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The Cardiovascular Risk in Young Finns Study

Emmi Lyyra (née Suomela)

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To My Family

ABSTRACT

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Determinants of fatty liver from childhood to adulthood

The Cardiovascular Risk in Young Finns Study

Background: Fatty liver not caused by excess alcohol intake or medicinal, viral or autoimmune causes is the most common cause of chronic liver disease in Western countries. Fatty liver is defined as a condition in which more than 5-10% of hepatocytes exhibit steatosis. Fatty liver is often accompanied by metabolic disturbances. It is a reversible condition, but it may progress into the more severe inflammatory condition, steatohepatitis, and in some cases result in permanent damage, liver cirrhosis. This is the rationale for assessing the determinants and risk factors of fatty liver.

Aims: The aims of this thesis were: to study risk factor levels for fatty liver in the Finnish adult population; investigate childhood risk factors and genetic predictors for adult fatty liver; and study the prediction between circulating metabolites and a fatty liver.

Participants and methods: This thesis uses data from the Cardiovascular Risk in Young Finns Study, an ongoing population-based multicenter follow-up study in Finland initiated in 1980. The 31-year follow-up was conducted in 2011 with a total of 2,063 participants; of these, a liver ultrasound to assess fatty liver was performed on 2,042 (mean age 41.4 years and 55% women).

Results: Prevalence of fatty liver was 19% among study participants. Adulthood risk factors of fatty liver were increased waist circumference and BMI, high alanine aminotransferase, male sex, increased apolipoprotein B concentration, elevated systolic blood pressure, high alcohol intake, high serum fasting insulin concentration and low physical activity index. Childhood risk factors for adult fatty liver included low birth weight, high BMI, and high serum insulin concentration. In addition, genetic variants and certain circulating small molecular weight metabolites, including lipids, fatty acids and amino acids, measured in adulthood were associated with fatty liver.

Conclusions: Fatty liver relates to components of metabolic syndrome both in childhood and adulthood. By utilizing knowledge of genetic variants and circulating metabolites, individuals at a high risk for fatty liver could be better recognized.

Keywords: longitudinal study, fatty liver, risk factors, population study, prevalence, metabolomics

TIIVISTELMÄ

EMMI LYYRA OS. SUOMELA

TURUN YLIOPISTO

Lääketieteellinen tiedekunta, Kardiologia ja kardiovaskulaarilääketiede, Turun kliininen tohtoriohjelma, Sydäntutkimuskeskus

Rasvamaksan riskitekijät lapsuudesta aikuisuuteen - Lasten Sepelvaltimotaudin Riskitekijät -tutkimus

Tausta: Alkoholiin liittymätön rasvamaksatauti on yleisin kroonisen maksasairauden syy länsimaissa. Maksaa pidetään rasvoittuneena, kun vähintään 5-10 %:iin maksasoluista on kertynyt rasvaa. Rasvamaksa on palautuva tila, johon liittyy metabolisia muutoksia. Se voi kuitenkin johtaa edenneeseen maksasairauteen, kuten rasvamaksatulehdukseen tai maksakirroosiin, johon ei ole parantavaa hoitoa. Siksi olisi tärkeää tunnistaa varhain henkilöt, joilla on riski sairastua tai joilla jo on rasvamaksatauti.

Tavoite: Väitöskirjatyöni tavoitteena oli selvittää rasvamaksan riskitekijätasoja suomalaisessa aikuisväestössä. Toisena tavoitteena oli tutkia lapsuuden riskitekijöitä aikuisuuden rasvamaksalle ja selvittää, onko geneettisellä tiedolla vaikutusta rasvamaksariskissä olevien tunnistamiseen. Kolmas tavoite oli selvittää verenkierron metaboliittien ja rasvamaksan yhteyttä.

Menetelmät: Väitöskirjatutkimus perustuu Lasten Sepelvaltimotaudin Riskitekijät (LASERI) -tutkimukseen, joka on vuonna 1980 alkanut kansallinen seurantatutkimus, johon osallistui alun perin 3 596 tutkittavaa. Vuonna 2011 toteutettiin 31-vuotis seuranta, jolloin tehtiin myös maksan ultraäänitutkimus 2 042 osallistujalle (keski-ikä 41,4 vuotta, 55 % naisia).

Tulokset: 19 %:lla osallistujista oli ultraäänellä diagnosoitu rasvamaksa. Rasvamaksa oli aikuisuudessa yhteydessä suurempiin vyötärönympärykseen ja painoindeksiin, kohonneisiin alaniiniaminotransferaasi- ja apolipoproteiini B -arvoihin, miessukupuoleen, korkeampaan systoliseen verenpaineeseen, runsaampaan alkoholin käyttöön, kohonneisiin insuliinitasoihin ja matalaan liikuntaindeksiin. Lapsuudessa rasvamaksan itsenäisiä ennustekijöitä olivat kohonnut insuliinitaso, suurempi painoindeksi ja matala syntymäpaino. Lisäksi geenivariantit ja verenkierron metaboliitit, kuten rasvat, rasvahapot ja aminohapot, olivat yhteydessä rasvamaksaan.

Johtopäätökset: Rasvamaksa on sekä lapsuudessa että aikuisuudessa yhteydessä metabolisen oireyhtymän osatekijöihin. Geeni- ja metaboliittitietoa hyödyntämällä voidaan paremmin löytää henkilöt, joilla on korkea riski sairastua rasvamaksaan. Näin elämäntapaohjaus ja seuranta osataan kohdentaa oikeisiin henkilöihin.

Avainsanat: pitkittäinen tutkimus, rasvamaksa, riskitekijät, väestötutkimus, prevalenssi, metabolomiikka

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Abbreviations

BMI	body mass index
GCKR	glucokinase regulator
HDL	high density lipoprotein
IDL	intermediate-density lipoprotein
LDL	low density lipoprotein
LYPLAL1	lysophospholipase-like 1
NAFLD	non-alcoholic fatty liver disease
NMR	nuclear magnetic resonance
OR	odds ratio
PNPLA3	patatin-like phospholipase domain-containing 3
SD	standard deviation
TM6SF2	transmembrane 6 superfamily member 2
VLDL	very low-density lipoprotein

List of original publications

This thesis is based on the following original publications, which are referred to in the text by Roman numerals I-III.

I **Suomela E**, Oikonen M, Virtanen J, Parkkola R, Jokinen E, Laitinen T, Hutri-Kähönen N, Kähönen M, Lehtimäki T, Taittonen L, Tossavainen P, Jula A, Loo BM, Mikkilä V, Younossi Z, Viikari JS, Juonala M, Raitakari OT. Prevalence and determinants of fatty liver in normal-weight and overweight young adults. The Cardiovascular Risk in Young Finns Study. *Annals of Medicine* 2015;47:1:40-46

II **Suomela E**, Oikonen M, Pitkänen N, Ahola-Olli A, Virtanen J, Parkkola R, Jokinen E, Laitinen T, Hutri-Kähönen N, Kähönen M, Lehtimäki T, Taittonen L, Tossavainen P, Jula A, Loo BM, Mikkilä V, Telama R, Viikari JS, Juonala M, Raitakari OT. Childhood predictors of adult fatty liver. The Cardiovascular Risk in Young Finns Study. *Journal of Hepatology* 2016;65:4:784-790

III Kaikkonen JE, Wurtz P, **Suomela E**, Lehtovirta M, Kangas AJ, Jula A, Mikkilä V, Viikari JS, Juonala M, Rönnemaa T, Hutri-Kähönen N, Kähönen M, Lehtimäki T, Soininen P, Ala-Korpela M, Raitakari OT. Metabolic profiling of fatty liver in young and middle-aged adults: Cross-sectional and prospective analyses of the Young Finns Study. *Hepatology* 2017;65:2:491-500

1 Introduction

Fatty liver due to increased liver steatosis not caused by excess alcohol intake or medicinal, viral or autoimmune causes is the most common cause of chronic liver disease in Western countries. Generally, the term non-alcoholic fatty liver disease (NAFLD) is used to describe this condition. Globally, approximately 25% of adults have NAFLD (Younossi et al., 2016), and its prevalence is between 70-90% in people who are obese or have type 2 diabetes (Younossi et al., 2016). NAFLD is defined as a condition in which more than 5-10% of hepatocytes exhibit macroscopic steatosis by light microscopy in the absence of other etiologies of liver disease (Younossi et al., 2016). Fatty liver covers a spectrum from simple steatosis to non-alcoholic steatohepatitis and cirrhosis. Fatty liver is frequently associated with risk factors of cardiovascular disease (Angulo, 2002), which include obesity, type 2 diabetes and hyperlipidemia (Souza, Diniz Mde, Medeiros-Filho, & Araujo, 2012; Yki-Järvinen, 2014). In addition, physical inactivity has a role in the pathogenesis of fatty liver (Yki-Järvinen, 2014). These risk factors are part of the metabolic syndrome criteria, and the metabolic syndrome belongs to the fatty liver risk factors (Yki-Järvinen, 2014).

