Non-Alcoholic Fatty Liver Disease

Aspects on Diagnosis and Long-term Prognosis

Patrik Nasr

Linköping University Medical Dissertations No. 1690

Non-Alcoholic Fatty Liver Disease

Aspects on Diagnosis and Long-term Prognosis

Patrik Nasr

Division of Gastroenterology and Hepatology
Department of Medical and Health Sciences
Linköping University, Sweden
Linköping 2019

©Patrik Nasr, 2019

Published articles have been reprinted with the permission of the copyright holder.

Printed in Sweden by LiU-Tryck, Linköping, Sweden, 2019

ISBN 978-91-7685-038-1

ISSN 0345-0082

Till Anna och Lova

"All opinions are not equal. Some are a very great deal more robust,
sophisticated and well supported in logic and argument than others"

Douglas Adams

CONTENTS

ABSTRACT	1
SVENSK SAMMANFATTNING	5
LIST OF PAPERS	9
ABBREVIATIONS	11
ACKNOWLEDGEMENTS	13
1. INTRODUCTION	15
1.1 Background	15
1.2 Definition of Non-Alcoholic Fatty Liver Disease	15
1.3 Pathogenesis	17
1.3.1 Fat Accumulation	18
1.3.2 From Fat to Inflammation to Fibrosis	21
1.4 Diagnosis of NAFLD	24
1.4.1 Alcohol Consumption	24
1.4.2 Abnormal Liver Enzymes	26
1.4.3 Elastography	28
1.4.4 Magnetic Resonance Techniques	30
1.4.5 Liver Biopsy	33
1.5 Histological Course of NAFLD	35
1.5.1 Non-Alcoholic Steatohepatitis	36
1.5.2 Inflammation and Ballooning Degeneration	38
1.5.3 Fibrosis Stage	39
1.5.4 Steatosis	40
1.6 Prognosis – morbidity and mortality	41
1.6.1 Obesity	41
1.6.2 Type 2 Diabetes Mellitus	42
1.6.3 Cardiovascular Disease	44
1.6.4 Liver Transplantation	45

1.6.5 Mortality.....	46
2. AIMS	47
3. METHOD	49
3.1 Procedures	49
3.1.1 Enrolment of Patients (Papers I, II, III, IV)	49
3.2 Data Collection	51
3.2.1 Biochemical Investigation (Papers I, III, IV)	51
3.2.2 Collection of Biochemical Variables (Paper II)	52
3.2.3 Clinical Assessment (Papers I, III, IV).....	52
3.2.4 Suspected Diagnosis of NAFLD Before Liver Biopsy (Paper I)	53
3.2.5 Collection of Baseline Clinical Characteristics (Paper II).....	53
3.2.6 Assessment of Alcohol Consumption (Papers I, III, IV).....	54
3.2.7 Assessment of Significant Alcohol Consumption (Paper II)	54
3.2.8 Liver Biopsy (Papers I, II, III, IV).....	54
3.3 Histopathological Evaluation (Papers I, II, III, IV)	55
3.4 Quantitative Assessment of Hepatic Steatosis (Papers I, IV)	56
3.4.1 Stereological Point Counting (SPC) (Papers I, III, IV)	56
3.4.2 Proton Magnetic Resonance Spectroscopy – Proton Density Fat Fraction (¹ H-MRS PDFF) (Paper I)	56
3.5 Statistics (Paper I, II, III, IV)	57
3.6 Ethical considerations (Papers I, II, III, IV)	58
4. RESULTS	59
4.1 The Non-Invasive Liver Biopsy (NILB) study (Paper I).....	59
4.2 Long-term Retrospective Study (Paper II).....	61
4.3 Long-term Follow-up Study (Paper III, IV)	64
4.3.1 Histological Outcome of The Study Group (Paper III).....	64
4.3.2 Follow-up of Patients With NAFLD and Isolated Steatosis (Paper III).....	65
4.3.3 Quantitative Steatosis and Prediction of T2DM and Mortality (Paper IV).....	65
5. DISCUSSION	69
6. CONCLUSIONS	75
7. REFERENCES	77

ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease affecting approximately 25% of the global population. NAFLD is commonly recognized as the hepatic manifestation of the metabolic syndrome, *i.e.* abdominal adiposity, dyslipidaemia, hypertension, and type 2 diabetes mellitus (T2DM). Most individuals with NAFLD will develop T2DM, and vice versa – making the two conditions highly intertwined.

The histological spectrum of NAFLD ranges from simple steatosis to non-alcoholic steatohepatitis (NASH), which is defined by hepatocellular injury and inflammation, with risk of developing fibrosis and subsequent cirrhosis and hepatocellular carcinoma.

The gold standard for diagnosing NAFLD is liver biopsy. However, because of its invasive nature, liver biopsy entails some risk of adverse events and even death. Also, there is a high risk of sampling error, as well as intra- and interobserver variability, making the results unreliable.

Therefore, several non-invasive methods have been developed and validated in evaluating presence of fat and absence of fibrosis in patients with NAFLD. However, evaluation of inflammation and hepatocellular injury (*i.e.* NASH) or staging of fibrosis, still requires liver biopsy.

Liver fat content can be assessed using various methods. The conventional histopathological method consists of a visual semiquantitative approach in which the pathologist uses a four-point scale: grade 0 corresponds to fat deposition in <5% of hepatocytes and grade 1–3 corresponds to ≥5% (grade 1=5–33%, 2=34–66%, and 3=67–100%). A diagnosis of NAFLD requires that at least 5% of hepatocytes contain fat vacuoles. An alternate approach is to quantitatively assess steatosis using stereological point counting (SPC). Both the semiquantitative histological method and SPC rely on biopsies, however, *in vivo* proton magnetic resonance spectroscopy (¹H-MRS) is a reliable non-invasive method that can be used to quantitatively assess total hepatic lipid content, or proton density fat fraction (PDFF).

In Paper I we compared the conventional semiquantitative histological method (grade 0–3) with SPC and ¹H-MRS. We found a strong positive correlation between ¹H-MRS and SPC, whereas the correlations between ¹H-MRS or SPC and histopathological grading were substantially weaker. Using the widely used cut-off value of PDFF ≥5%, all participants were found to have steatosis (specificity 100%, sensitivity 53%). Reducing the cut-off

value to 3% maintained 100% specificity while increasing sensitivity to 79%.

In Paper IV we evaluated quantitative steatosis, by SPC, in 106 biopsy-proven NAFLD patients during a 20-year follow-up. SPC was independently associated with an increased risk of all-cause mortality and development of T2DM. Moreover, in the 59 patients with sequential biopsies (approximately 10 years apart), a reduction of quantitative hepatic steatosis decreased the all-time risk of developing T2DM.

NASH is commonly seen as a histological feature portending a worse prognosis in NAFLD. Interestingly, no dual biopsy study has ever shown that NASH predicts fibrosis progression. Yet, NASH is seen as a surrogate marker in pharmaceutical trials – were resolution in NASH is equivalent to future resolution of fibrosis.

Recently, two studies, investigating the impact of NASH on mortality in patients with biopsy-proven NAFLD, were published. Both studies presented similar results; that only fibrosis, and no other histological features (including NASH) predicts all-cause and disease-specific mortality. However, in one study the cohort was small (n=229) but had a long follow-up (26 years), whilst the other study had a larger cohort (n=619) but shorter follow-up (12 years).

In a collaboration with Karolinska Institute, we conducted a long-term follow-up study (20 years) in a large cohort of biopsy-proven NAFLD patients (n=646). As previously shown, we could not ascertain that NASH had any effect on all-cause, or disease-specific mortality. However, the study was set in a retrospective manner, with all patients included through the respective academic medical centre's pathologic records.

Nevertheless, in Paper III, we present 129 patients (also included in Paper II), in which we had prospective, longitudinal data. They were included between 1988 and 1993, after performing liver biopsy because of chronically elevated liver enzymes. All patients alive, were re-invited between 2003 and 2005 and between 2013 and 2015. Dual biopsies were present in 68 patients, and three consecutive biopsies were available in 33 patients. Results showed that NAFLD is a highly heterogeneous disease, with 9.3% developing end-stage liver disease and 16% progressing to advanced stages of fibrosis, and without any clinically significant baseline data predicting disease progression.

In summary, when using ^1H -MRS as a diagnostic method for NAFLD, the cut-off for diagnosing hepatic steatosis should be reduced from 5% to 3%. Furthermore, quantitative amount of hepatic steatosis (either by ^1H -MRS or SPC) could be used to stratify patients with NAFLD related to future risk of developing T2DM. Moreover, we have shown that NASH does not predict future all-cause or disease-specific mortality nor end-stage liver

disease, therefore a different surrogate marker should be used in clinical trials when assessing NAFLD improvement, so to not imbue false reliance in new therapies. Also, we have shown that NAFLD has a more dismal prognosis than previously reported, and that it is unexpectedly difficult to predict fibrosis progression in individual NAFLD patients, emphasizing the need for robust non-invasive biomarkers suitable to monitor large number of patients.

SVENSK SAMMANFATTNING

Fettinlagring är ett vanligt fynd när levern undersöks med ultraljud, skiktröntgen, magnetkamera (MR) eller vävnadsprovtagning.

Tidigare har den vanligaste underliggande orsaken ansetts vara överkonsumtion av alkohol men på senare tid har dock icke-alkoholorsakad fettlevversjukdom visat sig vara den dominerande orsaken.

Icke-alkoholorsakad fettlevversjukdom, från engelskans non-alcoholic fatty liver disease (NAFLD), är en kronisk leversjukdom som förekommer hos cirka var fjärde person i världen. Tillståndet anses vara manifestationen av det metabola syndromet i levern. Metabola syndromet definieras av bukfetma, typ 2 diabetes, högt blodtryck och förhöjda blodfetter. Framförallt finns ett intrikat samspel mellan typ 2 diabetes och NAFLD. Majoriteten av individer med NAFLD utvecklar förr eller senare typ 2 diabetes, och vice versa. Således är sambandet otvetydigt, däremot är än så länge orsakssambandet mellan de två tillstånden oklart.

Referensmetoden för diagnostik och bedömning av leverskadans omfattning vid NAFLD är idag histologisk och därmed behöver man utföra vävnadsprovtagning från levern med en nål (leverbiopsi) för att granska vävnaden i mikroskop. Denna metod är invasiv, det vill säga att man behöver tränga in i kroppen, och medför vissa risker. Man har därför försökt utveckla och utvärdera nya, icke-invasiva, metoder för att diagnostisera och följa upp patienter med NAFLD. En sådan metod är MR-undersökning som visat sig kunna mäta fettmängden i levern med stor noggrannhet – där undersökningen ger en exakt siffra mellan 0 och 100%. När man undersöker levern med MR har man hittills ansett att NAFLD föreligger om levern innehåller mer än 5% fett.

Histologiskt innefattar NAFLD ett spektrum av förändringar från enbart fettinlagring till tillkomst av inflammation, celldöd och ärrvävnad (bindvävsinlagring) samt slutligen utveckling av skrumplever (cirros) med risk för tillkomst av leversvikt och/eller levercancer (hepatocellulär cancer). Förekomst av de tre histologiska förändringarna inflammation, celldöd och fettinlagring, brukar benämnas icke-alkoholorsakad steatohepatit, från engelskans non-alcoholic steatohepatitis (NASH). Ofta ses vid NASH också tillkomst av bindvävsinlagring, som vid progression kan leda till cirros och ovan nämnda komplikationer.

NASH anses förutspå en sämre prognos hos patienter med NAFLD både med avseende på framtida risk för leverrelaterade komplikationer men även avseende ökad risk att dö i förtid. Därför har de europeiska och amerikanska läkemedelsmyndigheterna valt att betrakta NASH som en surrogatmarkör för bindvävsinlagring under läkemedelsprövningar. Med

andra ord – en förbättring av NASH (det vill säga återgång av de histologiska parametrarna: inflammation, celldöd och fettinlagring) anses framgent medföra minskad bindvävsinlagring.

Vid granskning av leverbiopsi hos patienter med NAFLD bedöms oftast graden av fettinlagring i levern semikvantitativt genom att använda en fyrgradig skala (0–3). Denna bedömning motsvarar: ingen (<5%), mild (5–33%), måttlig (34–66%) och uttalad (67–100%) fettinlagring. En alternativ metod är att bedöma leverbiopsin kvantitativt med kvantitativ histologisk bedömning, eller stereological point counting (SPC). En SPC-beräkning anger hur stor del av vävnadsytan som belamras av fett – värdet, likt MR, återges som en exakt siffra mellan 0 och 100%.

Leverbiopsi och efterföljande granskning av levervävnad ger mycket information som kan vara av värde för handläggningen av NAFLD, emellertid föreligger vissa nackdelar. Utöver riskerna med att utföra en leverbiopsi, representerar en leverbiopsi endast en bråkdel av levern, därmed finns risk att man missar eller övertolkar fynd då de histologiska, eller vävnadsspecifika, förändringarna kan vara heterogent fördelade i levern. Samtidigt har man sett att det föreligger en stor variation i bedömningar av samma vävnadsprov både av samma patolog (som undersöker samma vävnadsprov vid två tillfällen) samt av olika patologer (som undersöker samma vävnadsprov vid samma tillfälle). Således är det av yttersta vikt att man finner ett standardiserat sätt att bedöma histologiska parametrar i levern för att undvika variation i bedömningarna.

I studie I jämförde vi histologisk semikvantitativ bedömning (0–3) med de kvantitativa bedömningarna: MR och SPC. Vi fann att MR och SPC hade en mycket hög överensstämmelse. Samtidigt noterade vi att det tidigare accepterade referensvärdet för fettinlagring mätt med MR (5%) resulterade i att man missade många patienter som bedömts som NAFLD vid granskning av leverbiopsi enligt semikvantitativ bedömning. Vi föreslår att gränsen för avvikande fettmängd i levern mätt med MR reduceras från 5% till 3%, något som skulle medföra att fler patienter med NAFLD kan diagnostiseras med MR.

I studie IV utvärderade vi värdet av kvantitativ bedömning av mängden fett på 106 patienter med biopsiverifierad NAFLD under en lång uppföljningstid (ca 20 år). Vi visade att stor mängd leverfett mätt med SPC ökade risken för att i framtiden utveckla diabetes, oberoende av andra riskfaktorer. Fentionio (59) studiepatienter genomgick leverbiopsi vid två tillfällen med ca 10 års mellanrum. SPC-beräkning utfördes på leverbiopsierna vid de två tillfällena, och skillnaden mellan första och andra leverbiopsin kunde således nyttjas för att beräkna risken för framtida diabetesutveckling. Vi noterade att en minskning av fett i levern, mellan första och andra leverbiopsin, minskade risken för att utveckla framtida diabetes.

Nyligen publicerades två multicenterstudier som visade att NASH inte förutspådde risken för död i förtid eller utveckling av leverrelaterade komplikationer. Den ena av dessa studier innehöll relativt få patienter (229 st.) som följdes under lång tid (26 år), medan den andra innehöll många patienter (619 st.) men som följdes under en kortare tid (12 år).

I studie II valde vi därmed att, i samarbete med kollegor från Karolinska Institutet, genomföra en studie med ett stort antal patienter (646 st.) som följdes under lång tid (20 år). Vi kunde inte påvisa att NASH var någon riskfaktor för framtida leverrelaterade komplikationer (skrumplever, leversvikt eller levercellscancer) eller för död i förtid. Därutöver, fann vi inte att individer med NAFLD, som helhet, löpte en ökad risk att dö i förtid jämfört med en kontrollgrupp. Dock löpte man ökad risk att dö i förtid eller drabbas av leverrelaterade komplikationer om man hade svårare form av bindvävsinlagring i levern.

På 129 av dessa patienter hade vi prospektiva, longitudinella data. En prospektiv, longitudinell studie definieras av att mätdata samlas in framåt i tiden samt att mätdata insamlas mer än en gång. De inkluderades mellan 1988 och 1993 då de genomgick leverbiopsi på grund av förhöjda leverblodprover och har sedan följts till dags dato. De har bjudits in för uppföljning och förnyad leverbiopsi vid två tillfällen: mellan 2003 och 2005 samt mellan 2013 och 2015. Vi fann att NAFLD, som sjukdom, är väldigt heterogen i hur den fortskrider. Vi kunde inte finna några kliniskt användbara parametrar för sjukdomsprogression. Samtidigt noterade vi även att de med godartade histologiska uttryck (enbart fett i levern utan inflammation, celledöd eller bindvävsinlagring) också riskerade att utveckla svår bindvävsinlagring och skrumplever/leversvikt.

Sammanfattningsvis bör man, om man väljer MR som diagnosmetod vid NAFLD, sänka gränsen för avvikande fettmängd i levern från 5% till 3% för att inte missa patienter som annars, vid granskning av leverbiopsi, skulle bedömts som NAFLD. Samtidigt bör MR ej enkom användas som diagnosmetod utan även användas för riskstratifiering, eftersom kvantiteten av fett förutsäger framtida risk för diabetesutveckling. Vidare har vi visat att NASH inte förutsäger risk för leversvikt eller förtida död och man bör således finna en ny surrogatmarkör vid läkemedelsprövningar för att inte ingjuta falsk övertro på nya läkemedel. Och avslutningsvis, verkar sjukdomsprogressen vid NAFLD i nuläget vara svår att förutspå, och de patienter som bör följas, svåra att identifiera. Man bör därmed finna och utvärdera nya parametrar som förutspår sjukdomsprogress vid NAFLD.

LIST OF PAPERS

- I. **Using a 3% Proton Density Fat Fraction as a Cut-off Value Increases Sensitivity of Detection of Hepatic Steatosis, Based on Results from Histopathology Analysis**
Nasr P*, Forsgren MF*, Ignatova S, Dahlström N, Cedersund G, Dahlqvist Leinhard O, Norén B, Ekstedt M, Lundberg P, and Kechagias S
Gastroenterology 2017 Jul;153(1):53-55.e7.
- II. **Fibrosis stage but not NASH predicts mortality and time to development of severe liver disease in biopsy-proven NAFLD**
Hagström H*, **Nasr P***, Ekstedt M, Hammar U, Stål P, Hultcrantz R, and Kechagias S
Journal of Hepatology 2017 Dec;67(6):1265-1275
- III. **Natural history of nonalcoholic fatty liver disease : A prospective follow-up study with serial biopsies.**
Nasr P, Ignatova S, Kechagias S*, Ekstedt M*
Hepatology communications 2017 Dec 27;2(2):199-210.
- IV. **The quantitative amount of histological liver fat associates with development of type 2 diabetes in nonalcoholic fatty liver disease.**
Nasr P, Fredrikson M, Ekstedt M*, Kechagias S*
Submitted to JHEP Reports

* Authors contributed equally

ABBREVIATIONS

γ GT	Gamma-glutamyltransferase
^1H -MRS	Proton magnetic resonance spectroscopy
AdipoR1/2	Adiponectin receptor 1 and 2
AFLD	Alcoholic fatty liver disease
ALT	Alanine aminotransferase
ApoB	Apolipoprotein B100
APRI	AST to platelet ratio index
AST	Aspartate aminotransferase
AUDIT	Alcohol use disorder identification test
AUDIT-C	AUDIT-Consumption
BARD	BMI, AST/ALT-ratio, diabetes
BMI	Body mass index
CDR	Cause of Death Register
CDT	Carbohydrate deficient transferrin
ChREBP	Carbohydrate-responsive element-binding protein
CLD	Chronic liver disease
CSE-MRI	Chemical shift encoded-MRI
CVD	Cardiovascular disease
DNL	De novo lipogenesis
FFA	Free fatty acid
Fib4	Fibrosis-4
FXR	Farnesoid X receptor
HCC	Hepatocellular carcinoma
HTGC	Hepatic triglyceride content
ICD	International Classification of Disease
IL-6	Interleukin-6
IR	Insulin resistance
IRS-1	Insulin receptor substrate-1
JAK	Janus kinase
JNK	c-Jun NH ₂ -terminal kinase
LDL	Low-density lipoprotein
MCP-1	Monocyte chemoattractant protein-1

MR	Magnetic resonance
MRE	MR elastography
MRI	MR imaging
NAFL	Non-alcoholic fatty liver
NAFLD	Non-alcoholic fatty liver disease
NAS	NAFLD activity score
NASH	Non-alcoholic steatohepatitis
NF- κ B	Nuclear factor- κ B
NFS	NAFLD fibrosis score
NPR	National Patient Register of Hospital Discharge
PDFF	Proton density fat fraction
PEth	Phosphatidylethanol
PIN	Personal identification number
ROS	Reactive oxygen species
SAF	Steatosis, Activity, Fibrosis
SCR	Swedish Cancer Register
SFA	Saturated fatty acid
SPC	Stereological point counting
SREBP	Sterol regulatory element-binding protein
STAT	Signal Transducer and Activator of Transcription proteins
T2DM	Type 2 diabetes mellitus
TE	Transient elastography
Tg	Triglycerides
TNF- α	Tumor necrosis factor- α
VLDL	Very low-density lipoprotein

ACKNOWLEDGEMENTS

To begin with, I would like to extend my outmost gratitude to all the patients who participated in these studies.

Furthermore, I would be remiss if I didn't acknowledge that one merely stands on the shoulder of giants – in the minuscular and the major. Therefore, I would like to thank everyone who made this thesis possible. And particularly, I would like to thank:

My main supervisor, colleague, mentor and friend, **Stergios Kechagias**. The vast knowledge, terrifyingly eidetic memory, formulating skills, to-the-point criticism and boundless humour made research fun, and nothing ever seemed unattainable. And even though you had your hands full – articles were read, mails were sent, and phones were answered – nonetheless late at night... I'm certain that you will continue to play an important role for me as a clinician and researcher in the future.

My assistant (or second, main?) supervisor, colleague, fearless and innovative research mentor and friend **Mattias Ekstedt**. For enthusiastically introducing me to NAFLD (by sending me to the basement archives for months...) and for networking inexhaustibly for the benefit of our research team. You always had an open-door policy and inclusive personality – never letting on how busy you are. For that I am grateful. Är det OK att spela squash nu?

My assistant supervisor **Peter Lundberg**. I'm proud and glad to have had you as my second assistant supervisor. You filled a gap of the vastness known as magnetic resonance, you created a course suitable for a clinician and, you always took time to reflect beyond reflection – a valuable trait.

My co-authors (Paper II) and fellow “*Naffel-Dées*” at Huddinge, Stockholm, **Hannes Hagström**, **Per Stål** and **Rolf Hultcrantz**. *Fettleverpojnkarnas LTU* is hopefully a never-ending project.

All my co-authors at CMIV (Paper I), and especially **Mikael Forsgren**. I agree, the combination of a “technologist” and “physiologist” was very successful. And it was fun! Also, a special thanks to **Nils Dahlström**, for always going that extra mile.

I would also like to thank **Simone Ignatova**, our tireless co-author (Paper I, III). You are the best liver pathologist I know. And, **Mats Fredrikson** (Paper IV), for diligently trying to explain the (philosophical?) field known as biostatistics.

Carola Fagerström and **Helen Hernandez**. What can I say? Thank you both for all your help, competence, smiles and laughter's. Also, thank

you for keeping us NAFLD:ers on track – without you we would've succumbed to the workload.

My boss and fellow *skåning*, **Henrik Hjortswang**, for teaching me that best patient care is given by seeking knowledge as a researcher, carrying it forward as a teacher and applying it as a clinician. Also, for giving me all opportunities to research, participate on courses and attend congresses on available time.

My clinical tutor and mentor, **Rikard Svernlöv**, for helping me realize that there is a life outside of work, helping me cope with clinical dilemmas, and for being a role model in all things, big and small, in the role as a physician. I only have 40 years left until retirement, and then I promise you'll be rid of me!

The Faculty of Medicine and Health at Linköping University, and its co-workers, for supporting me through a MD and a PhD.

Everyone working at the department of Gastroenterology and Hepatology. There's no other workplace I would rather be a part of.

Mina föräldrar, **Dzovinar** och **Joseph** som alltid stöttat, älskat och uppmuntrat mig. Alltid intresserade, alltid lyssnande. **Roni** och **Nathalie**, mina syskon och absolut bästa vänner. För att ni alltid tar er tid, alltid inkluderat mig och accepterat mig för den jag var, är och blivit. Det kan inte ha varit lätt... We ride, we die...

Lil'Ron, tack för att du alltid svarar när man ringer. Att prata med dig är som att koppla bort hjärnan för en stund – på ett positivt sätt. Alltid välbehövligt. Du kan förvänta dig dagligt skitsnack så länge jag har en transportsträcka till jobbet som är > 1 min.

Eva och **Lars**, för att ni ställer upp i vått och torrt. Stundom skulle vi nog havererat utan er hjälp.

Alla vänner!

Samt, mitt livs kärlek och närmaste vän, **Anna**, som av någon underlig anledning står ut med mig. Och sist, yngst men inte minst, **Lova**, som visat mig vad meningen med livet är. Att komma hem till er är det enda jag ser fram emot om dagarna. Älskar er ofantligt.

1. INTRODUCTION

1.1 Background

In 1980, Ludwig *et al* published a landmark paper that described 20 middle-aged patients without any alcohol consumption, with elevated liver enzymes and histological evidence of alcoholic hepatitis, *i.e.* moderate to severe steatosis with signs of inflammation.¹ The disease was coined non-alcoholic steatohepatitis (NASH). Albeit the study by Ludwig *et al* is often referred to as the initial report on NASH, the histopathological features seen in NASH had been described earlier.^{2, 3}

The most common cause of abnormal liver function tests is hepatic lipid accumulation (steatosis), which is present in up to 30% of the population.⁴ A common cause of hepatic steatosis among adults is non-alcoholic fatty liver disease (NAFLD),^{5, 6} with an estimated global prevalence of 25%.⁷

NAFLD was initially considered a benign disease with only a small proportion progressing to cirrhosis with risk of developing hepatocellular carcinoma. However, because of its high prevalence, among both overweight, normal weight and lean subjects, NAFLD has incited scientists, whom now predict a dismal future, with a high disease and economic burden and an increased need of liver transplantation.⁸

1.2 Definition of Non-Alcoholic Fatty Liver Disease

Hepatic fatty infiltration can arise in a variety of medical conditions and can also be triggered by drugs and nutritional alterations (Table 1). However, in most patients, hepatic steatosis is caused by either alcoholic fatty liver disease (AFLD) or NAFLD.

