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FROM FATTY LIVER TO END-STAGE LIVER DISEASE THROUGH TYPE 2 DIABETES

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Stockholm 2021

Published by Karolinska Institutet.
Printed by Universitetsservice US-AB, 2021

ISBN 978-91-8016-240-1

From fatty liver to end-stage liver disease through type 2 diabetes
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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The thesis will be defended in public at Karolinska Institutet, Huddinge, Erna Möllersalen, 2021-06-18, 9:00 AM.

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The first principle is that you must not fool yourself, and you are the easiest person to fool.

- Richard Feynman

POPULAR SCIENCE SUMMARY OF THE THESIS

In healthy individuals, the liver only stores small amounts of fat. Alongside the surge of obesity and type 2 diabetes that have occurred over the past decades, the proportion of people who store large amounts of fat in their liver have increased dramatically. Today, up to a quarter of the global adult population have a condition called NAFLD, short for nonalcoholic fatty liver disease. The vast majority of individuals that have NAFLD do not experience any symptoms from the disease, but a subset of the patients goes on to develop inflammation and scarring in the liver tissue. While it is known that type 2 diabetes and NAFLD often co-exist, it is not clear how they interact. In its most severe form, NAFLD can lead to liver cirrhosis, impaired liver function and liver cancer. How to effectively identify the individuals with NAFLD and type 2 diabetes that are at the highest risk of developing severe liver disease is not known.

In the first part of this doctoral thesis, we investigated which microscopic features in the liver tissue that are associated with an increased risk of type 2 diabetes in patients with NAFLD. We found that patients with NAFLD diagnosed on liver biopsy, where a small tissue sample is extracted from the liver and analyzed under a microscope, have a high risk of type 2 diabetes. Higher level of scarring correlated to risk of type 2 diabetes. For patients with lower amounts of scarring, the amount of fat in the biopsy was associated with risk of type 2 diabetes.

In the second part, we used population-based registries to assess the risk of severe liver disease - such as failure of liver function or liver cancer - in patients with type 2 diabetes and found that the risk appears to be increased compared to individuals without diabetes. We also identified several risk factors in patients with type 2 diabetes that are associated with risk of severe liver disease. This can help to identify which patients with diabetes that could be considered for specific investigations of the liver to diagnose NAFLD and cirrhosis.

In the third part, we examined a group of patients with type 2 diabetes that underwent a treatment program at a specialist clinic to achieve better control of their diabetes. The treatment program was a part of routine clinical care, and we studied how this treatment affected the liver health of the patients. Using a non-invasive technique called transient elastography, we found a high frequency of NAFLD and liver scarring in the patients with type 2 diabetes. We also found that the treatment program was associated with a reduced amount of fat in the patients with NAFLD after three months.

In the fourth part, we again used population-based registries, and compared the risk of cancer in patients with NAFLD to the risk in individuals without NAFLD. We found NAFLD to be associated with a slightly increased risk of cancer in general. The strongest association was found between NAFLD and risk of the most common form of liver cancer, hepatocellular carcinoma. Patients with NAFLD had a slightly increased risk of bladder, kidney and uterine cancer. Further, male patients with NAFLD had a slightly increased risk of colorectal cancer.

ABSTRACT

The past decades have seen a marked increase in the incidence of type 2 diabetes and nonalcoholic fatty liver disease (NAFLD). Epidemiological studies have clearly demonstrated that there is an association between type 2 diabetes and NAFLD. Further, it has been established that patients with type 2 diabetes are at an increased risk of severe liver disease. While liver biopsy remains the gold standard for diagnosing NAFLD, it is not clear how - and if - the histological features of NAFLD are associated with an increased risk of type 2 diabetes. It is unclear how to identify the subset of patients with type 2 diabetes that have the highest risk of severe liver disease. Likewise, it is not known how the currently practiced treatment strategies for type 2 diabetes affect the progression of NAFLD. Finally, the risk of cancer in the broader population of patients with NAFLD is not well known.

In the first paper, we investigated the risk of type 2 diabetes in a cohort of patients with biopsy-proven NAFLD and found that higher stages of fibrosis were associated with an increased risk of type 2 diabetes. In patients with small amounts of fibrosis, the amount of steatosis on biopsy was associated with an increased risk of type 2 diabetes. These results indicate that fibrosis and simple steatosis are useful histological variables when estimating risk of type 2 diabetes in patients with NAFLD.

In the second paper, we assessed the risk of severe liver disease (defined as a composite outcome of diagnoses correlated to cirrhosis) in a population-based cohort of patients with type 2 diabetes. We observed type 2 diabetes to be associated with an increased risk of severe liver disease compared to controls free of diabetes, and identified several risk factors for severe liver disease in patients with type 2 diabetes. These results motivate further studies to better characterize the patients with type 2 diabetes that are at a high risk of severe liver disease.

In the third paper, we studied how a personalized 4-day treatment program for type 2 diabetes currently used in clinical practice at the Karolinska University Hospital in Stockholm affects the progression of NAFLD. Patients were examined with transient elastography at baseline and at a follow-up visit after three months. The prevalence of NAFLD and increased liver stiffness was high. Improved glycemic control seen after the treatment program was associated with a reduction in hepatic steatosis in patients with NAFLD at baseline. These results indicate that improving glycemic control in type 2 diabetes *per se* can also be effective in treating NAFLD.

In the fourth paper, we examined the risk of cancer in a population-based cohort of patients with NAFLD compared to matched reference individuals. We observed an association between NAFLD and a slightly increased risk of any cancer. Of the specific types of cancer we investigated, the strongest association was found between NAFLD and risk of HCC. A slightly increased risk was also observed for bladder, kidney and uterine cancer. Further, male patients with NAFLD had a slightly increased risk of colorectal cancer. These results do not motivate general screening for cancer in patients with NAFLD.

LIST OF SCIENTIFIC PAPERS

I. Karl Björkström, Per Stål, Rolf Hultcrantz, Hannes Hagström.
Histologic Scores for Fat and Fibrosis Associate With Development of Type 2 Diabetes in Patients With Nonalcoholic Fatty Liver Disease.
Clinical Gastroenterology and Hepatology 2017;15:1461–1468

II. Karl Björkström, Stefan Franzén, Björn Eliasson, Mervete Miftaraj, Soffia Gudbjörnsdottir, Ylva Trolle-Lagerros, Ann-Marie Svensson, Hannes Hagström.
Risk Factors for Severe Liver Disease in Patients With Type 2 Diabetes
Clinical Gastroenterology and Hepatology 2019;17:2769–2775

III. Karl Björkström, Per Stål, Magnus Holmer, Bonnie Bengtsson, Johan Hoffstedt, Hannes Hagström.
A personalized treatment program in persons with type 2 diabetes is associated with a reduction in liver steatosis
European Journal of Gastroenterology & Hepatology: August 10, 2020 - Published Online Ahead of Print

IV. Karl Björkström, Linnea Widman, Hannes Hagström.
Risk of hepatic and extra-hepatic cancer in NAFLD - a population-based cohort study
Manuscript

CONTENTS

LIST OF ABBREVIATIONS

ACC	acetyl-CoA-carboxylase
ALT	alanine aminotransferase
AST	aspartate aminotransferase
ALP	alkaline phosphatase
BMI	body mass index
CAP	controlled attenuation parameter
CDR	causes of death registry
CI	confidence interval
CT	computed tomography
COPD	chronic obstructive pulmonary disease
DAG	diacylglycerol
FFA	free fatty acids
FIB-4	fibrosis-4
gamma-GT	gamma-glutamyltransferase
GFR	glomerular filtration rate
GLP-1	glucagon like peptide-1
HCC	hazard ratio
HDL	high density lipoprotein
HR	hepatocellular carcinoma
ICD	international classification of diseases
IR	incidence rate
IRR	incidence rate ratio
IRS	insulin receptor substrate
kPa	kilopascal
LDL	low density lipoprotein
LSM	liver stiffness measurement
LXRα	liver receptor x alpha
MRI	magnetic resonance imaging
MRS	magnetic resonance spectroscopy
NAFLD	nonalcoholic fatty liver disease

NAS	NAFLD activity score
NASH	nonalcoholic steatohepatitis
NDR	national diabetes registry
NFS	NAFLD fibrosis score
NPR	national patient registry
NPV	negative predictive value
OR	odds ratio
PKCε	protein kinase C epsilon
PNPLA3	patatin-like phospholipase domain-containing protein 3
PPV	positive predictive value
SAF	steatosis activity fibrosis
SCR	Swedish cancer registry
SGLT2	sodium glucose co-transporter 2
sHR	sub-distribution hazard ratio
SREBP-1	sterol regulatory element-binding protein 1
TM6SF2	transmembrane 6 superfamily 2
US	ultrasonography

1 INTRODUCTION

In the recent decades, the incidence of type 2 diabetes and obesity have increased globally, with the worldwide prevalence of type 2 diabetes now estimated to around 8% (1, 2). With the development of both type 2 diabetes and obesity, various mechanisms lead to increased storage of fat in the liver, resulting in nonalcoholic fatty liver disease (NAFLD). Thus, alongside the increase in type 2 diabetes and obesity, an epidemic of NAFLD has emerged. In a meta-analysis from 2016 of 45 studies in which imaging techniques were used to diagnose NAFLD, Younossi and colleagues reported a global prevalence of 25% (3). The highest frequency was reported in two studies from South America (30%), and the lowest in two studies from Africa (13%). In Europe, Asia and North America, where a larger number of studies were available, a prevalence of 24%, 27% and 24% was reported, respectively.

The liver does not physiologically store large amounts of fat, and the diagnostic threshold for NAFLD is presence of steatosis in ≥ 5% of hepatocytes on liver biopsy (4). For the diagnosis of NAFLD to be accurate, competing causes of liver disease such as excessive alcohol intake (≥20 g per day in women, ≥30 g per day in males), intake of medications with liver steatosis as a side effect, viral hepatitis and autoimmune liver disease need to be excluded (5). Among individuals with NAFLD, a subset have inflammatory changes in the liver tissue (4). This type of inflammatory damage, steatohepatitis, is highly similar to the pathologic finding induced by alcohol-related liver disease, and it wasn't until 1980 in a study by Ludwig and colleagues that the term *nonalcoholic* steatohepatitis (NASH) was coined (6). The 20 individuals described in the study by Ludwig and colleagues had steatohepatitis in their liver tissue that occurred in the absence of excessive alcohol intake (6). A majority of the study participants, the authors noted, were obese and many of them had other pathologies related to obesity (6). As NASH is diagnosed by liver biopsy the true prevalence is somewhat difficult to approximate, but a widespread estimate is that around 20% of individuals who today have NAFLD also have NASH (7). A recent study from the United States by Harrison and colleagues reported the prevalence of NAFLD and NASH in a cohort of asymptomatic individuals referred for colonoscopy (8). In the study Harrison and colleagues, the study participants had a mean age of 56 years and a mean BMI of 30.5 kg/m^2. Interestingly, 38% of the cohort had NAFLD (diagnosed by magnetic resonance imaging (MRI) proton density fat fraction), and 14% had NASH (diagnosed by liver biopsy). Most commonly, NAFLD and NASH are seen as asymptomatic disorders as long as liver function is retained (9). However, in a study by Younossi and colleagues comparing patient reported outcomes between patients with NASH and patients with chronic hepatitis C infection, it was reported that patients with NASH had worse outcomes regarding fatigue, physical pain and vitality (10). While it is not clear that this observation was due to the NASH diagnosis per se, or due to other factors such as higher levels of obesity, the results from the study by Younossi and colleagues indicate that patients with NASH can have a markedly reduced quality of life.

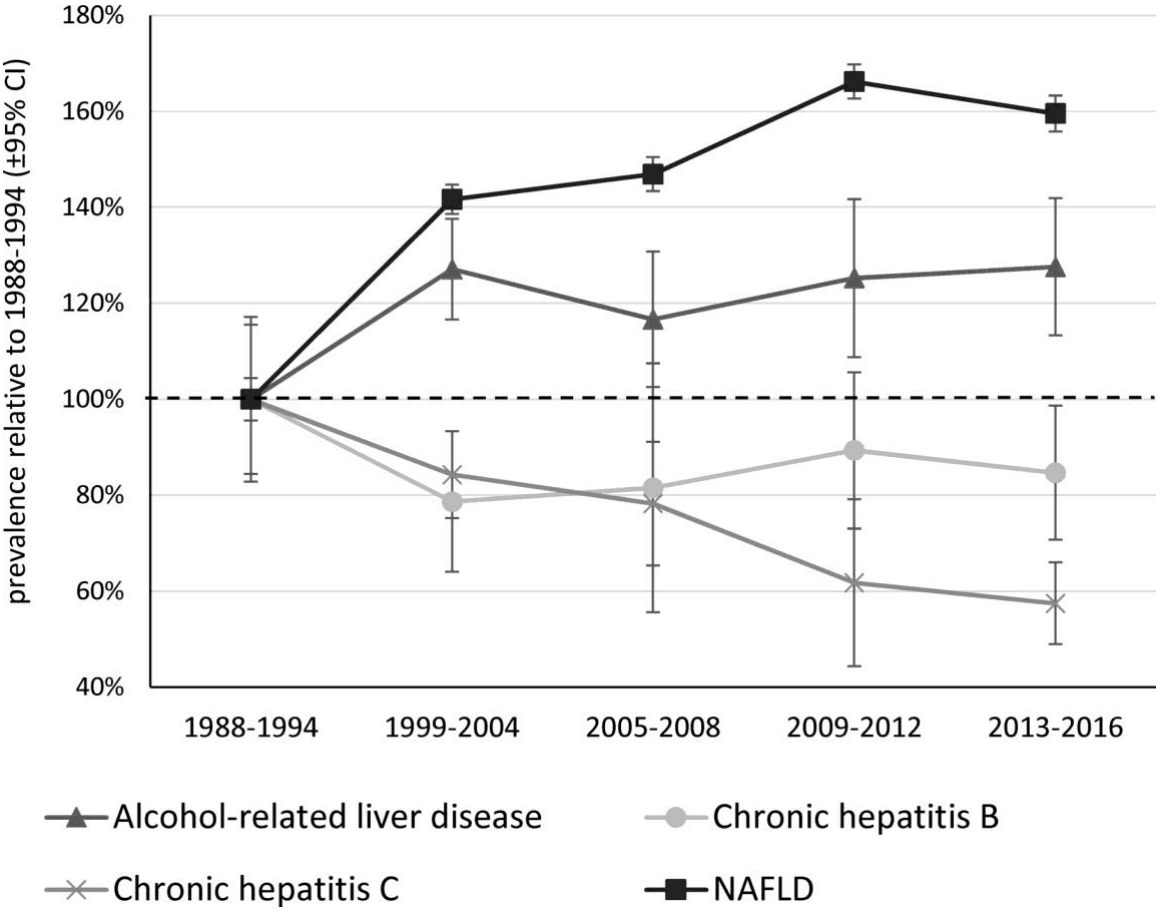

Figure 1. Changes in etiologies of liver disease in the US relative to 1988-1994. From Younossi et al. Gut 2020 Mar;69(3):564-568. Published with permission.

While liver biopsy remains the gold standard for diagnosing NAFLD, it can also be diagnosed using imaging techniques such as ultrasonography (US), MRI, magnetic resonance spectroscopy (MRS) and computed tomography (CT) (11). Liver enzymes, while commonly used in primary care settings, are however poor markers for the presence of NAFLD. In a 2015 study by Portillo-Sanchez and colleagues, 103 patients with type 2 diabetes and normal aspartate aminotransferase (AST) and alanine aminotransferase (ALT), without clinically significant chronic kidney disease or cardiovascular disease were examined with MRS to determine the prevalence of NAFLD and NASH (12). A NAFLD frequency of 50% was found, and out of the patients with NAFLD 56% had NASH, demonstrating the marked unreliability of liver enzymes for detection of NAFLD and NASH (12).

Since the introduction of the term NASH, different scoring methodologies to define presence of NASH via assessing liver biopsies have been defined. One method, primarily constructed to function as a feasible outcome parameter in clinical trials, is the NAFLD activity score (NAS) proposed in a study by the NASH clinical research network in 2005 (4). The NAS system is based on scoring on the separate histological features: steatosis (stage 0-3), lobular inflammation (stage 0-3), and ballooning (stage 0-2). In all, the maximum score is 8.

Another standardized method for diagnosing NASH, called the Steatosis Activity Fibrosis (SAF) score, was first proposed in morbidly obese patients in 2012, and validated in 2014 in patients with metabolic syndrome (13, 14). In the SAF score, liver biopsies are graded on steatosis (scale 0-3), lobular inflammation (scale 0-2) and hepatocyte ballooning (scale 0-2), and a score of at least one in all three variables is necessary for a NASH diagnosis. Additionally, on liver biopsies, fibrosis is usually graded on a scale from 0-4 (4). It was recently reported that while all-cause mortality is increased in all patients with NAFLD, a gradual increase in risk is seen from simple steatosis, to NASH, to fibrosis, to cirrhosis (where scarring of the liver has become widespread, and liver function might decrease) (15).

Transient elastography is an ultrasound based, non-invasive method where a probe is placed on the skin in the intercostal space over the liver. By generating a shear wave, the probe can produce a measurement of liver stiffness, expressed in kiloPascal (kPa). The kPa value can then be used as an indication of presence (or absence) of significant fibrosis. Further, the transient elastography can be used to estimate the controlled attenuation parameter (CAP), which provides an indication of the degree of hepatic steatosis (16).

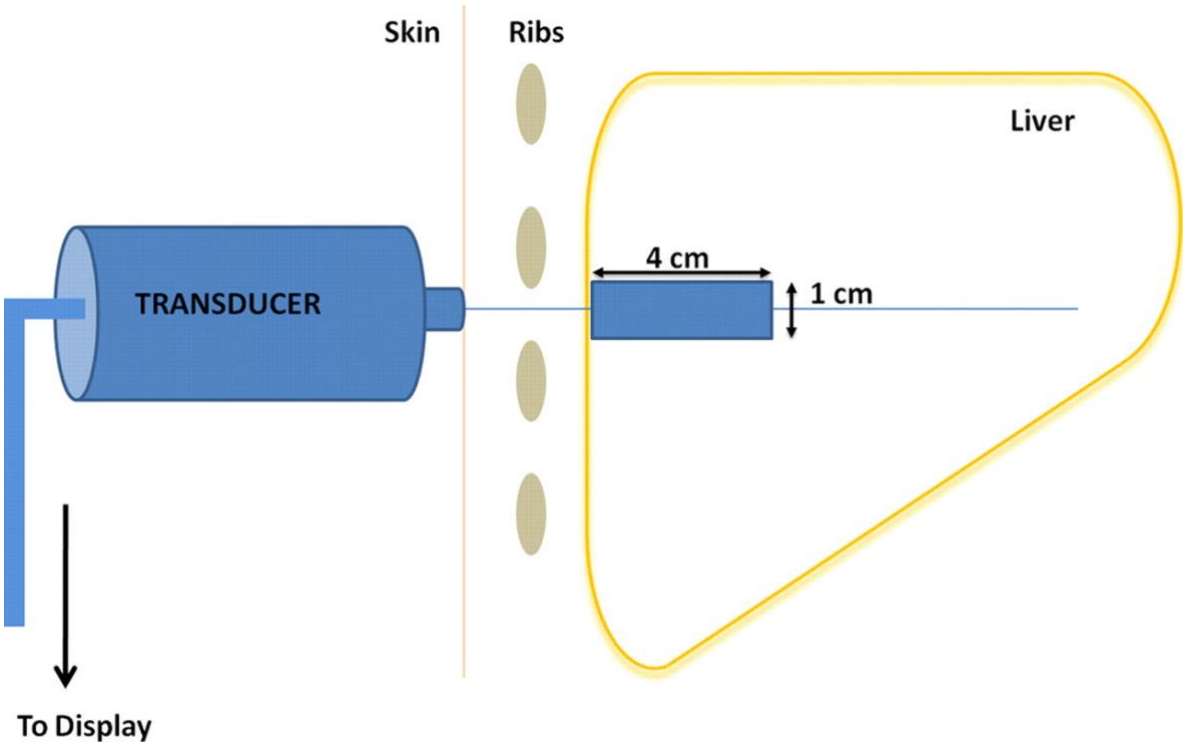

Figure 2. Schematic presentation of the transient elastography technique, where a probe is placed in the intercostal space to assess liver stiffness and steatosis. From Jaffer et al. Ultrasound. 2012;20(1):24-32. Published with permission.

Since it is not feasible to perform a liver biopsy in all patients with NAFLD, several non-invasive testing models for detecting advanced fibrosis in individuals with NAFLD have been developed (17). In general, the non-invasive models are characterized by high negative predictive value (NPV) and lower positive predictive value (PPV), hence limiting the clinical use to identifying patients eligible for referral to specialist centers and additional testing, rather than diagnosing advanced fibrosis (defined as stage 3-4 on biopsy) (17). A commonly

used non-invasive model to predict presence of advanced fibrosis is the fibrosis-4 (FIB-4) score. The FIB-4 score was initially developed to predict fibrosis in patients with co-infection of hepatitis C virus (HCV) and human immunodeficiency virus (HIV), and incorporates AST, ALT, platelets and age (18). In 2007, Angulo and colleagues proposed the NAFLD fibrosis score (NFS), which incorporates age, hyperglycemia/type 2 diabetes, body mass index (BMI), thrombocyte particle count, albumin and the ratio of AST to ALT, to non-invasively estimate the presence of advanced fibrosis (19). McPherson and colleagues published a study in 2010 where they tested the ability of different non-invasive models to predict advanced fibrosis in a cohort of 145 patients with biopsy-proven NAFLD, and found that the FIB-4 score had a NPV of 95% and a PPV of 36% at a cut-off of 1.3 (17). In the same study, NFS was found to have a NPV of 92% and a PPV of 30% at a cut-off of -1.455 (17). Due to its comparable simplicity and repeatedly reported high NPV for advanced fibrosis, the FIB-4 score has been proposed as a simple, first-stage screening tool for advanced fibrosis in primary care in recent studies (20).

