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Non-alcoholic Fatty Liver Disease:

The Role of Insulin Resistance, Inflammation and the PNPLA3 I148M Variant

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NON-ALCOHOLIC FATTY LIVER DISEASE: The role of insulin resistance, inflammation and the PNPLA3 I148M variant

Susanna Lallukka

ACADEMIC DISSERTATION

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LIST OF ABBREVIATIONS

36B4	Acidic ribosomal phosphoprotein 36B4
ADIPOQ	Adiponectin
ALT	Alanine aminotransferase
APTT	Activated partial thromboplastin time
AST	Aspartate aminotransferase
AUROC	Area under the receiver operating characteristic
BMI	Body mass index
CCR2	Receptor for MCP-1
CD68	Cluster of differentiation 68
CRP	C-reactive protein
CVD	Cardiovascular diseases
DNL	*De novo* lipogenesis
E	Lysine
F	Coagulation factor
FFA	Free fatty acid
FGG	Fibrinogen gamma-chain
GGT	Gamma-glutamyltransferase
GWAS	Genome-wide association study
HbA_{1C}	Glycosylated hemoglobin A_{1C}
HCC	Hepatocellular carcinoma
HDL	High-density lipoprotein
^{1}H-MRS	Proton magnetic resonance spectroscopy
HOMA-IR	Homeostatic model assessment of insulin resistance
HUSLAB	Helsinki University Hospital Laboratory
I	Isoleusine
IL-6	Interleukin 6
IR	Insulin-resistant
IRS-1	Insulin receptor substrate-1
IS	Insulin-sensitive
K	Glutamic acid
kPa	Kilopascal
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
M	Methionine
MBOAT7	Membrane bound O-acyltransferase domain-containing 7
MCP-1	Monocyte chemoattractant protein 1
NAFL	Non-alcoholic fatty liver/simple steatosis
NAFLD	Non-alcoholic fatty liver disease
NAS	NAFLD activity score
NASH	Non-alcoholic steatohepatitis
NF-κB	Nuclear factor-kappaB
NHANES III	Third National Health and Nutrition Examination Survey
OGTT	2-hour oral glucose tolerance test with 75g of glucose
PAI-1	Plasminogen activator inhibitor-1
PNPLA3	Patatin-like phospholipase domain-containing 3
$PNPLA3^{148II}$	Non-carriers of the PNPLA3 I148M variant

$PNPLA3^{148MM/MI}$	Carriers of the PNPLA3 I148M variant
$PNPLA3^{148MM}$	Homozygous for the PNPLA3 I148M variant
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
PT	Prothrombin time
qPCR	Quantitative real-time polymerase chain reaction
ROC	Receiver operating characteristic
SDHA	Succinate dehydrogenase complex, subunit A
SNP	Single-nucleotide polymorphism
TE	Echo time
TFPI	Tissue factor pathway inhibitor
TM	Mixing time
TM6SF2	Transmembrane 6 superfamily member 2
TNF-α	Tumor necrosis factor alpha
t-PA	Tissue-type plasminogen activator
TR	Repetition time
Twist1	Twist-related protein 1
VLDL	Very low-density lipoprotein
VWF	von Willebrand factor
VWF:RCo	von Willebrand factor ristocetin cofactor activity

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I. **Lallukka S**, Yki–Järvinen H. Non-alcoholic fatty liver disease and risk of type 2 diabetes. *Best Practice & Research Clinical Endocrinology & Metabolism*. 2016; 30:385–95.

II. **Lallukka S**, Sevastianova K, Perttilä J, Orho–Melander M, Lundbom N, Hakkarainen A, Olkkonen VM, Yki–Järvinen H. Adipose tissue is inflamed in NAFLD due to obesity but not due to genetic variation in PNPLA3. *Diabetologia*. 2013; 56:886–92.

III. **Lallukka S**, Luukkonen PK, Zhou Y, Isokuortti E, Leivonen M, Juuti A, Hakkarainen A, Orho–Melander M, Lundbom N, Olkkonen VM, Lassila R, Yki–Järvinen H. Obesity/insulin resistance rather than liver fat increases coagulation factor activities and expression in humans. *Thrombosis and Haemostasis*. 2017; 117:286–294.

IV. **Lallukka S**, Sädevirta S, Kallio MT, Luukkonen PK, Zhou Y, Hakkarainen A, Lundbom N, Orho–Melander M, Yki–Järvinen H. Predictors of NAFLD and liver stiffness – an 11-year prospective study. *Scientific Reports.* 2017; 7:14561.

The publications are referred to in the text by their Roman numerals.

As my own contribution, I participated in designing studies as well as in acquisition and interpretation of the data in all of studies I through IV. I wrote manuscripts and produced figures and tables (studies I–IV). In addition, in study I, I conducted the systematic review of the literature of interest. In studies II and III, I measured the expression of pro- and anti-inflammatory genes in adipose tissue and that of coagulation factor genes in the liver using a qPCR technique. In study IV, I also participated in the clinical examination of study subjects by performing liver stiffness measurements using a transient elastography.

ABSTRACT

Background: Non-alcoholic fatty liver disease (NAFLD) may result from obesity accompanied by insulin resistance and adipose tissue inflammation or from the common genetic variants in *PNPLA3* (rs738409, C>G/I148M), *TM6SF2* (rs58542926, C>T/E167K) and *MBOAT7* (rs641738, C>T). These variants increase the liver fat content and the severity of NAFLD without features of insulin resistance.

Aims: This thesis aimed to determine the following: i) whether NAFLD predicts type 2 diabetes independent of obesity and other known risk factors; ii) whether adipose tissue is inflamed in subjects homozygous for the PNPLA3 I148M variant; iii) whether obesity and insulin resistance rather than liver fat content increase coagulation factor activities and expression, and if adipose tissue inflammation is related to the plasma activities of coagulation factors in subjects not carrying the I148M variant; and iv) which factors predict NAFLD and liver stiffness during an 11-year follow-up period.

Subjects and methods: The present thesis includes a systematic review, two cross-sectional studies and a longitudinal study. Study subjects comprised adult men and women. For aim i), we conducted a systematic review of prospective longitudinal studies to determine if NAFLD predicts type 2 diabetes (study I). We examined a group of 82 subjects for aim ii) (study II), two groups consisting of 92 non-diabetic subjects and 26 morbidly obese patients for aim iii) (study III), and a group of 97 subjects for aim iv) (study IV). In these studies, clinical and biochemical parameters (studies II–IV) and plasma coagulation factor activities (study III) were determined during clinical visits. Liver fat was measured by proton magnetic resonance spectroscopy (studies II–IV) and we used transient elastography to measure liver stiffness as an estimate of fibrosis (study IV). Liver biopsies were performed if clinically indicated (studies III–IV). Needle biopsies of adipose tissue were performed for all subjects in study II and for 26 subjects in study III. In study IV, subjects were examined twice over an interval of 11 years. The gene expression of inflammatory markers in adipose tissue and that of coagulation factors in the liver were measured using quantitative real-time PCR (studies II and III).

Results: Several longitudinal studies, particularly among Asian populations, have shown that ultrasound-diagnosed NAFLD and liver enzymes predict type 2 diabetes independent of confounders such as age and obesity (study I). We found no studies indicating that NAFLD associated with genetic risk variants predicting future risk of type 2 diabetes (study I). The liver fat content was similarly increased in obese/insulin-resistant subjects and in carriers of the I148M variant compared to non-obese subjects and non-carriers of this variant (studies II and III). In obese subjects, the adipose tissue expression of pro-inflammatory chemokine *MCP-1* was increased and anti-inflammatory *ADIPOQ* and *TWIST1* were decreased compared with non-obese subjects, while these were comparable between carriers and non-carriers of the I148M variant (study II). Coagulation factor activities (FVIII, FIX, FXIII, fibrinogen and VWF:RCo) were increased, and the prothrombin time and activated partial thromboplastin time were shortened in insulin-resistant subjects when compared to insulin-sensitive

subjects; yet, these factors were similar in carriers and non-carriers of the I148M variant (study III). The hepatic gene expression of *F8*, *F9* and fibrinogen gamma-chain were higher in insulin-resistant subjects with NAFLD compared with equally obese insulin-sensitive subjects without NAFLD (study III). Furthermore, adipose tissue inflammation was related to plasma coagulation factor activities (study III). The baseline liver fat content independently predicted NAFLD and an increased liver stiffness at 11.3 years more accurately than routinely available clinical and biochemical parameters (study IV).

Conclusions: NAFLD predicts type 2 diabetes independent of age and obesity. However, while obese and insulin-resistant subjects have an increased liver fat content accompanied by features of insulin resistance, adipose tissue inflammation and increased coagulation factor activities, carriers of the PNPLA3 I148M variant have a high liver fat content without these abnormalities. Obesity and the associated inflammation and insulin resistance rather than excess hepatic fat *per se* are related to a procoagulant plasma profile in NAFLD. The liver fat content emerges as more important than the associated metabolic abnormalities as a predictor of liver fibrosis. These data support the view that NAFLD is heterogeneous. Therefore, subjects with 'Metabolic/obese' NAFLD are at an increased risk of type 2 diabetes, while NAFLD due to either insulin resistance or the genetic variants predisposes to liver fibrosis.

1. INTRODUCTION

The present series of studies is a part of larger entirety of studies on the pathogenesis and characteristics of non-alcoholic fatty liver disease (NAFLD). This thesis spefically focus on the role of insulin resistance, adipose tissue inflammation and genetic risk variants in NAFLD. For this aim, we performed one systematic review and three original articles (studies I–IV). This section serves as a short introduction followed by a review of the essential literature focused on to the topic of this thesis. The specific aims are introduced in the section 3 and the comprehensive description of study subjects, study design and methods in the section 4. Thereafter, the results and critical discussion are represented in sections 5 and 6 followed by summary and conclusions.

NAFLD is a rapidly increasing liver disease encompassing a range of conditions from non-alcoholic fatty liver (NAFL) to non-alcoholic steatohepatitis (NASH) with or without fibrosis, which can lead to cirrhosis and hepatocellular carcinoma (European Association for the Study of Diabetes *et al.* 2016). The increasing prevalence of NAFLD parallels that of obesity (Younossi *et al.* 2011). This common form of NAFLD (that is, 'Metabolic/obese NAFLD') is closely associated with all features of the metabolic syndrome (Kotronen *et al.* 2007).

A single-nucleotide polymorphism (rs738409; C>G/I148M) in the patatin-like phospholipase domain-containing 3 (*PNPLA3*, *adiponutrin*) gene predisposes individuals to NAFLD (Romeo *et al.* 2008). In hepatocytes expressing the PNPLA3 I148M variant, the hydrolysis of triglycerides is impaired (Huang *et al.* 2011, He *et al.* 2010) and the activity of lysophosphotidic acid acyltransferase increases resulting in an increased triglyceride synthesis (Kumari *et al.* 2012). A meta-analysis of 16 studies demonstrated that subjects homozygous for the I148M variant allele ($PNPLA3^{148MM}$) have on average a 73% higher liver fat content than weight-matched subjects not carrying the I148M variant ($PNPLA3^{148II}$) (Sookoian & Pirola 2011). $PNPLA3^{148MM}$ subjects also have more steatosis, fibrosis and inflammation in their liver biopsies than $PNPLA3^{148II}$ subjects (Sookoian & Pirola 2011). Features of the metabolic syndrome, such as hyperinsulinemia, hypertriacylglycerolemia and a low concentration of high-density lipoprotein (HDL) cholesterol, characterize obese and insulin-resistant NAFLD patients but not subjects carrying the I148M variant (Kotronen *et al.* 2007, Kotronen *et al.* 2009a), although genetic and metabolic risks of NAFLD may coexist (Luukkonen *et al.* 2016b).

In addition to the PNPLA3 I148M variant, two other genetic variants increase the risk of NAFLD. Genetic variation in the transmembrane 6 superfamily member 2 (TM6SF2; rs58542926, C>T/E167K) (Kozlitina *et al.* 2014, Liu *et al.* 2014, Dongiovanni *et al.* 2015) and in the membrane-bound O-acyltransferase domain-containing 7 (MBOAT7; rs641738, C>T) (Mancina *et al.* 2016, Luukkonen *et al.* 2016a) are associated with liver fat accumulation and an increased risk of NASH and fibrosis. Insulin resistance, however, is not a characteristic of these two conditions (Anstee & Day 2015, Luukkonen *et al.* 2016a, Luukkonen *et al.* 2017).

In obese insulin-resistant subjects, adipose tissue is inflamed compared to non-obese subjects (Xu *et al.* 2003b, Weisberg *et al.* 2003) and is characterized by macrophage infiltration and the increased expression of pro-inflammatory chemokines such as tumor necrosis factor α (TNF-α) and monocyte chemoattractant protein 1 (MCP-1) (Xu *et al.* 2003b, Weisberg *et al.* 2003, Kolak *et al.* 2007). The expression of anti-inflammatory markers such as adiponectin and Twist-related protein 1 (Twist1) is significantly lower in obese insulin-resistant subjects compared to non-obese insulin-sensitive subjects (Pettersson *et al.* 2011, Pietilainen *et al.* 2006). These changes are related to development of hepatic steatosis and insulin resistance (Zeyda & Stulnig 2009, Polyzos *et al.* 2010). In addition, NAFLD is associated with an increased risk of atherothrombotic cardiovascular diseases (CVDs) (Targher *et al.* 2016) and venous thromboembolism (Di Minno *et al.* 2010), possibly reflecting a hypercoagulable condition. Indeed, the activities of several coagulation factors are increased in patients with NAFLD compared to healthy subjects (Kotronen *et al.* 2011, Cigolini *et al.* 1996, Targher *et al.* 2005). It remains unknown whether this is a consequence of hepatic steatosis and increased production from the liver.

If adipose tissue inflammation were important for insulin resistance and the accumulation of triglycerides in 'Metabolic/obese' NAFLD, then subjects with the I148M variant would not have inflamed adipose tissue although they will exhibit an increased liver fat content. Yet, the PNPLA3 I148M variant is not associated with an increased risk for CVD. Therefore, we hypothesize that the carriers of the I148M variant with an increased liver fat content have neither inflamed adipose tissue nor increased activities among coagulation factors.

Furthermore, it remains unclear whether hepatic steatosis and the associated hypoxia and cell death lead to liver fibrosis (Jungermann & Kietzmann 2000, Nath & Szabo 2012) or whether features of insulin resistance associated with 'Metabolic/obese' NAFLD are important for fibrogenesis (Kotronen *et al.* 2007, Ratziu *et al.* 2003). In support of the role of steatosis alone, patients with a fatty liver due to genetic risk variants in *PNPLA3*, *TM6SF2* and *MBOAT7* develop the full spectrum of NAFLD, but are not insulin resistant (Simons *et al.* 2017, Kozlitina *et al.* 2014, Mancina *et al.* 2016, Romeo *et al.* 2008).

The present series of studies aimed to investigate through a systematic review whether NAFLD predicts type 2 diabetes independent of obesity and other known risk factors. We also sought to determine whether adipose tissue is inflamed and coagulation factor activities increased in subjects with an increased liver fat content due to the PNPLA3 I148M variant compared to non-carriers of this variant or whether these features merely characterize patients with hepatic steatosis and insulin resistance. Finally, we examined baseline factors predicting the liver fat content and increased liver stiffness as a measure of fibrosis in an 11-year follow-up study.

2. REVIEW OF THE LITERATURE

2.1. Introduction to NAFLD

2.1.1. Definitions

NAFLD is defined as hepatic steatosis (macroscopic steatosis in >5–10% of hepatocytes determined by histology) not caused by the excess use of alcohol (>20 g/day in women, >30 g/day in men), viruses such as hepatitis B or C, autoimmune hepatitis, the use of hepatotoxic drugs or other compounds or rare genetic liver diseases (European Association for the Study of Diabetes *et al.* 2016). As such, NAFLD encompasses a range of conditions from NAFL to NASH with or without fibrosis. In addition, NAFLD can lead to cirrhosis and hepatocellular carcinoma (HCC).

NAFLD is strongly associated with obesity, insulin resistance and other features of a metabolic syndrome ('Metabolic/obese NAFLD', **Figure 1**) (Kotronen *et al.* 2007). In addition to this type of NAFLD, three common genetic variants increase the risk of NAFLD. A variant in the patatin-like phospholipase domain-containing 3 (PNPLA3; rs738409, C>G/I148M) confers with a NAFLD susceptibility by increasing the liver fat content, the risk of inflammation and fibrosis ('PNPLA3 NAFLD', **Figure 2**) (Romeo *et al.* 2008, Valenti *et al.* 2010b). Genetic variation in the transmembrane 6 superfamily member 2 (TM6SF2; rs58542926, C>T/E167K) ('TM6SF2 NAFLD') (Kozlitina *et al.* 2014, Liu *et al.* 2014, Dongiovanni *et al.* 2015) and in the membrane-bound O acyltransferase domain-containing 7 (MBOAT7; rs641738, C>T) ('MBOAT7 NAFLD') (Mancina *et al.* 2016, Luukkonen *et al.* 2016a) are also associated with liver fat accumulation and an increased risk of NASH and fibrosis. Insulin resistance is not a characteristic of these three conditions (Anstee & Day 2015, Luukkonen *et al.* 2016a), although genetic and metabolic causes of NAFLD may coexist (Luukkonen *et al.* 2016b).

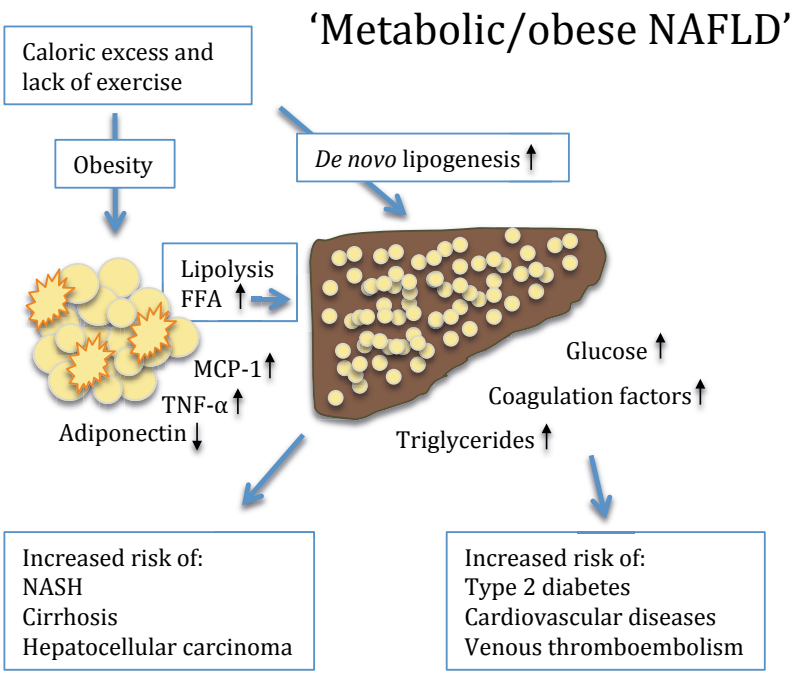

Figure 1. In 'Metabolic/obese NAFLD', excessive energy intake can lead to obesity, which is accompanied by adipocyte hypertrophy, inflammation and insulin resistance in adipose tissue. Thus, insulin is unable to properly inhibit lipolysis leading to FFA delivery to the liver. In addition, *de novo* lipogenesis is enhanced leading to steatosis. An insulin-resistant liver overproduces glucose and triglycerides. Plasma coagulation factor activities are increased under this type of NAFLD. Patients with 'Metabolic/obese NAFLD' have an increased risk of cirrhosis, hepatocellular carcinoma, type 2 diabetes and cardiovascular diseases. **Abbreviations:** FFA, free fatty acid; MCP-1, monocyte chemoattractant protein 1; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; TNF-α, tumor necrosis factor α.

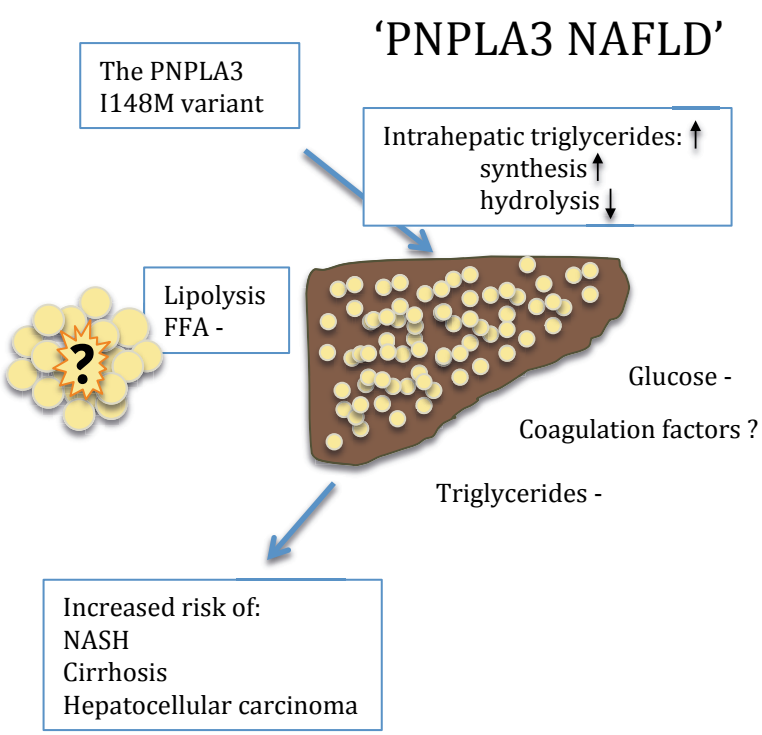

Figure 2. In 'PNPLA3 NAFLD', the exact mechanism of increased hepatic triglyceride content remains unknown. In mice, the data are controversial: triglyceride synthesis is increased while hydrolysis is decreased. Adipose tissue lipolysis and serum FFA concentration are normal in 'PNPLA3 NAFLD'. The PNPLA3 I148M variant increases the risk of cirrhosis and hepatocellular carcinoma but is not associated with features of metabolic syndrome, insulin resistance or cardiovascular diseases. **Abbreviations:** FFA, free fatty acid; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; -, normal.