It has been difficult to draw a line on excessive alcohol consumption to separate the two conditions; AFLD and NAFLD. The cut-off level for what is considered excessive alcohol consumption has ranged in studies from abstinence^{1, 9, 10} to 40 g/week,^{11, 12} 140 g/week,¹³⁻¹⁵ 210 g/week^{16, 17} and up to 252 g/week.¹⁸ Nevertheless, a consensus was reached in the European association for the study of liver disease in 2016 suggesting a cut-off of 210 g/week for men and 140 g/week for women.¹⁹ However, in 2018, the American association for the study of liver disease suggested cut-offs of 294 g/week for men and 196 g/week for women over a 2-year period preceding baseline liver histology.²⁰

In the presence of hepatic steatosis and after exclusion of excessive alcohol consumption as well as pre-existing medical conditions (specified in Table 1), and other chronic or drug-related liver diseases, the diagnosis is probably NAFLD.

Table 1. Causes of hepatic steatosis other than NAFLD and alcohol.

Nutritional	Drugs and toxins	Inborn errors of metabolism	Other conditions
GI surgery for obesity	5-Fluorouracil	Abetalipoproteinemia	AFLP
Malnutrition	Acetylsalicylic acid	Galactosemia	Environmental toxins
Rapid weight loss	Amiodarone	Glycogen storage disease	-Toxic mushrooms
Starvation	Carbamazepine	Hereditary fructose intolerance	-Phosphorus
TPN	Cocaine	Homocysteinuria	-Petrochemicals
	Dicloethylene	LAL-D/CESD/WD	-Organic solvents
	Didanosine (NRTI)	LCAT deficiency	HELLP syndrome
	DH	Systemic carnitine deficiency	Hepatitis C
	Diltiazem	Tyrosinemia	HIV
	Estrogen	Weber-Christian syndrome	IBD
	Ethionine	Wilson's disease	Lipodystrophia
	Ethyl bromide		Reye's syndrome
	Glucocorticoids		Severe anemia
	Hydrazine		SIBO
	Hypoglycin		
	Interferon		
	Irinotecan		
	Margosa oil		
	Methotrexate		
	NSAID		
	Perhexeline maleate		
	Protease inhibitors		
	Safrole		
	Stavudine (NRTI)		
	Tamoxifen		
	Tetracycline		
	Valproic acid		
	Vitamin A		
	Zidovudine (NRTI)		

Abbreviations: AFLP, acute fatty liver of pregnancy; CESD, cholesterol ester storage disease; DH, diethylaminoethoxy-hexestrol; GI, gastrointestinal; HELLP, hemolysis, elevated liver enzymes, low platelet count; HIV, human immunodeficiency virus; IBD, inflammatory bowel syndrome; LAL-D, lysosomal acid lipase deficiency; LCAT, lecithin-cholesterol acetyltransferase; NRTI, nucleoside reverse transcriptase inhibitors; NSAID, nonsteroidal anti-inflammatory drug; SIBO, small intestinal bacterial overgrowth; TPN, total parenteral nutrition; WD, Wolman's disease.

Fatty liver can be defined as an accumulation of fat, largely triglycerides, exceeding 5% of the liver weight.²¹ Although this definition is appealing, it is not applicable in a clinical setting. More commonly, liver biopsy is performed, where the diagnosis of NAFLD is set if >5% of the hepatocytes contain fat vacuoles.

NAFLD includes a histological spectrum ranging from isolated steatosis (*i.e.* only steatosis and no other features) to steatosis and mild inflammation (*i.e.* NAFL; non-alcoholic fatty liver) to steatohepatitis (*i.e.* NASH), fibrosis, cirrhosis and hepatocellular carcinoma (Figure 1).²²⁻²⁵ Steatohepatitis, or NASH, is defined as steatosis, lobular inflammation and ballooning of hepatocytes. Until recently NASH has been considered the more progressive form of NAFLD with increased mortality and risk of developing liver-related events.

Figure 1. The histological progression of the disease ranging from non-alcoholic fatty liver (NAFL), through non-alcoholic steatohepatitis (NASH) and to cirrhosis with risk of developing hepatocellular carcinoma (HCC). Estimated progression rate in %.

The gold standard for diagnosing NAFLD is liver biopsy. However, liver biopsy is invasive with rare, and potentially life-threatening complications. Liver biopsy is also prone to sampling errors as well as inter- and intraobserver variability.

The inherent limitation of liver biopsy has spurred the development of non-invasive techniques. Initially ultrasonography was used with excellent sensitivity and specificity for moderate and severe steatosis, but less sensitive for lower grades of steatosis. However, measurement of hepatic triglyceride content (HTGC) and fibrosis non-invasively has evolved in the last decade, with the utilization of magnetic resonance (MR) and elastographic techniques as promising methods for diagnosing and quantifying hepatic steatosis and fibrosis for both research and in clinical settings.

1.3 Pathogenesis

The hallmark of NAFLD is the accumulation of fat in hepatocytes, in the form of lipid droplets, containing triglycerides. However, the pathway from intracellular lipid storage to inflammation is not fully understood.

In 1998 Christopher Day and Oliver James postulated the “two-hit” hypothesis.²⁶ According to this model, the “first hit” would be the development of hepatic steatosis and the assumed “second hit” would lead to inflammation and consequently fibrosis. However, this view is now considered old-fashioned.^{27, 28} Instead, triglyceride accumulation in the form of lipid droplets is assumed to be “innocent bystanders” in the process leading

to cellular injury, a theory presented as early as in 1975 by pathologist Heribert Thaler who postulated that “the cause of steatosis, and not the fat accumulation itself, produces cirrhosis”.²⁹

Many molecular pathways leading from steatosis, to inflammation and subsequently to fibrosis have been studied. Nevertheless, it is not certain how fibrosis is preceded by inflammation. Thus, NAFLD is still seen as a highly heterogenous disease.

A useful theoretical framework in describing the pathogenesis of NAFLD is that the capacity of the liver to manage the main metabolic energy substances (*i.e.* carbohydrates and fatty acids) is overwhelmed, leading to an accumulation of toxic lipid species. These metabolites induce hepatocellular stress, injury and, eventually, cell death – resulting in fibrogenesis, cirrhosis and hepatocellular carcinoma.

1.3.1 Fat Accumulation

The accumulation of fat, mostly triglycerides, in the liver of NAFLD patients, is multifactorial and results from an imbalance in the hepatic lipid turnover. Triglycerides derive from esterification of glycerol and free fatty acids (FFAs), which once esterized enter storage or secretory pools with distinct rates of turnover. Free fatty acids, in turn, stem from either diet, adipose tissue (via lipolysis), or from hepatic *de novo* lipogenesis. Apart from esterification, FFAs can also be catabolized by entering β -oxidation. Further, decreased triglyceride secretion, either by decreased incorporation of triglycerides into very low-density lipoprotein (VLDL), or decreased secretion of VLDL from the liver, increases hepatic triglyceride content (Figure 2).

However, triglyceride accumulation in the liver is not hepatotoxic *per se*, and is rather seen as a defensive mechanism to balance FFA excess.³⁰⁻³² Therefore, increased triglyceride content should be seen as an epiphenomenon which happens simultaneously with generation of toxic metabolites, and subsequently liver injury.³³

1.3.1.1 Triglycerides

Increased delivery of FFAs from insulin resistant adipose tissue (through lipolysis), hepatic *de novo* lipogenesis, and dietary fat are the major reasons for triglyceride accumulation.³⁴ Although, triglycerides represent the major component of lipid droplets in hepatocytes in NAFLD, this form of accumulation is currently considered protective.

Approximately 25% of triglycerides stored in the liver of patients with NAFLD is produced by *de novo* lipogenesis, compared to circa 5% in patients without NAFLD.³⁴ In NAFLD mouse models, the activity of two transcriptional factors, sterol regulatory element-binding protein (SREBP) and

carbohydrate response element-binding protein (ChREBP), are increased. Both aforementioned factors regulate gene expression resulting in increased de novo lipogenesis. Moreover, it has recently been demonstrated that glucose and fructose increase ChREBP, however, fructose also specifically increases SREBP. This leads to increased hepatic steatosis and reduced hepatic insulin signalling.³⁵ Nonetheless, inactivation of SREBP in animal models abolishes the increase in lipogenesis while fatty acid β -oxidation remains leading to accumulation of lipid intermediates and increased energy drain which collectively results in oxidative stress, inflammation and liver damage.³⁶ Hence, the presence of SREBP seems to help redirect fatty acids towards more beneficial actions.

1.3.1.2 Free Fatty Acids

In plasma, there is a pool of FFAs, that contributes to the majority of fatty acids that flow to the liver in the fasted state.³⁴ Insulin resistance (IR) in adipose tissue results in attenuated suppression of hormone sensitive lipase in adipocytes, which contributes to an increased lipolysis within adipose tissue resulting in an influx of FFA to the liver.³⁷ However, the notion that IR is secondary to accumulation of triglycerides (as lipid droplets in muscle, liver and other tissues), is now obsolete, rather, they are thought to be parallel phenomena.³⁸

Free fatty acids exist in a variety of lengths and shapes, the latter is determined by the number of carbon chain double bonds. Some studies have shown that the unsaturated fatty acids palmitate and stearate, which are major components of our diet, and can be synthesized de novo, have toxic effects and induce apoptosis and inflammation.^{39, 40} On the other hand, polyunsaturated fatty acids have been shown to decrease hepatic steatosis in NAFLD patients without affecting inflammation or fibrosis.⁴¹

1.3.1.3 Very Low-Density Lipoprotein

Triglycerides are transported out of the liver in the form of VLDL. Each VLDL is coupled with an apolipoprotein (ApoB; apolipoprotein B100) and secreted into the blood stream.⁴² The synthesis of ApoB is crucial for the synthesis of mature VLDL particles.^{42, 43} High levels of insulin, seen in insulin resistance, decreases the synthesis of ApoB while hepatic steatosis seems to disturb hepatic ApoB production.⁴³ Moreover, decreased lipolysis reduces ApoB.⁴⁴ Albeit the mechanisms surrounding hepatic steatosis is

uncertain, reduced ApoB seems to play a role in decreasing triglyceride output and increasing lipid accumulation.

Figure 2. The metabolism of TG in the liver. The three major sources of FFAs are diet, peripheral (*i.e.* adipose) tissue and endogenous synthesis. FFAs have different possible routes, they can either be metabolized through β -oxidation in the mitochondria, stored as TG in lipid droplets in hepatocytes (*i.e.* hepatic steatosis) or packaged with ApoB into VLDL. Processes that increase TG input and reduces TG output, cause hepatic steatosis. Dietary carbohydrate increases glucose, fructose and insulin levels, which activate the transcription factors ChREBP and SREBP. Both increase *de novo* lipogenesis, while SREBP also decreases β -oxidation. Excessive adipose tissue and dietary intake of fat increases peripheral FFA as well as increases insulin resistance and subsequently increases mobilization of FFA. Increased hepatic steatosis and insulin resistance may disturb ApoB synthesis and ApoB production which in turn decreases VLDL transport out of the liver. Abbreviations: ApoB, apolipoprotein B100; ChREBP, carbohydrate response element-binding protein; Chylo, chylomicron; FFA, free fatty acid; SREBP, sterol regulatory element-binding protein; TG, triglyceride; VLDL, very low-density lipoprotein.

1.3.1.4 Bile Acids and Nuclear Receptors

Bile acids are amphipathic, meaning they have both hydrophilic and hydrophobic parts, and are synthesized from cholesterol in the hepatocytes. The primary bile acids (cholic acid and chenodeoxycholic acid) are later conjugated to glycine or taurine and secreted into the biliary tract. On reaching the small intestine, biliary acids enable emulsification and absorption of alimentary fats, cholesterol and fat-soluble vitamins. Almost all bile acids (~95%) are actively reabsorbed in the terminal ileum and later transported

back to the liver. The remaining bile acids (~5%) reach the colonic microbiota and are then metabolized into secondary bile acids (deoxycholic acid and lithocholic acid), which reach the liver via passive absorption into the portal circulation. The liver then recycles and reconstitutes the bile acids and secretes them back into the bile tract. This process is known as the enterohepatic circulation.⁴⁵

For many years, it was thought that biliary acid function was mainly limited to aiding digestion and absorption of fats from the intestine. However, over the past years, research has shown that bile acids may function as signalling molecules through different receptors, to regulate their own synthesis as well as other processes, such as the metabolism of glucose and lipids, as well as regulation of energy homeostasis.⁴⁶

Nuclear receptors are transcription factors that play important roles in embryogenesis, development and metabolism.⁴⁶ Bile acids effectively activate nuclear receptors, especially the farnesoid X receptor (FXR). The FXR is a highly expressed nuclear receptor in hepatocytes and enterocytes, which is activated by primary bile acids, and to some extent, secondary bile acids.⁴⁷ Activation of FXR regulates plasma triglyceride levels by inhibiting hepatic lipogenesis and stimulating peripheral triglyceride clearing.⁴⁷

1.3.2 From Fat to Inflammation to Fibrosis

The pathogenesis of NAFLD was first conceptualized as a disease of two consecutive hits (the “two-hit” hypothesis): the accumulation of fat in the hepatocytes (*i.e.* steatosis) triggering a cascade of tissue damage (*i.e.* inflammation), resulting in fibrosis.²⁶ However, there is now a broad consensus that more complex processes, involving multiple metabolic hits (the “multiple-hit” hypothesis) are responsible for tissue injury.⁴⁸ Fundamental in understanding the pathogenesis of inflammation and fibrosis in NAFLD is the notion of adipokines/cytokines, lipotoxicity and the influence of insulin resistance and oxidative stress.

1.3.2.1 Adipo(cyto)kines

In 1993, two research groups first showed that the proinflammatory cytokine, tumour necrosis factor- α (TNF- α), could induce IR.^{49, 50} This was revolutionary, though a substance produced in adipose tissue, and overproduced in excess of such,⁵¹ had both local and systemic effects on metabolism. In the upcoming decade, other cytokines were found to influence metabolism, including (but not limited to) adiponectin, leptin, interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1).⁵²⁻⁵⁵ While leptin and adiponectin are the only true adipokines (*i.e.* only produced by adipocytes), TNF- α , IL-6, and MCP-1 (and others) are commonly referred to as adipokines or adipo(cyto)kines (Figure 3).

TNF- α and IL-6: Under physiological circumstances, insulin binds to the insulin receptor which activates insulin receptor substrate-1 (IRS-1). The activation of IRS-1 leads to activation of the downstream signalling cascade which results in an insulin response. However, TNF- α and IL-6 disrupt this signalling pathway.

TNF- α , a proinflammatory cytokine produced in macrophages, causes IR by stimulating the proinflammatory factors c-Jun NH₂-terminal kinases (JNK) and Nuclear Factor- κ B (NF- κ B).⁵⁶⁻⁵⁹ Further, TNF- α can increase JNK expression through adenosine monophosphate activated protein kinase (AMPK), which causes glucose uptake in adipocytes.⁴⁸ Similarly, IL-6 induces IR by activating Janus kinase-Signal Transducer and Activator of Transcription proteins (JAK-STAT).^{56, 60} The net result of TNF- α , on JNK and NF- κ B, and IL-6, on JAK-STAT, is the serine/threonine phosphorylation of IRS-1, which causes downregulation of IRS-1, decreased response by insulin, and, ultimately, triggering insulin resistance.

Furthermore, the transcription factor NF- κ B, has many roles. Once activated it initiates an inflammatory response/cascade causing the upregulation of different cytokines, disrupting apoptosis, enforcing cell survival, and mobilizing an immunological response.⁶¹

Leptin: Leptin, an anti-inflammatory cytokine derived primarily from white adipose tissue, is involved in the homeostasis of appetite and energy expenditure – roles associated with the progression of IR.⁶² Leptin is essential for modulating glucose metabolism and pancreatic β -cell function,⁶³ and improves glucose metabolism, insulin sensitivity and lipid metabolism.^{64, 65} Furthermore, leptin stimulates β -oxidation, therefore, adipocytes and hepatocytes would be catalysing, rather than accumulating fat, if the endogenous leptin acted correctly.^{66, 67} However, in obese individuals, a state called leptin resistance occurs, resulting in increasing levels of leptin with attenuated effect.

Adiponectin: Like leptin, adiponectin is produced mainly by white adipose tissue, however, its levels are reduced in individuals with IR,⁶⁸ where it functions as an anti-inflammatory cytokine.⁶⁹ In the improvement of insulin resistance, two distinct receptors seem involved, the adiponectin receptor 1 (AdipoR1) and receptor 2 (AdipoR2), which are both highly expressed in skeletal muscles and liver. AdipoR1 reduces expression of genes that encode hepatic gluconeogenic enzymes, while AdipoR2 increases expression of genes that contribute to glucose consumption by activating peroxisome proliferator activated receptor- α .⁷⁰ The net sum of the effects of adiponectin on its two receptors is ameliorated insulin resistance by reducing glycogenesis and lipogenesis and increasing glucose consumption.^{70, 71}

MCP-1. MCP-1 is a proinflammatory chemokine, produced by adipocytes, macrophages and epithelial cells, that recruits immune cells.^{55, 56}

MCP-1 levels rise with increasing adiposity, which leads to recruitment of macrophages and dendritic cells, initiating an inflammatory cascade (*e.g.* upregulation of TNF- α and subsequent activation of NF- κ B) with decreasing insulin sensitivity and is therefore seen as a culprit in the development of insulin resistance, particularly in the liver.^{55, 59, 72}

1.3.2.2 Insulin Resistance

As mentioned above, dysregulation and overexpression of key adipokines and cytokines play a key role in the development of insulin resistance. Nevertheless, one of the essential metabolites, imperative for insulin resistance, and subsequently inflammation, is fatty acids. Free fatty acids are primarily delivered to the liver from the blood via the portal vein, following triglyceride lipolysis in adipose tissue, a process that is regulated by insulin. Reduced receptor signalling (through phosphorylation and downregulation of IRS-1) of adipose tissue contributes to hepatic inflammation through dysregulated lipolysis which results in excessive FFA influx to the liver.^{73, 74} Similarly, FFAs in hepatocytes may cause defect insulin signalling and contribute to IR – creating a vicious cycle.^{56, 58} Moreover, insulin suppresses adipose tissue lipolysis and increases hepatic *de novo* lipogenesis. Nevertheless, in individuals with IR, suppression by insulin signalling is impaired, which results in an increased efflux of FFAs to the liver.⁷⁵

Insulin resistance is highly intertwined with NAFLD and features of NASH is more prevalent in patients with IR.⁷⁶ Also, patients with NAFLD/NASH have decreased insulin sensitivity even in the absence of type 2 diabetes.^{37, 77} Therefore, IR is seen as an influential pathogenic factor in NAFLD and its progression to NASH. It is crucial for the establishment of lipotoxicity, oxidative stress and subsequent inflammatory cascade.⁷⁸

1.3.2.3 Lipotoxicity and Oxidative Stress

Lipotoxicity occurs in the setting of an excess of FFAs, especially saturated fatty acids^{79, 80}, rather than due to triglyceride accumulation.^{30, 81} Instead, triglyceride accumulation seems to be a protective mechanism to counteract lipotoxicity in the liver.⁸²

The current theory of lipotoxicity centres on an increased influx of FFAs to the hepatocytes. This is caused by increased dietary intake of fatty acids as well as *de novo* lipogenesis and adipose lipolysis in the setting of IR and attenuation of the compensatory mechanism of oxidative stress.³³ This results in generation and accumulation of toxic lipid metabolites, such as ceramides, diacylglycerols, lysophosphatidyl choline, and oxidized cholesterol, which act as reactive oxygen species (ROS).^{33, 83}

Figure 3. Multiple hit model for the development of steatosis, inflammation and fibrosis. Dietary factors, together with obesity lead to increased levels of serum FFA, development of insulin resistance through multiple factors. Also, secondary to obesity a subsequent adipocyte proliferation takes place, with augmentation of insulin resistance and increased levels of proinflammatory cytokines (TNF- α , IL-6, and MCP-1) with decreasing or desensitized adipokines (adiponectin and leptin). The dysregulated adipokine and cytokine balance creates and maintains an inflammatory cascade and vicious circle, respectively, and maintains the insulin resistance state. In the liver, insulin resistance amplifies de novo lipogenesis (DNL), decreases VLDL assembly and disrupts β -oxidation. The net sum, together with previously mentioned causes of raised serum FFA, is increased hepatic FFA influx. This leads to synthesis and accumulation of TG (also, see Figure 1) and toxic levels of FFAs. High levels of FFAs in the absence of β -oxidation causes lipotoxicity and subsequently generation of ROS. This process is further enhanced in the presence of cytokines and attracted immune cells caused by the inflammatory milieu, causing inflammation and cellular repair systems with secondary fibrosis. Abbreviations: DNL, de novo lipogenesis; FFA, free fatty acid; IL-6, interleukin-6; JAK-STAT, janus kinase-signal transducer and activator of transcription proteins; JNK, c-Jun NH₂-terminal kinase; MCP-1, monocyte chemoattractant protein-1; NASH, non-alcoholic steatohepatitis; NF- κ B, nuclear factor- κ B; ROS, reactive oxygen species; TG, triglycerides; TNF- α , tumor necrosis factor- α .

1.4 Diagnosis of NAFLD

1.4.1 Alcohol Consumption

Excluding excessive alcohol consumption is paramount for the diagnosis of NAFLD. However, the upper limit of “allowed” alcohol consumption has increased over time, from initially abstinence (0 g/week) to 2-3 standardized glasses per day (140-294 g/week).

The recommended tool for excluding excessive alcohol consumption when diagnosing NAFLD is AUDIT, with a test-retest kappa (κ) agreement of 0.7.^{84, 85} The AUDIT, or the Alcohol Use Disorder Inventory Test, has 10 questions that explore consumption (Q1-3), dependence (Q4-6), and alcohol related problems (Q7-10).⁸⁶ However, shorter versions have been developed. The most commonly used tool for excluding excessive alcohol consumption is the AUDIT-Consumption (AUDIT-C) questionnaire which includes only the three first questions of the AUDIT.⁸⁷

Moreover, in addition to AUDIT(-C), occasionally indirect alcohol markers, such as gamma-glutamyltransferase (γ GT), mean corpuscular volume, aspartate and alanine transaminase (AST and ALT) have been used. However, they all rely on chronic excessive drinking over a long period of time and they are encumbered with several confounders which results in low sensitivity and specificity.⁸⁸⁻⁹² Furthermore, carbohydrate deficient transferrin (CDT), is sometimes used to prove the presence of excessive alcohol consumption.⁹³ However, CDT only indicates heavy alcohol consumption (50-80 g/day or 350-560 g/week) over a period of >1-2 weeks – a threshold way above the limit for NAFLD. Similarly, as with all indirect alcohol markers, CDT is prone to erroneous values, mainly because of other liver diseases as confounding factor.⁹⁴⁻⁹⁶

In comparison to the questionnaires and indirect alcohol markers, the direct alcohol markers have a much higher specificity since they are all direct products of ethanol. Furthermore, in comparison to determination of ethanol in blood or exhaled air, they have a much larger window of detection. There are a variety of markers with phosphatidylethanol (PEth) showing high sensitivity and specificity.⁹⁷ In a study by Schröck *et al*, 16 volunteers received a single dose of alcohol (vodka), corresponding to 34-72 g of alcohol, in order to reach an estimated blood alcohol concentration of 1 g/kg, after abstaining from alcohol for 2 weeks.⁹⁸ PEth was measured every 2 hours from intake (up to 8 h after intake), and the maximum PEth value was reported. The maximum PEth values ranged from 0.06-0.31 $\mu\text{mol/L}$. In Sweden, 0.05-0.30 $\mu\text{mol/L}$ is clinically considered as moderate alcohol consumption. Also, in a study by Kechagias *et al*, 44 subjects were randomized to abstinence or consumption of 16 g (female) or 32 g (male) of alcohol (wine) per day for 12 weeks.⁹⁸ A majority of the subjects in the wine-group had PEth values below 0.04 $\mu\text{mol/L}$ (<0.05 $\mu\text{mol/L}$ is clinically considered as no or low alcohol consumption) while three subjects had 0.07, 0.12 and 0.17 $\mu\text{mol/L}$, indicating moderate alcohol consumption.⁹⁹

Both studies suggest that an occasional or chronic intake of up to 30 g alcohol per day results in classification in the low(-moderate) alcohol consumption group according to current clinical decision cut-offs for PEth, while consumption >70 g per day most probably will result in PEth values

$>0.30 \mu\text{mol/L}$, which in clinical practice is considered as heavy alcohol consumption. This is corroborated by Walther *et al* who also showed an almost linear correlation between reported alcohol consumption and PETH.¹⁰⁰

Alcohol consumption in NAFLD patients is not uncommon, on the contrary, it has been reported that nearly 66% of adults in the United States drink alcohol (~ 4 drinks/week)¹⁰¹ and 90% of adults in Sweden (~ 9 drinks/week).^{102, 103} There is a long-lasting controversy on the impact of alcohol consumption on the prognosis of NAFLD, with reports suggesting positive effects¹⁰⁴⁻¹⁰⁹ and others suggesting negative effects¹¹⁰⁻¹¹³, and with some of the studies suggesting a J-shaped curve where modest alcohol consumption is associated with decreased mortality. This is in concordance with previous studies showing that modest alcohol consumption is associated with decreased risk of cardiovascular mortality¹¹⁴ – which is reasonable since people with NAFLD are more likely to die from cardiovascular disease. However, recently, a study including 28 million individuals, suggested that there is no safe limit for alcohol use.¹¹⁵ Moreover, two recent studies demonstrated that alcohol use (mostly moderate use) was associated with fibrosis progression in NAFLD.^{116, 117} Furthermore, in another report, more than 3 (for men) or 1.5 (for women) drinks/day was associated with increased mortality – an effect that was more profound amongst individuals with the metabolic syndrome.¹¹⁸

In conclusion, there is an on-going debate if modest amounts of alcohol is beneficial, or if any amount of alcohol is detrimental in NAFLD.¹¹⁹⁻¹²² Also, proposing recharacterization of NAFLD as only present in abstainers has been suggested.¹²³

1.4.2 Abnormal Liver Enzymes

In clinical practice, patients with NAFLD are usually identified by the presence of chronically elevated liver enzymes or coincidentally during a radiological exam (*i.e.* ultrasonography or computed tomography) of the liver.¹²⁴⁻¹²⁷ However, NAFLD patients often present with normal liver enzymes, and moreover, ultrasonography and computed tomography have a low sensitivity and specificity for detecting low stages of hepatic steatosis.^{128, 129}

Several diagnostic panels have been proposed for detecting hepatic steatosis in NAFLD, among them, the Steatotest, the Fatty Liver Index (FLI), and the NAFLD Liver Fat Score. The Steatotest includes 12 different variables in an undisclosed formula¹³⁰, with an AUROC of 0.81, a sensitivity of 90% and a specificity of 45% in detecting hepatic steatosis ($>5\%$) at a cut-off of 0.38.¹³¹⁻¹³³ However, the specificity is inadequate, it has a limited AUROC, it is only validated in French cohorts, and, because of the undisclosed formula, a fee is imposed for each test applied. Moreover, the FLI showed

an AUROC of 0.84, and a sensitivity and specificity of 87% and 64%, respectively, at a cut-off of 30. Albeit the results have been confirmed in other studies (AUROC 0.78-0.84), only ultrasonography has been used as gold standard, and therefore the results should be interpreted carefully.¹³⁴⁻¹³⁷ Nevertheless, the FLI is used in epidemiological studies in an attempt to avoid ultrasonography.¹³⁸⁻¹⁴³

Recently, a Finnish team proposed the NAFLD Liver Fat Score, which was evaluated using magnetic resonance spectroscopy as gold standard. The NAFLD Liver Fat Score yielded an AUROC of 0.87 and a sensitivity of 95% and a specificity of 52% using a cut-off of -1.413.¹⁴⁴ These results were later confirmed by a different study group from the Netherlands with similar results.¹⁴⁵

The detection of suspected NAFLD often requires exclusion of other causes of hepatic steatosis. To some extent, this can easily be done non-invasively, by taking a full medical history and performing a full bloodwork. However, one must also diagnose the presence of advanced fibrosis (including cirrhosis), since early detection of advanced fibrosis is critical in the management and surveillance of patients with NAFLD.

