *et al.* These authors studied the regulation of apolipoprotein B (apoB) secretion from hepatocytes. It was shown that ferritin heavy and light chains in the absence of iron, post-translationally inhibit the secretion of apolipoprotein B.[10] This caused significantly elevated amount of lipids in cells loaded with both heavy and light chain ferritins.[10] This can contribute to NAFLD progression by increase ROS due to enhanced free fatty acid metabolism via mitochondrial β-oxidation.[3,11] Future longitudinal research projects are needed and should include the patterns of hepatic iron depositions, to elucidate the relation between inflammation, iron and NAFLD severity.

Extrahepatic involvement

Even though the term NAFLD refers to a disease of the liver, there is growing evidence to support the concept of NAFLD as a multisystem disease. To date,

many studies have pointed to associations of NAFLD with cardiovascular disease and type 2 diabetes mellitus. Also associations of NAFLD with colorectal cancer, obstructive sleep apnoea, chronic kidney disease and hypothyroidism have been suggested.[12] Since the prevalence of NAFLD is higher among patients with type 2 diabetes mellitus, it is interesting to study the skeletal muscle because of its role in glucose regulation. Insulin resistance is known to play a role in the pathophysiology of NAFLD, as is also suggested by both the distinct hit and the multiple parallel hit theory.[2,4] Intramyocellular lipid storage (myosteatosis) has been associated with the development of type 2 diabetes mellitus.[13] It has been suggested that alterations in the muscle-liver axis, due the hepatic steatosis, initiate a vicious circle in which liver disease favours defective muscle protein growth and in which muscle loss favours metabolic alterations as well as hepatic steatosis and inflammation.[14]

In our well characterised cohort of NAFLD patients, myosteatosis did not correlate with the degree of hepatic steatosis or fibrosis, but a positive correlation was shown with total body fat percentage and BMI (**chapter 5**) Furthermore, insulin resistance was not correlated with the amount of myosteatosis in our study.[15]

In contrast to our study, other studies found a positive association between myosteatosis and the severity of hepatic steatosis[16] or NASH.[17] The differences in results compared to our study could partially be explained by differences in the characteristics of the study population. Our population had less diabetics, was younger, and comprised a smaller number of patients with elevated ALAT levels compared to previous studies.[17,18] Unfortunately, we were not able to investigate the link between hepatic inflammation and myosteatosis as liver biopsies were not available. It is possible that myosteatosis is an important hallmark in hepatic inflammation but its relevance with respect to the degree of hepatic steatosis or fibrosis appears to be minimal. These results might suggest that myosteatosis is an "innocent bystander", a way to safely store triglycerides. Furthermore, in our study myosteatosis was not related to insulin resistance. This is in line with the "athlete's paradox", in which athletes show levels of myosteatosis exceeding those of diabetics. This indicates that myosteatosis per se does not lead to insulin resistance, and lipid species, fat oxidative capacity and lipid turnover might play a role.[13]

NAFLD research: human versus animal studies

NAFLD is a world-wide, multifactorial, lifestyle related disease resulting from a complex interplay between different factors such as diet, genetics, metabolic factors, and intestinal microbiome perturbations. Due to this complex interplay, the involvement of several organs and inter-individual variation in disease presentation, human research on NAFLD is important. This can be facilitated by a well-characterised cohort of NAFLD patients with bio-sampling, which can be followed over a longer period of time. This will help to get a better and more complete understanding of the natural disease course and factors that influence the development of progressive disease. Therefore, we have set up a longitudinal NAFLD cohort, in which extensive phenotyping of a NAFLD population was combined with bio-sampling, at 3 time points during a 10 year follow-up.

This type of clinical human research is very demanding, because it takes time and requires liver histology obtained by (repeated) liver biopsies to be able diagnose NASH. Therefore, non-invasive tools are urgently needed for accurate diagnosis and for monitoring over time. For this reason, human studies will be supplemented with animal studies. Several well-accepted animal models are now available, such as the dietary methionine choline deficient (MCD) model. In this model, normal mice (ea. C57/BL6) fed the MCD diet, develop a histologic appearance similar to NASH. Other frequently used mouse models in NAFLD research are the genetic leptin-deficient (ob/ob) or leptin resistant (db/db) mouse.[19]

Although relevant lessons about underlying mechanisms have been obtained from animal models, it should be noted that these models do not fully replicate the human NASH phenotype.[19,20] In the dietary MCD model, mice lose rather than gain weight and do not develop insulin resistance. Furthermore, patients with NASH are not choline deficient.[19] In the present thesis, defenestration was studied in human liver samples. Defenestration has also been studied in animal models. A study in mice deficient of Plasmalemma Vesicle Associated Protein (PLVAP), a protein coding gene required for the formation of fenestrae in liver sinusoidal endothelial cells (LSECs), showed not only development of extensive multivesicular steatosis, but also steatohepatitis and fibrosis at around three weeks of life.[6,7] In contrast, our study in humans showed defenestration was more common in patients with simple steatosis compared to patients with NASH. Genetic mouse models (such as leptin-deficient [ob/ob]) are suitable to study isolated pathways, but one should keep in mind that these gene mutations

are generally not present in patients.[21] Furthermore, it should be noted that most mouse models fail to develop important morphologic key features of NASH, namely hepatocyte ballooning[22] and do not reflect the large inter-individual differences with regard to host genetics, the intestinal microbiota composition and exposure to lifestyle factors. Even though, none of the existing models reflect the entire NAFLD phenotype as encountered in humans, animal models are important in elucidating mechanisms and pathways involved in the pathogenesis of the NAFLD spectrum. It remains challenging to translate the results from these models to humans.[19,20] It is essential that key findings from animal experiments are confirmed in the human clinical studies. To improve translation into clinical setting, researchers are now using omics data from both patients and from animal models, where clinical phenotype, genomic heterogeneity, transcriptomics, and metabolomic changes are combined to be able to identify the most suitable NAFLD animal model for a specific scientific question or to test a particular drug.[21,23]

Alcohol in relation to NAFLD: Dual Aetiology Fatty Liver Disease (DAFLD)

NAFLD is defined as hepatic fat accumulation of >5% in the absence of secondary causes, including daily alcohol consumption.[24] Although, the disease is called non-alcoholic fatty liver disease, many patients are not total abstainers. A daily alcohol consumption of <30 gram for men and <20 gram for women is allowed in case of NAFLD. Many studies on NAFLD are very strict when it comes to alcohol use. On the other hand, one may argue whether such differentiation is useful in daily clinical practice. Interestingly, administration of a moderate amount (20 grams) of alcohol can increase both small bowel and colon permeability,[25] which has been suggested to play a role in NAFLD.[26] Therefore, the term Dual Aetiology Fatty Liver Disease has been introduced, and is defined as having a fatty liver and consuming >20g/day of alcohol.[27]

Studying moderate alcohol use in NAFLD has shown contradictory results.[28,29] It has been associated with a lower prevalence of NASH and fibrosis when compared to abstinence,[30,31] whereas another cohort showed less NASH resolution in relation to moderate alcohol use.[32] Sinn et al. found that moderate alcohol use (<20 grams/day) was associated with decreased odds of carotid plaque and stenosis on duplex ultrasonography compared to non-drinkers in patients with NAFLD.[33] However, alcohol intake has also been shown to lead to weight gain and obesity, further exacerbating the risk factors for NAFLD.[27] Not

only the amount of alcohol consumed but also the drinking pattern should be taken into account, as binge drinking does not appear to have protective effects.[30] In addition, the type of beverage appears to be relevant: while wine consumption is beneficial, beer consumption is not.[30,34] This raises the question whether the protective effect is due to ethanol itself or other components in the beverage, such as antioxidants. With regard to recommendations on alcohol use in NAFLD patients, moderate alcohol use as a possible approach to reduce risk of disease progression in NAFLD does seem questionable. Apart from hepatotoxic effects, alcohol has been shown to disrupt the intestinal barrier, and changes the intestinal microbiota composition and activity. With the existing evidence, clinicians should advocate against the use of alcohol in health promotion.[29] Although, the importance of studying NAFLD pathophysiology in populations that do not consume alcohol for a clear overview of factors involved in NAFLD is argumentative, it would be interesting for clinical practice to include DAFLD patients in studies on NAFLD, since alcohol use is associated with the lifestyle that is related to NAFLD.