2.1.2. Diagnostic methods

The liver fat content can be quantified non-invasively using proton magnetic resonance spectroscopy (^{1}H-MRS). Using this technique, the upper limit of normal of the hepatic triglyceride content reached 5.56% among 345 non-obese and non-diabetic subjects with no known liver disease in the population-based Dallas Heart Study (Szczepaniak *et al.* 2005). Thus, NAFLD is often defined as a triglyceride content exceeding 5.56%.

The liver fat content above 20% to 30% can be observed using ultrasound (Hernaez *et al.* 2011). Ultrasound technique is widely available and less expensive than ^{1}H-MRS measurement, and can detect focal lesions. In addition, liver enzymes, ALT, AST and GGT, are routinely used as surrogates of hepatocellular injury. NAFLD is the most common cause of elevation in these liver enzymes both in the US (41% of increased ALT and 34% of increased AST) (Lazo *et al.* 2013) and in Finland (75% of increased ALT) (Kotronen *et al.* 2010). Yet, serum ALT is a poor marker of NAFLD. In a cohort of 222 patients with biopsy-proven NAFLD, 23% of those had normal ALT levels, and 38% of patients with normal ALT levels had NASH or advanced fibrosis (Verma *et al.* 2013). Furthermore, AST is not particularly helpful either, since it is less liver-specific compared to ALT (Giannini *et al.* 2005).

Liver fibrosis is diagnosed using an invasive liver biopsy but can be estimated as liver stiffness measured using non-invasive transient elastography (Yoneda *et al.* 2008, Wong *et al.* 2010a) or magnetic resonance elastography (Venkatesh *et al.* 2013, Kim *et al.* 2013a). In addition, several risk scores can be used to assess the risk of advanged fibrosis in NAFLD. For example, NAFLD fibrosis score based on routine clinical parameters including age, BMI, type 2 diabetes or impaired fasting glucose,

AST:ALT ratio, the platelet count and the albumin concentration identify patients with stage 3 to 4 fibrosis (see sections 2.3.2.1.) (Angulo *et al.* 2007, Musso *et al.* 2011). As well, NASH can be detected exclusively through a liver biopsy, which, therefore, remains the gold standard for the assessment of the severity and progression of disease (Brunt 2004). Several histological features are assessed in liver biopsies, including steatosis, lobular inflammation, ballooning and fibrosis, to estimate disease severity in NAFLD (Kleiner *et al.* 2005).

2.1.3. Prevalence

NAFLD is currently the most common liver disorder (Younossi *et al.* 2015). Worldwide prevalence is estimated at 25%, although this figure largely depends on the method used to diagnose NAFLD and the population studied (Younossi *et al.* 2015). The highest NAFLD prevalence diagnosed using ultrasound was found in South America and the Middle East. In Europe, the pooled prevalence of NAFLD diagnosed by imaging reached 24% in a meta-analysis of 11 studies (Younossi *et al.* 2015). Using elevated concentrations of the serum liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) as the diagnostic criteria, in Finland NAFLD prevalence stands at 21% (Kotronen *et al.* 2010). ALT is normal in 48% of subjects with NAFLD diagnosed using ^{1}H-MRS (Kotronen *et al.* 2009b) and, thus, liver enzymes may underestimate the NAFLD prevalence.

In a population-based study in the US, the Third National Health and Nutrition Examination Survey (NHANES III), the prevalence of NAFLD was higher in men than in women (Lazo *et al.* 2013). In that study, the prevalence of NAFLD increased by age and obesity among both men and women and among different ethnic groups (Lazo *et al.* 2013). Among morbidly obese subjects undergoing bariatric surgery, 74% to 97% had NAFLD (Dixon *et al.* 2001, Luyckx *et al.* 1998) and 11% had advanced fibrosis (Dixon *et al.* 2001).

2.1.4. Significance

2.1.4.1. Advanced liver disease

NAFLD encompasses a range of conditions from NAFL to NASH with or without fibrosis, potentially leading to cirrhosis and increasing the risk of HCC (**Figure 1**). Furthermore, NAFLD is the second most common cause necessitating a liver transplant in the US (Wong *et al.* 2015a) and the most common cause of HCC in both the US (Sanyal *et al.* 2010) and the UK (Dyson *et al.* 2014). An increased risk of advanced liver disease accompanies both metabolic/obese NAFLD and NAFLD due to genetic variants (see section 2.2.2 'Genetic NAFLD', below).

2.1.4.2. Atherothrombotic cardiovascular disease (CVD)

NAFLD, particularly 'Metabolic/obese NAFLD', is associated with several risk factors for atherothrombotic CVD such as obesity (Church *et al.* 2006), dyslipidemia (Adiels *et al.* 2006), insulin resistance (Kotronen *et al.* 2007), increased coagulation factor activity (Kotronen *et al.* 2011) and impaired fibrinolysis (Verrijken *et al.* 2014, Tripodi *et al.* 2014) (**Figure 1**). CVD represents the leading cause of death in subjects with NASH and fibrosis (Ekstedt *et al.* 2006, Musso *et al.* 2011). In a meta-analysis consisting of eight prospective population- and community-based studies, the incidence of CVD and fatal CVD events were approximately twofold higher in subjects with ultrasound or biopsy-proven NAFLD than in the general population even after adjusting for the presence or features of the metabolic syndrome (Musso *et al.* 2011). In another meta-analysis of 16

prospective and retrospective studies in which NAFLD was diagnosed using imaging or biopsy (Targher *et al.* 2016), NAFLD independently increased the combined risk of fatal and non-fatal CVD events 1.6-fold (Targher *et al.* 2016).

In contrast to 'Metabolic/obese NAFLD', NAFLD associated with the PNPLA3 I148M variant appears not to increase the risk of CVD, although sufficient data remain sparse (Simons *et al.* 2017). Study results on the impact of the I148M variant on subclinical CVD findings, such as carotid artery intima-media thickness and the prevalence of carotid plaques, contradict one another. In an Italian cohort of patients under 50 years of age with histological-confirmed NAFLD, the prevalence of carotid plaques and thick intima media were higher in subjects homozygous for the I148M variant compared to subjects lacking or heterozygous for the variant (Petta *et al.* 2013). By contrast, in another Italian study, carotid intima-media thickness was greater among subjects with NAFLD accompanied with features of the metabolic syndrome but not carrying the I148M variant than in subjects homozygous for the I148M variant (Di Costanzo *et al.* 2017).

In a cohort of 1,201 Italians and Finns, carriers of the TM6SF2 E167K variant (n = 157) exhibited lower concentrations of total cholesterol and triglycerides, but a higher prevalence of biopsy-proven NASH and fibrosis than non-carriers (n = 1,044) (Dongiovanni *et al.* 2015). In a cohort of 1,819 Swedish subjects, carriers of the E167K variant had a lower cumulative incidence of fatal and non-fatal CVD events during a median follow-up of 14 years after adjusting for age, gender, body mass index (BMI) and smoking (Dongiovanni *et al.* 2015). Thus, the TM6SF2 E167K variant increases the risk for liver disease, but may protect against CVD. To date, no data exist on the effects of the *MBOAT7* gene variant on CVD risk except for a letter suggesting that this variant does not influence risk (Simons *et al.* 2017).

2.1.4.3. Type 2 diabetes

The liver triglyceride content strongly correlates with features of the metabolic syndrome, such as fasting triglycerides, glucose and low HDL cholesterol concentrations, waist circumference and blood pressure (Kotronen *et al.* 2007). The liver is the main source of endogenous glucose after an overnight fast (Yki-Jarvinen 1993). In addition, the liver secretes triglycerides in very low-density lipoprotein (VLDL) particles. Once the liver is fatty and insulin resistant, such as that which accompanies type 2 diabetes, it overproduces both glucose and VLDL (Yki-Jarvinen 2014). Glucose stimulates insulin secretion, leading to hyperinsulinemia. Furthermore, diabetes develops if β-cells fail to sustain hyperinsulinemia in the face of insulin resistance (Yki-Jarvinen 2014). The increase in VLDL lowers the concentration of HDL cholesterol. These changes are often observed in obese subjects, but also appear independently of obesity (Kotronen *et al.* 2007). Although genetic variants in *PNPLA3*, *TM6SF2* and *MBOAT7* associate with an increased liver fat content, they neither promote insulin resistance nor are related to a high prevalence of type 2 diabetes as shown in various *in vivo* human studies (Romeo *et al.* 2008, Kotronen *et al.* 2009a, Kantartzis *et al.* 2009, Valenti *et al.* 2010b, Kozlitina *et al.* 2014, Zhou *et al.* 2015, Mancina *et al.* 2016).

Depending on the method of diagnosis, 65% to 87% of patients with type 2 diabetes have NAFLD (Doycheva *et al.* 2016, Saponaro *et al.* 2015). As such, the liver fat content was 54% higher in

subjects with type 2 diabetes than in age-, gender- and BMI-matched non-diabetic subjects (Kotronen *et al.* 2008). In addition, in a cross-sectional biopsy study among 1,069 study subjects, the prevalence of NASH, fibrosis and advanced fibrosis increased among patients with type 2 diabetes and among subjects with a positive family history of type 2 diabetes even after adjusting for age, gender and BMI (Loomba *et al.* 2012).

2.2. Pathogenesis of NAFLD

2.2.1. 'Metabolic/obese NAFLD'

2.2.1.1. Sources of intrahepatocellular triglycerides

Intrahepatocellular triglycerides originate from three major sources including adipose tissue lipolysis, *de novo* lipogenesis (DNL) and dietary fatty acids (**Figure 1**). After an overnight fast, lipolysis becomes the major source and covers approximately 60% of fatty acids in the hepatic triglycerides (Donnelly *et al.* 2005). When adipose tissue is insulin resistant, insulin does not sufficiently suppress lipolysis resulting in an increased free fatty acid (FFA) influx to the liver (Kotronen *et al.* 2008, Sanyal *et al.* 2001). In NAFLD, DNL produces approximately one-quarter of the fatty acids in intrahepatocellular triglycerides after an overnight fast (Donnelly *et al.* 2005). Among 13 obese subjects with NAFLD, DNL was increased threefold compared to equally obese subjects without NAFLD (Lambert *et al.* 2014). In addition, DNL produces exclusively saturated fatty acids such as palmitic acid, which can be desaturated to monounsaturated fatty acid (Chong *et al.* 2007). Following a meal, the contribution of meal-derived fatty acids to the synthesis of intrahepatocellular triglycerides increases, while fatty acids originating from DNL and lipolysis decrease (Donnelly *et al.* 2005).

2.2.1.2. Inflammation and insulin resistance in adipose tissue

Excessive energy intake can lead to adipocyte hypertrophy, hypoxia and cell death in adipose tissue (Cinti *et al.* 2005, Trayhurn 2013). Adipocyte hypertrophy is associated with increased lipolysis, the secretion of pro-inflammatory cytokines such as TNF-α and MCP-1 as well as NAFLD (Acosta *et al.* 2016, Laurencikiene *et al.* 2011, Petaja *et al.* 2013), and it may mediate the association between down-regulation of genes involved energy metabolism in adipose tissue and liver fat content shown among 32 individuals (Cheng *et al.* 2015). Once adipose tissue is insulin resistant, insulin is unable to properly inhibit lipolysis, leading to an increase in FFA delivery to the liver (Sanyal *et al.* 2001, Kotronen *et al.* 2008) (**Figure 1**). FFAs can cause insulin resistance independently by inhibiting insulin signaling (Pereira *et al.* 2014). Saturated FFAs in particular induce ceramide synthesis (Hu *et al.* 2011, Sanyal *et al.* 2001, Donnelly *et al.* 2005), which characterizes the insulin-resistant human liver in 'Metabolic/obese' NAFLD (Luukkonen *et al.* 2016b).

Obesity-induced inflammation consists primarily of the activation of the innate immune system, particularly macrophages (Weisberg *et al.* 2003). Macrophages are activated phagocytic monocytes, which can be detected by measuring the cluster of differentiation 68 (CD68) marker (Holness & Simmons 1993). In inflamed adipose tissue, macrophages surround dead adipocytes forming crown-like structures (Cinti *et al.* 2005, Kolak *et al.* 2007). The mRNA expression of CD68 correlates positively with the number of macrophages in adipose tissue and the liver fat content among obese subjects (Kolak *et al.* 2007) and inversely with insulin sensitivity among non-diabetic subjects (Westerbacka *et al.* 2006). The number of macrophages in

the subcutaneous adipose tissue is higher in morbidly obese subjects with NASH than in equally obese subjects without NASH (Tordjman *et al.* 2012), thus supporting the view that adipose tissue inflammation plays a role in the pathogenesis of NAFLD (Polyzos *et al.* 2010).

Macrophages are heterogeneous and can be divided into distinct subgroups, roughly as M1- and M2-polarized macrophages (Lumeng *et al.* 2007). M2 macrophages are characterized by the production of anti-inflammatory cytokine IL-10, while M1 macrophages produce pro-inflammatory cytokines such as MCP-1 and TNF-α (Lumeng *et al.* 2007). An increased ratio of M1:M2 macrophages is a hallmark of obesity-related inflammation in adipose tissue and associates with insulin resistance in mice (Lumeng *et al.* 2007).

Monocyte chemoattractant protein-1. MCP-1, also known as CCL2, is a chemokine crucial for the recruitment of macrophages into adipose tissue(Kanda *et al.* 2006, Kamei *et al.* 2006). It is expressed primarily in macrophages, but also in other cell types such as in adipocytes (Deshmane *et al.* 2009, Kamei *et al.* 2006). Transgenic mice overexpressing MCP-1 in adipose tissue display increased macrophage infiltration into adipose tissue. These mice are also more insulin resistant and have a higher hepatic triglyceride content than wild-type mice with a similar body weight (Kanda *et al.* 2006). Furthermore, an MCP-1 deficiency protects against macrophage infiltration into adipose tissue and insulin resistance (Kanda *et al.* 2006). Similar to an MCP-1 deficiency, a deficiency in the receptor for MCP-1 (CCR2) reduces macrophage accumulation and inflammation in adipose tissue and increases the adiponectin gene and protein expression and its concentration in adipose tissue (Weisberg *et al.* 2006).

In humans, the mRNA expression of *MCP-1* in adipose tissue correlates with the MCP-1 protein concentration in adipose tissue (Kolak *et al.* 2007) and in plasma, as well as with BMI (Christiansen *et al.* 2005). The concentration of the circulating MCP-1 decreases with weight loss (Christiansen *et al.* 2005, Schernthaner *et al.* 2006). In addition to obesity, the expression of *MCP-1* associates with insulin resistance and the liver triglyceride content since it is higher in insulin-resistant subjects with a high liver fat content than in equally obese insulin-sensitive subjects with a low liver fat content (Kolak *et al.* 2007). In a recent study by du Plessis *et al*., expression of *MCP-1* in subcutaneous adipose tissue was increased in subjects with biopsy-proven NAFL and NASH with fibrosis as compared to equally obese subjects without NAFLD (du Plessis *et al.* 2015).

Tumor necrosis factor α. TNF-α is an extensively studied pro-inflammatory cytokine. Macrophages stand as the major source of TNF-α in adipose tissue (Weisberg *et al.* 2003, Di Gregorio *et al.* 2005, Fain *et al.* 2004). For instance, the expression of TNF-α in adipose tissue is increased in obese and insulin-resistant rodents compared to lean rodents (Hotamisligil *et al.* 1993). In obese mice, TNF-α deficiency protects against diet-induced insulin resistance and lowers the concentration of FFA circulating compared to wild-type mice (Uysal *et al.* 1997).

TNF-α decreases insulin sensitivity and increases lipolysis *in vitro* through several mechanisms. In 3T3-L1 adipocytes, TNF-α decreases the expression of genes involved in insulin action, such as glucose transporter GLUT-4 and insulin signaling molecule insulin receptor substrate 1 (IRS-1) (Stephens *et al.* 1997). TNF-α deteriorates insulin-signaling pathways by inhibiting autophosphorylation and the

activity of the insulin receptor (Hotamisligil *et al.* 1994a, Hotamisligil *et al.* 1994b) and IRS-1 (Hotamisligil *et al.* 1996). In addition, TNF-α inhibits the expression of transcription factors regulating insulin sensitivity such as PPARγ in 3T3-L1 adipocytes (Zhang *et al.* 1996). In human adipocytes, TNF-α decreases the IRS-1 activity, glucose uptake (Liu *et al.* 1998) and the expression of adiponectin (Bruun *et al.* 2003), while increasing lipolysis (Zhang *et al.* 2002, Ryden *et al.* 2002). In addition to insulin resistance, TNF-α increases the mRNA expression of pro-inflammatory interleukin 6 (IL-6) during the differentiation of 3T3-L1 preadipocytes (Stephens *et al.* 1992) and in murine L-TK$^-$ and HeLa S3 cells meadiated by the nuclear factor kappa B (NF-κB) –like transcription factor (Shimizu *et al.* 1990).

In human adipose tissue, the gene expression of *TNF-α* is increased in obese and insulin-resistant subjects compared to lean and insulin-sensitive subjects (Hotamisligil *et al.* 1995, Kern *et al.* 1995, Pietilainen *et al.* 2006). In a cohort of 2356 subjects, the serum concentration of TNF-α correlated with insulin resistance measured using the homeostasis model assessment of insulin resistance (HOMA-IR) (Hivert *et al.* 2008). Infusing TNF-α in healthy subjects increases lipolysis and plasma FFA (Plomgaard *et al.* 2008). As such, serum TNF-α is higher among obese subjects with NAFLD than among leaner subjects without NAFLD (Wigg *et al.* 2001). In addition, plasma TNF-α is increased among NASH patients with fibrosis and correlates with the NAFLD activity score (NAS) and the concentration of the liver enzymes ALT and AST (du Plessis *et al.* 2015).

Adiponectin. Adiponectin is an insulin-sensitizing and anti-inflammatory cytokine encoded by the *ADIPOQ* gene in mature adipocytes (Scherer *et al.* 1995, Hu *et al.* 1996). Receptors mediating the effects of adiponectin, consisting of AdipoR1 and AdipoR2, have been found in the liver, the skeletal muscles (Yamauchi *et al.* 2003) and in adipose tissue (Rasmussen *et al.* 2006). In mice, adiponectin deficiency results in insulin resistance and atherosclerosis (Kubota *et al.* 2002), while also increasing the expression of *Mcp-1* and *Tnf-α* in adipose tissue and the concentration of circulating TNF-α (Maeda *et al.* 2002, Ohashi *et al.* 2010). The overexpression of *Adipoq* prevents macrophage infiltration and the expression of *Tnf-α* in adipose tissue, decreases the serum concentrations of insulin, glucose and FFAs and decreases the liver triglyceride content in morbidly obese mice (Kim *et al.* 2007). Similar effects occur with an injection and infusion of adiponectin in obese, diabetic and wild-type mice (Berg *et al.* 2001, Fruebis *et al.* 2001, Xu *et al.* 2003a). Treatment with adiponectin and the overexpression of AdipoR1 and AdipoR2 in the liver decreases the hepatic ceramide content in diet-induced obese mice (Holland *et al.* 2011). By contrast, the hepatic ceramide content increased among adiponectin-deficient mice compared to wild-type mice (Holland *et al.* 2011).

In isolated rat hepatocytes, adiponectin enhances the insulin-induced suppression of hepatic glucose production (Berg *et al.* 2001). A study on human endothelial cells suggested that adiponectin inhibits the TNF-α-mediated activation of NF-κB (Ouchi *et al.* 2000). Treatment with adiponectin increases the anti-inflammatory M2 phenotype of macrophages isolated from adiponectin-deficient mice (Ohashi *et al.* 2010).

In humans, the expression of adiponectin in adipose tissue and the concentration of circulating adiponectin are lower in obese and insulin-resistant individuals compared to non-obese and insulin-sensitive subjects (Pietilainen *et al.* 2006). Similarly, adiponectin expression in adipose tissue is decreased in insulin-resistant subjects with a high liver fat content measured by ^{1}H-MRS compared with equally obese subjects with a lower liver fat content (Kolak *et al.* 2007). Furthermore, serum adiponectin inversely correlates with insulin resistance in healthy and type 2 diabetic subjects (Andersson *et al.* 2016). In a meta-analysis of 27 studies including 1545 patients with biopsy-proven NAFLD and 698 controls without a known hepatic disorder, adiponectin was lower in subjects with NAFLD, particularly among those with NASH, compared to controls independent of age, gender, BMI and type 2 diabetes (Polyzos *et al.* 2011).

Twist-related protein 1. Twist-related protein 1 (Twist1) is an evolutionarily conserved basic helix–loop–helix transcription factor expressed in mesenchymal cell lines (Wang *et al.* 1997). In immortalized murine fibroblast, TNF-α induces the expression of *Twist1* via the NF-κB-dependent pathway (Sosic *et al.* 2003). In mice, Twist1 may decrease the expression of pro-inflammatory cytokines such as *Tnf-α* by creating a negative feedback loop through an interaction with NF-κB (Sosic *et al.* 2003). In human Twist1-silenced adipocytes, the TNF-α-induced expression of MCP-1 is approximately 20% higher than in control adipocytes (Pettersson *et al.* 2011).