Although liver enzymes are readily available and cheap, their sensitivity and specificity for diagnosing the presence of advanced fibrosis in NAFLD are poor, however their possibility in excluding advanced fibrosis is adequate.^{146, 147} Therefore, efforts have been made to create different scoring systems and evaluate their diagnostic accuracy as well as their prognostic value. The most commonly used scores are APRI, FIB-4, BARD and NAFLD Fibrosis Score (NFS) with high negative predictive values (Table 2).

Table 2. Sensitivity, specificity, PPV, NPV and AUROC for APRI, FIB-4, BARD and NAFLD Fibrosis Score (NFS) in NAFLD for detecting advanced fibrosis.

Score	Cut-off	No. of patients	AUROC	Sensitivity	Specificity	PPV	NPV
APRI*	1.00	1101	0.77	43%	86%	34%	90%
FIB-4†	1.3	2759	0.80	78%	71%	40%	93%
BARD‡	2	3057	0.76	75%	62%	38%	89%
NFS§	-1.455	3057	0.78	73%	74%	50%	92%

*AST to Platelet Ratio (APRI) = AST [U/L]/ALT [U/L]. FIB-4† = age [years] x AST [U/L]/(platelets [x10⁹/L] x ALT [U/L])^{1/2}. BARD‡ = (BMI ≥ 28 = 1; AST/ALT ratio > 0.8 = 2; Diabetes = 1). NAFLD Fibrosis Score (NFS) = -1.675 + 0.037 x age [years] + 0.094 x BMI [kg/m²] + 1.13 x IFG/Diabetes (yes = 1, no = 0) + 0.99 x AST/ALT ratio + 0.013 x platelet count (x10⁹/L) - 0.66 x albumin [g/dL]. Abbreviations: AUROC, Area Under the Receiver Operating Characteristics; PPV, positive predictive value; NPV, negative predictive value. Adapted from.¹⁴⁸

All the scoring systems have defined cut-off values for advanced fibrosis (fibrosis stage 3 and 4) and, to some extent, cirrhosis (fibrosis stage 4). However, with a low positive predictive value there is a high probability (usually >50%) of incorrectly diagnosing patients with suspected advanced fibrosis, especially in a primary health care setting. Therefore, these scoring systems, with high negative predictive values (89-93%), are mainly applied to exclude presence of advanced fibrosis.

1.4.3 Elastography

Imaging-based elastography is an emerging technology that uses imaging to non-invasively assess mechanical tissue properties. Three different techniques have evolved and been implemented in clinical routine: magnetic resonance elastography (MRE), shear wave elastography and vibration controlled transient elastography (VCTE). They assess stiffness indirectly by measuring the speed of shear waves propagating in the tissue of interest (*e.g.* the liver). The underlying concept is that shear wave speed is related to tissue stiffness; thus, shear waves travel faster in stiff tissue and slower in soft tissue. Shear waves may be generated either by applying a mechanical vibration to the surface of the body or by focusing an acoustic radiation force inside the tissue.

The most widely used and validated technique is VCTE, which is not solemnly user friendly, but has great reproducibility and high performance for ruling out advanced fibrosis with a negative predictive value of 96% if liver stiffness measure is <8 kPa.^{148, 149} A diagnostic algorithm for risk stratification of NAFLD patients non-invasively has been proposed (Figure 4).¹⁵⁰⁻¹⁵²

Magnetic resonance elastography, or MRE, also has high sensitivity for excluding advanced fibrosis in NAFLD when applying a cut-off of 3 kPa.¹⁵² Even though MRE has significantly better sensitivity than VCTE it is not as readily available and therefore mostly used for research purposes.¹⁵³

Figure 4. A suggested algorithm for the use of non-invasive tests for risk stratification of patients with NAFLD and without signs of decompensation. *Patients with an VCTE LSM of <8 kPa have a low risk of advanced fibrosis (NPV 94-100%). However, patients with an intermediate (8-9.9 kPa) or high (>9.9 kPa, PPV 47-70%) VCTE LSM should be considered for biopsy. †Caution should be taken when VCTE is used in patients with ascites, congestive heart failure or severely elevated ALT, though it could render false positive results. ‡There is still no clear consensus on repeat evaluation in patients with LSM <8 kPa, with some suggesting 1 year and others 3 years. Similarly, there is no clear consensus on when to repeat evaluation in patients with LSM >8 kPa without biopsy or biopsy confirmed fibrosis stage 3, however, repeat evaluation after 1 year has been suggested. Abbreviations: Fib-4, fibrosis-4; HL, hyperlipidemia; HTN, hypertension; LSM, liver stiffness measure; NFS, NAFLD fibrosis score; T2DM, type 2 diabetes mellitus; VCTE, vibration controlled transient elastography.

1.4.4 Magnetic Resonance Techniques

1.4.4.1 Basic Physics

Magnetic resonance techniques (most often) measure hydrogen-1 nuclei (protons or ^1H) signals as a function of their resonance frequency.¹⁵⁴ The hydrogen (or 'proton') signals of interest mainly come from fat and water, which both contains an abundance of hydrogen nuclei.

Hydrogen protons are positively charged (therefore often called just protons) but they also have a magnetic moment, meaning that they have a north and south pole. Furthermore, they 'rotate around their own axis' (hence they are also called 'nuclear spins') – which creates microscopic magnetic moments, or small microscopic magnetic fields. Hydrogen-1 (protons) can be found in an abundance in the body, mainly in water (about 60% of body weight) and fat (about 20% of body weight). Normally, the microscopic magnetic moments of all hydrogen (proton) spins are randomly oriented and therefore cancel each other out, thus not creating any macroscopic net magnetic moment. However, when put into a strong magnetic field (as a magnetic resonance imaging scan) the protons orient with the magnetic field (low-energy state) or against the magnetic field (high-energy state) in a state of precession (*e.g.* 64 MHz at 1.5 T). Therefore, tissues containing protons are associated with a macroscopic net longitudinal magnetization, which also depends on the orientation of the spins. Despite the vertical alignment of hydrogen spins in a magnetic field, they may all have a different 'horizontal' orientation; thus, they do not spin synchronously – often referred to as being 'out of phase'. However, if one exposes the tissue to a very brief high-powered radiofrequency pulse, it is possible to cancel the *longitudinal* (or '*vertical*') *magnetization* entirely, and thereby forcing all spins to 'rotate together' in the 'horizontal plane'. This will thus create a net magnetic force oriented *horizontally* (this is called *transverse magnetization*) which can be detected using an MR-detection coil (head coil, abdominal coil, knee coil etc). The reason is that the transverse spins that are in phase create a detectable current – or a resonance signal – in the detection coil. When the radiofrequency pulse is turned off inside the MR-scanner, the microscopic spins will tend to lose their synchrony and the transverse macroscopic net magnetization will therefore decrease; this is due to a natural process which is called the *spin-spin* – or *T₂* – *relaxation*. Eventually, spins will fall back into the baseline state (*i.e.*, the net magnetization will then tend to orient along the vertical axis). The previously absorbed (radiofrequency) energy will be converted as heat into the surrounding tissue in that process. Thus this relaxation process restores, the original longitudinal macroscopic net magnetization, and it is called *spin-lattice* – or *T₁* – *relaxation*.^{155, 156}

Because water and fat hydrogen protons experience different local environments, they have characteristic T1 and T2 relaxation values (measured in [s] or [ms]). You can enhance these characteristic differences by altering the rate of the application of radiofrequency energy – or in other words changing the repetition time (TR) – and, how quickly you choose to pick up the signals coming back from the transverse magnetization – or the echo time (or TE). The entire protocol which describes which timing parameters that are suitable and most optimal for a particular MR-examination is referred to as the *pulse sequence*.^{155, 156}

Conventional magnetic resonance imaging (MRI) is used to produce anatomical grey-scale images by exploiting the differences in relaxation properties, such as water and fat. The scans usually containing many thousands of volume elements (voxels) reflecting the net magnetic property of each fragment of the tissue, which are presented as darker or lighter areas.¹⁵⁷

Furthermore, radiofrequency energy is part of the electromagnetic spectrum that include visible lights and x-rays. All waves are defined as a wavelength (distance between peaks of wave) and frequency (how many cycles the wave completes every second in Hz). The signals from water and fat are not always in phase with each other and can therefore be separated depending on the specific choice of echo time.¹⁵⁵

Moreover, when a spin, is part of a molecule, it is slightly shielded from the large magnetic field by chemical bonds (which are entirely composed by *negatively* charged electrons). The amount of which it is shielded depends on the position of the spin inside the molecule. Therefore, spins in different chemical compounds (*i.e.* fat vs. water) will experience slightly different magnetic field strengths and will therefore resonate at different frequencies. In a magnetic field, hydrogen protons resonate at a frequency of 42.58 MHz per 1 Tesla (for example at 1.5 T the resonance frequency will be about 64 MHz). This is called the Larmor (or precession) frequency. The different resonance frequency of one hydrogen proton in one compound compared to another is known as the *chemical shift*.¹⁵⁷

Quantification of fat in the liver with MRI can be done using different techniques. In-phase and opposed-phase (or alternatively 'out of phase') MRI is based on the analysis of the signal loss in the opposed-phase images compared to the in-phase images to detect liver fat. For calculating the liver PDFF, the images are often used to create water-only and fat-only images. This technique is commonly called chemical shift encoded (CSE-)MRI, or 'Dixon imaging'.^{157, 158}

As with MRI, proton magnetic resonance spectroscopy (¹H-MRS) uses the differences in the resonance frequencies between water and fat. The

signal intensity at frequencies corresponding to water or fat can be quantified, and the fat-signal fraction can be calculated.^{157, 158} MRS is still the gold standard among imaging based procedures for determining PDFF, but it can only be used at one location at a time in contrast to imaging based techniques.

1.4.4.2 Proton Density Fat Fraction (PDFF)

MR-techniques have emerged as a rapid and convenient tool for measuring hepatic steatosis. Even though MR is not as widely available as other radiological techniques (*i.e.* ultrasonography and computed tomography), it surpasses them with a much higher specificity and sensitivity, even for lower amount of steatosis.

¹H-MRS and MRI are non-invasive methods that can be used to quantitatively assess the total HTGC, or the PDFF.^{158, 159} ¹H-MRS is widely considered to be the most accurate non-invasive method for measuring liver fat content, and it is therefore the reference standard in determining the PDFF.¹⁶⁰ However, CSE-MRI (Dixon imaging) is commonly used to assess hepatic steatosis. An excellent correlation has been shown between ¹H-MRS and CSE-MRI, and the two methods are commonly thought to be equal in assessment of hepatic steatosis.¹⁶¹⁻¹⁶³ Moreover, ¹H-MRS also correlates excellently with total lipid quantification in specimens of liver tissue.¹⁶⁴ Thus, some researchers have suggested that ¹H-MRS or CSE-MRI should replace liver biopsy as the standard method for assessment of liver fat content.^{164, 165}

When HTGC is measured with MRI-derived PDFF, a cut-off value of 5% or 5.56% is frequently used to define steatosis.^{159, 166} The cut-off of 5% is extrapolated from the cut-off based on liver biopsies, even though MRI-PDFF and liver biopsies give two distinctly different estimates of steatosis. Liver biopsy measures the fraction of hepatocytes with fat vacuoles whilst MRI-PDFF measures the amount of triglycerides of a given volume of tissue.

The latter value (5.56%) is based on the results from Szczepaniak *et al*, who examined the variance of HTGC using ¹H-MRS in 345 subjects at low risk for hepatic steatosis (*i.e.*, in lean subjects with no glucose intolerance or excessive alcohol consumption, and normal serum liver enzyme levels), but without specific knowledge of the subjects histopathology.¹⁵⁹ However, hepatic steatosis can be present in lean subjects¹⁶⁷ as well as in individuals with normal serum liver enzyme levels.^{4, 128, 168} Hence, histopathology should be used as the reference standard to define the optimal cut-off value for the definition of hepatic steatosis by MR.

In two recent studies, Tang *et al* evaluated the diagnostic accuracy of using an MRI-PDFF technique to distinguish between the absence (grade

o) and the presence (grade 1-3) of hepatic steatosis using histopathology as gold standard.^{169, 170} However, only 11 patients without steatosis were included in these two studies, yielding a threshold of 6.4% and 6.9%, respectively, for distinguishing between absence and presence of steatosis according to conventional histopathological method. The authors concluded that the cut-off to diagnose steatosis with MRI-PDFF should be evaluated in a cohort with a higher number of subjects free of hepatic steatosis. Moreover in other studies¹⁷¹⁻¹⁷⁴ that evaluated the diagnostic performance of MRI-PDFF for distinguishing absence from presence of steatosis, all, or the majority of the subjects, had confirmed or a high probability of NAFLD, and thus it may be difficult to assess an adequate cut-off value of HTGC with MR in these type of cohorts.

1.4.5 Liver Biopsy

The gold standard for diagnosing liver pathology is a morphological and histological examination of the liver in whole. Nevertheless, this is impossible in living subjects. Therefore, the best reference to gold standard is liver biopsy. However, liver biopsy only assesses 0.00002% of the liver, thus sometimes it is referred to as the best standard instead of the gold standard.¹⁷⁵

The first mention of liver biopsy was by von Frerichs, attributing the introduction to Ehrlich in 1884.¹⁷⁶ However, a hallmark study in professionalization of the technique came after the publication by Menghini, and his famous needle, in 1958.¹⁷⁷ Albeit being a useful tool in diagnosis, the mortality rate ranged from 0.02-0.05% with adverse events (*i.e.* major bleeding, pneumothorax, peritonitis) ranging from 0.3-0.4%.¹⁷⁸ The introduction of real time ultrasonography guided liver biopsy as well as new biopsy needles seems to have decreased the risk of pneumothorax, however the risk of adverse events and mortality are stationary.¹⁷⁹

Moreover, liver biopsy is prone to high sampling and observer variability, making the results good, but not gold (Table 3).

Table 3. Sampling, intraobserver and interobserver agreement in NAFLD

	Coefficient of concordance (κ)		
	Sampling agreement	Intraobserver agreement	Interobserver agreement
Steatosis grade	0.64-0.91 ^{a, c, e, f}	0.74-0.98 ^{a, b, f, h}	0.10-0.65 ^{c, d, h, i}
Ballooning grade	0.20-0.87 ^{a, c, e, f}	0.56-0.68 ^{a, b, f, h}	0.01-0.50 ^{d, h, i}
Lobular inflammation grade	0.13-0.58 ^{a, f}	0.37-0.58 ^{a, b, f, h}	0.14-0.29 ^{d, h, i}
Fibrosis stage	0.47-0.96 ^{a, c, e, f}	0.68-0.80 ^{a, b, f, h}	0.32-0.60 ^{c, d, h, i}
NAFLD Activity Score (NAS)	0.69 ^f	0.61-0.86 ^{b, f, h}	0.18-0.62 ^{g, h}
Diagnosis of NASH	0.69-0.91 ^{c, e, f}	0.81-0.86 ^{b, f}	0.18-0.90 ^{c, g}

^aRatziu et al¹⁸⁰, ^bVuppalanchi et al¹⁸¹, ^cArun et al¹⁸², ^{d, g}Younossi et al^{183, 184}, ^eLarson et al¹⁸⁵, ^fMerriman et al¹⁸⁶, ^hPournik et al¹⁸⁷ and ⁱJung et al¹⁸⁸.

This is further hampered by the normal fluctuation of inflammation and the inherent heterogeneity of the disease (Figure 5).¹⁸⁹ The sampling agreement is usually adequate when biopsy is performed in the same lobe, however biopsies from different lobes, usually renders high viarability.^{180, 182, 185, 186}

Nevertheless, liver biopsy is still of high value when:

- I. Diagnostic uncertainty exists (*e.g.* in patients with atypical features)
- II. Coexisting disorders are present (*e.g.* Human immunodeficiency virus and hepatitis C virus [HCV] infection, or alcoholic liver disease and HCV)
- III. An overlapping syndrome is suspected (*e.g.* Primary biliary cholangitis and autoimmune hepatitis)
- IV. Hereditary disorder is assumed (*e.g.* Hemochromatosis, alpha-1 antitrypsin deficiency, Wilson´s disease)
- V. Suspected advanced fibrosis stage needs to be confirmed

In the presence of fatty liver disease or chronically elevated liver function tests, different blood panels can exclude other liver diseases. If NAFLD is suspected one must differentiate between the presence, or the absence, of advanced fibrosis. This can partly be done non-invasively. However, although previously mentioned non-invasive techniques show high validity and accuracy in excluding severe liver disease, many of the non-invasive techniques tend to give unreliable or intermediate results when being used to display presence of advanced fibrosis. Hence, in a selected group of patients with NAFLD, liver biopsy is still required.

Figure 5. The new view of fibrosis progression in NASH with repetitive inflammation and cumulative accumulation of scar tissue with progression towards cirrhosis.

1.5 Histological Course of NAFLD

The histopathological spectrum of NAFLD ranges from isolated steatosis and NAFL to NASH with progressive fibrosis leading to cirrhosis and subsequently hepatocellular carcinoma (HCC).

Even though NAFLD encompasses a wide range of morphological differences, the hallmark of NAFLD is macrovesicular steatosis, which predominantly affects perivenular regions, and in severe cases extending to a panacinar distribution. Moreover, necroinflammatory changes and hepatocyte injury, expressed as hepatocellular ballooning degeneration, can be observed and is thought to portend a more progressive disease. Lobular inflammation with a mixed infiltration pattern (*i.e.* with neutrophils, lymphocytes and macrophages) can be detected although the severity is typically mild.

The characteristic pattern of fibrosis is typically perisinusoidal and/or pericellular. Eventually bridging fibrosis may develop and in some, progression to cirrhosis occurs.

1.5.1 Non-Alcoholic Steatohepatitis

Non-alcoholic steatohepatitis is thought to be the more progressive form of NAFLD with a more progressive histological outcome and with an increased mortality and an increased risk of end-stage liver disease. This was portrayed in a meta-analysis by Musso *et al* where subjects with NASH had an almost two-fold increase in overall mortality and six-fold increase in liver-related mortality.¹⁹⁰ However, the definition of NASH has varied since the discovery of NAFLD – making studies hard to compare. The diagnosis of steatohepatitis has long been the prerogative of pathologists, without any clear consistency.

However, in 1999, Brunt *et al* attempted to characterize the histological definition of NASH, and it has since then been widely accepted. It unified the lesions of steatosis and inflammation into a grade (0-3; ranging from none to mild, moderate and severe) and those of fibrosis, into a stage (ranging from 0-4).¹⁹¹ Although this scoring system was appealing, it was developed for NASH and did not encompass the entire spectrum of NAFLD. Hence, a multicentre cooperative called the NASH Clinical Research Network (NASH-CRN) was created¹⁹² and developed a scoring protocol comprised of 14 individual histopathological features to encompass the entire spectrum of NAFLD.¹⁹³ The developed scoring system was coined NAFLD Activity Score (NAS), where the unweighted sum of steatosis, lobular inflammation, and hepatocellular ballooning presented the severity of NAFLD. NASH was defined as a $NAS \geq 5$, “borderline NASH” as a NAS of 3 or 4, and “not NASH” as a $NAS \leq 2$. However, the authors also stated that NAS is not intended to replace the pathologist’s diagnostic determination of steatohepatitis (*i.e.* NASH). Despite this, NAS is recommended to be used to define and quantify disease in clinical trials of NAFLD/NASH.¹⁹⁴ When change in NAS is used as a primary outcome, it is recommended that ≥ 2 point improvement in total score be achieved, alongside no worsening of fibrosis.

In 2011, Younossi *et al*, studied the biopsies of 209 NAFLD patients and measured the agreement between Kleiner and Brunt’s classification of NASH, which yielded a κ agreement of 0.178, indicating a slight agreement.¹⁸⁴ In the same study, only advanced fibrosis demonstrated the best independent association with liver related mortality. This study provoked a commentary in the *Hepatology* journal questioning the NAS. The authors, Brunt and Kleiner, replied, and stated yet again with clarification, “When we described the NAS, we did note that there was a statistical correlation between $NAS \geq 5$ and an independent diagnosis of steatohepatitis by a pathologist. Unfortunately, this observation has been misinterpreted by some as a proposal for a diagnosis with the score, even though we clearly stated otherwise in our study”.¹⁹⁵

However, in 2015, two articles, by Angulo *et al* and Ekstedt *et al* showed that liver fibrosis, and no other histological features, predicted disease specific and all-cause mortality in NAFLD patients.^{196, 197} The impact of NASH, according to NAS, did not seem to have any effect on disease specific or all-cause mortality when adjusting for fibrosis. However, a new score named SAF (Steatosis, Activity, Fibrosis) was made with excellent interobserver agreement for the diagnosis of NASH ($\kappa=0.80$).¹⁹⁸ In the SAF score, the grading and staging of steatosis and fibrosis, respectively, is equal to that in NAS, however, activity is defined as the unweighted sum of ballooning grade and inflammation grade (with a slight difference compared to NAS). Moreover, the SAF score classifies you as having mild (activity <2 and fibrosis <2) or significant (activity >2 or fibrosis >2) disease severity. In parallel with SAF, the Fatty Liver Inhibition of Progression (FLIP) algorithm was presented. In the FLIP algorithm, all patients with at least 1 point in steatosis, ballooning and lobular inflammation are diagnosed as NASH. This new algorithm differs from NAS in several ways. When NASH is diagnosed with NAS, a patient with steatosis grade 3 and lobular inflammation grade 2 can be determined as having NASH (NAS=5). However, according to the FLIP algorithm, this patient would be classified as having NAFLD ("not NASH"). Hence, the FLIP algorithm depends on ballooning and inflammation to diagnose NASH (Figure 6).

In 2017, Hagström *et al*, used data from 139 NAFLD patients with up to 41 years of follow-up (median, 25.3). All patients' biopsies were graded according to SAF, and after adjusting for fibrosis, the SAF score was not associated with increased mortality in NAFLD patients.

The notion that inflammation surpasses fibrosis in NAFLD is not contested. However, there is no objective evidence that NASH, either defined according to Brunt and Kleiner (NASH-CRN) or SAF (FLIP-algorithm), predicts fibrosis progression or substitutes as a surrogate marker for fibrosis in pharmacological trials.¹⁹⁹

Figure 6. The FLIP algorithm for diagnosing NASH.

1.5.2 Inflammation and Ballooning Degeneration

Ballooning degeneration, or solely ballooning, is considered a form of hepatocyte apoptosis which results in hepatocyte swelling with nuclei shrinkage and fragmentation. Albeit ballooning alone does not define steatohepatitis, its presence is considered to represent a more progressive form of NAFLD.

In a hallmark meta-analysis by Argo *et al*, the presence of any type of inflammation (*i.e.* lobular, portal or necroinflammatory) predicted the development of advanced fibrosis.²⁰⁰ Moreover, Singh *et al* showed a more rapid fibrosis progression rate in patients with NASH.²⁴ In the study by Angulo *et al*, NASH, defined by NAS, showed no association with long term outcome in NAFLD when stratifying for fibrosis. However, ballooning and portal inflammation portrayed an increased risk of end-stage liver disease and transplantation.¹⁹⁷ Also, a trend of ballooning as a predictor of fibrosis progression was seen in the study by McPherson *et al*²⁰¹ with a p-value of 0.08. This was later corroborated by Sanyal *et al*¹⁹⁹, where baseline levels of ballooning was associated with fibrosis progression (HR 4.83, 95%CI 1.45-16.07).

Even though earlier paired biopsy studies have not been able to show that NASH predicts *progression* of fibrosis, NASH, and especially ballooning, seem to predict both *presence* of fibrosis and of clinical features such as insulin resistance, suggesting a high collinearity between NASH and fibrosis stage.

1.5.3 Fibrosis

There are 14 studies with paired biopsies in NAFLD patients^{10, 11, 15, 16, 22, 23, 199, 201-207}, including in total 996 patients with a median follow-up time between biopsies ranging from 2 to 13.8 years. However, because the definition of NASH and the staging of fibrosis have changed over time, comparisons are difficult to make. Moreover, a majority of the studies are retrospective with patient cohort selected through pathology records and with NASH dominating the histopathological diagnosis. In 10 studies, including 416 patients^{10, 15, 16, 22, 201-203, 205-207}, fibrosis stage at baseline and follow-up are reported; with 37% having fibrosis progression (153/416) and 12% progressing from low (Fo-F2) to advanced stages of fibrosis (F3-F4) (Table 4). The number of patients who developed advanced fibrosis does not include patients in whom biopsy was withheld secondary to clinical signs of end-stage liver disease.

Table 4. NAFLD studies with paired biopsies.