The causal mechanistic network involved in the development of NAFLD and NASH is complicated and not fully understood, but in principle consists of both lifestyle factors, such as high caloric intake from refined sugars also implicated in the pathogenesis of type 2 diabetes and obesity, and genetic factors such as mutations in the patatin-like phospholipase domain-containing protein 3 gene (PNPLA3) and the transmembrane 6 superfamily 2 human gene (TM6SF2) (21-23). In a study published in 2008, Romeo and colleagues investigated the association between genetic variants and NAFLD in the Dallas Heart Study cohort (22). In 2011 individuals of varying ethnic descent they found the rs738409(G)-allele of the PNPLA3 gene to be associated with increased hepatic fat content, even after adjusting for confounders such as BMI, alcohol intake and diabetes (22). Interestingly, the rs738409(G)-allele does not appear to be associated with other features of the metabolic syndrome, such as increased BMI, elevated triglycerides, low high density lipoprotein (HDL) or presence of type 2 diabetes (24, 25). On the other hand, the rs738409(G)-allele appears associated with an increased risk of development of hepatocellular carcinoma (HCC) caused by NAFLD, especially for homozygotic carriers (26).

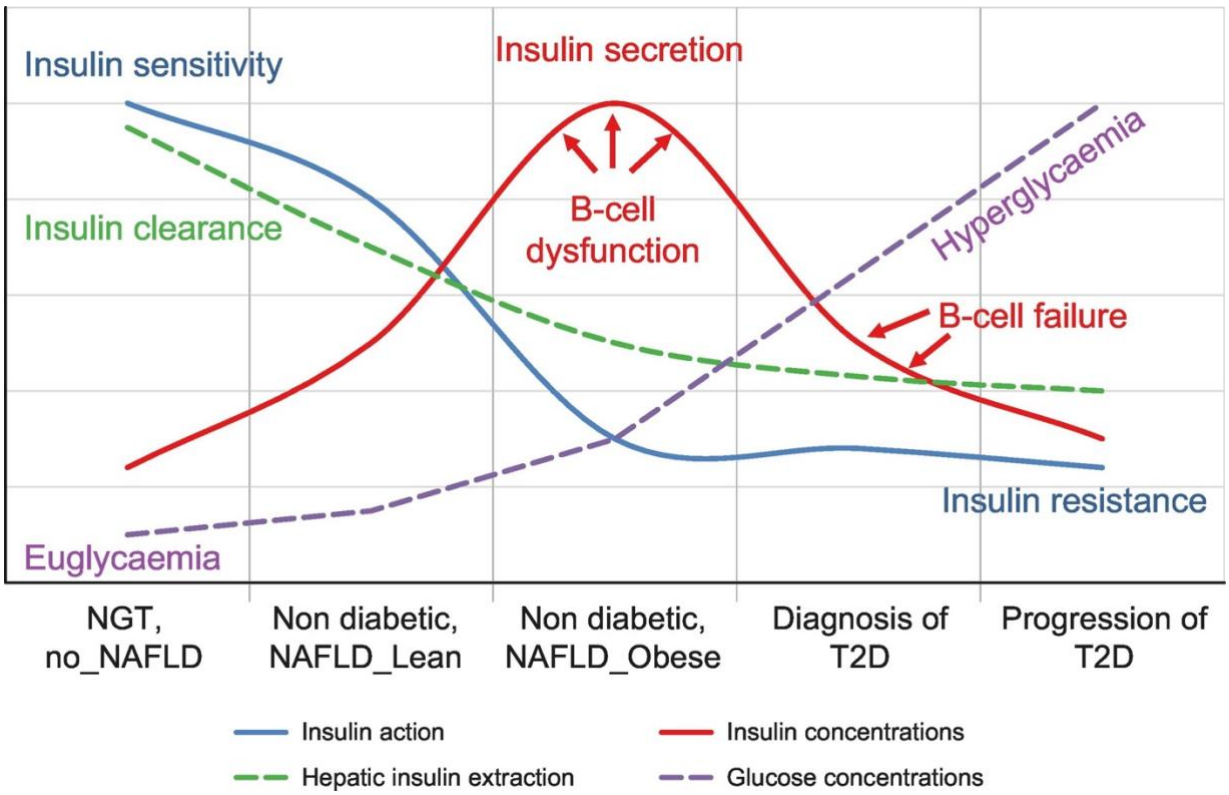

Figure 3. The progression from normal glucose tolerance and normal liver, to NAFLD and type 2 diabetes. Abbreviations: NGT=normal glucose tolerance, T2D=type 2 diabetes, B-cell=beta cell. From Gastaldelli et al. JHEP Rep 2019 Jul 19;1(4):312-328. doi: 10.1016/j.jhepr.2019.07.002 https://creativecommons.org/licenses/by-nc-nd/4.0/. Published with permission.

While a majority of patients with NAFLD are treated in primary care settings and not in specialized centers, the epidemics of NAFLD and NASH have been reported to be under-recognized among both general practitioners and endocrinologists (27-30). In a study from the UK published in 2018, 133 healthcare professionals, mainly consulting endocrinologists and resident endocrinologists specializing in diabetes, were queried on their knowledge of NAFLD (29). A vast majority of the responders underestimated the prevalence of both NAFLD and NAFLD-related fibrosis in patients with type 2 diabetes (29). Further, a majority of the responders reported not having used any non-invasive scoring system, such as the FIB-4 score, in the last year (29). In another study published in 2018, Australian researchers investigated the knowledge about NAFLD and NASH in primary health care professionals, mainly general practitioners, and found that half of them underestimated the prevalence of NAFLD in the general population to <10% (30). Additionally, a majority were uncertain about the clinical applicability of the FIB-4 score (30). In a recent study by Rashu and colleagues, the referral patterns to the Gastro Unit at the Copenhagen University hospital were studied. Of the 1 735 referred patients between January 2017 and June 2020, 323 (18.6%) ended up receiving a diagnosis of NAFLD (31). Of the patients with NAFLD, a majority (62.5%) were referred from general practitioners. Though not all patients underwent liver biopsy, significant fibrosis (stage 2-4) was found in 71 (22% of referred patients with

NAFLD) of the 110 patients that were biopsied. At referral, the FIB-4 score had not been calculated in any of the patients (31).

1.1 PATHOPHYSIOLOGICAL LINK BETWEEN TYPE 2 DIABETES AND NAFLD

The pathophysiological interplay between type 2 diabetes and NAFLD is complex and involves several biochemical pathways and feedback loops, and it has been a longstanding question how the two disorders interact (32). In a study by Bugianesi and colleagues from 2005, it was demonstrated that even non-diabetic, non-obese patients with NAFLD show signs of both hepatic and peripheral (both muscle and adipose tissue) insulin resistance compared to individuals without NAFLD (33). Likewise, it has been demonstrated that insulin resistance is an early detectable, independent predictor of impending development of type 2 diabetes (34).

It has long been established that the association of increased fat deposits in the body and the risk of metabolic syndrome is dependent on the distribution of fat throughout the body. The tendency to store fat in the upper parts of the body, and especially in and around the visceral organs, is associated with a higher risk of developing the metabolic syndrome (35, 36). It has been proposed that this observation between visceral adiposity and an increase in risk of metabolic syndrome is due to an increased delivery of free fatty acids to the liver (37). In individuals without obesity, the proportion of free fatty acids (FFA) delivered to the hepatocytes of the liver coming from visceral fat stores is around 5-10%, whereas a larger part originates from subcutaneous adipose tissue. In individuals with visceral obesity, however, the proportion of FFA originating from visceral adipose tissue is around 30% (37).

Animal models have shown that increased hepatic fat content, through diacylglycerol (DAG), can increase the activity of the enzyme protein kinase C epsilon (PKCε) (38). Once PKCε is activated it can impair insulin signaling in the hepatocyte by binding to and blocking the activity of the insulin receptor tyrosine kinase (38). Under normal physiological conditions, increased insulin signaling decrease gluconeogenesis in the liver and upregulate glycogen storage. When insulin action is inhibited, hepatocytes produce glucose in disproportionate amounts, resulting in hyperglycemia, initiating further increase in insulin production. In a 2012 study by Magkos and colleagues of 16 obese humans, out of which 13 had NAFLD, intrahepatic DAG content was shown to positively correlate with degree of insulin resistance in the liver (39).

The pathophysiological link between type 2 diabetes and NAFLD appears to be bidirectional in the sense that just as mechanisms by which increased fat content in the liver promotes insulin resistance have been described, a number of mechanisms by which elevated glucose and insulin levels over time can promote increased fat content in the liver have been proposed (40). When insulin production is increased in a person with insulin resistance, it can upregulate the activity of a transcription factor protein called sterol regulatory element-binding protein 1 (SREBP-1) (41). Interestingly, SREBP-1 is upregulated via insulin receptor substrate-1 (IRS-1), which remains augmented even in insulin resistant hepatocytes of

patients with NAFLD, as opposed to IRS-2 which instead appears downregulated (41). When SREBP-1 is upregulated, it in turn upregulates the activity of Acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS), which are enzymes increasing de novo lipogenesis (DNL) (41).

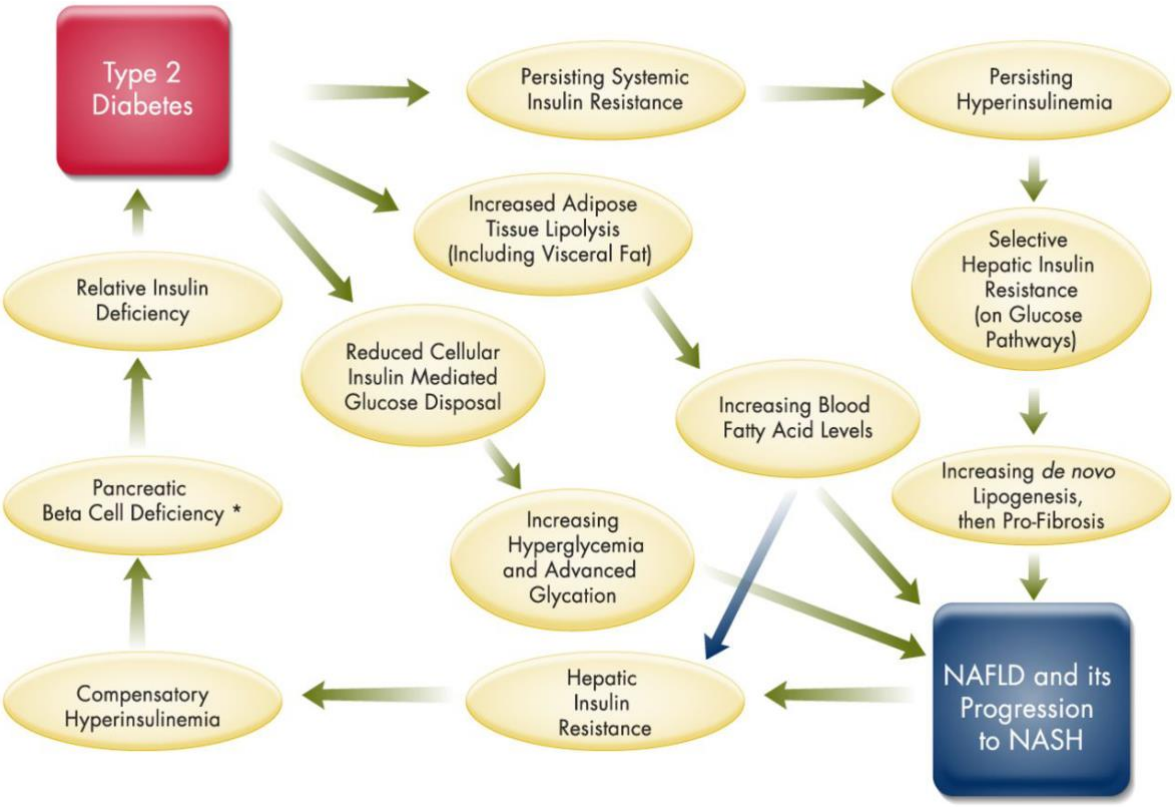

Figure 4. The pathophysiological interplay between NAFLD and type 2 diabetes. From Williams et al. Endocrine Review 2013 Feb;34(1):84-129. Published with permission.

Increased glucose levels can increase the activity of an enzyme called xylulose-5-phosphate (Xu-5-P) (42). As Xu-5-P is upregulated, this in turn promotes activity of the protein phosphatase 2A (PP2A). The increased activity of PP2A leads to promotion of a transcription factor called carbohydrate responsive element binding protein (ChREBP) (42). By upregulation of genes that code for enzymes such as liver type pyruvate kinase, ACC and FAS, ChREBP promotes DNL (42). A further mechanism through which increased glucose levels can stimulate DNL is through by increasing the activity of nuclear receptor liver X receptor alpha (LXRα) (43). When activated, the LXRα stimulates activity in SREBP-1, ACC and FAS, resulting in increased DNL (40).

1.2 RISK OF TYPE 2 DIABETES IN NAFLD

Several epidemiological studies on the risk of development of type 2 diabetes in patients with NAFLD have been performed (44-63). Due to the different methods and criteria available to diagnose NAFLD and type 2 diabetes, a direct comparison between studies is complicated. Further, the commonly asymptomatic disease progression of both NAFLD and type 2 diabetes often makes assessment of which of the two conditions that developed first difficult.

In one of the earliest studies of risk of type 2 diabetes in patients with NAFLD diagnosed by liver US, Okamoto and colleagues examined 840 individuals free of diabetes with liver US, and followed them for 10 years (44). At baseline, 120 of the 840 study participants were diagnosed with NAFLD. The authors reported an odds ratio (OR) of 2.62 (95% confidence interval [CI]=1.58-4.34) for a diagnosis of hyperglycemia at the 10-year follow-up visit in patients with NAFLD at baseline compared to individuals free of NAFLD (44). However, when adjusting for family history of diabetes, alcohol intake, frequency of medical check-ups, hemoglobin A1c (HbA1c), fasting glucose, BMI and age, presence of fatty liver at baseline was not associated with an increased risk of hyperglycemia at follow-up (adjusted OR [aOR] 1.83, 95% CI=0.95-3.51). The finding that NAFLD was not independently associated with an increased risk of type 2 diabetes is in contrast with the vast majority of other studies testing the same hypothesis. This discrepancy is possibly explained by the relatively small sample size, leading to an underpowered study (44).

In 2007, Shibata and colleagues investigated the risk of type 2 diabetes in 3 189 male workers older than 40 years from a company-based cohort in Japan (45). At baseline, 802 (25%) participants were diagnosed with NAFLD on liver US. During a mean follow-up of up 4 years, 44 cases (1.8%) of type 2 diabetes occurred in the group without NAFLD at baseline, and 65 cases (8.1%) occurred in the group with NAFLD at baseline (45). Adjusted for age and BMI, the hazard ratio (HR) for development of type 2 diabetes in the NAFLD group compared to the non-NAFLD group was 5.5 (95% CI=3.6-8.5, $p<0.001$) (45).

Several studies have reported that patients with more severe NAFLD appears to be at a greater risk of type 2 diabetes than do patients with less severe NAFLD. In a study from 2008, Kim and colleagues examined 5 372 South Korean individuals with a mean age of 46.8 years who underwent a routine health check-up, including liver US (46). At baseline, 1 790 (33%) study participants had NAFLD. Over a follow-up of 5 years, 233 (4.3%) individuals developed type 2 diabetes and presence of NAFLD was associated with an increased relative risk (RR) of 1.51 (95% CI=1.04-2.20) for development of type 2 diabetes compared to individuals free of NAFLD (46). The patients with NAFLD were also divided into groups based on severity of NAFLD on liver US (mild, moderate or severe) (46). Interestingly, when excluding patients with excess alcohol intake and adjusting for sex, age, family history of diabetes, smoking, components of the metabolic syndrome and ALT, mild NAFLD was not associated with an increased risk of development of type 2 diabetes compared to individuals free of NAFLD (RR 1.49, 95% CI=0.82-2.71, p=0.19) (46). In the same analysis, moderate to severe NAFLD was associated with an increased risk of development of type 2 diabetes (RR 2.29, 95% CI=1.13-4.63, p=0.02) (46). Similarly, in a 2013 study, Park and colleagues investigated the risk of type 2 diabetes in a cohort of over 25 000 men from South Korea, with a mean age of 42.5 years, who underwent a mandatory health check-up (47). At baseline, 8 858 (35%) patients had NAFLD. Over a mean follow-up of 3.7 years, 2 108 (8.4%) participants developed type 2 diabetes (47). Adjusting for age, waist circumference, triglycerides, high density lipoprotein (HDL) cholesterol, systolic blood pressure, homeostatic model assessment of insulin resistance (HOMA-IR), C-reactive protein (CRP),

creatinine, family history of diabetes, exercise and metabolic syndrome, an increased risk of type 2 diabetes was found in patients with moderate to severe steatosis (aHR 1.73, 95% CI=1.00-3.01, p<0.001), but not in patients with mild steatosis (aHR, 1.09, 95% CI=0.81-1.48), compared to individuals without NAFLD at baseline (47).

The reduction of liver steatosis in patients with NAFLD has also been reported to associate with a decreased risk of development of type 2 diabetes (48). In a Japanese study of 3 074 individuals examined with liver US, Yamazaki and colleagues examined the association between improvement of NAFLD and risk of development of type 2 diabetes (48). Of the 3 074 participants included in the study, 728 (23.7%) had NAFLD at baseline. Over a mean follow-up of 11.3 years, 189 (6.1%) developed type 2 diabetes. Adjusted for age, BMI, exercise, family history of diabetes, impaired fasting glucose, hypertension and dyslipidemia, NAFLD was associated with an increased risk of development of type 2 diabetes (aOR 2.37, 95% CI=1.60-3.52, p<0.001) (48). The increased risk of type 2 diabetes in patients with NAFLD was more pronounced in women (aOR 3.01, 95% CI=1.18-7.68) than in men (aOR 2.27, 95% CI=1.47-3.51). Of the patients with NAFLD at baseline, 110 (15.1%) had reduced their amount of liver steatosis at the second examination. In multivariable regression adjusted for the above mentioned covariates, improvement of NAFLD was associated with a decreased risk of development of type 2 diabetes (aOR 0.27, 95% CI=0.12-0.61, p<0.01) compared to no improvement (48).

In a study from 2017, using one of the larger cohorts ever examined when investigating the risk of type 2 diabetes in patients with NAFLD, Chen and colleagues reported findings from a study of 132 377 individuals examined with liver US (50). At baseline, 42 410 (32%) patients had NAFLD. Over a mean follow-up of 5.8 years, 6 555 individuals developed type 2 diabetes. Adjusted for age ≥ 65 years, family history of diabetes, hypertension, BMI ≥ 27 kg/m^2, smoking, alcohol intake, physical activity, cholesterol, triglycerides, HDL, ALT, AST, gamma-glutamyltransferase (gamma-GT) and alkaline phosphatase (ALP), NAFLD was independently associated with an increased risk of development of type 2 diabetes (aHR 2.08, 95% CI=1.93-2.23) (50). Further, ALT, AST, gamma-GT and ALP were also independently associated with an increased risk of development of type 2 diabetes (50).

Summarizing 19 observational studies investigating the risk of type 2 diabetes in NAFLD, Mantovani and colleagues published a meta-analysis in 2018 (64). They included only studies that had used liver US or CT to diagnose NAFLD, and no studies with fewer participants than 500 were included. In total, 296 439 individuals, out of which 30.1% had NAFLD, were included. Over a median follow-up of 5 years, 15 751 patients developed type 2 diabetes. In the pooled estimate of the HR for development of type 2 diabetes in patients with NAFLD compared to individuals without NAFLD, 16 studies were included (three were excluded due to no HR for type 2 diabetes in patients with NAFLD being reported) (64). After exclusion, four studies from China, four studies from Japan, four studies from South Korea, two studies from the US, one study from Taiwan and one study from Sri Lanka were included in the analysis. The pooled hazard ratio for development of type 2 diabetes in patients with NAFLD

compared to individuals without NAFLD was 2.22 (95% CI=1.84-2.60), though a substantial heterogeneity between studies (I^2=79%) was reported (64). In a sensitivity analysis, using only the studies deemed to be of high-quality according to the Newcastle-Ottawa scale, ten studies were included and the resulting aHR was 1.85 (95% CI=1.47-2.22) (64). The heterogeneity between the high quality studies included in the sensitivity analysis (I^2=68%) was, although still substantial, somewhat lower than the heterogeneity between the studies in the main analysis (I^2=79%) (64).

In 2006, Ekstedt and colleagues published a study where they examined 129 patients with biopsy proven NAFLD (65). The patients in the cohort had been referred to specialist centers due to persistently elevated liver enzymes. The mean follow-up was 13.7 years and the study cohort was compared to an age- and sex-matched background population. While 8.5% of the cohort had been diagnosed with diabetes before baseline, the authors were not able to ascertain baseline prevalence of type 2 diabetes as fasting glucose was not assessed at baseline. At the follow-up visit, 78% of the cohort had been diagnosed with either diabetes or impaired glucose tolerance. Interestingly, 71% of patients with NASH at baseline had been diagnosed with diabetes at the follow-up visit, while 46% of patients without NASH had diabetes at the follow-up visit (p=0.01) (65).

In summary, hitherto performed studies have demonstrated an increased risk of type 2 diabetes in patients with NAFLD. This increase in risk appears to be independent of other common risk factors, and a number of studies have reported an association between the severity of NAFLD and the magnitude of the increased risk of type 2 diabetes. Nonetheless, there's a lack of studies investigating the risk of development of type 2 diabetes in patients with NAFLD using cohorts of patients with NAFLD who have been diagnosed using liver biopsy. While a large number of studies have been performed, a majority of them have had follow-up times ≤ 10 years, which is somewhat problematic as type 2 diabetes is highly correlated with aging. Indeed, the pathophysiological progression from obesity and increased ectopic fat accumulation, to emerging insulin resistance, to pre-diabetes with impaired glucose regulation, to a clinical diagnosis of type 2 diabetes can occur over decades. In addition, most studies have been done on cohorts from Asian populations. Hence, the potentially increased risk of type 2 diabetes in northern European populations with NAFLD have not been thoroughly investigated.

1.3 RISK OF LIVER DISEASE IN TYPE 2 DIABETES

Several studies have demonstrated that patients with type 2 diabetes have an increased risk of severe liver disease, such as HCC (66-70), cirrhosis (68-74), hepatic failure (74-76) and death from liver disease (77, 78). While other etiologies of HCC and cirrhosis such as excessive intake of alcohol and viral hepatitis are still major causes, NAFLD is now emerging as a frequent etiology of severe liver disease. Indeed, up to a third of all cases of HCC are today caused by metabolic disease (including NAFLD, obesity and type 2 diabetes), and NAFLD is projected to become the leading cause of liver transplantation in the coming decade in the US

(79-81). Likewise, liver transplantations due to NAFLD have increased in the Nordic countries during the last decades (82).

