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Ville Männistö

Biomarkers for non-alcoholic steatohepatitis with special emphasis on lipid metabolism

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VILLE MÄNNISTÖ

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ABSTRACT:

Obesity and type 2 diabetes are serious global health threats. They associate closely with non-alcoholic fatty liver disease (NAFLD). Already 20-30% of individuals worldwide have non-alcoholic fatty liver (NAFL), which is the primary phenotype of NAFLD. However, it is not known why some people will develop non-alcoholic steatohepatitis (NASH), which can further progress to liver cirrhosis. NAFLD increases not only liver-related morbidity and mortality, but also cardiovascular diseases and cancers. The real prevalence of NASH is not known, since diagnosis of NASH needs a liver biopsy. A non-invasive method would be helpful not only for diagnostics, but also in population based studies, where liver biopsies cannot be obtained. Attempts have been made to develop different non-invasive diagnostic methods. Novel biomarkers could be related to pathogenesis of NASH. For example, changes in cholesterol and lipid metabolism have been observed in those with NASH.

In this thesis, a non-invasive score was developed in a cohort of 296 obese individuals with all essential NASH-associated measurements and parameters available. A score consisting of *PNPLA3* rs738409 genotype, aspartate aminotransferase (AST) and fasting insulin found NASH with a sensitivity of 72% and specificity of 74%. Next, the score was validated in an Italian population of 380 obese individuals. Finally, in the Finnish D2D population study the score estimated the prevalence of 5% for NASH in Finnish adult individuals, when used. Cytokeratin 18 (CK-18) has been stated to be the best biomarker for NASH. CK-18 found NASH rather well also in this study, but it did not improve the predictive value of the score. The potential of CK-18 as a marker of intervention effect in the liver was further tested in HEPFAT dietary intervention study. The value of CK-18 in predicting the effect of intervention in the liver was modest.

Different lipid subclasses in lipoproteins and low molecular weight molecules were measured with nuclear magnetic resonance (NMR) spectroscopy in a population of 116 massively obese individuals. Total cholesterol concentration of large, medium and small VLDL, and large and medium LDL was associated with liver inflammation independently of steatosis. Ketone bodies were lower in those with NASH than in those with fatty liver. This suggests lower lipid oxidation and decreased mitochondrial function in those with NASH.

The non-invasive score formed in this thesis could be used as a screening method of NASH together with serum CK-18 measurement. Awareness of the prevalence of NASH should put more focus on prevention and treatment of NAFLD in Finland. The association of cholesterol metabolism with NASH suggests that cholesterol metabolism should be the target of treatment in NASH. Finally, decreased lipid oxidation and increased ketolysis in those with NASH were observed. In summary, this thesis indicates that common NASH is associated with multiple alterations in lipid metabolism.

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TIIVISTELMÄ:

Lihavuus ja tyypin 2 diabetes ovat suuria terveysongelmia maailmanlaajuisesti. Niillä on läheinen yhteys ei-alkoholiperäiseen rasvamaksatautiin (NAFLD). Jopa 20-30 %:lla väestöstä on ei-alkoholiperäinen rasvamaksa (NAFL), joka on tautikirjon pääasiallinen esiintymismuoto. On epäselvää, miksi osalla tämä tauti etenee ei-alkoholiperäiseksi maksatulehdukseksi (NASH), joka voi johtaa jopa maksakirroosiin. NAFLD lisää maksaperäistä sairastavuutta ja kuolleisuutta, mutta myös sydän- ja verenkiertoelimistön sairauksia ja syöpiä. NASH:n todellista esiintyvyyttä ei tiedetä, koska sen diagnosointiin vaaditaan maksakoepalan otto. Ei-kajoava diagnostinen menetelmä auttaisi paitsi taudin toteamisessa, myös väestötutkimuksissa, joissa maksakoepaloja ei voida ottaa. Tämän takia on kehitetty erilaisia ei-kajoavia diagnoosikeinoja. NASH:n uusia merkkiaineita voisi löytyä taudin patogeneesia tutkimalla. Esimerkiksi kolesteroli- ja rasva-aineenvaihdunnan muutosten ajatellaan liittyvän NASH:n kehittymiseen.

Tässä väitöskirjatyössä muodostettiin NASH:a ennustava yhtälö 296 lihavan henkilön kohortissa, jossa olennaiset NASH:iin liittyvät määritykset olivat saatavilla. Yhtälö koostui *PNPLA3* rs738409 genotyypistä, aspartaattiaminotransferaasista (ASAT) ja paastoinsuliinipitoisuudesta ja löysi NASH:n 72 % herkkyydellä ja 74 % tarkkuudella. Yhtälö toimi yhtä hyvin 380 italialaisen kohortissa. Yhtälön avulla arvioitiin NASH:n esiintyvyydeksi suomalaisilla aikuisilla n. 5 % D2D-väestötutkimuksessa. Sytokeratiini-18 (CK-18) ei lisännyt yhtälön tarkkuutta, vaikka se on arvioitu parhaaksi NASH-merkkiaineeksi. CK-18:n hyötyä interventioiden maksavaikutuksen merkkiaineena tutkittiin HEPFAT-dieetti-interventiotutkimuksessa, jossa CK-18:n hyöty intervention maksavaikutuksen mittaamissa oli heikko.

Ydinmagneettiseen resonanssiin perustuvaa spektroskopiaa (NMR) käytettiin mittaamaan lipoproteiini-alaluokkien erilaiset lipidit ja pienen molekyylipainon omaavat molekyylit 116:lta sairaalloisesti lihavalta henkilöltä. Kokonaiskolesterolimäärä suurissa, keskisuurissa ja pienissä VLDL:ssa sekä suurissa ja keskisuurissa LDL:ssa olivat yhteydessä maksatulehdukseen maksan rasvoittumisesta riippumatta. Ketoainetasot olivat matalammat henkilöillä, joilla oli NASH verrattuna niihin, joilla oli pelkkä rasvamaksa. Tämä viittaa alentuneeseen lipidien hapettumiseen ja mitokondrioiden toimintahäiriöön niillä, joilla on NASH.

Tässä väitöskirjatyössä muodostettua ennusteyhtälöä voidaan käyttää NASH:n seulontaan yhdessä CK-18:a kanssa. NASH:n yleisyyden takia sen ehkäisemiseen ja hoitamiseen tulisi kiinnittää enemmän huomiota. NASH:iin liittyvien kolesteroli-aineenvaihdunnan muutosten tulisi olla taudin hoitokohteita. Lisäksi tässä työssä todettiin vähentynyt lipidien hapetus ja lisääntynyt ketoaineiden hajoaminen niillä, joilla on NASH. Tämä väitöskirjatyö osoittaa, että yleiseen NASH-sairauteen liittyy monenlaisia rasva-aineenvaihdunnan muutoksia.

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Yleinen Suomalainen Asiasanasto: rasvamaksa; maksatulehdus; lihavuus; aineenvaihdunta; diagnostiikka, kolesteroli; lipidit; lipoproteiinit; merkkiaineet

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Kuopio, November 2015

Ville Männistö

List of the original publications

This dissertation is based on the following original publications:

I Hyysalo J*, Männistö VT*, Zhou Y, Arola J, Kärjä V, Leivonen M, Juuti A, Jaser N, Lallukka S, Käkelä P, Venesmaa S, Simonen M, Saltevo J, Moilanen L, Korpi-Hyövalti E, Keinänen-Kiukaanniemi S, Oksa H, Orho-Melander M, Valenti L, Fargion S, Pihlajamäki J, Peltonen M, Yki-Järvinen H. A population-based study on the prevalence of NASH using scores validated against liver histology. *J Hepatol. 2014 Apr;60(4):839-46*.

II Männistö VT, Walle P, Simonen M, Kärjä V, Heikkinen M, Bjermo H, Iggman D, Kullberg J, Tuomilehto H, Risérus U, Pihlajamäki J. Serum cytokeratin-18 as a marker of NASH in interventions. *Submitted*

III Männistö VT, Simonen M, Soininen P, Tiainen M, Kangas AJ, Kaminska D, Venesmaa S, Käkelä P, Kärjä V, Gylling H, Ala-Korpela M, Pihlajamäki J. Lipoprotein subclass metabolism in nonalcoholic steatohepatitis. *J Lipid Res. 2014 Dec;55(12):2676-84.*

IV Männistö VT, Simonen M, Hyysalo J, Soininen P, Kangas AJ, Kaminska D, Matte AK, Venesmaa S, Käkelä P, Kärjä V, Arola J, Gylling H, Cederberg H, Kuusisto J, Laakso M, Yki-Järvinen H, Ala-Korpela M, Pihlajamäki J. Ketone body production is differentially altered in steatosis and non-alcoholic steatohepatitis in obese humans. *Liver Int. 2014 Dec 22*. doi: 10.1111/liv.12769.

* Equal contribution

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Abbreviations

AA	Acetoacetate
AASLD	American Association for the Study of Liver Diseases
ABCA1	ATP-binding cassette transporter A1
ABCG5/8	ATP-binding cassette transporters G5 and G8
ACAT	Acyl CoA-cholesterol acyltransferase
ACC	Acetyl CoA carboxylase
ACSS2	Acyl-CoA synthetase short-chain family member 2
Acyl-CoA	Acetyl coenzyme A
AGA	American Gastroenterological Association
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
AUROC	Area under the ROC curve
BDH1	β -hydroxybutyrate dehydrogenase, type 1
β -OHB	β-hydroxybuturate
BMI	Body mass index
CD-36	Fatty acid translocase
CE	Cholesterol ester
ChREBP	Carbohydrate-responsive element-binding protein
CI	Confidence interval
CK-18	Cytokeratin-18
CT	Computed tomography
DGAT2	Diacylglycerol O-acyltransferase 2
ER	Endoplasmic reticulum
FASN	Fatty acid synthase
FDA	The Food and Drug Administration
FDR	False discovery rate
FFA	Free fatty acid
FXR	Farnesoid X receptor
GB	Gastric banding
GCKR	Glucokinase regulatory
GLC	Gas-liquid chromatography
GLP-1	Glucagon-like peptide-1
GWAS	Genome-wide association study
HCC	Hepatocellular carcinoma
HMGCR	HMG-CoA reductase
HMGS	Hydroxymethylglutaryl-CoA synthase
IDF	International Diabetes Federation
IL-1β	Interleukin-1 beta
IR	Insulin resistance
IRE1α	Inositol requiring enzyme 1 alpha
JNK	c-Jun N-terminal kinase
KOBS	Kuopio Obesity Surgery Study
LDLR	LDL-receptor
LMWM	Low-molecular weight molecules
LXR	Liver X receptor
LYPLAL1	Lysophospholipase-like 1
METSIM	Metabolic Syndrome in Men
MRI	Magnetic resonance imaging
mRNA	messenger RNA
MRS	Magnetic resonance spectroscopy
MTTP	Microsomal triglyceride transfer protein
NAFL	Non-alcoholic fatty liver
NAFLD	Non-alcoholic fatty liver disease
NAS	NAFLD activity score
NASH	Non-alcoholic steatohepatitis

NCAN	Neurocan
NCEH	Neutral cholesterol ester hydrolase
NEFAs	Non-esterified fatty acids
NMR	Nuclear magnetic resonance
NPC1	Niemann-Pick disease, type C1
NPC1L1	Niemann-Pick C1-like 1
NPV	Negative predictive value
OR	Odds ratio
PCSK9	Proprotein convertase subtilisin/kexin type 9
PNPLA3	Patatin-like phospholipase domain-containing protein 3
PPP1R3B	Protein phosphatase-1 regulatory subunit 3b
PPV	Positive predictive value
PUFA	polyunsaturated fatty acid
ROC	Receiver operator curve
ROS	Reactive oxygen species
RQ	Respiratory quotient
RYGB	Roux-en-Y gastric bypass
SCD1	Stearoyl-CoA desaturase
SD	Standard deviation
SFA	Saturated fatty acid
SNP	Single nucleotide polymorphism
SOD2	Superoxide dismutase 2
SREBP	Sterol regulatory binding protein
TCA	Tricarboxylic acid cycle
TG	Triglyceride
TLR	Toll-like receptor
TM6SF2	Transmembrane 6 superfamily member 2
TNF-α	Tumor necrosis factor alpha
UCP2	Uncoupling protein 2
UPR	Unfolded protein response
US	Ultrasound
VLCD	Very low calorie diet
XBP1	X-box binding protein 1

1 Introduction

The most common liver disease in the Western world, non-alcoholic fatty liver disease (NAFLD), associates strongly with obesity (1,2). NAFLD presents primarily as non-alcoholic fatty liver (NAFL; fatty liver, simple steatosis), which has been described as a phenotype of the metabolic syndrome in the liver (3). Worldwide 20-30% of individuals have simple steatosis (4), which can progress to non-alcoholic steatohepatitis (NASH) in some subjects (5). Development of scar tissue (fibrosis) may occur in NASH (6), further leading to liver cirrhosis and ultimately to end stage liver disease and death (7). NAFLD increases the risk of cardiovascular complications and death (8,9). Furthermore, both simple steatosis (10) and NASH cirrhosis (11) increases the risk of liver cell carcinoma.

The major unanswered question is why some individuals with fatty liver will develop steatohepatitis. A multiple hits theory has been suggested (12). Cholesterol synthesis is proposed to be involved in the pathogenesis of simple steatosis (13) and NASH (14). Furthermore, mitochondrial dysfunction (15,16) and endoplasmic reticulum (ER) stress (17,18) have been suggested to contribute to the pathogenesis of NASH. Lipid oxidation takes mainly place in the mitochondria (19), and impaired lipid oxidation could lead to ER stress (20). However, controversial results about lipid oxidation in individuals with NAFLD and NASH have been published (16,21-24).

The diagnosis of NASH requires a liver biopsy (6). Thus, a biomarker or a non-invasive score predicting NASH would be very useful. It could be used in clinical practice to find those at risk for NASH and would be beneficial in population-based studies, where liver biopsy is not possible to obtain.

In the present study a non-invasive score predicting NASH was developed, and the prevalence of NASH in Finnish adults was estimated. In addition, serum CK-18 was tested as a marker of NASH in obesity surgery and dietary intervention studies. The metabolic changes in individuals with NASH were investigated with focus on the changes in lipid metabolism. The following literature review summarizes the scientific literature of NAFLD with the main emphasis on the pathogenesis of the disease, the changes in lipid metabolism and the diagnostic challenges of the disease.

2 Review of literature

2.1 OBESITY AND METABOLIC SYNDROME

2.1.1 Obesity

Overweight is defined as body mass index (BMI defined as weight in kilograms divided by the square of the height in meters) equal to or over 25 kg/m^2, and obesity as equal to or over 30 kg/m^2 (25). Obesity is defined as abnormal or excess fat accumulation, which may cause problems to individual´s health (26). In the last five years, average BMI has increased in the vast majority of countries around the World (27) (Figure 1). Worldwide over one billion people are overweight (28) and in the USA 70% of adults are overweight and 36% of those are obese (29). In Europe over 50% of adults are now either overweight or obese (30). Obesity is a serious problem in developed countries (28), but it is also a major health challenge in many middle-income countries and its prevalence is increasing also in developing countries (31,32). Actually, most of the world population lives in countries where obesity causes more deaths than underweight (33).

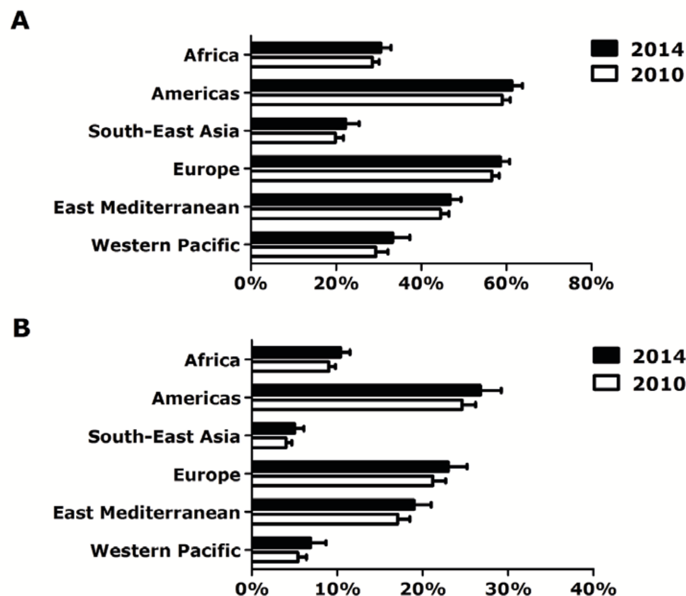

Figure 1. The prevalence of (A) overweighted and (B) obese individuals in different parts of the World in the years 2010 and 2014. Adapted from (34).

Obesity causes many problems. It increases the overall mortality at least with BMI ≥ 35 kg/m^2 when comparing to those with BMI < 25 kg/m^2 and those with BMI 25-30 kg/m^2 (35). Furthermore, obesity is risk factor for many diseases such as type 2 diabetes (36), arterial hypertension, and cardiovascular diseases (37) (including coronary artery disease (38) and stroke (39)), arthrosis (40), gout (41), and cancer (42). In fact, it has been estimated that overweight and obesity account for approximately 20% of all cancers (42).

2.1.2 Metabolic consequences of obesity

Obesity is closely linked to metabolic syndrome, which is also known as insulin resistance (IR) syndrome (43,44). Metabolic syndrome has been described with at least six different criteria (45). The most recent definition of the syndrome requires at least any three of the following five components (46): 1) Increased fasting plasma glucose (≥ 5.6 mmol/l) or type 2 diabetes, 2) hypertriglyceridemia (> 1.7 mmol/L), 3) low HDL cholesterol (< 1.0 mmol/L for males and < 1.3 mmol/L for females), 4) increased waist circumference (≥ 94 cm for males and ≥ 80 cm for females for Caucasians) and 5) hypertension (≥ 130 mmHg for systolic or ≥ 85 mmHg for diastolic blood pressure), or antihypertensive drug treatment, or history of hypertension.

However, some obese individuals can be metabolically healthy (47,48), and some lean individuals can have metabolic syndrome (49,50). It has been estimated that as much as 30% of obese people are metabolically healthy predominantly because of preserved insulin sensitivity (47,50). These individuals are suggested to have more subcutaneous fat but less liver fat than unhealthy obese individuals (48). It has been suggested that metabolically healthy individuals do not have increased cardiovascular mortality (51), although this has been criticized. A meta-analysis evaluated all-cause mortality and cardiovascular events in over 61000 individuals finding that also obese subjects with no metabolic abnormalities were at increased risk for adverse long-term outcomes. (52) A problem with studies about metabolically healthy obesity is that they have had varied criteria for the definition of the entity (48,53).

2.1.3 Obesity surgery

Obesity surgery has rapidly become a more frequent option in the treatment of severe obesity. It can induce massive and sustained weight loss of more than 30% two years after the operation (54), and sustained weight loss of more than 20% weight loss 20 years after the operation (55). European guidelines for the criteria of the surgery are 1) BMI ≥ 40 kg/m^2 or 2) BMI 35-40 kg/m^2 and comorbidities like type 2 diabetes, cardiorespiratory disease or severe joint disease. BMI for the evaluation may be the current or previous maximum BMI. (56) Other guidelines such as those from U.S. National Institutes of Health (NIH) (57) and American College of Cardiology (58) have similar criteria. The Food and Drug Administration (FDA) has approved gastric banding (GB) as a treatment option in subjects with BMI > 30 and at least one obesity associated disease (57). The International Diabetes Federation (IDF) suggests that if subject has BMI 30-35 and diabetes cannot be adequately controlled by optimal medication, surgery could be considered (59).