To develop fatty liver there must be an imbalance in hepatic lipid homeostasis between fatty acid inputs and outputs. Inputs include fatty acids from dietary sources, adipose breakdown and de novo lipogenesis, while outputs include fatty acid oxidation and fatty acid exported in the form of very low-density lipoprotein (VLDL) (Duwaerts & Maher, 2014). A major source of fatty acids is adipose tissue breakdown. The liver also takes up dietary fat and produces fatty acids, for example, from sugars via de novo lipogenesis (Donnelly et al., 2005). De novo lipogenesis is the most increased source of fatty acids in NAFLD (Donnelly et al., 2005; Lambert, Ramos-Roman, Browning, & Parks, 2014). Fatty liver is associated with insulin resistance (Yki-Järvinen, 2014), as in fatty liver, insulin is not able to suppress glucose and triglyceride production in the liver. This leads to hyperglycemia, hypertriglyceridemia and hyperinsulinemia (Yki-Järvinen, 2014). This so-called metabolic fatty liver is associated with a higher risk of type 2 diabetes, cardiovascular disease, non-alcoholic steatohepatitis and cirrhosis (Anstee & Day, 2013).

Although fatty liver frequently coexists with obesity, insulin resistance and type 2 diabetes, common genetic causes also exist (Anstee & Day, 2013). A variant in patatin-like phospholipase domain-containing 3 (*PNPLA3*) was one of the first variants identified as increasing a predisposition to fatty liver, but not to insulin resistance (Romeo et al., 2008). Around 30% of people carry this gene variant (Romeo et al., 2008). This gene variant leads triglyceride accumulation to the liver by preventing the lipolysis of triglycerides. Another genetic variant affecting liver fat content is the transmembrane 6 superfamily member 2 (*TM6SF2*) (Kozlitina et al., 2014), which is carried by approximately 7% of people (Petäjä & Yki-Järvinen, 2016). This gene variant does not have an association with insulin resistance (Kozlitina et al., 2014). Besides *PNPLA3* and *TM6SF2,* there are multiple other genetic pathways contributing to the pathogenesis of fatty liver (Anstee & Day, 2013) including genetic variants in glucokinase regulator (*GCKR*) and lysophospholipase-like 1 (*LYPLAL1*).

Ultrasound is a relatively inexpensive and widely available tool to visualize the liver and its fat content (Hernaez et al., 2011). Fatty liver appears as a diffuse increase in parenchymal brightness and echogenicity on ultrasound images and is often compared to hypoechogenity of the kidney cortex. Ultrasound lacks sensitivity in obese subjects and in subjects with low liver fat content (Saadeh et al., 2002). The sensitivity of fatty liver diagnose increases from 55% to 80% when liver fat increases from 10–20% to over 30% (Saadeh et al., 2002).

The Cardiovascular Risk in Young Finns Study is an on-going multicenter follow-up study in Finland. It was initiated to assess the biological and lifestyle factors underlying cardiovascular disease and its risk factors in children and young adults. The original cross-sectional survey was conducted in 1980 when 3,596 individuals aged 3-18 years participated. Since then, follow-ups have been conducted in 1983, 1986, 1989, 1992, 2001, 2007, and 2011. The latest follow-up was performed in 2011, when 2,063 participants (then aged 34 – 49 years) participated in clinical examinations. Ultrasound imaging of the liver was performed in 2011 on 2,040 of the study participants. In this thesis, the main objectives were to study the prevalence and determinants of a fatty liver in the population of the Cardiovascular Risk in Young Finns Study and to investigate childhoods genetic and lifestyle risk factors for adult fatty liver.

2 Review of literature

2.1 Origin and pathophysiology of fatty liver

Fatty liver results from triglyceride accumulation in the liver. Triglycerides consist of a glycerol backbone and three variable fatty acyl groups. The origin of these fatty acids is from de novo lipogenesis, adipose tissue lipolysis or dietary fat (Aarsland & Wolfe, 1998; Jacome-Sosa & Parks, 2014). Fatty acids are carboxylic acids with a long aliphatic chain with several carbon atoms (Aro, Aantaa, Mutanen, & Uusitupa, 2012). Fatty acids can be divided to three groups according to the amount of carbon–carbon double bonds in their chains: saturated, monounsaturated and polyunsaturated, and they are usually derived from triglycerides or phospholipids (Aro et al., 2012). In fatty liver, saturated fatty acids are elevated due to increased de-novo lipogenesis (Donnelly et al., 2005; Oresic et al., 2013). In healthy individuals, high-fat diets and fasting suppress the lipogenesis (Schwarz, Neese, Turner, Dare, & Hellerstein, 1995), whereas in insulin-resistant individuals with fatty liver, the suppression of de-novo lipogenesis during fasting fails to take place appropriately (Donnelly et al., 2005; Lambert et al., 2014). Higher liver-fat content might be associated with decreased concentrations of polyunsaturated fatty acids -containing phospholipids and ether lipids in addition to increased serum concentrations of triglycerides containing saturated or monounsaturated fatty acids (Oresic et al., 2013). Triglycerides with a low carbon number and a double-bond content are enriched in VLDL particles of insulin-resistant individuals (Kotronen, Velagapudi et al., 2009).

In fatty liver, more than half of the hepatic triglycerides originate from adipose tissue lipolysis (Donnelly et al., 2005). After an overnight fast, adipose tissue lipolysis is a major source of fatty acids. Lipolysis is increased in fatty liver because insulin has an inability to inhibit lipolysis normally with an increased intake of fatty acids to the liver (Berlanga, Guiu-Jurado, Porras, & Auguet, 2014; Bugianesi, Moscatiello, Ciaravella, & Marchesini, 2010). In the presence of obesity related insulin resistance, free fatty acid levels are high due to their resistance to the anti-lipolytic action of insulin (Bugianesi et al., 2005; Petta et al., 2016). These circulating free fatty acids represent the major source of hepatic fat accumulation in fatty liver (Petta et al., 2016). Insulin resistance also promotes adipose tissue dysfunction which is due to the altered production and secretion of adipokines and

inflammatory cytokines (Guilherme, Virbasius, Puri, & Czech, 2008). In addition to adipose tissue lipolysis, de novo lipogenesis is also increased in fatty liver (Donnelly et al., 2005; Lambert et al., 2014), and it produces saturated fatty acids. This might lead to the intrahepatic triglycerides being enriched with saturated fatty acids in fatty liver.

Moreover, genetic variants are associated with the pathogenesis of fatty liver. These genetic factors predispose to a condition in which there is an accumulation of triglycerides in the hepatocytes. They also influence hepatic free fatty acid flow, oxidative stress, response to endotoxins and cytokine production and activity, and are determinants of fatty liver development and progression (Anstee & Day, 2013; Eslam, Valenti, & Romeo, 2018).

Obesity is a major risk factor of fatty liver and obesity related fatty liver is called non-alcoholic fatty liver disease (Yki-Järvinen, 2014). Besides that alcohol is an important factor that influences liver damage (Cao, Yi, Liu, Wang, & Tang, 2016), and obesity and alcohol might overlap causing fatty liver (Kotronen et al., 2010). Persons with metabolic risk factors might be sensitive to alcohol liver injury (Åberg, Helenius-Hietala, Puukka, Farkkila, & Jula, 2018). In a Finnish FIN-D2D survey with 2,766 participants, the prevalence of NAFLD was 21% and prevalence of alcoholic fatty liver disease 7% (Kotronen et al., 2010). Metabolic syndrome and type 2 diabetes were prevalent both in NAFLD (70% and 25%) and alcoholic fatty liver disease (73% and 24%) (Kotronen et al., 2010). In a Finnish study of 6,732 participants, 49% of alcohol risk users had also metabolic syndrome (Åberg et al., 2018). In this study, term fatty liver is used because persons with possible alcoholic fatty liver disease are not excluded.