Study	N	Follow-up time, years (median (range))	Baseline		
			NASH	T2DM	Predictors of fibrosis progression
Lee ¹⁰	13	3.3 (1.2-6.9)	100%*	54%	None
Powell ¹¹	13	3 (1.5-6.5)	100%†	46%	None
Teli ¹⁶	12	7.6-16	0%‡	N/A	N/A
Ratziu ²⁰³	14	5 (1.5-15)	29%§	N/A	N/A
Evans ²⁰²	7	7 (5.5-14)	N/A*	43%	N/A
Harrison ²⁰⁴	22	5.7 (1.4-15.7)	41%**	41%	AST
Fassio ²⁰⁵	22	4.3 (3-14.3)	100%††	36%	BMI
Adams ²⁰⁶	103	3.2 (0.7-21.3)	93%‡‡	42%	BMI, FS, T2DM
Hui ²⁰⁷	17	6.1 (3.8-8)	35%§§	24%	None
Ekstedt ¹⁵	68	13.8 (10.3-16.3)	49%¶¶	9%	None
Wong ²²	52	3	33%***	50%	LDL
Pais ²³	70	3.4 (1-12)	64%†††	35%	N/A
McPherson ²⁰¹	108	6.6 (1.3-22.6)	75%‡‡‡	48%	Fib4
Sanyal ¹⁹⁹	475	1.8	71%¶¶¶	68%	FS

NASH defined as, *NASH according to pathologist, †steatosis+lobular inflammation, (†all patients had isolated steatosis), §steatosis+necroinflammation (=lobular necrosis and/or piecemeal necrosis), ¶NASH according to Brunt¹⁹¹, **NASH according to Brunt, ††steatosis(>10%)+lobular inflammation+ballooning/Mallory hyaline fibrosis/sinusoidal fibrosis/a combination thereof, ††steatosis+lobular inflammation+ballooning OR steatosis+any stage of fibrosis, §§NASH according to Brunt, ¶see Adams, ***steatosis+ballooning/intralobular hepatocyte necrosis, †††steatosis+lobular inflammation+ballooning/advanced fibrosis, †††steatosis+inflammation+ballooning ALSO 3 other patients defined as NASH (1 had steatosis+advanced fibrosis and 2 had steatosis+lobular inflammation+fibrosis stage 2), ¶¶ According to NASH CRN. Abbreviations: NASH, non-alcoholic steatohepatitis; T2DM, type 2 diabetes mellitus; AST, aspartate aminotransferase; BMI, body mass index; LDL, low-density lipoprotein; Fib4, fibrosis-4 (biochemical score for prediction of liver fibrosis); FS, fibrosis stage; N/A, not applicable.

A recent systematic review and meta-analysis by Singh *et al* compared 11 paired biopsy studies which included 411 patients with 2145.5 person years of follow-up (Table 4). They showed that 33.6% progressed in fibrosis

stage.²⁴ The overall annual fibrosis progression was found to be 0.07 stages for NAFL and 0.14 stages for NASH, corresponding to one stage of fibrosis progression over a median of 14.3 years and 7.1 years, respectively.

Nonetheless, predicting fibrosis progression using baseline histological clinical, biochemical or anthropometric parameters is difficult. Presence of NASH or NAS at baseline has never correlated with progression of fibrosis.

Conversely, NASH (or high NAS) has been more prevalent in the follow-up biopsies in patients with fibrosis progression. However, the notion that the presence of NASH correlates with presence of fibrosis does not mean that NASH equals the prediction of fibrosis progression. And therefore, resolution of NASH is not likely to be synonymous with regression of fibrosis.

In a recent study by Sanyal *et al*, 475 patients with NASH and advanced fibrosis had a second liver biopsy performed after 96 weeks during clinical trials.¹⁹⁹ Baseline high NAS did not predict fibrosis progression. However, among baseline factors, the presence of advanced fibrosis was associated with fibrosis progression. The findings by Sanyal *et al* correlate with previous studies and meta-analyses using paired biopsies. This was validated in an abstract, by McPherson *et al*, including 321 patients with NAFLD who underwent sequential biopsies conducted over a median follow-up of 4.1 years (range, 1-22.6). In this abstract, fibrosis progression was present in 35% of the subjects, with no difference in fibrosis progression based on fibrosis stage or NASH/NAFL at baseline. Interestingly, moderate to severe steatosis seemed associated with fibrosis progression.²⁵

1.5.4 Steatosis

When hepatic steatosis grade 1-3 is present without lobular inflammation, ballooning or fibrosis, it is usually referred to as isolated steatosis. However, when steatosis is present with mild inflammation, the term NAFL or simple steatosis is commonly used.

Since isolated steatosis has been considered a benign condition, few studies have focused on the natural history of patients with isolated steatosis. In a study by Teli *et al*¹⁶ 12 patients with isolated steatosis were followed for a period of 7.6 to 16 years. One, out of the 12 patients, progressed and did so from F0 to F1. In a recent study from the United Kingdom, 108 patients underwent serial biopsies – 17 were diagnosed with isolated steatosis of whom 4 (24%) progressed in fibrosis stage.²⁰¹

Although our knowledge of simple steatosis is limited, new studies on NAFL have recently emerged which show that fibrosis progression occurs in a significant proportion of NAFL patients.^{22, 23, 201} In a prospective study by Wong *et al*, 8 out of 29 NAFL patients (defined as NAS <3), with two

sequential liver biopsies 3 years apart, showed fibrosis progression.²² Similarly, in a study by McPherson *et al*, 10 out of 27 patients with NAFL showed fibrosis progression.²⁰¹ This was corroborated by Pais *et al*, wherein 6 out of 25 patients with NAFL progressed to bridging fibrosis.²³ However, in the two abovementioned studies by McPherson *et al* and Pais *et al*, NAFL was defined as isolated steatosis or steatosis with mild inflammation (*i.e.* grade 1), without ballooning but with fibrosis stage <3.

Even though NAFL seems to exhibit a slower progression rate compared to NASH (14.3 years vs. 7.1 years for 1 stage of fibrosis), there does not seem to be any difference between NAFL and NASH in the amount of patients progressing from fibrosis stage 0 to advanced fibrosis during a 6 year period (5 out of 29 patients with NAFL and baseline fibrosis stage 0 [17%] vs. 2 out of 11 patients with NASH and baseline fibrosis stage 0 [18%]) – as was shown in the meta-analysis by Singh *et al*.²⁴

1.6 Prognosis – morbidity and mortality

Chronic liver disease (CLD) is one of the leading causes of public health burden in the western world.²⁰⁸ During the last decade, the global burden of liver disease has increased with 10.3%, and with a consequential increase in liver cancer of 11.5%.²⁰⁹ Moreover, mortality from liver cirrhosis has increased over the last three decades, now accounting for approximately 2% of all deaths.²¹⁰ Albeit this is mostly related to alcohol and viral related hepatitis, data from the National Health and Nutrition Examination Surveys has shown that the overall prevalence of CLD in the United States has risen from 12% to 15% between 1988 and 2008, mainly attributed to NAFLD, whilst alcohol and viral related hepatitis remain stable.⁵ The prevalence of clinically significant liver fibrosis in the population is estimated at between 2-13%,²¹¹ mainly attributed to NAFLD.²¹²⁻²¹⁴

NAFLD is thought to be the hepatic manifestation of the metabolic syndrome^{38, 215, 216} (type 2 diabetes [T2DM], hypertension, obesity and dyslipidemia). And, with an epidemic trend in obesity in parallel with rising prevalence of T2DM, the prevalence of NAFLD is expected to rise.²¹⁷

1.6.1 Obesity

Obesity (body mass index [BMI] ≥ 30 kg/m²) has increased six fold over the last 4 decades, affecting over 600 million individuals in 2016, with another 1.3 billion individuals being overweight (BMI 25.0-29.9 kg/m²).²¹⁸ Even though there is an ongoing discussion whether overweight decreases or increases mortality²¹⁹ – non refute the fact that obesity increases all-cause mortality.²²⁰⁻²²² However, in a recent study by Nordström *et al* investigating 4,046 monozygotic twins, they were not able to verify that overweight

increased overall death or risk of myocardial infarction after consideration of genetic factors.²²³ Nonetheless, the risk of incident T2DM was significantly more frequent amongst heavier twins (OR 1.94, 95%CI 1.51-2.48).

As Nordström *et al* refuted a long, believed axiom of causality of overweight as a risk factor for myocardial infarction, the correlation between the two is still present. In the same way, the causality of the relationship between overweight and NAFLD is not clear, however, the correlation is existing with the prevalence of NAFLD increasing with rising BMI. Lazo *et al* showed that the prevalence of NAFLD increased almost exponentially with increasing BMI, with a prevalence of 57% and 44% in men and women with a BMI >35 kg/m².²²⁴ Moreover, patients with NAFLD commonly have higher BMI in cross-sectional studies compared with patients without NAFLD.^{225, 226}

In the Coronary Artery Risk Development in Young Adults Study, weight gain during young adulthood seem to predict future development of NAFLD.²²⁷ Furthermore, weight loss, either by lifestyle intervention²²⁸⁻²³⁰ or bariatric surgery,²³¹⁻²³³ seem to resolve both NAFLD and insulin resistance.

While overweight and obesity is highly associated with NAFLD and aspects of the metabolic syndrome, whether it is causal, or correlative is still controversial. However, obesity, and especially visceral adiposity, seem to predict development of severe liver disease both in the general population and in patients with NAFLD. In a study by Ioannou *et al*, obese patients followed for a period of 12.9 years showed an increased risk of cirrhosis related death or hospitalization compared to normal-weight subjects.²³⁴ These findings were corroborated by Liu *et al* and Hagström *et al* who showed that increasing BMI was associated with an increased incidence of severe liver disease.^{235, 236} Moreover, in 2003, Calle *et al* showed that for every 5 unit increase in BMI >25 kg/m², a parallel increase in mortality from cancer was seen.²³⁷ Also, they showed an exponential increase in the risk of liver cancer (hepatocellular carcinoma; HCC) in male subjects with obesity.

In a second study by Hagström *et al*, 1.2 million men enlisted for military conscription in Sweden, were followed for a mean period of 28.5 years.²³⁸ During follow-up, more than 5,000 cases of severe liver disease and 251 cases of HCC were identified. Overweight and obesity increased the risk of developing both severe liver disease and HCC.

1.6.2 Type 2 Diabetes Mellitus

During the last four decades the age-standardised prevalence of T2DM in adults has doubled in men and increased with 60% in women.²³⁹ The prevalence of T2DM in NAFLD ranges from 45%-75% in hospital based studies

and from 30-60% in population based studies.²⁴⁰ Equivalently, the overall prevalence of NAFLD in patients with T2DM is 55.5%.²⁴¹ However, it is not clear if NAFLD precedes or succeeds T2DM, though the relationship seems bidirectional.^{242, 243}

In a recent meta-analysis, patients with NAFLD had a two-fold increase in the risk of incident T2DM.²⁴⁴ This was later corroborated by Chen et al in a study with 132,000 subjects over a period of 6 years. They showed that NAFLD patients (diagnosed with ultrasonography), adjusted for age, sex, BMI and other common risk factors, had more than a two-fold increase for T2DM over time (aHR 2.08, 95%CI 1.93-2.33 for men and aHR 2.65, 2.43-2.88 for women).²⁴⁵

To date there are 21 studies investigating the association between NAFLD, diagnosed with ultrasonography or computed tomography, and T2DM.²⁴⁶⁻²⁶⁶ The incidence of T2DM between non-NAFLD and NAFLD subjects were 2.4% and 8.0%, respectively, with an overall relative risk of 1.72 (Figure 6).

There is an ongoing discussion whether the presence of steatosis predicts diabetes development or if it is an epiphenomenon. In a study by Sung *et al* resolution of hepatic steatosis diagnosed by ultrasonography during a

Figure 7. The incident risk of type 2 diabetes in NAFLD patients diagnosed with ultrasonography (n=20) or computed tomography (n=1).

5-year follow-up was not associated with decreased incident T2DM (aOR 0.95, 95%CI 0.5-1.9).²⁵⁷ Nevertheless, in a recent study by Yamazaki *et al*, hepatic steatosis resolution during a 11.3-year follow-up showed a reduction of incident T2DM (aOR 0.27, 95%CI 0.12-0.61).²⁵⁸

However, there are few studies examining the relationship between biopsy-proven NAFLD and the risk of incident T2DM. In a study by Ekstedt *et al*, 8.5% of patients with biopsy-proven NAFLD had T2DM at inclusion. After almost 14 years of follow-up 58% had T2DM.¹⁵ These study results have been corroborated by two other Scandinavian studies.^{267, 268}

Interestingly, the main histological findings predicting development of T2DM seem to be steatosis and fibrosis.²⁶⁹ The presence of a more severe histological form of NAFLD (*i.e.* inflammation and advanced fibrosis) is more prominent in NAFLD patients with T2DM.^{168, 270, 271} In a recent meta-analysis, 17% of all patients with T2DM and biopsy-proven NAFLD had advanced fibrosis.²⁴¹ Moreover, patients with newly diagnosed T2DM seem to be at increased risk of incident cirrhosis (aHR 2.55, 95%CI 2.35-2.76).²⁷² However, no paired biopsy studies have confirmed that patients with T2DM at baseline portend a worse prognosis (*i.e.* fibrosis progression).

In a recent epidemiological study by Björkström *et al*, patients with T2DM had more than a two-fold increased risk of developing severe liver disease (HR 2.28, 95%CI 2.21-2.36).²⁷³ Similarly, in a study by Zoppini *et al*, diabetic patients had more than a two-fold increased risk in dying secondary to a chronic liver disease, mainly NAFLD.²⁷⁴ Furthermore, T2DM is associated with both an increased risk of hospitalization secondary to NAFLD²⁷⁵, and an increased risk of developing HCC.^{276, 277}

1.6.3 Cardiovascular Disease

In a seminal article by Jepsen *et al* the relationship between cardiovascular disease (CVD) and fatty liver disease was established.²⁷⁸

The risk of incident cardiovascular disease and the risk of developing cardiovascular disease over time seems increased in patients with ultrasonography diagnosed NAFLD.²⁷⁹⁻²⁸¹ This increase is probably related to an increase in comorbidities. The Framingham Risk Score (FRS), which includes age, gender, total cholesterol, HDL, smoking, T2DM, and hypertension, is a gender specific algorithm used to estimate 10-year cardiovascular risk in an individual. In a study by Treeprasertuk *et al*, NAFLD patients had a higher FRS compared to the general population, also, FRS was the only variable significantly associated with new onset CVD (OR 1.13, 95%CI 1.05-1.21).²⁸² Similar results was observed in a study by Hagström *et al* where 608 biopsy-proven NAFLD patients had a significantly increased risk of developing a CVD event compared to a control population (HR 1.54, 95%CI

1.30-1.83). Amongst the NAFLD patients, age, male gender, smoking, T2DM and triglycerides were associated with incident CVD.²⁸³

In a prospective study by Fracanzani *et al*, the risk of developing major cardiovascular events over a 10-year period was higher in patients with NAFLD compared to controls.²⁸⁴ Albeit this was somewhat explained by the higher incidence of plaques and increased carotid intima media thickness, presence of histologically or ultrasonography confirmed hepatic steatosis was an independent predictor. Additionally, in a recent cohort study, 612 patients undergoing coronary angiography, were evaluated for the presence of hepatic steatosis measured by ultrasonography, and the majority were diagnosed with NAFLD.²⁸⁵ Coronary artery stenosis was significantly more common in NAFLD patients (84.6% vs. 64.1%) and the need of percutaneous coronary intervention significantly more frequent (68.3% vs. 43.4%). Conversely, NAFLD patients had a lower risk of CVD death ($p=0.006$) but equal risk of cardiovascular events ($p=0.054$) compared to the control group.

Even though increased incident CVD in NAFLD is established the association between NAFLD and CVD mortality is somewhat unclear. In a systematic review and meta-analysis including 34 studies of patients with NAFLD (mainly diagnosed by ultrasonography), no increase in CVD mortality was observed (HR 1.10, 95%CI 0.86-1.41).²⁸⁶ A supporting meta-analysis showed equivalent results without any overall increase in CVD mortality in NAFLD.²⁸⁷ However, in most studies including biopsy-proven NAFLD cohorts, mortality is increased in NAFLD patients compared to controls, with CVD being the main cause of death.^{196, 206}

1.6.4 Liver Transplantation

NAFLD is now the second leading aetiology of CLD among patients awaiting liver transplant, viral related hepatitis (mainly HCV) still being the dominating reason. Although the epidemic of obesity is a strong contributing factor for NAFLD, the prevalence of NAFLD among lean subjects is estimated to 16-20%.²⁸⁸⁻²⁹⁰ Also, new effective treatment for HCV, will subsequently decrease the number of patients in need of transplant secondary to end-stage liver disease caused by viral hepatitis.²⁹¹ Therefore, it is proposed that NAFLD will be the leading cause of liver transplant in a near future. However, due to the high prevalence of NAFLD the need for liver transplant will not only increase, but the number of healthy donors will furthermore decrease.²³ From an economic point of view the current and future burden of NAFLD in the Western world is expected to be vast.⁷

1.6.5 Mortality

In biopsy-proven NAFLD, the main cause of death is cardiovascular disease (HR 1.55-1.85, ~35%). Moreover, liver-related complications usually rank as the third leading cause of death (HR 3.2-6.5, ~8%).^{196, 197} Increased overall mortality has been shown in studies comparing biopsy-proven NAFLD with reference populations.^{14, 15, 196, 292} These findings were strengthened in a meta-analysis that showed increased risk of all-cause mortality in patients with biopsy-proven NAFLD (OR 1.40, 95%CI 1.23-1.60).¹⁹⁰ However, a newly published meta-analysis was unable to corroborate this difference (HR 1.14, 95%CI 0.99-1.32). Noteworthy is that, in the latter study the majority of patients were diagnosed with ultrasonography.²⁸⁶ This could reflect the selection bias that systematically permeate studies that include patients with biopsy-proven NAFLD, which usually are patients referred to specialists in secondary or tertiary care, coordinated by large academic centres.

2. AIMS

- To assess whether hepatic steatosis can be reliably detected non-invasively with magnetic resonance spectroscopy (^1H -MRS PDFF).
- To assess the accuracy of ^1H -MRS PDFF and stereological point counting (SPC) in diagnosing hepatic steatosis using histopathological grading as the reference standard.
- To determine appropriate cut-off values for diagnosing steatosis using both ^1H -MRS PDFF and SPC.
- To investigate the long-term prognosis in NAFLD patients compared to controls and to specifically study the effect of NASH on the outcomes of mortality and liver-specific morbidity.
- To describe the clinical, biochemical and histological disease progression of patients with NAFLD and to assess predictors of disease progression.
- To assess if severity of hepatic steatosis, assessed either with histopathological grading or SPC, is associated with future development of T2DM in NAFLD.

3. METHOD

3.1 Procedures

3.1.1 Enrolment of Patients (Papers I, II, III, IV)

This thesis is based on three different cohorts. In paper I we prospectively included 94 patients who accepted liver biopsy, irrespective of liver disease aetiology, for a cross-sectional study – comparing biochemical, clinical, histological and radiological parameters. In paper II we conducted a retrospective, long-term follow-up study including 646 patients with liver-biopsy confirmed hepatic steatosis to investigate the long-term prognosis of patients with NAFLD, and how fibrosis stage and steatohepatitis effect prognosis. In paper III and IV, an extended prospective, follow-up study was conducted based on a cohort previously published in 2006 by Ekstedt *et al.* All patients alive, were re-invited for a second follow-up. Out of the originally 129 NAFLD patients included at baseline, 59 patients, of the 79 still alive, accepted a second follow-up.

3.1.1.1 Quantitative Assessment of Liver Steatosis and Diagnostic Accuracy of ^1H -MRS PDFF. The Non-Invasive Liver Biopsy (NILB) study (Paper I)

This prospective study was performed on patients referred to the Department of Gastroenterology and Hepatology at University Hospital in Linköping, Sweden, for evaluation of chronically (≥ 6 months) elevated levels of serum alanine aminotransferase (ALT; defined as >71 U/L for men and >45 U/L for women) and/or aspartate aminotransferase (AST; defined as >45 U/L for men and >36 U/L for women) and/or serum alkaline phosphatase (ALP; defined as >106 U/L for both sexes). A diagnostic work-up was performed and all patients whom, on clinical indication, needed a liver biopsy where asked to participate in our study, which consisted of adding an MR examination to the routine diagnostic work-up.

We recruited from patients referred to our department between 2007-2014. The final cohort constituted of 94 patients. Of the 94 patients, 37 patients were diagnosed with NAFLD, 49 with other liver diseases and 8 had normal liver biopsy, and hence no explanation for their elevated liver enzymes. All liver biopsies were graded for steatosis according to Brunt and evaluated for the quantitative amount of hepatic steatosis by stereological point counting.

3.1.1.2 Long-term Retrospective Cohort Study of Biopsy-Proven NAFLD (Paper II)

This retrospective cohort study included all patients initially diagnosed with biopsy-proven NAFLD, at the Karolinska University Hospital, Huddinge and University Hospital in Linköping, during 1971 to 2009. All biopsies were categorized by a pathologist at the time of biopsy using the systemized nomenclature of medicine (SNOMED)²⁹³ to identify all liver biopsies with hepatic steatosis. All patients' medical charts were scrutinized in detail. Patients with other causes for steatosis than NAFLD or diagnosed with any concurrent liver disease at biopsy or during follow-up were excluded. Patients that reported significant alcohol consumption were excluded. Patients on treatment with drugs associated with hepatic steatosis or hepatotoxicity at the time of biopsy or patients with a diagnosis of either hepatocellular carcinoma or decompensated liver disease at/within 6 months from baseline were excluded.

After exclusions, the cohort constituted of 646 NAFLD patients of whom 402 were male (62%). Mean age at baseline was 48.2 ± 13.7 years and BMI was 28.3 ± 4.1 kg/m². Type 2 diabetes had previously been diagnosed in 93 patients (14%).

3.1.1.3 Long-term Prospective Study (Papers III, IV)

This prospective longitudinal cohort study included patients referred between 1988 and 1993 to the Department of Gastroenterology and Hepatology, University Hospital in Linköping, or to the Department of Internal Medicine, Östergötland County Hospital, for evaluation of chronically (>6 months) elevated serum ALT (defined as >41 U/L for both sexes), and/or AST (defined as >41 U/L for both sexes), and/or serum ALP (defined as >106 U/L for both sexes). At inclusion, a diagnostic work-up was performed, including physical examination, biochemical investigation, and liver biopsy, as well as an extensive review of patients' medical charts. After excluding other chronic liver diseases and significant alcohol consumption, 137 patients were diagnosed with NAFLD. During follow-up, 8 patients were reclassified as alcoholic fatty liver disease and were excluded.

The final cohort constituted of 129 patients with NAFLD of whom 87 (67%) were male. Mean age at baseline was 51.0 ± 12.9 years and mean BMI 28.3 ± 3.8 kg/m². Type 2 diabetes and cardiovascular disease had previously been diagnosed in 11 (8.5%) and 14 (11%) patients, respectively. A previous follow-up study of this cohort (first follow-up) was conducted between 2003 and 2005 (details of data collection and results have been reported elsewhere¹⁵). The current follow-up (second follow-up) was conducted between 2013 and 2015.

3.1.1.4 Reference Population (Paper II)

Every person in Sweden has a unique personal identification number (PIN), which was used to create a control population from Statistics Sweden, using ten controls per individual ($n=6,345$ controls after exclusions). Matching was performed for sex, age and municipality. Patients and controls ($n=6,991$) were then linked to the three different national, population-based registers; the National Patient Register of Hospital Discharge (NPR), the Swedish Cancer Register (SCR), and the Cause of Death Register (CDR).

The registers were used to ascertain all cause of death and all cases of severe liver disease during follow-up. Severe liver disease was defined as ICD-code (International Classification of Disease) for liver failure, cirrhosis, HCC or decompensated liver disease. Moreover, decompensated liver disease was in turn defined as an ICD-code of oesophageal varices, ascites or hepatic encephalopathy.

3.2 Data Collection

All patients that accepted enrolment (Paper I) or follow-up (Papers III, IV) were seen at the Department of Gastroenterology and Hepatology, University Hospital in Linköping (Papers I, III, IV), or at the Department of Internal Medicine, Östergötland County Hospital (Papers III, IV) by either Patrik Nasr, Mattias Ekstedt or Stergios Kechagias.

All patients that were found to have hepatic steatosis in the pathology register had their medical charts reviewed and scrutinized by Patrik Nasr (Linköping) or Hannes Hagström (Stockholm) for enrolment (Paper II).

3.2.1 Biochemical Investigation (Papers I, III, IV)

Subjects had blood drawn in the fasting state for a complete blood count, analysis of prothrombin, iron, transferrin, transferrin saturation, ferritin, ALT and AST, ALP, γ GT, bilirubin, thyroid stimulating hormone, anti-transglutaminase antibodies, total cholesterol, low- and high-density lipoprotein, glucose, serum insulin, ceruloplasmin, and plasma protein electrophoresis, including, among others, albumin, α_1 -antitrypsin, and immunoglobulins. All patients also underwent assessment of alcohol consumption with CDT and/or PEth. Patients who had not previously been diagnosed with T2DM underwent a 75 g oral glucose tolerance test. In addition, blood was drawn for detection of hepatitis B surface antigen, hepatitis B virus DNA, anti-hepatitis C virus antibodies, hepatitis C virus RNA, anti-nuclear antibodies (ANA), smooth muscle antibodies (SMA), and antimitochondrial antibodies (AMA). In addition, in Papers III and IV all patients had blood obtained to identify the C282Y, H63D, and S65C mutations in the

human hemochromatosis (HFE) gene as well as the S and Z mutations in the serine protease inhibitor A1 (SERPINA1 [previously known as the protein inhibitor, or Pi locus]) gene. However, in Paper I, only patients with elevated transferrin saturation or low levels of serum levels of α_1 -antitrypsin, were analysed for mutations in the HFE gene or the SERPINA1 gene, respectively.

3.2.2 Collection of Biochemical Variables (Paper II)

Biochemical variables within one month of liver biopsy were registered. These included ALT, AST, albumin, bilirubin, ALP, and γ GT levels. Complete blood count, fasting cholesterol and triglycerides, fasting glucose, ANA, SMA, AMA and α_1 -antitrypsin levels. In cases with missing data, multiple imputation was used. Analysis for detection of hepatitis B surface antigen was performed in all cases and anti-hepatitis C virus antibodies were analysed in cases evaluated after 1991 when testing became available.

3.2.3 Clinical Assessment (Papers I, III, IV)

A structured case report form was used to assess past and present clinical history, both acute and chronic, during inclusion (Papers I, III, IV) and follow-up (Papers III, IV). Also, an extensive review of the medical chart was done, including past and present medical treatments.