Nowadays, most widely used operation technique is Roux-en-Y gastric bypass (RYGB) followed by sleeve gastrectomy and adjustable gastric banding (60). Recently, sleeve gastrectomy is becoming the most used procedure in the USA (61). In RYGB, a small ventricle pouch is made and part of small bowel is by-passed with alimentary loop. Sleeve gastrectomy is based on a technique that shrinks the ventricle loop. (Figure 2) RYGB induces more weight loss (approximately 25% more) than less invasive purely restrictive GB (7,62). Sleeve gastrectomy brings about successful weight loss, although it is maybe not as effective as RYGB (63)

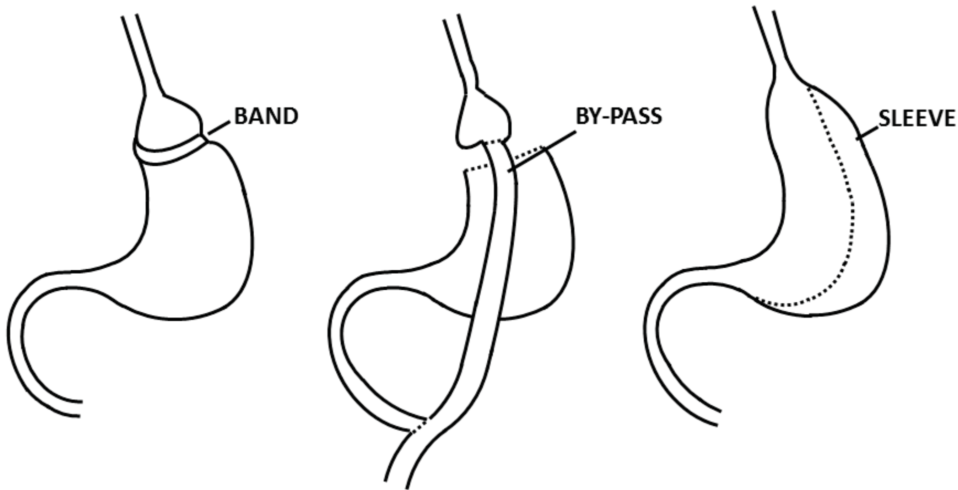

Figure 2. Most widely used methods for obesity surgery.

RYGB also causes rapidly efficient improvement in plasma glucose, which is thought to result from the stimulation of insulin secretion by increased levels of gut peptides; especially postprandial levels of glucagon-like peptide-1 (GLP-1) (64,65). Increased levels of GLP-1 together with weight loss are considered as main reasons for the resolution of type 2 diabetes (65). In fact, RYGB ameliorates type 2 diabetes in approximately 60% of patients (64,66). Sleeve gastrectomy has metabolic effects similar to RYGB. However, RYGB might provide better glycemic control and more sustained resolution of type 2 diabetes. (63) In addition, obesity surgery lowers mortality after the operation (67,68), and has also an effect on NASH (69,70-74).

Common complications after obesity surgery are bleeding, surgical site infection, deep venous thrombosis, line leakage and nutritional deficiencies such as B12 vitamin deficiency (75). Minor early complications are more frequent in those operated with RYGB compared to those having sleeve gastrectomy. No difference in major complications has been noted. (76) Up to 50% of patients operated with adjustable GB can have a complication reqiring re-operation. This is accompanied with a 29% band loss rate. (77)

2.2 NON-ALCOHOLIC FATTY LIVER DISEASE (NAFLD)

2.2.1 Definition and natural course of the disease

NAFLD is the most common reason for chronic liver disease in the Western world (5). NAFLD can present as liver steatosis i.e. NAFL, where triglyceride (TG) content is over 5-10% in the liver (78). Some individuals will develop inflammatory state described as NASH. Importantly, in NAFLD alcohol consumption (defined in European and American guidelines) is < 20g of alcohol daily for women and < 30g for men (78). When diagnosing NASH, other common causes for liver disease need to be excluded. Usually laboratory tests are used to exclude at least viral hepatitis B and C, autoimmune hepatitis and hemochromatosis (78,79).

Table 1. Nomenclature associated with non-alcoholic fatty liver disease. Adapted from (78).

Non-alcoholic fatty liver disease	NAFLD	- The whole spectrum of the fatty liver disease from simple steatosis to steatohepatitis and cirrhosis - No significant alcohol consumption (women < 20g/day, men < 30g/day)
Non-alcoholic fatty liver	NAFL	- Hepatic steatosis (simple steatosis, fatty liver) without hepatocellular injury (ballooning), inflammation and fibrosis
Non-alcoholic steatohepatitis	NASH	- Hepatic steatosis with hepatocellular injury (ballooning) - Fibrosis may be present
NASH cirrhosis		- Liver cirrhosis - Previous or current (histological) evidence about NAFLD
Cryptogenic cirrhosis		- Presence of cirrhosis without known etiology, metabolic risk factors may be present
NAFLD activity score	NAS	- An unweighted sum of steatosis, inflammation and ballooning scores in histological analysis - Developed for measuring changes in liver histology in clinical trials

Approximately 10-20% of those with simple steatosis have also NASH (4,8). Approximately 2-3% of patients with steatosis develop NASH during five year follow-up, and up to 8% of those with NASH will develop liver cirrhosis in five years. (4,8) Fibrosis has been thought to develop in individuals with NASH, but not in those with fatty liver (7,80-82). However, lately it has been suggested that liver fibrosis can occur similarly both in simple steatosis and in NASH (83). In that study, approximately 40% of individuals had progression in fibrosis, but another 40% had not. The presence of type 2 diabetes at baseline was risk factor for fibrosis progression. However, the definition of steatosis included also steatosis with mild inflammation. (83) This could explain results of fibrosis progression, because mild inflammation can be a sign of early NASH. Interestingly, liver fibrosis can also decrease during follow-up. Hamaguchi et al. reported that liver fibrosis improved in 31%, progressed in 28% and remained unchanged in 41% during a median follow-up of 2.4 years (84). Similar results are reported also by Wong et al. (85).

Both obesity (86) and diabetes (87) are known risk factors for hepatocellular carcinoma (HCC). Furthermore, NAFLD also increases the risk of HCC. It is suggested to be underlying reason in 35% of HCC cases making it the most common liver disease associated with HCC (88). Usually, HCC develops in the cirrhotic liver with the yearly risk of 2-3% (11). Alarming finding is that HCC might develop even directly from the fatty liver without previous hepatitis or fibrosis (10).

2.2.2 Prevalence of NAFLD and NASH

One third of the population in the USA has NAFLD (89). In the many parts of the world, the population prevalence is estimated to be at least 20%. In Europe, ultrasound (US)

studies have suggested a prevalence of 20-33% (90-95) and 35% of those over 70 years (96). In histological post mortem analysis, fatty liver was diagnosed in 31% of individuals (n=498, age 3-94 years) in Greece (94). In other parts of the world, the prevalence has been estimated to be 19-35% in South America (97,98), 13-17% in China (99,100), 32% in India (101,102), 29% in Japan (103,104) and 19-33% in other parts of Asia (105-107). NAFLD is much more common in those with type 2 diabetes, with prevalence of 42-94% (108-110). In severely obese patients a prevalence of 85-98% has been reported based on the liver biopsy (1,111-113).

The current consensus about the prevalence of NASH is based on the selected groups of patients, who have been biopsied for different reasons. The prevalence of NASH has been 16% in individuals with elevated transaminases with an unknown etiology (114), and 30% in those with fatty liver based on US examination (110,115). NASH was found in 40% of individuals examined post mortem in Greece (94). This population had mean age of 65 years, and half of them died of coronary artery disease (94). In addition, in liver donors the prevalence of NASH has estimated to be 15% (116). In those evaluated for liver transplantation, the prevalence of NASH increased from 1.2% to 9.7% in ten years (117,118). Based on these studies, the prevalence of NASH has been suggested to be 1-16%.

In obese and diabetic subjects the prevalence of NASH is higher. A prevalence of 62-80% in type 2 diabetics has been reported (119,120). However, in these studies only those with fatty liver in the US examination were biopsied. Thus, the prevalence could be even higher, because of limitations of the US (121). In patients undergoing obesity surgery, the prevalence estimates have ranged from 23 to 56% (112,113,122-124).

2.2.3 NAFLD and morbidity and mortality

NAFLD increases both morbidity and mortality. It increases the risk of chronic renal disease (125) and cardiovascular diseases such as atherosclerosis (126), stroke (127) and atrial fibrillation (128). NASH increases liver-related mortality five to sixfold (129,130) when comparing to those without NASH. The most common reasons for death are liver-related diseases and cardiovascular diseases (9,131). In fact, NAFLD is already the third most common cause for liver transplantation in individuals over 65 years old, and is suggested to be the most common in the near future (132). Recent publication with 33 year follow-up reported that NASH increases risk of death from cardiovascular disease, hepatocellular carcinoma, infectious diseases and cirrhosis (9). Previous studies have reported that the occurrence of deaths due to cardiovascular disease, liver disease and malignity are almost equal (8,131). However, Ekstedt et al. found cardiovascular diseases to be a clearly more common cause of death in those with NASH, followed by non-gastrointestinal malignancies, HCC and infections. Interestingly, those who had mild fibrosis (0-2) at baseline were not at an increased risk of death. (9)

2.2.4 Risk factors for NAFLD

Obesity and physical inactivity. Obesity is the strongest risk factor for NAFLD (1,2), although NAFLD can be sometimes present also in lean individuals (133). Physical inactivity is suggested to be a risk factor based on the finding that exercise decreases liver fat content independently of weight change (134,135).

Metabolic syndrome and type 2 diabetes. Liver steatosis is closely related to components of the metabolic syndrome (1,43,89,136,137). In fact, steatosis can be considered a hepatic

manifestation of metabolic syndrome (44). Furthermore, steatosis has been suggested to be the most accurate marker of metabolic syndrome, because it is the cause for many metabolic components of the metabolic syndrome (79). Type 2 diabetes is a strong predictor of NAFLD (108,109). In fact, NAFLD has an association with IR and type 2 diabetes independent of obesity (43,138) suggesting that fat accumulation in the liver is essential in the development of type 2 diabetes. Finally, lean individuals with NAFLD are also at increased risk for diabetes compared to lean controls (133), which highlights the crucial role of the liver in glucose metabolism.

Dietary factors. Excess intake of energy is a risk for NAFLD (139). In addition, the quality of diet also affects the risk. A high fat diet increases and low fat diet decreases liver fat (140). In addition, excess consumption of carbohydrates is associated with NAFLD (141). Higher intake of soft drinks and meat is associated with NAFLD independently of age, gender, BMI and total calories. Moreover, a tendency towards lower intake of fish (rich in omega-3 fatty acids) has been suggested. (142) Lately, the role of simple sugar intake has been discussed actively. High simple sugar intake and especially fructose intake has been associated with fatty liver in animal studies (143,144), with type 2 diabetes in humans (145), and with liver fibrosis in humans with NASH (146). However, a recent meta-analysis suggested that harmfulness of fructose might be simply because of excess energy intake (147). Furthermore, a high glucose diet is known to increase cholesterol synthesis more than a high fructose diet (148). On the other hand, there are studies reporting that fructose intake has an inverse association with the development of fatty liver (146,149). However, in these two studies diet was assessed with questionnaires, and intake of fructose was from both fruits and sugar-sweetened products (146,149). In the study by Kanerva et al. fruits were a major source of fructose for study subjects, which could overcome the possible harmful effect of added fructose.

Age, gender and race. Higher age is associated with the risk of NAFLD (97,105). However, older people often have more risk factors for fatty liver (150). Some reports suggest that NAFLD is more common in females (122,151,152), but based on newer studies NAFLD is more frequent in males (91,105,115,153). However, in lean individuals, NAFLD is more common in females (154).

There is racial difference in the prevalence of NAFLD. The disease is most frequent in East Asian Indians (155) and in Hispanics (89). African Americans are reported to less often have fatty liver (89). These findings were confirmed in a study with over 9000 individuals in the USA (156). Racial differences could be partly explained by differences in genetics such as in the frequencies of the known risk genotypes, e.g. Patatin-like phospholipase domain-containing protein 3 (*PNPLA3*) rs738409 genotype (157).

2.2.5 Genetics of NAFLD

A population based genome-wide association study (GWAS) suggested that 27% of hepatic steatosis is explained by genetic factors (158). *PNPLA3* (adiponutrin) gene was associated with NAFLD in year 2008 (157). Romeo et al. found single nucleotide polymorphism (SNP) variant at rs738409 representing a substitution from cytosine to guanine, which results in a switch from isoleucine to methionine at residue 148 (I148M). This polymorphism is associated with increased liver fat content and aminotransferase levels, but not with BMI and type 2 diabetes (157). Interestingly, individuals with NAFLD who have *PNPLA3* rs738409 variant have fewer metabolic abnormalities than those with “common NAFLD”

(79,159). *PNPLA3* is expressed predominantly in the liver and retina in humans (160), but not in adipose tissue as in mice (161). Wild type form of *PNPLA3* hydrolyses TGs and the I148M substitution abolishes this activity (162,163) explaining the role of *PNPLA3* I148M variant in increasing liver fat content and the risk of NASH (79).

Prevalence of *PNPLA3* rs738409 heterozygous gene variant is 35-40% and homozygous variant approximately 5% in Western populations (164). After finding the association of *PNPLA3* rs738409 with NAFLD, the genotype has been associated with simple steatosis and NASH in many studies. Based on the meta-analysis with 16 studies, homozygous variant carriers have 73% higher liver fat content than non-carriers. Those with risk variant have also greater risk for liver inflammation and fibrosis. (165) Additionally, *PNPLA3* rs738409 gene variant is associated with increased risk of HCC in those with NAFLD (166), probably because it causes more aggressive steatohepatitis and more often fibrosis (167).

The transmembrane 6 superfamily member 2 (*TM6SF2*) gene variant at rs58542926 has also been associated with NAFLD in a GWAS (168). This variant is an adenine for guanine substitution encoding nucleotide 499, which replaces glutamate at residue 167 with lysine (E167K) (168). E167K leads to higher hepatic TG content, elevated serum aminotransferases, and lower lipid content in serum lipoproteins. (168-170) Prevalence estimate of this variant is 7% in Europeans, 3% in Africans, and 5% in Hispanic Americans (168). In a recent study, the *TM6SF2* rs58542926 genotype was associated with increased risk of NASH. However, because this gene variant causes lipid accumulation in the liver, serum lipoprotein lipid levels are lower and variant carriers have lower cardiovascular morbidity. (171)

Other genotypes. Other gene variants have been associated with NAFLD in GWAS and confirmed in case-control studies. Neurocan (*NCAN*) variant at rs2228603 has been associated with steatosis, lobular inflammation and fibrosis (158,172). However, *TM6SF2* variant is most likely the causal variant in the same locus with *NCAN* (168). The glucokinase regulatory gene (*GCKR*) has an essential role in regulating glucose level balance (173). Variant rs780094 in *GCKR* has been associated with NAFLD, NASH and fibrosis (158). Variant rs12137855 near the lysophospholipase-like 1 (*LYPLAL1*) gene has been associated with NASH (158), and rs4240624 near protein phosphatase-1 regulatory subunit 3b (*PPP1R3B*) has been associated with steatosis (158).

Various other genes have also been suggested to associate with NAFLD based on case-control studies, but their association with the disease needs validation. For example, manganese superoxide dismutase is a mitochondrial enzyme taking part in detoxication of reactive oxygen species (ROS). This is encoded by superoxide dismutase 2 (*SOD2*). Lower hepatic levels of manganese superoxide dismutase have been associated with NASH (174). However, this was a rat study, and the finding could also result from decreased mitochondrial function in NASH. Uncoupling protein 2 (UCP2) is a regulator of mitochondrial lipid flux and also a regulator of ROS production by the respiratory chain. Recently, *UCP2* -866 A/A genotype was associated with decreased risk of higher grade steatosis and NASH (175).

2.2.6 Pathogenesis of simple steatosis

In simple steatosis lipids in hepatocytes are mainly TGs that are synthesized from free fatty acids (FFA) (176) (Figure 3). Three major sources providing free fatty acids to the liver are: 1) Most (up to 60%) are from plasma non-esterified fatty acids (NEFAs), deriving mainly from lipolysis in adipose tissue. When the storage capacity of adipose tissue is exceeded,

serum FFAs will increase (Figure 3). In addition, in IR syndrome insulin does not suppress adipose tissue lipolysis enough (13), increasing the influx. Fatty liver also further induces IR causing a vicious cycle (177,178). 2) 25% of fatty acids are normally from *de novo* lipogenesis in the liver (13), which is increased threefold in NAFLD (179) (Figure 3). 3) 15% of fatty acids derive from dietary fatty acids, which are absorbed from the gut. They are then transported in chylomicrons (without esterification) via lymphatic system and vessels, and finally chylomicron remnants are taken into the liver. The fatty acid amount deriving from the gut increases after a high-fat diet. (13)

In the liver fatty acids can have three different destinations. First, they can be oxidized (i.e. β-oxidation) for energy, which mainly takes part in liver cell mitochondria, but in a smaller amount in peroxisomes. Second, fatty acids can be packed as TGs and secreted in very low-density lipoproteins (VLDL) from the liver. Third, formed TGs can be stored in the liver as lipid droplets. (136) (Figure 3). Because TGs are formed from FFAs, liver steatosis can be thought as a protective mechanism from FFA caused lipotoxicity (180).

2.2.7 Pathogenesis of NASH

Although the pathogenesis of simple steatosis is well known, it is not clear why some individuals with simple steatosis will develop NASH. Previously, a two hit hypothesis was used to describe the development of NASH (181). Lipid accumulation in the liver has been considered as a key element for the pathogenesis of steatosis and being the "first hit" of the NAFLD. A "second hit" could be for example lipotoxicity (180,182), mitochondrial dysfunction (16), different adipocytokines (183,184), endoplasmic reticulum (ER) stress (185), and bacterial endotoxins (186) (Figure 3).

It is unclear why lipid accumulation is harmful only for some individuals, whereas others will never develop NASH. Thus, another yet unclearly understood mechanism is needed for development of NASH. Because not just one obvious hit has been found to cause NASH, the concept of "first and second hit" has been moved towards "multiple hits" theory. It has also been suggested that simple steatosis is a benign process for majority of individuals, and NASH could be a separate disease (12). However, this theory is challenged by recent findings of fibrosis progression in the liver of those with simple steatosis (83).

Whatever is the second hit, Kupffer cells and hepatic stellate cells are important mediators of the cell injury in NASH. Kupffer cells are resident macrophages that can detect damage messages from injured cells and trigger an inflammatory response with inflammasome activation. (187,188) Mice studies suggest that Toll-like receptor (TLR) activation promotes Kupffer cells to secrete cytokines such as IL-1β (189) and TNF-α (190). Furthermore, increased Kuppfer cell content and TNF-α level have been reported also in the liver of humans with NASH (191). Stellate cells are activated in the fibrinogenesis in NASH (192), in which also Kupffer cells are suggested to participate (193). Stellate cells also secrete cytokines and growth factors, which contribute to scar tissue formation in fibrinogenesis (187).