In 2002, El-Serag and colleagues published a study where they examined the risk of acute hepatic failure (i.e. a rapid decrease of liver function occurring over days to weeks) in a cohort of military veterans in the United States (75). Using hospital registries, the authors identified 173 643 individuals with a discharge diagnosis of diabetes between 1985 and 1990 and compared them to 650 620 individuals free of diabetes. The study participants were followed until 2001. No differentiation was made between patients with type 1 and type 2 diabetes, and 98% of the individuals in the study cohort were male. Excluding patients with prior liver disease, including hepatitis B and C, and adjusting for age, sex, ethnicity, having served in the Vietnam war and comorbidities, the aHR for development of acute hepatic failure was 1.43 (95% CI=1.25-1.67) for patients with diabetes compared to individuals without diabetes (75).

Investigating the leading causes of HCC in the US population, Welzel and colleagues published a study in 2013 on the population attributable fractions of HCC (81). Using data from insurance claims along with data from the United States National Cancer Institute, the authors identified 6 991 patients aged ≥68 years diagnosed with HCC in the US from 1994 to 2007. Further, 255 792 reference individuals without HCC were included. While almost a third (31.2%) of the individuals who had developed HCC had a prior diagnosis of liver disease caused by alcohol, almost two thirds (61.5%) had a prior diagnosis of obesity and/or diabetes (81). Adjusting for viral hepatitis, alcohol-related liver disease, rare metabolic disorders, ethnicity and age, a diagnosis of obesity and/or diabetes was associated with HCC in both males (aOR 2.48, 95% CI=2.32-2.65) and females (aOR 2.43, 95% CI=2.21-2.66). The estimated population attributable fraction of obesity and diabetes for HCC was 36.6% (95% CI=34.6-38.6%). In a similar study from 2016, Makarova-Rusher and colleagues estimated the population attributable fractions of leading causes of HCC in the US (80). The authors combined diagnoses of NAFLD, obesity, metabolic syndrome, pre-diabetes and diabetes into one composite category representing risk factors related to the metabolic syndrome. Individuals aged ≥68 years diagnosed with HCC between 2000 and 2011 were included in the study. In total 10 708 patients with HCC and 332 107 reference individuals free of HCC were included (80). Adjusting for age, socioeconomic status, ethnicity, viral hepatitis, rare genetic disorders, alcohol-related conditions and smoking, the composite category of risk factors associated to the metabolic syndrome was associated with HCC in both males (aOR 2.8, 95% CI=2.6-2.9) and females (aOR 2.7, 95% CI=2.5-2.9). The estimated population attributable fraction of metabolic disorders for HCC was 32% (95% CI=30.5-33.5%) (80).

Using the previously described cohort of military veterans, El-Serag and colleagues published a study in 2004 where they examined the risk of chronic liver disease and HCC in individuals with diabetes (66). Excluding patients with hepatitis C and B, and adjusting for age, sex, having served in the Vietnam war and comorbidities, the aHR for development of chronic

nonalcoholic liver disease including cirrhosis was 1.98 (95% CI=1.88-2.09) for patients with diabetes compared to individuals free of diabetes. Adjusting for the same variables, the hazard ratio for development of HCC was 2.13 (95% CI=1.79-2.53) for patients with diabetes (66).

In a study published in 2016, Wild and colleagues compared the incidence of liver disease in patients with type 2 diabetes to that of individuals free of diabetes (67). They used the Scottish National Diabetes Registry along with the Scottish hospital admission records, cancer records and causes of death records to ascertain cases of liver disease (67). Cases of alcoholic liver disease, autoimmune liver disease, haemochromatosis, HCC, NAFLD and viral hepatitis were ascertained during 1.8 million person years in patients with type 2 diabetes and during 24 million person years in individuals free of diabetes (67). The reported rate ratio, adjusted for age and socio-economic status, for HCC in patients with type 2 diabetes compared to individuals free of diabetes was 3.36 (95% CI=2.97-3.81) in men and 3.55 (95% CI=3.02-4.17) in women. In the same study, the authors reported a rate ratio of NAFLD in patients with type 2 diabetes compared to individuals free of diabetes of 3.03 for men and 5.11 for women (67). However, while a NAFLD-diagnosis per se most often does not represent a clinically significant liver disease and might be an "innocent bystander" in patients hospitalized for cardiovascular disease or diabetes, international classification of diseases (ICD) codes for more severe forms of liver disease, such as cirrhosis and portal hypertension were included in the NAFLD-category of ICD codes, rendering the results somewhat difficult to interpret (67).

Summarizing studies of how components of the metabolic syndrome affect the risk of severe liver disease, Jarvis and colleagues published a meta-analysis of 18 studies in 2020 (83). In the analysis of risk of severe liver disease in patients with type 2 diabetes, 12 studies were included. In total, 22.8 million individuals with a median follow-up of 10 years were included. Combining studies on both fatal and non-fatal severe liver disease in individuals with no other liver disease etiology than NAFLD, type 2 diabetes was associated with an increased risk of severe liver disease (HR 2.25, 95%=1.83-2.76). It should be noted that the heterogeneity between included studies was considerable (I^2=99%) (83).

Due to the poor sensitivity of blood tests for aminotransferases for detecting NAFLD, a very large proportion of patients with type 2 diabetes and concurrent NAFLD are not diagnosed with NAFLD in the routine clinical practice (12). Examining the prevalence of NAFLD and cirrhosis in a Chilean cohort of patients with type 2 diabetes and no known liver disease, Arab and colleagues published a study in 2016 (84). Individuals with other causes of liver disease than NAFLD were excluded. In total, 133 participants older than 55 years, with a mean BMI of 29.6 kg/m^2 were included in the study and underwent MRI. Around two thirds (64%) of the cohort had findings of steatosis on MRI, and 6% had findings indicative of cirrhosis on MRI. Using the NFS to estimate presence of fibrosis, the authors found evidence of advanced fibrosis in 13% of the cohort (84).

A number of studies have investigated the use of transient elastography to detect NAFLD and elevated liver stiffness in patients with type 2 diabetes (85-93). Kwok and colleagues published a study in 2016 where they performed transient elastography on 1 918 patients with type 2 diabetes (86). Study participants were included at a specialist center in Hong Kong where patients underwent screening for complications of type 2 diabetes, and individuals with other liver disease than NAFLD were excluded. Cut-offs used to ascertain steatosis were 222-232 dB/m for steatosis grade 1 (S1), 233-289 dB/m for S2 and ≥290 dB/m for S3. To ascertain fibrosis stage 3 (F3) a liver stiffness measurement (LSM) of 9.6 kPa was used, and for F4 a LSM of ≥11.5 kPa. On average, participants were aged 61 years and their average BMI was 26.6 kg/m^2. Of 1 799 patients with sufficient quality for steatosis measurements, 73% had S1 or higher. Of 1 884 patients with sufficient quality LSM, 334 (18%) had values indicating at least advanced fibrosis (≥F3), of which 224 patients (12%) had kPa values indicating cirrhosis. In total, 94 patients, of which 74% had LSM indicating advanced fibrosis or cirrhosis, went on to have a liver biopsy. The PPV and NPV of the transient elastography to detect advanced fibrosis or cirrhosis were 59% and 84%, respectively (86).

The studies on risk of severe liver disease in patients with type 2 diabetes that have been conducted so far have had some methodological problems that limits implementation of generated findings into clinical care. In many earlier studies, differentiation between type 1 and type 2 diabetes was not possible. Since patients with type 1 diabetes have a lower risk of severe liver disease than patients with type 2 diabetes, the risk-estimates are likely falsely low in studies that have included both patients with type 1 and type 2 diabetes. Further, study cohorts have in many instances been rather selected, for example including a majority of male participants, and have not been population based. The outcomes of interest in many studies have either been specific diagnoses of severe liver disease (for example cirrhosis), mortality from specific diagnoses (for example mortality from cirrhosis), or a combination of severe liver disease and early stage, less clinically relevant liver disease. Finally, there is a lack of studies investigating risk factors for severe liver disease in patients with type 2 diabetes. Thus, interpreting results from previous studies with regards to the population-based risk of severe liver disease in general in patients with type 2 diabetes is somewhat difficult.

1.4 RISK OF CANCER IN NAFLD

Given the local effect of hepatic fat accumulation and subsequent inflammatory damage to the hepatic tissue, it is not surprising that an association between NAFLD and an increased risk of HCC has been reported across a number of studies (94-97). Further, the risk of several other forms of cancers, such as colorectal (98-104), bladder (105) and breast cancer (106-108) in patients with NAFLD have also been examined.

To investigate the risk of HCC in patients with NAFLD, Younossi and colleagues examined cases of HCC occurring from 2004 to 2009 in the United States. Individuals with HCC were identified using data from the National Cancer Institute and Medicare insurance claims (97). The database used contained data on cancer incidence in 28% of the United States population. Of the 4 979 cases of HCC included in the study, 701 occurred in individuals

with NAFLD. As controls, 14 937 individuals (1 243 with NAFLD) without HCC were included, out of which 83% had no known liver disease. Adjusting for sex, ethnicity and comorbidities, NAFLD was associated with HCC (aOR 2.62, 95% CI=2.28-3.00) (97).

Assessing the risk of HCC in patients with NAFLD, Kanwal and colleagues published a study in 2018 (96). The authors included patients diagnosed with NAFLD from 2004 to 2008 in healthcare facilities organized by the Veterans Health Administration. Study participants were considered as having NAFLD if competing causes of hepatic steatosis were lacking, and their ALT levels were ≥ 40 IU/ml for men and ≥ 31 IU/ml for women on at least two separate occasions with at least 6 months having passed in between. In total, 296 707 individuals with NAFLD, and the same number of controls free of NAFLD matched for sex and age, were included in the study. Due to participants being included from the Veterans Health Administration, 94.4% of the cohort were men. Over a mean follow-up of 9 years, 490 patients with NAFLD and 55 controls developed HCC. In a multivariable analysis adjusted for age, ethnicity, BMI, diabetes and hypertension, NAFLD was associated with risk of HCC (aHR 7.62, 95% CI=5.76-10.09, p<0.01). Study participants with NAFLD but no cirrhosis had an incidence rate (IR) of HCC of 0.08 cases per 1000 person-years, whereas participants with NAFLD and cirrhosis had an IR of 10.6 cases per 1000 person-years. Thus, the absolute risk of HCC in patients with NAFLD without cirrhosis was low (96).

The risk of colorectal cancer in patients with NAFLD was investigated in a 2011 study by Stadlmayr and colleagues (98). Including individuals who underwent routine screening with colonoscopy for colorectal cancer at a single center in Austria between 2007 and 2009, the authors compared the prevalence of colorectal cancer in 632 patients with NAFLD compared to 579 individuals free of NAFLD. Excluding patients with liver disease other than NAFLD, and individuals with excess alcohol consumption, NAFLD was diagnosed liver US. Colorectal cancers were significantly more common in male patients with NAFLD (1.6%) than in males free of NAFLD (0.4%), whereas no significant difference was found in females. In a logistic regression analysis adjusted for impaired glucose regulation, BMI, age and sex, NAFLD was independently associated with presence of colorectal adenomas (OR 1.47, 95% CI=1.08-2.00, p=0.02) (98).

In a study published by Lin and colleagues in 2014, the authors examined the association between NAFLD and prevalence of colorectal cancer in a Chinese community cohort (101). Individuals were excluded if they had other liver disease than NAFLD, and NAFLD was diagnosed using liver US. After exclusion, 2 315 study participants, of which 263 had NAFLD, were included. The study participants with NAFLD had a significantly higher prevalence (29.3%) of colorectal cancer than the individuals free of NAFLD (18%). In a logistic regression analysis adjusted for several important confounders including BMI, blood lipids, and liver blood markers, NAFLD was independently associated with colorectal cancer (aOR 1.87, 95% CI=1.36-2.57, p<0.01) (101).

Examining the risk of colorectal adenomas and colorectal cancer in patients with NAFLD, Mantovani and colleagues published a systematic review and meta-analysis of 11 studies in

2018 (64). The authors included studies of 300 or more participants, and only studies on asymptomatic individuals undergoing screening colonoscopy. Only studies where NAFLD was diagnosed using liver biopsy (one study) or some form of imaging technique (ten studies) were included, and both cross-sectional and longitudinal studies were included. Of the 11 studies analyzed, eight were cross-sectional and three were longitudinal. Five studies were performed on South Korean cohorts, four were performed on Chinese cohorts, one was performed on an Austrian cohort and one was performed on a Taiwanese cohort (64). The prevalence of NAFLD in the included cohorts ranged from 11.4% to 45.7%. In total, 91 124 individuals were included in the meta-analysis, out of which 32.1% had NAFLD. All included studies performed regression analyses adjusted for several confounders, and a majority adjusted for important confounders such as smoking, BMI and diabetes. In the combined analysis of cross-sectional studies where NAFLD was diagnosed using radiology, NAFLD was associated with an increased prevalence of colorectal cancer (aOR 1.56, 95% CI=1.25-1.94). Likewise, in the one study where NAFLD was diagnosed using liver biopsy, an association with colorectal cancer was found (aOR 3.04, 95% CI=1.29-7.18). One longitudinal study included in the meta-analysis investigated risk of incident colorectal cancer, and found NAFLD to be independently associated with risk of colorectal cancer (aHR 3.08, 95% CI=1.02-9.03) (64). This longitudinal study, however, was performed on a cohort consisting only of females, and had a relatively short mean follow-up of 4.5 (64).

Studying the risk of several extrahepatic cancers in patients with NAFLD, Allen and colleagues published a study on a community based cohort of 4 772 patients with NAFLD and 14 441 reference individuals matched for age and sex (95). An increased risk of colorectal cancer was found in male patients with NAFLD compared to reference individuals (incidence rate ratio (IRR) 2.4, 95% CI=1.6-3.9). No increased risk of colorectal cancer was found, however, in female patients with NAFLD compared to reference individuals (IRR 1.3, 95% CI=0.8-2.1) (95). The authors further examined how NAFLD interacts with obesity regarding risk of cancer and reported that while an increase in risk was observed in patients with NAFLD compared to both non-obese (IRR 2.0, 95% CI=1.5-2.9) and obese controls (IRR 2.0, 95% CI=1.5-2.7) without a diagnosis of NAFLD, no increased risk was observed in obese patients without a diagnosis of NAFLD compared to non-obese controls (IRR 1.0, 95% CI=0.8-1.4. One interpretation of these results could be that further research should test the hypothesis that NAFLD is a driver of the generally increased risk of cancer in the obese population, but residual confounding cannot be ruled out partly due to the register-based nature of the study.

The research on the risk of breast cancer in patients with NAFLD has been performed primarily in the last five years. In 2017, Nseir and colleagues published a study where they investigated the results of examinations undertaken at mammography screening center in Israel from 2008 to 2011 (106). To assess the frequency of NAFLD, the authors used the results from abdominal CT-scans performed within one month after the diagnosis of breast cancer. Individuals matched for age and BMI who underwent mammography and breast US with normal results and performed an abdominal CT-scan within three months, were used as

controls. In total, 73 patients with breast cancer and 73 controls were included. No significant differences in age, BMI, smoking, diabetes or metabolic syndrome were found between cases and controls. Adjusting for age > 25 years at first delivery and use of estrogen, NAFLD was associated with an increased risk for breast cancer (aOR 2.82, 95% CI=1.20-5.50, p=0.02) (106).

Following a cohort of individuals who had underwent a health check-up at a hospital in South Korea from 2004 to 2005, Kim and colleagues investigated the risk of breast cancer in 11 981 women (94). Around one fifth (21%) of the women included in the cohort had NAFLD (diagnosed with liver US) at baseline and study participants were followed for a median of 7.5 years. Patients who developed cancer within one year of their health check-up were not included in the study. After adjusting for smoking, sex, diabetes, hypertension, gamma-GT, LDL and HDL cholesterol and triglycerides, NAFLD was associated with an increased risk of developing breast cancer (aHR 1.92, 95% CI=1.15-3.20, p=0.01) (94). In the same study by Kim and colleagues, the authors also reported associations between NAFLD and risk of HCC (aHR 16.73, 95% CI=2.09-133.85, p<0.01). Further, an association between NAFLD and risk of colorectal cancer was reported in males (aHR 2.01, 95% CI=1.10-3.68, p=0.02), but not in females (aHR 0.63, 95% CI=0.21-1.89, p=0.41). No association was found between NAFLD and gastric cancer.

In a case-control study published in 2019, Kwak and colleagues examined the association between NAFLD and risk of breast cancer in 270 patients with breast cancer and 270 controls (107). Study participants (both cases and controls) were included from 2008 to 2017 while undergoing a voluntary health check-up at a hospital in South Korea. The health check-up included both liver US and mammography. In addition to excluding patients with competing causes of liver steatosis, individuals aged younger than 30 years and patients with previous breast cancer were excluded. Controls were matched to cases on age and BMI. Among cases, 30% had NAFLD, and among the controls 20% had NAFLD. In a multivariable logistic regression model adjusted for age at menarche, blood pressure, triglycerides, gamma-GT, metabolic syndrome, waist circumference and BMI, NAFLD was associated with breast cancer (aOR 1.63, 95% CI=1.01-2.62, p=0.046). The authors further investigated the association between NAFLD and breast cancer after stratifying the cohort on obesity (BMI ≥ 25 kg/m^2). Interestingly, NAFLD was associated with breast cancer in the fully adjusted model for the non-obese patients (aOR 3.04, 95% CI=1.37-4.32, p<0.01), but not for the obese patients (aOR 0.40, 95% CI=0.11-1.45, p=0.16) (107).

In a recent study by Simon and colleagues, the risk of cancer in Swedish patients with biopsy-proven NAFLD was investigated (109). All patients in Sweden who had received a diagnosis of NAFLD confirmed by biopsy between 1966 and 2016 were included. During a median follow-up of 13.8 years, a total of 1 691 cases of cancers were identified in 8 892 included patients with NAFLD. For each patient with NAFLD, at least five reference individuals without a diagnosis of NAFLD from the general population were included. The reference individuals were matched to NAFLD patients on age, sex, calendar year and county of

residence. A total of 6 733 cases of cancer were observed in the 39 907 reference individuals. Adjusting for alcohol abuse (as a time-varying covariate), cardiovascular disease, diabetes, hypertension, dyslipidemia, hypertension, obesity, end-stage renal disease, family history of cancer (younger than 50 years), number of hospital visits and level of education, an association with an increased risk of any cancer (aHR 1.27, 95% CI=1.18-1.36) was found in patients with NAFLD compared to reference individuals. The association between NAFLD and an increased risk of cancer was found across the spectrum of disease severity in patients with NAFLD and simple steatosis, NASH but no fibrosis, fibrosis but no cirrhosis, and cirrhosis. While the increase in risk of cancer in patients with NAFLD appeared largely driven by the association to risk of HCC (aHR 17.08, 95% CI=11.56-25.25), an association between NAFLD and risk of extra-hepatic solid organ cancer was also observed (aHR 1.12, 95% CI=1.04-1.20). A limitation of the study is that liver biopsy is seldom performed in NAFLD. Hence, some degree of selection bias is likely present.

Taken together, studies performed to test the hypotheses of an association between NAFLD and an increased risk of development of cancer indicate that this association does exist, and that it is primarily driven by an increased risk of HCC in patients with NAFLD compared to individuals without NAFLD. Regarding the risk of other common cancers in patients with NAFLD, the observed results vary between different studies. This variation likely stems from methodological differences. For example, the way to define a diagnosis of NAFLD differ between studies, ranging from use of non-invasive models incorporating BMI and blood markers, to liver US, to liver biopsy. In the studies investigating cohorts were NAFLD have been defined using less sensitive diagnostic modalities, such as liver transaminases, the estimated prevalence of NAFLD is likely falsely low. In the studies investigating cohorts where NAFLD have been identified by liver biopsy, one can expect the patient population to be rather selected and likely to include more severe cases of NAFLD. Thus, the capture rate of the exposure is bound to differ between the different studies, rendering comparison of results difficult. Further, the characteristics of the cohorts - and the information on important confounders in the studied cohorts - vary between studies. Some of the largest studies have been done on selected cohorts that are not derived from population-based databases, lowering the external validity.

1.5 TREATMENT OF NAFLD

Due to the strong association between obesity and NAFLD, weight loss has been examined as a possible treatment for NAFLD. In a study by Vilar-Gomez and colleagues, 293 patients with biopsy-proven NASH undertook a one-year life-style intervention where they were instructed to reduce their caloric intake to 750 kcals lower than their daily need of energy (110). Further, 200 minutes/week of walking was recommended. On average, study participants lost 4.6kg (SD +/-3.2kg). At the end of the study a new biopsy was performed, where 25% of study participants were free of NASH, and the degree of weight loss was independently associated with histological improvement (110).

Different macronutrient compositions in diets have been studied in patients with NAFLD (111-116). In 2011, Haufe and colleagues published a study where they compared the effects of calorie restricted low-carbohydrate and low-fat diets on 170 overweight and obese subjects (111). Over a period of six months, 102 patients completed the study protocol where their baseline energy intake was lowered by 30%. The subjects were instructed to consume ≤90 grams of carbohydrates/day in the low-carbohydrate group, and fat equaling to ≤20% of total energy intake in the low-fat group. Both groups saw similar relative reductions in hepatic fat content; -47% for the low carbohydrate group, and -42% for the low fat group with a non-significant difference between groups (111). A mechanism by which the low-carbohydrate, ketogenic diet can reduce hepatic fat content in spite of an increase in circulating fatty acids was proposed in a 2020 study by Luukkonen and colleagues (115). In the study, 10 obese or overweight individuals consumed a hypocaloric ketogenic diet (≤25 grams/day) for 6 days. The diet resulted in an increased hepatic insulin sensitivity, a reduction in circulating insulin and an increase in the breakdown of hepatic fat to be used for ketone production (115). The effect of intermittent calorie restriction and a low-carbohydrate diet on NAFLD compared to standard treatment was tested in a recent study by Holmer and colleagues (116). In total, 74 patients with NAFLD were randomized to reduced caloric intake two days per week (600 kilo calories/day for men and 500 kilo calories/day for women), a low-carbohydrate high-fat diet (less than 10% from carbohydrates and up to 80% from fat), or standard of care (which included recommendations on reduced intake of saturated fat and sweets) for 12 weeks. Both the intermittent calorie restriction diet (-6.1%) and the low-carbohydrate high-fat diet (-7.2%) generated a larger absolute reduction in hepatic steatosis than the standard of care treatment (-3.6%). The average relative reductions in hepatic steatosis were -16.8% for participants randomized to standard of care, -50.9% for participants randomized to intermittent calorie restriction, and -53.1% for participants randomized to the low-carbohydrate high-fat diet. (116).