Alcohol affects the progression of fatty liver by causing liver cell damage, which leads to liver steatosis and more advanced liver diseases (Cao et al., 2016). Alcohol can affect direct damage to the liver cells as liver is the main organ responsible for ethanol metabolism (Seth, Haber, Syn, Diehl, & Day, 2011). The end products of hepatic ethanol metabolism include acetaldehyde, which damages the liver by directly triggering inflammation, extracellular matrix remodeling and fibrogenesis (Mello, Ceni, Surrenti, & Galli, 2008). In addition to this, it binds covalently to proteins and DNA leading to the production of immunogenic adducts in the hepatocytes (Niemelä, 2001). Finally, acetaldehyde stimulates transforming growth factor-beta signaling in hepatic stellate cells that acquire a pro-fibrogenic and pro-inflammatory profile (Chen, 2002). Alcohol consumption results in hypoxia of the perivenous hepatocytes, which is the first sign showing evidence of damage from chronic alcohol consumption (Ishak, Zimmerman, & Ray, 1991). The product of

ethanol metabolism is burned in the mitochondria and this burning requires an additional amount of oxygen, therefore the hepatocytes take up more oxygen from arterious blood, however, this uptake is not enough to adequately supply all liver regions (Beier & McClain, 2010). Cytochrome P450s are up-regulated in conditions of chronic alcohol abuse and assists alcohol dehydrogenase in converting alcohol to acetaldehyde (Seth et al., 2011). Reactive oxygen species generated by cytochrome P450s are responsible for the pro-inflammatory profile of the alcohol-related liver injury (Seth et al., 2011).

2.2 Risk factors of fatty liver in children and young adults

Fatty liver is the most prevalent form of chronic liver disease in childhood and adolescence, and it affects approximately 10-20% of the pediatric population (Temple, Cordero, Li, Nguyen, & Oben, 2016). The greatest risk factor for pediatric fatty liver is obesity (Alisi, Manco, Vania, & Nobili, 2009; Patton et al., 2006). The estimated prevalence of fatty liver in overweight and obese children is 50-80% compared to 2-7% in normal weight children (Anderson et al., 2015; Ozhan, Ersoy, Kiremitci, Ozkol, & Taneli, 2015). In addition to this, several studies have found an association between intrauterine growth restriction and fatty liver (Alisi, Panera, Agostoni, & Nobili, 2011; Alisi, Cianfarani, Manco, Agostoni, & Nobili, 2012). The pathogenic mechanisms are possibly associated with fetal epigenetic programming (Cordero, Li, & Oben, 2015).

In young adults, fatty liver is frequently associated with factors of metabolic syndrome: obesity, hypertension, insulin resistance, high level of triglycerides and low level of high density lipoprotein (HDL) cholesterol (Angulo, 2002; Targher, Day, & Bonora, 2010; Weiss, Rau, & Geier, 2014). Metabolic syndrome is also associated with a higher risk of nonalcoholic steatohepatitis and more progressive diseases (Rinella, 2015).

Several studies have shown that fatty liver is a cardiovascular risk factor (Ballestri et al., 2014; Bhatia, Curzen, Calder, & Byrne, 2012; Bonapace et al., 2012; Brea & Puzo, 2013; H. Liu & Lu, 2014; Perseghin et al., 2008; Pisto, Santaniemi, Bloigu, Ukkola, & Kesäniemi, 2014; Targher et al., 2010). According to the results of the prospective, population-based, cohort study: Oulu Project Elucidating Risk of Atherosclerosis, fatty liver predicted the future risk for death from cardiovascular disease and cardiovascular events (Pisto et al., 2014). There probably are multiple

pathways between fatty liver and increased risk for cardiovascular disease including lipid metabolism disorders, the deposition of fat outside of the adipocytes, insulin resistance, metabolic syndrome and a heightened systemic pro-inflammatory state (Brea & Puzo, 2013).

2.3 Genetics of fatty liver disease

Although fatty liver commonly coexists with obesity, insulin resistance and type 2 diabetes, common genetic causes also exist. A variant in *PNPLA3* (rs738409 [G], encoding I148M) confers susceptibility to fatty liver. Genetic variation in *TM6SF2* (rs58542926 [T], encoding E167K) also increases the risk of fatty liver. Besides *PNPLA3* and *TM6SF2* there are multiple other genetic pathways contributing to the pathogenesis of fatty liver (Anstee & Day, 2013; Eslam et al., 2018) including genetic variants in the glucokinase regulator (*GCKR*) and lysophospholipase-like 1 (*LYPLAL1*). The variants included to this study are widely studied and important determinants of liver fat content (Anstee & Day, 2013; Eslam et al., 2018; Kozlitina et al., 2014; Leon-Mimila et al., 2015; Sookoian & Pirola, 2011; Sookoian et al., 2015).

2.3.1 Patatin-Like Phospholipase Domain-Containing 3

Approximately 30% of people in Western countries carry the *PNPLA3* I148M variant (Romeo et al., 2008) and it is the major genetic determinant of hepatic fat content (Dongiovanni, Romeo, & Valenti, 2015). The single nucleotide polymorphism is called rs738409 C>G and it is in the *PNPLA3* gene. This single nucleotide polymorphism encodes the isoleucine to a methionine variant at the protein position 148. In humans *PNPLA3* encodes a 481 amino acid membrane protein localized in the endoplasmic reticulum and at the surface of lipid droplets (He et al., 2010). This protein has the highest expression in hepatic stellate cells, retina, and hepatocytes. The functional *PNPLA3* 148I variant allows optimal triglycerides hydrolysis in combination with other lipases and cofactors. In contrast, the loss-of-function of the 148M variant determines the accumulation of dysfunctional *PNPLA3* on the surface of lipid droplets, preventing the access of other lipases to the triglycerides, and this leads to triglyceride accumulation in the liver. In a genome-wide association study by Romeo et al. (Romeo et al., 2008), the *PNPLA3* rs738409 variant was associated with increased hepatic fat levels across European American, African American, and Hispanic American ancestries. Since

the discovery of this variant, other groups have replicated this association. A meta-analysis of 2,937 individuals with histologically measured fatty liver revealed that individuals with GG at rs738409 in *PNPLA3* had 73% higher lipid fat content compared with CC individuals (Sookoian & Pirola, 2011). In a study of Danish and American participants, the effect of gene variants on fatty liver increased with increasing body mass index (BMI) (Stender et al., 2017). In obese (BMI >35 kg/m^2), 84% of rs738409 GG participants had fatty liver versus only 42% in participants with CC *PNPLA3*. In the normal weight participants with a BMI of less than 25 kg/m^2, 18% of the rs738409 homozygous participants had fatty liver versus 9% in participants with wild-type *PNPLA3*. The human liver lipidome markedly differs between a metabolic fatty liver and a *PNPLA3* fatty liver (Luukkonen et al., 2016).

PNPLA3 associated fatty liver does not always feature metabolic abnormalities (Yki-Järvinen, 2014). In several studies, *PNPLA3* associated fatty liver was not accompanied by insulin resistance, hyperglycemia, hypertriglyceridemia, low HDL cholesterol concentration, or inflammation in adipose tissue (Kantartzis et al., 2009; Kitamoto et al., 2013; Kotronen, Johansson et al., 2009; Krarup et al., 2012; Lallukka et al., 2013; Norris & Rich, 2012; Romeo et al., 2008; Speliotes et al., 2011). However, an individual might have fatty liver caused by a combination of obesity and insulin resistance and the *PNPLA3* variant. *PNPLA3* fatty liver is characterized by defects in distinct circulating triacylglycerols (Hyysalo et al., 2014). Metabolic fatty liver, when compared with *PNPLA3* fatty liver, is associated with multiple changes in absolute and relative triacylglycerol concentrations (Hyysalo et al., 2014).

2.3.2 Transmembrane 6 Superfamily Member 2

Approximately 7% of people carry the *TM6SF2* E167K variant (Petäjä & Yki-Järvinen, 2016). The single nucleotide polymorphism is called rs58542926 C>T and it is located in the *TM6SF2* gene, and it encodes the lysine to glutamic acid variant at protein position 167. *TM6SF2* is a membrane protein with 7 to 10 predicted transmembrane domains (Kozlitina et al., 2014). The amount of the mutant form of *TM6SF2* protein is lower than the wild-type *TM6SF2* protein due to miss-folding and degradation (Kozlitina et al., 2014). Knocking down the *TM6SF2* gene in the livers of mice resulted in increased liver lipid accumulation and decreased serum triglycerides and low density lipoprotein (LDL) (Kozlitina et al., 2014). A recent meta-analysis showed that carriers of the *TM6SF2* gene variant

have a 2.1-fold higher risk of fatty liver than non-carriers (Pirola & Sookoian, 2015). They also had a lower circulating total cholesterol, lower LDL cholesterol, and triglyceride concentrations than non-carriers (Pirola & Sookoian, 2015). The mechanism is related to reduced secretion of VLDL resulting in intrahepatic retention of triglycerides and steatosis (Holmen et al., 2014). This genetic variant is associated with the full spectrum of liver damage associated with fatty liver (Y. L. Liu et al., 2014). *TM6SF2* associated fatty liver does not appear to be characterized by insulin resistance (Petäjä & Yki-Järvinen, 2016).