Non-invasive diagnosis of NASH: the importance of early diagnosis

NAFLD is usually asymptomatic, in general resulting in silent disease progression. To date, liver biopsy is the gold standard to diagnose NASH, but this is not a suitable tool for diagnosing large populations or disease monitoring.[35] In clinical practice only subjects at increased risk of having NASH, usually based on elevated aminotransferase levels, undergo a liver biopsy. This may lead to underdiagnosis of NASH.[36] Non-invasive diagnosis of NASH enables screening of large numbers of patients and early identification of patients at risk for disease progression, so early interventions can be taken to prevent progression. Therefore, a non-invasive diagnostic tool is urgently needed. It has been questioned whether it is necessary to develop a non-invasive tool to diagnose NASH, since fibrosis stages 1-4 were found to be the strongest predictor for disease-specific mortality in NAFLD.[37,38] It should be mentioned that NASH is a recognized driver of liver fibrosis and fibrosis progression.[39] Furthermore, NASH is associated with a low grade systemic inflammation and with an increased risk of developing type 2 diabetes mellitus compared to patients with simple steatosis. In addition, associations have been shown with cardiovascular disease and colorectal cancer.[40,41]

A non-invasive diagnostic tool for NASH could benefit both clinical and research purposes to help the diagnosing and monitoring of the disease in large patient groups. Due to the complexity of the pathogenesis of NASH, as discussed above, it is unlikely that a single non-invasive marker will be able to correctly diagnose NASH. We showed that none of the single blood markers that were included in the meta-analyses, had both a sensitivity and specificity >80%. However, several scoring systems and –omics approaches that combined markers for different pathophysiological processes in NASH, such as inflammation, lipid metabolism and fibrosis, showed promising findings. For example the microRNA profile combined with CK18-asp396[42] and the clinical and phosphoproteomic model.[43] However, most of the studied markers were not validated and therefore a non-invasive marker for NASH is not yet available for clinical practice (**chapter 6**).[44] It is important to perform validation studies, preferably in large unselected populations, to avoid selection- and work-up-bias.[45,46]

Interphase between primary and secondary care

General practitioners (GPs), as gatekeepers of our health care system, are going to play a major role in the challenge to adequately identify and manage NAFLD patients at population level. Due to the high prevalence and overall low to moderate severity of NAFLD, GPs will encounter most of these patients and become actively involved in the screening and management of patients. Therefore, GPs should be trained to identify subgroups of patients at risk for progressive liver disease and refer these patient to secondary care. Moreover, adequate education on the diagnosis, follow-up and treatment of low to moderate risk patients should be provided, taking into account cost-effectiveness. Only by doing so, we will be able to manage this healthcare issue in the coming years. A better knowledge and awareness of NAFLD in primary care is needed. This was endorsed by 84% of the GPs in our survey (**chapter 8**),[47] which is in line with Australian[48] and urban Western US data.[49] Due to the lack of non-invasive diagnostic tools to identify patients at risk for disease progression, it is difficult for GPs to decide which patients should be referred and which patients can be followed in primary care. Both the EASL[24] and the BASL guideline[50] now provide a flow-chart for the proposed assessment and monitoring of suspected NAFLD and metabolic risk factors, which can help GPs make these decisions.

The EASL guideline advises risk-based case finding for NAFLD by use of an abdominal ultrasound or steatosis biomarkers, like the Fatty Liver Index (FLI, including BMI, waist circumference, gamma-glutamyl transferase, triglycerides) in individuals with obesity, type 2 diabetes mellitus, metabolic syndrome, or incidentally discovered abnormal aminotransferase concentrations.[24] The BASL guideline advises screening for NAFLD using the FLI in high-risk populations based on the presence of the metabolic syndrome or its components (obesity, type 2 diabetes mellitus or patients with a history of ischemic cardiovascular disease).[50] In case significant fibrosis cannot be ruled out non-invasively using fibrosis scores, such as NAFLD Fibrosis Score (NFS) or FIB-4, a patient should be referred to a secondary care specialist.[24,50] In case no significant fibrosis is present, patients should be monitored and periodically retested. The EASL recommends a follow-up every 2 years for patients with steatosis, but without significant fibrosis. In case steatosis and fibrosis are not present, but patients are considered as a high risk population, a 3-5 year follow-up is recommended.[24] The BASL guideline suggests shorter follow-up intervals, being one year in case of steatosis without fibrosis and every two years in case of absence of steatosis and fibrosis. An easy-to-use web application based on the proposed algorithm by the BASL is freely available at www.antwerpnafldguide.com.[50]

Until early non-invasive diagnostics have been identified, the flowcharts provided by the EASL and BASL guideline could be used in primary care to help route patients to the right healthcare professional. In the Netherlands, flowcharts like these are not yet part of the Dutch Guidelines for GPs (NHG-standaarden) and therefore not yet used in primary care practice. With the ever-increasing prevalence of NAFLD, more emphasis should be put on using these flowcharts and incorporate them in the Dutch GP guidelines.

With regards to management of NAFLD in primary care, lifestyle interventions with diet and exercise are the key first step for all patients.[24,39] Furthermore, NAFLD management in primary care should include the treatment of metabolic comorbidities, such as dyslipidaemia and elevated glucose levels, to reduce cardiovascular risk, which can also prevent the future development of NASH and fibrosis.[39] Enhancing patient participation and adherence to lifestyle intervention and medication is important and might be improved by patients being aware of a more progressive state of their disease. Moreover, gathering data on the NAFLD population in the primary care will increase the insight in the

entire NAFLD population and risk factors, including the group that does not need referral to secondary care. This can help direct specific interventions to the right patients, being high versus low risk on progressive disease.

NAFLD: Is it all in the name?

The increasing knowledge, the heterogeneity of the population with NAFLD and pathogenic drivers not being identical among patients[20] has led to a discussion whether the term NAFLD is still up-to-date and truly reflects the current understanding of this disease.