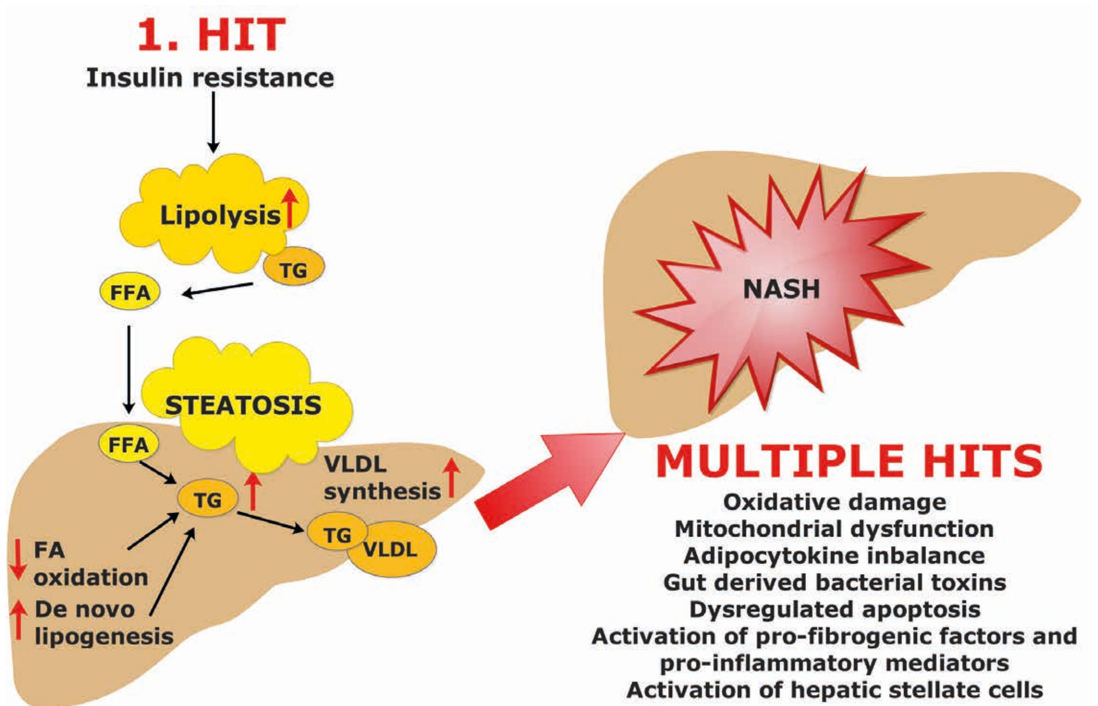

Figure 3. The multiple hit hypothesis in the pathogenesis of NAFLD. Adapted from (194).

Impaired lipid oxidation. One possible reason for the development of NASH is impaired lipid oxidation. Each hepatocyte has about 800 mitochondria, which are mainly responsible for lipid oxidation and energy production (19), although a smaller amount of lipid oxidation takes place in peroxisomes (195). In mitochondria, fatty acids and glycosylated sugars are oxidized (through β-oxidation) to produce acetyl coenzyme A (Acyl-CoA), which enters citric acid cycle a.k.a tricarboxylic acid cycle (TCA) (Figure 4). Decreased FFA influx in the postprandial state decreases fatty acid oxidation in mitochondria. Insulin and glucose also favour *de novo* lipogenesis regulating the entry of fatty acids inside of the mitochondria. (196) When in fasting state or during physical activity blood glucose level decreases, fatty acids are oxidized. β-oxidation then produces the ketone bodies acetoacetate (AA) and β-hydroxybuturate (β-OHB), which thus reflect the rate of lipid oxidation. Ketone bodies provide fuel to organs (especially to the brain) (195).

Impaired lipid oxidation has been reported in those with NAFLD and NASH in some, but not in all studies. Low (21), high (16,22,23) and unaltered (24) rate of lipid oxidation has been reported based on ketone body levels (Table 2). Impaired lipid oxidation has been thought to be caused by abnormal liver cell mitochondrial function (15) or morphology (16). Recently, it was found out that obese individuals with or without simple steatosis were found to have higher mitochondrial function rates than lean individuals even though the liver mitochondrial mass was similar. However, those with NASH had higher mitochondrial mass, but 31-40% lower maximal respiratory function than in obese subjects without or with simple steatosis. This suggests that mitochondria can adapt their function in the early stages of obesity and fatty liver, but this is lost in those with NASH. (20)

Table 2. Lipid oxidation in NAFLD based on the β-OHB measurement.

Name	**Year**	**Patients N**	**Liver biopsies**	**Hepatic lipid oxidation**	**Reference**
Sanyal et al.	2001	18	Yes	Increased in NASH vs. normal liver	(16)
Chalasani et al.	2003	37	Yes	Increased in NASH vs. controls	(23)
Bugianesi et al.	2005	18	Yes	Increased in NAFLD vs. controls	(22)
Kotronen et al.	2009	58	No	Unaltered in NAFLD vs. controls	(24)
Croci et al.	2012	35	Yes	Decreased in NAFLD vs. controls	(21)

Endoplasmic reticulum stress. Lipid accumulation and altered lipid oxidation can disturb ER function. ER is a membranous organelle that has important functions such as folding and modification of proteins, synthesis of phospholipids and a function as an enzyme such as cytochrome P450 (197,198). Both obesity (199) and hepatic steatosis (17) are known to induce ER stress in the liver. Interestingly, ER stress can induce hepatic steatosis (200) and inhibit VLDL secretion in experimental models (201), leading to lipid accumulation and more ER stress. Thus, ER stress is both a cause and consequence for lipid accumulation in the liver.

When ER stress is present, the unfolded protein response (UPR) pathway is activated, aiming to restore homeostasis and reduce the transfer of proteins into ER lumen (202). The UPR pathway also has an important role in hepatic lipid homeostasis (203). The UPR pathway is activated both in steatosis and NASH (204). When the pathway cannot restore normal ER balance, it induces apoptosis. (182) Inositol requiring enzyme 1 alpha (IRE1α) is one of the three ER transmembrane receptors (182). The IRE1α pathway takes part in ER stress-induced apoptosis. It can also activate c-Jun N-terminal kinase (JNK) (205,206), which promotes inflammation and apoptosis in the liver (182). IRE1α also links to lipid metabolism via activating X-box binding protein 1 (XBP1), which can directly activate key lipogenic genes (203). When XBP1 is suppressed, *de novo* lipogenesis is decreased (207). Active spliced XBP1 protein is decreased in individuals with NASH (204), suggesting a link between lipid metabolism and ER stress.

2.2.8 Lipid metabolism in NAFLD

Lipid metabolism has a key role in the pathogenesis of NASH. As described above (2.2.6), increased FFA influx and lipogenesis in the liver may contribute to NAFLD (13). Increased FFA influx and lipogenesis have also been associated with NASH (14,208,209). Additionally, cholesterol synthesis is increased in those with NAFLD (210,211) and NASH (212). Furthermore, secretion of VLDL has been reported to be increased in those with NAFLD (137,213). Interestingly, decreased secretion of VLDL was reported in NASH (213). These mechanisms contribute to TG and cholesterol accumulation and lipotoxicity in the liver. Genetic studies of *PNPLA3* and *TM6SF2* also support the idea that the balance

between lipid accumulation and secretion in VLDL is crucial in the development of NASH (157,171,214). Importantly, Fujita et al. reported similar liver TG content in those with steatosis and in NASH (213) suggesting that factors beyond TG accumulation are needed to cause inflammation and cellular damage. In the following, normal fatty acid and cholesterol synthesis pathways in the liver, mostly in the hepatocytes, are described with references to disturbances in these pathways in NASH.

Fatty acid metabolism. Fatty acids and cholesterol have separate metabolic pathways in the liver hepatocytes (Figure 4). Fatty acid translocase (CD36) is responsible for FFA uptake into liver (together with fatty acid transport proteins). Fatty acid synthesis is regulated by carbohydrate-responsive element-binding protein (ChREBP) activated by glucose independently of insulin (215), whereas sterol regulatory binding protein-1c (SREBP-1c) is activated by insulin (216). *SREBP-1c* is a master regulator of fatty acid metabolism with important target genes, such as fatty acid synthase (*FASN*), acetyl CoA carboxylase (*ACC*) and stearoyl-CoA desaturase (*SCD1*) (215,216), regulating fatty acid synthesis. Increased *SREBP-1c* expression in the liver has been associated with NAFLD in mice (217) and in humans (218), potentially because of hyperinsulinemia (13). However, *SREBP-1c* is not upregulated in those with NASH (219). In fact, Nagaya et al. found decreased *SREBP-1c* expression in those with advanced fibrosis (219). *SCD1* activity is negatively associated with liver fat content after high sugar diet in healthy subjects, suggesting increased fatty acid desaturation. (220)

Fatty acids are converted to TGs by diacylglycerol O-acyltransferase 2 (*DGAT2*), which has been suggested to be have an important role linking simple steatosis and IR possibly because of altered amounts of fatty acid metabolites regulating insulin sensitivity (221). If TGs are not formed normally, it decreases hepatic steatosis, but causes FFA accumulation, which can cause inflammation and liver injury. (180) After TG formation, microsomal triglyceride transfer protein (MTTP) packs TGs into VLDL (222). The function of MTTP may be decreased in those with NASH compared to those with simple steatosis. (213)

Cholesterol metabolism. LDL cholesterol from the circulation is taken into the liver via LDL-receptor (LDLR) -mediated endocytosis regulated by Proprotein convertase subtilisin/kexin type 9 (PCSK9) (222). Cholesterol synthesis takes place in the ER, where the rate-limiting enzyme is HMG-CoA reductase (HMGCR) (223). Both messenger RNA (mRNA) expression and protein levels of HMGCR are increased in those with NASH (224). Sterol regulatory binding protein-2 (*SREBP-2)* and liver X receptor (*LXR)* are major regulators in cholesterol metabolism (218,222,225). *SREBP-2* is a transcriptional regulator of *HMGCR*, and it also activates LDLR (225).

LXR is an important sterol sensor regulating cholesterol homeostasis (226), but it also has a role in fatty acids metabolism. *LXR* regulates cholesterol catabolism and secretion into bile (Figure 4 and 5) (227). Increased *LXR* expression has been associated with NASH and fibrosis in human liver (218). Normally, excess intracellular cholesterol inhibits *SREBP-2* and activates *LXR*, leading to cholesterol export and elimination. Moreover, a decrease in intracellular cholesterol content causes *SREBP-2* induced cholesterol synthesis and uptake. (222) However, increased mRNA expression (228) and SREBP2 protein content (224) in the liver have been reported in those with NASH, suggesting increased cholesterol synthesis, even though liver cholesterol content is increased (210,211) Interestingly, dietary cholesterol and synthetic *LXR* agonists have been reported to increase *SREBP-1c* expression by LXR, suggesting a link between hepatic cholesterol and TG metabolism (226).

Mice with null *LXR* have shown activation of stellate cells, which could participate in the development of liver cell fibrosis. In *LXR* null mice retinoid (storage form of vitamin A) levels are increased in the stellate cells suggesting possible role of retinoid metabolism in the progression of NASH. (229). Recently, *PNPLA3* was found to function as a lipase responsible for retinyl-palmitate hydrolysis in human stellate cells (160). Interestingly, the expression of *PNPLA3* is regulated by *ChREBP* and by *SREBP-1c* (215), supporting the major importance of *PNPLA3* in liver lipid metabolism.

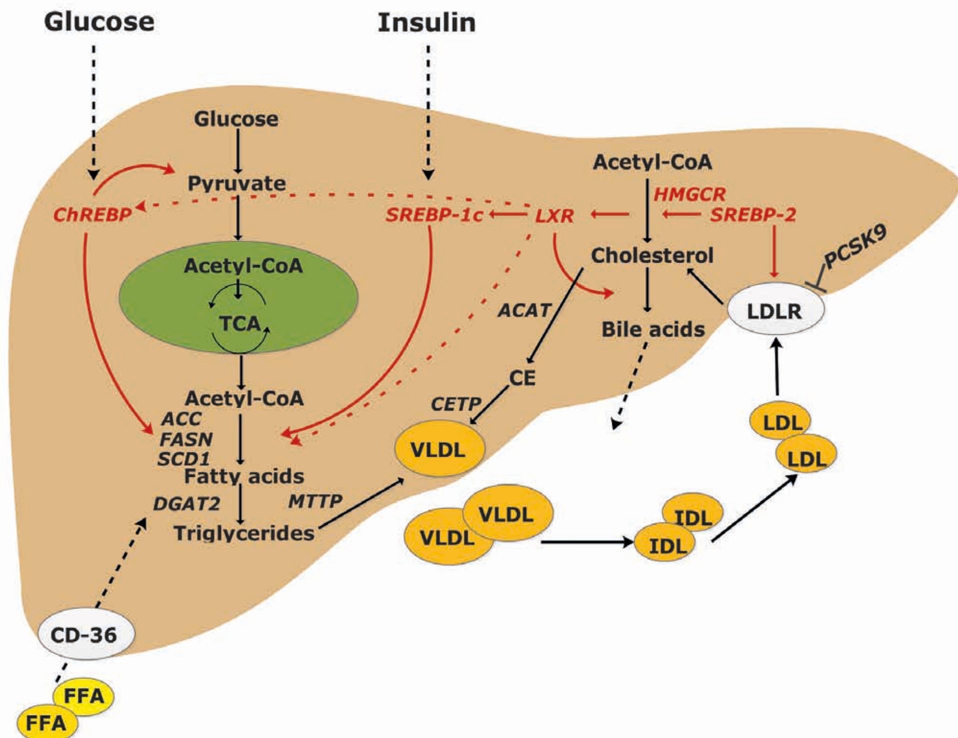

Figure 4. Lipid and lipoprotein pathways in the liver, modified from (222). Glucose and insulin activate transcription factors *SREBPs* and *ChREBP*, which are needed for synthesis of fatty acids and cholesterol. Increased lipogenesis leads to an increase in VLDL production. Liver uptake of LDL is via LDLR. Metabolic genes are in italics and black lines demonstrate metabolic pathways. Red lines demonstrate transcriptional regulation. Green ovals symbolize mitochondria.

Liver free cholesterol accumulation in NASH. Together with increased cholesterol synthesis (14,210,211), liver free cholesterol accumulation has a major role in NASH (210,228,230). Reduced cholesterol intake has been reported to decrease hepatic free cholesterol levels and NASH in mice (230), supporting the importance of cholesterol metabolism in the development of NASH. Intracellular cholesterol content is normally tightly regulated by mechanisms affecting cholesterol uptake, synthesis, catabolism and export (222). If these are disturbed, free cholesterol can accumulate in the cells and cause injury (231). In the following, several possible reasons for free cholesterol accumulation are covered:

1) Disturbed intracellular cholesterol trafficking. Inside the liver cell LDL cholesterol is hydrolyzed by Niemann-Pick disease, type C1 (NPC1) and free cholesterol is released (232) Next, cholesterol is carried to Acyl CoA-cholesterol acyltransferase (ACAT) in the endoplasmic reticulum, where cholesterol is esterified to the storage form (cholesterol

esters, CEs) (Figure 5). However, increased cholesterol de-esterification in NASH is suggested by increased expression of hepatic neutral cholesterol ester hydrolase (*NCEH*) (224,231). This can increase the free cholesterol content. Additionally, ATP-binding cassette transporter A1 (ABCA1) controls the cholesterol absorption and secretion (233) and its expression is reduced in those with NASH (224).

2) Altered cholesterol excretion into bile. Bile acids are important in cholesterol metabolism. Even under unlimited availability cholesterol is excreted into bile (234). Tumor necrosis factor alpha (TNF-α) can activate cholesterol synthesis, but also inhibit cholesterol elimination through bile acids in mice (235), suggesting a possible role also in NASH. Niemann-Pick C1-like 1 (NPC1L1) receptor regulates the amount of cholesterol excreted into bile together with ATP-binding cassette transporters G5 and G8 (ABCG5/8) (234,236). NPC1L1 is also important in cholesterol uptake from the gut (234). Liver *NPC1L1* gene expression correlates negatively with liver cell inflammation (and with *LXR* expression) in humans (218). In mice with IR, ABCG5/8 expression is increased, leading to increased biliary cholesterol excretion (237), but this has not been confirmed in humans (224). The farnesoid X receptor (FXR) is a nuclear receptor that is activated by elevated levels of bile acids. FXR attempts to limit accumulation of toxic metabolites (Figure 5). It has an important function regulating hepatic de novo lipogenesis, VLDL-TG export and plasma TG turnover. (238) Liver FXR function is defective in those with NASH, leading to increased bile acid levels (209), which could cause liver injury. However, also decreased bile acid synthesis in those with NASH has been suggested. *CYP7A1* and *CYP27A* are needed for the catabolism of cholesterol to bile acids and these are down-regulated in those with NASH (224), which could increase cholesterol content in the liver.

3) Increased cholesterol excretion (14,210,211) together with decreased cholesterol intake via LDLR has been reported in those with NASH (224). However, defective VLDL secretion has also been found in those with NASH (213), suggesting that increased cholesterol synthesis (212) together with decreased lipid outflow from the liver could lead to cholesterol accumulation (Figure 4).

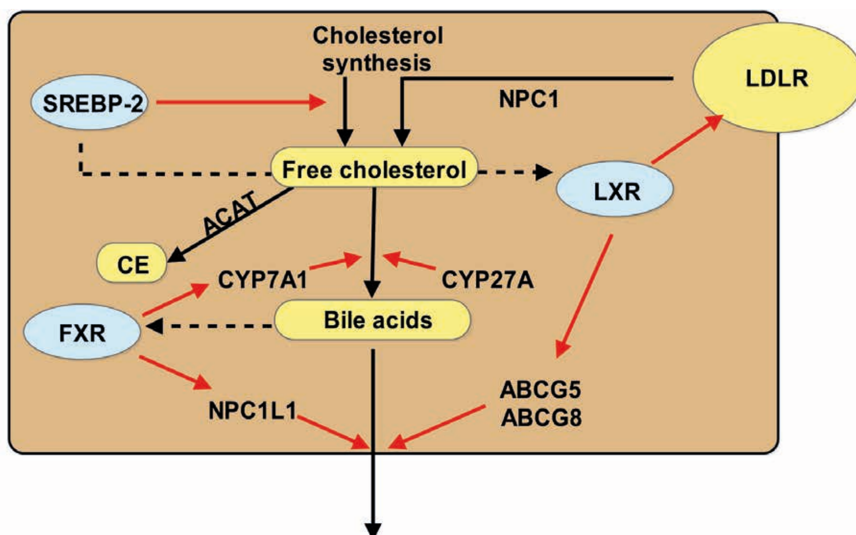

Figure 5. Cholesterol trafficking in the liver cell and excretion into the bile. Modified from (239). Red arrow demonstrates transcriptional activity of genes regulating cholesterol metabolism. Black dotted line demonstrates activation of FXR and LXR.

Free cholesterol toxicity in NASH. It is unclear why free cholesterol is toxic for the liver. It has been suggested that it can directly cause apoptosis and liver cell necrosis (240). Free cholesterol accumulation in stellate cells can possibly activate inflammatory pathway and lead to NASH. (241) Additionally, free cholesterol can activate TNF-α and Fas ligand (242), which promote hepatic inflammation (243,244). Free cholesterol accumulation links also with JNK activation, oxidative stress and mitochondrial membrane alterations in mice with NASH (240).

Free cholesterol toxicity could also be explained by its effect on mitochondria. Cholesterol concentration in the mitochondria is normally much lower (3-5% of the total cellular cholesterol) than in the plasma membrane. Mitochondria are very sensitive for the increase in the cholesterol content, which can disrupt the membrane function (245). Free cholesterol accumulation (but not TG or FFA accumulation) can cause mitochondrial stress and ROS (243,244). This is accompanied by defective mitochondrial function (15,20,21). Steroidogenic acute regulatory protein (StAR) takes part in cholesterol transport to mitochondria. Interestingly, liver *StAR* expression has been reported to be higher in those with NASH than in those with steatosis. (228)

Importantly, mitochondria are responsible for producing the major part of ROS (246). When mitochondrial ROS production exceeds cell´s antioxidative capacity, it can damage cell lipids, proteins and nucleic acids. This leads to oxidative stress and liver cell apoptosis (197). The intact mitochondrial glutathione pool is essential for controlling the formation of ROS (245,247). However, increased mitochondrial free cholesterol content impairs glutathione transport (242), which could further lead to increased mitochondrial ROS generation and cell injury. Furthermore, free cholesterol accumulation modifies the free cholesterol to phospholipid ratio in ER membrane and makes the membrane too stiff. This leads to ER stress and impaired ER function. (197) Thus, free cholesterol can also directly disturb ER function in addition to disrupting mitochondrial function and causing ER stress (18).

2.2.9 Diagnosis of NAFLD

NAFLD is the most common cause for elevated liver enzymes such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (4). However, if NAFLD diagnosis is based on elevated transaminase levels, up to 80% of patients with NAFLD can remain undiscovered (89).