2.3.3 Glucokinase Regulator

The *GCKR* gene is located at the chromosome locus 2p23. The single nucleotide polymorphism is called rs780094 C>T. The protein product of *GCKR* has been proposed to interfere with glucose and lipid homeostasis by modulating hepatic glucokinase activity (Peter et al., 2011). *GCKR* is a regulator of the cellular location of glucokinase. When low levels of glucose are present, *GCKR* binds to and keeps glucokinase localized in the nucleus where it is not active. High glucose concentrations lead to the release of glucokinase from *GCKR*, allowing glucokinase to be released into the cytoplasm where it can mediate phosphorylation of the glucose to glucose 6-phosphate. The glucose 6-phosphate can be used either in glycogen synthesis or converted via the pentose phosphate shunt to the precursors required for de novo lipogenesis (Peter et al., 2011). The *GCKR* variant that associates with fatty liver results in increased activity of glucokinase which phosphorylates glucose and, in this way, may promote glucose to triglyceride shifts (Bechmann et al., 2012). It has been hypothesized that the association of the rs780094 *GCKR* polymorphism with hepatic fat accumulation can be explained by the linkage disequilibrium with rs1260326, encoding for the P446L protein variant (Beer et al., 2009). The P446L variant affects *GCKR* ability to negatively regulate glucokinase in response to fructose-6-phosphate, thereby determining constitutive activation of hepatic glucose uptake (Beer et al., 2009). The genome-wide association study, consisting of 592 patients with fatty liver was the first study to report the role of rs780094 C>T SNP in the *GCKR* gene in fatty liver (Speliotes et al., 2011). Furthermore, in a meta-analysis from five case-control studies, rs780094 was associated with fatty liver with the pooled effect of a 1.25-fold increased risk, when the T-allele was compared with those with C-allele (Zain, Mohamed, & Mohamed, 2015). In a study of 308 participants with type 2 diabetes, 71% of participants with T allele had fatty liver versus 55% of participants with homozygote C allele (Petit et al., 2016).

2.3.4 Lysophospholipase-like 1

LYPLAL1 gene is located at chromosome locus 1q41. The single nucleotide polymorphism located in *LYPLAL1* gene is called rs12137855 C>T. The *LYPLAL1* gene encodes a protein with a sequence similarity to APT1, a member of the lysophospholipase family of proteins that have acyl protein thioesterases activity (Burger et al., 2012). In a study by Ahn et al. (Ahn et al., 2016), *LYPLAL1* inhibition led to an increase in glucose production in human hepatocytes proposing that *LYPLAL1* functions in the hepatic glucose metabolism. Biochemical and X-ray crystallographic studies indicate that *LYPLAL1* functions as a lysophospholipase (Burger et al., 2012). A 2011 published genome-wide association study was first to show that C>T SNP in *LYPLAL1* is associated with fatty liver (Speliotes et al., 2011). However, in a study by Yuan et al., significant associations between *LYPLAL1* and fatty liver were not found (C. Yuan et al., 2015) and also other studies have given inconsistent results (Flores et al., 2016; Leon-Mimila et al., 2015; X. Wang et al., 2016).

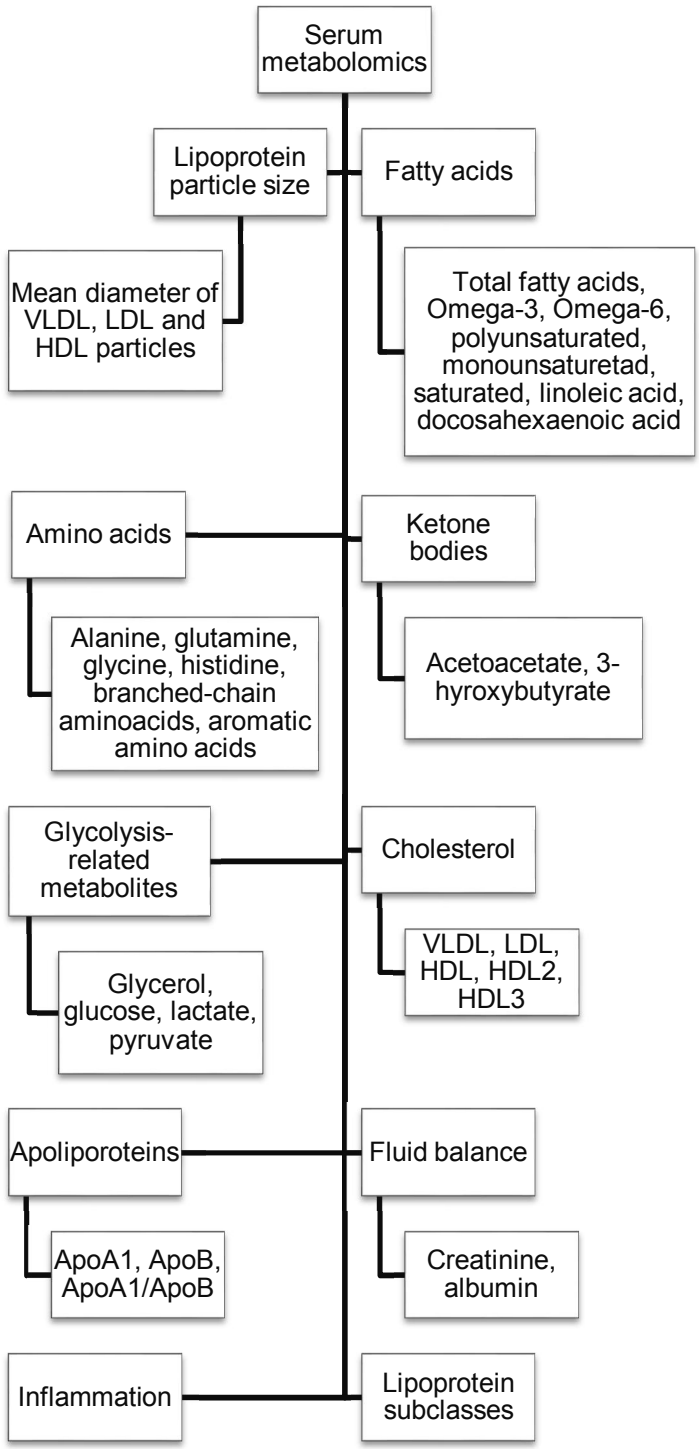
2.4 Serum metabolomics of fatty liver

Fatty liver is associated with many changes in the metabolome. The classification of serum metabolomics used in this thesis is presented in **Figure 1**. The more fat there is in the liver, the greater is the relative number of triglycerides containing saturated or monounsaturated fatty acids (Westerbacka et al., 2010). These changes could reflect an increase in de novo lipogenesis, which is increased in metabolic fatty liver (Donnelly et al., 2005) and produces saturated fatty acids, especially palmitic and stearic acids, which are then converted to respective monounsaturated fatty acids via stearoyl-CoA desaturase-1 (Aarsland & Wolfe, 1998). In a study of 679 individuals, higher liver-fat content was associated with increased serum concentrations of triglycerides containing saturated or monounsaturated fatty acids, and decreased concentrations of polyunsaturated fatty acid -containing phospholipids and ether lipids (Oresic et al., 2013). In an American study of 60 participants, significant changes were found in bile acids, glutathione metabolism, lipid and amino acid metabolism in participants with fatty liver (Kalhan et al., 2011). In a study of bariatric surgery patients from Finland, levels of β-hydroxybutyrate ketone bodies were higher in individuals with fatty liver when compared to individuals with a normal liver (V. T. Männistö et al., 2015). In a study of 125 participants, the total plasma monounsaturated fatty acids driven by

palmitoleic and oleic acids content were significantly increased in participants with fatty liver (Puri et al., 2009). Furthermore, in a recent nuclear magnetic resonance (NMR) study of 173 Mediterranean participants (Amor et al., 2017), the fatty liver index was directly related to the VLDL particle number and VLDL size. The amount of cholesterol within HDL particles, and the HDL size decreased as the fatty liver index increased. A high fatty liver index was associated with a higher proportion of small LDL particles and lower LDL size. In addition, in another recent study, urinary metabolomics found differences in 31 metabolites between fatty liver and steatohepatitis groups, including differences in nucleic acids and amino acids (Dong et al., 2017). In a population-based study with 555 participants from Germany, the metabolomics score explained 24% of the variation in liver signal intensity when studied using magnetic resonance imaging (Koch et al., 2017).

Two leading ways to measure serum metabolomics are NMR spectroscopy and mass spectrometry (Markley et al., 2017). NMR spectroscopy takes advantage of the magnetic properties of certain nuclei, such as ^{1}H (Brennan, 2014). This spectroscopy consists of arousing nuclear spins located in a magnetic external field through rapid changes of the magnetic field, and then recording the electromagnetic radiation occurring because of nuclear spins relaxation (Brennan, 2014). Mass spectrometry produces electrically charged species, which are sorted according to the mass-to-charge ratios (Urban, 2016). NMR spectroscopy has lower sensitivity than mass spectrometry (Markley et al., 2017). With NMR spectroscopy it is possible to observe and quantify all more abundant compounds in specimen without the need of sample preparation (Markley et al., 2017). NMR-based approach is non-destructive and allows further analysis to be performed (Brennan, 2014).