Hip and waist circumference as well as length and weight were measured in all patients and BMI was calculated ($\text{BMI} = \text{weight (kg)} / \text{length (m)}^2$). Overweight was defined as $\text{BMI} \geq 25 \text{ kg/m}^2$ but $< 30 \text{ kg/m}^2$, obesity as $\text{BMI} \geq 30 \text{ kg/m}^2$, T2DM as fasting plasma glucose $\geq 126 \text{ mg/dL}$, requiring treatment or plasma glucose $\geq 200 \text{ mg/dL}$ 2h after oral administration of 75 g of glucose, impaired glucose tolerance as fasting plasma glucose $< 126 \text{ mg/dL}$ and plasma glucose $\geq 140 \text{ mg/dL}$ but $< 200 \text{ mg/dL}$ 2h after oral administration of 75 g of glucose, impaired fasting glucose as fasting plasma glucose between 110 mg/dL and 125 mg/dL and plasma glucose $< 140 \text{ mg/dL}$ 2h after oral administration of 75 g of glucose, hypertension as blood pressure $\geq 130/85 \text{ mmHg}$ or requiring treatment, and hypertriglyceridemia as fasting triglycerides $\geq 150 \text{ mg/dL}$.

Cardiovascular disease was defined as having previous ischemic heart disease or known angina pectoris, a previous stroke or intermittent claudication.

Metabolic syndrome was defined as having a $\text{BMI} > 30 \text{ kg/m}^2$ or waist circumference $\geq 94 \text{ cm}$ in men or $\geq 80 \text{ cm}$ in women plus two of the following four factors: (1) fasting triglycerides $\geq 150 \text{ mg/dL}$ or treatment for this lipid abnormality; (2) reduced fasting high-density lipoprotein $< 40 \text{ mg/dL}$ in men or $< 50 \text{ mg/dL}$ in women or treatment for this lipid abnormality; (3) systolic blood pressure $\geq 130 \text{ mmHg}$ or diastolic blood pressure $\geq 85 \text{ mmHg}$

or treatment for previously diagnosed hypertension; (4) fasting plasma glucose ≥ 100 mg/dL or previously diagnosed type 2 diabetes mellitus.

3.2.4 Suspected Diagnosis of NAFLD Before Liver Biopsy (Paper I)

In Paper I, the subjects were initially, after clinical work-up but *a priori* to liver biopsy, divided into two groups, probable NAFLD and non-NAFLD, depending on whether their elevated liver enzyme levels were primarily attributed to NAFLD or to other liver diseases. The inclusion criteria for the NAFLD group were the following:

- I) alcohol consumption < 20 grams per day,
- II) absence of hepatic steatosis associated medication,
- III) undetectable hepatitis B surface antigens and anti-HCV antibodies,
- IV) absence of autoantibodies against cell nuclei, smooth muscle, and mitochondria
- V) serum transferrin saturation $< 45\%$,
- VI) serum ceruloplasmin level > 0.20 g/L,
- VII) serum α_1 -antitrypsin level > 0.86 g/L for men and > 0.94 g/L for women, and
- VIII) ALT/ULN $>$ ALP/ULN.

Moreover, at least one of the following criteria had to be fulfilled:

- 1) BMI > 25 kg/m²,
- 2) waist circumference ≥ 94 cm for men and ≥ 80 cm for women, or
- 3) presence of type 2 diabetes mellitus, impaired fasting glucose or impaired glucose tolerance.

3.2.5 Collection of Baseline Clinical Characteristics (Paper II)

Type 2 diabetes mellitus was defined as a registered diagnosis in patient charts, a plasma glucose of ≥ 180 mg/dL or a fasting plasma glucose of ≥ 126 mg/dL or requiring treatment. Hypertension was defined as a registered diagnosis, a resting blood pressure of $\geq 140/90$ mmHg or treatment for previously diagnosed hypertension. Cardiovascular disease was defined as having previous ischemic heart disease or known angina pectoris, a previous stroke or intermittent claudication, hyperlipidaemia as fasting total cholesterol value of ≥ 240 mg/dL or requiring treatment. Smoking was defined as being a current smoker or having smoked previously. Weight and height were objectively measured by hospital staff and used to calculate BMI.

3.2.6 Assessment of Alcohol Consumption (Papers I, III, IV)

A questionnaire was constructed using the three questions in the AUDIT-C questionnaire⁸⁷: “How often did you have a drink containing alcohol during the last three months?”, “How many drinks containing alcohol did you have on a typical day during the last three months when you were drinking?”, and “How often did you have 5 (for men)/4 (for women) drinks or more on one occasion during the last three months?”

After completion of the questionnaire and review of the biochemical results (including PEth and/or CDT) as well as performing an extensive chart review, the physicians (P.N, M.E, or S.K) conducted an oral interview to confirm that the questionnaire had been answered correctly. The patient was asked to describe his or her alcohol consumption during a typical week and changes during the years.

The information obtained during the interview was compared to the AUDIT-C questionnaire. When the results of the questionnaire, biochemical findings, chart review and the interview deviated or had discrepancies, the questionnaire was scrutinized together with the patient and misunderstandings were corrected to obtain the most reliable information on actual alcohol consumption at the time of enrolment. The number of drinking occasions were multiplied by the number of drinks (*i.e.* 12 g ethanol) consumed on each occasion. A consumption >20 g/day (or >140 g/week) was defined as overconsumption of alcohol.

3.2.7 Assessment of Significant Alcohol Consumption (Paper II)

During the extensive chart review, patients that reported a daily alcohol consumption of more than 30 g for men or 20 g for women at baseline or during follow-up were defined as alcohol overconsumption. Patients that reported binge drinking defined as 60 g or more for men and 48 g or more for women on the same occasion were also defined as alcohol overconsumption.

3.2.8 Liver Biopsy (Papers I, II, III, IV)

Liver biopsy was performed percutaneously with ultrasonography guidance using a 16-gauge (1.6-mm) BioPince needle (BioPince Full Core Biopsy Instrument; Argon Medical Devices, Plano, TX, USA), on an outpatient basis. In Paper III and IV baseline biopsies were performed blindly using a Menghini needle. All patients were monitored at an out-patient clinic for six hours after the liver biopsy was performed.

3.3 Histopathological Evaluation (Papers I, II, III, IV)

All liver biopsies were re-evaluated by two experienced hepatopathologists (R.H. and S.I.), who were blinded to patient details. Baseline biopsies for papers III and IV were evaluated by R.H. However, 74 liver biopsies were not available for reassessment but had previously been reassessed by an experienced liver pathologist (Lennart E. Franzén [L.E.F.]) as part of a prior follow-up study.¹⁵ There was a low agreement ($\kappa=0.062$) for hepatocellular ballooning and lobular inflammation between the two pathologists (R.H. and L.E.F.). Therefore, in Paper II, these 74 patients were excluded from the analysis of NAS. Nevertheless, they were still included in the analysis of fibrosis stage, as the agreement was substantially higher ($\kappa=0.73$). The old slides included in Paper II were usually well preserved, but, in those with faded staining, new sections and staining was performed. However, biopsies with insufficient quality or size were excluded.

Baseline biopsies in Paper I and all follow-up biopsies in Papers III and IV were assessed or reassessed by S.I.

All liver biopsies were scored according to the NAFLD activity score.¹⁹³ Steatosis was graded as 0–3, corresponding to none (<5%), mild (5–33%), moderate (34–66%) or severe (>66%), respectively. Lobular inflammation was graded as 0–3. Grades 0–3 correspond to none, fewer than 2 foci, 2–4 foci, and more than 4 foci per 200 x field, respectively. Hepatocellular ballooning was graded as 0–2. Grades 0–2 correspond to none, few ballooned cells, and many cells/prominent ballooning, respectively. The unweighted sum of steatosis, lobular inflammation and ballooning was calculated as the NAS (0–8). Fibrosis was staged on a 5-point scale: stage 0, no fibrosis; stage 1, zone 3 perisinusoidal/perivenular fibrosis; stage 2, zone 3 and periportal fibrosis; stage 3, septal/bridging fibrosis; and stage 4, cirrhosis. Patients with end-stage liver disease that did not undergo liver biopsy, were defined as having fibrosis stage 4 (*i.e.* cirrhosis).

In Paper II, the fatty liver inhibition of progressions (FLIP) algorithm was used to define the presence of NASH.^{198, 294}

In Paper III, two different definitions of disease progression were used; fibrosis progression and clinically significant disease progression. Fibrosis progression was defined as an increase of ≥ 1 in fibrosis stage between first and second/third liver biopsy. Moreover, clinically significant disease progression was defined as: (1) an increase of fibrosis stage from none (F0) to significant (F2); (2) development of advanced fibrosis (F3–F4); (3) development of end-stage liver disease; (4) NAFLD fibrosis score >0.676 or, (5) liver stiffness measurement >7.2 kPa as measured with transient elastography.

3.4 Quantitative Assessment of Hepatic Steatosis (Papers I, IV)

3.4.1 Stereological Point Counting (SPC) (Papers I, III, IV)

A Nikon Eclipse E800 microscope with a Nikon DS-Ri1 digital camera (Nikon Corporation, Tokyo, Japan) was used to capture all images for histopathologic analysis. Up to ten images from each sample were captured and stored using NIS-Elements BR v 4.0 (Nikon Corporation). The first field of view was selected at the outmost end of the tissue sample. After capturing the first image, the second field of view was selected by shifting 1.25 fields of view along the length axis of the biopsy to attain non-overlapping images for future assessment. This procedure was continued until at least ten tissue images had been captured or until all parts of the tissue sample had been photographed. A point grid, consisting of 221 crosses, 35 μm apart, was thereafter superimposed onto each image with a final magnification on the computer screen of 515 times, when counting. The number of fat vacuoles in the hepatocytes were manually counted. Ballooned hepatocytes could almost always be distinguished from hepatocyte fat vacuoles. All ballooned cells were omitted. Also, if distinguishing of ballooned cells were not possible, they were omitted. Crosses overlying damaged tissue or larger areas of fibrosis were excluded. The number of hits on fat vacuoles divided by the total number of hits on liver tissue represented the ratio of steatotic area (expressed as %).

Images from twenty randomly chosen specimens were later recounted by the original SPC assessor (P.N.) and a co-author (M.E.) to evaluate the reproducibility of the method. The inter- and intraobserver Pearson correlation coefficients were 0.92 and 0.94, respectively.

3.4.2 Proton Magnetic Resonance Spectroscopy – Proton Density Fat Fraction (^1H -MRS PDFF) (Paper I)

^1H -MRS was performed within 2 months of the clinical work-up and immediately before the liver biopsy. A Philips Achieva 1.5 T MR scanner (Philips Healthcare, Best, The Netherlands) was used to perform the ^1H -MRS. Two volumes of interest were acquired in succession at the same location using identical sequence parameters. To correct for attenuation caused by the presence of excess hepatic iron deposition, the T2 relaxation time of the water protons in the liver was measured.

The T2 relaxation times of the water protons in the liver were quantified and the mean values were derived from regions of interest placed in approximately the same anatomic region as the ^1H -MRS volumes of inter-

est by a radiologist (N.D.) who was blinded to patient details. The amplitudes of the water resonances and the lipid resonances were quantified and corrected for ^1H -MRS sequence parameters (echo time and repetition time) as well as water and lipid proton relaxation times (T_1 and T_2). The lipid content (*i.e.* PDFF) was quantified, as previously described by Longo *et al*, and reported as the mean of the repeated spectroscopic acquisitions.²⁹⁵

3.5 Statistics (Paper I, II, III, IV)

Data were expressed as mean $\pm$ standard deviation (SD) if continuous variables, median and range if categorical or count variable, or total numbers with percentages if applicable.

For continuous variables, the difference between two groups was evaluated using a two-sided independent Student's t test when the data was normally distributed, and with the Mann-Whitney U test when the assumption of normality was not met or if the variable was nonparametric. Longitudinal changes were assessed using the paired-sample t test for normally distributed continuous data, or the Wilcoxon rank-sum test if data was not normally distributed or nonparametric. For dichotomous values, differences were tested using the chi-square (χ^2) test or the Fischer's exact test.

For continuous variables, the difference between three or more groups were evaluated using the one-way analysis of variance (ANOVA) when the data was normally distributed, and with the Kruskal-Wallis one-way analysis of variance when the assumption of normality was not met or if the variable was nonparametric or dichotomous.

The Pearson correlation coefficient (r) and linear regression was used when comparing correlation between continuous variables. Cohen's kappa (κ) was used when comparing the agreement between categoric and stratified continuous variables. The Spearman rank correlation coefficient (ρ ; ρ) was used when comparing categoric and continuous variables.

Area under the receiver-operating characteristics (AUROC) curves were constructed to assess the overall diagnostic accuracy, and to identify the optimal cut-off values.

Survival curves were made according to the Kaplan-Meier method, and the log-rank test was applied for the determination of difference in survival between groups.

Cox regression models were used to estimate crude and adjusted hazard ratios (HR) for mortality and disease specific outcome. In Paper II a LaPlace regression was used to estimate the time to first event of severe liver disease for controls and according to fibrosis stage (F0-F3) for the 10th percentile of patients. In patients with cirrhosis (F4), time to development of decompensated liver disease or hepatocellular carcinoma was estimated.

In Paper II a competing risk regression for the outcome of severe liver disease was performed.

A *P* value <0.05 was considered statistically significant for all analyses.

3.6 Ethical considerations (Papers I, II, III, IV)

Written informed consent was contained from all participating subjects when needed (Paper I, III, IV). The study designs were approved by the Regional Ethical Review Board at the Karolinska Institutet in Stockholm (Paper II) and Linköping University (Paper I, II, III, IV).

4. RESULTS

4.1 The Non-Invasive Liver Biopsy (NILB) study (Paper I)

NAFLD was considered the probable cause of elevated liver function tests (LFTs, *i.e.* AST, ALT or ALP) in 41 subjects after diagnostic work-up but before ^1H -MRS and liver biopsy. Histopathological work-up allowed us to establish that 36 of these subjects could be confidently diagnosed with NAFLD (Figure 8). Liver biopsy samples showed normal histological features in the remaining five patients from the group with probable NAFLD before liver biopsy. However, among the 53 patients in the non-NAFLD group (*i.e.* where the suspected aetiology was not NAFLD prior to liver biopsy), one further patient was identified with NAFLD.

Figure 8. Suspected and final diagnoses of study subjects with division into analyzed groups. AAT, a1-antitrypsin; AIH, autoimmune hepatitis; DILI, drug-induced liver disease; HCV, hepatitis C virus; PSC, primary sclerosing cholangitis; PBC, primary biliary cirrhosis. *Gastroenterology* 2017. Published with permission.

Thus, after clinical work-up and histopathological evaluation, elevated LFTs were attributed to NAFLD in 37 patients and other liver diseases in 49 patients. Eight patients had liver biopsy samples with normal histological features and no evident cause of elevated liver enzyme levels. Among the 49 patients with other liver diseases, 10 had hepatic steatosis at histopathologic evaluation (two with HCV infection, two with primary sclerosing cholangitis, one with autoimmune hepatitis, one with primary biliary cirrhosis, one with α_1 -antitrypsin deficiency, one with Wilson's disease, and one with alcoholic liver disease).

There was a significant correlation between the ^1H -MRS and SPC results ($r = 0.92$, $p < 0.001$, Figure 9). The kappa correlation was 0.82. The kappa correlations between SPC and histopathological grading and between ^1H -MRS and histopathological grading were significantly lower ($\kappa = 0.38$ and $\kappa = 0.26$, respectively).

Figure 9. Correlation between proton density fat fraction (PDFF; %) as quantified with proton magnetic resonance spectroscopy (^1H -MRS) and the steatotic area of liver biopsy sample (%) as measured with stereological point counting (SPC). For values below 5% the correlation is shown with a higher resolution in the inset. *Gastroenterology* 2017. Published with permission.

Of all subjects enrolled, the pathologist graded 47 biopsies as grade 0, 24 as grade 1, 17 as grade 2 and 6 as grade 3. If SPC had been used as the reference standard to grade steatosis, 63 subjects would have been classified as having grade 0 steatosis, and 31 as having grade 1 steatosis. If $^1\text{H-MRS}$ had been used as the reference standard, 69 subjects would have been classified as having grade 0 steatosis, and 25 as having grade 1 steatosis. No subject would have been diagnosed with grade 2 or 3 steatosis if either SPC or $^1\text{H-MRS}$ had been used for the grading.

The AUROCs for $^1\text{H-MRS}$ and SPC were similar (Table 5). These values indicate that both methods have a high diagnostic accuracy. Of the 63 patients with grade 0 steatosis, as measured by SPC, 16 were diagnosed as grade 1 or above by the histopathologist. Of the 69 subjects with PDFF $<5.0\%$, as quantified with $^1\text{H-MRS}$, 22 were diagnosed with steatosis by the histopathologist. The sensitivity and specificity of SPC and $^1\text{H-MRS}$ grading based on lower cut-off values (3.0%, 2.0%, and 1.4%) are shown in Table 5.

Table 5. Diagnostic accuracy of $^1\text{H-MRS}$ PDFF and SPC for the absence (grade 0) versus the presence (Grades 1–3) of Histopathologic Steatosis (n = 94). *Gastroenterology 2017. Published with permission.*

Cut-off value	$^1\text{H-MRS}$ PDFF			SPC		
	Sensitivity	Specificity	AUROC (95%CI)	Sensitivity	Specificity	AUROC (95%CI)
			0.969 (0.933–1.000)			0.977 (0.955–1.000)
5.0%	53%	100%		64%	100%	
3.0%	79%	100%		81%	100%	
2.0%	87%	94%		87%	94%	
1.4%	98%	89%		96%	81%	

4.2 Long-term Retrospective Study (Paper II)

In total, 646 biopsy-proven NAFLD cases and 6,345 matched controls were included. Mean age and BMI were 42.2 years and 28.3 kg/m², respectively. The cohort was followed for a mean period of 19.9 years.

During follow-up a total of 214 NAFLD-cases and 1,903 controls died. Liver-related (7.9% in cases vs. 1.4% in controls, $p < 0.001$) and endocrine-related mortality including diabetes (5.1% vs. 2.7%, $p = 0.02$) were significantly more common in NAFLD-cases than controls. There was no significant difference in cardiovascular mortality between cases and controls (36.9% vs. 39.3%, $p = 0.74$).

Compared to age-, sex- and municipality matched controls, NAFLD cases showed no significant higher risk of mortality ($p = 0.07$). However,

those with significant fibrosis (F2-F4) showed both an increased risk of mortality and development of severe liver disease with increasing HRs per stage of fibrosis (Table 6).

Kaplan-Meier plots for mortality and severe liver disease stratified on fibrosis stage and compared to controls are shown in Figure 10 and Figure 11. Log-rank test is significant for both plots ($p < 0.001$ for both).

Table 6. Univariate hazard ratios for overall mortality and development of severe liver disease compared to matched controls. *Journal of hepatology 2017. Published with permission.*

NAFLD vs. controls	Overall mortality			Severe liver disease		
	HR	95%CI	P value	HR	95%CI	P value
NAFLD (yes)	1.14	0.99-1.32	0.07	4.25	3.09-5.84	<0.001
Controls	(ref)					
NAFLD-Cases						
F0	0.87	0.65-1.16	0.33	1.92	0.90-4.10	0.09
F1	0.88	0.70-1.12	0.3	1.65	0.84-3.24	0.15
F2	1.36	1.02-1.80	0.03	5.48	3.10-9.70	<0.001
F3	2.54	1.79-3.60	<0.001	14.28	7.90-25.8	<0.001
F4	5.19	3.06-8.79	<0.001	104.5	57.2-191-1	<0.001

Figure 10. Overall mortality stratified on fibrosis stage and compared to matched controls. Log-rank test $p < 0.001$. *Journal of hepatology 2017. Published with permission.*

Figure 11. Development of severe liver disease stratified on fibrosis stage and compared to matched controls. Log-rank test $p < 0.001$. *Journal of hepatology 2017. Published with permission.*

Using NAFLD-cases with absence of fibrosis (*i.e.* stage 0) as reference, and adjusting for age, sex, T2DM and NASH, a significant increase in mortality and development of severe liver disease was seen for advanced fibrosis (F3-F4)

Table 7. Multivariate hazard ratios for overall mortality and development of severe liver disease within the NAFLD group. Comparisons are made against NAFLD-cases with fibrosis stage 0 as reference. *Journal of hepatology* 2017. Published with permission.

Within NAFLD group	Overall mortality			Severe liver disease		
	aHR*	95%CI	P value	aHR*	95%CI	P value
NAFLD-Cases						
F0 (n=163)	(ref)			(ref)		
F1 (n=255)	0.91	0.61-1.36	0.66	0.76	0.26-2.25	0.63
F2 (n=149)	1.11	0.70-1.76	0.66	2.08	0.70-6.14	0.19
F3 (n=58)	1.76	1.02-3.06	0.04	3.82	1.15-12.7	0.03
F4 (n=20)	3.75	1.81-7.73	<0.001	58.12	17.3-195.4	<0.001

*Adjusted for age, sex, T2DM and NASH.

At the end of the study, 76 patients (11.8%) and 139 controls (2.2%) had developed severe liver disease ($p < 0.001$). When stratified on fibrosis stage, severe liver disease was diagnosed in 7.4% in patients with F0, 6.3% in F1, 12.1% in F2 and 25.9% in F3. Moreover, decompensated liver disease occurred in 3.7% in F0, 4.3% in F1, 8.7% in F2, 12.1% in F3 and in 45% of patients with F4. After performing a Laplace regression, time until the first 10% of the patients had developed severe liver disease was 30.5 years in F0 (95%CI 21.5–39.6), 35.6 years in F1 (95%CI 25.6–45.4), 19.4 years in F2 (95%CI 9.3–29.5) and 6.0 in F3 (95%CI 2.3–9.6). Furthermore, time until 10% of the patients had developed decompensated liver disease was 33.4 years for F0 (95%CI 24.2–42.6), 34.1 years for F1 (95%CI 25.1–43.2), 22.7 years for F2 (95%CI 13.7–31.7), 11.8 years for F3 (95%CI 4.3–19.4) and 5.6 years for F4 (95%CI 0.9–10.3) (Figure 12).

Figure 12. Time until >10% of the NAFLD-cases, stratified on fibrosis stage, developed severe liver disease (light blue) or decompensated liver disease (dark blue).

4.3 Long-term Follow-up Study (Paper III, IV)

In total, 129 patients with biopsy-proven NAFLD were prospectively enrolled between 1988 and 1993. Mean age and BMI were 51.0 years and 28.3 kg/m², respectively. The cohort was followed for a mean period of 19.8 years.

At the first follow-up, 88 out of 104 (85%) patients still alive accepted follow-up, of whom 68 patients underwent a second biopsy. At second follow-up, 59 out of 79 (75%) patients still alive, accepted follow-up, of whom 33 underwent liver biopsy (Figure 11). The mean time between first and second, second and third and first and third liver biopsies were 13.7 ± 1.5 years, 9.3 ± 1.0 years and 22.8 ± 1.3 years. In total, 29 patients had three consecutive biopsies.

Figure 13. Flow-chart for the study patients. ^aBoth patients were diagnosed with hepatocellular carcinoma at follow-up; 1 patient died shortly after diagnostic work-up at follow-up. ^bOne patient developed hepatocellular carcinoma and underwent liver transplantation during follow-up. ^cOne patient developed hepatocellular carcinoma, 1 developed ascites, and 1 developed gastric antral vascular ectasia. *Hepatology communications* 2018. Published with permission.

4.3.1 Histological Outcome of The Study Group (Paper III)

At the end of follow-up, 12 patients (9.3%) had developed symptoms of end-stage liver disease; 5 presented with ascites, 4 with hepatocellular carcinoma, 2 with variceal haemorrhage, and 1 with gastric antral vascular ectasia. Albeit 4 of these 12 patients had baseline advanced fibrosis (3 with

F3 and 1 with F4), the remaining 8 had initial low stages of fibrosis (2 with F0, 2 with F1, and 4 with F2).

In total, 34 patients (26%) were diagnosed with advanced fibrosis at inclusion or developed advanced fibrosis during the study period of whom 28 patients had biopsy-proven advanced fibrosis and 6 patients showed symptoms of end-stage liver disease.

Moreover, in the 113 patients with initially low stage fibrosis (F0-F2), 18 patients (16%) developed advanced fibrosis or end-stage liver disease during the study period. There were no clinical or biochemical differences at baseline in the 18 that developed advanced fibrosis compared to the 95 patients who did not. However, there was a difference in the baseline fibrosis stage in those who developed advanced fibrosis compared to those who did not (1 (0-2) vs. 0 (0-2), $p=0.015$).

In patients that underwent repeat liver biopsy or showed symptoms of end-stage liver disease (and were considered fibrosis stage 4), 40 (52.6%) progressed during the study period. In patients with F0, 23 (59% of all with F0) patients progressed histologically; the corresponding numbers for F1, F2, and F3 were 8 (40%), 7 (58.3%) and 2 (50%) patients, respectively. There was no significant difference between the groups ($p=0.374$).

4.3.2 Follow-up of Patients With NAFLD and Isolated Steatosis (Paper III)

Of the 129 patients in the initial cohort, 56 patients (43%) had isolated steatosis (*i.e.* only steatosis and no presence of lobular inflammation and/or ballooning and/or fibrosis). In general, the 56 patients were younger and had lower liver enzymes and ferritin levels.

In total, 5 out of 56 patients (9%) with isolated steatosis at baseline developed advanced fibrosis or clinical signs of end-stage liver disease. At baseline, these patients presented with more severe steatosis grade (3 (2-3) vs. 2 (1-3), $p=0.032$) and also higher prevalence of T2DM (20% vs. 2%, $p=0.04$).

4.3.3 Quantitative Steatosis and Prediction of T2DM and Mortality (Paper IV)

Quantitative steatosis measured with SPC was performed on almost all liver biopsies at baseline and follow-up. Of the 129 patients included at baseline, 14 were excluded because of baseline diabetes being present and furthermore, 9 were excluded because of missing SPC data on baseline liver biopsies. After exclusion 106 patients remained (Figure 14).

Figure 14. Flowcharts for patients included in the cohort.

Mean follow-up time was 17.5 years or 1859 person-years. During this period 66 (62%) developed T2DM, corresponding to an incidence rate of 40 cases per 1000 person-years.