Liver steatosis can be diagnosed with easily accessible abdominal US, with sensitivity of 60-94% and specificity of 66-97% (248-250). Nonetheless, conventional US examination does not reveal mild steatosis (steatosis under 30%) (121), although newer US devices have better accuracy. Quantitative US models have been developed, and they detect mild stetosis better than conventional US (251). Computed tomography (CT) detects steatosis well, and in fact detects local steatosis better than US. However, CT cannot be used as a screening method, because of radiation exposure. (252) Of the standard methods, magnetic resonance imaging (MRI) is the best and can reliably detect steatosis of 3% (253).

Magnetic resonance spectroscopy (MRS) has become a non-invasive gold standard for finding liver steatosis. MRS may even be more reliable than liver biopsy for this purpose, because it assesses the whole liver volume (254). MRS diagnosed steatosis correlates well, but not perfectly with liver biopsy-assessed steatosis grading (255). However, it should be remembered that MRS measures the amount of TGs in the parenchyma and histological

evaluation is based on the number of hepatocytes affected by steatosis (254). Sensitivity of MRS is so high that it can detect even very small amounts of TGs, which are perhaps not seen on histological analysis (252). The use of MRS is limited because of the expensive and not widely available methodology. Additionally, liver cell inflammation and fibrosis (254) cannot be currently detected by MRS. Therefore, MRS cannot replace liver biopsy when evaluating NASH.

2.2.10 Non-invasive diagnosis of NASH

Diagnosis of NASH is challenging without histological analysis Elevated ALT and AST levels may raise the suspicion of NASH (4), although transaminases can be normal in those with NASH (89). When a cut-off value < 35 IU/L for ALT is selected, a sensitivity of 89% and specificity of 29% for finding NASH have been achieved. When selecting cut-off values of 53-70 IU/L, sensitivity is 50-72% and specificity is 51-61%. (256) AST to ALT ratio has been used to differentiate alcoholic and non-alcoholic steatohepatitis. A ratio < 1 suggests non-alcoholic disease, and a ratio > 2 is strongly suggestive for alcoholic disease (257), although ratio > 1 predicts advanced fibrosis in those with NASH (258).

Serum cytokeratin-18 (CK-18) for finding NASH. Since liver transaminases are far from optimal to find subjects with NASH (89,256) other non-invasive markers have been studied. Liver cell apoptosis activates intracellular proteases such as caspase-3 and caspase-7 (259). These proteases can cleave intracellular substrates, such as CK-18, which is the major filament protein in the liver (259). Thus, it has been suggested that serum CK-18 could be used as a marker of liver cell injury. Both serum total CK-18 (uncleaved, M65 antigen) and its fragments (cleaved, M30 antigen) have been tested in finding those with NASH (260).

Increased concentrations of serum CK-18 M30 have been associated with liver cell injury in those with NASH (261,262). Diab et al. reported sensitivity of 82% and specificity of 77% with a CK-18 cut-off value of 252 U/L. When the cut-off was elevated to 275 U/L, sensitivity of 77% and specificity of 100% were achieved. (261) Feldstein et al. published similar results (262) and CK-18 M30 was suggested to be the best biomarker for finding those with NASH, although in another study the accuracy was not very good (sensitivity and specificity of 66%) (263). The Guidelines of American Gastroenterological Association (AGA) and American Association for the Study of Liver (AASLD) state that CK-18 is a promising biomarker for finding those with NASH, but it is too early to recommend CK-18 in clinical practice (78). Since these guidelines, its sensitivity to separate NASH from simple steatosis has been questioned (264,265). Cusi et al. reported that CK-18 is a good predictor for steatosis and fibrosis, but not for NASH (sensitivity of 58% and specificity of 68%) (264). However, a meta-analysis with total of 838 individuals reported a sensitivity of 83% and specificity of 71% for CK-18 M30 for diagnosing NASH. Total CK-18 concentration had a sensitivity of 77% and specificity of 71% for finding those with NASH. This suggests that CK-18 measurement is useful, but it works better in screening than in diagnosing NASH. (260)

Non-invasive scores. At least 15 non-invasive scores have been developed to detect NASH (112,124,266-278). These scores have tested how the combination of NASH-related biomarkers associates with histological diagnosis of NASH. Scores are composed of routine measures such as age, components of the IR/metabolic syndrome (BMI, TGs, glucose, insulin, hypertension), liver enzymes, platelets, albumin, CK-18, circulating collagen peptides and various inflammatory markers (254).

A NASH score consisting alpha-2-macroglobulin, haptoglobin, apolipoprotein A1, total bilirubin, gamma-glutamyl transferase (GGT), ALT, AST, TGs, cholesterol, age, gender, height and weight (267) is one of those few that have been externally validated, but it does not find NASH accurately enough (279). In addition, many scores for finding fibrosis have been developed, of which the NAFLD Fibrosis Score (258) is the most studied. The score is formed using age, BMI, hyperglycemia, platelet count, albumin, and AST/ALT ratio (258). The guidelines of the AASLD/AGA consider it as a clinically useful tool for identifying those with a higher likelihood of having bridging fibrosis and cirrhosis among those with NAFLD (78). However, critical comments have been made considering its accuracy for predicting NASH (280).

A problem with many of these scores is that they can perform well in the discovery cohort, but cannot find NASH equally well in validation cohorts. In addition, many of those that have worked well in discovery cohorts are complicated and not easy to implement in daily practice. Glolam´s model is composed of easily available variables (AST and presence of diabetes), but the accuracy of this score is not excellent (124). (Table 3)

Imaging methods. In recent years, elastography methods have been developed as non-invasive procedures to find liver fibrosis based on changes in liver elasticity. Transient elastography (like Fibroscan, Echosens, Paris, France) can be performed rapidly at the bedside. It is based on an US method measuring liver stiffness, and has primarily been used in hepatitis B and C patients to evaluate the grade of liver fibrosis. (281) However, there are promising results also in NAFLD patients for detecting higher grade fibrosis (grade 3 and 4) (281,282). US examination with bubble contrast media has shown excellent accuracy for finding all grades of fibrosis, and may potentially be used for detecting fibrosis in the future (283). Finally, MR elastography has better accuracy for finding liver fibrosis than US elastography suggesting that it could be used more widely in the future (284). However, it should be highlighted that these methods are for finding scarring fibrosis, and none of the imaging procedures can detect liver cell inflammation.

Table 3. Non-invasive scores for NASH.

Name	Individuals (n)	Country	Model	AUROC	Internal validation	External validation	Reference
HAIR	105	Australia	1. hypertension, 2. increased ALT (>40 IU/L), 3. Insulin resistance (index>5)	≥ 2 parameters 0.90	no	no	(112)
Palekar´s score	80	USA	Sum of: 1. age≥50 years, 2. female, 3. elevated AST (≥45 IU/L), 4. BMI≥30 kg/m², 5. AST/ALT ratio≥0.8, 6. hyaluronic acid≥55 ug/L	0.76	no	no	(266)
NASH test	160 (discovery) 97 (validation) 383 (controls)	France	a2-MG, haptoglobin, apolipoprotein A1, total bilirubin, GGT, ALT, AST, triglycerides, cholesterol, age, gender, height, weight	0.79 and 0.79	yes	in other French cohorts (n=494)	(267)
Gholam´s model	97	USA	-2.627 x lnAST + 2.13 if type 2 DM	0.82	no	no	(124)
NASH diagnostic	69 (discovery) 32 (validation)	USA	Cleaved and total CK-18, adiponectin, resistin	0.908	yes	same group re-evaluated (AUROC 0.70)	(268)
OxNASH	73 (discovery) 49 (validation)	USA	Ratio of 13-HODE to LA, age, BMI, and AST	0.83 and 0.74	yes	no	(272)
the Nice Model	310 (discovery) 154 (validation)	France	-5.654 + 3.780E-02 x ALT (IU/L) + 2.215E-03 x CK-18 fragments (IU)L) + 1.825 x (metabolic syndrome: yes=1, no=0)	0.83 and 0.88	yes	no	(271)
NASH diagnostic panel	79	USA	Formula: DM, gender, BMI, triglycerides, M30 and M65 antigens	0.81	no	no	(269)
Apoptosis panel	95 (discovery) 82 (validation)	USA	−6.4894 + 0.0078 × CK-18 fragments (U/L) + 0.4668 × sFas (ng/ml)	0.93	yes	no	(270)
NAFIC score	177 (discovery) 442 (validation, other centers)	Japan	Weighted sum of ferritin≥200 ng/ml (female) or ≥300 ng/ml (male) 1 point, IRI≥10.0 uU/ml 1 point and type IV collagen 7S≥5.0 ng/ml 2 points	0.85 and 0.78	yes	no	(276)
	66	Brazil	Cholesterol, ALT, AST/ALT, GGT ± US evaluation	0.73 and 0.82	no	no	(273)
	50	Indonesia	AST > 25 IU/L and TNF-α > 3.28 pg/ml	0.84	no	no	(274)
Antwerp score	200 (discovery) 113 (validation)	Belgium	ALT, fS-C-peptide, and ultrasound steatosis scores	0.85 and 0.84	yes	no	(277)
NASH-score	60	Romania	BMI, ALT, AST, ALP, HOMA-IR, M65	0.96	no	no	(275)
ION Score	4458 (ultrasound) 152 (biopsy)	USA	1.33 waist-to-hip ratio + 0.03 TG (mg/dL) + 0.18 ALT (U/L) + 8.53 HOMA − 13.93 in men; and NAFLD = 0.02 TG (mg/dL) + 0.24 ALT (U/L) + 9.61 HOMA − 13.99 in women	0.88	no	no	(278)

2.2.11 Histological diagnosis of NASH

Although imaging procedures can detect steatosis and possibly also fibrosis in the future, liver inflammation can currently be diagnosed only with liver biopsy (6,285). In liver biopsy, the main measures are steatosis, liver cell ballooning as a marker of hepatocellular injury, and lobular inflammation. In more detail, steatosis is evaluated primarily from perivenular area (Figure 6). Steatosis is graded as mild (<33%), moderate (33-66%) and severe (>66%) based on percentage of liver cells with fat accumulation. Severe steatosis can be present in the whole acinus (Figure 6). (6) Lobular inflammation is usually mild, and the inflammatory cells that are present are lymphocytes, eosinophils and possibly neutrophils. Inflammatory cells in lobular area (no foci = 0, <2 inflammatory foci per 200 x field = 1, 2-4 foci per 200 x field = 2 and >4 foci per 200 x field = 3) are evaluated. In addition, portal mononuclear inflammation can be present as a feature of more advanced disease (286). Kupffer cells can aggregate and form microgranulomas, which can be present in addition to lipogranulomas (6). Liver cell ballooning is an important feature in NASH, and it is associated with more aggressive disease (287). Ballooning is graded as minimal (0), present (1) or marked (2).

Although fibrosis is considered the unwanted outcome of NASH, its presence is not required for the diagnosis at the earlier stages of the disease. It usually starts from acinar zone 3 (Figure 6) and has a "chicken wire" pattern. In addition, portal fibrosis can be present. In more severe disease bridging fibrosis and liver cirrhosis can occur. (6) Cirrhosis in NASH is normally macronodular or mixed, but not micronodular (288). In addition, other NASH-associated features might be found in histological evaluation. These include Mallory-Denk bodies (289), which associate with the severity of NASH (6) and strengthen the NASH diagnosis, but they can be seen also in other forms of steatohepatitis (such as alcoholic steatohepatitis) (288). Mallory bodies contain misfolded keratins like CK-18 (290). There can be megamitochondria and glycogenated nuclei, which are only rarely seen in alcoholic steatohepatitis (288).

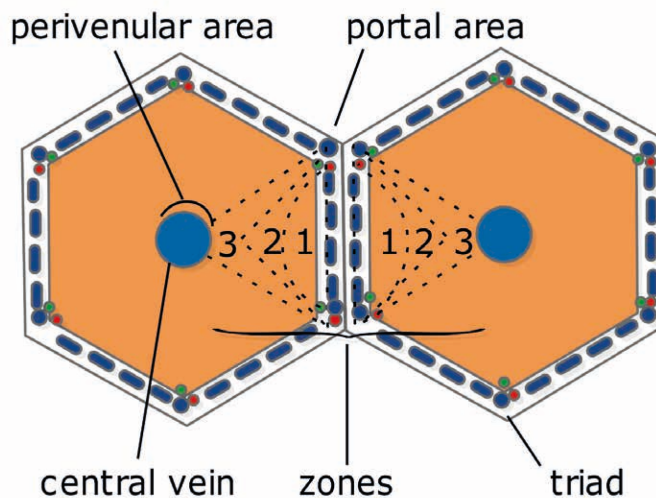

Figure 6. Liver acinus, the functional unit of the liver.

The NAFLD activity score has been developed to detect NASH (NAS; Table 4) combining evaluation of steatosis, hepatocellular ballooning and lobular inflammation. It should be noted that NAS was primarily formed as a tool for finding NASH in intervention studies (285). NAS over 5 suggests NASH, and score under 3 suggests the absence of NASH. Later, the prognostic value and the benefit of the score have been questioned (291-293). Additionally, a NASH activity grade combining the evaluation of steatosis, ballooning and lobular and portal inflammation has been used (6).

The liver biopsy is a relatively safe procedure, but can have even fatal complications, with a death rate of 0.01% (294). It also has some other limitations. First, adequate sized liver biopsy for histological analysis with approximately 10 portal tracts (295), is still only 1:50 000-1:65 000 of the whole liver size and thus only local analysis of lipid accumulation in the liver is performed (6). Accordingly, inflammatory lesions may be scattered in the liver, which could wrongly exclude NASH in as many as one third of cases (296,297). Second, interpretation of liver biopsy depends on the pathologist, leading to potential bias (6). Therefore, analysis should be made by an experienced pathologist.

Table 4. Histological variables analyzed in diagnosing NASH, also showing coding for the NAFLD activity score.

	Definition	**Score/code**
Steatosis		
	Grade	
	<5 %	0
	5-33 %	1
	33-66 %	2
	>66 %	3
	Location	
	Zone 3	0
	Zone 1	1
	Azonal	2
	Panacinar	3
	Microvesicular Steatosis	
	Not present	0
	Present	1
Fibrosis		
	Stage	
	None	0
	Perisinusoidal or periportal	1
	Mild, zone 3, perisinusoidal	1A
	Moderate, zone 3, perisinusoidal	1B
	Portal/periportal	1C
	Perisinusoidal and portal/periportal	2
	Bridging fibrosis	3
	Cirrhosis	4
Inflammation		
	Lobular inflammation	
	None	0
	<2 foci per 200x field	1
	2-4 foci per 200x field	2
	>4 foci per 200x field	3
	Microgranulomas	
	Absent	0
	Present	1
	Large Lipogranulomas	
	Absent	0
	Present	1
	Portal inflammation	
	None to minimal	0
	Greater than minimal	1
Liver cell		
	Ballooning	
	None	0
	Few balloon cells	1
	Many cells/prominent ballooning	2
	Acidophil bodies	
	None to rare	0
	Many	1
	Pigmented macrophages	
	None to rare	0
	Many	1
	Megamitochondria	
	None to rare	0
	Many	1
Other		
	Mallory's hyaline	
	None to rare	0
	Many	1
	Glycogenated nuclei	
	None to rare	0
	Many	1

2.2.12 Management of NAFLD

The most important treatment for NAFLD is lifestyle modification, because it is easily available and most effective. Lifestyle changes aim for weight reduction. Weight loss of 5% has resolved NAFLD in as many as 75% of subjects. (298) Similar weight loss has improved IR and liver steatosis in NASH, but weight loss of 7-9% is needed for improvement in inflammation and ballooning (299,300). Thus, a weight loss of 3-5% in those with simple steatosis, and 7-10% in those with NASH is recommended (78). It seems that weight loss of at least 10% is needed for fibrosis improvement (301).

Calorie restriction is essential for weight loss. A diet should contain 600 Kcal less than is needed for remaining weight stable with a target of 0.5-1.0 kg weight reduction per week. (302) The amount of dietary fat should be reduced, because high fat diet increases and low fat diet decreases liver fat (140). In addition, the quality of fatty acids may be important, because high amount of monounsaturated fatty acids in a diet reduces hepatic steatosis (303). Both omega-3 (304) and omega-6 fatty acids (305) have also shown potential in reducing liver fat.

Higher level of physical inactivity is associated with lower levels of steatosis (306,307). Interestingly, physical activity improves liver enzymes and decreases liver fat independently of weight loss (308,309). Physical activity also improves NASH (299,310).

Massive weight loss induced by obesity surgery has been shown to lead to resolution of NASH. Steatosis (311,312), inflammation (71) and fibrosis (70,71,311-313) have been reported to decrease after surgery. In a Cochrane analysis, authors could not find any randomised clinical trials fulfilling the inclusion criteria. However, 21 prospective or retrospective cohort studies reported improvement on steatosis or inflammation (74). Randomized studies are still needed. However, the challenge in these studies is to justify taking follow-up liver biopsies in a setting where clinical improvement is very likely and thus biopsies do not provide additional information.

The quality of the diet is also important. The role of fructose in the development of NAFLD is unclear (143,144,146,147,149). However, excess amounts of simple sugars should be avoided, because they cause weight gain (314) and increase liver fat content (315). Small amounts of alcohol might reduce the risk of NASH (112), but the safe limit is not known. Surely, heavy drinking is not recommended (78), and it might be wise to advise these individuals to avoid alcohol. Coffee drinking might protect from disease progression, at least for severe fibrosis (316), so it should not be forbidden.

Drug treatment has only a small role in treatment of NASH. In special occasions, like in young people with NASH or advanced fibrosis, vitamin E and pioglitazone could be considered as a treatment option (78,317). However, it should be noted that there are no Food and Drug Administration (FDA) approved drugs for the treatment of NASH (318). Table 5 summarizes the current knowledge about the drug treatment of NAFLD.

Table 5. Drug therapy for NAFLD. Modified from (317)

Benefit	Agent	Results
Potential benefit – could be considered for the treatment	Pioglitazone	Improved liver histology both in non-diabetic (319) and diabetic individuals Caused weight gain (320) Risk of congestive heart failure (321), osteoporosis (322) and possible increase in the risk for bladder cancer (323)
	Vitamin E	Improved liver histology (dose 800 IU/d) (320) Increased risk for stroke (324), prostate cancer (325) and overall mortality (326)
No clear benefit –should be studied further	Metformin	Effect in steatosis (327) and inflammation (328) in mice studies Unclear effect based on human studies (329-331)
	Statins	Improvement in transaminases and decrease in liver fat content (332) Those with statin have less steatosis, inflammation and fibrosis (333)
	Ezetimibe	Decreased fibrosis and ballooning. Worsened insulin sensitivity (334)
	Pentoxifylline	In small studies improved histology (335,336) and ALT (335,336)
	UDCA	No improvement in liver histology (dose 23-28 mg/kg/d). However, lobular inflammation seemed to improve (337)
New treatment ideas – should be studied further	GLP-1 analogues	Decreased intrahepatic lipid content in humans (338)
	Anti-TNF-α-agents	Improved NASH in mice (339)
	Probiotics	Improved NASH in mice (340) and decreased AST in humans (339)
	Resveratrol	SIRT1-activator has showed potential of decreasing NASH in mice (341)
	LXR-inverse agonist	Reversion of NASH in mice (342)
	Obeticholic acid	Farnesoid X nuclear receptor agonist reduced steatosis, inflammation and fibrosis in humans, associated with the development of dyslipidemia (343)

3 *Aims of the Study*

The aim of this thesis was to investigate metabolic disturbances in individuals with NASH and compare them to those with normal liver and those with simple steatosis for the purpose of finding biomarkers for NASH. The more specific aims were to:

1. Form a non-invasive score for predicting NASH and estimate the population prevalence of NASH in Finnish adult individuals (**I**)

2. Investigate serum CK-18 as a biomarker of NASH in dietary and surgical intervention studies (**I and II**)

3. Analyze lipoprotein subclass metabolism in NASH (**III**)

4. Evaluate lipid oxidation and ketone body metabolism in NASH (**IV**)

4 Methods

4.1 STUDY SUBJECTS

4.1.1 Kuopio Obesity Surgery Study (KOBS)

The primary study population of this thesis was 116 subjects from Kuopio Obesity Surgery Study (KOBS) (studies III and IV). In study II, 124 individuals from the KOBS were included. Patients were recruited amongst Kuopio University Hospital patients eligible for laparoscopic gastric bypass operation. Criteria for the surgery were (1) BMI > 40 kg/m^2 or (2) BMI 35-40 kg/m^2 and a comorbidity or its risk factor, such as type 2 diabetes, hypertension, sleep apnea, osteoarthritis of weight bearing joints or polycystic ovarian syndrome; and (3) previous conservative treatment for obesity was proven to be ineffective. These criteria are in line with European guidelines (56). Written informed consent was obtained. Chronic hepatitis B and C were excluded using serologic tests if ALT values were elevated prior to surgery. Alcohol consumption < 20 g per day was required as an inclusion criterion. Every subject participated in one-day visit including an interview on the history of previous diseases and current drug treatment. During the week preceding the elective obesity surgery operation, blood samples were taken after an overnight fast. Control samples were obtained 12 months after the surgery

4.1.2 Helsinki-Kuopio cohort

In studies I and IV an extended cohort with 296 obesity surgery patients from Kuopio and Helsinki was also included (n=129 in Kuopio and n=167 in Helsinki). Of these, 89 were same individuals as in the KOBS described above. Criteria for surgery were similar to KOBS cohort. Inclusion criteria were (1) age 18–60 years; (2) no known acute or chronic disease except for obesity or type 2 diabetes, hypertension, sleep apnea, osteoarthritis of weight bearing joints or polycystic ovarian syndrome based on history, physical examination, standard laboratory tests (blood counts, serum creatinine, thyrotropin, and electrolyte concentrations) and electrocardiogram; (3) alcohol consumption < 20 g per day.