Figure 1. Classification of serum metabolomics used in this thesis.



2.4.1 Lipoprotein particles

Fatty liver is believed to be a liver manifestation of the metabolic syndrome and it is driven by insulin resistance (D. E. Cohen & Fisher, 2013). Insulin resistance in the peripheral adipose tissue increases lipolysis and the delivery of adipose-derived free fatty acids to the liver (Berlanga et al., 2014). Dyslipidemia associated with fatty liver is pro-atherogenic (Chatrath, Vuppalanchi, & Chalasani, 2012; Targher et al., 2010), and the characteristic findings of fatty liver related dyslipidemia are increased plasma concentrations of VLDL triglycerides and decreased HDL cholesterol (Speliotes et al., 2010). Although LDL cholesterol levels may not be different in persons with fatty liver, there are important differences in the subpopulations of LDL particles (Chatrath et al., 2012). Higher levels of small, dense LDL particles are seen in patients with fatty liver (Nseir, Shalata, Marmor, & Assy, 2011; Targher et al., 2010).

In fatty liver related dyslipidemia, HDL cholesterol levels are decreased. This might be explained via insulin resistance because it reduces the formation of HDL (Nonomura, Arai, Mitani, Abe-Dohmae, & Yokoyama, 2011). The purpose of HDL particles is to collect excess cellular cholesterol and to transport it to the liver (Lewis & Rader, 2005; Silver, Jiang, Arai, Bruce, & Tall, 2000). This process is called reverse cholesterol transport (Fisher, Feig, Hewing, Hazen, & Smith, 2012; Glomset, 1968), and it begins with the production of nascent HDL particles by the liver (Timmins et al., 2005). The particles mature within the intravascular space, take up excess cholesterol from cells and then return to the liver where lipids are removed from HDL (Zannis, Chroni, & Krieger, 2006).

2.5 Measurement of fatty liver

Liver fat can be measured using various modalities (Schwenzer et al., 2009). One of them is histology by liver biopsy, which is the gold standard for the diagnosis of fatty liver (Bhatia et al., 2012). With histology, the complete range of the disease can be measured including steatosis, inflammation and fibrosis. Because liver biopsies involve a risk of complications, they are not usually performed without an indication (Al Knawy & Shiffman, 2007; Ratziu et al., 2005). Liver fat content can also be measured by using noninvasive techniques, but these imaging modalities are not very sensitive for detection of steatohepatitis or early fibrosis (Saadeh et al., 2002). Magnetic resonance spectroscopy is the most sensitive and specific for

measuring liver steatosis but performing it requires specialized expertise and it is not widely used in clinical practice (Lee et al., 2010). Ultrasound imaging, computed tomography, and magnetic resonance imaging are affordable, widely available, and confer minimal risk to the patient. Thus, they are most often used in clinical practice and in the population studies for evaluating for the presence of fatty liver (Lee et al., 2010). The sensitivity and specificity of magnetic resonance imaging is greater for quantifying fatty liver than computed tomography, which in turn is greater than ultrasound (**Table1**) (Lee et al., 2010). Ultrasound imaging has certain limitations: while the test shows high specificity, it has low sensitivity. It has been estimated that conventional ultrasound imaging can only detect steatosis when more than 30% of the liver is affected (Saadeh et al., 2002). In a study of 161 participants, the sensitivity of ultrasound to detect more than 5% liver fat was 53-62% and specificity was 81-93% (Lee et al., 2010). Both magnetic resonance imaging (sensitivity of 77%) and magnetic resonance spectroscopy (sensitivity of 80%) had better sensitivity than ultrasound. Between various imaging modalities, there were no differences in the specificity; it was 87% in magnetic resonance imaging and 80% in magnetic resonance spectroscopy (Lee et al., 2010). Other studies have shown that the sensitivity of ultrasound is between 60-94% and specificity between 66-95% in detecting fatty liver (Debongnie, Pauls, Fievez, & Wibin, 1981; Foster, Dewbury, Griffith, & Wright, 1980; Graif et al., 2000; Saverymuttu, Joseph, & Maxwell, 1986; Steinmaurer, Jirak, Walchshofer, & Clodi, 1984). In contrast computed tomography has a specificity of 100% and a sensitivity of 82% when used in histologic analysis as the reference standard (Park et al., 2006). However, ultrasound imaging is non-invasive, widely accessible, and cost-effective (Hernaez et al., 2011).

Table1. Sensitivity and specificity of various imaging modalities for quantifying fatty liver.

Imaging modality	Sensitivity (%)	Specificity (%)		N
Ultrasound imaging				
	53-62	81-93	Lee et al., 2010	161
	94	84	Saverymuttu et al., 1986	450
	80	100	Debongnie et al., 1981	104
	70	84	Steinmaurer et al. 1984	127
Magnetic resonance spectroscopy				
	80	80	Lee et al., 2010	161
Computed tomography				
	50	77	Lee et al., 2010	161
	73-82	100	Park et al., 2006	154
Magnetic resonance imaging				
	77	87	Lee et al., 2010	161

3 Aims of the study

The Cardiovascular Risk in Young Finns Study is an ongoing longitudinal follow-up study of atherosclerosis risk factors from childhood to adulthood. At baseline, in 1980 3,596 participants aged 3 to 18 years participated. The present thesis is based on the findings from the baseline and from the 31-year follow-up study performed in 2011 when participants where aged 34 to 49 years. In addition, liver ultrasound data from the 31-year follow-up study and metabolomics data were included in the analyses. The specific aims of this thesis include:

1. To report the prevalence and determinants of fatty liver in Finnish young adults (I).
2. To examine childhood characteristics and genetic predictors of adult fatty liver (II).
3. To study the relationship of circulating metabolites with a present or future fatty liver (III).

4 Participants and methods

4.1 Description of the Cardiovascular Risk in Young Finns Study

The Cardiovascular Risk in Young Finns Study is an ongoing multicenter follow-up study located in five cities and their rural surroundings in cooperation with the university hospitals in Finland: Turku, Tampere, Helsinki, Kuopio and Oulu. The aim is to evaluate atherosclerotic risk factors from childhood into adulthood (Åkerblom et al., 1985; Raitakari et al., 2008). The study began in 1980 when a total of 4,320 Finnish children and adolescents aged 3, 6, 9, 12, 15 and 18 were invited and of which 3,596 (83.2%) participated. Participants were randomly chosen from the national register. The progression of the Cardiovascular Risk in Young Finns Study is shown in **Table 2.** In addition, two pilot studies were conducted in 1978 and 1979. The latest follow-up was performed in 2011 with total of 2,063 participants (57.4% from the original cohort). The ultrasound imaging of the liver was performed in 2011. Childhood and youth data from the 1980, 1983 and 1986 follow-ups, adulthood data from the 2001, 2007 and 2011 follow-ups and liver ultrasound data from the year 2011 were included in the analysis

Table 2. Design and participation rates of the Cardiovascular Risk in Young Finns Study.

Year	N	Age														
1980	3,596	3	6	9	12	15	18									
1983	2,991		6	9	12	15	18	21								
1986	2,799			9	12	15	18	21	24							
1989	2,737				12	15	18	21	24	27						
1992	2,370					15	18	21	24	27	30					
2001	2,284								24	27	30	33	36	39		
2007	2,204										30	33	36	39	42	45
2011	2,063											34	37	40	43	46

4.2 Study design

Study I examined the prevalence of fatty liver in the Finnish population, associations of cardiovascular risk factors with fatty liver, and determinants of fatty liver separately in normal weight and overweight participants in order to discover whether fatty liver consists of different components according to weight. The cross-sectional study included individuals who had data on their fatty liver status, liver enzyme and cardiovascular risk factor levels measured in 2011. A total 1,998 participants aged 34-49 were included in this analysis.

In study II, childhood lifestyle and clinical and genetic determinants of adult fatty liver were studied. This study included 2,042 participants who had ultrasound liver scans in 2011.

In study III, the associations of circulating metabolites in present and future fatty livers were investigated. In cross-sectional analyses, a total of 2,002 participants with an assessment of their liver fat status and metabolite measures quantified by NMR metabolomics were included. In the prospective analyses 1,575 participants with serum samples collected in 2001 and liver fat scans in 2011 were included.