The effects of different types of exercise and respiratory fitness on NAFLD and NASH have also been studied. In a 2009 study from Australia, St George and colleagues published a study where 141 patients with NAFLD were assigned to a low intensity lifestyle intervention, a moderate intensity lifestyle intervention or a control group during three months (117). As a marker for improvement of NAFLD, the authors used liver enzymes. The patients who were physically active achieved an improvement in their liver enzymes compared to individuals who were sedentary (117). In 2011, Hallsworth and colleagues studied the effects of an eight-week training program focused on resistance exercise in 11 patients with NAFLD compared to ten patients with NAFLD who underwent no training program. Amount of intrahepatic fat was ascertained using MRS. After eight weeks, the intervention group achieved a relative reduction in liver fat content of 13% ($p=0.01$). Interestingly, this reduction in liver fat was achieved in absence of significant reductions in BMI or total body fat mass (118).

While no medication has yet been approved for the treatment of NAFLD, several substances have been investigated. Due to the commonalities between type 2 diabetes and NAFLD it has been hypothesized that various forms of anti-diabetic medications could be effective in

treating NAFLD, and several studies have examined this hypothesis in the past couple of decades (119-139).

In one of the earliest randomized controlled trials of the insulin sensitizer thiazolidinedione in patients with NASH and type 2 diabetes or impaired glucose tolerance, Belfort and colleagues compared the effect of six months of treatment with Pioglitazone with placebo (120). In total, 55 patients were randomized to receive either treatment or placebo. All study participants underwent liver biopsy to diagnose NASH, MRI to assess amount of hepatic steatosis and an oral glucose tolerance test to ascertain type 2 diabetes or impaired glucose tolerance. In addition to receiving either placebo or 45 mg of Pioglitazone per day, all subjects were instructed to lower their daily caloric intake by 500 kilo calories. At the end of the treatment period, participants who received Pioglitazone had gained on average 2.5 kg's, compared to a reduction of on average 0.5 kg's in the placebo group. Liver fat content measured by MRS reduced on average 54% in the treatment group, while it did not change in the placebo group. The Pioglitazone treatment was associated with improvement in histologic markers of necroinflammation, but not with reduced fibrosis (120). While some subsequent trials of thiazolidinediones have shown reduction in fibrosis (121, 130), others have shown improvements in steatosis, but not in fibrosis (122, 124, 125). In a study by Bril and colleagues, an association between treatment with thiazolidinedione and reduction in fibrosis was observed in patients with type 2 diabetes, but not in patients with pre-diabetes (139). There are, however, concerns about the side effects of thiazolidinediones, which limits their clinical applicability (140).

To examine the effect of the glucagon like peptide (GLP)-1 analogue Liraglutide on NAFLD, Armstrong and colleagues enrolled 52 patients with biopsy-proven NASH across four centers in the UK in a study published in 2016. The study participants were randomized to 48 weeks of treatment with Liraglutide or placebo. The treatment was significantly associated with resolution of NASH, but not with improvement of fibrosis. There was, however, a significant association between treatment with Liraglutide and no progression of fibrosis (131). In a recent study of the effect of the GLP-1 analogue Dulaglutide on hepatic fat content in patients with NAFLD and type 2 diabetes, Kuchay and colleagues randomized 64 patients to either 24 weeks with Dulaglutide or continued standard of care (137). Liver fat was measured using MRI, and liver stiffness was assessed using transient elastography. A significant association between treatment with Dulaglutide and reduction in liver fat was reported, but no association with reduction in liver stiffness was observed (137). In a phase 2 trial published in 2021, Newsome and colleagues randomized 320 patients with NASH to receive either placebo, or daily Semaglutide treatment of either 0.1 mg, 0.2 mg or 0.4 mg for 72 weeks (141). In the group that received the 0.4 mg dose 59% achieved resolution of NASH, whereas 33% in the placebo group achieved resolution of NASH ($p<0.001$). No significant difference between treatment and placebo groups were found regarding improvement of fibrosis (141).

The effect of the sodium glucose co-transporter 2 (SGLT2) inhibitor Dapafliglozin on hepatic steatosis and liver stiffness assessed by transient elastography was investigated in a study by

Shimizu and colleagues published in 2019 (136). In total, 63 participants with NAFLD and type 2 diabetes were randomized to either Dapafliglozin or continued standard of care for 24 weeks. The authors used a LSM cut-off of ≥8 kPa to indicate fibrosis. At the end of the study, treatment with Dapafliglozin was significantly associated with reduced CAP (reflecting hepatic steatosis), but not with a reduction in LSM (reflecting hepatic fibrosis) (136).

The results from the studies on treatment of NAFLD indicate that what was previously known to lower the risk of other diseases related to the metabolic syndrome - weight loss and to some degree exercise - is also effective in treating NAFLD. Unfortunately, sustainable weight loss is difficult to achieve for most patients. Alternative paths to treating NAFLD should therefore be explored. The studies on treatment of NAFLD with different anti-diabetic agents show some promise. Several studies indicate that agents such as SGLT-2 inhibitors and GLP-1 analogues can reduce hepatic steatosis. The effects on fibrosis and long term severe liver disease, however, remain unclear. Due to the fact that a majority of patients with type 2 diabetes have NAFLD, and that type 2 diabetes is a risk factor for more severe disease progression in NAFLD, it is of clinical importance to understand how the modern-day treatment strategies for improving type 2 diabetes affects the progression NAFLD. This is, however, not currently known.

2 RESEARCH AIMS

The aims of this doctoral thesis were to:

1. Investigate the incidence of type 2 diabetes in a cohort of patients with biopsy-proven NAFLD and examine if any histological characteristics associate with risk of development of type 2 diabetes (study 1).

2. Examine the risk of severe liver disease in patients with type 2 diabetes compared to controls free of diabetes, and to identify risk factors associated with development of severe liver disease (study 2).

3. Investigate the prevalence of NAFLD and increased liver stiffness in patients with type 2 diabetes, and to assess the effects of a personalized treatment program to improve glycemic control on liver health (study 3).

4. Study the risk of cancer in patients with NAFLD compared to individuals free of NAFLD and investigate how presence of cirrhosis affects this (study 4).

3 MATERIALS AND METHODS

3.1 STUDY DESIGN

In study 1, we assessed the risk of type 2 diabetes in patients with NAFLD. Participants were included from a cohort of patients with retrospectively collected data, who had been examined with liver biopsy at Karolinska University Hospital from 1971 to 2009. The indication for performing liver biopsies on patients in this cohort was predominantly a finding of persistently elevated liver transaminases. At baseline, the patients included also had metabolic parameters such as fasting glucose, blood lipids, blood pressure and BMI documented. All individuals with type 2 diabetes at baseline were excluded. For a patient to be classified as having type 2 diabetes, a diagnosis had to be present in the medical chart or in the NPR (defined as an ICD-code of E11), or a prescription of oral anti-diabetic medications or insulin had to be present in the patients' medical chart. The liver biopsies were all re-evaluated by one pathologist who assessed the quality of the biopsies and scored them to identify NASH (according to the SAF score) and fibrosis (as described by Kleiner and colleagues)(4, 13). The outcome of interest, development of type 2 diabetes, was identified through manual review of participants' medical charts. For individuals who had migrated from Stockholm we could not access their medical charts, hence the National Patient Registry (NPR) was used to identify diagnoses of type 2 diabetes. The NPR contains information on diagnoses for all patients admitted to hospitals in Sweden have received since 1964 and has a PPV for the majority of chronic disease between 85% and 95%. Further, it includes information on all diagnoses patients have received in out-patient visit in specialized care since 2001. Study participants were followed up until February 20, 2016.

In study 2, we examined the risk of severe liver disease in patients with type 2 diabetes. The National Diabetes Registry (NDR) was used to assemble the cohort. In the NDR, Swedish patients with diabetes are documented. Coverage has increased throughout the last decades to the point where the registry now includes the majority (around 90%) of patients with diabetes in Sweden. When a patient is included in the NDR, healthcare personnel documents information on metabolic parameters, anthropometric variables and medications. All individuals above 18 years of age with type 2 diabetes registered in the NDR between 1998 and 2012, without pre-existing liver disease - other than NAFLD - were included in the study. Other pre-existing liver disease than NAFLD were excluded through identification of relevant ICD-codes in the NPR. We defined all patients in the NDR with dietary treatment (with or without antidiabetic oral medication) and all patients older than 39 years when they were first diagnosed with diabetes as having type 2 diabetes. As controls free of diabetes, five individuals from the background population, matched for age, sex and living location were obtained from Statistics Sweden and included in the study. Baseline data on metabolic parameters, anthropometric variables and medications were not available for the reference individuals. The outcome of interest in study was severe liver disease. We chose to construct a composite outcome variable representing severe liver disease, including cirrhosis, decompensation events, hepatic failure, liver transplantation, HCC and death from any of

these diagnoses. The combination of several liver-related severe outcomes into one composite outcome variable was done because we thought the implications from our results for the clinical management of patients with type 2 diabetes would be more impactful with the combined outcome than if we had chosen to analyze separate severe liver-related diagnoses as main outcomes. This approach was also recently recommended by a large international panel of experts (142). As secondary outcomes, development of HCC and death from liver disease were analyzed separately. Outcomes were ascertained through the NPR, the Causes of Death Registry (CDR) and the Swedish Cancer Registry (SCR). The CDR documents the cause of death for all citizens of Sweden since 1961, and the SCR was established in 1958, and cover approximately 96% of all cancer cases in Sweden. Study participants were followed up until December 31, 2014.

In study 3, we investigated the effects of measures taken to improve the glycemic control in patients with type 2 diabetes on their liver health. To assemble the cohort for study 3, we invited patients with type 2 diabetes who were attending a 4-day treatment program at the endocrinology clinic at the Karolinska University Hospital. Individuals lacking understanding of the Swedish language were not considered for participation in the study as informed consent required sufficient skills in the Swedish language. Moreover, patients with a co-existing liver disease or those who had previously undergone a liver transplantation were excluded. All individuals who attend the program have been referred by their general practitioner due to difficulties managing the patient's glycemic control at the primary care level, or some other component of the patient's metabolic health, such as blood pressure or kidney damage, being difficult to manage. The treatment program is comprised of different measures aimed at improving the glycemic control and associated metabolic parameters. In addition to individual consultations by an endocrinologist, participants in the program are offered lectures by an endocrinologist, a specialist nurse and a dietician. Further, a seminar with a physiotherapist is included. At the start of the week, all patients were invited to participate in our study. At inclusion, baseline blood markers reflecting liver health and metabolic status were collected, other markers of metabolic health (e.g. blood pressure, BMI, waist-to-hip ratio) were documented, and a transient elastography examination to identify steatosis and increased liver stiffness was performed. To assess the effect of the treatment program on the liver health of the study participants, all patients were invited to a follow-up visit at approximately three months after the baseline examination. We chose the time period of three months as we deemed this an appropriate time interval for assessing effects of implemented lifestyle changes. At the follow-up visit, a second transient elastography examination was performed, and all markers of metabolic and liver health documented at baseline were collected again.

In study 4, we evaluated the risk of cancer in patients with NAFLD. The cohort was constructed from identification of all patients above 18 years of age, without any prior diagnosis of cancer, who received a code for NAFLD in the NPR (ICD-9: 578.1, ICD-10: K75.8 or K76.0) between January 1, 1987 and December 31, 2016. As controls, up to 10 reference individuals matched for age, sex and living location were obtained from Statistics

Sweden and included in the study. The primary outcome of interest, any cancer (except non-melanoma skin cancer), was identified through the SCR. Secondary outcomes were HCC, colorectal, stomach, kidney, bladder, cervix, ovary, uterus, breast, lung and esophagus cancer. Outcomes were identified from one year after baseline up until December 31, 2016. Thus, cancers that occurred earlier than one year after baseline were not counted as outcomes. The reason for introducing a one year-lag on the identification of the outcome was that we were interested in the effect of NAFLD on the risk of development of cancer. The lag was thought to mitigate the risk that the cancer, while undiagnosed, had actually been present in the study participant before baseline.

As excess accumulation of fat in the liver can result from multiple different causes, the diagnosis of NAFLD is ascertained when competing causes such as over-consumption of alcohol or intake of medications known to cause liver steatosis have been ruled out. This can be difficult to achieve as many real world patients have for example a condition of co-existing obesity and excess consumption of alcohol, which both contributes to development of liver steatosis. This problem is also present when trying to construct a cohort of patients with "true" diagnoses of NAFLD. In studies 1 and 3, participants with competing causes of liver disease were excluded by manual review of medical charts and by questionnaires, and in all patients in study 3 specifically by blood sampling of phosphatidylethanol. In studies 2 and 4, exclusions were based on presence of a diagnosis of a competing cause in the population-based registries.

3.2 STATISTICS

In studies 1, 3 and 4 we used Fisher's exact test and Wilcoxon rank sum test to investigate the differences between baseline variables of the different groups of the cohorts. To assess the association between baseline variables and risk of development of type 2 diabetes, we used a Cox regression model in study 1. The cohort was also divided into patients with or without advanced fibrosis at baseline. This was done as the risk of mortality is higher in patients with significant fibrosis, which in turn would've rendered the results difficult to interpret had all patients in the cohort been included in the same Cox regression model.

In study 2, Cox regressions were used for different purposes. First, the risk of development of the main outcome (severe liver disease) and secondary outcomes (HCC and death from severe liver disease) were compared between patients with type 2 diabetes and controls from the general population. As the controls were matched on sex, age and living location, and no other baseline variables were known regarding the controls, the Cox regression was univariable. Second, including only patients with type 2 diabetes, for whom several baseline variables were available, a Cox regression was performed to investigate which variables were associated with an increased risk of severe liver disease. Initially, univariable models were constructed for each baseline variable, where after a multivariable model where all baseline variables were included was constructed. In study 3, we used linear regression to investigate if any associations between the primary outcomes (liver steatosis and stiffness) and other parameters were present. As we were interested in evaluating a potential change in liver

steatosis and stiffness occurring from baseline (before the intervention program) to follow-up (three months after the intervention program), we used delta values (i.e. the change from baseline to follow-up visits) in the linear regression. The linear regression thus generated a beta-value, representing the change in liver steatosis and/or liver stiffness per change in other parameters, and an adjusted R^2-value, representing how much of the change in the outcome of interest is predicted by the other parameter. Further, as we were also interested in assessing the applicability of the FIB-4 score in this cohort, we calculated the sensitivity and specificity of the FIB-4 score for presence of elevated liver stiffness at baseline. To assess the broader clinical implications of the intervention program, differences between baseline and follow-up in parameters reflecting the metabolic health of the study participants were evaluated with Fisher's exact test for categorical variables and Wilcoxon ranksum test for continuous variables.

In study 4, we performed Cox regression analyses to assess the association between the exposure (NAFLD) and primary (any cancer excepts non-melanoma skin cancer) and secondary outcomes (specific cancers specified above). As we did obtain information regarding some co-morbidities chosen to reflect the metabolic health of study participants as well as information regarding presence of chronic obstructive pulmonary disease (as a proxy for smoking), we calculated both non-adjusted and adjusted risk estimates. Owing to previous studies having reported a difference in the association between NAFLD and risk of cancers in male and female subjects, we performed Cox regression analyses stratified on sex for the cancers where an association with NAFLD was observed in the main analysis.

3.2.1 SENSITIVITY ANALYSES

Sensitivity analyses were conducted in all studies. These were performed to test if any of the main analyses performed in our different studies contained biases which would be exposed when applying different criteria for the construction of the models.

In study 1, we performed two kinds of sensitivity analyses. First, the risk of development of type 2 diabetes was compared between groups of patients based on presence of NASH and fibrosis, where the group with no fibrosis and no NASH was used as reference. Second, we performed the same calculation of risk estimates as in the main analysis while excluding study participants where the NPR had been used to identify presence of type 2 diabetes. This was done to assess whether the inclusion of data from the NPR skewed the results in the main analysis.

In study 2, we performed a sensitivity analysis where all participants with a duration of follow-up under 1 year were excluded. This analysis was performed to assess whether individuals who had an outcome identified before 1 year of follow-up (and possibly had started developing severe liver disease before their diagnosis of type 2 diabetes) significantly altered the results in the main analysis. To assess how the risk of severe liver disease varies across different age groups of patients with type 2 diabetes, we performed the main analysis

separately in age-stratified groups of patients, ranging from under 40 years all the way up to above 80 years in 10-year strata, thus producing six different age groups.

In study 3, we performed a sensitivity analysis including only patients who had been examined with the same size of transient elastography probe at baseline and follow-up visits (and not, for example, the medium sized probe at the baseline visit and the extra-large sized probe at the follow-up visit). We did this to examine if use of different probes at baseline and follow-up introduced an inconsistency in measurement results which might have affected the main analysis.

In study 4, we performed one sensitivity analysis restricted only to patients who did not have a diagnosis of cirrhosis at baseline. This was done as previous studies had indicated that presence (or absence) of cirrhosis could be of great importance in assessing the risk of cancer in patients with NAFLD. In a second sensitivity analysis, we were interested in finding out if a potentially increased risk of the combined outcome of any cancer in individuals with NAFLD was primarily due to an increased risk of HCC specifically. To examine this, we performed an analysis where the only cancer cases we included were participants who did not have a diagnosis of HCC as their first cancer. In a third analysis, we examined whether a possible increased risk of development of HCC during follow-up in patients with NAFLD but no cirrhosis at baseline was influenced by that they might have developed cirrhosis during follow-up. To assess this, we included only participants that did not develop cirrhosis during follow-up. Further, we aimed to investigate if the probable increased risk of overall mortality in patients with NAFLD would influence the estimates of cumulative incidence from our Cox regression analysis. One method to approach this problem is by performing a competing risk analysis. Thus, we applied a competing risk regression where the competing risk event was death from other causes than cancer.

3.3 ETHICAL CONSIDERATIONS

In all scientific research, especially research involving animals or humans, careful consideration has to be given to potentially harmful effect of performing experiments or observations. Moreover, one needs to consider the implications of the results that might be produced, in that the conclusions drawn from generated results might impact the world outside the context of scientific research. While we performed blood tests and transient elastography examinations on the patients in the third study, we did not expose participants to any type of experimental treatment in our studies. Nonetheless several ethical questions need to be considered in the studies we've performed. In studies 1, 2 and 4, we included data from patients who were deceased, which can pose an ethical dilemma. Individuals who are still alive can, if they for example consider registry-based research unethical, try to persuade policy makers and build public opinion against the allowance of this type of research. Deceased individuals obviously can no longer voice their opinion and try to influence policy makers regarding participating in the study. However, never including deceased patients in studies would render testing of hypotheses involving mortality impossible, and the potential future benefits of preventing premature death would be lost. Thus, we argue that the benefits

of studying the disease course of deceased individuals outweigh the negatives. When we included data from population-based registries, we did not ask each participant for consent to participate in the study. Studying individuals without their consent also poses a clear ethical dilemma. Asking each individual in a population-based registry (many times covering millions of patients) for their consent regarding participation in a study would, however, demand enormous resources and render epidemiologic studies on such large cohorts practically impossible. As no intervention is performed on a patient that is included in a registry-based study, the risk for the patient can be considered to be largely related to questions of personal integrity. Thus, proper management of patient data to avoid harm to personal integrity is a crucial part of registry-based research and which we strictly adhered to in these studies. On the patients we studied in the clinic, in study 3, we performed an examination on their liver (transient elastography) and took blood tests that they otherwise would not have been exposed to, had they not participated in the study. Even if the patients who did have elevated liver stiffness did benefit from finding this out, it could be argued that the examinations were not performed in line with evidence based standards and routine clinical management, and therefore was not in the best interest of the patients, but rather an experimental practice in our scientific pursuit of falsifying a hypothesis. However, all patients gave written informed consent to participate after reading a clear description of the study. The line between routine medical practice and scientific endeavors needs to be clear. While many patients are well informed, their conceptual understanding of the aim and importance of participating in a study comes from physician who is also acting as a clinical researcher. Therefore, striving for objectiveness and clear communication about the aims of performing examinations within a scientific study is highly important.

4 RESULTS

4.1 STUDY 1

In study 1, a total of 396 patients with biopsy-proven NAFLD were included and followed for a median of 18.4 years. One third of the cohort (n=132, 33%) developed type 2 diabetes during follow-up. Comparing the group that developed type 2 diabetes during follow-up to the group that did not, we found a number of differing baseline variables. At baseline, the group that developed type 2 diabetes during follow-up had a statistically significant ($p<0.05$) higher proportion of individuals with hypertension (34.8% vs 20.8%) and a higher proportion of individuals with advanced fibrosis (stage 3-4) than the group that did not develop type 2 diabetes (15.2% vs 7.2%).

Table 1. Baseline characteristics of the cohort in study 1.

Variable	**Developed T2DM (N=132)**		**Free of T2DM (N=264)**			
	Mean/N	**SD**	**Mean/N**	**SD**	**P-value**	**Complete data (%)**
Sex, N male (%)	82 (62.0)		175 (66.2)		0.44	396 (100)
Age (years)	47.4	12.0	45.0	14.4	0.11	396 (100)
Smoking, ever N (%)	49 (37)		91 (34)		0.92	352 (88.9)
Follow-up time (years)	15.2	8.2	20.0	8.9	<0.001	395 (99.7)
Hypertension, N (%)	46 (34.8)		56 (20.8)		<0.001	381 (96.2)
BMI (kg/m^2)	28.1	3.8	27.3	4.2	0.03	379 (95.7)
Thrombocytes ($10^9/L$)	247.4	64.7	260.7	82.2	0.23	323 (81.6)
Haemoglobin (g/dL)	14.9	1.2	14.8	1.2	0.39	377 (95.2)
WBC ($10^9/L$)	6.8	1.8	6.7	2.1	0.46	324 (81.8)
Ferritin (µg/L)	248.8	167.7	235.2	218.5	0.18	187 (47.2)
ALT (U/L)	131.1	523.1	114.3	400.3	0.87	392 (99.0)
AST (U/L)	73.3	249.2	63.6	170.4	0.68	389 (98.2)
ɣGT (U/L)	109.7	143.7	110.8	124.5	0.53	332 (83.8)

Bilirubin (µmol/L)	0.7	0.5	0.7	0.5	0.48	367 (92.7)
Albumin (g/dL)	4.3	0.5	4.2	0.4	0.02	344 (86.9)
Cholesterol (mmol/L)	234.3	54.9	223.5	45.8	0.21	275 (69.4)
Triglycerides > 150mg/dL	60 (45.5)		60 (33)		0.03	251 (63.4)
Glucose (mg/dL)	96.2	17.5	94.5	17.9	0.37	307 (77.5)
NASH, N (%)	95 (72)		176 (67)		0.22	396 (100)
NAS (0-8)	4.7	1.85	4.4	2.0	0.10	396 (100)
Fibrosis (stage 3-4, %)	20 (15.2)		19 (7.2)		<0.001	396 (100)

Table 1. Baseline characteristics of the cohort in study 1. Abbreviations: ALT= alanine aminotransferase; AST= aspartate aminotransferase; γGT=gamma-glutamyltransferase; SD=standard deviation; WBC=white blood cells.