4.1.3 Validation cohort from Italy

In study I an Italian cohort with 380 patients from the Metabolic Liver Diseases Outpatient Service (Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico) was also used. These patients were diagnosed of having NAFLD between January 2008 and January 2010. A liver biopsy was performed in 309 (81%) of patients because of persistently abnormal liver enzymes or serum ferritin, or a long lasting history of steatosis associated with severe metabolic abnormalities. Seventy-one (19%) of patients were obesity surgery patients. Other causes of liver disease were excluded, including excess alcohol intake (>30 g/day for men and 20 g/day for women), viral and autoimmune hepatitis, hereditary hemochromatosis, and alpha1-antitrypsin deficiency.

4.1.4 D2D population study

In study I a cohort from national type 2 diabetes prevention programme (FIN-D2D) was used to assess the prevalence of NAFLD based the non-invasive score developed. D2D study was carried out in three hospital districts in Finland in year 2007 (344). 4500 subjects were stratified according to gender, 10-year age groups (45-54, 55-64 and 65-74 years) and three geographical areas. Total number of participating individuals was 2849 (63% of the original sample). Men consuming ≥ 40 g and women ≥ 20 g of ethanol per day based on the past week data were excluded from the study I. The health examination was carried out according to the WHO MONICA project protocol (345). This cohort had detailed routine measurements and *PNPLA3* rs738409 genotyping available.

4.1.5 METSIM population study

A total of 8749 non-diabetic men from the population-based cross-sectional METSIM (Metabolic Syndrome in Men) study (346) were included in study IV. The METSIM study was performed in 2005-2010. Subjects aged from 45 to 70 years were randomly selected from the population register of the town of Kuopio, Eastern Finland (population of 95 000). Their age was 57.2 ± 7.1 years and BMI 26.8 ± 3.8 kg/m^2. Every participant had a 1-day outpatient visit to the Clinical Research Unit at the University of Kuopio, including an interview on the history of previous diseases and current drug treatment and an evaluation of glucose tolerance and cardiovascular risk factors. Fasting blood samples were drawn after 12 h of fasting followed by an oral glucose tolerance test. Serum ALT was used as the marker of liver disease in this study. Ketone body levels were determined with serum NMR analysis (347). Consumption of alcohol in this population was 14 ± 19 g per day.

4.1.6 HEPFAT dietary intervention

In study II, diet intervention study population was included. The Role of Dietary Fatty Acids in Fatty Liver and Insulin Resistance (HEPFAT) Trial was a randomized, 10-week, parallel-group study conducted in Uppsala, Sweden, between February 2009 and April 2010 (305). The study group consisted of 67 individuals. Participants were randomly assigned to either a polyunsaturated fatty acid (PUFA) or saturated fatty acid (SFA) diet. The primary outcome was change in liver fat content measured by MRI and by MRS. Those 56 participants who had CK-18 measured and liver fat assessed by MRS before and after the intervention were included in the study II. Of these, 27 had been randomized to the n-6 PUFA diet and 29 to the SFA diet. When study subjects were divided to those with low liver fat (<5%) and those with high liver fat (>5%) content, 35 individuals were in low liver fat (mean 1.9 ± 1.3%) group and 21 individuals in high liver fat group (mean 11.7±7.2%).

4.1.7 VLCD intervention

In study II, population from very low calorie diet (VLCD) intervention study was included. It was a randomized clinical 1-year follow-up trial including individuals with mild obstructive sleep apnea. Study was conducted during October 2004 and December 2006. (348) The inclusion criteria were: (1) age 18–65 years; (2) BMI 28–40 kg/m^2; and (3) apnea–hypopnea index 5–15 events/hour. Serum ALT was used as the marker of liver disease in this study. Those 63 participants who had ALT and CK-18 measured before and after the intervention were included in the study II. Intervention group included 33 individuals, who received a 1-year lifestyle intervention including an initial weight reduction program with a

12-week VLCD of 600–800 kcal/day. Control group included 30 individuals, who received a single general dietary and exercise counseling session.

Table 6. Clinical characteristics of the different populations used in this thesis (except in study II n=124 for KOBS).

	KOBS (n=116)	**Helsinki-Kuopio (n=296)**	**Italians (n=380)**	**D2D (n=2849)**	**METSIM (n=8749)**	**HEPFAT (n=56)**	**VLCD (n=63)**
Sex (male-female)	39/77	116/180	246/134	1357/1492	8749	20/36	44/19
Age (y)	47±9	47±9	48±12	6±8	57±7	55±9	52±9
BMI (kg/m^2)	45.1±6.1	43.7±8.6	31.7±8,4	27.5±4.8	26.8±3.8	30.8±3.5	32.6±3.0
fP-insulin (mU/L)	19.9±12.0	17.0±11.0	20±21	8.8±16.4	8.35.9±	10.5±6.6	12.7±6.2
Type 2 diabetes (%)	41	39	20	17	0	15	-
S-LDL cholesterol (mmol/L)	2.5±0.9	2.5±0.8	3.2±1.0	3.4±0.9	3.4±0.9	3.3±0.8	2.9±0.8
S-HDL cholesterol (mmol/L)	1.1±0.3	1.2±0.4	1.3±0.4	1.4±0.3	1.5±0.4	1.4±0.3	1.1±0.3
S-TGs (mmol/L)	1.6±0.7	1.6±0.9	1.6±1.4	1.4±0.8	1.4±1.0	1.4±0.8	1.7±1.1
P-ALT (IU/L)	45±27	52±39	48±33	27±17	31±20	29±15	41±25
P-AST (IU/L)	33±18	39±25	23±33	26±13	-	-	-
NASH (%)	22	29	45	-	-	-	-

4.2 CLINICAL METHODS

Body weight was recorded using a calibrated weighing scale and was measured with a 0.1 kg precision. Height was measured to the nearest 0.1 cm. BMI was calculated as weight (kilogram) divided by height (meter) squared. Diabetes was defined by WHO´s criteria of diabetes (349). For study IV the respiratory quotient (RQ) (350) was calculated by dividing the CO_2 eliminated by O_2 consumed measured with indirect calorimetry (Deltatrac; Datex, Helsinki, Finland). The rate of lipid oxidation was calculated (g/min) with a simplified equation without urinary nitrogen: 1.81 x (O_2 consumption – CO_2 production). Metabolism of carbohydrates was calculated with a formula (4.12 x CO_2 production) – (2.91 x O_2 consumption) (351).

4.3 LABORATORY METHODS

4.3.1 Routine laboratory methods

Plasma glucose was measured by enzymatic hexokinase photometric assay (Konelab Systems Reagents; Thermo Fischer Scientific, Vantaa, Finland). Insulin was determined by immunoassay (ADVIA Centaur Insulin IRI, no. 02230141; Siemens Medical Solutions Diagnostics, Tarrytown, NY). Cholesterol, HDL cholesterol and TG levels from the whole serum were assayed by standard automated enzymatic methods (Roche Diagnostics, Mannheim, Germany). LDL cholesterol was calculated using the Friedewald formula (352). Plasma ALT and AST concentrations were determined using kinetic International Federation of Clinical Chemistry methods (Roche Diagnostics, Mannheim, Germany). CK-18 M30 antibody fragments (U/L) were measured with the Apoptosense® ELISA (cat.no 10010) assay (PEVIVA AB, Sweden). Serum FFAs were analyzed by an enzymatic method from Wako Chemicals GmbH (Neuss, Germany). The adipose tissue IR index was calculated as FFA mmol/L x fasting plasma insulin pmol/L (353).

4.3.2 PNPLA3 genotyping

Genomic DNA was isolated from the blood mononuclear cells using the QIAamp DNA Blood kit (Qiagen, Hilden, Germany). The *PNPLA3* rs738409 and *TM6SF2* rs58542926 polymorphisms were genotyped using an allele-specific PCR assay and a TaqMan probe (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocols.

4.3.3 Liver gene expression

In studies III and IV, samples for gene expression were immediately frozen in liquid nitrogen. Total RNA from the liver tissue was extracted using Tri-Reagent (Applied Biosystems [ABI] Foster City, CA) and reverse-transcribed using the High Capacity cDNA Reverse Transcriptional KIT (ABI) according to the manufacturer´s protocol. Quantitative real-time polymerase chain reaction (PCR) was carried out with the Applied Biosystems 7500 Real Time PCR System using KAPA SYBR FAST qPCR Universal Master Mix (KAPA Biosystems, Woburn, MA). Relative expression was normalized to *RPLP0*.

4.3.4 Serum and liver NMR analysis

Fasting concentrations of lipoprotein subclass particles and their main lipid components as well as low-molecular weight molecules (LMWM) were analyzed by proton nuclear magnetic resonance (NMR) spectroscopy in native serum samples in studies III and IV (354-356). The NMR data were measured at 37°C using a Bruker AVANCE III spectrometer operating at 500.36 MHz. The methodology is based on three different molecular windows, two of which (LIPO and LMWM) are applied to native serum and one for serum lipid extracts (LIPID).

The LIPO window gives information, e.g., on the lipoprotein subclass distribution and lipoprotein particle concentrations for 14 lipoprotein subclasses. Fourteen lipoprotein subclasses were calibrated using high-performance liquid chromatography (357). The subclasses are as follows: chylomicrons and largest VLDL particles (average particle diameter at least 75 nm); five different VLDL subclasses: very large VLDL (average particle

diameter of 64.0 nm), large VLDL (53.6 nm), medium VLDL (44.5 nm), small VLDL (36.8 nm), and very small VLDL (31.3 nm); intermediate-density lipoprotein (IDL) (28.6 nm); three LDL subclasses: large LDL (25.5 nm), medium LDL (23.0 nm), and small LDL (18.7 nm); and four HDL subclasses: very large HDL (14.3 nm), large HDL (12.1 nm), medium HDL (10.9 nm), and small HDL (8.7 nm). The following components of the lipoprotein particles were quantified: phospholipids (PL), TG, and cholesterol. All of these components are not available for every subclass, because of the resolution issues (356). The VLDL cholesterol concentration was calculated by subtracting the LDL, IDL and HDL cholesterol from the total cholesterol. The total cholesterol content of chylomicrons and extremely large VLDL, very large VLDL and very small VLDL was calculated by subtracting the TG and phospholipid concentrations from the total lipids of each subclass. Lipid composition as a percentage of each available lipid of the total lipid content in each lipoprotein subclass was also calculated.

The LMWM window gives information on various metabolites like amino acids and ketone bodies. The LIPID window gives information on various serum lipids like free cholesterol, esterified cholesterol and omega-3 fatty acids. This platform has been applied in various large-scale epidemiological and genetic studies (352,356,358)

Liver free cholesterol content was analyzed from 45 individuals in study III. Liver samples (ca. 50 mg) were homogenized in 1.5 ml Eppendorf tubes in NaCl solution (150 µl of 150 mM NaCl in D_2O) by pestle. After homogenization, 300 µl of CD_3OD and 600 µl of $CDCl_3$ were added and samples were mixed vigorously using a vortex mixer and sonicated 15 min (indirect sonication) in an ice bath. After mixing, the samples were centrifuged (5000 x g, 10 min, 4°C) to separate the organic and water phase. The lower organic phase was recovered, and the aqueous layer was extracted again first with 600 µl and then with 300 µl $CDCl_3$ to standardize the yield. The separated organic layers were combined and evaporated to dryness under a gentle flow of dried air. Prior to NMR analysis, the extracted lipids were redissolved into 600 µl of $CDCl_3$ containing 0.03% of tetramethylsilane as a reference substance.

^{1}H NMR spectra of extracted lipids were recorded on a Bruker Avance III HD 600 NMR spectrometer operating at 600.28 MHz and equipped with Prodigy TCI 5 mm cryogenically cooled probe head. Standard 1D ^{1}H NMR spectra were recorded with 96k data points using 32 transients and applying a standard Bruker zg pulse sequence. The acquisition time was 5 s and the relaxation delay 15 s. The spectra were measured at 295 K.

For data processing, the free induction decays (FIDs) with 96k data points were zero-filled to 256k data points and multiplied by an exponential window function with a 0.3 Hz line-broadening. The areas of the known lipid resonances (359) in the spectra were determined using a constrained total-line-shape fitting approach to enable quantitative analysis of severely overlapping peaks and to increase the quantification accuracy (354). This methodology allowed us to get information on the amounts of several lipid components in the extracted samples, e.g., free cholesterol and total TGs. The PERCH NMR software was used for all the lineshape fitting analyses.

4.3.5 Liver total cholesterol content measurement

Total cholesterol content (per 100 mg liver tissue) was analyzed from 52 individuals in study III. It was quantified with gas-liquid chromatography (GLC) on a 50 m long capillary column (Ultra 2, Agilent Technologies, Wilmington, DE, USA) and 5α-cholestane was used as an internal standard (360).

4.4 HISTOLOGICAL ASSESSMENT OF THE LIVER SAMPLES

Liver biopsies were obtained using either Trucut needle (Radiplast AB, Uppsala, Sweden) or ultrasonic scissors during elective gastric bypass operation. Overall histological assessment of liver biopsy samples in KOBS was performed by one experienced pathologist according to standard criteria (6). Primarily, histological diagnosis was divided into 3 categories: 1. not NASH, 2. possible NASH and 3. definitely NASH. According to the NASH clinical research networking scores and definitions (285) steatosis was graded into 4 categories, fibrosis was staged from 0 to 4, and inflammation was defined as lobular inflammation (graded 0-3) (Table 7). Lobular inflammation was chosen as a marker of inflammation of liver because it was the most frequent inflammation variable. Subjects were also divided into categories based on clinical liver phenotype: 1. Normal liver without any steatosis, inflammation, ballooning or fibrosis, 2. simple steatosis (steatosis > 5%) without evidence of hepatocellular ballooning, inflammation or fibrosis, and 3. definite NASH (as described above). In studies III and IV 76 of 116 patients had clearly defined liver phenotypes (32 with normal liver, 19 with simple steatosis and 25 with NASH). In a study II, 82 of the 124 patients had a distinct liver phenotype (33 with normal liver, 20 with simple steatosis and 29 with NASH). Hemochromatosis was excluded by histological analysis of liver biopsies, and also by normal serum ferritin levels in subjects that had elevated serum ALT level.

In studies I and IV in Kuopio-Helsinki cohort liver histology was assessed by two experienced pathologists. They first analysed biopsies independently in a blinded fashion and after that together to obtain consensus. A 10% difference in fat content, 1 stage difference in fibrosis score or 1 grade difference in steatohepatitis activity was found in 15% of the samples. In Italian cohort (study I) liver histology was analysed by one experienced liver pathologist. In a study IV the liver phenotype was defined differently from KOBS population: Normal liver was defined when liver fat percent was <10% and there was no necroinflammation. In simple steatosis liver fat percent was >10% but there was no necroinflammation. NASH was defined as a combination of liver fat percent >10% with necroinflammation.

Table 7. Liver histology in different liver phenotypes (KOBS in studies III and IV).

	Definition	Score/code	Normal Liver n=32	Simple steatosis n=19	NASH n=25
Steatosis	*Grade*				
	<5 %	0	32	0	0
	5-33 %	1	0	15	10
	33-66 %	2	0	2	10
	>66 %	3	0	2	5
	Location				
	Zone 3	0	9	11	12
	Zone 1	1	0	1	3
	Azonal	2	21	5	6
	Panacinar	3	2	2	4
	Microvesicular steatosis				
	Not present	0	31	13	11
	Present	1	1	6	14
Fibrosis	*Stage*				
	None	0	32	19	4
	Perisinusoidal or	1	0	0	6
	Mild, zone 3,	1A	0	0	9
	Moderate, zone 3,	1B	0	0	1
	Portal/periportal	1C	0	0	3
	Perisinusoidal and	2	0	0	1
	Bridging fibrosis	3	0	0	0
	Cirrhosis	4	0	0	1
Inflammation	*Lobular inflammation*				
	None	0	32	19	0
	<2 foci per 200x field	1	0	0	17
	2-4 foci per 200x field	2	0	0	8
	>4 foci per 200x field	3	0	0	0
	Microgranulomas				
	Absent	0	31	19	15
	Present	1	1	0	10
	Large Lipogranulomas				
	Absent	0	32	18	22
	Present	1	0	1	3
	Portal inflammation				
	None to minimal	0	32	19	21
	Greater than minimal	1	0	0	4
Liver cell Injury	*Ballooning*				
	None	0	32	19	9
	Few balloon cells	1	0	0	12
	many cells/prominent	2	0	0	4
	Acidophil bodies				
	None to rare	0	32	19	24
	Many	1	0	0	1
	Pigmented macrophages				
	None to rare	0	32	19	25
	Many	1	0	0	0
	Megamitochondria				
	None to rare	0	32	19	25
	Many	1	0	0	0
Other findings	*Mallory's hyaline*				
	None to rare	0	32	19	24
	Many	1	0	0	1
	Glycogenated nuclei				
	None to rare	0	23	14	16
	Many	1	9	5	9

4.5 STATISTICAL METHODS

Characteristics of the study groups are given as means and standard deviations (SD). The unpaired t-test and the Kruskal-Wallis and Wilcoxon tests were used to compare normally and non-normally distributed data, respectively. The unpaired t-test with Bonferroni correction, when appropriate, was used to analyse intraindividual differences before and after surgery (false discovery rate = FDR). Chi-square test was used for analyzing categorical data. Spearman's rank correlation (studies III and IV) and Pearson's linear correlation (study II) were used for correlation analysis. All calculations were performed using SPSS 19.0 and 21.0 for Windows (SPSS, Chicago, IL) and the R 3.0.1 program (http://www.R-project.org, Vienna, Austria). Computational analysis of the NMR data was performed as described in NMR analysis chapter. GraphPad Prism 6 (GraphPad Software, Inc. La Jolla, California, USA) was used for drawing graphs.

Development of the non-invasive scores was made as follows. In the study I the biopsy subjects were randomly divided into discovery (n=195) and validation (n=97) groups to build and validate the new scores. All study subjects with available data for the NASH score (n=292) were used as a second validation group. The unpaired t-test was used to compare the differences between the discovery and validation groups. Next, univariate logistic regression analyses were used to calculate odds ratios (OR) and confidence intervals (CI) for NASH. Multivariate logistic regression analyses were used to build the NASH score. Variables significantly associated with NASH in univariate logistic regression analyses were included in multivariate backward logistic regression analyses to identify variables independently associated with NASH. The receiver operator curve (ROC) and the Youden index (361) were used to estimate the optimal cut-off. The sensitivity, specificity, positive predictive values (PPV) and negative predictive values (NPV) for relevant cut-offs were calculated as described (362).