The 66 patients who developed T2DM were compared to the 40 who did not develop T2DM during follow-up. Those that developed T2DM had higher BMI (29 vs. 27 kg/m², $p=0.032$) and steatosis grade (3 (1-3) vs 2 (1-3), $p=0.039$) with a trend for higher SPC (14.4 ± 10.2 vs. 10.6 ± 10.0 %, $p=0.065$).

Multivariate hazard ratios were calculated for overall mortality and the risk of developing T2DM are shown in Table 8.

Table 8. Multivariate hazard ratios for overall mortality and development of T2DM in NAFLD patients.

Variable	Overall mortality*			Development of T2DM**		
	aHR	95%CI	P value	aHR	95%CI	P value
Sex (male)				1.15	0.64-2.05	0.65
BMI (kg/m ²)	1.05	0.96-1.14	0.29			
Steatosis grade (0-3)	1.41	0.87-2.29	0.17	1.60	1.13-2.28	<0.01
1	(ref)			(ref)		
2	0.65	0.18-2.31	0.50	1.06	0.47-2.36	0.90
3	1.82	0.89-3.72	0.10	2.04	1.20-3.46	<0.01
SPC (%)	1.04	1.00-1.07	0.04	1.03	1.00-1.05	0.02
Lobular inflam. (0-3) [†]	1.27	0.52-3.13	0.60	0.44	0.15-1.27	0.13
Ballooning (0-2) ^{††}	0.52	0.24-1.15	0.11	1.58	0.76-3.29	0.22

*Adjusted for age, sex and fibrosis stage. **Adjusted for age, BMI, sex and fibrosis stage. [†]No patients had lobular inflammation grade >1. ^{††}1 patient had ballooning grade 2. Numbers in bold are significant. Abbreviations: BMI, body mass index; SPC, stereological point counting

Of the 106 patients included at baseline, 24 patients had steatosis grade 1 (SPC 1.1 ± 1.3 %), 23 patients had steatosis grade 2 (SPC 8.1 ± 4.8 %), and

59 patients had steatosis grade 3 (SPC 19.7 ± 8.1 %). BMI and hypertriglyceridemia was significantly associated with steatosis grade ($p=0.016$ and $p=0.01$, respectively). Mortality rate was 21, 22 and 41% in patients with steatosis grade 1, 2, and 3, respectively. Kaplan-Meier plots showed decreased survival rate and increased risk of development of T2DM in patients with grade 3 steatosis compared to steatosis grade 1 and 2 (Figure 15 and Figure 16).

Figure 15. Overall mortality stratified on steatosis grade 1-3. Log-rank test $p=0.03$).

Figure 16. Risk of development of type 2 diabetes mellitus, stratified on steatosis grade 1-3. Log-rank test $p<0.01$.

5. DISCUSSION

The most common cause of abnormal liver function tests is hepatic steatosis, which is present in up to 30% of the population⁴. The most common cause of this abnormality is NAFLD.⁷ Albeit steatosis is seen as a benign condition, some patients with NAFLD develop traits of the metabolic syndrome,²⁹⁶ with a subpopulation advancing to end-stage liver disease with need for liver transplantation. Hence, NAFLD is predicted to be a major future economical health burden.⁸

The need for effective treatment in NAFLD is highly warranted. Although several drugs with different targets are in pipeline and some have shown efficacy, there are currently no approved therapies for NAFLD. A major obstacle on therapeutic evaluation and advances to improve outcome in NAFLD is the innate, slowly progressive nature of NAFLD. It can take decades for individuals with NAFLD to develop cirrhosis (and sometimes decades for cirrhosis to portray symptoms of end-stage liver disease).

Nonetheless, there is a broad consensus that patients with NAFLD should be identified.¹⁹ However, which diagnostic modality to use, or which patients to follow-up is still unknown.

The diagnosis of NAFLD is based on the intracellular accumulation of lipid droplets in the hepatocytes. The gold standard for diagnosing NAFLD is liver biopsy. However, because of the high prevalence (25%), liver biopsy as a diagnostic modality is not feasible. Therefore, non-invasive methods for diagnosing NAFLD are being studied. A novel method for diagnosing hepatic steatosis non-invasively, with high accuracy (even for lower stages of steatosis), is magnetic resonance techniques.

The cut-off value for diagnosing NAFLD with magnetic resonance techniques has hitherto been 5% or 5.56 %. The latter value is based on the results from Szczepaniak *et al.*, who examined the distribution of HTGC using ¹H-MRS in 345 subjects at low risk for hepatic steatosis (*i.e.*, lean patients with no glucose intolerance or excessive alcohol consumption, and normal serum liver enzyme levels), but without knowledge of the subjects histopathology.¹⁵⁹ However, hepatic steatosis can be present in lean subjects¹⁶⁷ as well as in individuals with normal serum ALT levels.^{4, 128, 168} Also, with increasing fibrosis stage, steatosis grade seem to decrease.²⁹⁷

In two studies, Tang *et al* evaluated the diagnostic performance of using an MRI PDFF estimation technique to distinguish between the absence (grade 0) and the presence (grade 1–3) of hepatic steatosis using histopathology as gold standard^{169, 170}. In total only 11 patients without steatosis

were included in these two studies, yielding a threshold of 6.4% and 6.9%, respectively, for distinguishing between absence and presence of steatosis according to conventional histopathological method. The authors concluded that the threshold to diagnose steatosis with MRI should be evaluated in a cohort with a higher number of subjects free of steatosis. Also, in other studies, evaluating the diagnostic performance of MRI PDFF for distinguishing absence from presence of steatosis, all, or the majority of the subjects, had confirmed or a high probability of NAFLD.¹⁷¹⁻¹⁷⁴ Thus, it is difficult to assess an adequate cut-off value of HTGC with MR in this type of cohorts.

An important strength of our first study (Paper I) is that the subjects did not have a definitive diagnosis *a priori* at inclusion. Contrary to the studies by Tang *et al.*,^{169, 170} in which most subjects had steatosis, 47 out of 94 subjects in our study did not have steatosis, which suggests that this study is more suitable to define cut-off for hepatic steatosis with ¹H-MRS PDFF.

Recently published guidelines recommend screening for NAFLD amongst patients with obesity or traits of the metabolic syndrome (in particular T2DM) by the use of non-invasive methods relying on the accuracy of the method used.²⁹⁸ If the widely used threshold of 5% had been used to define hepatic steatosis with ¹H-MRS in our study, 12 out of 37 (32%) subjects with elevated liver enzyme levels due to NAFLD would have been incorrectly considered to not have steatosis and the reason for elevated liver enzyme levels would have been unexplained. Furthermore, four of these patients had advanced fibrosis (*i.e.* stages 3 or 4).

The threshold we estimated for the diagnosis of steatosis with ¹H-MRS was considerably lower than in previous studies. At a cut-off value of 3.0%, the specificity was 100%, and the sensitivity was 79%. A cut-off value of 2.0% yielded the greatest diagnostic accuracy, although there were also a few false positives (specificity 94% and sensitivity 87%). These results are in accordance with those of Rhem *et al* who recently reported that a cut-off value of 3.0% or 3.5 % is an optimal PDFF threshold for predicting presence of T2DM or the metabolic syndrome, respectively.²⁹⁹ The discordance between cut-offs could also be related to the use of different PDFF-techniques (¹H-MRS vs. CSE-MRI) in estimating HTGC. As shown by a novel study by Hong *et al*, CSE-MRI methods may have, on average, a 2% absolute error in PDFF estimation (especially in lower levels of steatosis) which may challenge the interpretation of quantitative hepatic steatosis in the lower PDFF range.³⁰⁰

Albeit several studies have shown that magnetic resonance techniques are accurate and safe diagnostic modalities for diagnosing NAFLD, liver biopsy is and will remain the clinical reference standard for the diagnosis of NAFLD. This is in part mainly because it provides information, not only on

steatosis, but also on other features such as NASH (steatosis + inflammation + ballooning), and fibrosis. However, NASH has never been shown to predict progressive liver disease nor mortality. And moreover, fibrosis can be excluded with high accuracy using non-invasive elastographic techniques.

To date, 14 paired liver biopsy studies with almost 1,000 NAFLD patients have not shown that NASH predicts fibrosis progression. Moreover, long-term follow-up studies with biopsy-proven NAFLD has not shown that NASH predicts liver related outcomes or mortality.

In our second study (Paper II), we studied the long-term outcomes in patients with biopsy-proven NAFLD based on histopathological traits. In person-years, it is the largest reported study of its kind, with 646 patients and 19.9 years of follow-up, corresponding to 12,631 person-years.

Moreover, we showed that patients with NASH had an increased risk for liver-specific morbidity and overall mortality compare to the reference population when using a univariate analysis. However, this was not significant when adjusting for age, sex, presence of T2DM and fibrosis stage.

There was a clear collinearity between NASH and advanced stages of fibrosis, with 94% of patients with F4 having NASH (compared to 35% of patients with Fo) and 17% of patients with NASH having stage 3–4 fibrosis (compared to 2% of patients without NASH). It is highly probable that the increased risk of NASH on mortality and liver-specific morbidity in our study is due to collinearity between NASH and higher stages of fibrosis. Hence, adjusting the models with fibrosis stage as an independent factor for NASH did not change the estimates significantly, however, adjusting models with NASH as an independent factor for fibrosis stage significantly reduced the estimates.

The notion that inflammation is a culprit in the pathogenesis of fibrosis development and progression is not contested. However, the notion that histological traits of lobular inflammation and ballooning are surrogates for fibrosis progression, has yet not been proven. Therefore, caution should be used in clinical trials before accepting improvements in these entities as surrogates for fibrosis resolution.

Nevertheless, we showed that advanced fibrosis ($\geq F3$), and no other histopathological features predicted overall mortality and disease-specific outcome over time. We also assessed the minimum time needed to develop clinically relevant hepatic endpoints in NAFLD per stage of fibrosis, which can be used to guide individual patient prognosis (*i.e.* a patient with fibrosis stage 0–2 will not develop signs of severe liver disease in the upcoming two decades). Our results support data from previous studies, including our recent publication from 2015 that used a smaller subset of this cohort,¹⁹⁶ and other studies.^{184, 197}

The slow progressive, heterogenous nature of fibrosis in patients with NAFLD is well known.²³ Studies with collectively up to 1,000 patients with paired biopsies exist. However, the heterogeneity of the study protocols and histopathological assessments makes it difficult to draw any conclusions using meta-analyses. Therefore, the need for large (*i.e.* many person-years or long-term follow-up) paired biopsy studies are needed to observe progression.

In our third study (Paper III) we followed 129 patients with biopsy-proven NAFLD for a mean follow-up time of 19.8 years, corresponding to 2,587 person-years. We had fibrosis stage on 32 NAFLD patients on three separate occasions with a mean time between first and third biopsy of 22.8 years, letting enough time pass for progression to occur.

There was no difference in progression rate between the different stages of fibrosis ($p=0.374$). Moreover, in the 113 NAFLD patients with baseline low stage fibrosis (F0-2), 18 patients (16%) developed advanced fibrosis. Furthermore, in those with isolated steatosis (*i.e.* only steatosis and no other histopathological features on liver biopsy), 3 developed advanced fibrosis and 2, end-stage liver disease.

Predicting fibrosis progression was difficult, which corroborates previous paired biopsy studies. In a recent study including 475 NAFLD patients with advanced fibrosis (F3-F4) who had undergone sequential biopsies, the primary determinant of clinical disease progression was fibrosis and its change over time.¹⁹⁹ Similarly, in an abstract by McPherson *et al*, including 321 NAFLD patients with sequential biopsies over a median follow-up time of 4.1 years, no difference in fibrosis progression over the different stages of fibrosis could be observed.²⁵ Moreover, NASH did not seem to predict progressive disease. However, moderate to severe steatosis seemed to be associated with fibrosis progression.

Since isolated steatosis has been considered a benign condition, few studies have investigated the natural history of this disease. However, in the study by McPherson *et al*, 108 patients with NAFLD underwent repeat liver biopsy. Seventeen patients were diagnosed with isolated steatosis of whom 4 (24%) showed signs of progression.²⁰¹

Although our knowledge of isolated steatosis is limited, new studies on NAFL have recently emerged. In a prospective study by Wong *et al*, 8 of the 29 patients (28 %) with non-alcoholic fatty liver (defined as NAS <3) showed fibrosis progression. Furthermore, these findings were similar to those of McPherson *et al*, were 16 out of 52 patients (31 %) with NAFL showed signs of progression.²⁰¹ Additionally, Pais *et al* showed that 6 out of 25 patients (24%) with NAFL progressed to bridging fibrosis (F3).

The importance of hepatic steatosis is not fully understood. As mentioned, few paired biopsy studies have focused on patients with isolated steatosis, or steatosis as predictor of fibrosis progression.

In our fourth study (Paper IV), 106 out of the 129 patients in Paper III had baseline stereological point counting (SPC) available. An additional 59 patients had two sequential biopsies with SPC available. Sixty-six patients (62%) developed T2DM during follow-up. Conventional histological grading of steatosis and SPC independently (adjusted for age, BMI and fibrosis stage) predicted development of T2DM (aHR 1.60, 95%CI 1.13-2.28 per grade and 1.03, 95%CI 1.00-1.05, $p = 0.02$ per percent, respectively). Interestingly, in patients that underwent repeat liver biopsy, reduction of liver fat measured with SPC was associated with decreased risk of developing T2DM. Moreover, SPC, but no other histological feature, independently predicted overall mortality in NAFLD with an adjusted (for sex, age and fibrosis stage) hazard ratio of 1.04 per percent. These results are in concordance with a recent study by Björkström *et al*, wherein NAFLD patients with low stage fibrosis showed an increased risk of developing T2DM per grade of steatosis (aHR 1.34, 95%CI 1.03-1.74).²⁶⁹ Interestingly, new light is being shed over hepatic fat content as a more important predictor of the metabolic syndrome compared to visceral adipose tissue.^{301, 302}

Because of the lack of sequential biopsies there are few data on the effect of resolution or progression in histological hepatic steatosis and subsequently decreased or increased risk of T2DM. However, there are few studies with ultrasonography confirmed NAFLD patients investigating this issue. In a study by Sung *et al* more than 13,000 patients underwent repeat ultrasonography during a follow-up time of 5 years. During the study period, fatty liver resolved in 828 patients and developed in 1,640 patients.²⁵⁷ Resolution of fatty liver was not associated with decreased risk of incident T2DM (aOR 0.95, 95%CI 0.46-1.96), however, development of fatty liver increased the risk of incident T2DM (aOR 2.49, 95%CI 1.49-4.14). Moreover, equal results were seen in a study by Cho *et al*, where patients that resolved had a decreased, but nonsignificant, risk of incident T2DM (aHR 0.44, 95%CI 0.16-1.20).²⁶⁶ Nevertheless, in a recent study by Yamazaki *et al*, hepatic steatosis resolution during a 11.3-year follow-up showed a reduction of incident T2DM (aOR 0.27, 95%CI 0.12-0.61).²⁵⁸

Our study is the only one showing that resolution of histological hepatic steatosis decreases the risk of T2DM. However, in previous studies, resolution of fatty liver has been shown to improve dyslipidemia³⁰³ and, furthermore, worsening of fatty liver has been shown to increase fibrosis progression.³⁰⁴

There are some limitations. First, as in all studies using liver biopsy as gold standard (Papers I-IV), sampling error as well as inter- and intraobserver variability is a fact.³⁰⁵ Furthermore, in Paper I, the ¹H-MRS voxels

(*i.e.* volume elements) and the liver biopsy were not colocalized, which may have caused a significant degree of variability.¹⁶⁵ Second, all patients were selected from academic medical centres, indicating that selection bias could be present. However, most patients were biopsied because of chronically elevated liver enzyme and not because of a suspected chronic liver disease. Noteworthy is that liver biopsy was more common before the turn of the century and reflects the fact that >80 % of all the cases in Paper II underwent biopsy during the 1970–2000. Third, in Papers II–IV cardiovascular disease was the main cause of death, which it always tends to be in observational NAFLD studies. It is therefore seen as a competing risk.³⁰⁶ Initially, Kaplan-Meier survival analysis and Cox proportional hazard regression were originally developed to describe all-cause mortality (rather than incident disease [Paper II, IV]).³⁰⁷ Therefore, when using Kaplan-Meier or Cox regression in any other reason than all-cause mortality, these methods may lead to biased results because of other (*e.g.* CVD) competing risk factors.^{308–310} Fourth, because of the high prevalence of NAFLD in the general population, the control group in Paper II could have been diluted which could have made our estimates falsely low.

In summary, NAFLD can be diagnosed with magnetic resonance technique, albeit the threshold for diagnosis should be reduced from 5% to 3% in order to identify more NAFLD patients when using ¹H-MRS. Moreover, quantitative steatosis measurement should not only be used for diagnosing NAFLD patients but also for monitoring, because the quantity of hepatic steatosis entails an increased risk of developing T2DM. The diagnosis of hepatic steatosis and exclusion of advanced fibrosis can be done accurately non-invasively. However, the need for liver biopsy is useful in patients with NAFLD when diagnosis of the presence of fibrosis is needed, or determination of the degree of NASH is necessary. However, determining NASH does not seem to predict fibrosis progression nor mortality. The prediction of fibrosis progression (which in higher stages predict mortality) is difficult to do. Few studies have found any interesting baseline characteristics of clinical use. Therefore, NASH should not be used as a surrogate of fibrosis progression, but rather new surrogates should be found and evaluated, so not to imbue false hope in coming therapies.

6. CONCLUSIONS

- Hepatic steatosis can be diagnosed accurately with ^1H -MRS.
- The cut-off for diagnosing NAFLD with ^1H -MRS PDFF should be reduced to 3% to increase sensitivity.
- There is an excellent correlation between SPC and ^1H -MRS in quantifying hepatic steatosis.
- Steatosis quantification with SPC independently predicts development of T2DM and overall mortality in NAFLD patients.
- Resolution of steatosis decreases the risk of developing T2DM.
- NAFLD is a heterogeneous disease and it is difficult to predict fibrosis progression.
- NAFLD patients with low fibrosis stage do not have increased mortality compared to the general population.
- NAFLD patients with fibrosis stage 0 to 2 remain free of severe liver disease for at least 20 years after diagnosis.
- NASH does not predict fibrosis progression or mortality in patients with NAFLD

7. REFERENCES

1. Ludwig J, Viggiano TR, McGill DB, et al. Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease. *Mayo Clin Proc* 1980;55:434-8.
2. WESTWATER JO, FAINER D. Liver impairment in the obese. *Gastroenterology* 1958;34:686-93.
3. THALER H. [The fatty liver and its pathogenetic relation to liver cirrhosis]. *Virchows Arch Pathol Anat Physiol Klin Med* 1962;335:180-210.
4. Browning JD, Szczepaniak LS, Dobbins R, et al. Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. *Hepatology* 2004;40:1387-95.
5. Younossi ZM, Stepanova M, Afendy M, et al. Changes in the prevalence of the most common causes of chronic liver diseases in the United States from 1988 to 2008. *Clin Gastroenterol Hepatol* 2011;9:524-530.e1; quiz e60.
6. Loomba R, Sanyal AJ. The global NAFLD epidemic. *Nat Rev Gastroenterol Hepatol* 2013;10:686-90.
7. Younossi ZM, Koenig AB, Abdelatif D, et al. Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology* 2016;64:73-84.
8. Younossi ZM, Blissett D, Blissett R, et al. The economic and clinical burden of nonalcoholic fatty liver disease in the United States and Europe. *Hepatology* 2016;64:1577-1586.
9. Diehl AM, Goodman Z, Ishak KG. Alcohollike liver disease in nonalcoholics. A clinical and histologic comparison with alcohol-induced liver injury. *Gastroenterology* 1988;95:1056-62.
10. Lee RG. Nonalcoholic steatohepatitis: a study of 49 patients. *Hum Pathol* 1989;20:594-8.
11. Powell EE, Cooksley WG, Hanson R, et al. The natural history of nonalcoholic steatohepatitis: a follow-up study of forty-two patients for up to 21 years. *Hepatology* 1990;11:74-80.
12. Angulo P, Keach JC, Batts KP, et al. Independent predictors of liver fibrosis in patients with nonalcoholic steatohepatitis. *Hepatology* 1999;30:1356-62.
13. Bacon BR, Farahvash MJ, Janney CG, et al. Nonalcoholic steatohepatitis: an expanded clinical entity. *Gastroenterology* 1994;107:1103-9.
14. Adams LA, Lymp JF, St Sauver J, et al. The natural history of nonalcoholic fatty liver disease: a population-based cohort study. *Gastroenterology* 2005;129:113-21.

15. Ekstedt M, Franzén LE, Mathiesen UL, et al. Long-term follow-up of patients with NAFLD and elevated liver enzymes. *Hepatology* 2006;44:865-73.
16. Teli MR, James OF, Burt AD, et al. The natural history of nonalcoholic fatty liver: a follow-up study. *Hepatology* 1995;22:1714-9.
17. Matteoni CA, Younossi ZM, Gramlich T, et al. Nonalcoholic fatty liver disease: a spectrum of clinical and pathological severity. *Gastroenterology* 1999;116:1413-9.
18. Dam-Larsen S, Franzmann M, Andersen IB, et al. Long term prognosis of fatty liver: risk of chronic liver disease and death. *Gut* 2004;53:750-5.
19. (EASL) EAftSotL, (EASD) EAftSoD, (EASO) EAftSoO. EASL-EASD-EASO Clinical Practice Guidelines for the Management of Non-Alcoholic Fatty Liver Disease. *Obes Facts* 2016;9:65-90.
20. Chalasani N, Younossi Z, Lavine JE, et al. The diagnosis and management of nonalcoholic fatty liver disease: Practice guidance from the American Association for the Study of Liver Diseases. *Hepatology* 2018;67:328-357.
21. Sherlock S. *The Clinician Looks at Fatty Liver*. Boston, MA: Springer, 1989.
22. Wong VW, Wong GL, Choi PC, et al. Disease progression of non-alcoholic fatty liver disease: a prospective study with paired liver biopsies at 3 years. *Gut* 2010;59:969-74.
23. Pais R, Charlotte F, Fedchuk L, et al. A systematic review of follow-up biopsies reveals disease progression in patients with non-alcoholic fatty liver. *J Hepatol* 2013;59:550-6.
24. Singh S, Allen AM, Wang Z, et al. Fibrosis progression in nonalcoholic fatty liver vs nonalcoholic steatohepatitis: a systematic review and meta-analysis of paired-biopsy studies. *Clin Gastroenterol Hepatol* 2015;13:643-54.e1-9; quiz e39-40.
25. McPherson S, Pais R, Valenti L, et al. Further delineation of fibrosis progression in NAFLD: evidence from a large cohort of patients with sequential biopsies. *The International Liver Congress 2017*. Volume 66. Amsterdam: Journal of Hepatology, 2017:S593.
26. Day CP, James OF. Steatohepatitis: a tale of two "hits"? *Gastroenterology* 1998;114:842-5.
27. Choi SS, Diehl AM. Hepatic triglyceride synthesis and nonalcoholic fatty liver disease. *Curr Opin Lipidol* 2008;19:295-300.
28. Jou J, Choi SS, Diehl AM. Mechanisms of disease progression in nonalcoholic fatty liver disease. *Semin Liver Dis* 2008;28:370-9.
29. Thaler H. Relation of steatosis to cirrhosis. *Clin Gastroenterol* 1975;4:273-80.
30. Liao W, Hui TY, Young SG, et al. Blocking microsomal triglyceride transfer protein interferes with apoB secretion without causing retention or stress in the ER. *Journal of Lipid Research* 2003;44:978-985.

31. Koliwad SK, Streeper RS, Monetti M, et al. DGAT1-dependent triacylglycerol storage by macrophages protects mice from diet-induced insulin resistance and inflammation. *Journal of Clinical Investigation* 2010;120:756-767.
32. Yamaguchi K, Yang L, McCall S, et al. Inhibiting triglyceride synthesis improves hepatic steatosis but exacerbates liver damage and fibrosis in obese mice with nonalcoholic steatohepatitis. *Hepatology* 2007;45:1366-74.
33. Neuschwander-Tetri BA. Hepatic lipotoxicity and the pathogenesis of nonalcoholic steatohepatitis: The central role of nontriglyceride fatty acid metabolites. *Hepatology* 2010;52:774-788.
34. Donnelly KL, Smith CI, Schwarzenberg SJ, et al. Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. *J Clin Invest* 2005;115:1343-51.
35. Softic S, Gupta MK, Wang GX, et al. Divergent effects of glucose and fructose on hepatic lipogenesis and insulin signaling. *J Clin Invest* 2017;127:4059-4074.
36. Papazyan R, Sun Z, Kim YH, et al. Physiological Suppression of Lipotoxic Liver Damage by Complementary Actions of HDAC3 and SCAP/SREBP. *Cell Metab* 2016;24:863-874.
37. Sanyal AJ, Campbell-Sargent C, Mirshahi F, et al. Nonalcoholic steatohepatitis: association of insulin resistance and mitochondrial abnormalities. *Gastroenterology* 2001;120:1183-92.
38. Yki-Järvinen H. Non-alcoholic fatty liver disease as a cause and a consequence of metabolic syndrome. *Lancet Diabetes Endocrinol* 2014;2:901-10.
39. Ralston JC, Lyons CL, Kennedy EB, et al. Fatty Acids and NLRP3 Inflammasome-Mediated Inflammation in Metabolic Tissues. *Annu Rev Nutr* 2017;37:77-102.
40. Ogawa Y, Imajo K, Honda Y, et al. Palmitate-induced lipotoxicity is crucial for the pathogenesis of nonalcoholic fatty liver disease in cooperation with gut-derived endotoxin. *Sci Rep* 2018;8:11365.
41. Sanyal AJ, Abdelmalek MF, Suzuki A, et al. No significant effects of ethyl-eicosapentanoic acid on histologic features of nonalcoholic steatohepatitis in a phase 2 trial. *Gastroenterology* 2014;147:377-84.e1.
42. Tessari P, Coracina A, Cosma A, et al. Hepatic lipid metabolism and non-alcoholic fatty liver disease. *Nutrition, Metabolism and Cardiovascular Diseases* 2009;19:291-302.
43. Cohen JC, Horton JD, Hobbs HH. Human Fatty Liver Disease: Old Questions and New Insights. *Science* 2011;332:1519-1523.
44. Gilham D, Ho S, Rasouli M, et al. Inhibitors of hepatic microsomal triacylglycerol hydrolase decrease very low density lipoprotein secretion. *FASEB J* 2003;17:1685-7.
45. Arab JP, Karpen SJ, Dawson PA, et al. Bile acids and nonalcoholic fatty liver disease: Molecular insights and therapeutic perspectives. *Hepatology* 2017;65:350-362.