Mean baseline values of BMI (28.1 vs 27.3 kg/m^2) and albumin (4.3 vs 4.2 g/dL) were higher in the group that developed type 2 diabetes than in the group that did not. Further, the proportion of patients with triglycerides higher than 150 mg/dL at baseline was significantly higher (45.5% vs 33%) in the group that developed type 2 diabetes than in the group that did not. In the group of patients with advanced fibrosis (stage 3-4) at baseline, 51.2% developed type 2 diabetes during follow-up, while 31.3% of the group of patients with fibrosis stage 0-2 developed type 2 diabetes. Adjusting for age, BMI, sex and triglycerides >150 mg/dL, fat score on biopsy was associated with risk of development of type 2 diabetes during follow-up (aHR 1.36, 95% CI 1.03-1.79, p=0.03) for patients with fibrosis stage 0-2. In the same model, no associations were found for NASH or scores for ballooning, inflammation or NAS.

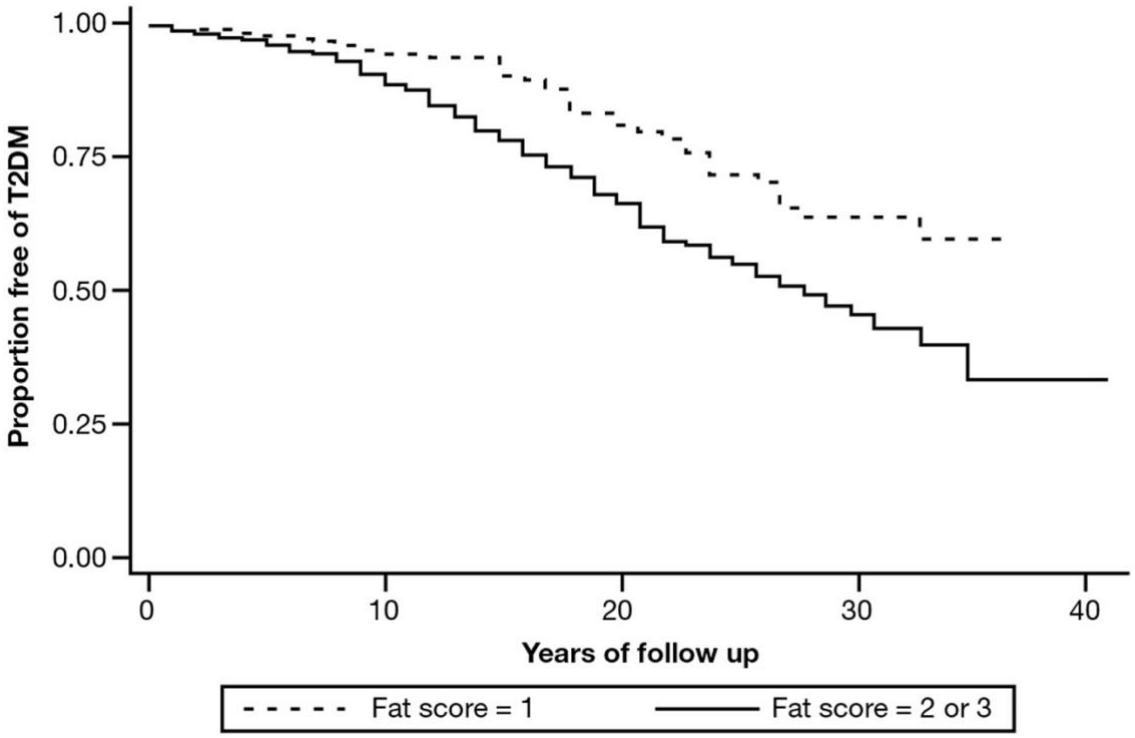

Figure 5. Kaplan-Meier curve for risk of type 2 diabetes in patients with fibrosis stage 0–2, stratified by histologic fat score of 1 vs 2–3. Log-rank < .001. Published with permission.

No associations between development of type 2 diabetes and histological markers were found in the group of patients with advanced fibrosis at baseline. The group with advanced fibrosis was more likely to die during follow-up, with 25.6% dying in the group with fibrosis stage 0-2, and 55.9% dying in the group with advanced fibrosis. In the sensitivity analysis evaluating the risk of development of type 2 diabetes in patients with NASH and no fibrosis, NASH and fibrosis stage 1-2, and NASH and fibrosis stage 3-4 compared to the group of patients with simple steatosis and no fibrosis, we found that only the group with NASH and fibrosis stage 1-2 had an increased risk in the fully adjusted model. Of note, the group with advanced fibrosis lacked data on baseline levels of triglycerides, which was included in the fully adjusted model, in 51.2% of patients. In the model adjusted for age, BMI and sex, the group with advanced fibrosis had an increased risk compared to the group with simple steatosis and no fibrosis (aHR 3.07, 95% CI=1.35-6.96, p<0.01).

4.2 STUDY 2

In study 2, we included 406 770 patients with type 2 diabetes who were followed for a median of 7.7 years. On average, the patients with type 2 diabetes were overweight, with a mean BMI of 29.8 kg/m2, and just over half the group were men (53.8%). Hypertension was present in 61.7%, and 12.1% were smokers. Close to one in five (19.0%) were on insulin treatment, and 52.5% were on anti-diabetic oral medication at the start of the study. Baseline microalbuminuria and macroalbuminuria was present in 8.6% and 4.7% of patients, respectively. A little more than 1% (5092 individuals, 1.3%) of patients with type 2 diabetes developed the main outcome, severe liver disease, before the end of the study. As reference individuals free of diabetes, we included 2 033 850 controls matched for age, sex and living

location. Other baseline variables were not obtained for reference individuals. Severe liver disease occurred in 11 619 (0.6%) of controls during follow-up. Type 2 diabetes was associated with an increased risk (HR 2.28, 95% CI=2.21-2.36, p<0.01) of severe liver disease compared to not having diabetes. Similarly, type 2 diabetes was associated with an increased risk of both secondary outcomes, development of HCC (HR 3.18, 95% CI=2.94-3.44) as well as dying from severe liver disease (HR 2.29, 95% CI=2.17-2.41, p<0.01), compared to not having diabetes. While an increased risk of severe liver disease across all age groups of patients with type 2 diabetes compared to their respective reference individuals was found, differences were observed in the absolute risks between the different age groups. In the group under 40 years, and the 40-49-year group, the proportion of patients with type 2 diabetes who developed severe liver disease was 0.18% and 0.56%, respectively. In the 50-59-year group, the proportion of patients with type 2 diabetes who developed severe liver disease was 1.08%, increasing with age to 1.39% in the 60-69-year group, to 1.62% in the 70-79-year group.

Table 2. Risk factors for severe liver disease in patients with type 2 diabetes in study 2.

Variable	HR (95% CI)	P-value	aHR (95%CI)	P-value
Age (years)	1.03 (1.03-1.03)	<0.001	1.05 (1.04-1.05)	<0.001
Sex (female)	0.77 (0.71-0.83)	<0.001	0.72 (0.63-0.82)	<0.001
Times since first diagnosis of T2DM (years)	1.01 (1.01-1.02)	<0.001	1.00 (0.99-1.01)	0.762
HbA1c (mmol/mol)	1.00 (1.00-1.01)	0.07	1.01 (1.00-1.01)	0.151
Hypertension (mmHg)	1.63 (1.47-1.80)	<0.001	1.24 (1.05-1.46)	0.01
BMI (kg/m^2)	1.02 (1.01-1.03)	<0.001	1.04 (1.02-1.05)	<0.001
LDL (mg/dL)	0.83 (0.79-0.88)	<0.001	0.79 (0.74-0.85)	<0.001
HDL (mg/dL)	0.82 (0.72-0.94)	0.004	0.87 (0.73-1.03)	0.099
Triglycerides (mg/dL)	1.01 (0.97-1.06)	0.50	0.99 (0.92-1.06)	0.756
GFR (ml/min/1.73m^2)	1.00 (1.00-	0.709	1.01 (1.00-	<0.001

	1.00)		1.01)	
Statins	0.84 (0.77-0.91)	<0.001	0.67 (0.59-0.77)	<0.001
Smoking at baseline (yes)	1.25 (1.12-1.39)	<0.001	1.58 (1.35-1.86)	<0.001
Microalbuminuria	1.41 (1.27-1.56)	<0.001	1.25 (1.01-1.45)	0.005
Macroalbuminuria	1.85 (1.49-2.31)	<0.001	1.17 (0.80-1.73)	0.419

Table 2. Risk factors for severe liver disease in patients with type 2 diabetes in study 2. Cox regression models were performed to estimate hazard ratios. The multivariable model is adjusted for all other variables. Abbreviations: aHR=adjusted hazard ratio; BMI=body mass index; CI=confidence interval; DBP=diastolic blood pressure; GFR=glomerular filtration rate; HbA1c=glycosylated hemoglobin; HDL=high-density lipoprotein; HR=hazard ratio; LDL=low-density lipoprotein; SBP=systolic blood pressure; T2DM=type 2 diabetes mellitus. Published with permission.

In the multivariable Cox regression analysis of risk factors for severe liver disease performed separately in patients with type 2 diabetes, we found several baseline variables to be significantly associated with an increased risk: age (aHR 1.05, 95% CI=1.05-1.06), smoking (aHR 1.58, 95% CI=1.35-1.86), BMI (aHR 1.04, 95% CI=1.02-1.05), GFR (aHR 1.01, 95% CI=1.00-1.01) and microalbuminuria (aHR 1.25, 95% CI=1.01-1.45)). In the sensitivity analysis where we wanted to examine if patients having severe liver disease diagnosed during the first year of follow-up influenced the results in the main analysis, and therefore excluded these individuals, we obtained similar results as in the main analysis.

4.3 STUDY 3

After excluding patients who were previously liver transplanted, who had excessive alcohol intake, who were not fluent in Swedish, and patients with unreliable transient elastography examinations, 91 participants were enrolled in study 3 (flowchart in Figure 6). The study cohort had a majority of male participants (58%) and a median age of 59 years. Median baseline levels of HbA1c and BMI was 71 mmol/mol and 30.1 kg/m^2, respectively. Baseline hypertension was present in 69.2% of the cohort, while 63.7% were on statin treatment, 61.5% were on insulin treatment, 73.6% were on metformin treatment, 15.4% were on GLP-1-analogue treatment and 6.6% were on SGLT-2-inhibitor treatment at baseline. Due to technical difficulties performing the transient elastography exam and due to the malfunctioning of the software on the machine used to perform the transient elastographies, reliable data on liver steatosis and liver stiffness could not be obtained in all enrolled individuals at baseline or follow-up. At baseline, 76% of patients with reliable steatosis

measurements had results indicating presence of NAFLD (CAP >268 dB/m), and 24.2% had results indicating presence of significant fibrosis (kPa >7.9). Stiffness levels signifying advanced fibrosis (> 13.9 kPa), were found in 8.8% of patients at baseline. Of the 91 patients enrolled at baseline, 13 individuals chose not to participate at the follow-up examination. Over the course of the study, reductions in steatosis were seen in 64% of participants with CAP values that indicated NAFLD at baseline, and in which reliable CAP values were obtained at follow-up (n=39). This translated into an average decrease of 18.3 dB/m in patients with NAFLD at the start of the study. Similarly, 67% of participants with elevated liver stiffness at baseline and reliable stiffness measurements at follow-up (n=15) achieved a decrease in liver stiffness during the study. An average decrease in liver stiffness of 2.6 kPa was observed during the course of the study in participants where an increased liver stiffness was recorded at the start of the study.

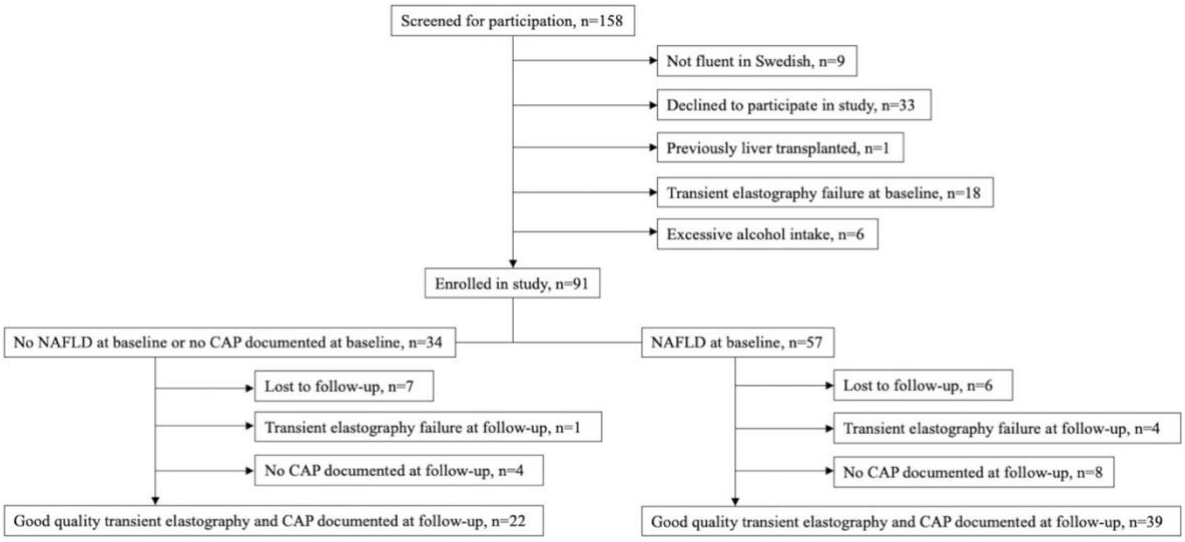

Figure 6. Flowchart for patients included in study 3.

We found that improvement in fasting glucose from baseline to follow-up was associated with improvement in steatosis in patients with NAFLD in linear regression. The reduction in CAP per one unit mg/dl reduction in fasting glucose, or beta, was 0.48 dB/m. The R2 value, denoting the proportion of change in CAP that could be ascribed to changes in fasting glucose, was 0.35. This association was stable after adjusting for changes in BMI and if patients had been started on treatment with GLP-analogues during the intervention week (beta=0.45 dB/m, R2=0.41). Further, the association remained in the sensitivity analysis including only patients who had been examined with the same size of probes at both study visits.

Table 3. FIB-4 score as a predictor of elevated liver stiffness at baseline in study 3.

	≥8 kPa	<8 kPa	
Intermediate or high FIB-4	7	6	PPV 54%

Low FIB-4	14	58	NPV 81%
	Sensitivity 33%	Specificity 91%	

Table 3. Sensitivity, specificity, PPV and NPV of the FIB-4 score for elevated liver stiffness measurement on transient elastography in study 3. Abbreviations: FIB-4=fibrosis 4 score, kPa=kilopascal, NPV=negative predictive value, PPV=positive predictive value.

Regarding changes in liver stiffness, we found no association with changes in other variables. In the calculations of the PPV and NPV of the FIB-4 score regarding prediction of increased liver stiffness (defined as a value of ≥8 kPa), we found a PPV of 54% and a NPV of 81%

4.4 STUDY 4

In the fourth study, we included 8 415 patients with an ICD-based diagnosis of NAFLD, without a previous diagnosis of cancer. As in study 3, the study cohort had a slight majority of male patients (56%). In addition to the patients with NAFLD, we included 70 934 controls matched for age, sex and county. The median age of the study participants was 53 years. Close to one fifth (19.1%) of patients with NAFLD had a recorded diagnosis of diabetes, one fourth (25.6%) had hypertension, one tenth had hyperlipidemia (11.1%) and one in thirty had COPD (3.3%) at baseline. In reference individuals, co-morbidities were less common, with 3.4% having diabetes, 7.4% having hypertension, 2.5% having hyperlipidemia and 0.9% having COPD at baseline.

Over the course of the study (median follow-up 6.0 years), we observed 527 occurrences of cancer in the NAFLD group (6.3%, incidence rate [IR] 9.7 per 1000 person-years), while 4716 (6.6%, IR 8.6 per 1000 person-years) cases occurred in the control group. Both the analysis in which patients with cirrhosis at baseline (n=183) were excluded and the analysis where additional patients who obtained a diagnosis of cirrhosis during the study (n=10) period were excluded generated results where an association between NAFLD and risk of HCC was observed.

Table 4. Baseline characteristics of the cohort in study 4

	Entire cohort			**Individuals with NAFLD**		
	Individuals with NAFLD, n=8415	Reference individuals, n=70934		Cirrhosis at baseline, n=183	No cirrhosis at baseline, n=8232	
Age, years, median (IQR)	54 (23)	53 (23)	p<0.01	62 (12)	54 (24)	p<0.01

Females (%)	3 844 (45.7)	31 222 (44.0)	p<0.01	81 (44.3)	4490 (54.5)	p<0.01
Baseline comorbidities						
Cirrhosis, n (%)	183 (2.2)	0 (0.0)	-	183 (100)	-	-
Diabetes, n (%)	1 607 (19.1)	2 407 (3.4)	p<0.01	105 (57.4)	1502 (18.2)	p<0.01
COPD, n (%)	280 (3.3)	614 (0.9)	p<0.01	12 (6.6)	268 (3.3)	p=0.02
Hypertension, n (%)	2 184 (25.6)	5 218 (7.4)	p<0.01	85 (46.4)	2086 (25.3)	p<0.01
Hyperlipidemia, n (%)	937 (11.1)	1 804 (2.5)	p<0.01	35 (19.1)	902 (11.0)	p<0.01
Liver biopsy within 1 year prior to baseline, n (%)	481 (5.7)	2 (0.0)	p<0.01	24 (13.1)	457 (5.6)	p<0.01
Year of diagnosis						
1987-1989, n (%)	160 (1.9)	-	-	3 (1.6)	157 (1.9)	p=1.00
1990-1999, n (%)	397 (4.7)	-	-	4 (2.2)	393 (4.8)	p=0.11
2000-2009, n (%)	2 298 (27.3)	-	-	31 (16.9)	2 267 (27.5)	p<0.01
2010-2016, n (%)	5 560 (66.1)	-	-	145 (79.2)	5 415 (65.8)	p<0.01

Table 4. Baseline characteristics for the study population. Individuals with cirrhosis at baseline had a concurrent or previous diagnosis of cirrhosis before receiving a diagnosis of NAFLD. Differences between baseline variables of individuals with NAFLD with and without cirrhosis at baseline were calculated using Wilcoxon rank sum test for continuous variables and Fisher's exact test for categorical variables. Abbreviations: COPD=chronic obstructive pulmonary disease.

In the competing risk regression analysis, where we treated death from other causes than cancer as a competing risk to the event of interest, a diagnosis of any cancer, we found a higher risk for cancer in the NAFLD group compared to reference individuals (sub-distribution HR [sHR] 1.10, 95% CI=1.02-1.20). This pattern was also observed when a

diagnosis of HCC was analyzed as the event of interest in the competing risk analysis (sHR 8.16, 95% CI=5.68-11.75). In male patients with NAFLD (n=4 571), a total of 265 cases (5.8%) of cancer was observed during the study, while the 39 712 male reference individuals experienced 2 568 cases (6.5%) of cancer. In female patients with NAFLD (n=3 844), a total of 262 cases (6.8%) of cancer was observed, while the 31 222 female reference individuals experienced 2 148 (6.9%) of cancer. In both male and female patients with NAFLD, the IR per 1000 person-years was higher than in their respective reference individuals without NAFLD (IR 8.5 vs 8.1 in males, and IR 11.2 vs 9.3 in females). The risk of any cancer was slightly higher in female patients with NAFLD compared to female reference individuals, than in male patients with NAFLD compared to male reference individuals (aHR 1.18 vs aHR 1.26). For HCC, we observed 30 cases (0.7%) in males with NAFLD, compared to 25 cases (0.1%) in male reference individuals. The IR of HCC per 1000 person-years was 0.9 in males with NAFLD, and 0.1 in male reference individuals, corresponding to an aHR of 14.54 (95% CI=7.35-28.74) for HCC in males with NAFLD compared to male reference individuals. In females with NAFLD, we observed 17 cases of HCC (0.4%), compared to 9 cases (0.0%) in female reference individuals. The IR of HCC per 1000 person-years was 0.7 in females with NAFLD, and 0.0 in female reference individuals, corresponding to an aHR for HCC of 8.74 (95% CI=3.39-22.55) in females with NAFLD compared to female reference individuals.

Table 5. Risk of cancer in men and women compared to controls without NAFLD.