NAFLD used to be described as a condition of exclusion, meaning it exists only when other conditions, such as viral hepatitis, autoimmune disease or excessive alcohol intake are absent. The use of this definition has resulted in a one-size-fits-all approach in treating this disease, despite the heterogeneity seen in people affected by the disease. With advancements in our understanding of the pathological processes involved, it is now considered a disease that must be defined by inclusion, rather than by exclusion. Furthermore, given its high prevalence, fatty liver disease is recognized to coexist with other conditions such as viral hepatitis, autoimmune disease and alcohol and will have synergistic effects on disease progression. Furthermore, linking NAFLD, a distinct entity, to alcohol in its name is problematic. Therefore, a new name has been suggested, namely 'MAFLD' or metabolic associated fatty liver disease.[51-53] MAFLD is present if hepatic steatosis is accompanied by either obesity or overweight (BMI >25 kg/m^2 in white and >23 kg/m^2 in Asian individuals), type 2 diabetes mellitus or evidence of metabolic dysregulation. At least two metabolic risk factors should be present for definition of metabolic dysregulation: waist circumference ≥102/88 cm in white men and women or ≥90/80 cm in Asian men and women; prediabetes; inflammation with elevated high-sensitive serum C-reactive protein level; elevated blood pressure or specific drug treatment; decreased HDL-cholesterol levels; increased plasma triglycerides levels; and homeostasis model assessment (HOMA)-insulin resistance score ≥2.5.[51,53]

Societal impact and future perspectives

NAFLD is an intensive research field given its public health implications. The knowledge on NAFLD is rapidly expanding. It is very likely that certain paradigms about the disease will be modified in the next decade. NAFLD shares common pathogenic features with cardiovascular disease and type 2 diabetes mellitus; disorders which are all related to the Western lifestyle, characterised

by excess caloric intake and a sedentary lifestyle. Changing this lifestyle will demand both a broader societal change in our way of living as well as personalised education/counselling for individuals who are at increased risk of health problems due to this lifestyle.

In complicated, progressive liver disease, liver transplantation is the only treatment left. Not only will there be an increased need for liver transplantations in the near future, with the rapidly growing prevalence of NAFLD, also the pool of potential liver donors is impacted, as it is expected that a higher proportion of potential liver donors will have steatosis or steatohepatitis and might be declined for liver transplantation use.[54] This will not only affect those in need of a liver transplantation due to NASH, but also patients suffering from liver diseases due to other aetiologies. Assuming the actual trends in the prevalence of diabetes and obesity, it has been estimated that the overall liver graft utilization in 2030 will fall from 78% to 44% .[55] Not only will NAFLD shrink the pool of potential liver donors if our lifestyle does not change, but patients develop recurrent NAFLD after LT, which also puts an extra strain on the system.

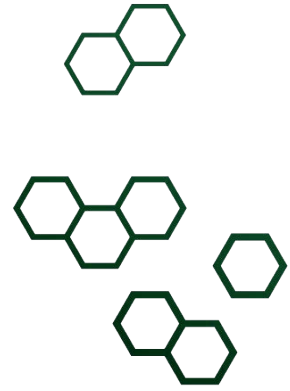
To reduce the burden of NAFLD, or should we say MAFLD, and related diseases, a broad effort on social, economic, cultural and medical level is required to solve the origin of these problems: "globesity".

References

1. Day CP, James OF. Steatohepatitis: a tale of two "hits"? Gastroenterology 1998; 114:842-845.
2. Tilg H, Moschen AR. Evolution of inflammation in nonalcoholic fatty liver disease: the multiple parallel hits hypothesis. Hepatology 2010;52:1836-1846.
3. Bessone F, Razori MV, Roma MG. Molecular pathways of nonalcoholic fatty liver disease development and progression. Cell Mol Life Sci 2019;76:99-128.
4. Yilmaz Y. Review article: is non-alcoholic fatty liver disease a spectrum, or are steatosis and non-alcoholic steatohepatitis distinct conditions? Aliment Pharmacol Ther 2012;36:815-823.
5. Neuschwander-Tetri BA. Hepatic lipotoxicity and the pathogenesis of nonalcoholic steatohepatitis: the central role of nontriglyceride fatty acid metabolites. Hepatology 2010;52:774-788.
6. Hammoutene A, Rautou PE. Role of liver sinusoidal endothelial cells in non-alcoholic fatty liver disease. J Hepatol 2019;70:1278-1291.
7. Herrnberger L, Hennig R, Kremer W, Hellerbrand C, Goepferich A, Kalbitzer HR, et al. Formation of fenestrae in murine liver sinusoids depends on plasmalemma vesicle-associated protein and is required for lipoprotein passage. PLoS One 2014;9:e115005.
8. Fraser R, Cogger VC, Dobbs B, Jamieson H, Warren A, Hilmer SN, et al. The liver sieve and atherosclerosis. Pathology 2012;44:181-186.
9. Evstatiev R, Gasche C. Iron sensing and signalling. Gut 2012;61:933-952.
10. Hevi S, Chuck SL. Ferritins can regulate the secretion of apolipoprotein B. J Biol Chem 2003;278:31924-31929.
11. Gehrke N, Schattenberg JM. Metabolic Inflammation-A Role for Hepatic Inflammatory Pathways as Drivers of Comorbidities in Nonalcoholic Fatty Liver Disease? Gastroenterology 2020;158:1929-1947 e1926.
12. Tariq R, Axley P, Singal AK. Extra-Hepatic Manifestations of Nonalcoholic Fatty Liver Disease: A Review. J Clin Exp Hepatol 2020;10:81-87.
13. Bosma M, Kersten S, Hesselink MK, Schrauwen P. Re-evaluating lipotoxic triggers in skeletal muscle: relating intramyocellular lipid metabolism to insulin sensitivity. Prog Lipid Res 2012; 51:36-49.
14. De Bandt JP, Jegatheesan P, Tennoune-El-Hafaia N. Muscle Loss in Chronic Liver Diseases: The Example of Nonalcoholic Liver Disease. Nutrients 2018;10.
15. De Munck TJI, Verhaegh P, Lodewick T, Bakers F, Jonkers D, Masclee AAM, et al. Myosteatosis in nonalcoholic fatty liver disease: An exploratory study. Clin Res Hepatol Gastroenterol 2020.
16. Kitajima Y, Eguchi Y, Ishibashi E, Nakashita S, Aoki S, Toda S, et al. Age-related fat deposition in multifidus muscle could be a marker for nonalcoholic fatty liver disease. J Gastroenterol 2010;45: 218-224.
17. Kitajima Y, Hyogo H, Sumida Y, Eguchi Y, Ono N, Kuwashiro T, et al. Severity of non-alcoholic steatohepatitis is associated with substitution of adipose tissue in skeletal muscle. J Gastroenterol Hepatol 2013;28:1507-1514.
18. Machado MV, Ferreira DM, Castro RE, Silvestre AR, Evangelista T, Coutinho J, et al. Liver and muscle in morbid obesity: the interplay of fatty liver and insulin resistance. PLoS One 2012;7:e31738.
19. Anstee QM, Goldin RD. Mouse models in non-alcoholic fatty liver disease and steatohepatitis research. Int J Exp Pathol 2006;87:1-16.
20. Friedman SL, Neuschwander-Tetri BA, Rinella M, Sanyal AJ. Mechanisms of NAFLD development and therapeutic strategies. Nat Med 2018;24:908-922.
21. Castro RE, Diehl AM. Mistakes in mouse models of nonalcoholic steatophepatitis and how to avoid them. In; 2018.
22. Denk H, Abuja PM, Zatloukal K. Animal models of NAFLD from the pathologist's point of view. Biochim Biophys Acta Mol Basis Dis 2019;1865:929-942.