For the NASH Liver Fat Score a new cut-off for NASH was calculated using the equation previously developed for diagnosis of NAFLD (363). The NAFLD Liver Fat Score was calculated as -2.89 + 1.18 × metabolic syndrome (yes=1/no=0) + 0.45 × type 2 diabetes (yes=2/no=0) + 0.15 × fS-insulin (mU/L) + 0.04 × AST (IU/L) – 0.94 × AST/ALT (363). A new cut-off value was defined for NASH, and the score was named the NASH Liver Fat Score.

In study IV, NASH predicting score was formed to find those with possible NASH in the METSIM study. First, the predictive value of age, weight, BMI, ALT, AST, bilirubin, alkaline phosphatase, INR, glucose, insulin, CK-18, β-OHB and serum FFAs was assessed with logistic regression for diagnosing NASH or ballooning in discovery cohort of 195 individuals in Helsinki-Kuopio biopsy cohort. First the association of these variables with NASH was evaluated with univariate logistic regression. Thereafter, variables that independently predicted NASH were identified by applying backward multivariate regression analysis. Fasting insulin, age and ALT remained in model to form a NASH finding score: -5.544 + 0.057 × age + 0.064 × fasting insulin + 0.014 × ALT.

The performance of the scores was assessed by ROC curves. The area under the ROC-curve (AUROC) was used to describe the diagnostic accuracy of the scores. Sensitivity analysis in study I for calculated NASH score and the NASH Liver Fat score were performed by taking into account stochastic false-positivity and false-negativity rates in a Bayesian model. JAGS and R package (rjags) were used to construct the model on the basis of 2 Markov chains, each

containing 5000 ''burn-in'' samples and 10000 retained samples to obtain the posterior estimates of prevalence (364).

4.6 APPROVALS

Studies of this thesis follow the recommendations for biomedical research involving humans (Declaration of Helsinki of the World Medical Association 1964 including the revisions up to Hong Kong 1989 and Edinburgh, Scotland 2000) as well as a Finnish law concerning information protection. The KOBS project has been approved by the Ethics Committee of the Northern Savo Hospital District (54/2005,104/2008 and 27/2010). All methods have been previously used in humans and are known not to form any risk for the participants. The study population consist individuals who were eligible for obesity surgery and were willing to take part in the study. The nature and potential risks of the study were explained to all subjects before obtaining their written informed consent.

5 Results

5.1 NON-INVASIVE SCORE FOR NASH (STUDY I)

The main finding of study I was that easily accessible laboratory measurements such as serum AST and fasting insulin levels together with *PNPLA3* rs738409 genotyping can be used to find those with NASH non-invasively. Furthermore, the prevalence of NASH in Finland was estimated for the first time.

Development of NASH Score. Non-invasive score for NASH was formed by first analysing which variables were associated with NASH in a cohort of 296 bariatric surgery subjects in the Helsinki-Kuopio cohort (Table 8).

Table 8. Odds ratios and confidence intervals of NASH in Helsinki-Kuopio cohort (OR = odds ratio, CI = confidence interval).

	All subjects OR (95% CI)	**P value**	**Discovery cohort**	**P value**	**Validation cohort**	**P value**
Age (years)	1.03 (1.01, 1.06)	0.02	1.04 (1.00, 1.07)	0.05	1.04 (0.99, 1.09)	0.15
Gender	1.97 (1.18, 3.30)	0.01	2.0 (1.04, 3.83)	0.04	1.9 (0.81, 4.49)	0.14
BMI (kg/m²)	0.98 (0.95, 1.00)	0.13	0.98 (0.94, 1.01)	0.22	0.97 (0.92, 1.02)	0.29
Type 2 diabetes	1.87 (1.44, 2.44)	<0.001	1.73 (1.24, 2.42)	0.001	2.10 (1.35, 3.33)	0.001
Metabolic syndrome	1.23 (0.65, 2.48)	0.54	1.31 (0.57, 3.28)	0.54	1.15 (0.40, 3.57)	0.80
P-ALT (IU/l)	1.01 (1.00, 1.02)	<0.001	1.01 (1.00, 1.02)	0.003	1.03 (1.01, 1.05)	0.001
P-AST (IU/l)	1.03 (1.02, 1.05)	<0.001	1.03 (1.02, 1.05)	<0.001	1.04 (1.02, 1.08)	0.003
fP-Insulin (mU/l)	1.06 (1.04, 1.09)	<0.001	1.06 (1.03, 1.10)	<0.001	1.07 (1.02, 1.11)	0.004
P-Albumin (g/l)	1.06 (1.00, 1.14)	0.06	1.07 (1.00, 1.17)	0.07	1.04 (0.91, 1.18)	0.60
B-Platelets ($x10^9$/l)	1.00 (0.99, 1.00)	0.08	1.00 (0.99, 1.00)	0.41	0.99 (0.99, 1.00)	0.06
TGs (mmol/l)	1.28 (0.97, 1.69)	0.08	1.20 (0.86, 1.68)	0.27	1.51 (0.90, 2.63)	0.13
S-HDL cholesterol	0.86 (0.41, 1.66)	0.66	1.15 (0.48, 2.57)	0.72	0.48 (0.12, 1.57)	0.26
S-LDL cholesterol	1.08 (0.71, 1.65)	0.70	1.08 (0.64, 1.80)	0.78	1.06 (0.51, 2.24)	0.88
S-CK-18 (U/L)	1.01 (1.00, 1.01)	0.001	1.01 (1.00, 1.01)	0.002	1.00 (1.00, 1.01)	0.21

Variables independently associating with NASH were put in logistic regression analysis. In the discovery cohort of 195 individuals, serum AST, *PNPLA3* rs738409 genotype and serum fasting insulin level were strongest predictors of NASH forming the NASH Score. Based on these

variables, backward logistic regression analyses revealed a score best predicting NASH: *-3.05 + 0.562 x PNPLA3 genotype (CC = 1 / GC = 2 /GG = 3) – 0.0092 x fS-insulin (mU/L) + 0.0023 x AST (IU/L) + 0.0019 x (fS-insulin x AST)*. The AUROC of the NASH Score was 0.776 (95% CI: 0.701, 0.852) in the discovery group. With optimal cut-off point -1.054 (determined with Youden index (361)), score had a sensitivity of 75% and specificity of 74% in the discovery group (Table 9). Next, the NASH Score was validated in the remaining 1/3 of Helsinki-Kuopio cohort (n=97) with AUROC of 0.758 (95% CI: 0.626, 0.891), and in the whole cohort and AUROC 0.774 (95% CI: 0.709, 0.839) (Figure 7), respectively. In the validation cohort sensitivity was 65% and specificity 73%, and in the whole cohort sensitivity was 72% and specificity 74% for predicting NASH (Table 9). Interestingly, the AUROC for CK-18 was 0.727 (95% CI: 0.660, 0.790) in the whole Helsinki-Kuopio cohort.

Furthermore, a previously developed NAFLD Liver Fat Score (363) was tested in the prediction of NASH using a newly estimated cut-off (2.122). This NASH Liver Fat Score had an AUROC of 0.734 (95% CI: 0.664, 0.805) in the whole Helsinki-Kuopio cohort (Figure 7).

Validation of the NASH Score. To further test developed NASH scores, they were validated in an Italian cohort consisting 380 moderately obese individuals. The NASH Score had AUROC of 0.759 (95% CI: 0.711, 0.807) with a sensitivity of 39% and specificity of 89% (Table 9). The NASH Liver Fat Score had AUROC of 0.737 (95% CI: 0.687, 0.787) (Figure 7) with sensitivity of 93% and specificity of 33%. After the validation, scores were utilized to estimate the prevalence of NASH in Finnish adults in the D2D cohort. Scores were used in D2D study population (n=2849). Prevalence of NASH based on NASH Score and NASH Liver Fat Score were 4.2% and 6.0%, respectively. Because the accuracy of the developed scores was rather good, but not excellent, sensitivity analyses were made using a Bayesian model. Model gave estimates of 3.1% (NASH Score) and 3.6% (NASH Liver Fat Score) for the prevalence of NASH. Finally, based on the primary and Bayesian analyses, the prevalence of NASH in Finnish 45-74 year old subjects was estimated to be approximately 5%.

Table 9. Comparison of the performances of the NASH score with a cut-off -1.054 in the Helsinki-Kuopio cohort (n=296) and in the Italian cohort (n=380).

Population	AUROC (95% CI)	Sens.%	Spec.%
Finnish biopsy cohort			
Discovery cohort	0.776 (0.701, 0.852)	75	74
Internal validation	0.758 (0.626,0.891)	65	73
Whole study group	0.774 (0.709, 0.839)	72	74
Italian validation cohort			
Whole study group	0.759 (0.711,0.807)	39	89

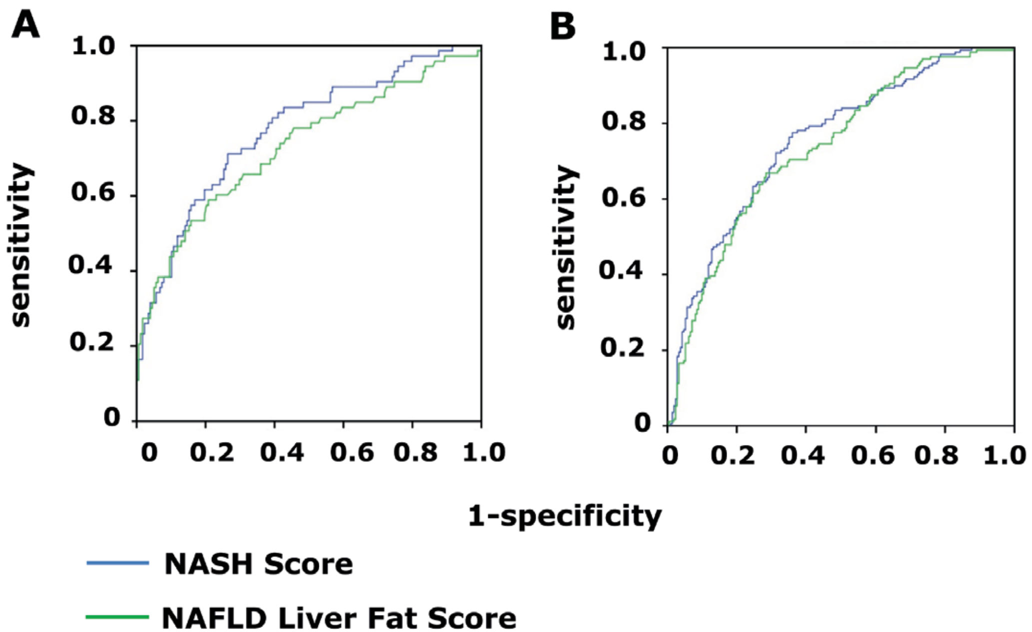

Figure 7. Comparison of NASH Score and NASH Liver Fat Score in Helsinki-Kuopio cohort (A) and in Italian validation cohort (B).

5.2 SERUM CK-18 AS A MARKER OF NASH IN INTERVENTIONS (STUDY II)

The main finding of study II was that serum CK-18 can be used for the screening of NASH in high-risk individuals, but it is not useful as a marker of intervention effect on NAFLD when the predicted probability of NASH is low.

KOBS. Serum CK-18 M30 fragments correlated with serum ALT and AST levels (r = 0.347, P = $9.2x10^{-5}$ and r_s = 0.695, P = $1.1x10^{-14}$; respectively), and also with liver steatosis, inflammation and fibrosis (r = 0.348, P = $7.7x10^{-5}$; r= 0.377, P = $1.6x10^{-5}$ and r = 0.210, P = 0.019; respectively) in the KOBS cohort. The concentration of serum CK-18 was significantly higher in those with NASH (284 ± 161 U/L in those with normal liver, 331 ± 200 U/L in those with simple steatosis and 554 ± 498 Ul/L in those with NASH, P = 0.004). CK-18 was also higher in those with NASH when pairwise compared to those with normal liver and also those with simple steatosis (P < 0.05). CK-18 had AUROC of 0.668 (95% CI: 0.555-0.781) for finding NASH in the KOBS population.

The change in serum CK-18 concentration correlated with ALT change (r = 0.454, P = $3.9x10^{-6}$) and CK-18 concentration decreased significantly after obesity surgery in the whole KOBS population (376 ± 303 U/L vs. 218 ± 187 U/L, P = $4.3x10^{-15}$) (Figure 8). There was a significant decrease in CK-18 concentration in all liver phenotype groups: in normal liver (284 ± 161 vs. 241± 167 U/L, P = 0.006), in simple steatosis (332 ± 200 U/L vs. 225 ± 145 U/L, P = 0.004) and in NASH (554 ± 498 U/L vs. 236 ± 298 U/L, P = $4.2x10^{-5}$) (Figure 9). The largest decrease was in those with NASH (-31 ± 97%). The decrease in CK-18 was not explained by weight loss, because weight loss was equal in the study groups (-20 ± 9% in those with normal liver, -26 ± 7% and -25 ± 7% in those with NASH; respectively, P = 0.116).

The *TM6SF2* rs58542926 genotype (171) associated with the CK-18 change (r = 0.225, P = 0.034) suggesting that carriers of E167K variant (CC vs. CT) had smaller decrease in CK-18 concentration after the surgery (-24% vs. -3%, respectively, P = 0.035). However, there was no association between CK-18 change and *PNPLA3*.

Diet intervention study. Serum CK-18 concentration was not different in HEPFAT study population before the intervention between n-6 PUFA and SFA groups (P = 0.431). Liver fat content measured with MRS correlated with serum CK-18 concentration (r = 0.317, P = 0.017) and serum ALT level (r = 0.183, P = 0.177) at baseline. Mean liver fat percent of the whole study group was 5.6 ± 6.6% and CK-18 concentration was 215 ± 230 U/L in those with low liver fat (< 5%) and 263±211 U/L in those with high liver fat (≥ 5%) (P = 0.117). Serum CK-18 concentration did not change significantly in the PUFA group (P = 0.190) or in the SFA group (P = 0.914), but tended to be lower after the n-6 PUFA diet compared to the SFA diet (192 ± 175 mmol/L and 271 ± 325 U/L, respectively, P = 0.064). There was no significant effect of the diets on CK-18 concentration in the subgroups with low and high liver fat content. However, there was a trend for increase in CK-18 after the intervention in those with high liver fat (P = 0.087). In addition, weight did not change significantly during the study (P = 0.210).

VLCD intervention study. Because ALT level at baseline and the effect of the intervention on the liver fat were mild in the HEPFAT study, it was investigated how more effective VLCD intervention, known to decrease liver fat content (365), affects serum CK-18 levels. Study subjects lost weight in both study groups (16.5±6 kg and 1.7±4 kg at 3 months, and 10.7±6 kg and 2.4±6 kg at 12 months, respectively). In the VLCD group, CK-18 decreased significantly at 3 months (352±144 U/L vs. 269±128 U/L, P = 0.003) and also at 12 months (352±144 U/L vs. 280±118

U/L, P = 0.029). In the control group serum CK-18 level decreased at 3 months (311±130 U/L vs. 249±92 U/L, P = 0.007), but not at 12 months (P = 0.673).

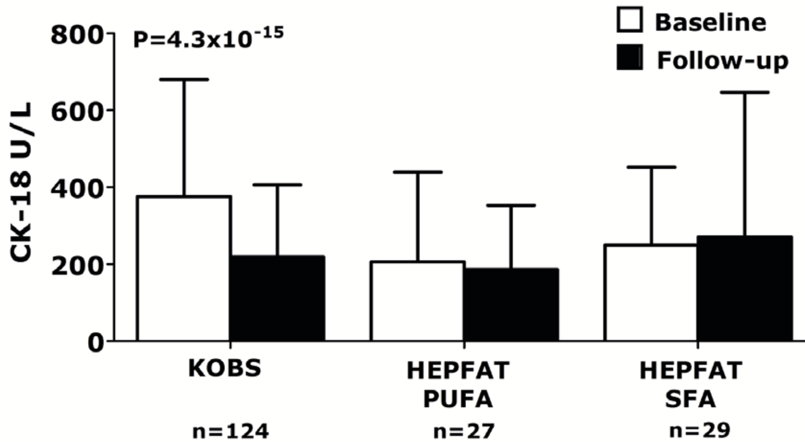

Figure 8. CK-18 levels before and after the intervention in the KOBS and in the HEPFAT study separately for the groups on PUFA and SFA diet. The Wilcoxon nonparametric test was used for statistical testing.

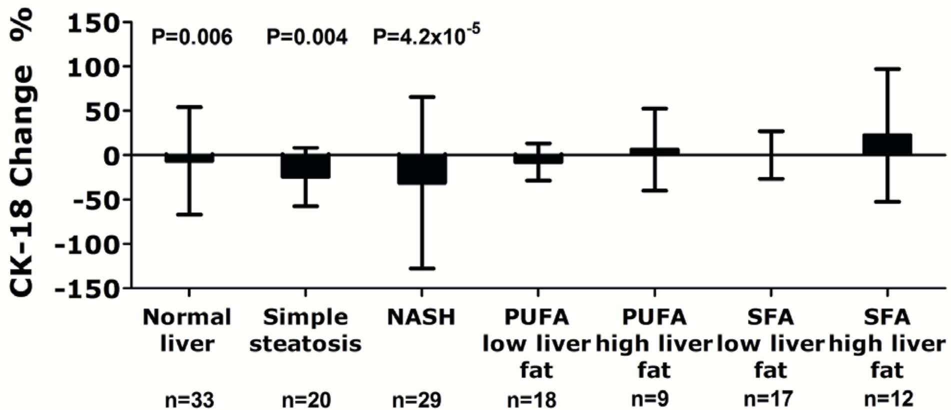

Figure 9. The change in CK-18 during the intervention in different study groups. The Wilcoxon nonparametric test was used for statistical testing.

5.3 LIPOPROTEIN SUBCLASSES ASSOCIATE WITH NASH INDEPENDENTLY OF STEATOSIS (STUDY III)

The main finding of study III was that cholesterol of lipoprotein subclasses associate with NASH independently of simple steatosis. Total and LDL cholesterol were higher in individuals with NASH compared to those with simple steatosis in traditional serum sample analyses ($P = 0.002$ and $P = 0.007$, respectively). The results were essentially same, when individuals using cholesterol lowering medication (n=21) were excluded. Steatosis grade associated with higher fasting insulin levels, but not with cholesterol levels. However, lobular inflammation and stage 1 fibrosis associated with serum total and LDL cholesterol levels. There was no difference when comparing individuals without fibrosis to those with grade 2-4 fibrosis.

Table 10. Clinical characteristics based on liver phenotype in the KOBS population (Study III and Study IV). Kruskal-Wallis test for continuous variables and Chi-Square test for categorical variables. #Normal liver vs. NASH $P < 0.05$, ▲Steatosis vs. NASH $P < 0.05$.