Type of cancer	Incident cases		Incidence rate (95% CI) per 1000 PY		HR (95% CI)	aHR (95% CI)
	NAFLD (n, %)	Reference individuals (n, %)	NAFLD	Reference individuals		
Women	n=3 844	n=31 222				
All cancers	262 (6.8)	2148 (6.9)	11.2 (10.0-12.7)	9.3 (8.9-9.7)	1.27 (1.13-1.43) (p<0.01)	1.26 (1.11-1.43) (p<0.01)
HCC	17 (0.4)	9 (0.0)	0.7 (0.4-1.0)	0.0 (0.0-0.1)	16.37 (7.61-35.23) (p<0.01)	8.74 (3.39-22.55) (p<0.01)
Colon and rectum	32 (0.8)	297 (1.0)	1.3 (0.9-1.8)	1.2 (1.1-1.4)	1.18 (0.83-1.67) (p=0.37)	1.21 (0.84-1.73) (p=0.31)
Kidney	9 (0.2)	32 (0.1)	0.4 (0.2-0.7)	0.1 (0.1-0.2)	3.27 (1.67-6.41) (p<0.01)	3.02 (1.50-6.05) (p<0.01)

Bladder	11 (0.3)	51 (0.2)	0.4 (0.2-0.8)	0.2 (0.2-0.3)	2.61 (1.41-4.84) (p<0.01)	2.81 (1.49-5.31) (p<0.01)
Men	n=4 571	n=39 712				
All cancers	265 (5.8)	2568 (6.5)	8.5 (7.6-9.6)	8.1 (7.8-8.5)	1.16 (1.03-1.31) (p=0.01)	1.18 (1.04-1.34) (p<0.01)
HCC	30 (0.7)	25 (0.1)	0.9 (0.6-1.3)	0.1 (0.0-0.1)	15.06 (8.70-26.08) (p<0.01)	14.54 (7.35-28.74) (p<0.01)
Colon and rectum	45 (1.0)	352 (0.9)	1.4 (1.1-1.9)	1.1 (1.0-1.2)	1.51 (1.12-2.04) (p<0.01)	1.54 (1.13-2.08) (p<0.01)
Kidney	12 (0.3)	78 (0.2)	0.4 (0.2-0.7)	0.2 (0.2-0.3)	1.84 (1.03-3.30) (p=0.04)	1.87 (1.02-3.43) (p=0.04)
Bladder	26 (0.6)	148 (0.4)	0.8 (0.6-1.2)	0.5 (0.4-0.5)	2.27 (1.54-3.35) (p<0.01)	2.43 (1.62-3.65) (p<0.01)

Table 5. Risk of cancer in male and female subjects with NAFLD compared to reference individuals without NAFLD. Cox regression models were performed to estimate hazard ratios. The multivariable model is adjusted for diabetes, hypertension, hyperlipidemia and chronic obstructive pulmonary disease. Abbreviations: aHR=adjusted hazard ratio, HR=hazard ratio, CI=confidence interval, NAFLD=nonalcoholic fatty liver disease, PY=person-years.

We found 9 cases (n=0.2%) of kidney cancer in female patients with NAFLD and 32 cases (0.1%) in female reference individuals. In male patients with NAFLD, we found 12 cases (0.3) of kidney cancer, and in male reference individuals we found 78 cases (0.2%). The IR per 1000 person-years for kidney cancer was increased in both female patients with NAFLD (IR 0.4 vs 0.1) and male patients with NAFLD (IR 0.4 vs 0.2) compared to their respective reference individuals, corresponding to an aHR for kidney cancer of 3.02 (95% CI=1.50-6.05) in female patients with NAFLD, and an aHR of 1.87 (95% CI=1.02-3.43) in male patients with NAFLD. For bladder cancer, we found 11 cases (0.3%) in females with NAFLD and 51 cases (0.2%) in female reference individuals, and 26 cases in males with NAFLD (0.6%) and 148 cases (0.4%) in male reference individuals. The IR's per 1000 person-years for bladder cancer were higher in both females with NAFLD (IR 0.4 vs 0.2) and males with NAFLD (IR 0.8 vs 0.5) compared to their respective reference individuals. This corresponded to an aHR for bladder cancer of 2.81 (95% CI=1.49-5.31) in females with NAFLD compared

to female reference individuals, and an aHR for bladder cancer of 2.43 (95% CI 1.62-3.65) in males with NAFLD compared to male reference individuals. In the analyses of risk of colorectal cancer, we found the risk to be increased in male patients with NAFLD compared to their reference individuals without NAFLD, but not in female patients with NAFLD.

5 DISCUSSION

The main finding in study 1 was that the risk of developing type 2 diabetes was high in a cohort of patients with NAFLD. Indeed, the IR of type 2 diabetes was 18 per 1000 person-years in our cohort, which is markedly increased compared to the IR of type 2 diabetes in the background population in Stockholm, which was reported to be 3.6 per 1000 person-years in 2010 (143). As previously mentioned, the vast majority of studies investigating the risk of type 2 diabetes in patients with NAFLD have used cohorts where patients have received their NAFLD diagnoses after undergoing liver US examinations, or have been classified as NAFLD using non-invasive models based on liver transaminases and BMI. The different diagnostic modalities for identifying NAFLD between our study and most previous studies renders direct comparison of results somewhat difficult. The finding of an increased risk of development of type 2 diabetes in patients with NAFLD compared to individuals free of NAFLD has been reported in the vast majority of studies investigating this subject.

In one of the largest studies to date, Chen and colleagues investigated the risk of type 2 diabetes in a cohort of 42 410 Korean individuals with NAFLD (50). In their study, over a mean follow-up of 5.8 years, 3 735 (8.8%) of patients with NAFLD went on to develop type 2 diabetes. This is somewhat lower than the proportion that developed type 2 diabetes in our study, which is plausible considering the follow-up in our study was significantly longer (mean 18.4 years). As the individuals with NAFLD in the study by Chen and colleagues had not undergone liver biopsy, the focus was not - as it was in our study - to assess if any liver histological parameters associated with an increased risk of developing type 2 diabetes. The Cox regression models we constructed in our study were also stratified on severity of fibrosis, with risk factors for developing type 2 diabetes in patients with advanced fibrosis being analyzed in a separate regression model. Further, Chen and colleagues included individuals without NAFLD in their cohort, thus including NAFLD as an independent variable in their Cox regression analysis with type 2 diabetes being the dependent variable. In their unadjusted estimates, they reported age, family history of diabetes, hypertension, BMI at or over 27 kg/m^2, cholesterol, elevated triglycerides, HDL, presence of NAFLD, ALP and liver transaminases to all be associated with an increased risk of type 2 diabetes. These results are somewhat similar to the results from our univariable Cox regression analyses in the group with fibrosis stage 0-2, where we found age, elevated triglycerides, hypertension, fat score on biopsy, inflammation score on biopsy and NAS to be associated with risk of development of type 2 diabetes.

In the 2006 study by Ekstedt and colleagues, a cohort of patients with biopsy proven NAFLD was examined regarding their risk of developing type 2 diabetes (65). The indications for why study participants underwent liver biopsies were similar in the study by Ekstedt and colleagues and in our study (i.e. due to persistently elevated liver enzymes), which enhances comparability between our studies. One important difference between our studies, however, was that the patients in the study by Ekstedt and colleagues were not examined regarding signs of type 2 diabetes at baseline. If the included patients had a diagnosis of diabetes

already ascertained at baseline, which was the case in 8.5% of the cohort, this was noted. The other patients were not examined with a test of fasting glucose at baseline, thus the baseline prevalence of type 2 diabetes in the study by Ekstedt and colleagues was unclear. It can be noted, though, that prevalence of disorders related to the metabolic syndrome was comparably high in the baseline cohort, with 56% being overweight, 29% obese, 72% hypertensive, and 57% suffering from hypertriglyceridemia. It is therefore plausible that the baseline prevalence of type 2 diabetes was higher than the ascertained 8.5%. At the time of the follow-up assessment, 88 of the 104 from the original study cohort (n=129) who were still alive chose to participate. At follow-up, 37% of participants had received a diagnosis of diabetes prior to the follow-up visit, and an additional 16% were diagnosed at the follow-up visit. Further, 20% were diagnosed with impaired glucose tolerance at the follow-up visit. Thus, in total, 78% of the participants that took part in the follow-up assessment had either impaired glucose tolerance or diabetes. The authors note that 71% of the patients with baseline NASH that attended follow-up had diabetes, compared to 46% of the patients without baseline NASH, and that the difference in follow-up prevalence of diabetes was found to be statistically significant. This finding is in line with the results from our study, indicating that histological severity of NAFLD could be of prognostic value when identifying risk of type 2 diabetes. However, the relatively small size of these histology-based cohorts did not allow for full adjustment of plausible confounders, why future studies in this field needs to be appropriately sized. Another note is that liver histology is unlikely to be a clinically valuable predictor of incident diabetes per se, given its invasive nature.

In the large meta-analyses published by Mantovani and colleagues in 2018, a total of 296 439 participants, out of which almost one third (30.1%) had NAFLD, from 19 different studies were included. Reflecting the active NAFLD research in Asia, a majority of studies included in the meta-analysis were performed on Asian cohorts. As the quality of the studies included varied, the authors performed a sensitivity analysis where they included only studies of in which risk-estimates had been adjusted for important confounders. In this sensitivity analysis of high quality studies, the pooled HR for risk of type 2 diabetes in NAFLD compared to reference individuals without NAFLD was 1.85 (95% CI=1.47-2.22). In an attempt to investigate if severity of NAFLD is of importance in assessing the risk of type 2 diabetes, the authors performed separate examinations of the four studies included in the meta-analyses that had reported severity of NAFLD, either from classification by liver US or by NFS. In the three studies where severity of NAFLD was assessed by liver US, an HR of 2.15 (95% CI=1.57-70) for risk of type 2 diabetes was reported, and in the one study where severity was assessed by the NFS, an HR of 4.74 (95% CI=3.54-5.94) was reported. While these estimates are derived from a small number of studies using diagnostic tools that are not considered gold standard, they indicate that severity of NAFLD is of importance when assessing risk of type 2 diabetes in patients with NAFLD. This conclusion is supported by the results from our study, where we were able to test this hypothesis using the gold standard diagnostic modality, liver biopsy, for ascertaining disease severity. Thus, our results

combined with results from previous studies indicate that physicians should take extra careful consideration in assessing the risk of type 2 diabetes in patients with more severe NAFLD.

In study 2, our main finding was that patients with type 2 diabetes are at a higher risk of severe liver disease than matched controls without diabetes after a median of 7.7 years of follow-up. Previous studies on the risk of different forms of severe liver disease in patients with diabetes have repeatedly reported an increased risk compared to patients without diabetes. As the diagnostic coding for diabetes previously did not separate between type 1 and type 2 diabetes, many of the earlier studies on risk of liver disease in patients with diabetes were not able to distinguish between type 1 and type 2 diabetes in their cohorts. In the previously mentioned studies from 2002 and 2004, El Serag and colleagues investigated the risk of acute hepatic failure (75) and HCC (66) in a cohort of more than 800 000 military veterans in the US, out of which 173 643 patients had a discharge diagnosis of diabetes between 1985 and 1990, and reported and association to an increased risk of both conditions (aHR 1.43 for acute hepatic failure, aHR 2.13 for HCC) in patients with diabetes compared to individuals without diabetes. These results are supported by our study, where we were able to differentiate between type 1 and type 2 diabetes and included a more diverse cohort (98% of participants in the studies by El-Serag and colleagues were male).

In a more recent study on the risk of liver disease in patients with type 2 diabetes, Wild and colleagues used the Scottish National Diabetes Registry and Scottish hospital admission records, cancer records and causes of death records to identify liver disease in the Scottish population. During a follow-up of 1.8 million person years in patients with type 2 diabetes, and 24 million person years in reference individuals free of diabetes, cases of several different types of liver disease were identified. Alike the findings in our study, Wild and colleagues reported an independent association between type 2 diabetes and risk of HCC in both men (rate ratio 3.36, 95% CI=2.97-3.81) and women (rate ratio 3.55, 95% CI=3.02-4.17), adjusted for age and socio-economic status. In a recent meta-analysis on the association between components of the metabolic syndrome and severe liver disease by Jarvis and colleagues, published in 2020, study 2 in this doctoral thesis was included. As is the case in many meta-analyses, the heterogeneity between studies included in the study by Jarvis and colleagues was considerable (I^2=99%), which motivates caution when considering the reported pooled estimates. Reporting results from studies that looked at both fatal and non-fatal severe liver disease in patients with no other liver disease etiology than NAFLD, an association between type 2 diabetes and risk of severe liver disease was identified (HR 2.25, 95% CI=1.83-2.76). The results from this meta-analysis (which included our own study) is in line with our findings.

One aspect on the risk of liver disease patients with type 2 diabetes that we analyzed in study 2, that was not reported in most previous studies of risk of liver disease in patients with type 2 diabetes, was the risk difference among different age groups. We found the absolute risk of severe liver disease in patients with type 2 diabetes under 50 years of age to be low. In the perspective of potentially formulating a more concise screening strategy, it can be argued that

information on the difference in risk across different age groups is highly valuable. Our study differs from previously published studies on the topic in several ways. First, our cohort is population-based. Second, we were able to differentiate between type 1 and type 2 diabetes Finally, we used a composite outcome of severe liver disease rather than single diagnoses - or mortality from single diagnoses. For health care providers, the results from study 2 can increase the awareness of the risk of severe liver disease in the large patient population with type 2 diabetes and increase the probability of earlier detection.

In study 3, the main findings were the high prevalence of elastography-defined NAFLD and elevated liver stiffness in patients with type 2 diabetes, and that an improvement in glycemic control after a 4-day personalized treatment program was associated with an improvement of hepatic steatosis. As we investigated a comparably small sample of study participants, the frequencies of NAFLD and elevated liver stiffness should be interpreted with caution. However, the observed results indicate a significant prevalence of clinically relevant liver disease in this cohort. In the previously mentioned study by Kwok and colleagues, where the prevalence of increased CAP and elevated liver stiffness in a cohort of patients with type 2 diabetes were evaluated, increased CAP was found in 72.8% of patients and elevated liver stiffness was found in 17.7% of patients. In our study we found a baseline prevalence of increased CAP of 76%, and a baseline prevalence of elevated liver stiffness of 24.2%. The similarities between our results and the results reported in the study by Kwok and our study indicate that the results we observed are somewhat representative of the general frequency of NAFLD and elevated liver stiffness in patients with type 2 diabetes. However, some significant differences between our studies should be noted. On the one hand, our cohort consisted of patients with difficult-to-treat type 2 diabetes, whereas the cohort in the study by Kwok and colleagues consisted of patients who were recruited to the study while undergoing screening for complications from type 2 diabetes. This difference would probably result in a higher prevalence of NAFLD and elevated liver stiffness in our cohort. On the other hand, the cut-off used for defining steatosis grade 1 in the study by Kwok and colleagues was 233 dB/m, whereas we used a cut-off of 268 dB/m. The broader classification of NAFLD in the study by Kwok and colleagues meant that individuals whom in our study would have been identified as not having NAFLD were classified as having NAFLD, thus increasing the prevalence in the study by Kwok and colleagues. A reasonable synthesis of these findings would be that the prevalence of NAFLD and advanced fibrosis increases with the severity of diabetes and insulin resistance.

The observation that a personalized treatment program aimed at improving metabolic control is associated with reduction in hepatic steatosis is in line with previously reported effects of diet, exercise and some anti-diabetic medications on NAFLD. The clinical implications of these findings could be that physicians treating patients with type 2 diabetes and improving their glycemic control can also improve the liver health of the patients. However, long-term effects regarding liver-related outcomes and mortality from this type of personalized treatment program in patients with type 2 diabetes remains uncertain.

In study 4, the main finding was that NAFLD was associated with an increased risk of cancer. The risk of HCC was comparably high, while the risk of other forms of cancer was only slightly elevated. Comparison to results from previously published studies is somewhat complicated by differences in the characteristics of study cohorts and differences in confounders adjusted for in multivariable models. In the 2018 study by Kanwal and colleagues, where an increased risk of HCC compared to patients without NAFLD was found primarily in patients with NAFLD and cirrhosis, for example, the diagnosis of NAFLD was made by identification of consecutively elevated ALT levels, and the cohort consisted of a majority of male subjects. We found a higher IR of HCC in patients with NAFLD but without cirrhosis than was reported in the study by Kanwal and colleagues. One explanation for this could be that we included only patients with NAFLD that had come in to contact with specialized care, and therefore belonged to a higher risk category than the patients in the study by Kanwal and colleagues, or that cirrhosis is under-coded in the NPR.

The recent results from a study by Simon and colleagues on Swedish patients with biopsy-proven NAFLD indicate a significant increase in risk HCC compared to individuals without NAFLD (aHR 1.27, 95% CI=1.18-1.36). These results are rather similar to results from study 4, in which we calculated an aHR for patients with NAFLD of 1.22 (95% CI=1.12-1.33) for any cancer compared to matched reference individuals. Regarding risk of HCC, Simon and colleagues reported an increased risk in patients with NAFLD (aHR 17.08, 95% CI=11.56-25.25), while we observed an aHR for HCC of 12.18 (95% CI=7.15-20.79) for patients with NAFLD. One would expect our results to fairly similar, as the cohorts of patients that we studied in large part probably consists of the same patients (Swedish individuals who have received a diagnosis of NAFLD in specialized care during the last decades). Important differences between the study by Simon and colleagues and study 4 include the diagnostic modality, where Simon and colleagues included only patients who had undergone liver biopsy, and therefore likely had more severe liver disease. This is reflected in the somewhat lower risk-estimates in our study.

In terms of clinical implications, the results in study 4 together with previous studies indicate that screening for cancer, except for HCC, in patients with NAFLD is unlikely to be necessary due to the low absolute risk of cancer. Regarding screening for HCC, it is likely of use in the group of patients with NAFLD with the highest risk of HCC. How to define this group, and how to outline a potential screening program, will be an area of future research. The results from our study indicates that the elevated risk of HCC is present in some patients with NAFLD even if they do not have cirrhosis.

5.1 LIMITATIONS

All four studies included in this thesis were of observational design. As the observational design lacks randomization and therefore inherently carries residual confounding, conclusions regarding causality cannot be drawn from generated results. Even in studies where reference individuals free of the exposure of interest are included, as was the case in studies 2 and 4 in this thesis, one must be careful to only draw conclusions about associations

between exposures and outcomes, and not about causal mechanisms. While causal inference is usually not possible from observational studies, observed results can still be of value both in clinical settings, in construction of screening strategies and in generating hypotheses ample for testing in randomized trials. Further, some hypotheses are not testable in a randomized setting. It would not be possible, for example, to randomize one group of study participants to develop type 2 diabetes and one group to remain free of diabetes and then examine the effects on development of severe liver disease. This ties in to one of the most fundamental aspects of science; the idea that the objective of scientific endeavor is not to prove one's hypothesis, but to try to refute it. In a scientific sense, there is no way to "prove" a hypothesis. Since no experiment in science can be carried out in a perfect, limitation-free manner, all observed results and subsequent conclusions carry caveats. Thus, it is inaccurate to speak in terms of "scientifically proving" something, or that something has been "scientifically proved". While a hypothesis cannot be proved, it can have more or less support. Where we as scientists and medical professionals choose to draw a line and postulate that a hypothesis has been researched enough, and has enough support to implemented in clinical practice, is a question that requires careful consideration on a case-by-case basis. The potential benefits of implementing a new routine in clinical practice must be weighed against the risk of acting on the hitherto generated evidence. The scientific mechanism of probabilistic reasoning has to be communicated in a pedagogical way to the general public, to policy makers, and - in the case of medical research - to patients that will be affected by implementing scientific findings in clinical practice. Overstating the certainty of one's findings in medical science could, in a worst case scenario, put patients at harm. Further, it could undermine the public trust of science. Thus, understanding and clarifying limitations in one's studies is a fundamental aspect of sound scientific research.

The inclusion of study participants using national, population-based registries has both benefits and drawbacks. The fact that all individuals in the population of Sweden, regardless of ethnic background, socioeconomic status, sex or place of living are included in the registries benefits the diversity of the generated cohorts. In turn, the diversity of the cohorts benefits the external validity of studies performed on the population-based cohorts. The reliance on patient registries for constructing cohorts confers a selection bias in that an individual has to come in contact with healthcare to be registered. If an individual suffers from NAFLD, the disease can go unrecognized for long periods of time if no examination is performed. The probability of inclusion in the registry in question is thus dependent on the propensity of the individual with the disease to seek healthcare (either for a general check-up or for evaluation of some specific symptom). The propensity of the individual to seek healthcare can be associated with a number of confounding factors that introduces a bias into generated risk-estimates. A similar impediment to external validity was present in study 3, where we investigated a cohort of patients with type 2 diabetes who enrolled in a 4-day program aimed at improving glycemic control and other metabolic parameters. As the referral of their primary care provider meant that the patients had to take active part in care provided at a specialist center in a university hospital, a selection was likely present were some patients

that were not inclined to take a more active part in the treatment of their condition did not enroll in the program and were thus left out of our cohort. Another limitation of using registries to study a disease like NAFLD is the internal validity, due to the disease being notoriously under-recognized in the registries. The under-recognition of NAFLD in the registries likely has several reasons. First, knowledge of the diagnosis is lacking in the broader spectrum of healthcare personnel, leading to a low likelihood of detecting the disease in its most common, non-symptomatic form. Second, easy to use, economically feasible and reliable diagnostic tools are lacking. Hence, even as knowledge about NAFLD is increasing among health care personnel, the diagnostic difficulties hamper real-world diagnosing in patients. The resulting effect of this misclassifications in studies using registry-based cohorts will most often be falsely low risk-estimates, as the effect is diluted by many individuals with NAFLD that are wrongly included as reference individuals.

In study 1, the size of the cohort was a limitation in that it possibly was not large enough to detect all true associations between exposure (histological features of NAFLD) and risk of developing the outcome (type 2 diabetes). Further, as identification of development of type 2 diabetes in a study participant is reliant either on the disease producing symptoms inferring the patient to seek healthcare, or on detection of abnormal blood glucose levels during a general health check-up, it is likely that some patients actually did develop type 2 diabetes during follow-up but went undiagnosed. This could potentially have led to both falsely low and falsely high risk-estimates. Another limitation in study 1 was the amount of missing data of baseline variables that we deemed important to include in Cox regression analysis. Namely, baseline documentation on fasting glucose and triglycerides were missing in 89 (22%) and 145 (37%) patients, respectively. This limitation could also introduce bias which could lead to both falsely low and falsely high risk-estimates. When participants are included due to having undergone some type of diagnostic test, it is paramount to consider the indications for the test, as this might introduce an important selection bias. The individuals included in study 1 had undergone liver biopsies due to persistently elevated liver transaminases. This compromises the external validity of study 1, in the sense that many patients with NAFLD does not have abnormal liver transaminases, and that conclusions drawn in the study therefore might not be relevant for the large population of patients with NAFLD and normal liver transaminases.