23. Goossens N, Jornayvaz FR. Translational Aspects of Diet and Non-Alcoholic Fatty Liver Disease. Nutrients 2017;9.
24. European Association for the Study of the L, European Association for the Study of D, European Association for the Study of O. EASL-EASD-EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease. J Hepatol 2016;64:1388-1402.
25. Elamin E, Masclee A, Troost F, Pieters HJ, Keszthelyi D, Aleksa K, et al. Ethanol impairs intestinal barrier function in humans through mitogen activated protein kinase signaling: a combined in vivo and in vitro approach. PLoS One 2014;9:e107421.
26. Miele L, Valenza V, La Torre G, Montalto M, Cammarota G, Ricci R, et al. Increased intestinal permeability and tight junction alterations in nonalcoholic fatty liver disease. Hepatology 2009;49:1877-1887.
27. Houghton D, Zalewski P, Hallsworth K, Cassidy S, Thoma C, Avery L, et al. The degree of hepatic steatosis associates with impaired cardiac and autonomic function. J Hepatol 2019;70:1203-1213.
28. Ajmera VH, Terrault NA, Harrison SA. Is moderate alcohol use in nonalcoholic fatty liver disease good or bad? A critical review. Hepatology 2017;65:2090-2099.
29. Weng G, Dunn W. Effect of alcohol consumption on nonalcoholic fatty liver disease. Transl Gastroenterol Hepatol 2019;4:70.
30. Mitchell T, Jeffrey GP, de Boer B, MacQuillan G, Garas G, Ching H, et al. Type and Pattern of Alcohol Consumption is Associated With Liver Fibrosis in Patients With Non-alcoholic Fatty Liver Disease. Am J Gastroenterol 2018;113:1484-1493.
31. Sookoian S, Castano GO, Pirola CJ. Modest alcohol consumption decreases the risk of non-alcoholic fatty liver disease: a meta-analysis of 43 175 individuals. Gut 2014;63:530-532.
32. Ajmera V, Belt P, Wilson LA, Gill RM, Loomba R, Kleiner DE, et al. Among Patients With Nonalcoholic Fatty Liver Disease, Modest Alcohol Use Is Associated With Less Improvement in Histologic Steatosis and Steatohepatitis. Clin Gastroenterol Hepatol 2018;16:1511-1520 e1515.
33. Sinn DH, Gwak GY, Cho J, Son HJ, Paik YH, Choi MS, et al. Modest alcohol consumption and carotid plaques or carotid artery stenosis in men with non-alcoholic fatty liver disease. Atherosclerosis 2014;234:270-275.
34. Dunn W, Xu R, Schwimmer JB. Modest wine drinking and decreased prevalence of suspected nonalcoholic fatty liver disease. Hepatology 2008;47:1947-1954.
35. Rockey DC, Caldwell SH, Goodman ZD, Nelson RC, Smith AD, American Association for the Study of Liver D. Liver biopsy. Hepatology 2009;49:1017-1044.
36. Verdam FJ, Dallinga JW, Driessen A, de Jonge C, Moonen EJ, van Berkel JB, et al. Non-alcoholic steatohepatitis: a non-invasive diagnosis by analysis of exhaled breath. J Hepatol 2013;58:543-548.
37. Angulo P, Kleiner DE, Dam-Larsen S, Adams LA, Bjornsson ES, Charatcharoenwitthaya P, et al. Liver Fibrosis, but No Other Histologic Features, Is Associated With Long-term Outcomes of Patients With Nonalcoholic Fatty Liver Disease. Gastroenterology 2015;149:389-397 e310.
38. Ekstedt M, Hagstrom H, Nasr P, Fredrikson M, Stal P, Kechagias S, et al. Fibrosis stage is the strongest predictor for disease-specific mortality in NAFLD after up to 33 years of follow-up. Hepatology 2015;61:1547-1554.
39. Tsochatzis EA, Newsome PN. Non-alcoholic fatty liver disease and the interface between primary and secondary care. Lancet Gastroenterol Hepatol 2018;3:509-517.
40. Adams LA, Anstee QM, Tilg H, Targher G. Non-alcoholic fatty liver disease and its relationship with cardiovascular disease and other extrahepatic diseases. Gut 2017;66:1138-1153.
41. VanWagner LB, Rinella ME. Extrahepatic Manifestations of Nonalcoholic Fatty Liver Disease. Curr Hepatol Rep 2016;15:75-85.
42. Becker PP, Rau M, Schmitt J, Malsch C, Hammer C, Bantel H, et al. Performance of Serum microRNAs -122, -192 and -21 as Biomarkers in Patients with Non-Alcoholic Steatohepatitis. PLoS One

2015;10:e0142661.
43. Younossi ZM, Baranova A, Stepanova M, Page S, Calvert VS, Afendy A, et al. Phosphoproteomic biomarkers predicting histologic nonalcoholic steatohepatitis and fibrosis. J Proteome Res 2010;9:3218-3224.
44. Verhaegh P, Bavalia R, Winkens B, Masclee A, Jonkers D, Koek G. Noninvasive Tests Do Not Accurately Differentiate Nonalcoholic Steatohepatitis From Simple Steatosis: A Systematic Review and Meta-analysis. Clin Gastroenterol Hepatol 2018;16:837-861.
45. Selection bias. In: Catalogue of Bias. https://catalogofbias.org/biases/selection-bias/
46. Verification bias. In: Catalogue of Bias. https://catalogofbias.org/biases/verification-bias/
47. van Asten M, Verhaegh P, Koek G, Verbeek J. The increasing burden of NAFLD fibrosis in the general population: Time to bridge the gap between hepatologists and primary care. Hepatology 2017;65:1078.
48. Patel PJ, Banh X, Horsfall LU, Hayward KL, Hossain F, Johnson T, et al. Underappreciation of non-alcoholic fatty liver disease by primary care clinicians: limited awareness of surrogate markers of fibrosis. Intern Med J 2018;48:144-151.
49. Wieland AC, Quallick M, Truesdale A, Mettler P, Bambha KM. Identifying practice gaps to optimize medical care for patients with nonalcoholic fatty liver disease. Dig Dis Sci 2013;58:2809-2816.
50. Francque S, Lanthier N, Verbeke L, Reynaert H, Van Steenkiste C, Vonghia L, et al. The Belgian Association for Study of the Liver Guidance Document on the Management of Adult and Paediatric Non-Alcoholic Fatty Liver Disease. Acta Gastroenterol Belg 2018;81:55-81.
51. Eslam M, Newsome PN, Sarin SK, Anstee QM, Targher G, Romero-Gomez M, et al. A new definition for metabolic dysfunction-associated fatty liver disease: An international expert consensus statement. J Hepatol 2020;73:202-209.
52. Eslam M, Sanyal AJ, George J, International Consensus P. MAFLD: A Consensus-Driven Proposed Nomenclature for Metabolic Associated Fatty Liver Disease. Gastroenterology 2020;158:1999-2014 e1991.
53. Tilg H, Effenberger M. From NAFLD to MAFLD: when pathophysiology succeeds. Nat Rev Gastroenterol Hepatol 2020;17:387-388.
54. Pais R, Barritt ASt, Calmus Y, Scatton O, Runge T, Lebray P, et al. NAFLD and liver transplantation: Current burden and expected challenges. J Hepatol 2016;65:1245-1257.
55. Orman ES, Mayorga ME, Wheeler SB, Townsley RM, Toro-Diaz HH, Hayashi PH, et al. Declining liver graft quality threatens the future of liver transplantation in the United States. Liver Transpl 2015;21:1040-1050.