In humans, *TWIST1* is expressed primarily by adipocytes (Pettersson *et al.* 2010). The gene and protein expression of Twist1 in human adipose tissue appears lower in obese subjects compared to non-obese subjects and can be increased with weight loss (Pettersson *et al.* 2011). The expression of *TWIST1* is directly related to the degree of insulin resistance measured using HOMA-IR (Pettersson *et al.* 2011). The low expression of *TWIST1* correlates with the increased secretion of TNF-α and MCP-1 (Pettersson *et al.* 2011). Similarly, in a study among 21 subjects with insulin resistance–associated polycystic ovary syndrome and 21 healthy controls, the expression of *TWIST1* in adipose tissue inversely correlated with BMI and positively with insulin sensitivity (Manneras-Holm *et al.* 2014). The latter association did not remain significant after adjusting for BMI (Manneras-Holm *et al.* 2014). Yet, studies examining Twist1 in NAFLD remain non-existent.

2.2.1.3. Coagulation factors

Hepatocytes and sinusoidal endothelial cells represent major sites for the production of coagulation factors (Marder *et al.* 2013, Wion *et al.* 1985). NAFLD is associated with an increased risk of atherothrombotic CVD (Targher *et al.* 2016) and venous thromboembolism (Di Minno *et al.* 2010), possibly reflecting a hypercoagulable state (**Figure 1**). Metabolic, immune and coagulation systems are interrelated processes regulating each other (Hotamisligil 2006, Margetic 2012). In lean mice, injecting TNF-α increases the tissue factor gene expression in adipose tissue threefold (Samad *et al.* 1998). The tissue factor triggers the extrinsic coagulation pathway, including coagulation factors prothrombin, FVII and FX (Owren 1959). Fibrinogen and coagulation factor FVIII, in addition to forming a part of the coagulation cascade, represent acute phase proteins and transcriptionally respond to NF-κB activation (Begbie *et al.* 2000, Albrecht *et al.* 2007). IL-6 stimulates the gene expression of *F8* (gene encoding for FVIII) in human HepG2 and Chang liver cells

(Stirling *et al.* 1998) and the gene and protein expression of fibrinogen in human HepG2 (Castell *et al.* 1990, Castell *et al.* 1989). Increased activities of fibrinogen and FVIII are associated with CVD (Meade *et al.* 1994, Fibrinogen Studies Collaboration *et al.* 2005) and insulin resistance (de Lange *et al.* 2003, Raynaud *et al.* 2000), while FVIII activity also is related to a higher risk of venous thromboembolism (Luxembourg *et al.* 2009).

The activities of several coagulation factors such as FVIII, FIX, FXI and FXII were higher in 54 subjects ^{1}H-MRS-diagnosed with NAFLD than among 44 subjects without NAFLD after adjusting for age, gender and BMI (Kotronen *et al.* 2011). Among 31 healthy men with ultrasound-confirmed liver steatosis, FVII and plasminogen activator inhibitor 1 (PAI-1) activities were increased and tissue-type plasminogen activator (t-PA) decreased compared to 33 subjects without steatosis (Cigolini *et al.* 1996). Adjusting for BMI carried no effect, although no differences existed between groups after adjusting for plasma triglyceride and insulin concentrations (Cigolini *et al.* 1996). In another study, fibrinogen, von Willebrand factor (vWF) and PAI-1 activities were increased among subjects ultrasound-diagnosed with NAFLD, where the amount of visceral fat correlated significantly with their activities (Targher *et al.* 2005). Similarly, fibrinogen and PAI-1 activities and high-sensitive C-reactive protein increased and adiponectin decreased at the fibrosis stage among 45 men with biopsy-confirmed NASH (Targher *et al.* 2008). Bilgir *et al.* found that the tissue factor pathway inhibitor (TFPI) and the concentration of vWF were increased while PAI-1 remained unchanged in biopsy-confirmed NAFLD compared to healthy controls (Bilgir *et al.* 2014). By contrast, Verrijken *et al.* (2014) found that PAI-1 increased as a function of the degree of steatosis, lobular inflammation, ballooning and NAS, but not by fibrosis independent of obesity and insulin resistance. The hepatic gene expression of *PAI-1* was increased among subjects with NASH compared to those without NASH (Verrijken *et al.* 2014).

It remains unknown whether an increase in the coagulation factors in NAFLD results from obesity, insulin resistance and a low-grade inflammation or due to the liver fat content *per se*. Coagulation factor activities remain unstudied across different types of NAFLD. Likewise, the mechanisms underlying the increased activities of coagulation factors in NAFLD remain unclear.

2.2.1.4. Interactions between adipose tissue and the liver

Adipose tissue and the liver are both active endocrine organs that interact with each other via hormones, lipids and cytokines. As discussed above (in sections 2.2.1.1 and 2.2.1.2) in the literature review, FFAs from adipose tissue constitute the primary source of fatty acids for intrahepatic triglycerides and can induce insulin resistance via several mechanisms. In rats, a lipid infusion with heparin, which increses plasma FFA concentration, during a euglycemic hyperinsulinemic clamp increases the hepatic diacylglycerol content, the activation of NF-κB, the gene expression of *Tnf-α* and the plasma concentration of MCP-1 (Boden *et al.* 2005).

In adipose tissue, macrophages and adipocytes produce several cytokines acting both in a paracrine and endocrine fashion (Mohamed-Ali *et al.* 1997). Subcutaneous adipose tissue appears to secrete IL-6, but not TNF-α via circulation in humans (Mohamed-Ali *et al.* 1997). IL-6

stimulates the gene and protein expression of the C-reactive protein (CRP) and the production of other acute phase proteins such as fibrinogen, serum amyloid A, haptoglobin and α_1-antitrypsin. TNF-α inhibits the expression of fibrinogen in hepatocytes (Castell *et al.* 1989, Castell *et al.* 1990). In human HepG2 cells, IL-6 increases the gene and protein expression of total fibrinogen (Rein-Smith *et al.* 2013) and the gene expression of *F8* (Stirling *et al.* 1998). In subjects with CVD, serum IL-6 positively correlates with circulating TNF-α, high-sensitivity CRP and ceramides independent of age, gender, BMI and HOMA-IR (de Mello *et al.* 2009).

Fibrinogen and fibrin can increase the production of TNF-α, IL-6 and MCP-1 in murine macrophages and monocytes and in human peripheral mononuclear cells (Szaba & Smiley 2002, Jensen *et al.* 2007). Tissue factor–activated FVII increases the gene expression of pro-inflammatory cytokines, including *TNF-α* and *IL-6* in lipopolysaccharide (LPS)-stimulated human macrophages (Muth *et al.* 2005). TNF-α induces the gene expression and activity of the tissue factor and increases the level of the tissue factor antigen via the NF-κB pathway in HUVEC cells (Chen *et al.* 2008). These effects can be inhibited by adipocyte-derived adiponectin in a dose-dependent manner (Chen *et al.* 2008).

Adiponectin binds to the AdipoR1 and AdipoR2 receptors and activates several intracellular pathways resulting in decreased gluconeogenesis, DNL, fibrinogenesis and the FFA influx, while increasing FFA oxidation in the liver (Yamauchi *et al.* 2014, Polyzos *et al.* 2010). In addition, adiponectin carries anti-inflammatory effects and inhibits the LPS-induced gene and protein expression of TNF-α in hepatic macrophages, the Kupffer cells, isolated from rats (Thakur *et al.* 2006). The expression of the *AdipoR1*, *AdipoR2*, *Irs-2* and *Ppar-a* genes in the liver are downregulated in obese Zucker rats with diet-induced NASH compared to equally obese rats without NAFLD (Matsunami *et al.* 2011). In adiponectin-deficient mice, the hepatic ceramide content increases compared to wild-type mice (Holland *et al.* 2011). *In vitro*, a deficiency of AdipoR1 and AdipoR2 leads to a decreased activity in the ceramidase in the HEK-293T cells, whereas *in vivo* the overexpression of these receptors increases the ceramidase activity resulting in a decreased hepatic ceramide content in diet-induced obese mice (Holland *et al.* 2011).

2.2.2. 'Genetic NAFLD'

2.2.2.1. The PNPLA3 I148M variant

A genome-wide association study (GWAS) identified a single-nucleotide polymorphism (SNP) in the *PNPLA3* gene that increased the risk of NAFLD diagnosed by ^{1}H-MRS (Romeo *et al.* 2008). Subsequently, several studies including meta-analyses validated this finding (Sookoian & Pirola 2011, Zhang *et al.* 2015). This variant is a non-synonymous cytosine-to-guanine nucleotide transversion that produces an isoleusine (I)-to-methionine (M) amino acid change at codon 148 (rs738409, C>G/I148M) (Romeo *et al.* 2008). While common, the prevalence of the PNPLA3 I148M variant differs somewhat between ethnicities paralleling the prevalence of NAFLD (Romeo *et al.* 2008). Among Hispanics, the frequency of this variant allele reaches 49%, standing at 23% among Caucasians and 17% among African Americans (Romeo *et al.* 2008). Among healthy Chinese, the frequency of this variant allele stands at 31%, climbing to 45% among ultrasound-diagnosed NAFLD patients (Li *et al.* 2012b). In a Finnish population-based study, the variant allele frequency reached

22%, whereby 39% of the population carried the PNPLA3 I148M variant (Hyysalo *et al.* 2013).

In humans, the PNPLA3 protein is expressed in several tissues, particularly in the liver and the retina (Pirazzi *et al.* 2014). In human liver cells, the expression of the *PNPLA3* gene is higher in hepatic stellate cells than in hepatocytes (Pirazzi *et al.* 2014). The PNPLA3 protein is located in the endoplasmic reticulum and in the intracellular lipid droplet (He *et al.* 2010). In hepatocytes expressing the I148M variant of PNPLA3, hydrolysis of triglycerides is impaired (Huang *et al.* 2011, He *et al.* 2010) and the activity of the lysophosphotidic acid acyltransferase increases resulting in an increased triglyceride synthesis (Kumari *et al.* 2012). In hepatic stellate cells, PNPLA3 hydrolyzes retinyl palmitate into retinol and palmitic acid, while the I148M variant decreases this activity (Pirazzi *et al.* 2014). Stellate cells represent dominant sources of the extracellular matrix and fibrosis in the liver, while PNPLA3 appears crucial for the activation of human stellate cells (Bruschi *et al.* 2017). The I148M variant increases the profibrinogenic features of these cells by promoting the cell proliferation and migration, and expression of pro-inflammatory cytokines (Bruschi *et al.* 2017). *In vivo*, in mice, neither the deficiency nor the overexpression of PNPLA3 affect the hepatic fat content (Chen *et al.* 2010, He *et al.* 2010), yet overexpressing the human *PNPLA3* I148M variant increases the hepatic fat content due to impaired triglyceride hydrolysis during a high-glucose diet (Li *et al.* 2012a).

The PNPLA3 I148M variant predisposes individuals to steatosis, NASH, fibrosis and HCC (**Figure 2**). Among 591 Caucasian subjects with NAFLD, the prevalence of NASH and fibrosis were higher among subjects with the I148M variant than non-carriers and correlated with the number of risk alleles (Valenti *et al.* 2010b). The *PNPLA3* genotype predicts grade 2 to 3 steatosis, NASH and stage 2 to 4 fibrosis independent of age, gender and BMI (Valenti *et al.* 2010b). A meta-analysis of 16 studies concluded that the liver fat content is approximately 73% higher in subjects homozygous for the I148M variant allele than among weight-matched non-carriers of the I148M variant (Sookoian & Pirola 2011). In addition, a meta-analysis of seven studies showed that the I148M variant increases the risk of cirrhosis 1.9-fold (Shen *et al.* 2015). In other meta-analyses, the I148M variant increased the risk of HCC in NAFLD, as well in other liver diseases such as alcohol-related liver disease (Trepo *et al.* 2014, Singal *et al.* 2014). Thus, the PNPLA3 variant increases the severity of the liver injury in several liver diseases, but is not associated with insulin resistance or metabolic complications such as CVD (see section 2.1.4.2, above).

2.2.2.2. The TM6SF2 E167K variant

In 2014, two research groups discovered an association between the TM6SF2 rs58542926 C>T/E167K variant and the liver fat content, serum alanine and aspartate aminotransferase concentrations, along with a lower serum triglyceride and low-density lipoprotein (LDL) cholesterol concentrations using GWAS (Kozlitina *et al.* 2014, Holmen *et al.* 2014). SNP at rs58542926 leads to a lysine (E)-to-glutamic acid (K) substitution at codon 167 (Holmen *et al.* 2014, Kozlitina *et al.* 2014). The frequency of the E167K variant is higher among European-ancestry Americans (7.2%) (Kozlitina *et al.* 2014) and European Caucasians (12%) (Liu *et al.* 2014) than among African Americans (3.4%) or Hispanics (4.7%) (Kozlitina *et al.* 2014) in population-based cohorts. Among

Chinese subjects, the frequency reached 6.6% in a case-control study (8.9% among subjects with NAFLD and 4.5% among a healthy population) (Wang *et al.* 2016) and 7.3% in a community-based study (Wong *et al.* 2014b).

In humans, *TM6SF2* is expressed primarly in the liver and the intestines, while the TM6SF2 protein is located in the endoplasmic reticulum in human hepatoma HuH7 and HepG2 cells (Mahdessian *et al.* 2014). The E167K variant is associated with a decreased protein expression of TM6SF2 (Kozlitina *et al.* 2014). TM6SF2 silencing in HuH7 and HepG2 cells increases the intracellular concentration and reduces the secretion of triglycerides, while the overexpression of TM6SF2 causes an opposite effect (Mahdessian *et al.* 2014). In mice, the hepatic knockdown of *Tm6sf2* increases liver triglycerides and cholesteryl esters (Kozlitina *et al.* 2014), the primary contents of VLDL particles. These data suggest that TM6SF2 functions (Mancina *et al.* 2016) as a part of the VLDL secretion, while the E167K variant diminishes the triglyceride secretion leading to the retention of lipids in hepatocytes.

The E167K variant increases the risk of hepatic steatosis, necroinflammation, ballooning and advanced fibrosis in NAFLD with a preserved insulin sensitivity (Dongiovanni *et al.* 2015, Liu *et al.* 2014, Zhou *et al.* 2015). In a study of 1074 Caucasian subjects, carriers of the E167K variant had a 1.9-fold higher risk of NAFLD-related HCC compared to non-carriers (Liu *et al.* 2014). However, the impact of the variant on the risk of HCC was not independent of known risk factors such as age, gender, BMI, type 2 diabetes and the presence of cirrhosis (Liu *et al.* 2014). In addition to NAFLD, the TM6SF2 variant increases the risk of cirrhosis (Buch *et al.* 2015) and HCC (Falleti *et al.* 2016) in alcohol-related liver disease. As such, a meta-analysis of ten studies showed lower serum concentrations of triglycerides and LDL as well as total cholesterol, but similar levels of HDL cholesterol in spite of a 2.2-fold higher hepatic fat content in carriers compared to non-carriers of the E167K variant (Pirola & Sookoian 2015). The prevalence of carotid plaques was lower among carriers than among non-carriers of the E167K variant with biopsy-confirmed NAFLD (Dongiovanni *et al.* 2015). Similarly, the incidence of fatal and non-fatal cardiovascular events was lower among 294 carriers than among 1525 non-carriers of the variant (hazard ratio, 0.61; 95% CI, 0.39–9.95) (Dongiovanni *et al.* 2015). Thus, data suggest that not all patients with NAFLD have an equal risk of CVD (Dongiovanni *et al.* 2015, Holmen *et al.* 2014).

2.2.2.3. The MBOAT7 rs641738 C>T variant

The rs641738 C>T single nucleotide polymorphism in the *MBOAT7* gene was first identified as predisposing to cirrhosis in alcohol-related liver disease (Buch *et al.* 2015). In addition, the rs641738 C>T variant influences the severity of NAFLD (Mancina *et al.* 2016). The frequency of the rs641738 C>T variant stands at 33% among African, 42% among European and 44% among Hispanic Americans in a population-based study (Mancina *et al.* 2016). Similarly, in 115 Finnish bariatric surgery patients, variant allele frequency is 43% (Luukkonen *et al.* 2016a).

In humans, *MBOAT7* is expressed in several tissues including the ovaries, the uterus, the brain and the liver, in which hepatocytes, hepatic sinusoidal endothelial cells and hepatic stellate cells express *MBOAT7* (Mancina *et al.* 2016). The

rs641738 C>T variant leads to a lower enzymatic activity of MBOAT7 by decreasing the gene expression and protein synthesis of MBOAT7 in humans (Mancina *et al.* 2016). MBOAT7, also known as lysophosphatidylinositol acyltransferase 1, catalyzes the desaturation of the second acyl-chain during the remodeling of phosphatidyl-inositols (D'Souza & Epand 2014). Furthermore, the MBOAT7 variant leads to alterations in certain plasma (Mancina *et al.* 2016) and hepatic (Luukkonen *et al.* 2016a) phosphatidyl-inositols, but not in other lipids such as triglycerides, ceramides and cholesterols (Mancina *et al.* 2016). A recent study suggested that this variant does not influence the risk of CVD (Simons *et al.* 2017).

In a cohort of 1149 Caucasian subjects with biopsy-confirmed NAFLD, the MBOAT7 rs641738 C>T variant associated with a higher grade of steatosis, necroinflammation and stage of fibrosis, but not with ballooning after adjusting for obesity and the presence of a higher fasting glucose or type 2 diabetes (Mancina *et al.* 2016). In a study consisting of 115 morbidly obese Finns, the prevalence of stage 2 to 4 fibrosis differed significantly according to the MBOAT7 genotype, reaching 0%, 5% and 25% among non-carriers of the variant, carriers of one variant allele and carriers of two variant alleles, respectively, whereas fasting insulin and glucose concentrations were comparable across groups (Luukkonen *et al.* 2016a). In addition, the rs641738 C>T variant increases inflammation and fibrosis in chronic liver diseases caused by the hepatitis B (Thabet *et al.* 2017) and C (Thabet *et al.* 2016) viruses. To date, no data exist on the effect of the MBOAT7 rs641738 C>T variant on the risk of cirrhosis or HCC in NAFLD.

2.3. Natural course of NAFLD

2.3.1. Liver fat

2.3.1.1. Diagnosis

The steatotic liver can be diagnosed using either a liver biopsy (macroscopic steatosis in >5–10% of hepatocytes) or via non-invasive imaging techniques such as ultrasound or proton magnetic resonance spectroscopy (^{1}H-MRS) (European Association for the Study of Diabetes *et al.* 2016). Liver biopsy is, however, an invasive procedure and can cause complications such as pain and bleeding (Bravo *et al.* 2001). Thus, it is unsuitable for screening purposes. In addition, a biopsy sample comprises only approximately 1/50,000 of the total liver mass and, hence, does not represent the overall histology of the liver (Bravo *et al.* 2001, Ratziu *et al.* 2005). Ultrasound represents the only semi-quantitative technique to estimate the degree of steatosis (Hernaez *et al.* 2011), although it remains unreliable among obese subjects (Mottin *et al.* 2004). Using the state-of-the-art technique ^{1}H-MRS, NAFLD is often defined as a triglyceride content exceeding 5.56% (Szczepaniak *et al.* 2005), corresponding to approximately 10% of the macroscopic steatosis of a liver biopsy (Petaja & Yki-Jarvinen 2016).

2.3.1.2. Progression and significance

NAFL has been considered a benign and non-progressive condition without an increased risk of liver-related mortality (Teli *et al.* 1995). Recently, paired-biopsy studies in NAFLD patients challenged this theory by showing that steatosis can indeed progress into NASH and fibrosis (Pais *et al.* 2013, McPherson *et al.* 2015, Wong *et al.* 2010b). A meta-analysis of 11 cohort studies consisting of 150 biopsy-confirmed patients with NAFL and 261 patients with NASH showed that the rate of disease progression is slower in patients with NAFL (stage 1 fibrosis over 14.3

years) compared to NASH patients (7.1 years) (Singh *et al.* 2015). The latter meta-analysis found that only hypertension predicted the progression of fibrosis (Singh *et al.* 2015).

2.3.2. Fibrosis

2.3.2.1. Diagnosis

Fibrosis can be diagnosed only by histological examination of a liver biopsy, although several non-invasive biomarkers and imaging methods have been developed to estimate the stage of fibrosis in the liver. For example, the NAFLD fibrosis score based on routine clinical parameters including age, BMI, type 2 diabetes or impaired fasting glucose, AST:ALT ratio, the platelet count and the albumin concentration (Angulo *et al.* 2007) identified patients with stage 3 to 4 fibrosis with an area under the receiver operating characteristic (AUROC) value of 0.85 in a meta-analysis of 3064 subjects with NAFLD (Musso *et al.* 2011). Liver stiffness measurements allow for the estimation of the presence and severity of fibrosis in the liver (Wong *et al.* 2010a, Park *et al.* 2017). While magnetic resonance elastography is expensive and not widely available, transient elastography (FibroScan) can be useful in screening patients at high risk of stage 2 to 4 fibrosis (Wong *et al.* 2010a, Park *et al.* 2017, Wong *et al.* 2012).

2.3.2.2. Progression and significance

During the early stages, fibrosis appears in the perisinusoidal or periportal area in zone 3 of the lobule (stage 1) and can progress to perisinusoidal and periportal (stage 2) and bridging fibrosis (stage 3). As the disease progresses toward cirrhosis (stage 4), steatosis can become irregularly distributed or disappear (Powell *et al.* 1990). NAFLD is the most important etiology for HCC in the US (Sanyal *et al.* 2010) and the UK (Dyson *et al.* 2014), while the incidence rate of NAFLD-related HCC is increasing by 9% annually in the US (Younossi *et al.* 2015). Although, the majority of HCC develop in the cirrhotic liver, HCC can be detected in NAFLD independent of fibrosis and cirrhosis (Margini & Dufour 2016).