In study 2, the relatively short follow-up time was a limitation. As patients were followed for a median of 7.7 years, and severe liver disease as previously mentioned can develop over decades, we most likely did not have enough time to fully observe a potential association between type 2 diabetes and development of severe liver disease. This limitation would generate a falsely low risk-estimate. A further limitation in study 2 is the probable misclassification of patients with undiagnosed type 2 diabetes as reference individuals free of diabetes. As is the case with NAFLD, type 2 diabetes is a disease that can be present in a patient for long time periods with no apparent symptoms. Thus, it is likely that some individuals included in study 2 as reference individuals in fact suffered from type 2 diabetes. This would lead to a dilution of the observed hypothesized effect of type 2 diabetes on the

risk of developing severe liver disease, and falsely low risk estimates. Another source of misclassification is that a substantial number of participants, both patients with type 2 diabetes and reference individuals, included in study 2 likely had an alcohol intake which could cause liver disease without having received a diagnosis of alcohol abuse. As we were aiming to investigate the effects of type 2 diabetes - and not excessive alcohol consumption - on liver disease this could reduce the internal validity of our study. It is not entirely obvious, though, that this misclassification would differ between exposed individuals and reference individuals, which would have made it more problematic. In the Cox regression analysis of risk factors for development of severe liver disease, we did not include reference individuals as we only had data on age, sex and living location on these individuals. Had we been able to obtain more baseline variables for the reference individuals free of diabetes, the conclusions we were able to draw from the study regarding specific risk factors for severe liver disease in patients with type 2 diabetes could have been more solid.

Participants in study 3 were included from group of patients that had been referred to an endocrinology clinic by primary care providers as they found it difficult to manage the participants' disease course. This limits the generalizability of the conclusions study 2, as the results are not generalizable to patients whose metabolic control is successfully managed in the primary care setting. Further, the fact that a number of individuals declined to participate in the study could introduce a selection bias as these individuals possibly differ from the individuals that chose participate. The individuals that did not participate in the study could, for example, have more severe disease. If this is the case, then we likely observed falsely low estimates of NAFLD and elevated liver stiffness in the cohort. As in study 1, the size of the cohort in study 3 warrants caution when interpreting the results. In all, 91 patients were enrolled in the study at baseline. Due to technical difficulties performing the transient electrography examination, software malfunctioning, and loss to follow-up, the number of participants with good quality baseline and follow-up measurements of steatosis was reduced to 61 patients, out of which 39 had NAFLD at baseline. With this relatively small number of individuals in the study cohort, the risk of obtaining results that makes one accept the null hypothesis when in fact the alternative hypothesis is correct (i.e. type 2 error) is increased. Another limitation to consider in study 2 is related to the technical difficulties of performing the transient electrography examination in severely obese patients. As this can be more difficult, a large proportion of failed examinations occurred in severely obese patients, thus skewing the cohort towards less obese, and likely metabolically more healthy patients. The observed 76% baseline prevalence of NAFLD and 24.2% baseline prevalence of kPa values indicating significant fibrosis were, therefore, likely falsely low estimates.

In addition to the above-mentioned limitation of under-diagnosing of NAFLD in study 4, a further limitation was the inclusion of patients with NAFLD from the NPR. As the NPR does not include patients that are followed in primary care, and this is where the overwhelming majority of patients with NAFLD are followed, we likely had a selection of comparably severe cases of NAFLD, who had for some reason encountered specialist care, in our study. However, the relatively low prevalence if cirrhosis argues against selection bias as a major

threat to the study's results. As in study 2, even though we did not include any individuals who had received a diagnosis of alcohol-related liver disease or alcohol abuse, we might have included individuals with liver disease due to alcohol-related liver disease. In the Cox regression models, we incorporated four baseline variables deemed to be confounders of interest: diabetes, COPD, hypertension and hyperlipidemia. To identify if these covariates were present at baseline we used the NPR. As earlier alluded to, these diagnoses can be present in patients for long periods of time, and only detected and diagnosed when the patient encounters healthcare. Further, they are only entered into the NPR when a patient encounters specialist care. Thus, it is highly likely that we under-diagnosed these conditions in our study cohort, and that a significant amount of residual confounding therefore was present.

6 CONCLUSIONS

The main findings from the four studies included in this doctoral thesis - and the subsequent conclusions generated from these findings - can be summarized as follows:

- The risk of type 2 diabetes is elevated in patients with NAFLD. Higher stage of fibrosis on liver biopsy associates with higher risk of type 2 diabetes. For patients with NAFLD but little fibrosis, higher amount of fat on liver biopsy associates with higher risk of type 2 diabetes.

- The risk of severe liver disease is increased in patients with type 2 diabetes compared with matched controls from the background population. While this increase in risk of severe liver disease is observed across all age groups of patients with type 2 diabetes, patients under 50 years of age have a low absolute risk. Among patients with type 2 diabetes, male sex, older age, smoking, higher BMI, presence of hypertension, and lower GFR is associated with an increased risk of severe liver disease.

- Patients with type 2 diabetes deemed difficult to treat by a primary care physician have a high prevalence of NAFLD and elevated liver stiffness, indicating presence of significant fibrosis. In these patients, reduction in fasting glucose achieved by a four-day personalized treatment program is associated with a decrease in hepatic steatosis during a three-month period.

- In the general population in Sweden, a diagnosis of NAFLD is associated with a somewhat increased risk of developing cancer. While a slight association between NAFLD and risk of bladder, kidney and uterine cancer, and between NAFLD and risk of colorectal cancer in men is present, the strongest association is seen for NAFLD and HCC. The results argue against specific screening for cancer in an unselected NAFLD population.

7 FUTURE RESEARCH

Several important questions regarding the interaction between type 2 diabetes and NAFLD, and the optimal clinical care of the large populations with these conditions, remain to be answered. One of the more pressing issues, which we've partly aimed to address in this doctoral thesis, is how to construct the optimal screening strategy for identification of NAFLD in patients with metabolic syndrome or type 2 diabetes. In Sweden, the absolute majority of with type 2 diabetes is managed in primary care. As previously mentioned, the majority of patients with NAFLD will not go on to develop end-stage liver disease. Nonetheless, the size of the patient population likely means that even if only a fraction of patients with NAFLD eventually develop severe liver disease, this will eventually place a heavy burden on an already strained health care system. Thus, a concise strategy implemented on the primary care level for identifying patients with a high risk of developing severe liver disease would likely be of great individual, systemic and - in the long run - societal benefit. As made clear by the last few decades of research on this topic, translating results from scientific studies on the matter into the desired strategy for finding high risk patients is very difficult. Using a screening strategy that is too inclusive would result in excess number of patients being referred from primary care to specialist centers, draining much needed resources quickly. Using a screening strategy that is too selective, on the other hand, would result in misclassification of high-risk patients as low-risk patients, potentially resulting in high levels of morbidity and mortality that could have been prevented. To clarify this question, future studies will need a number of high-quality characteristics compared to previous studies. First, capture rate of both exposure and outcomes will have to be higher. That is, NAFLD, as well as other traits related to the metabolic syndrome, have to be diagnosed with a higher accuracy in future study cohorts than what has been achieved to date. The higher diagnostic accuracy could be achieved either through a higher use of high-quality diagnostics already available (such as liver biopsy), or by using new diagnostic methods. Given the amount of resources demanded by the currently available high-quality diagnostic methods, some combination of these two paths will likely be needed.

Likewise, while potentially not as big of a problem in previous studies, the outcomes of severe liver disease will need to be identified with higher accuracy. Further, as both type 2 diabetes, NAFLD, and potential end-stage complications of NAFLD develop over extended time periods, sufficient follow-up will be of key importance in future studies of what characterizes high-risk patients. As mentioned previously, hitherto performed studies on both the risk of type 2 diabetes in patients with NAFLD, as well as studies on the risk of development of severe liver disease in patients with type 2 diabetes, have mostly had follow-up times spanning from five to ten years. Sufficient follow-up in the context of these conditions would likely require studies following cohorts of patients for several decades, reflecting the time it takes for a patient to (potentially) pass through the different stages of disease progression. Parallel to observational studies, further mechanistic studies on biochemical pathways and causal associations will most likely be paramount in detangling how type 2 diabetes, NAFLD, and severe liver disease interact.

The question of how treatment of a patients type 2 diabetes affects the often co-existing NAFLD also requires further research. With conditions that are so likely to co-exist, consideration has to be given to the possibility that treatment of one of the two condition worsens the other. Likewise, the frequent co-existence of type 2 diabetes and NAFLD hints that they are caused by similar mechanisms, and therefore possibly best treated with similar pharmacological and life-style strategies. Thus, further elucidating which treatments of type 2 diabetes that are potentially beneficial also for treating NAFLD will likely be a continued focus of future studies.

As with previously described regarding studies on the interactions between NAFLD and type 2 diabetes, future studies on the link between NAFLD and cancer will require higher capture rates of both exposures and outcomes, as well as longer follow-ups. Given the relatively low risk-estimates in previous studies examining risk of non-HCC cancer in patients with NAFLD, future studies will likely focus on clarifying how to distinguish patients with NAFLD who have a high risk of HCC from patients with NAFLD who a low risk of HCC. Again, this will be a joint effort between epidemiologic research and biochemical research aimed at finding mechanistic, causal pathways. Results from current studies of treatments for NAFLD are often evaluated on histological end-points. As medications are approved and put to use in the large patient population with NAFLD, subsequent studies with longer follow-up will also be able to evaluate the potential effect on reducing the risk NAFLD-related HCC.

8 ACKNOWLEDGEMENTS

All patients that were included in the studies of this thesis. For contributing to the advancement of health and knowledge.

Hannes Hagström, my main supervisor. For the all the guidance on a sometimes challenging journey, for sharing your deep knowledge of science and medicine, and for your everlasting, minute-by-minute feedback and encouragement on everything I've done.

Per Stål, my co-supervisor. For your kindness and calm wisdom, for your deep knowledge of hepatology, and for putting things in perspective.

Ylva Trolle-Lagerros, my co-supervisor. For all your upbeat advice on writing and science, for your deep knowledge of endocrinology, and for introducing me to your research group.

All my co-authors, Rolf Hultcrantz, Stefan Franzén, Björn Eliasson, Mervete Miftaraj, Soffia Gudbjörnsdottir, Ann-Marie Svensson, Magnus Holmer, Bonnie Bengtsson, Johan Hoffstedt and Linnea Widman. For great collaborations.

Everyone in the MILES and PSC research groups at Karolinska Institutet. For contributing to a creative and productive research environment, and for all the dinners.

My father, Anders. For helping me navigate through all aspects of life and for being an excellent role model.

My mother, Christina. For always being there for me and for inspiring me to take the right path, even when it was difficult.

My siblings, Simon, Rakel, Elin, Sofia and Julia. For setting great examples on how to be fundamentally good people, and for always showing me the way. **All the in-laws**, without you our family would not have been the same.

Anton, **Jonatan**, **Pete**, **Marcus**, **Markus,** the whole of "**matlaget"** and all other friends. For all your support, for speaking when I stayed quiet, and for listening when I had something to say.

Nicklas, my scientific mentor, brother-in-law and friend. For hundreds - if not thousands - of hours of conversations on curiosity, science, companionship and the meaning of life itself. For providing rock solid support to our family when we needed it the most.

Ani, my dear wife. For your wonderful laughter and charm, for your beauty and clever thoughts on life, and for never failing to stand by my side. "*Vad du anförtror åt mig, ska jag anförtro åt dig*". I love you.

9 REFERENCES

1. Collaboration NCDRF. Worldwide trends in diabetes since 1980: a pooled analysis of 751 population-based studies with 4.4 million participants. Lancet. 2016;387(10027):1513-30.
2. Collaboration NCDRF. Trends in adult body-mass index in 200 countries from 1975 to 2014: a pooled analysis of 1698 population-based measurement studies with 19.2 million participants. Lancet. 2016;387(10026):1377-96.
3. 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(1):73-84.
4. 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(6):1313-21.
5. Angulo P. Nonalcoholic fatty liver disease. N Engl J Med. 2002;346(16):1221-31.
6. Ludwig J, Viggiano TR, McGill DB, Oh BJ. Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease. Mayo Clin Proc. 1980;55(7):434-8.
7. Estes C, Razavi H, Loomba R, Younossi Z, Sanyal AJ. Modeling the epidemic of nonalcoholic fatty liver disease demonstrates an exponential increase in burden of disease. Hepatology. 2018;67(1):123-33.
8. Harrison SA, Gawrieh S, Roberts K, Lisanti CJ, Schwope RB, Cebe KM, et al. Prospective evaluation of the prevalence of non-alcoholic fatty liver disease and steatohepatitis in a large middle-aged US cohort. J Hepatol. 2021.
9. Sweet PH, Khoo T, Nguyen S. Nonalcoholic Fatty Liver Disease. Prim Care. 2017;44(4):599-607.
10. Younossi ZM, Stepanova M, Lawitz EJ, Reddy KR, Wai-Sun Wong V, Mangia A, et al. Patients With Nonalcoholic Steatohepatitis Experience Severe Impairment of Health-Related Quality of Life. Am J Gastroenterol. 2019;114(10):1636-41.
11. Miele L, Zocco MA, Pizzolante F, De Matthaeis N, Ainora ME, Liguori A, et al. Use of imaging techniques for non-invasive assessment in the diagnosis and staging of non-alcoholic fatty liver disease. Metabolism. 2020;112:154355.
12. Portillo-Sanchez P, Bril F, Maximos M, Lomonaco R, Biernacki D, Orsak B, et al. High Prevalence of Nonalcoholic Fatty Liver Disease in Patients With Type 2 Diabetes Mellitus and Normal Plasma Aminotransferase Levels. J Clin Endocrinol Metab. 2015;100(6):2231-8.
13. 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(5):1751-9.
14. Bedossa P, Consortium FP. Utility and appropriateness of the fatty liver inhibition of progression (FLIP) algorithm and steatosis, activity, and fibrosis (SAF) score in the

evaluation of biopsies of nonalcoholic fatty liver disease. Hepatology. 2014;60(2):565-75.

15. Simon TG, Roelstraete B, Khalili H, Hagström H, Ludvigsson JF. Mortality in biopsy-confirmed nonalcoholic fatty liver disease: results from a nationwide cohort. Gut. 2020.

16. Tapper EB, Loomba R. Noninvasive imaging biomarker assessment of liver fibrosis by elastography in NAFLD. Nat Rev Gastroenterol Hepatol. 2018;15(5):274-82.

17. McPherson S, Stewart SF, Henderson E, Burt AD, Day CP. Simple non-invasive fibrosis scoring systems can reliably exclude advanced fibrosis in patients with non-alcoholic fatty liver disease. Gut. 2010;59(9):1265-9.

18. Sterling RK, Lissen E, Clumeck N, Sola R, Correa MC, Montaner J, et al. Development of a simple noninvasive index to predict significant fibrosis in patients with HIV/HCV coinfection. Hepatology. 2006;43(6):1317-25.

19. 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(4):846-54.

20. Castera L, Friedrich-Rust M, Loomba R. Noninvasive Assessment of Liver Disease in Patients With Nonalcoholic Fatty Liver Disease. Gastroenterology. 2019;156(5):1264-81.e4.

21. 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(5):251-64.

22. Romeo S, Kozlitina J, Xing C, Pertsemlidis A, Cox D, Pennacchio LA, et al. Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease. Nat Genet. 2008;40(12):1461-5.

23. Kozlitina J, Smagris E, Stender S, Nordestgaard BG, Zhou HH, Tybjaerg-Hansen A, et al. Exome-wide association study identifies a TM6SF2 variant that confers susceptibility to nonalcoholic fatty liver disease. Nat Genet. 2014;46(4):352-6.

24. Kantartzis K, Peter A, Machicao F, Machann J, Wagner S, Konigsrainer I, et al. Dissociation between fatty liver and insulin resistance in humans carrying a variant of the patatin-like phospholipase 3 gene. Diabetes. 2009;58(11):2616-23.

25. Speliotes EK, Butler JL, Palmer CD, Voight BF, Consortium G, Consortium MI, et al. PNPLA3 variants specifically confer increased risk for histologic nonalcoholic fatty liver disease but not metabolic disease. Hepatology. 2010;52(3):904-12.

26. Liu YL, Patman GL, Leathart JB, Piguet AC, Burt AD, Dufour JF, et al. Carriage of the PNPLA3 rs738409 C >G polymorphism confers an increased risk of non-alcoholic fatty liver disease associated hepatocellular carcinoma. J Hepatol. 2014;61(1):75-81.

27. Blais P, Husain N, Kramer JR, Kowalkowski M, El-Serag H, Kanwal F. Nonalcoholic fatty liver disease is underrecognized in the primary care setting. Am J Gastroenterol. 2015;110(1):10-4.

28. 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(3):1078.

29. Marjot T, Sbardella E, Moolla A, Hazlehurst JM, Tan GD, Ainsworth M, et al. Prevalence and severity of non-alcoholic fatty liver disease are underestimated in clinical practice: impact of a dedicated screening approach at a large university teaching hospital. Diabet Med. 2018;35(1):89-98.

30. 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(2):144-51.

31. Rashu EB, Werge MP, Hetland LE, Junker AE, Jensen MK, Gluud LL. Referral Patterns for Patients with Nonalcoholic Fatty Liver Disease. J Clin Med. 2021;10(3).

32. Valenti L, Bugianesi E, Pajvani U, Targher G. Nonalcoholic fatty liver disease: cause or consequence of type 2 diabetes? Liver Int. 2016;36(11):1563-79.

33. Bugianesi E, Gastaldelli A, Vanni E, Gambino R, Cassader M, Baldi S, et al. Insulin resistance in non-diabetic patients with non-alcoholic fatty liver disease: sites and mechanisms. Diabetologia. 2005;48(4):634-42.

34. Lyssenko V, Almgren P, Anevski D, Perfekt R, Lahti K, Nissén M, et al. Predictors of and longitudinal changes in insulin sensitivity and secretion preceding onset of type 2 diabetes. Diabetes. 2005;54(1):166-74.

35. Kissebah AH, Vydelingum N, Murray R, Evans DJ, Hartz AJ, Kalkhoff RK, et al. Relation of body fat distribution to metabolic complications of obesity. J Clin Endocrinol Metab. 1982;54(2):254-60.

36. Fujioka S, Matsuzawa Y, Tokunaga K, Tarui S. Contribution of intra-abdominal fat accumulation to the impairment of glucose and lipid metabolism in human obesity. Metabolism. 1987;36(1):54-9.

37. Nielsen S, Guo Z, Johnson CM, Hensrud DD, Jensen MD. Splanchnic lipolysis in human obesity. J Clin Invest. 2004;113(11):1582-8.

38. Shulman GI. Ectopic fat in insulin resistance, dyslipidemia, and cardiometabolic disease. N Engl J Med. 2014;371(12):1131-41.

39. Magkos F, Su X, Bradley D, Fabbrini E, Conte C, Eagon JC, et al. Intrahepatic diacylglycerol content is associated with hepatic insulin resistance in obese subjects. Gastroenterology. 2012;142(7):1444-6.e2.

40. Musso G, Gambino R, Cassader M. Recent insights into hepatic lipid metabolism in non-alcoholic fatty liver disease (NAFLD). Prog Lipid Res. 2009;48(1):1-26.

41. Kohjima M, Higuchi N, Kato M, Kotoh K, Yoshimoto T, Fujino T, et al. SREBP-1c, regulated by the insulin and AMPK signaling pathways, plays a role in nonalcoholic fatty liver disease. Int J Mol Med. 2008;21(4):507-11.

42. Uyeda K, Repa JJ. Carbohydrate response element binding protein, ChREBP, a transcription factor coupling hepatic glucose utilization and lipid synthesis. Cell Metab. 2006;4(2):107-10.

43. Mitro N, Mak PA, Vargas L, Godio C, Hampton E, Molteni V, et al. The nuclear receptor LXR is a glucose sensor. Nature. 2007;445(7124):219-23.

44. Okamoto M, Takeda Y, Yoda Y, Kobayashi K, Fujino MA, Yamagata Z. The association of fatty liver and diabetes risk. J Epidemiol. 2003;13(1):15-21.

45. Shibata M, Kihara Y, Taguchi M, Tashiro M, Otsuki M. Nonalcoholic fatty liver disease is a risk factor for type 2 diabetes in middle-aged Japanese men. Diabetes Care. 2007;30(11):2940-4.

46. Kim CH, Park JY, Lee KU, Kim JH, Kim HK. Fatty liver is an independent risk factor for the development of Type 2 diabetes in Korean adults. Diabet Med. 2008;25(4):476-81.

47. Park SK, Seo MH, Shin HC, Ryoo JH. Clinical availability of nonalcoholic fatty liver disease as an early predictor of type 2 diabetes mellitus in Korean men: 5-year prospective cohort study. Hepatology. 2013;57(4):1378-83.

48. Yamazaki H, Tsuboya T, Tsuji K, Dohke M, Maguchi H. Independent Association Between Improvement of Nonalcoholic Fatty Liver Disease and Reduced Incidence of Type 2 Diabetes. Diabetes Care. 2015;38(9):1673-9.

49. Shah RV, Allison MA, Lima JA, Bluemke DA, Abbasi SA, Ouyang P, et al. Liver fat, statin use, and incident diabetes: The Multi-Ethnic Study of Atherosclerosis. Atherosclerosis. 2015;242(1):211-7.

50. Chen SC, Tsai SP, Jhao JY, Jiang WK, Tsao CK, Chang LY. Liver Fat, Hepatic Enzymes, Alkaline Phosphatase and the Risk of Incident Type 2 Diabetes: A Prospective Study of 132,377 Adults. Sci Rep. 2017;7(1):4649.

51. Bae JC, Rhee EJ, Lee WY, Park SE, Park CY, Oh KW, et al. Combined effect of nonalcoholic fatty liver disease and impaired fasting glucose on the development of type 2 diabetes: a 4-year retrospective longitudinal study. Diabetes Care. 2011;34(3):727-9.

52. Sung KC, Jeong WS, Wild SH, Byrne CD. Combined influence of insulin resistance, overweight/obesity, and fatty liver as risk factors for type 2 diabetes. Diabetes Care. 2012;35(4):717-22.

53. Kasturiratne A, Weerasinghe S, Dassanayake AS, Rajindrajith S, de Silva AP, Kato N, et al. Influence of non-alcoholic fatty liver disease on the development of diabetes mellitus. J Gastroenterol Hepatol. 2013;28(1):142-7.

54. Chang Y, Jung HS, Yun KE, Cho J, Cho YK, Ryu S. Cohort study of non-alcoholic fatty liver disease, NAFLD fibrosis score, and the risk of incident diabetes in a Korean population. Am J Gastroenterol. 2013;108(12):1861-8.