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This thesis focussed on the highly prevalent non-alcoholic fatty liver disease (NAFLD). NAFLD is a lifestyle related disease, associated with overnutrition and a sedentary lifestyle. This lifestyle is leading to an escalating global epidemic of overweight and obesity, which leads not only to NAFLD, but also to other serious non-communicable diseases, including diabetes mellitus, cardiovascular disease, hypertension and stroke and specific types of cancer.[1] These diseases undermine health, shorten life expectancy and cause enormous suffering, disability and economic costs.[2]

Currently, the global prevalence of NAFLD of more than 25%. The number of patients diagnosed and referred with NAFLD is expected to rise further in the near future. The term NAFLD comprises both simple steatosis and non-alcoholic steatohepatitis (NASH). Simple steatosis is a more benign form of fat accumulation in the liver, present in the majority of patients. NASH is a chronic inflammatory state that develops in about 30% of NAFLD patients and leads to an overall increase in morbidity and mortality due to the progression to fibrosis, cirrhosis and hepatocellular carcinoma (HCC). The complex pathophysiology of NAFLD is multifactorial and not yet completely clear. Furthermore, at this moment there are limited therapeutic options available for NAFLD. Lifestyle changes, including diet and physical exercise are considered the mainstay treatment for the majority of patients with NAFLD.[3,4] However, it is common knowledge that sustained weight loss is difficult to achieve and especially difficult to maintain. Therefore, other treatment options like pharmacotherapy also need to be considered as an add-on therapy. Up to now no drug has been approved for NASH by regulatory agencies, and any drug treatment is still off-label.

In case lifestyle changes and other interventions failed and end-stage liver disease has developed, a liver transplantation can be considered. It is thought that NAFLD will probably become the leading indication for liver transplantation due to the still rising prevalence of NAFLD.[5] Additionally, the rapidly growing prevalence of NAFLD will also impact the pool of potential donors, as it is expected that a higher proportion of potential liver donors will have NAFLD and might be declined for liver transplantation use. Not only the fact that NAFLD can shrink the pool of potential liver donors, but also patients developing recurrent NAFLD after liver transplantation is putting a strain on the system.

In order to counter these alarming trends, a better knowledge and understanding of factors associated with progressive disease in NAFLD is needed. These numbers underline the importance of properly routing NAFLD patients to the right healthcare professional, meaning referral of patients at risk of progressive liver disease to secondary care and follow-up of low-risk NAFLD patients in primary care, to lower both the economic and disease burden of this disease. In this thesis, we aimed to gain more insight the pathophysiology, non-invasive diagnostic options and the bridge to primary care, to improve healthcare.
Several pathophysiological factors were studied in this thesis, namely defenestration of liver sinusoidal endothelial cells, the role of ferritin and myosteatosis. Even though, interesting results were found, they do not explain the entire pathophysiology of NAFLD. This has to do with the fact that pathophysiology of NAFLD is multifactorial. Due to this complex interplay, such as the involvement of several organs and inter-individual variation in disease presentation, human research on NAFLD is important. A well-characterised cohort of NAFLD patients with bio-sampling, which can be followed over a longer period of time, can help to get a better and more complete understanding of the natural disease course and factors that influence the development of progressive disease. However, this type of clinical research is very demanding, therefore it is important to supplement these kind of studies with research, both human and animals studies, focussing on specific mechanisms. In this manner, the longitudinal studies could be used to validate findings and in this way benefit scientific impact.

To date, liver biopsy is the most sensitive test for detecting and staging NAFLD. So far, it is the only reliable method to diagnose NASH.[3] Due to its invasiveness, high costs and risk of complications, liver biopsy is not a diagnostic method that is suitable to apply in large populations or for monitoring the disease. In clinical practice, only subjects at high risk of NASH, usually based on elevated liver enzymes measured in the blood, will be considered to undergo a liver biopsy. This may lead to underdiagnoses of NASH, since a significant part of NASH patients do not have elevated liver enzymes. Even though, fibrosis stages 1-4 were found to be the strongest predictor for disease-specific mortality in NAFLD, there is an urgent need for non-invasive methods to detect NASH among the general population, since NASH is a recognized driver of liver fibrosis and fibrosis progression. A non-invasive diagnostic tool for NASH could benefit both healthcare and research purposes. This thesis provided an extensive overview of

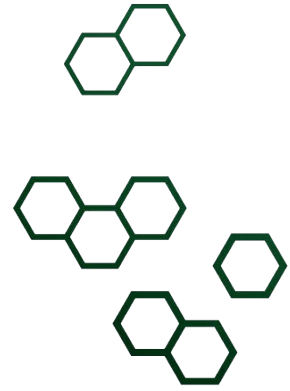
the available non-invasive diagnostics studied in recent years. We showed that several scores that combined different non-invasive markers showed promising results. However, none of the markers or combination of markers identified, was able to differentiate NASH from simple steatosis and a non-invasive marker for NASH is therefore not yet available in clinical practice. Due to the complexity of NAFLD, it is likely that a score consisting of multiple parameters may be able to diagnose NASH in the future. It would be of value to use -omics approaches to identify interesting parameters that could possibly be used for non-invasively diagnosing NASH. Omics aim at the collective characterization and quantification of pools of biological molecules that translate into the structure, function and dynamic of an organism or organisms. The goal is to find an affordable diagnostic tool that includes parameters that both easily as well as reliably diagnose NASH non-invasively. Once implemented in clinical care, it should enable diagnosing and monitoring of large groups, so the proper guidance/treatment can be given to the right patients. Finding and implementing such a tool would be of great benefit especially for general practitioners (GPs). Due to the high prevalence but low severity of NAFLD in the majority of patients, GPs, as gatekeepers of our health care system, are confronted with large numbers of NAFLD patients. However, until a non-invasive diagnostic tool is available, GPs need to be trained to identifying patients at risk of progressive liver disease for secondary care referral. Furthermore, adequate education on the follow-up and treatment of low-risk patients should be provided, taking into account cost-effectiveness. This thesis showed that there is a need for increased awareness and knowledge on NAFLD in the field of primary care. Until non-invasive diagnostics, to enable early diagnosis of NASH, are implemented in clinical care, the flowcharts provided by the EASL and BASL guideline should be used in primary care to help route patients to the right healthcare professional.[3,6]

In conclusion, this thesis proposes possible factors that play a role in the pathophysiology of NAFLD, these findings can provide foundation for future mechanistic research. Furthermore, we have provided an overview of the non-invasive diagnostics of NASH studied over the years. Even though a non-invasive diagnostic tool is not yet available in clinical healthcare, our overview has shown that combining various parameters is necessary in the development of a non-invasive diagnostic tool for NASH. This can provide leads for developing a test that can be implemented in clinical practice and can be used in future studies on the identification of risk factors for progressive disease.

Finally, this thesis has shown the importance of a collaboration between hepatologists and primary care physicians to guide patients to the right healthcare professionals, especially as the numbers of NAFLD patients are expected to rise even further the coming years.
A great effort to increase application of public health and interventions to reduce prevalence of poor diet and insufficient physical activity, increase awareness of NAFLD and promote the use of flowcharts until a non-invasive diagnostic tool is available, could substantially reduce the human and economic costs of this disease.

References

1. World Health Organisation (WHO) - Controlling the global obesity epidemic. In.
2. Eyre H, Kahn R, Robertson RM, Clark NG, Doyle C, Hong Y, et al. Preventing cancer, cardiovascular disease, and diabetes: a common agenda for the American Cancer Society, the American Diabetes Association, and the American Heart Association. Circulation 2004;109:3244-3255.
3. European Association for the Study of the L, European Association for the Study of D, European Association for the Study of O. EASL-EASD-EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease. J Hepatol 2016;64:1388-1402.
4. Rinella ME. Nonalcoholic fatty liver disease: a systematic review. JAMA 2015;313:2263-2273.
5. Samji NS, Verma R, Satapathy SK. Magnitude of Nonalcoholic Fatty Liver Disease: Western Perspective. J Clin Exp Hepatol 2019;9:497-505.
6. Francque S, Lanthier N, Verbeke L, Reynaert H, Van Steenkiste C, Vonghia L, et al. The Belgian Association for Study of the Liver Guidance Document on the Management of Adult and Paediatric Non-Alcoholic Fatty Liver Disease. Acta Gastroenterol Belg 2018;81:55-81.