Patients with NAFLD carry an increased risk for overall mortality, a risk already observed during fibrosis stage 1 (Dulai *et al.* 2017). Similarly, the risk of liver-related mortality increases when fibrosis exceeds stage 2, where the risk is exponentially higher during stages 3 and 4 (Dulai *et al.* 2017). NAFL or NASH without fibrosis has no effect on the mortality (Ekstedt *et al.* 2015, Angulo *et al.* 2015). Similar to fibrosis, liver stiffness measured using transient elastography predicted long-term outcomes in 360 French patients with NAFLD (Boursier *et al.* 2016). The overall mortality reached over 50% in patients with liver stiffness >12.0 kPa, while it fell to below 20% in patients with <8.8 kPa and less than 10% in patients with <4.6 kPa during a follow-up period of 10 years (Boursier *et al.* 2016). The survival of NAFLD patients was rather similar if predicted using transient elastography or histological assessment (Angulo *et al.* 2015, Boursier *et al.* 2016).

2.4. Summary

Taken together, this up-to-date review of the literature suggests that adipose tissue inflammation and insulin resistance are important for the accumulation of triglycerides in ‘Metabolic/obese NAFLD'. Although NAFLD is associated with increased risk of type 2 diabetes, it is unclear if all types of NAFLDs predispose to type 2 diabetes independent of other risk factors. In addition, it remains unknown whether subjects with the PNPLA3 I148M variant lack inflamed adipose tissue although they exhibit an

increased liver fat content. Yet, the PNPLA3 I148M variant is not associated with an increased risk for CVD. Therefore, we hypothesize that patients with 'Metabolic/obese NAFLD' but not the carriers of risk variants are at a risk for type 2 diabetes (study I), and that the carriers of the PNPLA3 I148M variant have neither inflamed adipose tissue (study II) nor increased coagulation factor activities (study III) despite increased liver fat content.

Furthermore, it remains unclear whether hepatic steatosis and the associated hypoxia and cell death result in liver fibrosis (Jungermann & Kietzmann 2000, Nath & Szabo 2012) or whether features of insulin resistance are important for fibrogenesis (Kotronen *et al.* 2007, Ratziu *et al.* 2003). In study IV, we aimed to examine which baseline factors predict the future liver stiffness as an estimate of fibrosis.

3. AIMS OF THE STUDY

The present series consisting of one systematic review (I), two cross-sectional studies (II and III) and one longitudinal study (IV) in adult men and women aimed to address the following questions:

I. Does NAFLD predict type 2 diabetes independent of obesity and other known risk factors?

II. Is adipose tissue inflamed in subjects homozygous for the PNPLA3 I148M variant?

III. A) Do obesity and insulin resistance rather than an increased liver fat content increase coagulation factor activities in humans?

B) Is hepatic gene expression of coagulation factors increased in insulin-resistant subjects with NAFLD?

C) Is adipose tissue inflammation related to coagulation factor activities in non-carriers of the PNPLA3 I148M variant?

IV. Which factors predict NAFLD and increased liver stiffness during an 11-year follow-up period?

4. SUBJECTS, STUDY DESIGN AND METHODS

4.1. Systematic review

In study I, we conducted a systematic review of prospective longitudinal studies among adult men and women examining whether NAFLD predicts type 2 diabetes. We searched for eligible articles as suggested by the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) group. (Moher *et al.* 2009). As such, we searched MEDLINE using the terms "fatty liver", "diabetes", "ultrasound", "magnetic resonance spectroscopy" and "biopsy" for publications on NAFLD confirmed diagnoses by ultrasound, ^{1}H-MRS and histological assessment in a liver biopsy. In addition, we used the terms "fatty liver", "steatosis", "liver enzymes", "transaminases", "alanine aminotransferase" (ALT), "aspartate aminotransferase" (AST), "gamma-glutamyltransferase" (GGT), and "diabetes incidence" while searching for publications in which liver enzymes were used to determine NAFLD. All searches were performed by the end of February 2016.

We included prospective longitudinal cohort studies investigating whether baseline NAFLD predicts the incidence of type 2 diabetes. We only included publications in English. We also excluded studies, in which subjects with other liver diseases such as hepatitis B and C or a malignancy were not excluded, and in which alcohol consumption was not assessed or the analysis did not adjust for alcohol consumption.

4.2. Study subjects

For studies II, III and IV, we recruited adult Finnish men and women for metabolic studies through newspaper advertisements, by contacting colleagues and occupational health services or from among individuals referred to the Department of Gastroenterology due to chronically elevated serum transaminase concentrations. We used the following inclusion criteria to study healthy subjects or patients with features of the metabolic syndrome or NAFLD (studies II–IV): i) aged 18 to 75 years; ii) no known acute or chronic disease except for obesity, hypertension, dyslipidemia, NAFLD or type 2 diabetes based on medical history, physical examination and standard laboratory tests and electrocardiogram; iii) alcohol consumption of less than 20 g per day for women and less than 30 g per day for men; iv) no pregnancy or lactation; and v) no a history of use of toxins or drugs associated with liver steatosis. Study physicians assessed the study subjects' self-reported alcohol intake by using a questionnaire addressing the quantity of different alcoholic drinks consumed during an average week. We explained the purpose, nature and potential risks of the study to potential study subjects before obtaining their written informed consent. The ethics committee of the Helsinki University Hospital approved the study protocols.

4.2.1. Study II subjects

Subjects for study II were recruited from among those who had been genotyped at rs738409 for the *PNPLA3* gene in our laboratory. We included all who were PNPLA3^{148MM} and excluded subjects who were heterozygous for the I148M variant allele (PNPLA3^{148MI}) or had type 2 diabetes. The corresponding number of non-carriers of the I148M variant (PNPLA3^{148II}) was selected at random. All

subjects eligible for inclusion based on the criteria mentioned above during a telephone interview were invited for a metabolic study.

4.2.2. Study III subjects

In study III, we examined the impact of the I148M variant on plasma coagulation factors in 92 subjects. We recruited these individuals based on the inclusion criteria mentioned above excluding subjects with type 2 diabetes. To measure the hepatic coagulation factor expression, we consecutively recruited morbidly obese subjects undergoing bariatric surgery who met the inclusion criteria mentioned above and in whom a liver biopsy was clinically indicated. Of those subjects, we included 26 non-carriers of the I148M variant consisting of 13 patients with NAFLD and 13 without NAFLD. To determine whether coagulation factor activities associate with adipose tissue inflammation, we obtained needle biopsies of abdominal adipose tissue and measured coagulation factor activities in 26 subjects selected randomly from PNPLA3^{148II} subjects in the group of 92 subjects mentioned above.

4.2.3. Study IV subjects

We contacted all individuals (n=191) who participated in metabolic studies on the liver fat content in our laboratory between 1998 and 2004 (Juurinen *et al.* 2007, Juurinen *et al.* 2008, Makkonen *et al.* 2007, Ryysy *et al.* 2000, Seppala-Lindroos *et al.* 2002, Tiikkainen *et al.* 2004a, Tiikkainen *et al.* 2004b, Vehkavaara *et al.* 2000, Westerbacka *et al.* 2006, Westerbacka *et al.* 2008), of whom 139 subjects were compliant to participate in the follow-up study visit. For a metabolic study visit, we invited all subjects who met the inclusion criteria mentioned above based on a telephone interview. Furthermore, we excluded 12 patients after the follow-up visit because of an excessive use of alcohol (n = 9), the use of herbal medicinal products (n = 1) and cortisone (n = 1), and on whom bariatric surgery was performed (n = 1). Thus, study IV consisted of 97 subjects.

4.3. Study designs

4.3.1. Design of study II

In this cross-sectional study, 82 subjects were divided into two groups based on median BMI (obese and non-obese groups) and *PNPLA3* genotype at rs738409 (PNPLA3^{148MM} and PNPLA3^{148II} groups). Clinical measurements and blood samples were obtained at the clinical research center the morning after an overnight fast. Needle biopsies of adipose tissue were performed during this visit. We used a quantitative real-time polymerase chain reaction (qPCR) technique to determine the gene expression of inflammatory markers in the adipose tissue and ^{1}H-MRS to measure the liver fat content.

4.3.2. Design of study III

In this cross-sectional study, we divided a cohort of 92 subjects into insulin-resistant (IR) and insulin-sensitive (IS) groups based on the median HOMA-IR value, and based on the *PNPLA3* genotype at rs738409 into carriers (PNPLA3$^{148MM/MI}$) and non-carriers (PNPLA3^{148II}) of the I148M variant. Since obesity, insulin resistance and the I148M variant are common, we also analyzed coagulation factors in a two-by-two fashion in four subgroups of subjects. These subgroups were as follows: individuals with both risk factors—that is, both insulin resistance and the I148M variant (IR and PNPLA3$^{148MM/MI}$); individuals with one risk factor—that is, with either insulin resistance without this variant (IR and PNPLA3^{148II}) or a carrier of the I148M variant without insulin resistance (IS and PNPLA3$^{148MM/MI}$); or individuals with neither risk factor (IS and PNPLA3^{148II}).

Clinical measurements and blood samples were obtained at the clinical research center the morning after an overnight fast. We used ^{1}H-MRS to measure the liver fat content and needle biopsies of adipose tissue were performed on 26 subjects at the clinical visit.

Morbidly obese subjects were invited to a separate clinical visit one week prior to bariatric surgery for a detailed metabolic characterization. After basal blood sampling and anthropometric measurements, a two-hour 75-g oral glucose tolerance test (OGTT) was performed during which both glucose and insulin concentrations at 0, 30 and 120 min were obtained. In addition, liver biopsies were obtained during surgery. After genotyping consecutive patients, we identified subjects lacking the PNPLA3 I148M variant (PNPLA3^{148II}) divided into groups based on a liver histology as either "Metabolic NAFLD" or "No NAFLD". We determined the hepatic gene expression of coagulation factors using qPCR.

4.3.3. Design of study IV

This longitudinal study consisted of two metabolic study visits: the baseline and follow-up visits. At the baseline visit, medical history was obtained, physical examination including measurement of body weight, height and waist circumference were completed, and fasting blood samples were taken after an overnight fast. ^{1}H-MRS was used to measure the liver fat content.

At the follow-up visit, medical history and physical examination were repeated. Fasting blood samples were taken to measure the same metabolic parameters in the same laboratory as at baseline. In addition, antibodies against hepatitis A (HAVAbG and HAVAbM), B (HBcAb) and C (HCVAb), transferrin saturation, anti-smooth muscle, anti-nuclear and anti-mitochondrial antibodies were measured. We obtained blood samples to genotype study subjects for the variants of *PNPLA3* at rs738409, *TM6SF2* at rs58542926 and *MBOAT7* at rs641738. Thereafter, OGTT was performed among non-diabetic subjects. The measurement of the liver fat content using ^{1}H-MRS was repeated. In a separate visit, liver stiffness was measured using transient elastography in 92 subjects after an overnight fast. Liver biopsies were performed if clinically indicated.

4.4. Methods

4.4.1. Body composition and blood pressure

Body weight was recorded to the nearest 0.1 kg using a calibrated digital scale (Soehnle, Monilaite-Dayton, Finland) with subjects barefoot wearing light indoor clothing. Height along with waist and hip circumferences were recorded to the nearest 0.5 cm using a non-stretchable tape. BMI was defined as weight/height^2 (kg/m^2). Waist circumference was measured midway between the lower rib margin and the iliac crest and hip circumference over the greater trochanters. The percentage of body fat was determined using bioelectric impedance analysis (BioElectrical Impedance Analyzer System model #BIA-101A, RJL Systems, Detroit, MI). Blood pressure was measured in a sitting position after a minimum of 15 min acclimatization and before blood sampling using an automatic sphygmomanometer (OMRON M7, Omron Healthcare Co. Ltd., Kyoto, Japan).

4.4.2. Measurement of liver fat by proton magnetic resonance spectroscopy (^{1}H-MRS)

In studies II through IV, the liver fat content was measured using three generations of 1.5 Tesla clinical scanners

(Magnetom Vision, Sonata and Avanto, Siemens Healthcare Diagnostics, Erlangen, Germany) and, thus, the intensity differences arising from various acquisition parameters and localization techniques had to be normalized. T1-weighted high-resolution magnetic resonance imaging scans were collected using a standard ^{1}H body coil. The ^{1}H-MRS voxel of interest (8 to 27 cm^{3}) was carefully located within the right lobe of the liver avoiding subcutaneous fat, large vessels, bile ducts and the gall bladder. Localization was performed using the STEAM sequence with echo time (TE)/mixing time (TM)/repetition time (TR) of 20/30/3000 ms and 32 acquisitions for Vision measurements and the PRESS sequence with TE/TR of 30/3000 ms and 16 acquisitions for Sonata and Avanto measurements. Subjects were breathing normally during the data collection. All spectra were analyzed with the MRUI/jMRUI software using VARPRO/AMARES (available at www.mrui.uab.es/mrui/). The intensities of the peaks resonating from the protons of water, and protons of methylene $(CH_2)_{n-2}$ groups in the fatty acid chains were determined using line-shape fitting with prior knowledge. Signal intensities were corrected for T1 and T2 relaxation using the equation $I_m=I_0$ exp(−TE/T2)* [1−exp(−(TR−TM−0.5TE)/T1)]*exp(−TM/ T1) for Vision data and the equation $I_m=I_0$ exp(−TE/T2) for Sonata and Avanto data. T1 of 600 ms (Stanisz *et al.* 2005) and 300 ms (Graham *et al.* 1999) and experimentally determined T2 of 46 ms and 58 ms were used for water and fat, respectively. Liver fat content was expressed as a ratio of signal from methylene group to total signal of methylene and water. Liver fat content was converted from signal ratio to a weight fraction, applying method validated by Longo *et al.* (Longo *et al.* 1995) and Szczepaniak *et al.* (Szczepaniak *et al.* 2005). The following experimentally determined factors were used: i) the ratio of the number of lipid protons in the fitted $(CH_2)_{n-2}$ signal to the total number of lipid protons is 0.6332 (Szczepaniak *et al.* 1999); ii) proton densities of fat and water are 111 and 111 mol/l, respectively; iii) 1 g liver tissue contains 711 mg water; iv) densities of the liver tissue, fat in the liver, and water are 1.051 g/ml, 0.900 g/ml, and 1.000 g/ml; respectively. A physicist unaware of any of the clinical data analyzed all spectra. NAFLD was defined as liver fat content exceeding 5.56% as in the Dallas Heart Study (Szczepaniak *et al.* 2005).

4.4.3. Measurement of liver stiffness using transient elastography

We measured liver stiffness using transient elastography (FibroScan, Echosens, Paris, France) in the right lobe of the liver when patients were lying supine with their right arm in maximal abduction (study IV). The tip of the probe transducer was covered with gel and placed on the skin level with the right lobe of the liver. The depth of the measurement was 25 mm to 65 mm below the skin surface using the M probe and 35 mm to 75 mm using the XL probe. The measurements were first performed with the M probe. The XL probe was used if obesity prevented adequate measurement. The cut-off for F3 to F4 fibrosis was 8.7 kPa with the M probe and 7.2 kPa with the XL probe as recommended (Wong *et al.* 2012). The results are reported as the median value from ten successful measurements in kilopascal (kPa).

4.4.4. Liver biopsies and histological assessment

Liver biopsies were performed if clinically indicated (studies III and IV). Wedge biopsies of the liver were obtained during bariatric surgery (study III). Percutaneous liver biopsies (study IV) were obtained under ultrasound guidance using a 16G

BioPince Full Core Biopsy instrument (Argon Medical Devices, Athens, TX, USA). Part of the biopsy was sent to the pathologist for histological assessment. The remainder of the biopsy was snap-frozen in liquid nitrogen for the subsequent isolation of RNA and analysis of the gene expression using qPCR (study III). The histology was analyzed by an experienced liver pathologist in a blinded fashion according to the criteria proposed by Brunt *et al.* (studies III and IV) (Brunt *et al.* 1999). NAFLD was diagnosed based on the histology as macroscopic steatosis in >10% of hepatocytes (study III) (Petaja & Yki-Jarvinen 2016).

4.4.5. Subcutaneous adipose tissue biopsies

A small area of the skin in the abdomen was anesthetized with 1% lidocain during the metabolic study visit. A 14G biopsy needle attached to a syringe was inserted through the skin and the adipose tissue sample was aspirated by vacuum suction (studies II and III). The adipose tissue samples were rinsed with sterile saline, frozen in liquid nitrogen and stored at –80°C until analyzed.

4.4.6. Gene expression

The relative mRNA concentrations of the genes of interest in adipose tissue and liver biopsies were quantified using qPCR (studies II and III). The total RNA was isolated from subcutaneous adipose tissue using the RNeasy Lipid Tissue Mini Kit (Qiagen) (studies II and III) and from liver tissue using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) (study III). The RNA (500 ng) was reverse-transcripted using the SuperScript VILO cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturers protocol. Each sample was amplified in duplicate for the quantification of the mRNA expression on a 7000 Sequence Detection System (study II) (Applied Biosystems/Life Technologies, Carlsbad, CA, USA) or a LightCycler® 480 (study III) (Roche Diagnostics, Rotkreuz, Switzerland) using the SYBR-Green kit (Roche Diagnostics). The adipose tissue gene expression of inflammatory markers was normalized relative to the expression of the housekeeping gene acidic ribosomal phosphoprotein 36B4 (*36B4*) in the adipose tissue. The hepatic gene expression of the coagulation factors was normalized relative to the expression of the housekeeping gene succinate dehydrogenase complex, subunit A (*SDHA*) in the liver. The primer sequences are listed in **Table 1**.

Table 1. Oligonucleotide primers for mRNA quantification using qPCR

Gene	Forward primer 5'-3'	Reverse primer 5'-3'
CD68	GCTACATGGCGGTGGAGTACAA	ATGATGAGAGGCAGCAAGATGG
MCP-1	CACTCACTCCACAACCCAAGA	CAAAGACCCTCAAAACATCCC
TNF-α	CCTCTCTCTAATCAGCCCTCTG	GAGGACCTGGGAGTAGATGAG
ADIPOQ	TATCCCCAACATGCCCATTCG	TAGGCAAAGTAGTACAGCCCA
TWIST1	GGCTCAGCTACGCCTTCTC	CCTTCTCTGGAAACAATGACATCT
F8	CACACTCACCCTATTCCCATTC	GTATGCTGAAATATCTTCATAACTG
F9	TTGGAAGCAGTATGTTGATGG	TGGTTCACAGGACTTCTGGT
F11	AGCCTGGAGCATCGTAACATTTG	CACAAAAGCATCGGGAGCCATG
F13A1	CTGACCTTCCTGTTGGATTTGG	CTTGATGGCTTGAACCGAGG
FGG	GATTCATTTGATAAGCACACAGT	TGAACATGGCATAGTCTGCAGTA
36B4	CATGCTCAACATCTCCCCCTTCTCC	GGGAAGGTGTAATCCGTCTCCACAG
SDHA	CATGCTGCCGTGTTCCGTGTGGG	GGACAGGGTGTGCTTCCTCCAGTGCTCC

Abbreviations: *36B4*, acidic ribosomal phosphoprotein 36B4; *ADIPOQ*, adiponectin; *CD68*, cluster of differentiation 68; *F8*, coagulation factor VIII; *F9*, coagulation factor IX; *F11*, coagulation factor XI; *F13A1*, coagulation factor XIII A chain; *FGG*, fibrinogen gamma chain; *MCP-1*, monocyte chemoattractant protein 1; *SDHA*, succinate dehydrogenase complex, subunit A; *TNF-α*, tumor necrosis factor α; *TWIST1*, Twist-related protein 1. Adapted from Lallukka *et al*. *Diabetologia.* 2013; 56:886–92 (study II). Reproduced with permission from the copyright holder.

4.4.7. Biochemical procedures

The fasting plasma glucose was measured using the hexokinase method on an autoanalyser (Roche Diagnostics Hitachi 917, Hitachi Ltd., Tokyo, Japan) (studies II–IV). The serum insulin concentration was determined by time-resolved fluoroimmunoassay using Insulin Kit (AUTOdelfia, Wallac, Turku, Finland) (studies II–IV). HOMA-IR was calculated using the following formula: fasting glucose (mmol/l) × fasting insulin (mU/l) / 22.5 (studies II–IV) (Matthews *et al.* 1985). The Matsuda insulin sensitivity index was applied as another measurement of the insulin sensitivity and was calculated from the insulin and glucose concentrations assessed at 0, 30 and 120 min during the oral glucose tolerance test (study III) (DeFronzo & Matsuda 2010). Glycosylated hemoglobin A_{1C} (HbA_{1C}) was measured by high-pressure liquid chromatography using a fully automated Glycosylated Hemoglobin Analyzer System (BioRad, Richmond, CA, USA) (studies II–IV) and HbA_{1c} (mmol/mol) was calculated using the following formula: $10.93 \times HbA_{1c}$ (%) – 23.50 (study II). The plasma total, LDL and HDL cholesterol and triglyceride concentrations were measured with respective enzymatic kits from Roche Diagnostics using an autoanalyzer (Roche Diagnostics Hitachi 917, Hitachi Ltd., Tokyo, Japan) (studies II–IV). Serum FFAs were measured by the enzymatic colorimetric assay (NEFA-HR(2), Wako Chemicals GmbH, Neuss, Germany) using a Konelab 60i analyzer (Thermo Electron

Corporation, Vantaa, Finland) (studies II and IV). Plasma ALT, AST, ALP, GGT and creatinine concentrations were determined as recommended by the European Committee for Clinical Laboratory Standards (studies II–IV). Serum adiponectin was measured using the Human Adiponectin ELISA kit from B-Bridge International (Cupertino, CA, USA) (studies II and III) and serum MCP-1 using an ELISA kit from Quantikine, R&D Systems (Minneapolis, MN, USA) (study III). The coefficience of variation values for serum adiponectin and MCP-1 are shown in **Table 2**. We calculated the NAFLD fibrosis score based on the age, BMI, impaired fasting glucose and diabetes status, concentrations of AST, ALT and albumin, and the platelet count as described (study IV) (Angulo *et al.* 2007).