4.3 Clinical characteristics

4.3.1 Baseline in 1980

Height and weight were measured, and BMI was calculated as weight in kilograms divided by height in meters squared. Blood pressure was measured from the

brachial artery with a standard mercury sphygmomanometer. The average of three measurements was used in statistical analysis. Questionnaires were used to obtain data on smoking, age at menarche, physical activity (Telama et al., 2005), birth weight, birth height, length of gestation, breastfeeding, family history of coronary heart disease, parental hypertension (self-reported diagnosis of hypertension in either parent at baseline) and parental occupational status. Preterm birth was defined as birth before 37 weeks' gestation. Small for gestational age was defined as a birth weight below the 10th percentile, appropriate birth weight for gestational age as a birth weight in the 50–90th percentiles and large for gestational age as a birth weight over the 90th percentile. Parental occupational status was divided into 3 categories: manual, lower-grade non-manual, and higher-grade non-manual. Data on birth weight and birth height was verified by well-baby clinic records. In 1980, 1983 and 1986, questionnaire information on cigarette smoking was collected in participants aged 12 years or older. Individuals who had reported daily smoking at any age between ages 12 and 18 were defined as smokers. Physical activity was available for participants aged 9 years or older.

4.3.2 Follow-up in 2011

Waist circumference, height, and weight were measured, and BMI calculated as kg/m^2. Normal weight was defined as a BMI lower than 25.0 kg/m^2 and overweight/obese as BMI 25.0 kg/m^2 or more. Normal waist circumference was defined as 85 cm or less in women and 90 cm or less in men. Blood pressure was measured as the average of three measurements taken at 2-minute intervals in a sitting position from the right arm brachial artery by random zero sphygmomanometer. The metabolic syndrome was defined according to the harmonized definition (Alberti et al., 2009). Food consumption during the last 12 months was assessed in 2007 using a self-administered 131-item food frequency questionnaire, developed and validated by the Institute of Health and Welfare (S. Männistö, Virtanen, Mikkonen, & Pietinen, 1996). The percentage proportion of energy was calculated for fat, protein, carbohydrates, and alcohol to reflect the composition of the participants' diet using the National Food Composition Database Fineli (The National Public Health Institute, Nutrition Unit, 2007). The alcohol energy percentage variable was categorized according to the Finnish Nutrition Recommendations: 1) the amount of alcohol less than 5 energy percent; and 2) the amount of alcohol more than 5 energy percent. Data regarding daily alcohol consumption, physical activity, and smoking were obtained by questionnaires. Alcohol consumption data were acquired by standardized

questionnaires and calculated in standard doses (12 grams pure ethanol) per day by dividing the total number of doses consumed per week (0.33 liter doses of beer or cider, 0.12 liter doses of wine, and 0.04 liter doses of hard liquor) by 7. The participants were categorized into four groups: 1) no alcohol intake during the last week; 2) >0 to <2 units of alcohol per day; 3) 2 to <4 units of alcohol per day; and 4) ≥4 units of alcohol per day (Juonala et al., 2009). Heavy drinking was defined as consuming 6 or more alcohol doses on the same occasion at least once a week. Moderate alcohol use was defined as consuming less than 1.67 standard drinks per day (corresponding to 20 grams of pure ethanol) according to the safety levels of drinking for women as endorsed by the European Association for the Study of the Liver and the American Association for the Study of the Liver (in men 30 grams and in women 20 grams of alcohol per day) (European Association for the Study of Liver, 2012; O'Shea, Dasarathy, McCullough, Practice Guideline Committee of the American Association for the Study of Liver Diseases, & Practice Parameters Committee of the American College of Gastroenterology, 2010). A physical activity index was calculated (Telama et al., 2005). The participants were classified as smokers if they smoked daily.

4.4 Biochemical analysis

4.4.1 Baseline in 1980

Venous blood samples were drawn after an overnight fast to determine serum lipid levels, insulin, and C reactive protein. Serum insulin was measured with an immunoassay. Standard enzymatic methods were used for serum total cholesterol, triglycerides, and HDL cholesterol. LDL cholesterol concentration was calculated by the Friedewald formula in subjects with triglycerides <4.0 mmol/L (Friedewald, Levy, & Fredrickson, 1972). Childhood serum samples taken in 1980 were stored at -20°C, and the childhood C reactive protein levels were analyzed in 2005. During the storage, the samples were not thawed or refrozen. In 2005, serum high-sensitive C reactive protein was analyzed by an automated analyzer (Olympus AU400) using a turbidimetric immunoassay kit (“CRP-UL”-assay, Wako Chemicals, Neuss, Germany).

4.4.2 Follow-up in 2011

Venous blood samples were drawn after an overnight fast and serum was separated, aliquoted, and stored at -70°C until analysis. Serum alanine aminotransferase, aspartate aminotransferase, gamma-glutamyltransferase, glucose, total cholesterol, and triglyceride concentrations were measured by enzymatic methods (ALAT, ASAT, GGT, Glucose, Cholesterol and Triglycerides System Reagent, Beckman Coulter Biomedical, O'Callaghan's Mills, Ireland) on an automatic analyzer (AU400, Olympus, Tokyo, Japan). Apolipoprotein A1, apolipoprotein B, and C reactive protein were determined immunoturbidimetrically (ApoA1 and B assay reagent, Orion Diagnostica, Espoo, Finland and CRP Latex reagent, Beckman Coulter Biomedical) using the same analyzer. HDL cholesterol was measured with the cholesterol reagent after precipitation of LDL cholesterol and VLDL cholesterol with dextran sulfate-Mg^{2+} and LDL cholesterol calculated by the Friedewald formula (Friedewald et al., 1972). The concentration of insulin was determined with a chemiluminescent microparticle immunoassay (Architect insulin assay, Abbott Laboratories, Abbott Park, USA) and glycated hemoglobin A1c (Hemoglobin A1c assay, Abbott Laboratories) with an immunoturbidimetric method, both measured on an Architect ci8200 analyzer (Abbott Laboratories). Insulin resistance was estimated by the homeostasis model (Matthews et al., 1985).

4.5 Metabolite quantification

A high-throughput NMR metabolomics platform was used for the quantification of 68 lipid and metabolite measures that represent a broad molecular signature of systemic metabolism from serum samples collected in 2001, 2007 and 2011. All metabolites were measured using a single experimental setup that allows for the simultaneous quantification of routine lipids, lipoprotein subclass distributions, fatty acids and other low-molecular weight metabolites, for example amino acids and glycolysis-related metabolites in absolute concentration units.

The levels of serum metabolites were measured using NMR metabolomics. The NMR metabolomics platform measures the concentrations of standard biomarkers, such as various cholesterol measures, triglycerides, and creatinine, but in the same experiment also provides quantitative molecular data on lipoprotein subclasses, such as lipids, fatty acids, and apolipoproteins as well as on various low-molecular weight metabolites, including amino acids, glycolysis-related metabolites, and ketone bodies (Soininen, Kangas, Wurtz, Suna, & Ala-Korpela, 2015). All these metabolite measures are obtained from a single serum or plasma sample. NMR is currently the only methodology capable of offering reproducible high-throughput

metabolite quantifications in a cost-effective manner (Soininen et al., 2015). The NMR-based approach is non-destructive and allows further analysis to be performed (Brennan, 2014). NMR spectroscopy takes advantage of the magnetic properties of certain nuclei, such as ^{1}H, ^{13}C and ^{31}P (Brennan, 2014). This spectroscopy consists of arousing nuclear spins located in a magnetic external field through rapid changes of the magnetic field, and then recording the electromagnetic radiation occurring because of nuclear spins relaxation (Brennan, 2014). When a given frequency of the electromagnetic wave is used, only the nuclei with such a resonance frequency can absorb it. The immediate surroundings of the nucleus affects its resonance frequency, thus making it possible to distinguish nuclei which are surrounded by different atoms in a given compound (Kruk et al., 2017).

4.6 Genetic analyses

The SNPs rs738409 near the *PNPLA3* gene, rs58542926 near the *TM6SF2* gene, rs12137855 near the *LYPLAL1* gene and rs780094 near the *GCKR* gene were used as the genetic markers for susceptibility for fatty liver. Genotyping was performed with the custom-built Illumina BeadChip 670K.