	Normal liver (n=32)	**Simple steatosis (n=19)**	**NASH (n=25)**	**P value over groups**
Sex (male-female)	11/21	4/15	10/15	0.404
Age (y)	47.9 ± 9.7	45.8 ± 9.8	46.7 ± 8.0	0.725
Weight (kg)	127.2 ± 19.4	126.2 ± 14.8	132.1 ± 24.5	0.676
BMI (kg/m2)	44.1 ± 6.8	44.8 ± 4.3	44.3 ± 6.9	0.716
Fasting glucose (mmol/L)	6.1 ± 0.9	6.3 ± 1.2	6.5 ± 1.6	0.929
Fasting insulin (mU/L)	14.5 ± 9.0	19.7 ± 10.1	25.4# ± 17.1	**0.006**
Type 2 diabetes (%)	8 (25)	6 (31)	11 (44)	0.257
Total cholesterol (mmol/L)	4.23 ± 0.8	3.80 ± 0.9	4.74#▲ ± 1.0	**0.004**
LDL cholesterol (mmol/L)	2.48 ± 0.7	2.11 ± 0.8	2.89▲ ± 1.0	**0.010**
HDL cholesterol (mmol/L)	1.07 ± 0.3	1.02 ± 0.2	1.06 ± 0.4	0.539
Total triglycerides (mmol/L)	1.49 ± 0.7	1.46 ± 0.6	1.74 ± 0.6	0.103
ALT (IU/L)	40.6 ± 25.1	39.1 ± 15.9	56.8 ± 35.1	0.117
AST (IU/L)	28.3 ± 10.1	28.7 ± 8.1	42.7 ± 30.1	0.066
CK-18	455 ± 505	290 ± 185	362 ± 226	0.459
PNPLA3 rs738409 (n)				0.327
CC	23	11	16	
CG	9	6	5	
GG	0	2	3	

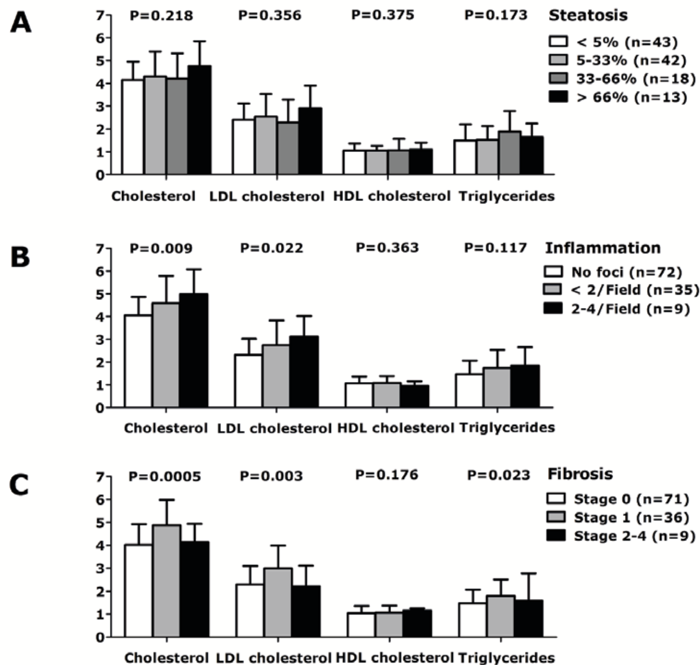

Figure 10. Serum lipid levels with traditional enzymatic method in individuals divided into groups by (A) steatosis (B) inflammation and (C) fibrosis. The Kruskal-Wallis test was used to assess differences among groups.

Serum NMR analysis. NMR spectroscopy analysis (354,358) revealed that total lipid concentration of all VLDL (excluding very small VLDL) together with medium and small LDL associated with NASH (FDR < 0.1). The total lipid concentrations in VLDL, IDL and LDL subclasses (excluding very small VLDL) did not associate with steatosis, but associated with inflammation and grade 1 fibrosis. In more advanced fibrosis, lipoprotein lipid concentrations were lower than in grade 1 fibrosis (Figure 11). The total lipid concentration of all VLDL and LDL particles also had an association with both the NAFLD activity score and ballooning (FDR < 0.1).

The cholesterol concentration of VLDL (except in small VLDL), IDL and LDL subclasses associated with inflammation and fibrosis (FDR < 0.05), but not with steatosis (Figure 12). Interestingly, association of cholesterol concentration of large, medium and small VLDL, and large and medium LDL with liver inflammation was independent of steatosis, total TGs and fasting insulin (all P < 0.05)

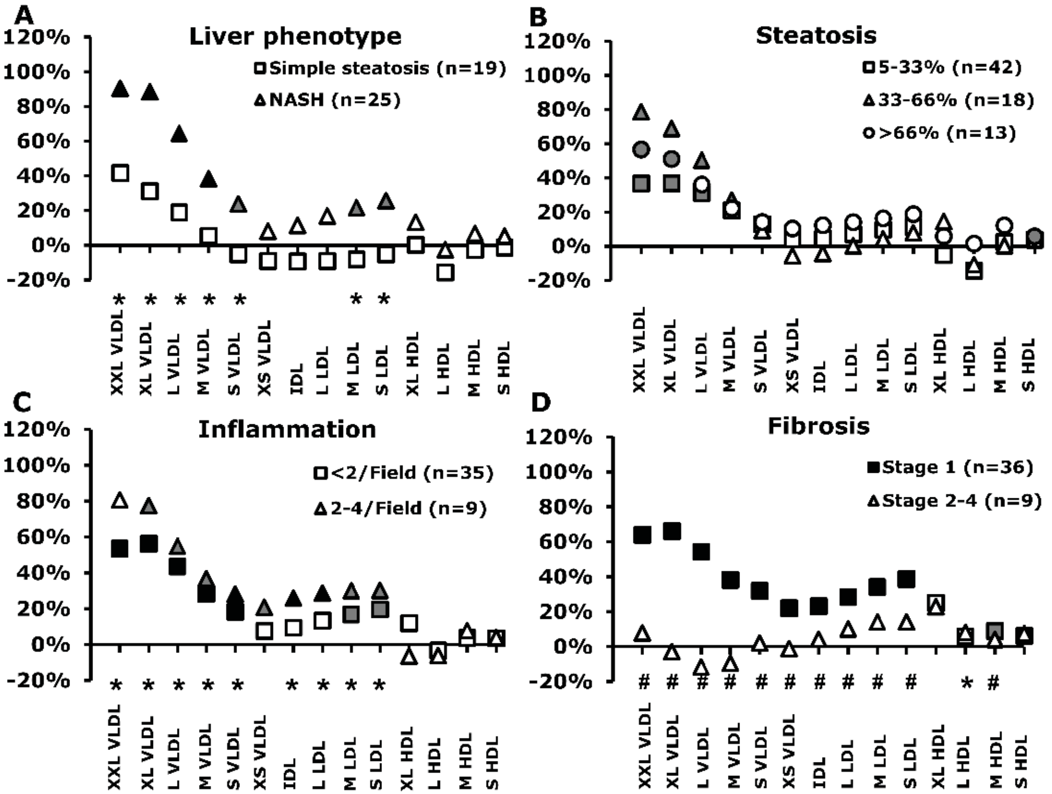

Figure 11. Lipoprotein subclass lipid concentration in individuals divided into groups by (A) liver phenotype, (B) steatosis grade, (C) lobular inflammation and (D) fibrosis stage. Percentage changes comparing to the group without the pathology (set to 0%) have been calculated. Statistical significance over all groups (normal and all degrees of pathology in each panel) are visualized with *= false discovery rate (FDR) < 0.05 and # = FDR < 0.01 compared to individuals without the pathology below the horizontal axis in each panel. Color of the symbol (white, gray, black) indicates subgroup analysis comparing given group to the group without pathology (gray indicates P < 0.05, black P < 0.01).

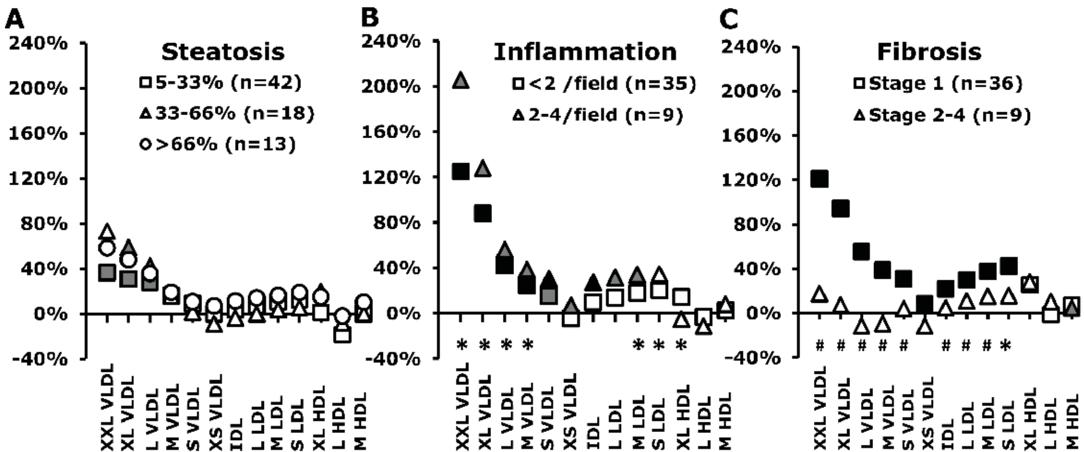

Figure 12. Lipoprotein subclass cholesterol content in individuals divided into groups by (A) steatosis grade, (B) inflammation and (C) and fibrosis stage. Description of symbols is similar to Figure 9.

Lipoprotein subclasses and liver cholesterol content. Liver total cholesterol content correlated positively with lobular inflammation (r_s = 0.393, P = 0.004), but not with steatosis (r_s = 0.258, P = 0.065) or fibrosis (r_s = -0.186, P = 0.221). Liver total cholesterol content (n=52) correlated with subclasses of VLDL cholesterol, but not with LDL and HDL cholesterol (Figure 13). Liver free cholesterol content (n=45) correlated with liver total cholesterol content (r_s = 0.419, P = 0.024), but not with steatosis, lobular inflammation or fibrosis. Furthermore, liver free cholesterol content correlated with cholesterol of all VLDL subclasses (r_s = 0.315-0.381, P < 0.005) except with small VLDL (r_s = 0.279, P = 0.063), but not with LDL and HDL cholesterol (Figure 13).

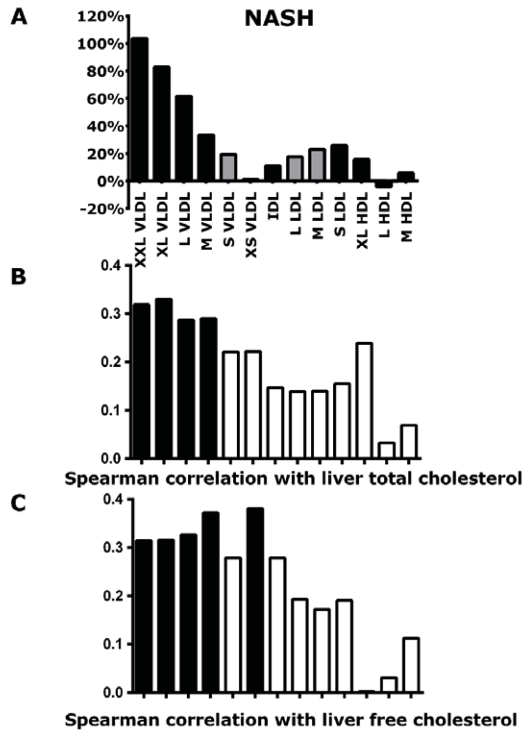

Figure 13. (A) Lipoprotein subclass cholesterol concentration in individuals with NASH compared to those with normal liver. Percentage changes comparing to the group without the pathology (set to 0%) have been calculated and color of the symbol indicates subgroup analysis comparing given group to the group without pathology (gray indicates P < 0.05, black P < 0.01). Spearman correlation between lipoprotein subclass cholesterol concentration and liver cholesterol content (B), and liver free cholesterol content (C) (black bars P < 0.05).

Gene expression in the liver. Because high VLDL cholesterol is linked with increased cholesterol synthesis in the liver (137), liver mRNA expression of genes regulating cholesterol synthesis and uptake was measured. These genes did not correlate with liver histology (all P > 0.3) and had only some correlations with different lipoprotein cholesterols. However, *XBP1* splicing correlated with lobular inflammation (r_s = 0.272, P = 0.014) and ballooning (r_s = 0.243, P = 0.029), but not with steatosis (r_s = 0.080, P = 0.479). *XBP-1* splicing also associated with cholesterol concentration of small VLDL, IDL, LDL subclasses and medium HDL (r_s = 0.220-0.313, P < 0.05).

5.4 LIPID OXIDATION AND ALTERED KETONE BODY METABOLISM IN NASH (STUDY IV)

The main finding of study IV was that individuals with NASH had lower ketone body levels and altered ketone body metabolism.

Levels of ketone bodies β-OHB and AA were significantly different between study groups (P = 0.011 and P = 0.017, respectively), and they were lower in individuals with NASH compared to those with simple steatosis (P = 0.004 and P = 0.018, respectively) (Figure 14). However, there was no difference between those with normal liver and those with simple steatosis (P = 0.106 and P = 0.267, respectively). Levels of ketone bodies also associated with liver cell ballooning (P = 0.0005 for β-OHB and P = 0.0003 for AA), but interestingly not with steatosis and fibrosis.

Both β-OHB and AA levels correlated with negatively with RQ (r_s = -0.614, P = 0.001 and r_s = -0.615, P = 0.001; respectively) and had a positive correlation with the rate of lipid oxidation (r_s = 0.663, P = 0.0004 and r_s = 0.688, P = 0.002; respectively). Serum citrate levels had a similar trend to ketone bodies. Citrate levels were lower in those with NASH than in those with normal liver (P = 0.009), or simple steatosis (P = 0.007). However, other energy metabolism-related LMWMs (like lactate and pyruvate) were not different between study groups.

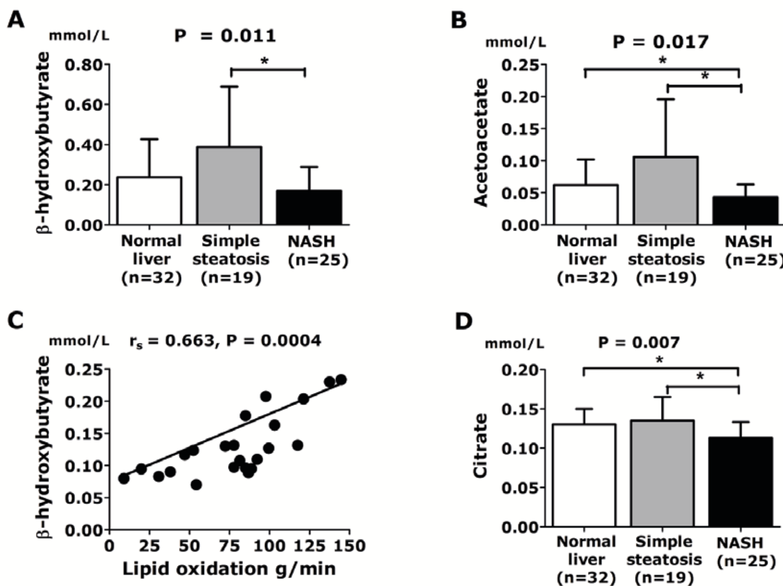

Figure 14. Levels of ketone bodies β-hydroxybutyrate (A) and acetoacetate (B) differed between the study groups (KOBS) and were lower in individuals with NASH than in those simple steatosis. Lipid oxidation had a positive correlation with β-hydroxybutyrate levels (C). Serum citrate levels were lower in individuals with NASH than in individuals with steatosis or normal liver. Asterisk denotes a P value < 0.05 (Kruskal-Wallis test).

Replication of the primary results. Finding of lower β-OHB levels was validated in the Helsinki-Kuopio cohort (study I). β-OHB was measured with an enzymatic method in 188 individuals with an overlap of 56 individuals from the KOBS cohort. β-OHB tended to be lower in individuals with steatosis combined with necroinflammation than in those with simple steatosis (P = 0.060). When normal weight individuals (BMI under 25 kg/m^2) were excluded, the

difference was statistically significant (n = 185 and P = 0.041). The result was also validated in the METSIM population-based study. The non-invasive NASH Score developed in this thesis found 5086 individuals below the cut-off and 3585 individuals above the cut-off, meaning possible NASH. β-OHB levels were lower in those below the cut-off (0.137 vs. 0.129 mmol/L, P = 0.001) (Figure 15).

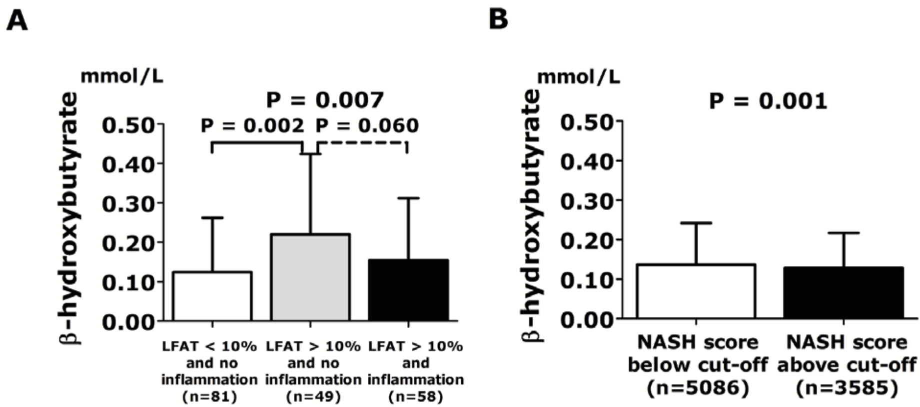

Figure 15. Ketone body levels in the Helsinki-Kuopio cohort (A) and in the METSIM population study population (B). The Kruskal-Wallis test was used to assess differences among groups.

FFAs and ketone bodies. Serum fasting FFAs correlated positively with β-OHB and AA (r_s = 0.559, P = $6.9x10^{-11}$ and r_s = 0.452, P = $3.5x10^{-7}$, respectively). They were also lower in NASH than in simple steatosis (0.74 vs. 0.55 mmol/L, P = 0.030). In multivariate logistic regression analysis, the predictive value of age, weight, BMI, ALT, AST, bilirubin, alkaline phosphatase, INR, fasting glucose, fasting insulin, CK-18, β-OHB and serum fasting FFAs in diagnosing NASH or ballooning was tested. After backwards logistic regression, β-OHB remained in the model, suggesting that it was the strongest independent predictor of NASH.

Gene expression in the liver. Liver gene expression of lipid and ketone body metabolism associated genes revealed that β-OHB and AA correlated negatively with SREBP-1c (r_s = -0.318, P = 0.004 and r_s = -0.308, P = 0.005; respectively). Both β-OHB and AA had a negative correlation with fatty acid synthase (FASN) (r_s=-0.355, P=0.0009 and r_s=-0.382, P=0.0003; respectively).

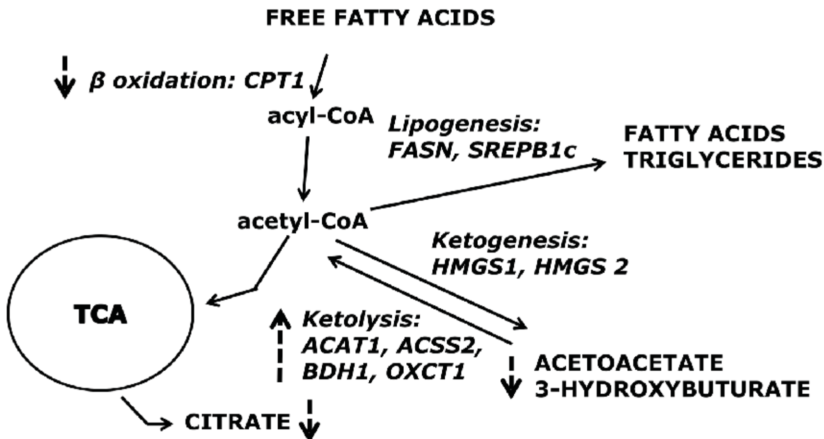

Figure 16. Genes in liver metabolism analysed in study III. Dashed arrows mark the direction of the change in metabolic pathways associated with NASH. TCA = tricarboxylic acid cycle.

Because lower FFA supply did not explain the lower ketone body levels in NASH, liver gene expression of genes regulating fatty acid synthesis, fatty acid oxidation, ketogenesis and ketolysis was analysed (Figure 16). There were no differences between liver phenotype groups in genes regulating fatty acid oxidation and ketogenesis. However, expression of ketolytic genes (*ACAT1* and *BDH1*) was higher in NASH. Furthermore, expression of *ACAT1*, *ACSS2* and *BDH1* correlated positively with lobular inflammation (r_s = 0.388, P = 0.0003, r_s = 0.379, P = 0.0006 and r_s = 0.385, P = 0.0004, respectively), but interestingly not with steatosis and fibrosis. Liver gene expression of *ACAT1* had also a positive correlation with ballooning (r_s = 0.262, P = 0.018).