Blood samples for the coagulation assays were collected into tubes containing 0.129 mol/l citrate after an overnight fast from an antecubital vein (study III). Plasma was separated by centrifugation for 20 min at 2245 g at room temperature, and frozen in aliquots stored at –70 °C until assayed. Prothrombin time (PT, % of activity from normal), activated partial thromboplastin time (APTT), while the activities of fibrinogen, VWF, FVII, FVIII, FIX, FXI, FXII and FXIII were measured using the BCS® XP coagulation analyzer (Siemens Healthcare Diagnostics, Marburg, Germany) at the coagulation laboratory of the Helsinki University Hospital (HUSLAB). PT was measured by the Owren method and the Nycotest® PT reagent (Axis-Shield, Oslo, Norway), APTT with the Actin® FSL reagent (Siemens Healthcare Diagnostics) and fibrinogen with a modification of the Clauss method (Multifibren® U; Siemens Healthcare Diagnostics). D-dimer was assessed using an immunoturbidimetric assay (Tina-quant D-Dimer®, Roche Diagnostics, Mannheim, Germany). VWF ristocetin cofactor (VWF:RCo) activity was measured using the BC® von Willebrand Reagent, FVII activity using Dade® Innovin® and Coagulation Factor VII Deficient Plasma and the FXIII activity using Berichrom® FXIII (all from Siemens Healthcare Diagnostics). For the one-stage FVIII, FIX, FXI and FXII activity measurements, we used Pathromtin SL® and the specific coagulation factor deficient plasma (all from Siemens Healthcare Diagnostics). The local reference ranges and the coefficient of variation values are shown in **Table 2**.

4.4.8. Genotyping of the risk variants

Study subjects were genotyped for the *PNPLA3* gene variant in studies II through IV and for *TM6SF2* and *MBOAT7* variants in study IV. Genomic DNA was extracted from whole blood using the Autopure LS (Qiagen, Hilden, Germany). All three SNPs (*PNPLA3* at rs738409, C>G/I148M; *TM6SF2* at rs58542926, C>T/E167K; and *MBOAT7* at rs641738, C>T) were genotyped using the TaqMan PCR method (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Post-PCR allelic discrimination was carried out measuring allele-specific fluorescence on an ABI Prism Sequence Detection System ABI 7900HT (Applied Biosystems). The success rate for genotyping was >95% for all three SNPs and the genotypes of all three SNPs were in the Hardy‑Weinberg equilibrium.

Table 2. The local reference ranges and the coefficients of variation for plasma coagulation factors and the coefficient of variation values for serum adiponectin and MCP-1 (studies II–III)

	The reference range	Intra-assay variation (%)	Inter-assay variation (%)
FVII (%)	76–170	4.8[a]	5.8[a]
FVIII (%)	52–148	6.6[a]	7.3[a]
FIX (%)	67–135	4.3[a]	6.0[a]
FXI (%)	60–120	3.3[a]	3.4[a]
FXII (%)	60–150	3.1[a]	4.2[a]
FXIII (%)	76–156	2.7[a]	3.2[a]
VWF:RCo (%)	44–183	4.2[a]	4.8[a]
Fibrinogen (g/l)	1.7–4.0	2.9[a]	4.6[a]
PT (%)	70–130	1.9[a]	3.4[a]
APTT (s)	20–33	2.8[a]	3.9[a]
D-dimer (mg/l)	<0.5	1.0[b]	3.4[b]
Adiponectin	–	3.5[c]	5.2[c]
MCP-1	–	5.8[d]	5.7[d]

[a]Data obtained from the method verification at the coagulation laboratory of the Helsinki University Hospital (HUSLAB). [b]Data obtained from the Tina-quant D-Dimer user manual (Roche Diagnostics, Mannheim, Germany). [c]Data obtained from the Human Adiponectin ELISA kit user manual (B-Bridge International, Cupertino, CA, USA). [d]Data obtained from the Human CCL2/MCP-1 ELISA product datasheet (Quantikine, R&D Systems, Minneapolis, MN, USA). **Abbreviations:** APTT, activated partial thromboplastin time; FVII, coagulation factor VII; FVIII, coagulation factor VIII, FIX, coagulation factor IX; FXI, coagulation factor XI; FXII, coagulation factor XII; FXIII, coagulation factor XIII; MCP-1, monocyte chemoattractant protein 1; PT, prothrombin time; VWF:RCo, von Willebrand factor ristocetin cofactor activity.

4.4.9. Statistical analyses

The distribution of continuous variables was tested for normality using the Kolmogorov–Smirnov test (study II), the D'Agostino–Pearson normality test (study III) and the Shapiro–Wilk's normality test (study IV). Normally distributed data were shown as mean ± the standard error of the mean, while non-normally distributed data were reported as the median followed by the 25th and 75th percentiles (studies II–IV). We applied the logarithmic transformation for non-normally distributed data when necessary (studies II–IV). The study groups were compared using the Fisher's exact test for categorical variables, and the unpaired t-test or the Mann–Whitney's test for continuous variables (studies II–IV). For three or more groups, we used the one-way analysis of variance with the Bonferroni post-hoc test and the Kruskal–Wallis H test (study III). Changes during follow-up within the study groups were analyzed using the paired t-test or Wilcoxon's matched pairs test, while McNemar's test was used for categorical variables (study IV). Correlation analyses were performed using the Pearson's correlation coefficient (studies II–IV), the Spearman's correlation coefficient (study III) and the partial correlation (study III). The Grubbs' test with an alpha-value of 0.05 was used to identify outliers that were then excluded (study III). A *p* value of less

than 0.05 was considered statistically significant (studies II–IV).

Linear and binary logistic regression analyses were used to identify predictors of liver fat and stiffness (study IV). Variables predicting liver fat content and stiffness at a significance level of $p<0.05$ in univariate analyses were entered into multiple linear or backward logistic regression analyses as appropriate. If variables were measures of the same biological process (weight, BMI or body fat percent; waist, hip or waist-to-hip ratio; glucose, HbA_{1C}, insulin or HOMA-IR), we only included the one most closely associated with the outcome of interest. The area under the receiver operating characteristic (ROC) curve (AUROC) of the logistic regression models was used to compare models applying the method of DeLong *et al.* (DeLong *et al.* 1988) (study IV). We used GraphPad Prism version 4.03 for PC (study II) and version 6.00 for Mac (studies III and IV) (GraphPad Software Inc., San Diego, CA, USA), IBM SPSS Statistics 22.0 (study III) and 24.0 (study IV) for Mac (IBM SPSS, Chicago, IL, USA) and the pROC package in R (http://www.R-project.org/) (study IV) for all statistical analyses.

5. RESULTS

5.1. Systematic review

5.1.1. Study selection

Systematic literature searches resulted in 1718 potentially relevant citations in which plasma or serum liver enzymes (n = 810 citations), ultrasound (n = 605), ^{1}H-MRS (n = 247) and liver biopsy (n = 56) were used to diagnose NAFLD. After evaluating the titles and abstracts from these citations, 54, 30, 1 and 4, respectively, publications tentatively met the inclusion criteria. Based on a full-text judgment, we excluded 30 articles which were not prospective cohort studies, 22 articles which did not adjust their analyses for alcohol use or exclude other liver diseases, 15 articles in which the above listed methods were not used to diagnose NAFLD and two citations for which only an abstract was available. Thus, we included a total of 20 publications listed in Tables 2 and 3 in study I. We identified no longitudinal studies aimed at examining if the liver fat content measured by ^{1}H-MRS or liver histology predicted type 2 diabetes.

5.1.2. Study characteristics

Table 3 provides the details of the six studies that used ultrasound to diagnose NAFLD. All except one small study included subjects of Asian origin (Table 2). The mean age of the study subjects ranged from 37 to 49 years and BMI ranges from 23 to 27 kg/m^2. The duration of the follow-up period varied from 3 to 10 years. In 4 of the 6 studies (Shibata *et al.* 2007, Park *et al.* 2013, Kasturiratne *et al.* 2013, Chang *et al.* 2013), type 2 diabetes was diagnosed as a fasting plasma or serum glucose ≥7.0 mmol/l, HbA_{1C} ≥6.5%, an OGTT ≥11.1 mmol/l and/or the use of glucose-lowering medication. Okamoto *et al*. defined hyperglycemia as a fasting plasma glucose concentration >6.1 mmol/l or HbA_{1C} >6.4% (Okamoto *et al.* 2003), while Zelber–Sagi *et al.* used cut-offs of ≥5.6 mmol/l and ≥5.7%, respectively (Zelber-Sagi *et al.* 2013).

Table 4 shows the details of the 14 studies in which NAFLD was diagnosed using liver enzymes. The mean age of study subjects ranged from 44 to 61 years and BMI ranged from 23 to 28 kg/m^2. The duration of the follow-up period ranged from 2 to 20 years. Half of these studies consisted of men only. Nine studies defined type 2 diabetes as a fasting glucose ≥7.0 mmol/L or the use of diabetes medication (Lee *et al.* 2003, Nakanishi *et al.* 2003, Nakanishi *et al.* 2004, Sattar *et al.* 2004, Nannipieri *et al.* 2005, Goessling *et al.* 2008, Adams *et al.* 2009, Nanditha *et al.* 2014, Ahn *et al.* 2014). OGTT was performed in three studies (Nannipieri *et al.* 2005, Cho *et al.* 2007, Nanditha *et al.* 2014) and HbA_{1C} was measured in one study (Ahn *et al.* 2014). Questionnaires or patient records were used for the diagnosis of type 2 diabetes in four studies (Perry *et al.* 1998, Wannamethee *et al.* 2005, Ford *et al.* 2008, Monami *et al.* 2008). Most of these studies compared the risk of diabetes between subjects in the highest quarter or fifth with subjects in the lowest quarter or fifth of liver enzyme measurements. Two studies with slightly different study designs included subjects from the same cohort (Nakanishi *et al.* 2003, Nakanishi *et al.* 2004).

Table 3. Prospective studies in which NAFLD was diagnosed by ultrasound.

Cohort	n (men, %)	Age (years)	BMI (kg/m²)	Follow-up (years)	Independent of confounders	Effect estimate (95% CI)
Asians (Okamoto *et al.* 2003)	840 (55.6%)	42.7	22.5	10	No (BMI, age, gender, FPG, HbA_{1c}, alcohol, family history of T2DM)	OR 2.62 (1.58–4.34)*
Asians (Shibata *et al.* 2007)	3189 (100.0%)	48.0	23.1	4	BMI, age	HR 4.8 (3.3–7.1)
Asians (Park *et al.* 2013)	25,232 (100.0%)	42.5	24.2	3.8	BMI, age, WC, TG, HDL, systolic BP, hsCRP, HOMA, creatinine, family history T2DM, exercise, MetS	Mild NAFLD HR 1.09 (0.81–1.48) Moderate/severe NAFLD HR 1.73 (1.00–3.01)
Asians (Kasturiratne *et al.* 2013)	1842 (43.2%)	– (52.5)	– (24.0)	3	BMI, age, gender, WC, ALT, family history of T2DM, hypertension	HR 1.64 (1.20–2.23)
Asians (Chang *et al.* 2013)	38,291 (62.5%)	36.8	23.3	5.1	BMI, age, gender, smoking, alcohol, exercise, family history of T2DM, cholesterol, TG, HDL, HOMA, hsCRP	Low NFS HR 1.81 (1.61–2.04) High NFS HR 3.84 (2.93–5.02)
Caucasian (Israelis) (Zelber-Sagi *et al.* 2013)	141 (50.4%)	48.8	26.6	6.8	BMI, age, gender, family history of T2DM, fS insulin, adiponectin, fS glucose, physical activity	Normal US OR 2.95 (1.03–8.44) HRI OR 7.77 (1.82–33.26)

Abbreviations: ALT, alanine transferase; BP, blood pressure; DM, diabetes mellitus, fS, fasting serum; FPG, fasting plasma glucose; HbA_{1c}, glycosylated hemoglobin A_{1c}; HDL, high-density lipoprotein cholesterol; HOMA, homeostasis model assessment for insulin resistance; HR, hazard ratio; HRI, hepato-renal ultrasound index; hsCRP, high-sensitivity C-reactive protein; IFG, impaired fasting glucose; MetS, metabolic syndrome; NAFLD, non-alcoholic fatty liver disease; NFS, NAFLD fibrosis score; OR, odds ratio; T2DM, type 2 diabetes; TG, triglyceride; US, ultrasonography; WC, waist circumference.

*Unadjusted estimate. Adapted from Lallukka *et al. Best Practice & Research Clinical Endocrinology & Metabolism*. 2016; 30:385–95 (study I). Reproduced with permission from the copyright holder.

Table 4. Prospective studies in which NAFLD was defined by liver enzymes.

Cohort	n (men, %)	Age (years)	BMI (kg/m²)	Follow-up (years)	iver enzyme	Independent of confounders	Effect estimate (95% CI)
Caucasian men (Perry *et al.* 1998)	7458 (100.0%)	40–59	–	12.8	GGT	BMI, age, physical activity, alcohol, smoking, prevalent CHD	Quintiles Q5 vs. Q1 RR 4.7 (2.4–9.4)
Asian men (Lee *et al.* 2003)	4088 (100.0%)	25–55	–	4	GGT, ALT	BMI, age, smoking, exercise, family history of T2DM, FPG, alcohol	Highest vs. lowest concentration group with alcohol <90g/week
Asian men (Nakanishi *et al.* 2003)	2918 (100.0%)	46.5	23.3	7	GGT	BMI, age, family history of T2DM, alcohol, smoking, physical activity, systolic BP, cholesterol, TG, FPG, white blood count	Quartiles Q4 vs. Q1 RR 3.44 (1.69–6.70)
Asian men (Nakanishi *et al.* 2004)	3260 (100.0%)	–	–	7	GGT, ALT	BMI, age, family history of T2DM, alcohol, smoking, physical activity, FPG, white blood count, other liver enzymes	Quintiles Q5 vs. Q1 GGT HR 2.44 (1.34–4.46)
Caucasian men (Sattar *et al.* 2004)	5974 (100.0%)	55.4	26.0	4.9	ALT	BMI, age, smoking, systolic BP, cholesterol/HDL ratio, TG, alcohol, FPG	Quartiles Q4 vs. Q1 HR 2.04 (1.16–3.58)
Hispanics (Nannipieri *et al.* 2005)	1441 (38.8%)	47.1	28.0	7	AST	BMI, age, gender, WC, alcohol, FSI	Quartile Q4 vs. Q1-3 OR 1.67 (1.06–2.64)
Caucasian men (Wannamethee *et al.* 2005)	3500 (100.0%)	60–79	–	5	GGT, ALT	BMI, age, social class, physical activity, smoking, alcohol, preexisting CHD/stroke, use of statins	Quartiles: Q4 vs. Q1 GGT RR 3.68 (1.68–8.04)
Asians (Cho *et al.* 2007)	8750 (46.6%)	51.8	24.4	2	GGT, ALT	BMI, age, systolic BP, family history of T2DM, smoking, alcohol, exercise, FPG, TG, HDL, HOMA, CRP	Quartiles Q4 vs. Q1 ALT RR 2.20 (1.28–3.73) in men RR 1.97 (1.03–3.77) in women

Caucasians (Goessling *et al.* 2008)	2812 (44.4%)	44.0	25.6	20	ALT, AST	BMI, age, gender, smoking, menopause, alcohol	Per +1 SD in logALT ALT OR 1.48 (1.30–1.69)
Caucasians (Ford *et al.* 2008)	2298 (38.3%)	49.5	26.0	7	GGT, ALT	BMI, age, gender, education, smoking, alcohol, physical activity, WC, systolic BP, cholesterol, HDL, CRP, FPG	Quintiles Q5 vs. Q1 GGT HR 2.61 (1.59–4.28)
Caucasians (Monami *et al.* 2008)	2662 (42.9%)	54.3	25.9	3.3	GGT, ALT, AST	Age, gender, alcohol, smoking	Per +10 U/l GGT HR 1.09 (1.04–1.15)
Caucasians (Adams *et al.* 2009)	358 (68.4%)	59.9	27.1	11.1	ALT	No (age, WC, HOMA, HDL, TG)	ALT >40 vs. <40 U/l RR 3.1 unadjusted
Asian men (Nanditha *et al.* 2014)	537 (100.0%)	46.0	25.8	2	GGT	BMI, age, family history of T2DM, smoking, alcohol, ALT, OGTT, FPG, HbA_{1c}, TG, HOMA	Above vs. below median GGT HR 1.78 (1.17–2.68)
Asians (Ahn *et al.* 2014)	6926 (37.6%)	61.4	24.3	4.2	GGT, ALT	BMI, age, WC, cholesterol, HDL, TG, alcohol, smoking, physical activity, follow-up time, CRP	Quartiles Q4 vs. Q1 GGT OR 2.13 (1.33–3.41) in men OR 2.69 (1.86–3.89) in women

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BP, blood pressure; CHD, coronary heart disease; FG, fasting plasma or serum glucose; FSI, fasting serum insulin; GGT, gamma-glutamyltransferase; HDL, high-density lipoprotein cholesterol; HOMA, homeostasis model assessment for insulin resistance; HR, hazard ratio; IFG, impaired fasting glucose; NAFLD, non-alcoholic fatty liver disease; OGTT, two-hour oral glucose tolerance test; OR, odds ratio; RR, risk ratio; T2DM, type 2 diabetes; TG, triglyceride; US, ultrasound; WC, waist circumference.

*This column provides the effect estimate of the best predictor of type 2 diabetes in the respective study. Adapted from Lallukka *et al. Best Practice & Research Clinical Endocrinology & Metabolism*. 2016; 30:385–95 (study I). Reproduced with permission from the copyright holder.

5.1.3. NAFLD diagnosed by ultrasound and the risk of type 2 diabetes

Each of the six studies relying on ultrasound showed that NAFLD predicted type 2 diabetes independent of age at baseline. In addition, NAFLD remained a significant predictor of type 2 diabetes in four out of the five studies after adjusting for BMI (**Table 3**). NAFLD also predicted type 2 diabetes independent of several other factors, such as gender, a family history of diabetes, HDL cholesterol, triglycerides, high-sensitivity CRP, insulin resistance, physical activity and smoking status (**Figure 3**).

5.1.4. Liver enzymes and risk of type 2 diabetes

In 12 of the 14 studies, either GGT, ALT, AST or a combination of these enzymes significantly predicted type 2 diabetes independent of age, BMI and alcohol consumption (**Table 4**). In addition, GGT significantly predicted type 2 diabetes in 10 of 11 studies, ALT in 10 of 13 studies and AST in three of seven studies (**Table 4**). GGT and ALT predicted type 2 diabetes even when concentrations fell within the normal range (**Figure 4**).