4.7 Ultrasound studies

Ultrasound imaging of the liver was performed on 2,042 study participants using a validated protocol (Edens et al., 2009) and Sequoia 512 ultrasound mainframes (Acuson, Mountain View, CA, USA) with 4.0 MHz adult abdominal transducers. Evaluation of fatty liver was performed according to liver-to-kidney contrast, parenchymal brightness, deep beam attenuation, bright vessel walls, and visibility of the neck of the gallbladder (National center for health statistics, centers for disease control and prevention. Hepatic steatosis. [accessed: July 24, 2011];ultrasound images assessment procedures manual. available from: Http://Www.cdc.gov/nchs/data/nhanes/nhanes3/Hepatic_Steatosis_Ultrasound_Procedures_Manual.pdf.). Liver-to-kidney contrast was defined as an evident ultrasonographic contrast between the hepatic parenchyma and the right renal cortex as visualized in the right intercostal space in the midaxillary line. The presence of similar echogenicity of the liver and cortex of the right kidney is indicative of normal hepatic parenchyma. Parenchymal brightness means bright liver, which is defined as abnormally intense, high-level echoes arising from the

hepatic parenchyma. Liver tissue is hyperechogenic with fine, tightly packed echoes on ultrasound examination, and it is considered characteristic of liver steatosis. Deep beam attenuation is the decreased ability of the ultrasound beam to penetrate the liver tissue causing posterior darkness and loss of definition of the diaphragm. Bright vessel walls were defined as the presence of bright walls of small intrahepatic vessels. Gallbladder wall definition is the degree of visualization of the gallbladder walls. Impaired visualization occurs in the presence of fatty infiltration in the areas surrounding the gallbladder. According to these criteria the presence of fatty liver was assessed visually from non-blinded images by a trained ultrasonographer masked to the participant's characteristics. The participants were classified into fatty liver and normal liver groups. In addition, a categorical fatty liver score was formed based on the above criteria.

4.8 Statistical analyses

An attrition analysis was performed to determine whether the representativeness of the baseline sample was maintained in the present cohort; baseline characteristics were compared between those who participated and those who did not participate at the 2011 follow-up. Variables with skewed distributions were log-transformed prior to the analyses. Statistical analyses were performed with the Statistical Analysis System 9.3 and 9.4 (SAS Institute, Inc., Cary, NC, USA). Two-tailed P values less than 0.05 were considered statistically significant.

Study I

The aim of Study I was to examine the prevalence and determinants of fatty liver in the study population. An additional interest was to clarify whether the determinants of fatty liver differ between participants with a different weight status to test the hypothesis that fatty liver may have different determinants in the normal weight and overweight study population. That is why four study groups were defined: 1) normal weight and normal liver; 2) normal weight and fatty liver; 3) overweight or obese and normal liver; and 4) overweight or obese and fatty liver. Analyses of covariance adjusted for sex and age were used to assess differences in continuous variables between the four study groups. The differences in categorical variables between the four study groups were examined by a chi-square test. Pairwise group comparisons were done by Tukey's test.

All variables were standardized to enable comparison between group effects. To study the independent correlates of fatty liver, standardized logistic multivariable regressions adjusted for significant variables from age- and sex-adjusted models were performed. Due to a strong correlation between alcohol intake and heavy drinking, only alcohol intake was included in the model. BMI and waist circumference were strongly correlated in all participants, and alcohol intake and smoking in normal weight participants, and thus these variables were studied with separate models. The results are expressed as odds ratios (OR) per 1 standard deviation (SD) increase in the variables.

To examine whether weight status modifies the associations between the predictor variables and fatty liver, weight status x liver fat interaction terms were included in regression models. The predictors independently associated with fatty liver in the normal weight and the overweight or obese participants were selected in the interaction analyses.

To test whether men and women can be analyzed together, interaction analyses were done to test whether the strength of associations between the predictor variables and fatty liver differed between sexes. In the overweight or obese participants, sex differences were observed in triglycerides, apolipoprotein A1, waist circumference, and homeostasis model index, and in the normal weight participants in BMI and waist circumference. Because no other interactions were observed between sexes, the sexes were analyzed together.

Study II

The aim of Study II was to examine the childhood determinants of adulthood fatty liver in the study participants. In addition, genetic variants were added to the analysis to discover the importance of these variants to fatty liver. The distribution of physical activity index was strongly skewed. Therefore, the physical activity index was divided to quartiles. Alcohol consumption data were calculated in standard doses (12 grams pure ethanol) per day by dividing the total number of doses consumed per week (0.33 liter doses of beer or cider, 0.12 liter doses of wine, and 0.04 liter doses of hard liquor) by 7.

Logistic regression was used to examine the ORs of adult fatty liver for a 1-SD increase for each of the continuous childhood variables. The significant variables from age-, sex- and BMI-adjusted analyses were then added to a multivariable

logistic regression model to determine the independent childhood predictors of adult fatty liver. To examine whether gene variant status modifies the associations between the predictor variables and fatty liver, we included predictor variable x gene variant interaction terms in regression models. The incremental value of adding risk variables to predict adult fatty liver was examined through the use of alcohol consumption adjusted multivariate logistic regression models. Additionally, birth weight was selected to the final multivariable model and model comparison analysis, and the ORs of birth height, small for gestational age and preterm birth were assessed in separate models. The ability of several models to predict fatty liver risk was estimated with alcohol consumption adjusted C statistics by calculating the area under the receiver-operating characteristic curve, the net reclassification improvement, and the integrated discrimination index (Pencina, D'Agostino RB, D'Agostino, & Vasan, 2008). The net reclassification improvement and integrated discrimination index were calculated to determine the extent to which incorporation of birth weight and the genetic variants in *PNPLA3* and *TM6SF2* reassigned individuals to risk categories that more correctly reflected whether the subjects developed fatty liver in adulthood.

To test the ability of multiple childhood risk factors to predict adult fatty liver the AUC-value and NRI were used. The AUC-value gives the probability that the predicted risk is higher for a participant with fatty liver than for a participant with a normal liver (Uhari & Nieminen, 2012). AUC-value varies from 0.5 to 1.0 where 1.0 means perfect discrimination. The AUC-values of two models can be compared to evaluate whether a new risk factor improves prediction of future fatty liver. A limitation of the AUC-value is that it does not quantify the absolute difference in risk (Cook, 2007). NRI attempts to quantify how well a new model reclassifies subjects compared to an old model (Pencina et al., 2008). It compares the movements in the scale of predicted probabilities when the new risk factor is added to the model. In cases, and in this case for participants with fatty liver, upward movements mean an improved prediction and downward movements a worse prediction. Therefore, NRI indicates how much more often a correct versus an incorrect reclassification occurs. The improvement in reclassification can be quantified as a sum of the differences in proportions of individuals moving up minus the proportion moving down for people who develop events, in this case fatty liver, and the proportion of individuals moving down minus the proportion moving up for people who do not develop events or in other words who have a normal liver. IDI compares the mean differences in predicted risk between diseased and non-diseased participants for the models with and without the new risk factor (Pencina et al., 2008). It indicates how far individuals move along the risk scale

with the addition of a new risk marker. IDI does not require categories. Furthermore, calibration of the model needs to be on an acceptable level. Calibration is a measure of the model´s goodness of fit, and it is usually tested with the Hosmer-Lemeshow test, $P<0.05$ generally indicating poor calibration (Hosmer & Hjort, 2002). The Hosmer-Lemeshow test identifies data by dividing it into deciles. It is a measure of how well a predicted risk approximates with the actual observed risk.

Study III

The aim of Study III was to examine how a panel of 68 serum lipid and metabolite markers assessed with the NMR metabolomics platform associate with fatty liver. This was done by using logistic regression models separately for each metabolite measure. Age and sex adjusted cross-sectional associations were performed, and the analyses were further assessed with additional adjustment for waist circumference or BMI, alcohol consumption, physical activity index and smoking status. Similar logistic regression models were done in a prospective analysis, where metabolite measures and covariates from the study years 2001 or 2007 were used as predictors of incident fatty liver in 2011. Participants having alanine aminotransferase of more than 30 U/L in 2001 or in 2007 were excluded from these analyses to exclude participants with a possible fatty liver at baseline. In order to facilitate comparison across different metabolite measures, ORs from the logistic regression models were scaled to 1 SD increment in metabolite concentration. Spearman correlations were performed to assess continuous associations between the 68 metabolite measures and the 0-9 categorical fatty liver score. Multiple testing correction was performed by accounting for 68 tests using the Bonferroni method, with $P < 0.0007$ considered statistically significant.

4.9 Ethics

The Cardiovascular Risk in Young Finns Study was approved by local ethics committees. Participants signed written informed consent forms during the follow-up studies and their parents gave their consent in 1980.

5 Results

5.1 Clinical characteristics of the participants

To determine whether the representativeness of the baseline sample was maintained in the present cohort, we compared the baseline characteristics between those who participated and those who did not participate in the 2011 follow-up. The non-participants were younger (9.9 versus 10.9 years; $p < 0.0001$, sex-adjusted analysis of variance) and more often male (54% versus 45%; $p < 0.0001$, Chi-squared test). No statistically significant differences were observed for other baseline study variables. Baseline (1980) and 2011 follow up clinical and biochemical characteristics of the study population according to fatty liver status are shown in **Table 3**. In 1980, there were significant differences between normal liver and fatty liver study participants in age, male sex, BMI, systolic blood pressure, total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides and insulin levels, birth weight, percentage of small for gestational age, preterm birth, breastfeeding, daily smoking, parental occupational status and parental hypertension prevalence, mother´s BMI and age at menarche. In 2011, there were significant differences between participants with fatty liver and with normal liver in age, male sex, BMI, waist circumference, total cholesterol, LDL cholesterol, HDL cholesterol, apolipoprotein A1, apolipoprotein B, triglyceride, glucose, glycated hemoglobin A1c and insulin levels, insulin resistance, systolic blood pressure, diastolic blood pressure, alanine aminotransferase, aspartate aminotransferase, gamma-glutamyltransferase, C reactive protein, alcohol intake, heavy drinking, physical activity index, metabolic syndrome and energy percent of protein, fat, carbohydrates and alcohol.