Summary

Summary

Non-alcoholic fatty liver disease (NAFLD) is the most widespread liver disease in Western society; about 25% of the global population has NAFLD. The term NAFLD comprises both simple steatosis and non-alcoholic steatohepatitis (NASH). Simple steatosis is a more benign form of fat accumulation, present in the majority of patients. NASH is a chronic inflammatory state that develops in about 30% of NAFLD patients and can progress to severe liver disease, such as cirrhosis, liver failure and hepatocellular cancer. Its pathophysiology is multifactorial and not yet fully understood. Overweight and obesity are major risk factors for NAFLD, as is also true for other related serious non-communicable diseases, including diabetes mellitus and cardiovascular disease. All these diseases are linked to each other and their prevalence is expected to increase even further the coming years due to the western lifestyle, that is characterised by overeating and sedentary behaviour, leading to an obesity epidemic. In this thesis we have focussed on the pathophysiology of NAFLD and non-invasive diagnosis of NASH.

The pathophysiology of NAFLD is complex and not yet completely understood. Several theories have been proposed to explain the progression of NAFLD. The most recent concept states that simple steatosis and NASH are two independent conditions caused by insulin resistance. In **chapter 2**, we studied the ultrastructural differences between NAFLD patients with and without NASH to find leads that might fill the gaps in the pathophysiology of NAFLD. Liver biopsy samples of 37 NAFLD patients were studied using light microscopy and transmission electron microscopy (TEM). TEM enables visualisation of the smallest details within liver cells, with a zoom mode of 1.000-1.500.000 times. Using preservation techniques recently developed by our department, we were able to study the fenestrae in liver sinusoidal endothelial cells. These are very small openings in the cells lining the smallest blood vessels in the liver, that serve as a sieve. We showed that patients without NASH had an increased loss of fenestration (*i.e.*, defenestration) compared to NASH patients. This study provides additional new insights in pathophysiology of NAFLD: one could speculate that defenestration might be a protective mechanisms seen in simple steatosis, which is lacking in NASH.

In **chapter 3** and **4,** we focussed on the role of serum ferritin in NAFLD. In **chapter 3**, we performed an extensive literature search to investigate whether

hyperferritinaemia in NAFLD is an expression of iron overload or inflammation. These data show that in the majority of cases hyperferritinaemia in NAFLD seems to be related to inflammation. However, a limitation of the studies included in this review was the heterogeneity with regards to objectives, study population and methods used. Therefore, we studied the relation between serum ferritin, NAFLD severity and iron in a group of biopsy proven NAFLD patient, in collaboration with the Universitair Ziekenhuis Antwerpen (UZA) (**chapter 4**). It was shown that serum ferritin was associated with having NASH with significant fibrosis (F2-4) and p.C282Y or p.H63D heterozygosity, in the presence and absence of hepatocellular iron.

Not only the liver is affected in NAFLD, extrahepatic organs can also be affected. In **chapter 5**, we studied fat accumulation in the skeletal muscle (myosteatosis) in relation to hepatic fat accumulation and the presence of fibrosis in a well-characterised cohort of NAFLD patients. Chemical shift Magnetic Resonance Imaging (MRI) was used to measure the muscle fat fraction (MFF). In addition, the hepatic fat fraction (using MRI), liver stiffness (using FibroScan®) and appendicular skeletal muscle mass (using Dual-energy X-ray absorptiometry) were measured. We found that myosteatosis did not correlate with the degree of hepatic steatosis or fibrosis, but it was positively correlated with total body fat percentage and body mass index (BMI). Insulin resistance (measured as HOMA-IR) was correlated with hepatic steatosis and fibrosis. These findings suggest that fat accumulation in the muscle does not correspond with hepatic disease severity.

To date, liver biopsy is the most sensitive test for detecting and staging steatosis, inflammation and fibrosis in NAFLD. Steatosis and fibrosis can also be reliably diagnosed using non-invasive techniques. However, liver biopsy is the only reliable method to diagnose NASH. Its invasiveness and risk of complications make it unsuitable for diagnosing large populations or for monitoring the disease. To investigate the reliability of non-invasive markers for NASH, we performed a systematic literature search and meta-analyses on available non-invasive diagnostic blood markers and scores for NASH (**chapter 6**). An extensive overview of all available markers/scores was provided with calculated pooled sensitivity and specificity. A reliable non-invasive marker or score to differentiate NASH from simple steatosis was not identified. Due to the multifactorial pathophysiology of NASH, it is unlikely that a single non-invasive marker will be able to diagnose NASH. Several scoring systems and – omics

approaches that combined markers that are specific for different pathophysiological processes in NASH, such as inflammation, lipid metabolism, fibrogenesis, showed promising findings (being a sensitivity and specificity >80%). However, for the time being, a non-invasive marker for NASH is not yet available in clinical practice.

NAFLD is a prevalent liver disease, which numbers will only continue to grow the coming years. Depending on the study population, the prevalence of NASH within a NAFLD population can vary. Within bariatric studies the prevalence of NASH has been shown to vary largely. In **chapter 7**, we studied a cohort of patients undergoing bariatric surgery showing zero patients had NASH. We suggest the preoperative crash diet (low-caloric diet) might be the cause of this. Preoperative diet interventions are common but not obligatory, therefore, this diet might at least partly explain the differences between prevalence of NASH in bariatric studies. We advocate proper mentioning of preoperative diet interventions in bariatric studies.

General practitioners (GPs), as gatekeepers of our health care system, are going to play a major role in the challenge to adequately identify and manage NAFLD patients at population level. Due to the high prevalence and overall low to moderate severity of NAFLD, GPs will encounter most of these patients and become actively involved in the screening and management of patients. In **chapter 8**, we describe the results of a survey conducted amongst GPs, to investigate the knowledge and practices concerning NAFLD in the Dutch GP practice. A total of 64 GPs completed the questionnaire. The acronyms NAFLD and NASH were not known by 34% and 53% of the GPs, respectively. Although 63% of the GPs acknowledged NAFLD as an important health problem, most GPs never (33%) or rarely (63%) screened for its presence. The results indicate the need for increased awareness and knowledge on NAFLD in primary care, which was endorsed by 84% of the GPs. Bridging the gap between hepatologists and primary care is necessary to improve detecting and managing NAFLD patients.

Finally, in **chapter 9**, we provide an overview of the findings of this thesis, discussing new insights and future perspectives, focussing on pathophysiology, non-invasive diagnosis and bridging the gap to primary care.

Samenvatting

Samenvatting

Niet-alcoholische vetleverziekte (NAFLD) is de meest voorkomende leverziekte in westerse landen. Wereldwijd komt NAFLD bij ongeveer 25% van de bevolking voor. De term NAFLD omvat zowel leververvetting (steatose) als leverontsteking (NASH; niet-alcoholische steatohepatitis). Bij leververvetting is er sprake van vetophoping in de levercellen. Als leververvetting aanhoudt kan dit leiden tot leverontsteking. Leverontsteking ontstaat bij ongeveer 30% van de patiënten met leververvetting en kan op den duur levercirrose en leverkanker tot gevolg hebben. Bij levercirrose is er ernstige schade aan de lever die ertoe kan leiden dat de lever op den duur minder goed functioneert. NAFLD is het gevolg van een ongezonde leefstijl, die gekenmerkt wordt door een calorierijke voeding en weinig lichaamsbeweging. Overgewicht en obesitas zijn dan ook belangrijke risicofactoren voor het ontwikkelen van leververvetting. De precieze manier waarop leverontsteking ofwel NASH, ontstaat in patiënten met leververvetting is echter nog niet helemaal bekend. In dit proefschrift worden drie onderwerpen met betrekking tot NAFLD besproken, namelijk i) factoren die mogelijk een rol spelen bij het ontstaan van leverontsteking, ii) het vaststellen van leverontsteking op een niet belastende manier en iii) de rol van de huisarts in het diagnosticeren van NAFLD.