55. Choi JH, Rhee EJ, Bae JC, Park SE, Park CY, Cho YK, et al. Increased risk of type 2 diabetes in subjects with both elevated liver enzymes and ultrasonographically diagnosed nonalcoholic fatty liver disease: a 4-year longitudinal study. Arch Med Res. 2013;44(2):115-20.

56. Ming J, Xu S, Gao B, Liu G, Ji Y, Yang F, et al. Non-alcoholic fatty liver disease predicts type 2 diabetes mellitus, but not prediabetes, in Xi'an, China: a five-year cohort study. Liver Int. 2015;35(11):2401-7.

57. Li WD, Fu KF, Li GM, Lian YS, Ren AM, Chen YJ, et al. Comparison of effects of obesity and non-alcoholic fatty liver disease on incidence of type 2 diabetes mellitus. World J Gastroenterol. 2015;21(32):9607-13.

58. Fukuda T, Hamaguchi M, Kojima T, Hashimoto Y, Ohbora A, Kato T, et al. The impact of non-alcoholic fatty liver disease on incident type 2 diabetes mellitus in non-overweight individuals. Liver Int. 2016;36(2):275-83.

59. Chen GY, Cao HX, Li F, Cai XB, Ao QH, Gao Y, et al. New risk-scoring system including non-alcoholic fatty liver disease for predicting incident type 2 diabetes in East China: Shanghai Baosteel Cohort. J Diabetes Investig. 2016;7(2):206-11.

60. Li Y, Wang J, Tang Y, Han X, Liu B, Hu H, et al. Bidirectional association between nonalcoholic fatty liver disease and type 2 diabetes in Chinese population: Evidence from the Dongfeng-Tongji cohort study. PLoS One. 2017;12(3):e0174291.

61. Ma J, Hwang SJ, Pedley A, Massaro JM, Hoffmann U, Chung RT, et al. Bi-directional analysis between fatty liver and cardiovascular disease risk factors. J Hepatol. 2017;66(2):390-7.

62. Liu M, Wang J, Zeng J, Cao X, He Y. Association of NAFLD With Diabetes and the Impact of BMI Changes: A 5-Year Cohort Study Based on 18,507 Elderly. J Clin Endocrinol Metab. 2017;102(4):1309-16.

63. Cho HJ, Hwang S, Park JI, Yang MJ, Hwang JC, Yoo BM, et al. Improvement of Nonalcoholic Fatty Liver Disease Reduces the Risk of Type 2 Diabetes Mellitus. Gut Liver. 2019;13(4):440-9.

64. Mantovani A, Dauriz M, Byrne CD, Lonardo A, Zoppini G, Bonora E, et al. Association between nonalcoholic fatty liver disease and colorectal tumours in asymptomatic adults undergoing screening colonoscopy: a systematic review and meta-analysis. Metabolism. 2018;87:1-12.

65. Ekstedt M, Franzen LE, Mathiesen UL, Thorelius L, Holmqvist M, Bodemar G, et al. Long-term follow-up of patients with NAFLD and elevated liver enzymes. Hepatology. 2006;44(4):865-73.

66. El-Serag HB, Tran T, Everhart JE. Diabetes increases the risk of chronic liver disease and hepatocellular carcinoma. Gastroenterology. 2004;126(2):460-8.

67. Wild SH, Morling JR, McAllister DA, Kerssens J, Fischbacher C, Parkes J, et al. Type 2 diabetes and risk of hospital admission or death for chronic liver diseases. J Hepatol. 2016;64(6):1358-64.

68. Alexander M, Loomis AK, van der Lei J, Duarte-Salles T, Prieto-Alhambra D, Ansell D, et al. Risks and clinical predictors of cirrhosis and hepatocellular carcinoma diagnoses in adults with diagnosed NAFLD: real-world study of 18 million patients in four European cohorts. BMC Med. 2019;17(1):95.

69. Nderitu P, Bosco C, Garmo H, Holmberg L, Malmström H, Hammar N, et al. The association between individual metabolic syndrome components, primary liver cancer and cirrhosis: A study in the Swedish AMORIS cohort. Int J Cancer. 2017;141(6):1148-60.

70. Pang Y, Kartsonaki C, Turnbull I, Guo Y, Clarke R, Chen Y, et al. Diabetes, Plasma Glucose, and Incidence of Fatty Liver, Cirrhosis, and Liver Cancer: A Prospective Study of 0.5 Million People. Hepatology. 2018;68(4):1308-18.

71. de Marco R, Locatelli F, Zoppini G, Verlato G, Bonora E, Muggeo M. Cause-specific mortality in type 2 diabetes. The Verona Diabetes Study. Diabetes Care. 1999;22(5):756-61.

72. Zoppini G, Fedeli U, Gennaro N, Saugo M, Targher G, Bonora E. Mortality from chronic liver diseases in diabetes. Am J Gastroenterol. 2014;109(7):1020-5.

73. Liu B, Balkwill A, Reeves G, Beral V, Collaborators MWS. Body mass index and risk of liver cirrhosis in middle aged UK women: prospective study. BMJ. 2010;340:c912.

74. Porepa L, Ray JG, Sanchez-Romeu P, Booth GL. Newly diagnosed diabetes mellitus as a risk factor for serious liver disease. CMAJ. 2010;182(11):E526-31.

75. El-Serag HB, Everhart JE. Diabetes increases the risk of acute hepatic failure. Gastroenterology. 2002;122(7):1822-8.

76. Liu TL, Trogdon J, Weinberger M, Fried B, Barritt ASt. Diabetes Is Associated with Clinical Decompensation Events in Patients with Cirrhosis. Dig Dis Sci. 2016;61(11):3335-45.

77. Stepanova M, Rafiq N, Younossi ZM. Components of metabolic syndrome are independent predictors of mortality in patients with chronic liver disease: a population-based study. Gut. 2010;59(10):1410-5.

78. Goh GB, Pan A, Chow WC, Yuan JM, Koh WP. Association between diabetes mellitus and cirrhosis mortality: the Singapore Chinese Health Study. Liver Int. 2017;37(2):251-8.

79. Wong RJ, Cheung R, Ahmed A. Nonalcoholic steatohepatitis is the most rapidly growing indication for liver transplantation in patients with hepatocellular carcinoma in the U.S. Hepatology. 2014;59(6):2188-95.

80. Makarova-Rusher OV, Altekruse SF, McNeel TS, Ulahannan S, Duffy AG, Graubard BI, et al. Population attributable fractions of risk factors for hepatocellular carcinoma in the United States. Cancer. 2016;122(11):1757-65.

81. Welzel TM, Graubard BI, Quraishi S, Zeuzem S, Davila JA, El-Serag HB, et al. Population-attributable fractions of risk factors for hepatocellular carcinoma in the United States. Am J Gastroenterol. 2013;108(8):1314-21.

82. Holmer M, Melum E, Isoniemi H, Ericzon BG, Castedal M, Nordin A, et al. Nonalcoholic fatty liver disease is an increasing indication for liver transplantation in the Nordic countries. Liver Int. 2018.

83. Jarvis H, Craig D, Barker R, Spiers G, Stow D, Anstee QM, et al. Metabolic risk factors and incident advanced liver disease in non-alcoholic fatty liver disease (NAFLD): A systematic review and meta-analysis of population-based observational studies. PLoS Med. 2020;17(4):e1003100.

84. Arab JP, Barrera F, Gallego C, Valderas JP, Uribe S, Tejos C, et al. High prevalence of undiagnosed liver cirrhosis and advanced fibrosis in type 2 diabetic patients. Ann Hepatol. 2016;15(5):721-8.

85. Casey SP, Kemp WW, McLean CA, Topliss DJ, Adams LA, Roberts SK. A prospective evaluation of the role of transient elastography for the detection of hepatic fibrosis in type 2 diabetes without overt liver disease. Scand J Gastroenterol. 2012;47(7):836-41.

86. Kwok R, Choi KC, Wong GL, Zhang Y, Chan HL, Luk AO, et al. Screening diabetic patients for non-alcoholic fatty liver disease with controlled attenuation parameter and liver stiffness measurements: a prospective cohort study. Gut. 2016;65(8):1359-68.

87. Sporea I, Mare R, Lupușoru R, Sima A, Sirli R, Popescu A, et al. Liver Stiffness Evaluation by Transient Elastography in Type 2 Diabetes Mellitus Patients with Ultrasound-proven Steatosis. J Gastrointestin Liver Dis. 2016;25(2):167-74.

88. Lai LL, Wan Yusoff WNI, Vethakkan SR, Nik Mustapha NR, Mahadeva S, Chan WK. Screening for non-alcoholic fatty liver disease in patients with type 2 diabetes mellitus using transient elastography. J Gastroenterol Hepatol. 2019;34(8):1396-403.

89. Mansour A, Mohajeri-Tehrani MR, Samadi M, Gerami H, Qorbani M, Bellissimo N, et al. Risk factors for non-alcoholic fatty liver disease-associated hepatic fibrosis in type 2 diabetes patients. Acta Diabetol. 2019;56(11):1199-207.

90. Sporea I, Mare R, Popescu A, Nistorescu S, Baldea V, Sirli R, et al. Screening for Liver Fibrosis and Steatosis in a Large Cohort of Patients with Type 2 Diabetes Using Vibration Controlled Transient Elastography and Controlled Attenuation Parameter in a Single-Center Real-Life Experience. J Clin Med. 2020;9(4).

91. Mikolasevic I, Domislovic V, Turk Wensveen T, Delija B, Klapan M, Juric T, et al. Screening for nonalcoholic fatty liver disease in patients with type 2 diabetes mellitus using transient elastography - a prospective, cross sectional study. Eur J Intern Med. 2020;82:68-75.

92. Lombardi R, Airaghi L, Targher G, Serviddio G, Maffi G, Mantovani A, et al. Liver fibrosis by FibroScan. Liver Int. 2020;40(2):347-54.

93. Mantovani A, Turino T, Lando MG, Gjini K, Byrne CD, Zusi C, et al. Screening for non-alcoholic fatty liver disease using liver stiffness measurement and its association with chronic kidney disease and cardiovascular complications in patients with type 2 diabetes. Diabetes Metab. 2020;46(4):296-303.

94. Kim GA, Lee HC, Choe J, Kim MJ, Lee MJ, Chang HS, et al. Association between non-alcoholic fatty liver disease and cancer incidence rate. J Hepatol. 2017.

95. Allen AM, Hicks SB, Mara KC, Larson JJ, Therneau TM. The risk of incident extrahepatic cancers is higher in non-alcoholic fatty liver disease than obesity - A longitudinal cohort study. J Hepatol. 2019;71(6):1229-36.

96. Kanwal F, Kramer JR, Mapakshi S, Natarajan Y, Chayanupatkul M, Richardson PA, et al. Risk of Hepatocellular Cancer in Patients With Non-Alcoholic Fatty Liver Disease. Gastroenterology. 2018;155(6):1828-37.e2.

97. Younossi ZM, Otgonsuren M, Henry L, Venkatesan C, Mishra A, Erario M, et al. Association of nonalcoholic fatty liver disease (NAFLD) with hepatocellular carcinoma (HCC) in the United States from 2004 to 2009. Hepatology. 2015;62(6):1723-30.

98. Stadlmayr A, Aigner E, Steger B, Scharinger L, Lederer D, Mayr A, et al. Nonalcoholic fatty liver disease: an independent risk factor for colorectal neoplasia. J Intern Med. 2011;270(1):41-9.

99. Wong VW, Wong GL, Tsang SW, Fan T, Chu WC, Woo J, et al. High prevalence of colorectal neoplasm in patients with non-alcoholic steatohepatitis. Gut. 2011;60(6):829-36.

100. Lee YI, Lim YS, Park HS. Colorectal neoplasms in relation to non-alcoholic fatty liver disease in Korean women: a retrospective cohort study. J Gastroenterol Hepatol. 2012;27(1):91-5.

101. Lin XF, Shi KQ, You J, Liu WY, Luo YW, Wu FL, et al. Increased risk of colorectal malignant neoplasm in patients with nonalcoholic fatty liver disease: a large study. Mol Biol Rep. 2014;41(5):2989-97.

102. Lee T, Yun KE, Chang Y, Ryu S, Park DI, Choi K, et al. Risk of Colorectal Neoplasia According to Fatty Liver Severity and Presence of Gall Bladder Polyps. Dig Dis Sci. 2016;61(1):317-24.

103. Ahn JS, Sinn DH, Min YW, Hong SN, Kim HS, Jung SH, et al. Non-alcoholic fatty liver diseases and risk of colorectal neoplasia. Aliment Pharmacol Ther. 2017;45(2):345-53.

104. Pan S, Hong W, Wu W, Chen Q, Zhao Q, Wu J, et al. The relationship of nonalcoholic fatty liver disease and metabolic syndrome for colonoscopy colorectal neoplasm. Medicine (Baltimore). 2017;96(2):e5809.

105. Chiang CL, Huang HH, Huang TY, Shih YL, Hsieh TY, Lin HH. Nonalcoholic Fatty Liver Disease Associated With Bladder Cancer. Am J Med Sci. 2020;360(2):161-5.

106. Nseir W, Abu-Rahmeh Z, Tsipis A, Mograbi J, Mahamid M. Relationship between Non-Alcoholic Fatty Liver Disease and Breast Cancer. Isr Med Assoc J. 2017;19(4):242-5.

107. Kwak MS, Yim JY, Yi A, Chung GE, Yang JI, Kim D, et al. Nonalcoholic fatty liver disease is associated with breast cancer in nonobese women. Dig Liver Dis. 2019;51(7):1030-5.

108. Park JH, Choi IS, Han KD, Park H, Kim KH, Kim JS. Association Between Fatty Liver Index and Risk of Breast Cancer: A Nationwide Population-Based Study. Clin Breast Cancer. 2020;20(4):e450-e7.

109. Simon TG, Roelstraete B, Sharma R, Khalili H, Hagström H, Ludvigsson JF. Cancer Risk in Patients With Biopsy-Confirmed Nonalcoholic Fatty Liver Disease: A Population-Based Cohort Study. Hepatology. 2021.

110. 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.

111. Haufe S, Engeli S, Kast P, Bohnke J, Utz W, Haas V, et al. Randomized comparison of reduced fat and reduced carbohydrate hypocaloric diets on intrahepatic fat in overweight and obese human subjects. Hepatology. 2011;53(5):1504-14.

112. Tendler D, Lin S, Yancy WS, Jr., Mavropoulos J, Sylvestre P, Rockey DC, et al. The effect of a low-carbohydrate, ketogenic diet on nonalcoholic fatty liver disease: a pilot study. Dig Dis Sci. 2007;52(2):589-93.

113. de Luis DA, Aller R, Izaola O, Gonzalez Sagrado M, Conde R. Effect of two different hypocaloric diets in transaminases and insulin resistance in nonalcoholic fatty liver disease and obese patients. Nutr Hosp. 2010;25(5):730-5.

114. Kontogianni MD, Tileli N, Margariti A, Georgoulis M, Deutsch M, Tiniakos D, et al. Adherence to the Mediterranean diet is associated with the severity of non-alcoholic fatty liver disease. Clin Nutr. 2014;33(4):678-83.

115. Luukkonen PK, Dufour S, Lyu K, Zhang XM, Hakkarainen A, Lehtimäki TE, et al. Effect of a ketogenic diet on hepatic steatosis and hepatic mitochondrial metabolism in nonalcoholic fatty liver disease. Proc Natl Acad Sci U S A. 2020;117(13):7347-54.

116. Holmer M, Lindqvist C, Petersson S, Moshtaghi-Svensson J, Tillander V, Brismar TB, et al. Treatment of NAFLD with intermittent calorie restriction or low-carb high-fat diet - a randomised controlled trial. JHEP Rep. 2021;3(3):100256.

117. St George A, Bauman A, Johnston A, Farrell G, Chey T, George J. Independent effects of physical activity in patients with nonalcoholic fatty liver disease. Hepatology. 2009;50(1):68-76.

118. Hallsworth K, Fattakhova G, Hollingsworth KG, Thoma C, Moore S, Taylor R, et al. Resistance exercise reduces liver fat and its mediators in non-alcoholic fatty liver disease independent of weight loss. Gut. 2011;60(9):1278-83.

119. Bugianesi E, Gentilcore E, Manini R, Natale S, Vanni E, Villanova N, et al. A randomized controlled trial of metformin versus vitamin E or prescriptive diet in nonalcoholic fatty liver disease. Am J Gastroenterol. 2005;100(5):1082-90.

120. 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(22):2297-307.

121. 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(4):1176-84.

122. Ratziu V, Giral P, Jacqueminet S, Charlotte F, Hartemann-Heurtier A, Serfaty L, et al. Rosiglitazone for nonalcoholic steatohepatitis: one-year results of the randomized placebo-controlled Fatty Liver Improvement with Rosiglitazone Therapy (FLIRT) Trial. Gastroenterology. 2008;135(1):100-10.

123. Haukeland JW, Konopski Z, Eggesbø HB, von Volkmann HL, Raschpichler G, Bjøro K, et al. Metformin in patients with non-alcoholic fatty liver disease: a randomized, controlled trial. Scand J Gastroenterol. 2009;44(7):853-60.

124. Omer Z, Cetinkalp S, Akyildiz M, Yilmaz F, Batur Y, Yilmaz C, et al. Efficacy of insulin-sensitizing agents in nonalcoholic fatty liver disease. Eur J Gastroenterol Hepatol. 2010;22(1):18-23.

125. 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(18):1675-85.

126. Torres DM, Jones FJ, Shaw JC, Williams CD, Ward JA, Harrison SA. Rosiglitazone versus rosiglitazone and metformin versus rosiglitazone and losartan in the treatment of nonalcoholic steatohepatitis in humans: a 12-month randomized, prospective, open- label trial. Hepatology. 2011;54(5):1631-9.

127. Sharma BC, Kumar A, Garg V, Reddy RS, Sakhuja P, Sarin SK. A Randomized Controlled Trial Comparing Efficacy of Pentoxifylline and Pioglitazone on Metabolic Factors and Liver Histology in Patients with Non-alcoholic Steatohepatitis. J Clin Exp Hepatol. 2012;2(4):333-7.

128. Bolinder J, Ljunggren Ö, Kullberg J, Johansson L, Wilding J, Langkilde AM, et al. Effects of dapagliflozin on body weight, total fat mass, and regional adipose tissue

distribution in patients with type 2 diabetes mellitus with inadequate glycemic control on metformin. J Clin Endocrinol Metab. 2012;97(3):1020-31.

129. Shao N, Kuang HY, Hao M, Gao XY, Lin WJ, Zou W. Benefits of exenatide on obesity and non-alcoholic fatty liver disease with elevated liver enzymes in patients with type 2 diabetes. Diabetes Metab Res Rev. 2014;30(6):521-9.

130. Cusi K, Orsak B, Bril F, Lomonaco R, Hecht J, Ortiz-Lopez C, et al. Long-Term Pioglitazone Treatment for Patients With Nonalcoholic Steatohepatitis and Prediabetes or Type 2 Diabetes Mellitus: A Randomized Trial. Ann Intern Med. 2016;165(5):305-15.

131. 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(10019):679-90.

132. Dutour A, Abdesselam I, Ancel P, Kober F, Mrad G, Darmon P, et al. Exenatide decreases liver fat content and epicardial adipose tissue in patients with obesity and type 2 diabetes: a prospective randomized clinical trial using magnetic resonance imaging and spectroscopy. Diabetes Obes Metab. 2016;18(9):882-91.

133. Yan J, Yao B, Kuang H, Yang X, Huang Q, Hong T, et al. Liraglutide, Sitagliptin, and Insulin Glargine Added to Metformin: The Effect on Body Weight and Intrahepatic Lipid in Patients With Type 2 Diabetes Mellitus and Nonalcoholic Fatty Liver Disease. Hepatology. 2019;69(6):2414-26.

134. Cusi K, Bril F, Barb D, Polidori D, Sha S, Ghosh A, et al. Effect of canagliflozin treatment on hepatic triglyceride content and glucose metabolism in patients with type 2 diabetes. Diabetes Obes Metab. 2019;21(4):812-21.

135. Eriksson JW, Lundkvist P, Jansson PA, Johansson L, Kvarnström M, Moris L, et al. Effects of dapagliflozin and n-3 carboxylic acids on non-alcoholic fatty liver disease in people with type 2 diabetes: a double-blind randomised placebo-controlled study. Diabetologia. 2018;61(9):1923-34.

136. Shimizu M, Suzuki K, Kato K, Jojima T, Iijima T, Murohisa T, et al. Evaluation of the effects of dapagliflozin, a sodium-glucose co-transporter-2 inhibitor, on hepatic steatosis and fibrosis using transient elastography in patients with type 2 diabetes and non-alcoholic fatty liver disease. Diabetes Obes Metab. 2019;21(2):285-92.

137. Kuchay MS, Krishan S, Mishra SK, Choudhary NS, Singh MK, Wasir JS, et al. Effect of dulaglutide on liver fat in patients with type 2 diabetes and NAFLD: randomised controlled trial (D-LIFT trial). Diabetologia. 2020;63(11):2434-45.

138. Kahl S, Gancheva S, Straßburger K, Herder C, Machann J, Katsuyama H, et al. Empagliflozin Effectively Lowers Liver Fat Content in Well-Controlled Type 2 Diabetes: A Randomized, Double-Blind, Phase 4, Placebo-Controlled Trial. Diabetes Care. 2020;43(2):298-305.

139. Bril F, Kalavalapalli S, Clark VC, Lomonaco R, Soldevila-Pico C, Liu IC, et al. Response to Pioglitazone in Patients With Nonalcoholic Steatohepatitis With vs Without Type 2 Diabetes. Clin Gastroenterol Hepatol. 2018;16(4):558-66.e2.

140. Rinella ME, Sanyal AJ. Management of NAFLD: a stage-based approach. Nat Rev Gastroenterol Hepatol. 2016;13(4):196-205.

141. Newsome PN, Buchholtz K, Cusi K, Linder M, Okanoue T, Ratziu V, et al. A Placebo-Controlled Trial of Subcutaneous Semaglutide in Nonalcoholic Steatohepatitis. N Engl J Med. 2021;384(12):1113-24.

142. Hagström H, Adams LA, Allen AM, Byrne CD, Chang Y, Grønbaek H, et al. Administrative coding in electronic health care record-based research of NAFLD: an expert panel consensus statement. Hepatology. 2021.

143. Andersson T, Ahlbom A, Magnusson C, Carlsson S. Prevalence and incidence of diabetes in Stockholm County 1990-2010. PLoS One. 2014;9(8):e104033.