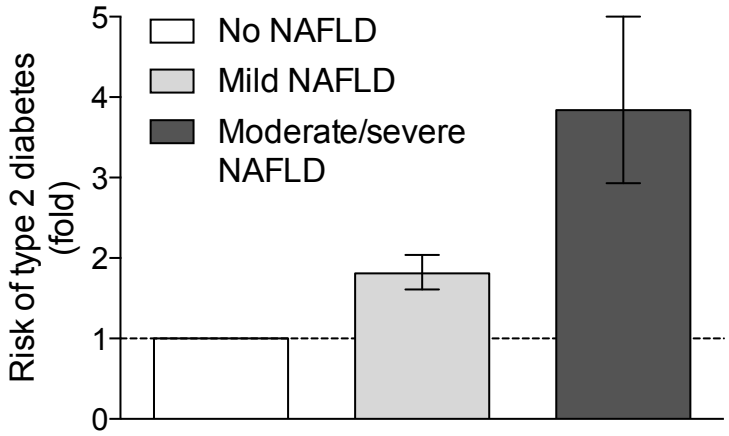

Figure 3. Risk of type 2 diabetes in the mild and moderate to severe NAFLD groups compared to controls without NAFLD (no NAFLD). NAFLD was diagnosed using ultrasound. The severity of NAFLD was defined based on the NAFLD fibrosis score using the cut-off points <–1.455 for mild and ≥–1.455 for moderate to severe NAFLD (Angulo *et al.* 2007). Data are modified from the study by Chang *et al*. (Chang *et al.* 2013) and are shown as the hazard ratios and 95% confidence intervals. Analyses were adjusted for age, gender, BMI, smoking, alcohol intake, exercise, family history of type 2 diabetes, HOMA-IR and the concentrations of total cholesterol, triglycerides, HDL cholesterol and high-sensitivity CRP. The *p* value for a trend was $p < 0.001$. Adapted from Lallukka *et al. Best Practice & Research Clinical Endocrinology & Metabolism. 2016; 30:385–95.* (study I). Reproduced with permission from the copyright holder

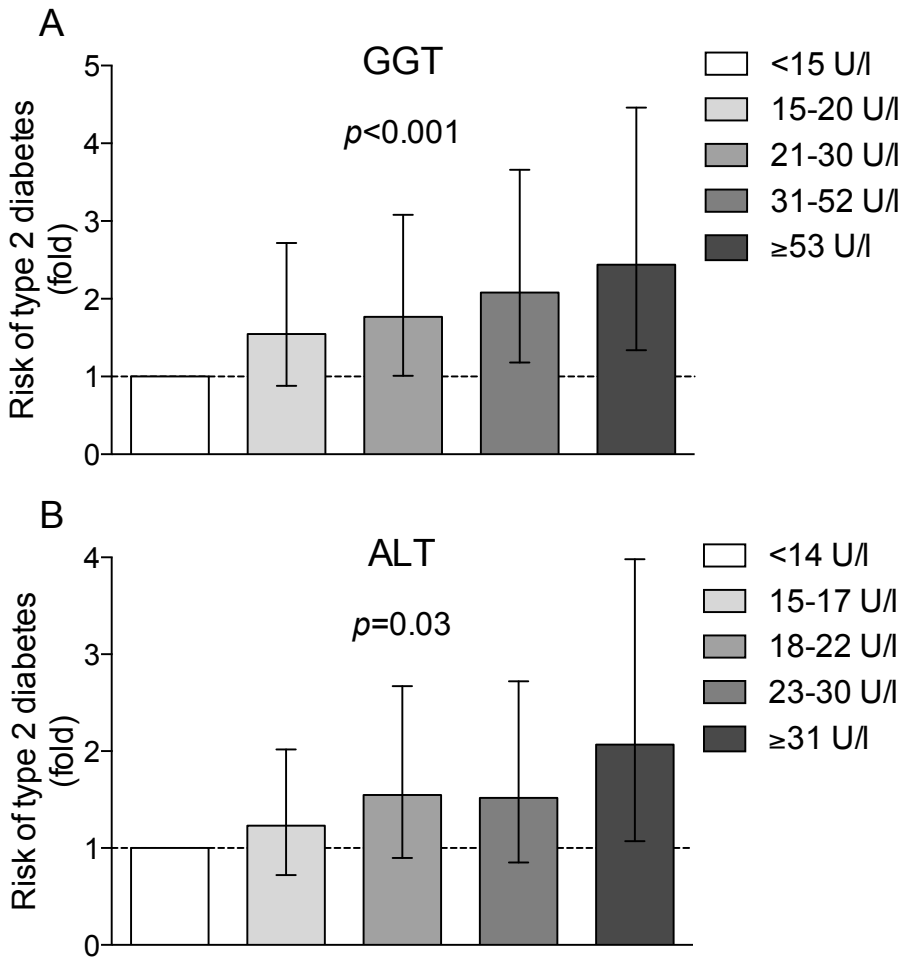

Figure 4. The risk of type 2 diabetes by quintiles of A) gamma-glutamyltransferase (GGT) and B) alanine aminotransferase (ALT) concentrations. Data were obtained from the study by Nakanishi *et al*. (Nakanishi *et al.* 2004) and are shown as the hazard ratios and 95% confidence intervals compared to the lowest quintile. Analyses were adjusted for age, BMI, smoking, alcohol intake, physical activity, a family history of type 2 diabetes, fasting serum or plasma glucose, blood leukocyte count and other liver enzymes including aspartate aminotransferase and alkaline phosphatase. Adapted from Lallukka *et al. Best Practice & Research Clinical Endocrinology & Metabolism. 2016; 30:385–95.* (study I). Reproduced with permission from the copyright holder

5.2. Characteristics of the study subjects (studies II–IV)

The physical and biochemical characteristics of the subjects in studies II through IV are shown in **Tables 5**, **6** and **7**.

Table 5. Characteristics of the study groups in study II

	Non-obese (n = 41)	**Obese (n = 41)**	**PNPLA3^{148II} (n = 55)**	**PNPLA3^{148MM} (n = 27)**
Men/women	13/28	6/35	12/43	7/20
PNPLA3^{148II}/PNPLA3^{148MM}	26/15	29/12	55/0	0/27$^{\#\#\#}$
Age (years)	47 (32–58)	50 (39–61)	48 (37–60)	50 (34–63)
Weight (kg)	75.3 ± 1.6	101.7 ± 2.4***	88.1 ± 2.6	89.5 ± 3.4
Waist-to-hip ratio	0.89 (0.84–0.96)	0.94 (0.90–1.00)**	0.92 (0.86–0.97)	0.92 (0.88–0.97)
Fasting glucose (mmol/l)	5.4 (5.1–5.8)	5.7 (5.4–6.2)	5.6 (5.2–5.9)	5.5 (4.9–5.9)
HbA_{1c} (%)	5.6 (5.2–5.8)	5.7 (5.5–5.9)	39 (37–41)	5.7 (5.2–5.9)
Fasting triglyceride (mmol/l)	0.9 (0.6–1.5)	1.3 (1.2–1.9)***	1.3 (0.8–1.8)	1.2 (0.8–1.7)
Fasting HDL cholesterol (mmol/l)	1.6 (1.2–2.1)	1.4 (1.2–1.7)	1.6 (1.2–1.8)	1.4 (1.2–1.7)
Fasting LDL cholesterol (mmol/l)	2.7 ± 0.1	3.2 ± 0.2*	2.9 ± 0.1	3.0 ± 0.2
AST (U/l)	27 (23–41)	26 (23–38)	25 (21–30)	31 (26–53) $^{\#\#\#}$
ALT (U/l)	21 (17–42)	28 (22–45)	23 (18–35)	38 (22–90) $^{\#}$
AST/ ALT	1.1 ± 0.1	0.9 ± 0.0	1.1 ± 0.1	0.9 ± 0.1
GGT (U/l)	24 (15–42)	27 (17–43)	26 (16–38)	23 (17–50)

Data are shown as mean ± standard error of the mean or median followed by the 25th and 75th percentiles. *p<0.05, **p<0.005, ***p<0.0005 for comparisons within groups. $^{\#}p$<0.05 $^{\#\#\#}p$<0.0005 for comparison to PNPLA3^{148II} group **Abbreviations:** ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; GGT, gamma-glutamyltransferase; HbA_{1C}, glycosylated hemoglobin A_{1C}; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; LDL, low-density lipoprotein. Adapted from Lallukka *et al. Diabetologia.* 2013; 56:886–92 (study II). Reproduced with permission from the copyright holder.

Table 6. Characteristics of the study groups in study III.

	Plasma coagulation factors				*Hepatic expression of coagulation factors*		*Adipose tissue inflammation*
	IS (n = 46)	**IR (n = 46)**	**PNPLA3^{148II} (n = 54)**	**PNPLA3$^{148MM/MI}$ (n = 38)**	**No NAFLD (n = 13)**	**Metabolic NAFLD (n = 13)**	**PNPLA3^{148II} (n = 26)**
HOMA-IR	1.0 (0.6–1.6)	3.6 (2.8–4.6)***	2.4 (1.0–3.3)	2.4 (1.1–3.7)	1.7 (1.2–3.2)	4.6 (2.9–6.9)^^	1.6 (0.7–3.3)
PNPLA3^{148II}/PNPLA3$^{148MM/MI}$	27/19	27/19	54/0	0/38###	13/0	13/0	26/0
Men/women	22/24	23/23	25/29	20/18	4/9	6/7	8/18
Age (years)	40 (26–52)	43 (34–54)	40 (27–50)	43 (31–56)	44 ± 1	44 ± 2	47 ± 2.6
BMI (kg/m^2)	28.2 ± 0.7	33.4 ± 0.6***	31.0 ± 0.7	30.5 ± 0.8	45.7 ± 1.7	45.7 ± 0.9	31.2 ± 1.0
Waist-to-hip ratio	0.91 ± 0.01	0.99 ± 0.01***	0.95 ± 0.01	0.95 ± 0.02	0.99 ± 0.04	1.02 ± 0.03	0.92 ± 0.02
Fasting insulin (mU/l)	4.2 (2.7–6.7)	14.1 (12.2–17.1)***	10.4 (3.9–13.4)	8.8 (4.5–14.3)	7.9 (5.5–12.5)	19.0 (12.1–26.6)^	8.6 ± 1.3
Fasting glucose (mmol/l)	5.4 ± 0.1	5.7 ± 0.1*	5.5 ± 0.1	5.6 ± 0.1	5.2 (4.7–5.9)	5.8 (5.3–6.2)	5.6 ± 0.1
HbA_{1c} (%)	5.5 ± 0.1	5.7 ± 0.1*	5.6 ± 0.1	5.6 ± 0.1	5.7 ± 0.2	6.2 ± 0.2	5.7 ± 0.1
Fasting triglyceride (mmol/l)	1.0 (0.7–1.5)	1.6 (1.2–2.2)***	1.3 (0.9–1.9)	1.2 (0.9–1.7)	1.1 (0.8–2.1)	1.5 (1.2–2.0)	1.3 (0.9–2.0)
Fasting HDL cholesterol (mmol/l)	1.5 (1.3–1.7)	1.3 (1.1–1.5)**	1.4 (1.1–1.6)	1.3 (1.1–1.6)	1.1 ± 0.1	1.0 ± 0.1	1.5 (1.2–1.7)
Fasting LDL cholesterol (mmol/l)	2.9 (2.3–3.4)	3.1 (2.6–3.5)	2.9 (2.3–3.4)	3.0 (2.5–3.5)	–	–	3.0 ± 0.2
Leukocyte count (10^9 /l)	5.5 (4.7–6.6)	6.5 (5.7–7.3)**	6.0 (5.0–7.0)	5.8 (5.0–7.2)	–	–	5.7 (4.7– 6.7)
AST (U/l)	25 (20–31)	30 (24–45)**	25 (21–33)	31 (25–46)#	31 (25–39)	35 (28–41)	25 (19–27)
ALT (U/l)	22 (17–46)	39 (24–81)***	26 (19–53)	34 (19–80)	34 ± 3	52 ± 6^	22 (18–42)
AST/ ALT	1.1 ± 0.1	0.8 ± 0.1***	0.9 ± 0.1	1.0 ± 0.1	–	–	1.0 ± 0.1

Data are shown as mean ± standard error of mean or median followed by the 25th and 75th percentiles. *p<0.05, **p<0.005, ***p<0.0005 for comparison to IS group. #p<0.05 ##p<0.005 for comparison to PNPLA3^{148II} group. ^p=0.05, ^^p<0.005 for comparison to No NAFLD group. **Abbreviations:** ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; HbA_{1C}, glycosylated hemoglobin A_{1C}; HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessment of insulin resistance; IR, insulin-resistant; IS, insulin-sensitive; LDL, low-density lipoprotein; NAFLD, non-alcoholic fatty liver disease. Adapted from Lallukka *et al. Thrombosis and Haemostasis. 2017; 26; 117:286–294.* (study III). Reproduced with permission from the copyright holder.

Table 7. Characteristics of the study subjects in study IV

	All (n = 97)
Men/women	46/51
PNPLA3 (148^{II}/148^{IM}/148^{MM})	54/33/10
TM6SF2 (CC/CT/TT)	74/17/1[a]
MBOAT7 (CC/CT/TT)	35/43/14[a]
Age (years)	44 ± 1
BMI (kg/m^2)	29.0 ± 0.5
Waist circumference	99.0 ± 1.3
Fasting glucose (mmol/l)	5.8 (5.3–6.4)
HbA_{1c} (%)	5.7 (5.3–6.1)
HOMA-IR	2.0 (1.2–3.5)
Fasting triglycerides (mmol/l)	1.3 (0.9–1.9)
Fasting HDL cholesterol (mmol/l)	1.3 (1.1–1.5)
Fasting LDL cholesterol (mmol/l)	3.1 (2.4–3.8)
AST (U/l)	28 (22–38)
ALT (U/l)	28 (20–48)
AST/ALT	0.9 (0.7–1.1)
GGT (U/l)	25 (15–46)

Data are shown as mean ± standard error of mean or median followed by the 25th and 75th percentiles. [a]genotyping failed in five subjects. **Abbreviations:** ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; GGT, gamma-glutamyltransferase; HbA_{1C}, glycosylated hemoglobin A_{1C}; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; LDL, low-density lipoprotein.

5.3. Adipose tissue inflammation in 'Metabolic/obese NAFLD' and 'PNPLA3 NAFLD' (study II)

5.3.1. Liver fat content and features of insulin resistance

The obese group exhibited a higher BMI than the non-obese group by definition (**Figure 5A**). In addition, liver fat content was significantly higher among obese (9.5% ± 1.3%) compared to non-obese subjects (5.1% ± 0.9%, p=0.007) (**Figure 5B**). The obese group also exhibited a significantly higher HOMA-IR (**Figure 5C**) and higher concentrations of serum insulin (10.9 ± 1 mU/l vs. 6.7 ± 1 mU/l; p<0.001) and triglycerides (**Table 5**) than the non-obese group. By contrast, the PNPLA3^{148MM} group had a significantly higher liver fat content (11.4% ± 1.7%) than the PNPLA3^{148II} group (5.3% ± 0.8%, p<0.001) (**Figure 5B**), while BMI (**Figure 5A**), HOMA-IR (**Figure 5C**) and concentrations of the serum insulin (8.7 ± 1 mU/l vs. 8.9 ± 1 mU/l) and triglycerides (**Table 5**) were comparable between groups.

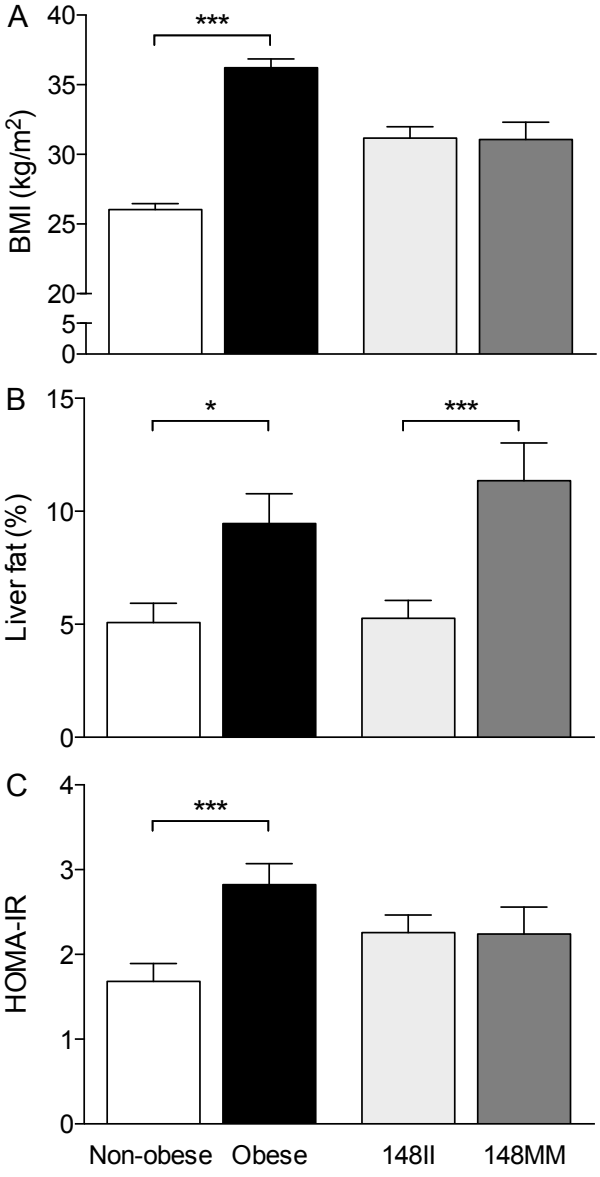

Figure 5. Body mass index (BMI; **A**), liver fat content (**B**) and HOMA-IR (**C**) in non-obese and obese groups and in PNPLA3^{148II} and PNPLA3^{148MM} groups. Data are shown as mean ± standard error of mean. *p<0.05, ***p<0.0005. Adapted from Lallukka *et al. Diabetologia.* 2013; 56:886–92 (study II). Reproduced with permission from the copyright holder.

5.3.2. Gene expression of inflammatory markers in subcutaneous adipose tissue

The expression of gene *MCP-1* was significantly higher among the obese group compared to the non-obese group (**Figure 6A** and Table 1 in original publication for study II). That of the macrophage marker *CD68* was also slightly higher in the obese compared to the non-obese group, although the difference was not significant (**Figure 6A** and Table 1 in original publication for study II). By contrast, the expression of the anti-inflammatory genes *TWIST1* and *ADIPOQ* were significantly lower in the obese group compared to the non-obese group (**Figure 6A** and Table 1 in original publication for study II). The PNPLA3^{148MM} and PNPLA3^{148II} groups appeared similar with respect to the expression of the pro-inflammatory and anti-inflammatory genes (**Figure 6B** and Table 1 in original publication for study II).

5.3.3. Serum FFA and adiponectin concentrations

The concentration of the fasting serum FFA did not differ significantly between the obese (515, 403–594 μmol/l) and the non-obese (450, 361–657 μmol/l) groups. In addition, the concentration appeared comparable between the PNPLA3^{148MM} (474, 367–582 μmol/l) and PNPLA3^{148II} (462, 384–635 μmol/l) groups. Fasting FFA correlated with the liver fat content among all subjects (r=0.28, p=0.02), particularly in the obese (r=0.41, p=0.01) and PNPLA3^{148II} (r=0.36, p=0.02) groups but not in the non-obese (r=0.10, p=0.59) or PNPLA3^{148MM} (r=0.22, p=0.32) groups.

The serum adiponectin concentration correlated inversely with liver fat content across all subjects (r=–0.41, p<0.001) and was slightly although not significantly lower in the obese (9.2 ± 0.7 μg/ml) compared to the non-obese (10.2 ± 1.0 μg/ml) group. The concentration appeared similar between the PNPLA3^{148MM} (10.1 ± 1.4 μg/ml) and PNPLA3^{148II} (9.5 ± 0.6 μg/ml) groups.

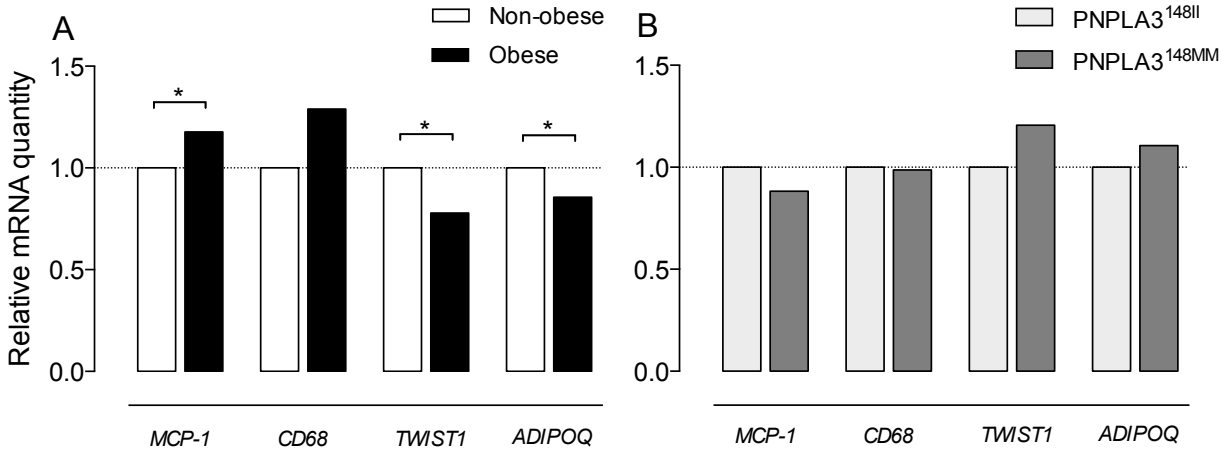

Figure 6. The gene expression of pro-inflammatory (monocyte chemoattractant protein-1, *MCP-1*; cluster of differentiation 68, *CD68*) and anti-inflammatory (Twist-related protein 1, *TWIST1*; adiponectin, *ADIPOQ*) genes relative to the expression of the housekeeping gene (acidic ribosomal phosphoprotein 36B4, *36B4*) in subcutaneous adipose tissue in non-obese and obese groups (**A**) and in PNPLA3^{148II} and PNPLA3^{148MM} groups (**B**). *p<0.05. Adapted from Lallukka *et al. Diabetologia.* 2013; 56:886–92 (study II). Reproduced with permission from the copyright holder.

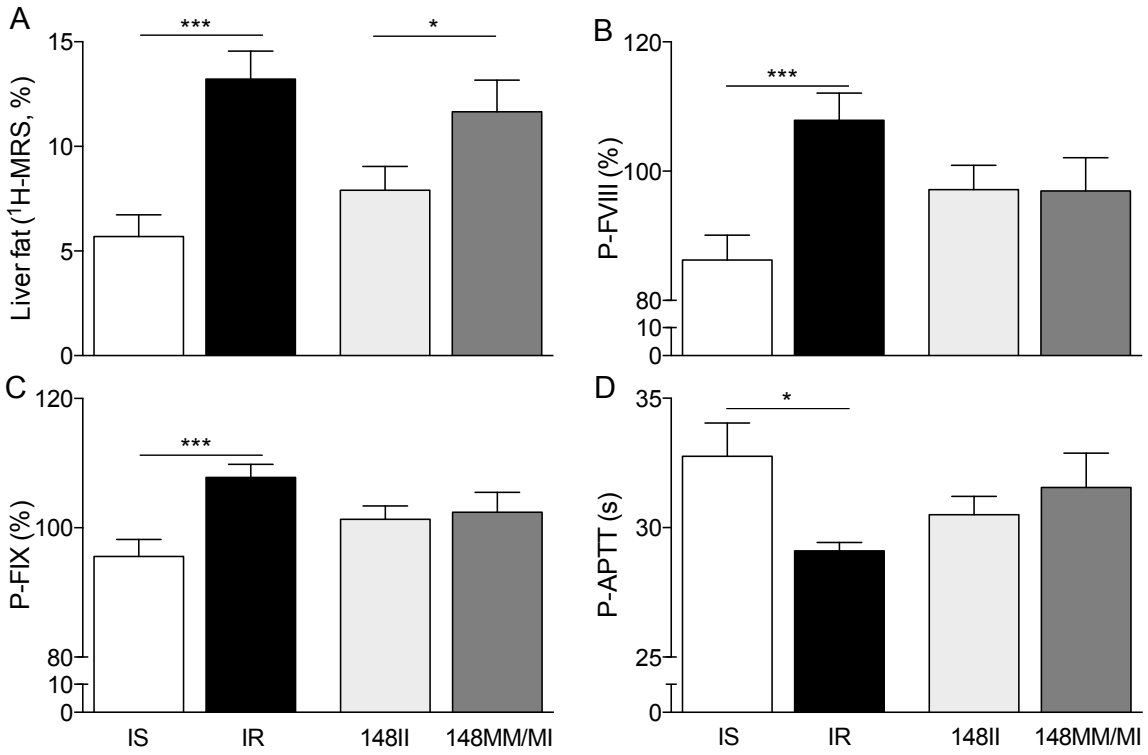

Figure 7. The liver fat content (**A**), activities of FVIII (**B**) and FIX (**C**), and APTT (**D**) in insulin-sensitive (IS) and insulin-resistant (IR) groups, and in the PNPLA3^{148II} (148II) and PNPLA3$^{148MM/MI}$ (148MM/MI) groups. Data are shown as mean ± standard error of mean. *p<0.05 and ***p<0.0005. **Abbreviations:** APTT, activated partial thromboplastin time; FIX, coagulation factor VIII; FIX, coagulation factor IX; ^{1}H-MRS, proton magnetic resonance spectroscopy; P, plasma. Adapted from Lallukka *et al. Thrombosis and Haemostasis. 2017; 26; 117:286–294.* (study III). Reproduced with permission from the copyright holder.