Het ontstaan van leververvetting en NASH is complex en nog niet volledig duidelijk. Verschillende theorieën zijn beschreven om het ontstaan van NASH te verklaren. De meest recente theorie stelt dat leververvetting en NASH twee onafhankelijke aandoeningen zijn, die veroorzaakt worden door insuline resistentie. In **hoofdstuk 2** hebben we de endotheelcellen van de lever bestudeert met behulp van een elektronenmicroscoop. Deze endotheelcellen bevatten kleine openingen ofwel fenestrae genaamd. Via deze fenestrae kan de lever stoffen opnemen en uitscheiden (als een soort zeef). Onderzoek van leverbiopten van patiënten met leververvetting en NASH met een elektronenmicroscoop, heeft aangetoond dat deze fenestrae vrijwel geheel afwezig zijn bij patiënten met alleen leververvetting. Dit duidt mogelijk op een beschermingsmechanisme van de lever tegen overtollige vetten en andere schadelijke stoffen, dat ontbreekt bij patiënten die NASH ontwikkelen.

In **hoofdstuk 3** en **4**, wordt de rol van ferritine in NAFLD onderzocht. Ferritine is een eiwit dat betrokken is bij de opslag van ijzer in de lichaamscellen, en komt in een kleine hoeveelheid voor in het bloed. Ferritine waardes kunnen niet alleen

verhoogd zijn door een teveel aan ijzer in het lichaam, ontsteking kan ook tot verhoogde ferritine waardes leiden. Een deel van de patiënten met NAFLD heeft een verhoogde ferritine waarde (hyperferritinemie). **Hoofdstuk 3** beschrijft een literatuuronderzoek naar de oorzaak van hyperferritinemie in NAFLD patiënten. Hieruit bleek dat hyperferritinemie in NAFLD patiënten, in de meerderheid van de gevallen, een uiting is van ontsteking. Het literatuuronderzoek had echter beperkingen, waaronder de variatie tussen de verschillende onderzoeken met betrekking tot gebruikte meetmethodes en studiepopulatie. Om deze reden hebben we de relatie tussen ferritine, de ernst van de NAFLD en ijzerstapeling in leverbiopten bestudeerd in een grote groep NAFLD patiënten in samenwerking met het Universitair Ziekenhuis Antwerpen (UZA) (**hoofdstuk 4**). Dit onderzoek toonde dat verhoogde ferritine waardes geassocieerd waren met het hebben van leverontsteking met verlittekening (fibrose) van de lever en met het hebben van een mutatie in de genen (p.C282Y en p.H63D heterozygotie) die een rol spelen bij erfelijke ijzerstapelingsziekten (hereditaire hemochromatose). De diagnostische nauwkeurigheid van ferritine is echter onvoldoende om als marker voor NASH met fibrose te kunnen dienen.

Niet alleen de lever is aangedaan bij NAFLD, ook andere organen dan de lever worden beïnvloed. In **hoofdstuk 5** werd de relatie tussen vetophoping in de spieren (myosteatose) en leversteatose en -fibrose onderzocht in een cohort van goed gekarakteriseerde NAFLD patiënten. Insuline resistentie speelt een belangrijke rol in de ontwikkeling van leververvetting en wordt gelinkt aan ophoping van vet in de spieren (myosteatose). Myosteatose werd gemeten middels een MRI scan. Leversteatose, -fibrose en skeletspiermassa werden gemeten met respectievelijk een MRI scan, FibroScan® en DEXA-scan. Wij vonden geen relatie tussen myosteatose en de mate van steatose of fibrose van de lever. Myosteatose was wel geassocieerd met het percentage totaal lichaamsvet en BMI (body mass index). Insuline resistentie (gemeten middels HOMA-IR) was geassocieerd met lever steatose en fibrose.

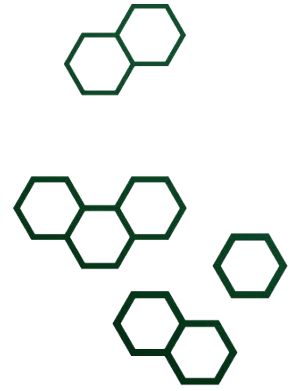
Tot op heden is het uitvoeren van een leverbiopt de meest betrouwbare manier om de mate van NAFLD vast te stellen. Leversteatose en -fibrose kunnen echter ook betrouwbaar met niet-belastende technieken vastgesteld worden, bijvoorbeeld de FibroScan®. NASH kan alleen op een betrouwbare manier worden vastgesteld met behulp van het leverbiopt. Een leverbiopt is echter een belastend (invasief) onderzoek en er bestaat een risico op complicaties. Hierdoor is het geen geschikte manier om grote groepen te onderzoeken of om patiënten

over de tijd te volgen. **Hoofdstuk 6** beschrijft een literatuuronderzoek met meta-analyse naar de betrouwbaarheid van niet-invasieve onderzoeken, met name bloedonderzoek, om NASH vast te stellen. Ondanks dat vele markers voor het vaststellen van NASH onderzocht zijn, bleek geen van deze onderzoeken in staat om NASH op een betrouwbare manier vast te stellen. Aangezien er vele verschillende factoren betrokken zijn bij het ontstaan van NASH lijkt het waarschijnlijk dat een combinatie van verschillende bloedonderzoeken wel kan leiden tot een betrouwbare diagnose van NASH. Aanvullend onderzoek is nodig om dit uit te zoeken.

NAFLD is een veelvoorkomende aandoening en gedacht wordt dat de aantallen alleen maar zullen groeien de komende jaren, aangezien overgewicht en obesitas steeds meer voorkomen. Het voorkomen van NASH in een groep van NAFLD patiënten varieert afhankelijk van de gekozen studiepopulatie. In **hoofdstuk 7** onderzoeken we het voorkomen van NASH in een groep patiënten met fors overgewicht die een operatie ondergaan om gewicht te verliezen (bariatrische ingreep). In deze groep bleek geen van de patiënten NASH te hebben. Andere onderzoeken in een gelijksoortige patiëntengroep toonden dat NASH vaker voorkwam in deze groep. Gedacht wordt dat het crash dieet dat in Nederland voorafgaand aan de bariatrische ingreep gevolgd wordt, het ontbreken van NASH in deze groep, ten minste deels, verklaard. Om deze reden is het belangrijk om preoperatieve diëten duidelijk te beschrijven in toekomstige studies.

Aangezien NAFLD vaak een goedaardig beloop heeft en slechts een deel van de patiënten NASH en de gevolgen van NASH (fibrose/cirrose en leverkanker) ontwikkelt, spelen huisartsen een belangrijke rol bij het identificeren en opvolgen van NAFLD patiënten. In **hoofdstuk 8** worden de resultaten van een vragenlijst over de kennis en werkwijze rondom NAFLD in de huisartsenpraktijk beschreven. De vragenlijsten werden ingevuld door 64 huisartsen in Zuid-Limburg. Het bleek dat de termen NAFLD en NASH onbekend waren bij respectievelijk 34% en 53% van de huisartsen. Hoewel huisartsen aangeven NAFLD een belangrijk gezondheidsprobleem te vinden, screenen de meeste huisartsen nooit (33%) of zelden (63%) naar de aanwezigheid van NAFLD. De resultaten laten zien dat er behoefte is aan meer bewustzijn en kennis rondom NAFLD in de huisartsenzorg. Dit werd onderschreven door 84% van de huisartsen. Het is nodig om het gat tussen huisartsen en hepatologen te verkleinen om de identificatie en opvolging van NAFLD patiënten te verbeteren.