6 Discussion

6.1 PATIENTS AND METHODS

6.1.1 Study design and subjects

The KOBS cohort was the primary study population in this thesis. It had over 100 well characterized subjects with detailed liver histology. Individuals in KOBS cohort had clinical and laboratory measurements taken at baseline and one year after surgery. This cohort provided large prospective study population to investigate metabolic changes associated with NASH. In addition, control measurements one year after the operation provided option to investigate changes associated with the improvement in liver disease. Individuals in KOBS were severely obese and thus observed results may not be generalized to normal weight subjects. However, obesity is a strong risk factor for NAFLD (1,2), and thus the KOBS cohort was an optimal population for studying NAFLD. However, NAFLD can be present also in lean individuals (133), and *PNPLA3* genotype at rs738409 is strongly associated with NAFLD in lean subjects (157). The association of *PNPLA3* with NASH would optimally be investigated in a cohort including both normal weight and obese individuals. This should be considered in the future, although it is challenging to justify taking liver biopsies if there is no suspicion of NASH.

The KOBS cohort has some limitations. First, there is no control group of lean individuals. However, characterization of different liver phenotypes gave an opportunity to compare results within the same BMI range. Furthermore, it should be kept in mind that the primary focus of these studies was liver disease. Unfortunately, follow-up liver biopsy was available only from 11 individuals. However, obesity surgery is known to ameliorate NASH (70,71,311-313). Second, the majority (108 of 116) of the patients had VLCD 4 weeks before the operation. This could have affected the results, especially in the study IV. A VLCD diet is essential before the obesity surgery to decrease the liver fat content. In addition, 21 out of 116 individuals had cholesterol lowering medication (statin), which regulate cholesterol metabolism. Importantly, results in the study III were essentially same if those having statin treatment were excluded.

Some measurements in KOBS cohort were not available from all study subjects. Indirect calorimetry was measured only in 15 individuals (5 with normal liver and 10 with NASH). However, when rates of lipid oxidation and metabolism of carbohydrates were calculated, clearly statistically significant results were achieved. Furthermore, liver total and free cholesterol were analysed in a limited number of subjects (n=52 and n=45, respectively) in the KOBS. Limited sample size might have had an effect on results. However, sample numbers are higher than (210) or similar to (224) previous studies.

The Helsinki-Kuopio cohort was a larger cohort including 89 individuals from the KOBS cohort. Subjects in the cohort had all the essential variables measured for the development of the non-invasive score. The majority of individuals in the Italian cohort have had liver biopsy because of persistently abnormal liver enzymes, representing real-life patients. CK-18 measurement was not available in the Italian cohort. Because CK-18 did not remain in the NASH Score, it was not essential. However, it would have been interesting to test the ability of

CK-18 for diagnosing NASH in a big Italian cohort. The Italian cohort subjects were only moderately obese, which can be considered a limitation when comparing to a severely obese primary cohort. However, the NASH Score proved to perform equally well also in Italian cohort.

D2D and METSIM study offered big, well characterized populations with comprehensive data to validate primarily observed results. Genotyping of *PNPLA3* at rs738409 in the D2D population gave an excellent opportunity to use the NASH Score in this cohort and evaluate the prevalence of NASH in Finland for the first time. The D2D and METSIM population based studies had mainly slightly overweight individuals, which can limit the comparability of their results with the results of the KOBS. However, these populations were used as validation and replication cohorts to strengthen observed results. ALT was used as a marker of liver disease in METSIM population. Because ALT is not optimal marker of NASH (89), non-invasive score was formed to find those with NASH. Unfortunately, AST was not measured in these individuals and thus the NASH score from study I could not be used.

The HEPFAT study was a randomized, parallel-group study. However, it was a quite small study with 67 individuals, and only 56 individuals had all measurements needed in study IV. In addition, the 10 week intervention was rather short to cause large changes. Nonetheless, it was a well conducted randomized study that demonstrated that dietary fatty acid composition has an effect on liver fat. HEPFAT study subjects were moderately obese (BMI 31) and those with BMI >40 were excluded from the study. Additionally, their mean liver fat content was only 5.6 ± 6.6%. Thus, it is probable that study subjects had mainly steatosis and not NASH. However, liver fat content decreased in HEPFAT trial (305), and thus measuring the change in CK-18 was justified. Low ALT and low liver fat content in HEPFAT study subjects might reflect lack of NAFLD and could explain the small change in CK-18 levels in response to the intervention. Thus, an additional VLCD study cohort with higher baseline ALT was included in the study II.

6.1.2 Clinical measurements and laboratory methods

Indirect calorimetry was used as an estimate of the energy expenditure based on oxygen consumption and carbon dioxide excretion. Direct measurement is not possible and indirect calorimetry is the best way to measure the energy consumption in clinical populations (366). Standardized and validated laboratory methods for blood sample analyses were used. NMR spectroscopy has been already applied in various large-scale epidemiological and genetic studies (367,368), and it has been proven to quantify serum lipids and lipoproteins reliable and cost-effectively (356). The main advantage of NMR spectroscopy is to measure several different particles in a single sample rapidly (356). This method offers a hypothesis-free approach to find associations in epidemiological studies. However, in the studies of this thesis, NMR spectroscopy was used as a normal laboratory measurement in a hypothesis driven fashion. NMR spectroscopy offered an optimal method to investigate lipoprotein lipid profile in those with NASH.

Liver biopsies offered unique opportunities also for analyses other than standard histological evaluation. These included liver gene expression, which gave essential information about metabolic pathways when combined with other results. Furthermore, possibility to measure liver total cholesterol content gave deeper understanding about cholesterol metabolism in those with NASH. Importantly, in study IV NMR spectroscopy was used to quantify liver cholesterol

content. NMR has been rarely used for that purpose and measurement was obvious strength in this thesis, because liver free cholesterol is strongly associated with NASH (210,228,230). Method for liver NMR cholesterol analysis was developed for this thesis and thus has not been validated before. However, total cholesterol measured with NMR had excellent correlation with the results from the GLC measurement.

Estimation of insulin sensitivity was based on levels of fasting insulin, which can be thought of as a non-optimal method. The hyperinsulinemic-euglycemic clamp has been considered as the gold standard for assessing insulin action in vivo. However, fasting insulin levels have a moderately high correlation with results achieved with the clamp and are widely used as a crude measure of insulin resistance (369).

6.1.3 Diagnosis of NASH

In the studies of this thesis, liver biopsy was used for diagnosing NASH. Liver biopsy is a gold standard for the diagnosis of NASH (295) and thus the optimal method was used. However, liver biopsy presents only a small amount of the liver, which makes limitations for histological analysis. Nonetheless, a better option for diagnosis is not available and multiple biopsies are usually not done, because of the risk of complication (294). Because liver biopsy analysis is pathologists dependent (295), in these studies a maximum of two pathologists per study were used to evaluate liver biopsies. The histological characterization was different in the Helsinki-Kuopio biopsy cohort and therefore the results cannot be directly compared to those with KOBS (study IV). A limitation of the population-based studies is that liver biopsies were not available. However, it is ethically impossible to obtain liver biopsies in population-based studies.

6.2 NON-INVASIVE SCORE TO FIND NASH (STUDIES I AND II)

Non-invasive NASH Score was carefully developed and validated and is one of the few, which have been both internally and externally validated (267). External validation is essential, before real benefits of the score can be evaluated. Study I gave novel information about the prevalence of NASH in Finnish adult subjects. Since NASH score worked equally well in Italian validation cohort, the prevalence estimate could be generalized for Europeans with a similar range in age and BMI. Results of study I are very important, because previous prevalence estimates of NASH are based on selected groups of patients (1,111,114-117,370,371) suggesting that their results cannot be generalized to the standard population. Results from study I suggest that NASH is very common disease. Since it increases both mortality and morbidity, more attention should be paid to decrease the prevalence of the main risk factor, obesity. Interventions should be focused also for adolescents and importantly even before individuals are obese. Based on the estimated prevalence, NASH will be more common reason for liver transplantation in the future also in Finland (132). Alcohol has been the major cause for liver cirrhosis in Finland (372). Nonetheless, it is probable that obesity together with high alcohol consumption causes liver injury earlier than each alone (373).

The development of the NASH Score revealed that easily available AST is one of the best routine markers to find NASH. AST might have been used too sparingly in Finnish health care system in recent years. Although transaminases might be normal in those with NASH (89), AST measurement should be considered when taking liver enzymes in obese individuals. In

addition, *PNPLA3* at rs738409 associated with the risk of NASH like previously published (157). *PNPLA3* genotyping option should be available more widely than it is today. It would help to plan follow-up for patients with NAFLD, because carriers of G/G genotype are at risk for more severe disease (167). Additionally, genotyping could strengthen the diagnosis when lean individual is having NAFLD.

The NASH Score is not accurate enough for being a diagnostic tool. It had a good, but not excellent AUROC (0.774) for finding NASH. However, the NASH Score could help to find patients with possible NASH when considering liver biopsy. Secondly, NASH Score could be used in population-based studies where liver biopsies cannot be obtained. The limitation for the NASH score use is that *PNPLA3* genotyping is not widely available, and insulin measurements have not been standardized. The AUROC of the NASH score was similar to that of other NASH-predicting tests in a meta-analysis of 494 severely obese individuals (279), but slightly worse than the Nice model (components CK-18, ALT and metabolic syndrome) (271). However, the Nice model has not been validated externally. Scores that have had good or excellent AUROCs (112,268,270,275) have small study populations, and have not been externally validated.

Results from study I and II suggest that CK-18 is a good, but not optimal biomarker for detecting NASH. Interestingly, CK-18 did not remain in the NASH Score (study I), even though it had an AUROC almost as high as the NASH Score (0.727). This might be because of the high standard deviation of values. The results suggest that CK-18 cannot be used as a diagnostic tool, but it could help as a screening method (260). With a low cut-off it could be used to screen for fatty liver, and with a higher cut-off to screen fibrosis (264). Option for CK-18 measurement would help in clinical decision-making, when considering the possibility of NASH. However, based on findings from study II, CK-18 is not useful as a marker of intervention effect for the liver if the predicted probability of NASH is low (low liver fat content) or the intervention effect on the liver is modest. Finally, it should be highlighted that the need for optimal biomarker is obvious. However, as long as the pathogenesis of NASH is unclear, it will be difficult to find an optimal biomarker for NASH.

6.3 LIPOPROTEIN SUBCLASSES ASSOCIATE WITH NASH INPENDENTLY OF STEATOSIS (STUDY III)

Study III supported the idea about the importance of cholesterol metabolism in NASH. Previously reported association of cholesterol metabolism with simple steatosis (13,137) and NASH (210,211) was further clarified. The major finding was the association of liver inflammation and lipoprotein subclass cholesterol independent of liver steatosis, TGs and fasting insulin.

Liver free cholesterol accumulation has been suggested to be a key factor in NASH causing mitochondrial stress (243,244), but it has still been limitedly studied in humans with NASH (210,224,228). In study III, liver total cholesterol content correlated with liver inflammation. Liver free cholesterol content correlated with liver total cholesterol, but not with liver steatosis, inflammation or fibrosis. However, liver free cholesterol content was associated with cholesterol concentration of majority of VLDL subclasses. This suggests that liver free cholesterol content reflects increased cholesterol synthesis, but does not support its individual

role in NASH (230,243). Nonetheless, this may be because of limited number of free cholesterol measurements in this study (n=45) and should be studied further.

Interestingly, the expression of cholesterol synthesis genes did not associate with steatosis, inflammation, or fibrosis. This suggests that increased cholesterol synthesis does not explain the role of cholesterol and its accumulation in NASH. However, liver gene expression of *XBP1* splicing associated with liver inflammation and ballooning, but not with steatosis. It also associated with the cholesterol concentration of small VLDL, IDL, LDL subclasses and medium HDL. *XBP1* splicing takes part in liver lipid metabolism (203) and has a role in ER stress in the liver (204). ER stress can cause liver steatosis (200), further disturb ER function (18) and promote liver injury. Upregulation of *XBP1* splicing may have a crucial role in starting this cycle.

Results from study III support previous findings that non-HDL cholesterol (VLDL+LDL) has been associated with NASH (374). It seems improbable that cholesterol subclass panel would work as a biomarker of NASH, because cholesterol levels vary widely also without NASH. However, the possible benefits of NMR lipidomics panel for finding NASH should be studied in a larger human population.

Finally, also based on results of study III, drug therapy focusing on cholesterol metabolism in NASH needs urgently more research. There are already studies about the benefits of statins (332,333), and some data about the ezetimibe treatment (334). The combination of statin and ezetimibe (blocking cholesterol synthesis and absorption) should be investigated, although cholesterol absorption is reported to be decreased in NAFLD (211). In addition, the possibility that *PNPLA3* I148M genotype limits the effect of statins on steatosis and NASH (333) should be further studied.

6.4 LIPID OXIDATION AND ALTERED KETONE BODY METABOLISM IN NASH (STUDY IV)

The most important finding from study IV was that serum ketone body levels (AA and β-OHB) were lower in NASH than in simple steatosis. This suggests altered ketone body metabolism in those with NASH. Previously, lipid oxidation rates in NAFLD has been controversial (16,21-24). Based on the levels of ketone bodies in study IV, oxidative metabolism is possibly increased in those with simple steatosis, but significantly decreased in those with NASH. Observed decrease in lipid oxidation (21) suggests mitochondrial dysfunction in NASH (20). Mitochondrial dysfunction could result from the changes in cholesterol metabolism. Possible reasons may be increased cholesterol synthesis and cholesterol accumulation (study III) and altered trafficking in the liver cell, leading to ER stress. This could further lead to free cholesterol accumulation in the liver and especially in the mitochondria (228,243,244). If mitochondrial oxidative function declines, also fatty acids can accumulate and cause lipotoxicity and NASH progression (180).

Decreased oxidative metabolism was further supported by decreased citrate levels in NASH. Lower citrate levels might be caused by less substrate for the TCA cycle, meaning decreased amounts of Acyl-CoA from β oxidation. Thus, ketone body levels might decrease, because ketolysis may compensatorily increase to provide more Acyl-CoA for TCA cycle. This was supported by overexpressed ketolysis genes *ACAT1* and *BDH1* in the liver of those with NASH.

Based on the results from study IV, ketone body levels cannot be considered biomarkers of NASH, because they seemed to be increased in simple steatosis (Helsinki-Kuopio cohort), but decreased in NASH. It would therefore be difficult to separate those with normal liver from those with NASH. However, ketone body measurement in those with fatty liver may be helpful revealing the development of higher grade inflammation. Thus, further studies about this subject are urgently needed. The possibility if ketone bodies and citrate levels could be used as biomarkers of NASH (maybe together with serum CK-18) should be considered.

Finally, drug development in NASH should be addressed for protection of mitochondrial function. There are already data from a study in mice suggesting that mild mitochondrial uncoupling reverses diabetes and NASH (375). However, data about cholesterol toxicity (study III) and cholesterol-associated mitochondrial disturbances (228,243,244) in NASH suggest that cholesterol metabolism should perhaps be the primary treatment target.

7 Conclusions

The main findings and conclusions of these series of studies were:

I *PNPLA3* rs738409 genotype together with routine laboratory tests could be used as a non-invasive method for screening NASH. The prevalence of NASH based on this screening in Finnish adults is approximately 5%.

II CK-18 could be used as a biomarker of NASH, if the predicted probability of the disease is high.

III Cholesterol metabolism is associated with the development of NASH independently of simple steatosis.

IV Lipid oxidation is decreased in NASH, suggesting mitochondrial dysfunction.

8 Future Perspectives

Serum CK-18 has been suggested as a screening tool for NASH (261-263), although its sensitivity to distinguish simple steatosis from NASH has been questioned (264). Studies of this thesis support those findings. However, the potential of CK-18 as a biomarker of NASH needs to be studied further. Because an optimal biomarker for NASH is still undiscovered, additional studies about biomarkers are urgently needed.

There are two interesting genotypes associated with NASH: *PNPLA3* rs738409 (157) and *TM6SF2* rs58542926 (168). Study II suggests that *TM6SF2* variant carriers may have smaller decrease in CK-18 after the surgery. Thus, it is important to investigate if the effect of obesity surgery on NASH differs based on NASH risk genotypes. Additionally, it should be studied if the usefulness of CK-18 for finding NASH depends on NASH associated genotypes.

Although the pathogenesis of NASH is still not thoroughly understood, cholesterol metabolism is strongly associated with the process (14,210,211,228). However, it is not totally clear if the changes in cholesterol metabolism (for example increased cholesterol synthesis and accumulation) are causes or consequences of NASH. Previously non-HDL cholesterol (374) and desmosterol (212) have been suggested as biomarkers of NASH. Further investigation of cholesterol metabolism in NASH should be given top priority. High quality studies are very likely to find more biomarkers of NASH.

Previously the role of free cholesterol in NASH has been studied combining liver gene expression analyses with cholesterol content measurements (224). However, there is an urgent need to find the primary mechanism for free cholesterol accumulation in the liver. This requires careful investigation of cholesterol uptake, synthesis, trafficking and excretion pathways in the liver together with other possible changes in liver lipid content. Furthermore, a study with follow-up liver biopsies with liver lipid content analysis would be highly valuable. It would help to figure out what liver lipids at baseline are associated with more advanced NASH in the follow-up. In addition, other lipids in the liver should be investigated in more detail. These include for example phosphatidylcholines, which are reported to be decreased in the liver of those with steatosis (210,376). Our preliminary data suggests that serum phosphatidylcholines are increased in those with NASH, but phosphatidylcholine content in the liver is decreased in those with NASH.

Although NAFLD and NASH are strongly associated with the metabolic syndrome (44), the *PNPLA3* rs738409 genotype associated NAFLD does not have similar metabolic abnormalities (79), suggesting lower risk for cardiovascular complications. Moreover, the *TM6SF2* rs58542926 genotype is associated with increased liver fat content (170) and increased risk of NASH (171), but decreased risk for cardiovascular disease (171). Metabolomic approach could be used to find metabolical differences between risk genotypes. In addition, subjects with these risk variants could be screened from the population-based study (such as METSIM) to be further evaluated. Examination including liver fat content measurement or liver biopsies (risk variant carriers are at increased risk for NASH) together with serum and liver NMR analyses could reveal differences in energy and cholesterol metabolism based on the risk genotypes.

New treatment options for NASH are needed to ameliorate liver disease and avoid cardiovascular complications. Understanding free cholesterol accumulation and the disturbances in mitochondria have great potential to lead to new clinical solutions in the treatment of NASH. Since mitochondrial dysfunction has critical role in NASH, restoring mitochondrial function is essential in the treatment of NASH. Furthermore, mitochondrial transplantation has been done in animal models in cardiac ischemia (377), and transplantation of autologous mitochondria could help in NASH reversion. Interestingly, administration of β-OHB has been shown to prevent PUFA-induced acute liver failure in an experimental animal model (378). In addition, ketone body supplementation decreases mitochondrial production of ROS in the brain (379). Thus, the administration of ketone bodies and normalization of ketone body metabolism should be tested as a treatment option of NASH. When disturbances of cholesterol metabolism in those with NASH are better understood, also gene therapy options (for example *LDLR* overexpression (380)) could be tested. Finally, human hepatocyte transplantations have already been made in treatment of the Crigler-Najjar syndrome (381), and this approach should be studied further.

9 References

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Ville Männistö

Biomarkers for non-alcoholic steatohepatitis with special emphasis on lipid metabolism

Non-alcoholic steatohepatitis (NASH) is a common cause of chronic liver disease. However, pathogenesis of NASH is still partly unclear. In this thesis, a non-invasive NASH score was developed. It estimated population prevalence of 5% for NASH in Finnish adults. Furthermore, alterations in cholesterol metabolism, lipid oxidation and ketone body metabolism were revealed in those with NASH. This thesis highlights the significance of NASH and relates it with risk factors for other common diseases.

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