5.4. Coagulation factors in 'Metabolic/obese NAFLD' and 'PNPLA3 NAFLD' (study III)

5.4.1. Plasma coagulation factors

5.4.1.1. Liver fat content and features of insulin resistance

Insulin-resistant subjects (the IR group) had a higher liver fat content (**Figure 7A**), fasting glucose and insulin concentrations, HOMA-IR and triglyceride concentration than insulin-sensitive subjects (the IS group) (**Table 6**). The PNPLA3$^{148MM/MI}$ group exhibited a higher liver fat content than the PNPLA3^{148II} group (**Figure 7A**), although no differences existed in the fasting glucose or insulin concentrations, HOMA-IR, or triglyceride concentration across these groups (**Table 6**).

5.4.1.2. Activities and concentrations of coagulation factors

The activities of FVIII (**Figure 7B**), FIX (**Figure 7C**), FXI, FXII, FXIII, VWF:RCo, fibrinogen and PT were all significantly higher, while APTT (**Figure 7D**) was significantly shorter in duration in the IR groups than in the IS group (**Table 8**). The FVII activity and D-dimer concentrations did not significantly differ between these groups. Despite the liver fat content distinction, all coagulation variables appeared comparable between the PNPLA3$^{148MM/MI}$ and PNPLA3^{148II} groups (**Table 8**).

The results were comparable in a two-by-two fashion (see Supplemental Table 1 and 2 in original publication for study III). The

activities of FVIII, FIX, FXI and fibrinogen were increased and APTT was shortened in IR subjects as compared to IS subjects without the I148M variant (IR and PNPLA3^{148II} vs. IS and PNPLA3^{148II}). The *PNPLA3* genotype did not influence the coagulation activities among either IS or IR subjects.

Table 8. Plasma coagulation variables in study III

	IS (n = 46)	IR (n = 46)	PNPLA3^{148II} (n = 54)	PNPLA3$^{148MM/MI}$ (n = 38)
FVII (%)	114 (100–129)	118 (110–132)	117 (106–130)	117 (97–128)
FXI (%)	96 ± 3	108 ± 2**	103 ± 2	102 ± 3
FXII (%)	100 ± 4	111 ± 3*	106 ± 3	105 ± 4
FXIII (%)	96 ± 3	105 ± 3*	99 ± 3	104 ± 4
VWF:RCo (%)	99 ± 7	124 ± 7*	110 ± 7	114 ± 7
Fibrinogen	3.6 ± 0.2	4.2 ± 0.2*	4.0 ± 0.2	3.9 ± 0.2
PT (%)	114 ± 3	124 ± 3*	117 ± 2	121 ± 4
D-dimer (mg/l)	0.08 (0.05–0.20)	0.15 (0.05–0.30)	0.1 (0.05–0.20)	0.1 (0.05–0.30)

Data are shown as mean ± standard error of mean or median followed by the 25th and 75th percentiles. *p<0.05, **p<0.005, ***p<0.0005. **Abbreviations:** APTT, activated partial thromboplastin time; F, coagulation factor; IR, insulin resistant; IS, insulin sensitive; PT, prothrombin time; VWF:RCo, von Willebrand factor ristocetin cofactor activity. Adapted from Lallukka *et al. Thrombosis and Haemostasis. 2017; 26; 117:286–294.* (study III). Reproduced with permission from the copyright holder.

Table 9. Pearson's correlation coefficient and partial correlation coefficient adjusted for age, gender, BMI and *PNPLA3* genotype between HOMA-IR and coagulation factors.

	HOMA-IR (log) (n = 92)	
	r	Partial *r*
FVII (%)	0.29**	0.21*
FVIII (%)	0.35***	0.24*
FIX (%)	0.50***	0.30**
FXI (%)	0.45***	0.36**
FXII (%)	0.33**	0.32**
FXIII (%)	0.23*	0.001
VWF:RCo (%)	0.28**	0.21*
Fibrinogen (g/l)	0.31**	–0.01
PT (%)	0.40***	0.29**
APTT (s)	–0.33**	–0.24*
D-dimer (IU/l)	0.17	0.17

*p<0.05, **p<0.01, ***p<0.001. **Abbreviations:** APTT, activated partial thromboplastin time; HOMA-IR, homeostatic model assessment of insulin resistance; FVII, coagulation factor VII; FVIII, coagulation factor VIII, FIX, coagulation factor IX; FXI, coagulation factor XI; FXII, coagulation factor XII; FXIII, coagulation factor XIII; P, plasma; PT, prothrombin time; VWF:RCo, von Willebrand factor ristocetin cofactor activity. Adapted from Lallukka *et al*. *Thrombosis and Haemostasis. 2017; 26; 117:286–294.* (study III). Reproduced with permission from the copyright holder.

5.4.1.3. Insulin resistance and coagulation activities

The degree of insulin resistance assessed using HOMA-IR positively correlated with the activities of FVII, FVIII, FIX, FXI, FXII, FXIII, VWF:RCo, fibrinogen and PT, and inversely correlated with APTT. The relationships between HOMA-IR and the activities of FVII, FVIII, FIX, FXI, FXII, VWF:RCo, PT and APTT remained significant after adjusting for age, gender, BMI and *PNPLA3* genotype. The correlation coefficients and *p* values are shown in **Table 9**.

5.4.2. The hepatic expression of coagulation factors

Patients with 'Metabolic NAFLD' had higher serum insulin, HOMA-IR and plasma ALT, and a lower Matsuda insulin sensitivity index (35, 24-59 vs. 79, 50-145; p<0.05) than subjects without NAFLD (**Table 6**). The groups were equally obese and did not differ with regards to age or gender. The hepatic gene expression of *F8*, *F9* and *FGG* (fibrinogen gamma-chain) was higher in insulin-resistant 'Metabolic NAFLD' than in insulin-sensitive subjects with no NAFLD (**Figure 8**). The expression of *F11* (p=0.11) and *F13A1* (p=0.28) did not differ significantly between these groups.

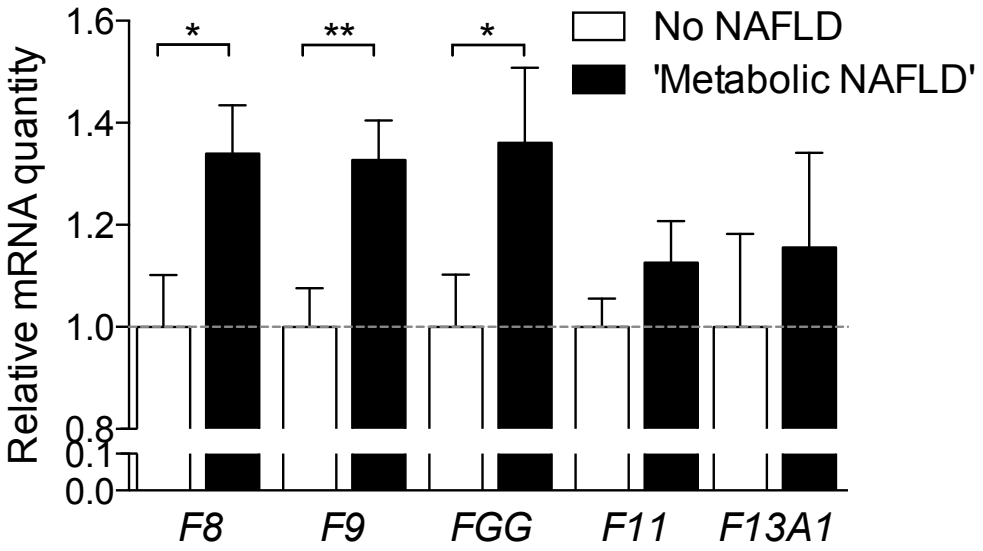

Figure 8. The hepatic expression of *F8*, *F9*, *FGG, F11* and *F13A1* relative to the housekeeping gene (*SDHA*) in subjects with no NAFLD and those with 'Metabolic NAFLD'. Data are shown as the mean ± standard error of mean. *p<0.05 and **p<0.005. **Abbreviations:** *F8*, coagulation factor VIII; *F9*, coagulation factor IX; *F11*, coagulation factor FXI; *F13A1*, coagulation factor XIII subunit A; *FGG*, fibrinogen γ chain; NAFLD, non-alcoholic fatty liver disease. Adapted from Lallukka *et al. Thrombosis and Haemostasis. 2017; 26; 117:286–294.* (study III). Reproduced with permission from the copyright holder.

5.4.3. Adipose tissue inflammation

The gene expression of pro-inflammatory markers in adipose tissue positively correlated with PT% (shortened PT), FVIII, FIX, FXI and VWF:RCo activities, as well as the concentration of fibrinogen in plasma. Yet, the expression of anti-inflammatory markers inversely correlated with PT% (prolonged PT), FIX activity and fibrinogen.

The correlation coefficients for all coagulation variables and inflammatory markers appear in **Table 10**. The concentration of serum MCP-1 positively correlated with VWF:RCo (r=0.47, p=0.02), while the serum adiponectin concentration inversely correlated with fibrinogen (r=–0.43, p=0.04).

Table 10. Pearson's correlation coefficient between adipose tissue expression of pro- and anti-inflammatory markers and plasma coagulation factor activities.

	Pro-inflammatory genes (n=26)			Anti-inflammatory genes (n=26)	
	CD68	*MCP-1*	*TNF-α*	*ADIPOQ*	*TWIST1*
PT (%)	0.45*	0.34	0.36	–0.38	–0.57
APTT (s)	–0.22	–0.062	–0.27	–0.18	–0.034
FVII (%)	0.35	0.15	0.20	–0.19	–0.33
FVIII (%)	0.38	0.53**	0.30	–0.22	–0.28
FIX (%)	0.54*	0.39	0.45*	–0.39*	–0.57**
FXI (%)	0.44*	0.29	0.47*	–0.20	–0.30
FXII (%)	0.32	0.28	–0.034	–0.28	–0.37
FXIII (%)	0.24	0.16	0.27	–0.28	–0.28
Fibrinogen (g/l)	0.42*	0.29	0.33	–0.41*	–0.38
D-dimer (IU/l)	0.59*	0.60**	0.22	–0.31	–0.48**
VWF:RCo (%)	0.40*	0.59**	0.25	–0.15	–0.35

*p<0.05, **p<0.01. **Abbreviations:** APTT, activated partial thromboplastin time; *CD68*, cluster of differentiation 68; FVII, coagulation factor VII; FVIII, coagulation factor VIII, FIX, coagulation factor IX; FXI, coagulation factor XI; FXII, coagulation factor XII; FXIII, coagulation factor XIII; *MCP-1*, monocyte chemoattractant protein 1; P, plasma; PT, prothrombin time; *TNF-α*, tumor necrosis factor α; *TWIST1*, Twist-related protein 1; VWF:RCo, von Willebrand factor ristocetin cofactor activity. Adapted from Lallukka *et al. Thrombosis and Haemostasis. 2017; 26; 117:286–294.* (study III). Reproduced with permission from the copyright holder.

5.5. Predictors of NAFLD and liver stiffness (study IV)

5.5.1. Liver fat content

The median duration of follow-up was 11.3 years (range 7.3–13.4 years). The liver fat content decreased slightly by 5% from a median of 6.1% (1.9–14.0%) at baseline to 5.8% (1.9–13.1%, p=0.02) at 11.3 years. The individual values were highly interrelated (r=0.81, p<0.0001; Figure 1 in original publication for study IV). Among subjects without NAFLD at baseline, 79% remained free of NAFLD at the end of the follow-up period, while 73% of those with NAFLD at baseline still had NAFLD after the 11.3-year follow-up period.

5.5.1.1. Baseline predictors of NAFLD

In the univariate analysis, among the baseline parameters, measures of obesity, concentrations of fasting glucose, insulin, triglycerides, HDL cholesterol and liver enzymes as well as the baseline liver fat content predicted liver fat content at 11.3 years (Table 2 in original publication for study IV).

In a multiple logistic regression model, the liver fat content remained the only independent predictor of NAFLD at 11.3 years (OR 1.22; 95% CI 1.11–1.34; $p<0.001$; Table 3 in original publication for study IV). To determine how well routinely available clinical variables at baseline predict NAFLD at 11 years, we included the significant baseline variables other than liver fat content into another logistic regression model. In this model, the baseline waist circumference and plasma ALT measurements emerged as independent predictors of NAFLD at 11.3 years (Table 3 in original publication for study IV). AUROC for the first model consisting of the baseline liver fat (0.84; 95% CI 0.76–0.92, $p<0.0001$) was significantly higher than that for the latter model consisting only of routinely available parameters (0.76; 95% CI 0.65–0.86, $p<0.0001$ for AUROC and $p=0.02$ for the difference between models) (Figure 2 in original publication for study IV).

5.5.1.2. Changes during follow-up

The relationship between changes in the clinical and biochemical parameters and the liver fat content at follow-up appear shown in Supplementary Table 1 in the original publication for study IV. When changes were included in the model containing the baseline parameters, only the change in BMI in addition to the baseline liver fat content remained an independent predictor of NAFLD at 11.3 years (Table 3 in original publication for study IV). AUROC for this model was 0.90 (95% CI 0.83–0.96, $p<0.0001$), which was not significantly better than the model for the baseline liver fat content alone (model 3 in Figure 2 in original publication for study IV).

5.5.2. Liver stiffness and NAFLD fibrosis score

At follow-up, 29% of study subjects had an increased liver stiffness. These subjects were significantly more obese, had wider waist circumferences and higher blood leukocyte counts at baseline than subjects whose liver stiffness remained below the cut-off values (Table 1 in original publication for study IV). The baseline liver fat content was significantly higher in subjects with an increased liver stiffness (10.6%, 4.0–20.0%) than those without an increased liver stiffness (4.1%, 1.4–9.7%, $p<0.001$).

The NAFLD fibrosis score increased significantly over the 11-year follow-up period from a median of –2.202 (–2.713 to –1.294) at baseline to –0.989 (–1.776 to –0.095, $p<0.001$) at follow-up. Aging explained 32% of this change, although the increase was significant even as age was kept constant (–1.379 (–2.152 to –0.510) at follow-up, $p<0.001$ for the change from baseline).

5.5.2.1. Baseline predictors of liver stiffness at 11 years

Those baseline parameters significantly associated with an increased liver stiffness in the univariate analysis (Table 2 in original publication for study IV) were entered into a multiple logistic regression analysis. The baseline liver fat content remained an independent predictor of an increased liver stiffness at follow-up (Table 3 in original publication for study IV). AUROC for this model was 0.73 (95% CI 0.60–0.85, $p=0.002$).

5.5.2.2. Liver biopsies

Seven patients with stiffness values of 6.6 kPa (#1), 7.0 kPa (#2), 10.2 kPa (#3) and 10.4 kPa (#4) measured using the XL probe

and 10.4 kPa (#5), 28.4 kPa (#6) and 72.0 kPa (#7) measured using the M probe underwent a liver biopsy. These patients had 60% macrovesicular steatosis, ballooning, lobular inflammation and stage 1 fibrosis (#1); 10% steatosis without fibrosis (#2); 80% steatosis, ballooning, lobular inflammation and stage 1 fibrosis (#3); 60% steatosis, ballooning, lobular inflammation and stage 2 fibrosis (#4); 30% steatosis, ballooning, lobular inflammation and stage 2 fibrosis (#5); 40% steatosis and stage 2 fibrosis without inflammatory activity (#6); and 30% steatosis and stage 4 fibrosis (#7), respectively.

6. DISCUSSION

The systematic review was performed to investigate whether NAFLD predicts type 2 diabetes independent of obesity and other known risk factors. To assess the roles of insulin resistance, adipose tissue inflammation and genetic variations in NAFLD, we examined two models of human NAFLD, 'Metabolic/obese NAFLD' (subjects with an increased liver fat content and obesity/insulin resistance) and 'PNPLA3 NAFLD' (subjects with an increased liver fat content carrying the PNPLA3 I148M variant).

The present series of studies found through a systematic review that 'Metabolic/obese NAFLD' predicts type 2 diabetes independent of obesity and other known risk factors. In addition, we determined that adipose tissue is inflamed and coagulation factor activities increased in subjects with an increased liver fat content due to obesity and insulin resistance, while these features did not characterized carriers of the PNPLA3 I148M variant with similarly increased liver fat content. Finally, we detected that the baseline liver fat content significantly predicts future liver fat content and increased liver stiffness, as a measure of fibrosis, in an 11-year follow-up study.

6.1. Systematic review (study I)

In the systematic review (study I), ultrasound-diagnosed NAFLD predicted type 2 diabetes in all of the publications included in our study, and in most publications even after adjusting for potential confounders. Most studies consisted of Asian subjects, with one exception involving a small study among Israelis. Asians, both thoses with NAFLD (Wong & Ahmed 2014) and with type 2 diabetes (Wong *et al.* 2014a, Weston *et al.* 2005) are leaner than Caucasians. Thus, it is uncertain whether these data apply to non-Asian subjects. Since ultrasound is unreliable and difficult to use in obese subjects (Mottin *et al.* 2004), this technique may be more sensitive to detecting a risk of type 2 diabetes among Asian subjects with NAFLD. In addition, ultrasound is inaccurate in quantifying liver fat content below 20% to 30% (Hernaez *et al.* 2011), possibly influencing the estimation of the risk. Ultrasound is, however, widely available and can detect focal lesions in addition to providing an estimate of liver fat content and, therefore, remains plausible for clinical use.

The liver enzyme GGT is more sensitive to alcohol intake than ALT (Sharpe *et al.* 1996, Kotronen *et al.* 2010). Nevertheless, both GGT and ALT predicted type 2 diabetes even when subjects with excessive alcohol use were excluded at baseline measurement (Nannipieri *et al.* 2005, Goessling *et al.* 2008, Adams *et al.* 2009) and when analyses adjusted for the amount of alcohol consumed (Ahn *et al.* 2014, Cho *et al.* 2007, Ford *et al.* 2008, Goessling *et al.* 2008, Lee *et al.* 2003, Monami *et al.* 2008, Nakanishi *et al.* 2003, Nakanishi *et al.* 2004, Nanditha *et al.* 2014, Nannipieri *et al.* 2005, Perry *et al.* 1998, Sattar *et al.* 2004, Wannamethee *et al.* 2005). The relationships between GGT and ALT concentrations and the risk of type 2 diabetes were linear and fell within the normal range of liver enzymes. Although ALT and GGT predicted type 2 diabetes on average, the correlation between liver fat content quantified using ^{1}H-MRS, a state-of-the-art technique, and ALT remained sex-dependent and weaker than between liver fat content and fasting insulin

concentration (Kotronen *et al.* 2009b). ALT appears normal in 48% of subjects with NAFLD diagnosed using ^{1}H-MRS, while 23% of subjects without NAFLD exhibit an elevated concentration of ALT (>30 U/l in women, >40 U/l in men) (Kotronen *et al.* 2009b). This suggests that liver enzymes are not optimal in predicting NAFLD or in predicting the associated risk of type 2 diabetes. Furthermore, AST is not particularly helpful either, since it is less liver-specific compared to ALT (Giannini *et al.* 2005).

NAFLD is related to the pathogenesis of the metabolic syndrome and insulin resistance, but can also associate with genetic variations in *PNPLA3* (Romeo *et al.* 2008), *TM6SF2* (Kozlitina *et al.* 2014) and *MBOAT7* (Mancina *et al.* 2016). At the time of study I, the *MBOAT7* rs641738 C>T variant was not yet identified as a NAFLD risk variant. Neither the PNPLA3 I1478M variant (Romeo *et al.* 2008, Romeo *et al.* 2010, Petit *et al.* 2010, Li *et al.* 2012b, Li *et al.* 2011, Sookoian *et al.* 2009, Valenti *et al.* 2010a, Krarup *et al.* 2012, Kitamoto *et al.* 2013, Hyysalo *et al.* 2014, Luukkonen *et al.* 2016b) nor the TM6SF2 E167K variant (Kozlitina *et al.* 2014, Zhou *et al.* 2015, Sookoian *et al.* 2015, Dongiovanni *et al.* 2015, Luukkonen *et al.* 2017) are associated with insulin resistance. We found no studies that examined whether NAFLD associated with these gene variants influenced the future risk of type 2 diabetes. Globally, these variants do predict NASH, fibrosis and HCC (Dongiovanni *et al.* 2015, Liu *et al.* 2014, Valenti *et al.* 2010b), but have not emerged as significant predictors in multiple GWAS attempting to identify genetic risk factors for type 2 diabetes (Prasad & Groop 2015).