46. Chiang JY. Bile acid metabolism and signaling. *Compr Physiol* 2013;3:1191-212.
47. Copple BL, Li T. Pharmacology of bile acid receptors: Evolution of bile acids from simple detergents to complex signaling molecules. *Pharmacol Res* 2016;104:9-21.
48. Tilg H, Moschen AR. Evolution of inflammation in nonalcoholic fatty liver disease: the multiple parallel hits hypothesis. *Hepatology* 2010;52:1836-46.
49. Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor- α : direct role in obesity-linked insulin resistance. *Science* 1993;259:87-91.
50. Feinstein R, Kanety H, Papa M, et al. Tumor necrosis factor- α suppresses insulin-induced tyrosine phosphorylation of insulin receptor and its substrates. *Journal of Biological Chemistry* 1993;268:26055-26058.
51. Sharma M, Vikram NK, Misra A, et al. Assessment of 11- β hydroxysteroid dehydrogenase (11- β HSD1) 4478T>G and tumor necrosis factor- α (TNF- α)-308G>A polymorphisms with obesity and insulin resistance in Asian Indians in North India. 2013;40:6261-6270.
52. Zhang Y, Proenca R, Maffei M, et al. Positional cloning of the mouse obese gene and its human homologue. 1994;372:425-432.
53. Fried SK, Bunkin DA, Greenberg AS. Omental and Subcutaneous Adipose Tissues of Obese Subjects Release Interleukin-6: Depot Difference and Regulation by Glucocorticoid1. *The Journal of Clinical Endocrinology & Metabolism* 1998;83:847-850.
54. Scherer PE, Williams S, Fogliano M, et al. A Novel Serum Protein Similar to C1q, Produced Exclusively in Adipocytes. 1995;270:26746-26749.
55. Kanda H. MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. 2006;116:1494-1505.
56. Shoelson SE. Inflammation and insulin resistance. *Journal of Clinical Investigation* 2006;116:1793-1801.
57. Hotamisligil G. Molecular mechanisms of insulin resistance and the role of the adipocyte. *International Journal of Obesity* 2000;24:S23-S27.
58. Hotamisligil GS. Role of Endoplasmic Reticulum Stress and c-Jun NH₂-Terminal Kinase Pathways in Inflammation and Origin of Obesity and Diabetes. *Diabetes* 2005;54:S73-S78.
59. Kahn SE, Hull RL, Utzschneider KM. Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature* 2006;444:840-846.
60. Rotter V, Nagaev I, Smith U. Interleukin-6 (IL-6) Induces Insulin Resistance in 3T3-L1 Adipocytes and Is, Like IL-8 and Tumor Necrosis Factor- α , Overexpressed in Human Fat Cells from Insulin-resistant Subjects. *Journal of Biological Chemistry* 2003;278:45777-45784.

61. Baeuerle PA, Henkel T. Function and activation of NF-kappaB in the immune system. *Annual review of immunology* 1994;12:141-179.
62. Petersen KF, Shulman GI. Etiology of Insulin Resistance. *The American Journal of Medicine* 2006;119:S10-S16.
63. Lee Y-H, Magkos F, Mantzoros CS, et al. Effects of leptin and adiponectin on pancreatic β -cell function. 2011;60:1664-1672.
64. Toyoshima Y, Gavrilova O, Yakar S, et al. Leptin Improves Insulin Resistance and Hyperglycemia in a Mouse Model of Type 2 Diabetes. 2005;146:4024-4035.
65. Yau SW, Henry BA, Russo VC, et al. Leptin Enhances Insulin Sensitivity by Direct and Sympathetic Nervous System Regulation of Muscle IGFBP-2 Expression: Evidence From Nonrodent Models. 2014;155:2133-2143.
66. Shimabukuro M, Koyama K, Chen G, et al. Direct antidiabetic effect of leptin through triglyceride depletion of tissues. 1997;94:4637-4641.
67. Zhou YT, Shimabukuro M, Koyama K, et al. Induction by leptin of uncoupling protein-2 and enzymes of fatty acid oxidation. 1997;94:6386-6390.
68. Kern PA, Di Gregorio GB, Lu T, et al. Adiponectin Expression From Human Adipose Tissue: Relation to Obesity, Insulin Resistance, and Tumor Necrosis Factor- Expression. *Diabetes* 2003;52:1779-1785.
69. Ouchi N, Walsh K. Adiponectin as an anti-inflammatory factor. 2007;380:24-30.
70. Yamauchi T, Nio Y, Maki T, et al. Targeted disruption of AdipoR1 and AdipoR2 causes abrogation of adiponectin binding and metabolic actions. 2007;13:332-339.
71. Crimmins NA, Martin LJ. Polymorphisms in adiponectin receptor genes ADIPOR1 and ADIPOR2 and insulin resistance. 2007;8:419-423.
72. Panee J. Monocyte Chemoattractant Protein 1 (MCP-1) in obesity and diabetes. *Cytokine* 2012;60:1-12.
73. Lomonaco R, Ortiz-Lopez C, Orsak B, et al. Effect of adipose tissue insulin resistance on metabolic parameters and liver histology in obese patients with nonalcoholic fatty liver disease. 2012;55:1389-1397.
74. Pal M, Febbraio MA, Lancaster GI. The roles of c-Jun NH 2-terminal kinases (JNKs) in obesity and insulin resistance. 2016;594:267-279.
75. Lewis GF, Carpentier A, Adeli K, et al. Disordered Fat Storage and Mobilization in the Pathogenesis of Insulin Resistance and Type 2 Diabetes. 2002;23:201-229.
76. Pagano G. Nonalcoholic steatohepatitis, insulin resistance, and metabolic syndrome: Further evidence for an etiologic association. 2002;35:367-372.
77. Cortez-Pinto H, Camilo ME, Baptista A, et al. Non-alcoholic fatty liver: another feature of the metabolic syndrome? 1999;18:353-358.

78. Peverill W, Powell L, Skoien R. Evolving Concepts in the Pathogenesis of NASH: Beyond Steatosis and Inflammation. *International Journal of Molecular Sciences* 2014;15:8591-8638.
79. Ntambi JM, Miyazaki M, Stoehr JP, et al. Loss of stearyl-CoA desaturase-1 function protects mice against adiposity. 2002;99:11482-11486.
80. Jiang G, Li Z, Liu F, et al. Prevention of obesity in mice by antisense oligonucleotide inhibitors of stearyl-CoA desaturase-1. 2005;115:1030-1038.
81. Monetti M, Levin MC, Watt MJ, et al. Dissociation of Hepatic Steatosis and Insulin Resistance in Mice Overexpressing DGAT in the Liver. 2007;6:69-78.
82. Flowers MT, Groen AK, Oler AT, et al. Cholestasis and hypercholesterolemia in SCD1-deficient mice fed a low-fat, high-carbohydrate diet. 2006;47:2668-2680.
83. Han MS, Park SY, Shinzawa K, et al. Lysophosphatidylcholine as a death effector in the lipoapoptosis of hepatocytes. 2008;49:84-97.
84. Thursz M, Gual A, Lackner C, et al. EASL Clinical Practice Guidelines: Management of alcohol-related liver disease. *Journal of Hepatology* 2018;69:154-181.
85. Selin KH. Test-Retest Reliability of the Alcohol Use Disorder Identification Test in a General Population Sample. 2003;27:1428-1435.
86. Saunders JB, Aasland OG, Babor TF, et al. Development of the Alcohol Use Disorders Identification Test (AUDIT): WHO Collaborative Project on Early Detection of Persons with Harmful Alcohol Consumption--II. *Addiction* 1993;88:791-804.
87. Bush K, Kivlahan DR, McDonell MB, et al. The AUDIT alcohol consumption questions (AUDIT-C): an effective brief screening test for problem drinking. Ambulatory Care Quality Improvement Project (ACQUIP). Alcohol Use Disorders Identification Test. *Arch Intern Med* 1998;158:1789-95.
88. Seitz HK. Additive effects of moderate drinking and obesity on serum γ -glutamyl transferase. *American Journal of Clinical Nutrition* 2006;83:1252-1253.
89. Gough G, Heathers L, Puckett D, et al. The Utility of Commonly Used Laboratory Tests to Screen for Excessive Alcohol Use in Clinical Practice. *Alcoholism: Clinical and Experimental Research* 2015;39:1493-1500.
90. Hock B, Schwarz M, Domke I, et al. Validity of carbohydrate-deficient transferrin (%CDT), γ -glutamyltransferase (γ -GT) and mean corpuscular erythrocyte volume (MCV) as biomarkers for chronic alcohol abuse: a study in patients with alcohol dependence and liver disorders of non-alcoholic and. *Addiction* 2005;100:1477-1486.
91. Reynaud M, Schellenberg F, Loiseux-Meunier MN, et al. Objective diagnosis of alcohol abuse: compared values of carbohydrate-

- deficient transferrin (CDT), gamma-glutamyl transferase (GGT), and mean corpuscular volume (MCV). *Alcohol Clin Exp Res* 2000;24:1414-9.
92. Staufer K, Andresen H, Vettorazzi E, et al. Urinary ethyl glucuronide as a novel screening tool in patients pre- and post-liver transplantation improves detection of alcohol consumption. *Hepatology* 2011;54:1640-1649.
 93. Helander A, Wielders J, Anton R, et al. Reprint of Standardisation and use of the alcohol biomarker carbohydrate-deficient transferrin (CDT). *Clinica Chimica Acta* 2017.
 94. Niemelä O. Biomarker-Based Approaches for Assessing Alcohol Use Disorders. 2016;13:166.
 95. Anton RF, Lieber C, Tabakoff B. Carbohydrate-Deficient Transferrin and gamma-Glutamyltransferase for the Detection and Monitoring of Alcohol Use: Results From a Multisite Study. 2002;26:1215-1222.
 96. Anton RF. Carbohydrate-deficient transferrin for detection and monitoring of sustained heavy drinking. *Alcohol* 2001;25:185-188.
 97. Hartmann S, Aradottir S, Graf M, et al. Phosphatidylethanol as a sensitive and specific biomarker? comparison with gamma-glutamyl transpeptidase, mean corpuscular volume and carbohydrate-deficient transferrin. *Addiction Biology* 2007;12:81-84.
 98. Schröck A, Thierauf-Emberger A, Schürch S, et al. Phosphatidylethanol (PEth) detected in blood for 3 to 12 days after single consumption of alcohol—a drinking study with 16 volunteers. *International Journal of Legal Medicine* 2017;131:153-160.
 99. Kechagias S, Dernroth DN, Blomgren A, et al. Phosphatidylethanol Compared with Other Blood Tests as a Biomarker of Moderate Alcohol Consumption in Healthy Volunteers: A Prospective Randomized Study. *Alcohol and Alcoholism* 2015;50:399-406.
 100. Walther L, De Bejczy A, Löf E, et al. Phosphatidylethanol is Superior to Carbohydrate-Deficient Transferrin and gamma-Glutamyltransferase as an Alcohol Marker and is a Reliable Estimate of Alcohol Consumption Level. *Alcoholism: Clinical and Experimental Research* 2015;39:2200-2208.
 101. Saad L. Majority in US drink alcohol, averaging four drinks a week. *Gallup Well-Being* 2012.
 102. Trolldal B, Leifman H. Alkoholkonsumtionen i Sverige 2017. CAN Rapport 175. Hämtad från Centralförbundet för alkohol-och narkotikaupplysnings webbplats: https://www.can.se/contentassets/00aedc7994c94a34b2f35880ba8e1083/alkoholkonsumtionen-i-sverige-2017_webb.pdf 2018.
 103. Eurobarometer S. EU citizens' attitudes towards alcohol. Brussels: European Commission 2010.
 104. Dunn W, Sanyal AJ, Brunt EM, et al. Modest alcohol consumption is associated with decreased prevalence of steatohepatitis in patients with non-alcoholic fatty liver disease (NAFLD). 2012;57:384-391.

105. Kwon HK, Greenson JK, Conjeevaram HS. Effect of lifetime alcohol consumption on the histological severity of non-alcoholic fatty liver disease. *Liver International* 2014;34:129-135.
106. Cao G, Yi T, Liu Q, et al. Alcohol consumption and risk of fatty liver disease: a meta-analysis. 2016;4:e2633.
107. Moriya A, Iwasaki Y, Ohguchi S, et al. Roles of alcohol consumption in fatty liver: A longitudinal study. 2015;62:921-927.
108. Hagström H, Nasr P, Ekstedt M, et al. Low to moderate lifetime alcohol consumption is associated with less advanced stages of fibrosis in non-alcoholic fatty liver disease. *Scandinavian Journal of Gastroenterology* 2017;52:159-165.
109. Hajifathalian K, Torabi Sagvand B, McCullough AJ. Effect of Alcohol Consumption on Survival in Nonalcoholic Fatty Liver Disease: A National Prospective Cohort Study. *Hepatology* 2018.
110. Ekstedt M, Franzén LE, Holmqvist M, et al. Alcohol consumption is associated with progression of hepatic fibrosis in non-alcoholic fatty liver disease. 2009;44:366-374.
111. Ascha MS, Hanouneh IA, Lopez R, et al. The incidence and risk factors of hepatocellular carcinoma in patients with nonalcoholic steatohepatitis. *Hepatology* 2010;51:1972-8.
112. Ajmera V, Belt P, Wilson LA, et al. Among patients with nonalcoholic fatty liver disease, modest alcohol use is associated with less improvement in histologic steatosis and steatohepatitis. *Clinical Gastroenterology and Hepatology* 2018;16:1511-1520. e5.
113. Kimura T, Tanaka N, Fujimori N, et al. Mild drinking habit is a risk factor for hepatocarcinogenesis in non-alcoholic fatty liver disease with advanced fibrosis. *World journal of gastroenterology* 2018;24:1440.
114. Ronksley PE, Brien SE, Turner BJ, et al. Association of alcohol consumption with selected cardiovascular disease outcomes: a systematic review and meta-analysis. *Bmj* 2011;342:d671.
115. Griswold MG, Fullman N, Hawley C, et al. Alcohol use and burden for 195 countries and territories, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016. *The Lancet* 2018;392:1015-1035.
116. Åberg F, Helenius-Hietala J, Puukka P, et al. Interaction between alcohol consumption and metabolic syndrome in predicting severe liver disease in the general population. *Hepatology* 2017.
117. Chang Y, Cho YK, Kim Y, et al. Nonheavy Drinking and Worsening of Noninvasive Fibrosis Markers in Nonalcoholic Fatty Liver Disease: A Cohort Study. *Hepatology* 2019;69:64-75.
118. Younossi ZM, Stepanova M, Ong J, et al. Effects of Alcohol Consumption and Metabolic Syndrome on Mortality in Patients With Nonalcoholic and Alcohol-Related Fatty Liver Disease. *Clinical Gastroenterology and Hepatology* 2018.

119. Ajmera VH, Terrault NA, Harrison SA. Is moderate alcohol use in nonalcoholic fatty liver disease good or bad? A critical review. *Hepatology* 2017;65:2090-2099.
120. Hagström H. Alcohol Consumption in Concomitant Liver Disease: How Much is Too Much? *Current Hepatology Reports* 2017.
121. Kechagias S, Blomdahl J, Ekstedt M. Alcohol consumption in non-alcoholic fatty liver disease—harmful or beneficial? *Hepatobiliary Surgery and Nutrition* 2019.
122. Brandman D, Terrault NA. Alcohol use in patients with non-alcoholic fatty liver: a tangled web of causality. *Hepatobiliary Surgery and Nutrition* 2019;8:277-279.
123. Eslam M, Sanyal AJ, George J. Are Non-alcoholic Fatty Liver Disease and Alcoholic Fatty Liver Disease More Than Just Semantics? *Gastroenterology* 2019.
124. Skelly MM, James PD, Ryder SD. Findings on liver biopsy to investigate abnormal liver function tests in the absence of diagnostic serology. *Journal of Hepatology* 2001;35:195-199.
125. Daniel S, Ben-Menachem T, Vasudevan G, et al. Prospective evaluation of unexplained chronic liver transaminase abnormalities in asymptomatic and symptomatic patients. *Am J Gastroenterol* 1999;94:3010-4.
126. Hultcrantz R, Glaumann H, Lindberg G, et al. Liver Investigation in 149 Asymptomatic Patients with Moderately Elevated Activities of Serum: Aminotransferases. 1986;21:109-113.
127. Williams CD, Stengel J, Asike MI, et al. Prevalence of Nonalcoholic Fatty Liver Disease and Nonalcoholic Steatohepatitis Among a Largely Middle-Aged Population Utilizing Ultrasound and Liver Biopsy: A Prospective Study. *Gastroenterology* 2011;140:124-131.
128. Mofrad P, Contos MJ, Haque M, et al. Clinical and histologic spectrum of nonalcoholic fatty liver disease associated with normal ALT values. *Hepatology* 2003;37:1286-92.
129. Lee SS, Park SH, Kim HJ, et al. Non-invasive assessment of hepatic steatosis: Prospective comparison of the accuracy of imaging examinations. *Journal of Hepatology* 2010;52:579-585.
130. Poynard T, Ratziu V, Naveau S, et al. The diagnostic value of biomarkers (SteatoTest) for the prediction of liver steatosis. *Comparative hepatology* 2005;4:10.
131. Lassailly G, Caiazzo R, Hollebecque A, et al. Validation of noninvasive biomarkers (FibroTest, SteatoTest, and NashTest) for prediction of liver injury in patients with morbid obesity. 2011;23:499-506.
132. Poynard T, Lassailly G, Diaz E, et al. Performance of Biomarkers FibroTest, ActiTest, SteatoTest, and NashTest in Patients with Severe Obesity: Meta Analysis of Individual Patient Data. 2012;7:e30325.

133. Munteanu M, Tiniakos D, Anstee Q, et al. Diagnostic performance of FibroTest, SteatoTest and ActiTest in patients with NAFLD using the SAF score as histological reference. 2016;44:877-889.
134. Bedogni G, Bellentani S, Miglioli L, et al. The Fatty Liver Index: a simple and accurate predictor of hepatic steatosis in the general population. *BMC Gastroenterology* 2006;6.
135. Koehler EM, Schouten JNL, Hansen BE, et al. External Validation of the Fatty Liver Index for Identifying Nonalcoholic Fatty Liver Disease in a Population-based Study. *Clinical Gastroenterology and Hepatology* 2013;11:1201-1204.
136. Kim JH, Kwon SY, Lee SW, et al. Validation of fatty liver index and lipid accumulation product for predicting fatty liver in Korean population. 2011;31:1600-1601.
137. Yang B-L, Wu W-C, Fang K-C, et al. External Validation of Fatty Liver Index for Identifying Ultrasonographic Fatty Liver in a Large-Scale Cross-Sectional Study in Taiwan. 2015;10:e0120443.
138. Gastaldelli A, Kozakova M, Højlund K, et al. Fatty liver is associated with insulin resistance, risk of coronary heart disease, and early atherosclerosis in a large European population. 2009;49:1537-1544.
139. Calori G, Lattuada G, Ragona F, et al. Fatty liver index and mortality: The cremona study in the 15th year of follow-up. *Hepatology* 2011;54:145-152.
140. Lerchbaum E, Pilz S, Grammer TB, et al. The fatty liver index is associated with increased mortality in subjects referred to coronary angiography. 2013;23:1231-1238.
141. Bozkurt L, Göbl CS, Tura A, et al. Fatty Liver Index Predicts Further Metabolic Deteriorations in Women with Previous Gestational Diabetes. 2012;7:e32710.
142. Khang AR, Lee HW, Yi D, et al. <p>The fatty liver index, a simple and useful predictor of metabolic syndrome: analysis of the Korea National Health and Nutrition Examination Survey 2010–2011</p>. *Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy* 2019;Volume 12:181-190.
143. Pais R, Redheuil A, Cluzel P, et al. Relationship Among Fatty Liver, Specific and Multiple-Site Atherosclerosis, and 10-Year Framingham Score. *Hepatology* 2019.
144. Kotronen A, Peltonen M, Hakkarainen A, et al. Prediction of Non-Alcoholic Fatty Liver Disease and Liver Fat Using Metabolic and Genetic Factors. *Gastroenterology* 2009;137:865-872.
145. Wlazlo N, Van Greevenbroek MMJ, Ferreira I, et al. The diagnosis of non-alcoholic fatty liver disease. 2012;35:204-205.
146. McPherson S, Stewart SF, Henderson E, et al. Simple non-invasive fibrosis scoring systems can reliably exclude advanced fibrosis in patients with non-alcoholic fatty liver disease. *Gut* 2010;59:1265-9.
147. Siddiqui MS, Patidar KR, Boyett S, et al. Performance of Non-invasive Models of Fibrosis in Predicting Mild to Moderate Fibrosis

- in Patients with Nonalcoholic Fatty Liver Disease (NAFLD). 2015:n/a-n/a.
148. Xiao G, Zhu S, Xiao X, et al. Comparison of laboratory tests, ultrasound, or MRE to detect fibrosis in patients with non-alcoholic fatty liver disease: A meta-analysis. *Hepatology* 2017.
 149. Cassinotto C, Boursier J, De Lédinghen V, et al. Liver stiffness in nonalcoholic fatty liver disease: A comparison of supersonic shear imaging, FibroScan, and ARFI with liver biopsy. *Hepatology* 2016;63:1817-1827.
 150. European Association for Study of L, Asociacion Latinoamericana para el Estudio del H. EASL-ALEH Clinical Practice Guidelines: Non-invasive tests for evaluation of liver disease severity and prognosis. *J Hepatol* 2015;63:237-64.
 151. Castera L, Friedrich-Rust M, Loomba R. Noninvasive Assessment of Liver Disease in Patients With Nonalcoholic Fatty Liver Disease. *Gastroenterology* 2019;156:1264-1281.e4.
 152. Tapper EB, Loomba R. Noninvasive imaging biomarker assessment of liver fibrosis by elastography in NAFLD. *Nature Reviews Gastroenterology & Hepatology* 2018;15:274-282.
 153. Castera L, Friedrich-Rust M, Loomba R. Noninvasive Assessment of Liver Disease in Patients With Nonalcoholic Fatty Liver Disease. *Gastroenterology* 2019;156:1264-1281 e4.
 154. Lundberg P, Harmsen E, Ho C, et al. Nuclear magnetic resonance studies of cellular metabolism. *Analytical biochemistry* 1990;191:193-222.
 155. Brix G, Kolem H, Nitz WR, et al. Basics of magnetic resonance imaging and magnetic resonance spectroscopy. *Magnetic Resonance Tomography: Springer*, 2008:3-167.
 156. McRobbie DW, Moore EA, Graves MJ, et al. *MRI from Picture to Proton*: Cambridge university press, 2017.
 157. Reeder SB, Sirlin CB. Quantification of Liver Fat with Magnetic Resonance Imaging. 2010;18:337-357.
 158. Reeder SB, Cruite I, Hamilton G, et al. Quantitative Assessment of Liver Fat with Magnetic Resonance Imaging and Spectroscopy. *J Magn Reson Imaging* 2011;34:729-749.
 159. Szczepaniak LS, Nurenberg P, Leonard D, et al. Magnetic resonance spectroscopy to measure hepatic triglyceride content: prevalence of hepatic steatosis in the general population. *Am J Physiol Endocrinol Metab* 2005;288:E462-8.
 160. Charatcharoenwitthaya P, Lindor KD. Role of radiologic modalities in the management of non-alcoholic steatohepatitis. *Clin Liver Dis* 2007;11:37-54, viii.
 161. Yokoo T, Bydder M, Hamilton G, et al. Nonalcoholic Fatty Liver Disease: Diagnostic and Fat-Grading Accuracy of Low-Flip-Angle Multiecho Gradient-Recalled-Echo MR Imaging at 1.5 T 1. 2009;251:67-76.

162. Yokoo T, Shiehorteza M, Hamilton G, et al. Estimation of Hepatic Proton-Density Fat Fraction by Using MR Imaging at 3.0 T. 2011;258:749-759.
163. Hernando D, Sharma SD, Aliyari Ghasabeh M, et al. Multisite, multivendor validation of the accuracy and reproducibility of proton-density fat-fraction quantification at 1.5T and 3T using a fat-water phantom. 2016.
164. Roldan-Valadez E, Favila R, Martínez-López M, et al. In vivo 3T spectroscopic quantification of liver fat content in nonalcoholic fatty liver disease: Correlation with biochemical method and morphometry. *J Hepatol* 2010;53:732-7.
165. Bannas P, Kramer H, Hernando D, et al. Quantitative magnetic resonance imaging of hepatic steatosis: Validation in ex vivo human livers. *Hepatology* 2015;62:1444-55.
166. Loomba R, Sirlin CB, Ang B, et al. Ezetimibe for the treatment of nonalcoholic steatohepatitis: Assessment by novel MRI and MRE in a randomized trial (MOZART Trial). *Hepatology* 2014.
167. Das K, Mukherjee PS, Ghosh A, et al. Nonobese population in a developing country has a high prevalence of nonalcoholic fatty liver and significant liver disease. *Hepatology* 2010;51:1593-602.
168. Portillo-Sanchez P, Bril F, Maximos M, 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;100:2231-8.
169. Tang A, Tan J, Sun M, et al. Nonalcoholic fatty liver disease: MR imaging of liver proton density fat fraction to assess hepatic steatosis. *Radiology* 2013;267:422-31.
170. Tang A, Desai A, Hamilton G, et al. Accuracy of MR imaging-estimated proton density fat fraction for classification of dichotomized histologic steatosis grades in nonalcoholic fatty liver disease. *Radiology* 2015;274:416-25.
171. Idilman IS, Aniktar H, Idilman R, et al. Hepatic steatosis: quantification by proton density fat fraction with MR imaging versus liver biopsy. *Radiology* 2013;267:767-75.
172. Nouredin M, Lam J, Peterson MR, et al. Utility of magnetic resonance imaging versus histology for quantifying changes in liver fat in nonalcoholic fatty liver disease trials. *Hepatology* 2013;58:1930-40.
173. Le TA, Chen J, Changchien C, et al. Effect of colesvelam on liver fat quantified by magnetic resonance in nonalcoholic steatohepatitis: a randomized controlled trial. *Hepatology* 2012;56:922-32.
174. Permutt Z, Le TA, Peterson MR, et al. Correlation between liver histology and novel magnetic resonance imaging in adult patients with non-alcoholic fatty liver disease - MRI accurately quantifies hepatic steatosis in NAFLD. *Aliment Pharmacol Ther* 2012;36:22-9.
175. Bedossa P, Carrat F. Liver biopsy: The best, not the gold standard. 2009;50:1-3.