Tot slot wordt in **hoofdstuk 9** een overzicht van de belangrijkste bevindingen van dit proefschrift gegeven. Daarnaast worden verschillende concepten geïntegreerd en worden toekomstperspectieven besproken.



List of publications

List of publications

Shami G, Cheng D, **Verhaegh P**, Koek G, Wisse E, Braet F. Three-dimensional ultrastructure of giant mitochondria in human non-alcoholic fatty liver disease. Sci Rep. 2021 Feb 8;11(1):3319.

Verhaegh P*, Wisse E*, de Munck T, Greve JW, Verheij J, Riedl R, Duimel H, Masclee A, Jonkers D, Koek G. Electron microscopic observations in perfusion-fixed human non-alcoholic fatty liver disease biopsies. Pathology. 2021 Feb;53(2):220-228.

Verhaegh P*, De Munck T*, Lodewick T, Bakers F, Jonkers D, Masclee A, Verbeek J, Koek G. Myosteatosis in nonalcoholic fatty liver disease: An exploratory study. Clin Res Hepatol Gastroenterol. 2020 Aug 19:S2210-7401(20)30195-9.

Verhaegh P*, Moris W*, Jonkers D, van Deursen C, Koek G. Hyperferritinemia in Nonalcoholic Fatty Liver Disease: Iron Accumulation or Inflammation? Semin Liver Dis. 2019 Nov;39(4):476-482.

De Munck T, **Verhaegh P**, Verbeek J, Verheij J, Greve JW, Jonkers D, Masclee A, Koek G. Crashing NASH in Patients Listed for Bariatric Surgery. Obes Surg. 2019 Mar;29(3):1012-1014.

Verhaegh P, Jonkers D, Koek G. Reply. Clin Gastroenterol Hepatol. 2018 Apr;16(4):596-597.

Verhaegh P, Bavalia R, Winkens B, Masclee A, Jonkers D, Koek G. Non-invasive Tests Do Not Accurately Differentiate Non-alcoholic Steatohepatitis From Simple Steatosis: A Systematic Review and Meta-analysis. Clin Gastroenterol Hepatol. 2018 Jun;16(6):837-861.

van Asten M, **Verhaegh P**, Koek G, Verbeek J. The increasing burden of NAFLD fibrosis in the general population: Time to bridge the gap between hepatologists and primary care. Hepatology. 2017 Mar;65(3):1078.

Verhaegh P, Moris W, Koek G, van Deursen C. Response to can modifier gene mutations improve the predictive value of the modified Iron Avidity Index in Type 1 Hereditary Haemochromatosis? Liver Int. 2016 Nov;36(11):1714.

Verhaegh P, Moris W, Koek G, van Deursen C. The modified iron avidity index: a promising phenotypic predictor in HFE-related haemochromatosis. Liver Int. 2016 Oct;36(10):1535-9.

Submitted

Eddie Wisse, Filip Braet, Gerry Shami, Bartlomiej Zapotoczny, Celien Vreuls, **Pauline Verhaegh**, Peter Frederik, Peter Peters, Steven Olde Damink, Ger Koek. Observations on fat and various cell types in human fatty liver disease

Toon De Munck; Markus Boesch; **Pauline Verhaegh**; Ad Masclee; Daisy Jonkers; Jos Van Pelt; Johannie du Plessis; Hannelie Korf; Frederik Nevens; Ger Koek; Schalk Van der Merwe; Jef Verbeek. Is there a role for neuregulin 4 in human nonalcoholic fatty liver disease?

Wenke Moris*, **Pauline Verhaegh***, Jef Verbeek, Dorine Swinkels, Coby Laarakkers, Ad Masclee, Ger Koek. Absorption of non-heme iron during gastric acid suppression in patients with hereditary hemochromatosis and healthy controls

Wenke Moris, **Pauline Verhaegh**, Dorine Swinkels, Coby Laarakkers, Ger Koek, Cees Van Deursen. Inflammation can increase hepcidin in HFE-hereditary hemochromatosis: a case report

To be submitted

Pauline Verhaegh, An Verrijken, Luisa Vonghia, Eveline Dirinck, Sara Diels, Wim van Hul, Ad Masclee, Daisy Jonkers, Ann Driessen, Sven Francque*, Ger Koek*. Serum ferritin is associated with non-alcoholic steatohepatitis with significant fibrosis

Toon De Munck*, **Pauline Verhaegh***, Corinne Spooren, Tobias Wienhold, Daisy Jonkers, Ad Masclee, Ger Koek, Jef Verbeek. Site-specific intestinal permeability in nonalcoholic fatty liver disease

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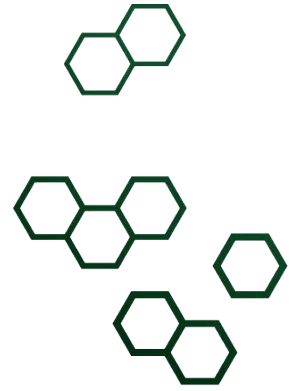
Digital ILC 2020, "young investigator full bursary" for the study: Electron microscopic observations in perfusion-fixed human non-alcoholic fatty liver disease biopsies.

Pélerin symposium 2016, Maastricht, "Nomination for the Pélerin science award" for the study: Non-invasive Tests Do Not Accurately Differentiate Non-alcoholic Steatohepatitis From Simple Steatosis: A Systematic Review and Meta-analysis.

ILC 2016, Barcelona, "registration bursary" for the study: Non-invasive Tests Do Not Accurately Differentiate Non-alcoholic Steatohepatitis From Simple Steatosis: A Systematic Review and Meta-analysis.

ILC 2015, Vienna, "young investigator full bursary" for the study: The modified Iron Avidity Index; A promising phenotypical predictor in HFE-Haemochromatosis



Dankwoord

Dankwoord

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Liefs, Pauline

Curriculum Vitae

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Pauline Verhaegh was born on April 28th 1990, in Venray, the Netherlands. After completing her pre-university education at Dendron College (Horst) in 2008, she studied Medicine at Maastricht University (Maastricht). During her bachelor she followed the course "Abdominal complaints outside Europe and North-America" at Universita degli Studi di Ferrara, Italy, under the supervision of late prof. dr. Stockbrügger. She was awarded the Top 3% award twice during the bachelor programme. She obtained her BSc degree in 2011. During her master, she did her internship ophthalmology at Kasturba Medical Collage in Manipal, India and her internship surgery at Port Augusta Hospital (University of Adelaide) in Port Augusta, Australia. She graduated with distinction for her MSc degree in 2014. After graduating, Pauline started as a PhD candidate at the department Internal Medicine, division of Gastroenterology and Hepatology within NUTRIM school of Nutrition and Translational Research in Metabolism at Maastricht University. She performed the research projects described in this thesis: "Non-alcoholic fatty liver disease: mind the gaps", supervised by prof. dr. A.A.M. Masclee, prof. dr. D.M.A.E. Jonkers, and dr. G.H. Koek. During her PhD candidacy, Pauline was a member of the NUTRIM PhD council. As of October 2017, she started her gastroenterology training at Maastricht University Medical Centre in Maastricht.