In conclusion, abundant longitudinal studies particularly among Asian populations using ultrasound found that NAFLD predicted type 2 diabetes independent of known risk factors such as age and obesity. In addition, the liver enzymes ALT and GGT independently predict type 2 diabetes. While these data support the notion that hepatic steatosis associated with insulin resistance serves as an important factor in the pathogenesis of type 2 diabetes, ultrasound and liver enzymes are not particularly useful in practice because of their diagnostic limitations related to NAFLD. This suggests that the measurement of features of the metabolic syndrome and the assessment of family history (Kathiresan *et al.* 2008) remain the gold standards for predicting the risk of type 2 diabetes. Conversely, physicians examining and treating type 2 diabetic patients should also remember the liver and measure at least the liver enzymes since these patients carry an increased risk of developing NASH (Adams *et al.* 2005) and cirrhosis (Caldwell *et al.* 1999) as well as HCC (El-Serag *et al.* 2006, Ratziu *et al.* 2010).

6.2. Study subjects and methods (studies II–IV)

In studies II and III, we examined non-diabetic subjects divided arbitrarily into either less and more obese groups (non-obese and obese subjects, study II) based on median BMI or less and more insulin-resistant groups (insulin-sensitive and insulin-resistant subjects, study III) based on HOMA-IR values. In addition, the same subjects were divided into those not carrying the PNPLA3 I148M variant ($PNPLA3^{148II}$) and homozygous for this this variant ($PNPLA3^{148MM}$, study II) or carriers of this variant ($PNPLA3^{148MM/MI}$, study III). Subjects in the obese group had higher HOMA-IR values and subjects in insulin-resistant group were more obese than subjects in the non-obese or insulin-sensitive groups. Thus, separating the effects of obesity and insulin resistance on adipose tissue inflammation and

coagulation factor activities is impossible in these groups. Although liver fat content was similarly high in the obese and insulin-resistant groups and in the group of the PNPLA3 I148M variant carriers compared to the non-obese and insulin-sensitive groups and the non-carriers of the variant, not all subjects in the first groups were diagnosed with NAFLD –that is, exhibiting a liver fat content exceeding 5.56%. However, dividing subjects into these groups provides a model to characterize how insulin resistance and liver fat content dissociate in the human liver.

We aimed to study NAFLD in this series of studies. By definition, this condition appears as an increased liver fat content not due to excessive use of alcohol, which has been defined as alcohol consumption of more than 20 g of alcohol per day for women and more than 30 g per day for men (European Association for the Study of Diabetes *et al.* 2016). We used a questionnaire to evaluate individuals' self-reported alcohol consumption, which may lead to an underestimation of alcohol consumption (Del Boca & Darkes 2003). However, we had no tools to identify and exclude study subjects not reporting their alcohol consumption truthfully.

In study IV, subjects were examined twice over an average interval of 11 years. These subjects included both patients with NAFLD and subjects with a normal liver fat content at baseline. The liver fat content was measured using three generations of magnetic scanners in studies II through IV. Thus, a systematic bias may exist in comparison of liver fat content at baseline and at follow-up visits in study IV. However, we aimed to avoid the bias by normalizing the intensity differences arising from various acquisition parameters and localization techniques as described in Methods (see section 4.3.3.).

In study III, one limitation is that plasma coagulation factor activities and hepatic expression were not examined in the same cohort of subjects. However, performing an invasive liver biopsy in apparently healthy subjects without a clinical indication would be unethical. For this reason, we used non-invasive imaging methods to measure liver steatosis using ^{1}H-MRS (studies II–IV) and to estimate the stage of fibrosis using transient elastography (study IV). Furthermore, we studied the hepatic expression of coagulation factors in two equally obese groups which included insulin-sensitive subjects without NAFLD and insulin-resistant subjects with NAFLD, allowing us to examine gene expression independent of obesity (study III).

The gene expression was measured using qPCR, the most sensitive method for the quantification of mRNA concentrations (Bustin 2000). However, the mRNA concentration does not necessarily reflect protein expression due to the post-transcriptional regulation of mRNA and the secondary and tertiary structuring and degradation of proteins. The gene expression in adipose tissue originates from cells residing in adipose tissue, such as adipocytes, preadipocytes, macrophages and endothelial cells, whereby the exact source cannot be differentiated using qPCR.

6.3. Results (studies II–IV)

6.3.1. Adipose tissue inflammation and coagulation factors in 'Metabolic/obese NAFLD' and 'PNPLA3 NAFLD' (studies II and III)

In studies II and III, we showed that adipose tissue is inflamed and plasma coagulation factor activities are increased in subjects with a high liver fat content due to obesity and insulin resistance (model for 'Metabolic/obese NAFLD') but not in subjects homozygous for or carrying the

PNPLA3 I148M variant (model for 'PNPLA3 NAFLD'). In addition, study III suggests that the expression of coagulation factors in the liver is upregulated in patients with 'Metabolic NAFLD', while adipose tissue inflammation is related to plasma coagulation factor activities.

Several studies have linked adipose tissue inflammation to insulin resistance and the pathogenesis of NAFLD. These metabolic, immune and coagulation systems represent interrelated processes and regulate one another (Hotamisligil 2006, Margetic 2012). We chose to examine the adipose tissue gene expression of chemokine *MCP-1*, macrophage marker *CD68*, cytokine *TNF-α*, transcription factor *TWIST1* and adipokine *ADIPOQ* (encoding for adiponectin), shown to associate with all of these conditions as discussed below.

The overexpression of MCP-1 in adipose tissue leads to increased macrophage infiltration into the adipose tissue, insulin resistance and hepatic triglyceride content in mice (Kanda *et al.* 2006). A deficiency in MCP-1 and its receptor protects against these changes (Weisberg *et al.* 2006). In humans, the gene expression of *MCP-1* in adipose tissue positively correlates with the plasma concentration of MCP-1 and with BMI (Christiansen *et al.* 2005). In addition to obesity, the expression of *MCP-1* is higher in insulin-resistant subjects with a high liver fat content than in equally obese insulin-sensitive subjects with a low liver fat content (Kolak *et al.* 2007). Similarly, we found that the gene expression of *MCP-1* in adipose tissue was significantly higher in obese than in non-obese subjects, while the expression was comparable between PNPLA3^{148MM} and PNPLA3^{148II} groups (study II).

The gene expression of *MCP-1* in adipose tissue correlated with the plasma activity of FVIII, whereas adipose tissue *MCP-1* and *CD68* expressions and the circulating MCP-1 protein correlated with the activity of the FVIII-binding protein VWF (study III). As such, an increased FVIII activity is associated with CVD (Meade *et al.* 1994), insulin resistance (de Lange *et al.* 2003)and venous thromboembolism (Luxembourg *et al.* 2009). FVIII is an acute-phase protein transcriptionally responsive to NF-κB activation by pro-inflammatory cytokines (Begbie *et al.* 2000). In line with that, *F8* gene expression in the liver was upregulated in patients with 'Metabolic NAFLD' (study III). In addition, the plasma activities of FVIII and VWF were elevated in insulin-resistant subjects with a high liver fat content, but not among carriers of the PNPLA3 I148M variant.

The gene expression of the macrophage marker *CD68* in adipose tissue emerged as higher in obese compared to non-obese subjects and correlated with the number of macrophages in the adipose tissue in both mice (Weisberg *et al.* 2003) and humans(Makkonen *et al.* 2007). Furthermore, the adipose tissue expression of *CD68* appears related to the liver fat content in humans (Makkonen *et al.* 2007). In study II, the expression of the macrophage marker *CD68* was slightly but not significantly higher in obese compared to non-obese subjects, and did not differ between the PNPLA3^{148MM} and the PNPLA3^{148II} groups. The expression did, however, correlate with several plasma coagulation factor activities, such as FIX, FXI, VWF and fibrinogen, D-dimer and a shortened PT (study III). Previous studies also reported a correlation between these factors and IL-6 and high-sensitivity CRP (Wannamethee *et al.* 2007, Kaye *et al.* 2012). In study III, FIX and FXI activities were increased among subjects with 'Metabolic/obese NAFLD' but not in

carriers of the PNPLA3 I148M variant, while the hepatic expression of *F9* but not *F11* was upregulated in patients with 'Metabolic' NAFLD.

In the murine fibroblast, TNF-α induces the expression of *Twist1* via an NF-κB-dependent pathway (Sosic *et al.* 2003). Yet, Twist1 decreases the expression of pro-inflammatory cytokines such as *Tnf-α* by creating a negative feedback loop through an interaction with NF-κB in mice (Sosic *et al.* 2003). The gene and protein expressions of Twist1 in human adipose tissue remain lower in obese subjects compared with non-obese subjects possibly related to the increased expression of pro-inflammatory cytokines and a decreased insulin sensitivity (Pettersson *et al.* 2011). Similarly, in study II, the gene expression of *TWIST1* was significantly lower in obese subjects compared to non-obese subjects, but did not differ between the PNPLA3^{148MM} and the PNPLA3^{148II} groups. The adipose tissue expression of *TWIST1* negatively correlated with the plasma coagulation factor FIX, yet *TNF-α* resulted in a positive correlation (study III).

Adiponectin deficiency leads to the activation of the macrophages in adipose tissue, the increased secretion of pro-inflammatory cytokines, hepatic insulin resistance and steatosis and the activation of Kupffer cells in mice (Ohashi *et al.* 2012, Fukushima *et al.* 2009, Kadowaki *et al.* 2006). In humans, the gene expression of *ADIPOQ* in adipose tissue and the circulating adiponectin concentration are decreased in obese and insulin-resistant subjects compared to non-obese and insulin-sensitive subjects (Westerbacka *et al.* 2006). In study II, obese subjects had a significantly lower gene expression of *ADIPOQ* in adipose tissue than non-obese subjects. By contrast, we found that the expression of *ADIPOQ* was similar between PNPLA3^{148II} and PNPLA3^{148MM} groups (study II). Furthermore, the gene expression of *ADIPOQ* in adipose tissue and the circulating adiponectin were inversely related to the plasma fibrinogen concentration (study III). Previous research identified a higher fibrinogen concentration in patients with NAFLD than in less obese subjects without NAFLD (Targher *et al.* 2005). We extended previous datasets by demonstrating that patients with 'Metabolic NAFLD' had an increased hepatic expression of the fibrinogen gamma-chain compared to equally obese subjects without NAFLD. These data are consistent with the idea that the expression of fibrinogen may reflect an inflammation in the extra-hepatic tissues rather than the liver fat content *per se* (Fish & Neerman-Arbez 2012), although the regulation of the coagulation factor activities is complex and cannot be determined in a cross-sectional *in vivo* study.

The extrinsic coagulation pathway including prothrombin, FVII and FX (Owren 1959) is triggered by the tissue factor, which is closely associated with atherosclerotic plaques (Tremoli *et al.* 1999). PT measures the activity of this pathway, and APTT assesses the activity of the intrinsic coagulation pathway consisting of prothrombin, FV, VIII, IX, X, XI and XII, as well as fibrinogen (Marder *et al.* 2013). In study III, subjects with an increased liver fat content and insulin resistance had a shortened PT (i.e. increased percentage of activity) and APTT, suggesting changes in individual coagulation factors translated into an enhanced clotting activity.

Inflammation and insulin resistance in adipose tissue can lead to hepatic steatosis via increased lipolysis and the release of

FFA (Kotronen *et al.* 2008). We found a positive correlation between fasting FFA and the liver fat content in obese subjects with an increased liver fat content and in the PNPLA3^{148II} group, but not in the PNPLA3^{148MM} group. This is consistent with previous studies, which showed by the glycerol turnover using stable isotopes and the effect of insulin on serum FFA that adipose tissue lipolysis does not increase among subjects with an increased liver fat content due to the PNPLA3 I148M variant (Kotronen *et al.* 2009a, Sevastianova *et al.* 2012). Thus, we found that insulin sensitivity, adipose tissue inflammation and coagulation factor activities did not differ between carriers and non-carriers of the PNPLA3 I148M variant, despite distinct liver fat contents in studies II and III. This finding is constant with multiple studies showing that the PNPLA3 I148M variant predisposes to liver disease but not to insulin resistance or CVD (Valenti *et al.* 2010b, Luukkonen *et al.* 2016b, Simons *et al.* 2017).

In conclusion, we extended previous knowledge by showing that 'Metabolic/obese NAFLD' but not 'PNPLA3 NAFLD' is associated with adipose tissue inflammation and increased plasma coagulation activities. Together with the mechanistic studies linking adipose tissue inflammation to insulin resistance, these data suggest that liver triglycerides *per se* may be metabolically harmless, while obesity, adipose tissue inflammation and insulin resistance contribute to the features of the metabolic syndrome and increased coagulation factor activities. In addition, we showed that increased plasma coagulation factor activities could reflect the enhanced gene expression of these factors in 'Metabolic/obese NAFLD'.

6.3.2. Predictors of NAFLD and liver stiffness (study IV)

In study IV, we measured the liver fat content using ^{1}H-MRS at the baseline and after a follow-up period of roughly 11 years. The liver fat content decreased by 5%, although the baseline and follow-up values were highly interrelated. Study IV shows that the baseline liver fat content and the change in BMI were the best predictors of the liver fat content at follow-up. At 11 years, 29% of subjects exhibited increased liver stiffness, and these subjects were more obese, had wider waist circumferences and had a higher liver fat content and blood leukocyte counts at baseline than subjects with less stiff livers. In a multivariate analysis, the baseline liver fat content remained the only independent predictor of liver stiffness.

The correlation coefficient between the baseline and the follow-up liver fat content values (r=0.81) remained identical to that observed in 76 obese adolescents followed for 1.9 years (Kim *et al.* 2013b). In the study in Hong-Kong, the correlation coefficient between the baseline and follow-up liver fat content values was 0.39 in subjects without NAFLD and 0.50 in patients with NAFLD at baseline (Wong *et al.* 2015b). Consistent with the data in study IV, a study using ultrasound found 81% of 147 subjects without steatosis at baseline remained free of NAFLD, while 64% of 66 subjects with NAFLD at baseline still had NAFLD after a 7-year follow-up period (Zelber-Sagi *et al.* 2012).

Although individuals' liver fat content values were closely correlated at baseline and follow-up, the liver fat content decreased by 5%, while fibrosis significantly exacerbated at least when evaluated using the NAFLD fibrosis score normalized for age during the follow-up period. This is consistent with paired-

biopsy studies in which the steatosis grade significantly decreased while fibrosis progressed over 3.2 years among 103 subjects of an unspecified ethnic origin (Adams *et al.* 2005), over 6.4 years among 132 Italian subjects (Sorrentino *et al.* 2010) and over 13.7 years among 68 Swedes (Ekstedt *et al.* 2006). By contrast, among 52 Chinese subjects with NAFLD, the steatosis grade increased significantly while the fibrosis stage remained stable over 3 years (Wong *et al.* 2010b).

In addition to the baseline liver fat content, several features of the metabolic syndrome such as obesity and concentrations of fasting glucose, insulin, triglycerides and HDL cholesterol predicted liver fat content at follow-up in univariate analyses. These data resemble findings among Chinese population (Wong *et al.* 2015b). In the latter study, subjects who developed NAFLD diagnosed using ^{1}H-MRS were more obese and had higher glucose and triglyceride concentrations and lower concentrations of HDL cholesterol than those without NAFLD after follow-up (Wong *et al.* 2015b). Most studies assessing steatosis by ultrasound have been performed among Asians (Li *et al.* 2013, Wang *et al.* 2013, Zhou *et al.* 2012, Kim *et al.* 2012). In these studies, baseline obesity (Zhou *et al.* 2012, Wang *et al.* 2013, Li *et al.* 2013), age (Wang *et al.* 2013, Zhou *et al.* 2012), components of the metabolic syndrome (Wang *et al.* 2013, Zhou *et al.* 2012) and the serum ferritin (Kim *et al.* 2012) predicted NAFLD. A change in BMI represented the only significant predictor of a change in liver fat content over an 11-year follow-up period in study IV. Similarly, a change in weight associated with the development and remission of NAFLD diagnosed by ultrasound among 213 Israeli subjects (Zelber-Sagi *et al.* 2012).

We found that the liver fat content emerges as an independent predictor of liver stiffness at 11 years. These data are consistent with paired-biopsy studies showing that steatosis predicts fibrosis (Singh *et al.* 2015, Pais *et al.* 2013, McPherson *et al.* 2015). The ability of the liver fat content, but not features of the metabolic syndrome, to predict stiffness 11 years later may provide clues of the pathogenesis of fibrosis. Patients with NAFLD associated with the genetic risk variants in *PNPLA3* (Sookoian & Pirola 2011), *TM6SF2* (Liu *et al.* 2014) and *MBOAT7* (Mancina *et al.* 2016) are at risk of fibrosis, but these patients are neither more obese nor more insulin resistant compared to non-carriers (Kotronen *et al.* 2009a, Luukkonen *et al.* 2017, Luukkonen *et al.* 2016a). This suggests that the liver fat content *per se* rather than insulin resistance could induce the development of fibrosis. In liver lobules, fat accumulates and hepatocytes undergo cell death (ballooning) around the central vein (Brunt *et al.* 1999), from where perisinusoidal collagen formation also begins (Brunt *et al.* 1999, Nath & Szabo 2012).

The limitations of this study include the lack of a population-based sample and the limited number of study subjects for to detect the effects of the genetic risk variants on the liver fat content.

In conclusion, the liver fat content decreased slightly, although the NAFLD status remained quite stable over a period of 11 years. As a novel finding, the baseline liver fat content stands as the best predictor of both liver fat content and stiffness during 11 years of follow-up. Routinely available clinical measurements and biochemical parameters appear significantly less accurate than baseline liver fat content in predicting liver. These

data support the view that liver fat content is important in the pathogenesis of fibrosis.

6.4. Future prospects

The results of this thesis as a whole support the view that NAFLD is heterogeneous. Not all the patients with NAFLD are at the similar risk of morbidity and mortality. Patients with 'Metabolic/obese NAFLD' are at an increased risk of type 2 diabetes and CVD, while NAFLD due to either insulin resistance or the genetic variants predisposes individuals to liver fibrosis and advanged liver disease. Thus, further studies are required for to determine patients who would benefit from the active pharmacological treatment for associated metabolic abnormalities, such as type 2 diabetes and dyslipidemia, and who would benefit from the active management for advanced liver disease. To date, however, only a limited number of pharmacological treatments are available specifically for NAFLD (Barb *et al.* 2016). Although several therapeutic agents have been tested, none have been approved by regulatory agencies (European Association for the Study of Diabetes *et al.* 2016). As liver fibrosis has a prognostic value for overall and liver-related mortality among NAFLD patients (Dulai *et al.* 2017), there is a demand for to find the patients with advanged liver fibrosis and to target prospective treatments for these NAFLD patients. Furthermore, triglyceride and ceramide concentrations in the liver are significantly different in 'Metabolic/obese NAFLD' and in 'PNPLA3 NAFLD' (Luukkonen *et al.* 2016b) giving an idea of different mechanisms behind these conditions. Yet, these mechanisms remain to be resolved.

7. SUMMARY

The results of studies I through IV are summarized as follows:

I. 'Metabolic NAFLD' predicts type 2 diabetes independent of obesity and other known risk factors. By contrast, we found no evidence suggesting that NAFLD associated with genetic risk variants predicts future risk of type 2 diabetes.

II. Adipose tissue is not inflamed in subjects homozygous for the PNPLA3 I148M variant despite an increased liver fat content.

III. A) Obesity and insulin resistance rather than excess hepatic fat increase coagulation factor activities in humans.

B) The hepatic gene expressions of coagulation factors FVIII and FIX and the gamma-chain of fibrinogen are increased in insulin-resistant subjects with NAFLD.

C) Adipose tissue inflammation is related to coagulation factor activities in subjects not carrying the PNPLA3 I148M gene variant.

IV. The baseline liver fat content and a chance in BMI independently predict NAFLD during an 11-year follow-up period, and baseline liver fat content predicts an increased liver stiffness better than routinely available clinical or biochemical parameters.

8. CONCLUSIONS

We examined two models of human NAFLD, 'Metabolic/obese NAFLD' (subjects with an increased liver fat content and obesity/insulin resistance) and 'PNPLA3 NAFLD' (subjects with an increased liver fat content carrying the PNPLA3 I148M variant). Analysis of the literature showed that 'Metabolic/obese NAFLD' predicted type 2 diabetes independent of age and obesity, thus supporting the notion that an increased liver fat content when associated with insulin resistance predicts type 2 diabetes. A similar association was not found for 'PNPLA3 NAFLD' or NAFLD due to other less common genetic variants. Subjects with 'Metabolic/obese NAFLD' were insulin resistant and exhibited adipose tissue inflammation, while neither of these features characterized 'PNPLA3 NAFLD'. Subjects with 'Metabolic/obese NAFLD' also exhibited increased activities of circulating liver-derived coagulation factors, which did not accur among subjects with 'PNPLA3 NAFLD'.

These findings support the view that NAFLD is heterogeneous and carries clinical implications. All subjects with hepatic steatosis are at an increased risk of liver fibrosis. Such individuals should be identified, for instance, by using the NAFLD fibrosis score, and subjects with a high-risk score could be examined using non-invasive transient elastography prior to liver biopsy. Patients with features of insulin resistance in addition to NAFLD should be carefully monitored and treated because of the increased risk of type 2 diabetes and atherothrombotic vascular disease.

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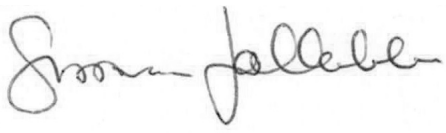

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