176. Frerichs FT. *Über den Diabetes*. 1884.
177. Menghini G. One-second needle biopsy of the liver. *Gastroenterology* 1958;35:190-9.
178. Poynard T, Ratziu V, Bedossa P. Appropriateness of Liver Biopsy. *Canadian Journal of Gastroenterology* 2000;14.
179. Boyum JH, Atwell TD, Schmit GD, et al. Incidence and Risk Factors for Adverse Events Related to Image-Guided Liver Biopsy. *Mayo Clin Proc* 2016;91:329-35.
180. Ratziu V, Charlotte F, Heurtier A, et al. Sampling Variability of Liver Biopsy in Nonalcoholic Fatty Liver Disease. *Gastroenterology* 2005;128:1898-1906.
181. Vuppalanchi R, Ünalp A, Van Natta ML, et al. Effects of Liver Biopsy Sample Length and Number of Readings on Sampling Variability in Nonalcoholic Fatty Liver Disease. *Clinical Gastroenterology and Hepatology* 2009;7:481-486.
182. Arun J, Jhala N, Lazenby AJ, et al. Influence of Liver Biopsy Heterogeneity and Diagnosis of Nonalcoholic Steatohepatitis in Subjects Undergoing Gastric Bypass. *Obesity Surgery* 2007;17:155-161.
183. Younossi ZM, Gramlich T, Liu YC, et al. Nonalcoholic fatty liver disease: assessment of variability in pathologic interpretations. *Mod Pathol* 1998;11:560-5.
184. Younossi ZM, Stepanova M, Rafiq N, et al. Pathologic criteria for nonalcoholic steatohepatitis: interprotocol agreement and ability to predict liver-related mortality. *Hepatology* 2011;53:1874-82.
185. Larson SP, Bowers SP, Palekar NA, et al. Histopathologic Variability Between the Right and Left Lobes of the Liver in Morbidly Obese Patients Undergoing Roux-en-Y Bypass. *Clinical Gastroenterology and Hepatology* 2007;5:1329-1332.
186. Merriman RB, Ferrell LD, Patti MG, et al. Correlation of paired liver biopsies in morbidly obese patients with suspected nonalcoholic fatty liver disease. *Hepatology* 2006;44:874-880.
187. Pournik O, Alavian SM, Ghalichi L, et al. Inter-observer and Intra-observer Agreement in Pathological Evaluation of Non-alcoholic Fatty Liver Disease Suspected Liver Biopsies. *Hepat Mon* 2014;14:e15167.
188. Jung ES, Lee K, Yu E, et al. Interobserver Agreement on Pathologic Features of Liver Biopsy Tissue in Patients with Nonalcoholic Fatty Liver Disease. *J Pathol Transl Med* 2016;50:190-6.
189. Schuppan D, Surabattula R, Wang XY. Determinants of fibrosis progression and regression in NASH. *Journal of Hepatology* 2018;68:238-250.
190. Musso G, Gambino R, Cassader M, et al. Meta-analysis: natural history of non-alcoholic fatty liver disease (NAFLD) and diagnostic accuracy of non-invasive tests for liver disease severity. *Ann Med* 2011;43:617-49.

191. Brunt EM, Janney CG, Di Bisceglie AM, et al. Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. *Am J Gastroenterol* 1999;94:2467-74.
192. Nonalcoholic steatohepatitis clinical research network. *Hepatology* 2003;37:244.
193. Kleiner DE, Brunt EM, Van Natta M, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* 2005;41:1313-21.
194. Sanyal AJ, Brunt EM, Kleiner DE, et al. Endpoints and clinical trial design for nonalcoholic steatohepatitis. *Hepatology* 2011;54:344-53.
195. Ballestri S, Lonardo A, Loria P. Nonalcoholic fatty liver disease activity score and Brunt's pathologic criteria for the diagnosis of nonalcoholic steatohepatitis: what do they mean and do they agree? *Hepatology* 2011;53:2142-3; author reply 2143.
196. Ekstedt M, Hagström H, Nasr P, et al. Fibrosis stage is the strongest predictor for disease-specific mortality in NAFLD after up to 33 years of follow-up. *Hepatology* 2015;61:1547-54.
197. Angulo P, Kleiner DE, Dam-Larsen S, et al. Liver Fibrosis, but No Other Histologic Features, Is Associated With Long-term Outcomes of Patients With Nonalcoholic Fatty Liver Disease. *Gastroenterology* 2015;149:389-397.e10.
198. Bedossa P, Poitou C, Veyrie N, et al. Histopathological algorithm and scoring system for evaluation of liver lesions in morbidly obese patients. *Hepatology* 2012;56:1751-9.
199. Sanyal AJ, Harrison SA, Ratziu V, et al. The Natural History of Advanced Fibrosis due to Nonalcoholic Steatohepatitis: Data from the Simtuzumab Trials. *Hepatology* 2019.
200. Argo CK, Northup PG, Al-Osaimi AM, et al. Systematic review of risk factors for fibrosis progression in non-alcoholic steatohepatitis. *J Hepatol* 2009;51:371-9.
201. McPherson S, Hardy T, Henderson E, et al. Evidence of NAFLD Progression from Steatosis to Fibrosing-Steatohepatitis Using Paired Biopsies: Implications for Prognosis & Clinical Management. *J Hepatol* 2014.
202. Evans CD, Oien KA, MacSween RN, et al. Non-alcoholic steatohepatitis: a common cause of progressive chronic liver injury? *J Clin Pathol* 2002;55:689-92.
203. Ratziu V, Giral P, Charlotte F, et al. Liver fibrosis in overweight patients. *Gastroenterology* 2000;118:1117-23.
204. Harrison SA, Torgerson S, Hayashi PH. The natural history of nonalcoholic fatty liver disease: a clinical histopathological study. *Am J Gastroenterol* 2003;98:2042-7.
205. Fassio E, Alvarez E, Domínguez N, et al. Natural history of nonalcoholic steatohepatitis: a longitudinal study of repeat liver biopsies. *Hepatology* 2004;40:820-6.

206. Adams LA, Sanderson S, Lindor KD, et al. The histological course of nonalcoholic fatty liver disease: a longitudinal study of 103 patients with sequential liver biopsies. *J Hepatol* 2005;42:132-8.
207. Hui AY, Wong VW, Chan HL, et al. Histological progression of non-alcoholic fatty liver disease in Chinese patients. *Aliment Pharmacol Ther* 2005;21:407-13.
208. Williams R, Aspinall R, Bellis M, et al. Addressing liver disease in the UK: a blueprint for attaining excellence in health care and reducing premature mortality from lifestyle issues of excess consumption of alcohol, obesity, and viral hepatitis. *Lancet* 2014;384:1953-97.
209. Mortality GBD, Causes of Death C. Global, regional, and national age-sex specific all-cause and cause-specific mortality for 240 causes of death, 1990-2013: a systematic analysis for the Global Burden of Disease Study 2013. *Lancet* 2015;385:117-71.
210. Mokdad AA, Lopez AD, Shahraz S, et al. Liver cirrhosis mortality in 187 countries between 1980 and 2010: a systematic analysis. *BMC Med* 2014;12:145.
211. Harris R, Harman DJ, Card TR, et al. Prevalence of clinically significant liver disease within the general population, as defined by non-invasive markers of liver fibrosis: a systematic review. *Lancet Gastroenterol Hepatol* 2017;2:288-297.
212. Setiawan VW, Stram DO, Porcel J, et al. Prevalence of chronic liver disease and cirrhosis by underlying cause in understudied ethnic groups: The multiethnic cohort. *Hepatology* 2016;64:1969-1977.
213. Roulot D, Costes JL, Buyck JF, et al. Transient elastography as a screening tool for liver fibrosis and cirrhosis in a community-based population aged over 45 years. *Gut* 2011;60:977-84.
214. Koehler EM, Plompen EP, Schouten JN, et al. Presence of diabetes mellitus and steatosis is associated with liver stiffness in a general population: The Rotterdam study. *Hepatology* 2016;63:138-47.
215. Marchesini G, Brizi M, Morselli-Labate AM, et al. Association of nonalcoholic fatty liver disease with insulin resistance. *Am J Med* 1999;107:450-5.
216. Marchesini G, Brizi M, Bianchi G, et al. Nonalcoholic fatty liver disease: a feature of the metabolic syndrome. *Diabetes* 2001;50:1844-50.
217. Wong RJ, Aguilar M, Cheung R, et al. Nonalcoholic steatohepatitis is the second leading etiology of liver disease among adults awaiting liver transplantation in the United States. *Gastroenterology* 2015;148:547-55.
218. Collaboration NCDRF. Worldwide trends in body-mass index, underweight, overweight, and obesity from 1975 to 2016: a pooled analysis of 2416 population-based measurement studies in 128.9 million children, adolescents, and adults. *Lancet* 2017;390:2627-2642.
219. Hughes V. The big fat truth. *Nature* 2013;497:428-30.

220. Prospective Studies C, Whitlock G, Lewington S, et al. Body-mass index and cause-specific mortality in 900 000 adults: collaborative analyses of 57 prospective studies. *Lancet* 2009;373:1083-96.
221. Berrington de Gonzalez A, Hartge P, Cerhan JR, et al. Body-mass index and mortality among 1.46 million white adults. *N Engl J Med* 2010;363:2211-9.
222. Flegal KM, Kit BK, Orpana H, et al. Association of all-cause mortality with overweight and obesity using standard body mass index categories: a systematic review and meta-analysis. *JAMA* 2013;309:71-82.
223. Nordstrom P, Pedersen NL, Gustafson Y, et al. Risks of Myocardial Infarction, Death, and Diabetes in Identical Twin Pairs With Different Body Mass Indexes. *JAMA Intern Med* 2016;176:1522-1529.
224. Lazo M, Hernaez R, Eberhardt MS, et al. Prevalence of nonalcoholic fatty liver disease in the United States: the Third National Health and Nutrition Examination Survey, 1988-1994. *Am J Epidemiol* 2013;178:38-45.
225. Xu C, Yu C, Ma H, et al. Prevalence and risk factors for the development of nonalcoholic fatty liver disease in a nonobese Chinese population: the Zhejiang Zhenhai Study. *Am J Gastroenterol* 2013;108:1299-304.
226. Wu J, He S, Xu H, et al. Non-alcoholic fatty liver disease incidence, remission and risk factors among a general Chinese population with a 6-year follow-up. *Sci Rep* 2018;8:7557.
227. VanWagner LB, Khan SS, Ning H, et al. Body mass index trajectories in young adulthood predict non-alcoholic fatty liver disease in middle age: The CARDIA cohort study. *Liver Int* 2018;38:706-714.
228. Centis E, Marzocchi R, Di Domizio S, et al. The effect of lifestyle changes in non-alcoholic fatty liver disease. *Dig Dis* 2010;28:267-73.
229. Zelber-Sagi S, Lotan R, Shlomai A, et al. Predictors for incidence and remission of NAFLD in the general population during a seven-year prospective follow-up. *J Hepatol* 2012;56:1145-51.
230. Vilar-Gomez E, Martinez-Perez Y, Calzadilla-Bertot L, et al. Weight Loss Through Lifestyle Modification Significantly Reduces Features of Nonalcoholic Steatohepatitis. *Gastroenterology* 2015;149:367-78 e5; quiz e14-5.
231. Mathurin P, Gonzalez F, Kerdraon O, et al. The evolution of severe steatosis after bariatric surgery is related to insulin resistance. *Gastroenterology* 2006;130:1617-24.
232. Mathurin P, Hollebecque A, Arnalsteen L, et al. Prospective study of the long-term effects of bariatric surgery on liver injury in patients without advanced disease. *Gastroenterology* 2009;137:532-40.
233. Rabl C, Campos GM. The impact of bariatric surgery on nonalcoholic steatohepatitis. *Semin Liver Dis* 2012;32:80-91.

234. Ioannou GN, Weiss NS, Kowdley KV, et al. Is obesity a risk factor for cirrhosis-related death or hospitalization? A population-based cohort study. *Gastroenterology* 2003;125:1053-9.
235. Liu B, Balkwill A, Reeves G, et al. Body mass index and risk of liver cirrhosis in middle aged UK women: prospective study. *BMJ* 2010;340:c912.
236. Hagstrom H, Stal P, Hultcrantz R, et al. Overweight in late adolescence predicts development of severe liver disease later in life: A 39years follow-up study. *J Hepatol* 2016;65:363-8.
237. Calle EE, Rodriguez C, Walker-Thurmond K, et al. Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. *N Engl J Med* 2003;348:1625-38.
238. Hagstrom H, Tynelius P, Rasmussen F. High BMI in late adolescence predicts future severe liver disease and hepatocellular carcinoma: a national, population-based cohort study in 1.2 million men. *Gut* 2018;67:1536-1542.
239. Collaboration NCDRF. Worldwide trends in diabetes since 1980: a pooled analysis of 751 population-based studies with 4.4 million participants. *Lancet* 2016;387:1513-30.
240. Lonardo A, Bellentani S, Argo CK, et al. Epidemiological modifiers of non-alcoholic fatty liver disease: Focus on high-risk groups. *Digestive and Liver Disease* 2015;47:997-1006.
241. Younossi ZM, Golabi P, de Avila L, et al. The Global Epidemiology of NAFLD and NASH in Patients with type 2 diabetes: A Systematic Review and Meta-analysis. *Journal of Hepatology* 2019.
242. Ma J, Hwang S-J, Pedley A, et al. Bi-directional analysis between fatty liver and cardiovascular disease risk factors. *Journal of Hepatology* 2017;66:390-397.
243. Li Y, Wang J, Tang Y, 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:e0174291.
244. Ballestri S, Zona S, Targher G, et al. Nonalcoholic fatty liver disease is associated with an almost two-fold increased risk of incident type 2 diabetes and metabolic syndrome. Evidence from a systematic review and meta-analysis. 2015:n/a-n/a.
245. Chen SC-C, Tsai SP, Jhao J-Y, et al. Liver Fat, Hepatic Enzymes, Alkaline Phosphatase and the Risk of Incident Type 2 Diabetes: A Prospective Study of 132,377 Adults. *Scientific Reports* 2017;7.
246. Okamoto M, Takeda Y, Yoda Y, et al. The Association of Fatty Liver and Diabetes Risk. *Journal of Epidemiology* 2003;13:15-21.
247. Fan J-G, Li F, Cai X-B, et al. Effects of nonalcoholic fatty liver disease on the development of metabolic disorders. *Journal of Gastroenterology and Hepatology* 2007;22:1086-1091.
248. Shibata M, Kihara Y, Taguchi M, et al. Nonalcoholic Fatty Liver Disease Is a Risk Factor for Type 2 Diabetes in Middle-Aged Japanese Men. *Diabetes Care* 2007;30:2940-2944.

249. Kim CH, Park JY, Lee KU, et al. Fatty liver is an independent risk factor for the development of Type 2 diabetes in Korean adults. *Diabetic Medicine* 2008;25:476-481.
250. Yamada T, Fukatsu M, Suzuki S, et al. Fatty liver predicts impaired fasting glucose and type 2 diabetes mellitus in Japanese undergoing a health checkup. *Journal of Gastroenterology and Hepatology* 2010;25:352-356.
251. Bae JC, Rhee EJ, Lee WY, 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 2011;34:727-729.
252. Sung KC, Jeong WS, Wild SH, et al. Combined Influence of Insulin Resistance, Overweight/Obesity, and Fatty Liver as Risk Factors for Type 2 Diabetes. 2012;35:717-722.
253. Park SK, Seo MH, Shin HC, et al. 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;57:1378-83.
254. Kasturiratne A, Weerasinghe S, Dassanayake AS, et al. Influence of non-alcoholic fatty liver disease on the development of diabetes mellitus. *Journal of Gastroenterology and Hepatology* 2013;28:142-147.
255. Chang Y, Jung H-S, Yun KE, et al. Cohort study of non-alcoholic fatty liver disease, NAFLD fibrosis score, and the risk of incident diabetes in a Korean population. *The American journal of gastroenterology* 2013;108:1861.
256. Choi JH, Rhee EJ, Bae JC, 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. *Archives of Medical Research* 2013;44:115-120.
257. Sung KC, Wild SH, Byrne CD. Resolution of fatty liver and risk of incident diabetes. *J Clin Endocrinol Metab* 2013;98:3637-43.
258. Yamazaki H, Tsuboya T, Tsuji K, et al. Independent Association Between Improvement of Nonalcoholic Fatty Liver Disease and Reduced Incidence of Type 2 Diabetes. *Diabetes Care* 2015;38:1673-1679.
259. Fukuda T, Hamaguchi M, Kojima T, et al. The impact of non-alcoholic fatty liver disease on incident type 2 diabetes mellitus in non-overweight individuals. *Liver International* 2016;36:275-283.
260. Ming J, Xu S, Gao B, 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;35:2401-7.
261. Li WD, Fu KF, Li GM, et al. Comparison of effects of obesity and non-alcoholic fatty liver disease on incidence of type 2 diabetes mellitus. *World J Gastroenterol* 2015;21:9607-13.

262. Shah RV, Allison MA, Lima JAC, et al. Liver fat, statin use, and incident diabetes: The Multi-Ethnic Study of Atherosclerosis. 2015;242:211-217.
263. Chang Y, Jung H-S, Yun KE, et al. Metabolically healthy obesity is associated with an increased risk of diabetes independently of nonalcoholic fatty liver disease. *Obesity* 2016;24:1996-2003.
264. Käräjämäki AJ, Bloigu R, Kauma H, et al. Non-alcoholic fatty liver disease with and without metabolic syndrome: Different long-term outcomes. *Metabolism* 2017;66:55-63.
265. Bae JC, Han JM, Cho JH, et al. The persistence of fatty liver has a differential impact on the development of diabetes: The Kangbuk Samsung Health Study. *Diabetes Research and Clinical Practice* 2018;135:1-6.
266. Cho HJ, Hwang S, Park JI, et al. Improvement of Nonalcoholic Fatty Liver Disease Reduces the Risk of Type 2 Diabetes Mellitus. *Gut Liver* 2019.
267. Friis-Liby I, Aldenborg F, Jerlstad P, et al. High prevalence of metabolic complications in patients with non-alcoholic fatty liver disease. *Scand J Gastroenterol* 2004;39:864-9.
268. Dam-Larsen S, Becker U, Franzmann MB, et al. Final results of a long-term, clinical follow-up in fatty liver patients. *Scand J Gastroenterol* 2009;44:1236-43.
269. Bjorkstrom K, Stal P, Hultcrantz R, et al. Histologic Scores for Fat and Fibrosis Associate With Development of Type 2 Diabetes in Patients With Nonalcoholic Fatty Liver Disease. *Clin Gastroenterol Hepatol* 2017;15:1461-1468.
270. Kwok R, Choi KC, Wong GL-H, 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;65:1359-1368.
271. Bazick J, Donithan M, Neuschwander-Tetri BA, et al. Clinical Model for NASH and Advanced Fibrosis in Adult Patients With Diabetes and NAFLD: Guidelines for Referral in NAFLD. 2015;38:1347-1355.
272. Porepa L, Ray JG, Sanchez-Romeu P, et al. Newly diagnosed diabetes mellitus as a risk factor for serious liver disease. 2010;182:E526-E531.
273. Bjorkstrom K, Franzen S, Eliasson B, et al. Risk Factors for Severe Liver Disease in Patients With Type 2 Diabetes. *Clin Gastroenterol Hepatol* 2019.
274. Zoppini G, Fedeli U, Gennaro N, et al. Mortality from chronic liver diseases in diabetes: Nature Publishing Group, 2014.
275. Wild SH, Morling JR, McAllister DA, et al. Type 2 diabetes, chronic liver disease and hepatocellular cancer: a national retrospective cohort study using linked routine data. 2016.
276. Davila JA. Diabetes increases the risk of hepatocellular carcinoma in the United States: a population based case control study. *Gut* 2005;54:533-539.

277. El-Serag HB, Tran T, Everhart JE. Diabetes increases the risk of chronic liver disease and hepatocellular carcinoma. *Gastroenterology* 2004;126:460-468.
278. Jepsen P, Vilstrup H, Møllehave L, et al. Prognosis of patients with a diagnosis of fatty liver--a registry-based cohort study. *Hepato-gastroenterology* 2003;50:2101-2104.
279. Hamaguchi M, Kojima T, Takeda N, et al. Nonalcoholic fatty liver disease is a novel predictor of cardiovascular disease. *World journal of gastroenterology: WJG* 2007;13:1579.
280. Targher G, Bertolini L, Rodella S, et al. Nonalcoholic Fatty Liver Disease Is Independently Associated With an Increased Incidence of Cardiovascular Events in Type 2 Diabetic Patients. 2007;30:2119-2121.
281. Stepanova M, Younossi ZM. Independent Association Between Nonalcoholic Fatty Liver Disease and Cardiovascular Disease in the US Population. 2012;10:646-650.
282. Treeprasertsuk S, Levarage S, Adams LA, et al. The Framingham risk score and heart disease in nonalcoholic fatty liver disease. 2012;32:945-950.
283. Hagström H, Nasr P, Ekstedt M, et al. Cardiovascular risk factors in non-alcoholic fatty liver disease. *Liver International* 2018.
284. Fracanzani AL, Tiraboschi S, Pisano G, et al. Progression of carotid vascular damage and cardiovascular events in non-alcoholic fatty liver disease patients compared to the general population during 10 years of follow-up. *Atherosclerosis* 2016;246:208-13.
285. Wong VW, Wong GL, Yeung JC, et al. Long-term clinical outcomes after fatty liver screening in patients undergoing coronary angiogram: A prospective cohort study. *Hepatology* 2016;63:754-63.
286. Wu S, Wu F, Ding Y, et al. Association of non-alcoholic fatty liver disease with major adverse cardiovascular events: A systematic review and meta-analysis. *Sci Rep* 2016;6:33386.
287. Targher G, Byrne CD, Lonardo A, et al. Non-alcoholic fatty liver disease and risk of incident cardiovascular disease: A meta-analysis. *J Hepatol* 2016;65:589-600.
288. Vernon G, Baranova A, Younossi ZM. Systematic review: the epidemiology and natural history of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis in adults. *Aliment Pharmacol Ther* 2011;34:274-85.
289. Wanless IR, Lentz JS. Fatty liver hepatitis (steatohepatitis) and obesity: an autopsy study with analysis of risk factors. *Hepatology* 1990;12:1106-10.
290. Kim D, Kim WR. Nonobese Fatty Liver Disease. *Clin Gastroenterol Hepatol* 2016.
291. Pais R, Barritt AS, Calmus Y, et al. NAFLD and liver transplantation: Current burden and expected challenges. *J Hepatol* 2016;65:1245-1257.

292. Adams LA, Harmsen S, St Sauver JL, et al. Nonalcoholic fatty liver disease increases risk of death among patients with diabetes: a community-based cohort study. *Am J Gastroenterol* 2010;105:1567-73.
293. Cote RA, Robboy S. Progress in medical information management. Systematized nomenclature of medicine (SNOMED). *Jama* 1980;243:756-62.
294. 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;60:565-75.
295. Longo R, Pollesello P, Ricci C, et al. Proton MR spectroscopy in quantitative in vivo determination of fat content in human liver steatosis. *J Magn Reson Imaging* 1995;5:281-5.
296. Byrne CD, Targher G. NAFLD: A multisystem disease. *Journal of Hepatology* 2015;62:S47-S64.
297. Imajo K, Kessoku T, Honda Y, et al. Magnetic Resonance Imaging More Accurately Classifies Steatosis and Fibrosis in Patients With Nonalcoholic Fatty Liver Disease Than Transient Elastography. *Gastroenterology* 2016;150:626-637.e7.
298. European Association for the Study of the L, European Association for the Study of D, European Association for the Study of O. EASL-EASD-EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease. *J Hepatol* 2016;64:1388-402.
299. Rehm JL, Wolfgram PM, Hernando D, et al. Proton density fat-fraction is an accurate biomarker of hepatic steatosis in adolescent girls and young women. *Eur Radiol* 2015;25:2921-30.
300. Hong CW, Hamilton G, Hooker C, et al. Measurement of spleen fat on MRI-proton density fat fraction arises from reconstruction of noise. *Abdominal Radiology* 2019.
301. Kantartzis K, Machann J, Schick F, et al. The impact of liver fat vs visceral fat in determining categories of prediabetes. 2010;53:882-889.
302. Gepner Y, Shelef I, Komy O, et al. The beneficial effects of Mediterranean diet over low-fat diet may be mediated by decreasing hepatic fat content. *Journal of Hepatology* 2019.
303. Sung K-C, Lee M-Y, Lee J-Y, et al. Resolution of fatty liver and weight loss: Independent associations with changes in serum lipids and apolipoproteins. *Atherosclerosis* 2018;272:47-53.
304. Ajmera V, Park CC, Caussy C, et al. Magnetic Resonance Imaging Proton Density Fat Fraction Associates With Progression of Fibrosis in Patients With Nonalcoholic Fatty Liver Disease. *Gastroenterology* 2018;155:307-310.e2.
305. Ratziu V, Charlotte F, Heurtier A, et al. Sampling variability of liver biopsy in nonalcoholic fatty liver disease. *Gastroenterology* 2005;128:1898-906.

306. Berry SD, Ngo L, Samelson EJ, et al. Competing Risk of Death: An Important Consideration in Studies of Older Adults. *Journal of the American Geriatrics Society* 2010;58:783-787.
307. Kalbfleisch JD, Prentice RL. Estimation of the Average Hazard Ratio. *Biometrika* 1981;68:105.
308. Gooley TA, Leisenring W, Crowley J, et al. Estimation of failure probabilities in the presence of competing risks: new representations of old estimators. *Stat Med* 1999;18:695-706.
309. Southern DA, Faris PD, Brant R, et al. Kaplan–Meier methods yielded misleading results in competing risk scenarios. *Journal of Clinical Epidemiology* 2006;59:1110-1114.
310. Kim HT. Cumulative Incidence in Competing Risks Data and Competing Risks Regression Analysis. *Clinical Cancer Research* 2007;13:559-565.

Papers

The papers associated with this thesis have been removed for copyright reasons. For more details about these see:

<http://urn.kb.se/resolve?urn=urn:nbn:se:liu:diva-160523>

FACULTY OF MEDICINE AND HEALTH SCIENCES

Linköping University Medical Dissertation No. 1690, 2019
Department of Medical and Health Sciences

Linköping University
SE-581 83 Linköping, Sweden

www.liu.se