Table 3. Characteristics of the participants with and without a fatty liver diagnosis in adulthood, in childhood in 1980 and in adulthood in 2011.

Adult fatty liver status in 2011	Normal liver	Fatty liver	*P-value*
Characteristics in 1980			
No.	1,657	385	
Age (years)	10.7±0.1	12.3±0.3	<0.0001
Male sex (%)	40.0	68.3	<0.0001
BMI (kg/m^2)	17.7±0.1	19.0±0.2	<0.0001
Systolic blood pressure (mmHg)	112±0	116±1	<0.0001
Diastolic blood pressure (mmHg)	68±0	70±1	0.01
Total cholesterol (mmol/L)	5.3±0.0	5.1±0.0	0.0006
LDL cholesterol (mmol/L)	3.5±0.0	3.3±0.0	0.0008
HDL cholesterol (mmol/L)	1.6±0.0	1.5±0.0	0.03
Triglycerides (mmol/L)	0.66±0.0	0.70±0.0	0.03
Fasting insulin (mU/L)	9.5±0.1	11.4±0.3	<0.0001
C reactive protein (mg/L)	1±0	1±0	0.9
Birth weight (grams)	3,529±15	3,441±32	0.01
Birth height (cm)	50.4±0.1	50.2±0.1	0.2
Large for gestational age (%)	9.4	8.8	0.7
Small for gestational age (%)	9.1	14.8	0.005
Appropriate for gestational age (%)	81.5	76.4	0.06
Preterm birth (%)	3.1	6.9	0.002
Breastfeeding (%)	85.9	78.8	0.005
Daily smoking (%)[a]	18.5	23.6	0.03
Physical activity index	8.4±0.1	8.6±0.1	0.3
Mother´s BMI (kg/m^2)	23.8±0.1	24.7±0.2	<0.0001
Father´s BMI (kg/m^2)	25.4±0.1	25.8±0.2	0.05
Parental occupational status (%)*	38/43/19	44/42/14	0.03
Parental hypertension prevalence (%)	13.6	20.1	0.003
Age at menarche (years)[a]	13.1±0.1	12.7±0.1	0.02
Characteristics in 2011			
No.	1,628	370	
Age (years)	41.5±0.1	43.2±0.3	<0.0001
Male sex (%)	40	68	<0.0001
BMI (kg/m2)	25.5±0.1	30.9±0.2	<0.0001
Waist (cm)	89±0	105±1	<0.0001
Total cholesterol (mmol/l)	5.1±0.0	5.4±0.0	<0.0001
LDL cholesterol (mmol/l)	3.2±0.0	3.4±0.0	0.001
HDL cholesterol (mmol/l)	1.4±0.0	1.2±0.0	<0.0001
Apolipoprotein A1(g/L)	1.6±0.0	1.5±0.0	<0.0001
Apolipoprotein B (g/L)	1.0±0.0	1.2±0.0	<0.0001
Triglycerides (mmol/l)	1.2±0.0	2.0±0.1	<0.0001
Glucose (mmol/l)	5.3±0.0	5.9±0.0	<0.0001
Fasting insulin (mU/l)	8.1±0.3	18.2±0.7	<0.0001
Insulin resistance	2.0±0.2	5.4±0.3	<0.0001
Glycated hemoglobin A1c (mmol/mol)	36±0	39±0	<0.0001

Systolic blood pressure (mmHg)	117±0	127±1	<0.0001
Diastolic blood pressure (mmHg)	73±0	82±1	<0.0001
Alanine aminotransferase (U/l)	15±0	32±1	<0.0001
Aspartate aminotransferase (U/l)	22±0	32±1	<0.0001
Gamma-glutamyltransferase (U/l)	28±1	63±2	<0.0001
C reactive protein (mg/L)	1±0	3±0	<0.0001
Smoking, daily (%)	14	18	0.08
Alcohol intake (drinks per day)	0.7±0.0	1.3±0.1	<0.0001
Heavy drinking (%)[b]	13	26	<0.0001
Physical activity index	9.2±0.0	8.4±0.1	<0.0001
Metabolic syndrome (%)	13	60	<0.0001
Protein (% of energy)	17.4±0.1	17.8±0.1	0.005
Fat (% of energy)	32.7±0.1	33.5±0.3	0.009
Carbohydrates (% of energy)	46.3±0.2	44.5±0.3	<0.0001
Alcohol (% energy)	2.4±0.1	3.1±0.2	0.0007

[a] Data available for 12-18 years old participants. [b] Heavy drinking was defined as consuming 6 or more alcohol doses on the same occasion at least once a week. * Parental occupational status was divided into 3 categories: manual, lower-grade non-manual, and higher-grade non-manual.

5.2 Cross-sectional risk factors for adult fatty liver

Altogether 1,998 participants with data on liver ultrasound and cardiovascular risk factors were included in the study. In total, 19% of participants showed evidence of fatty liver by ultrasound: 5% of normal weight participants and 29% of overweight or obese participants. Prevalence of fatty liver in 34- to 49-year-old participants is shown in **Figure 2a** and sex stratified in **Figure 2b**. Prevalence of fatty liver was 11% in 34 years old participants and reached 25% in 49 year old participants. In men, the prevalence of fatty liver was 28% and in women 11%. The sex difference remained after adjustment for alcohol consumption (OR 3.27 versus 2.70).

To study the multivariate determinants of fatty liver, multivariable models were analyzed (**Table 4**). Variables significant in age- and sex-adjusted univariate logistic regression were selected in the final models. Due to the strong correlation between BMI and waist circumference, these were analyzed in separate models. Independent predictors of fatty liver in all participants from the strongest to the weakest were waist circumference/BMI, alanine aminotransferase, male sex, apolipoprotein B, systolic blood pressure, alcohol consumption, insulin, and physical activity index (inverse correlation). In normal weight participants, the statistically significant predictors of fatty liver from the strongest to the weakest were alanine aminotransferase level, smoking, systolic blood pressure, and alcohol consumption. Whereas, in overweight participants, the independent predictors of fatty liver from the strongest to the weakest were waist circumference, alanine aminotransferase, male sex, triglycerides, systolic blood pressure, insulin, alcohol consumption, age, and physical activity index (inversely association).

The effects of alcohol intake, smoking, and triglycerides were significantly different between normal weight and overweight or obese participants (**Table 5**). Alcohol intake and smoking correlated more strongly with fatty liver in the normal weight participants, and triglycerides correlated more strongly with fatty liver in the overweight or obese participants.

The effects of alcohol consumption were analyzed to study whether the effect of moderate alcohol consumption would be different in the normal weight and the overweight or obese individuals. The OR for fatty liver of moderate alcohol consumption (maximum 1.7 standard drinks per day) was 1.47 in normal weight participants, and 1.20 in the overweight or obese participants. Thus, apparently,

moderate alcohol consumption may also markedly increase the risk of having fatty liver.

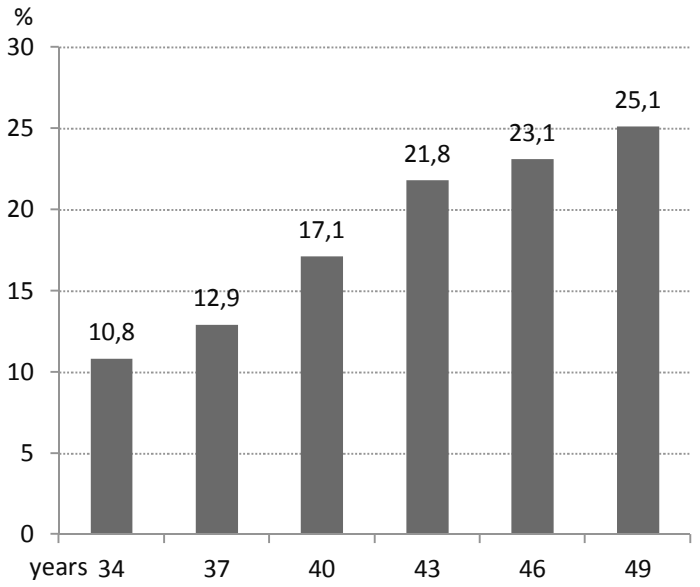


Figure 2a. Age stratified prevalence of fatty liver in the Cardiovascular Risk in Young Finns Study population aged 34-49 in 2011.

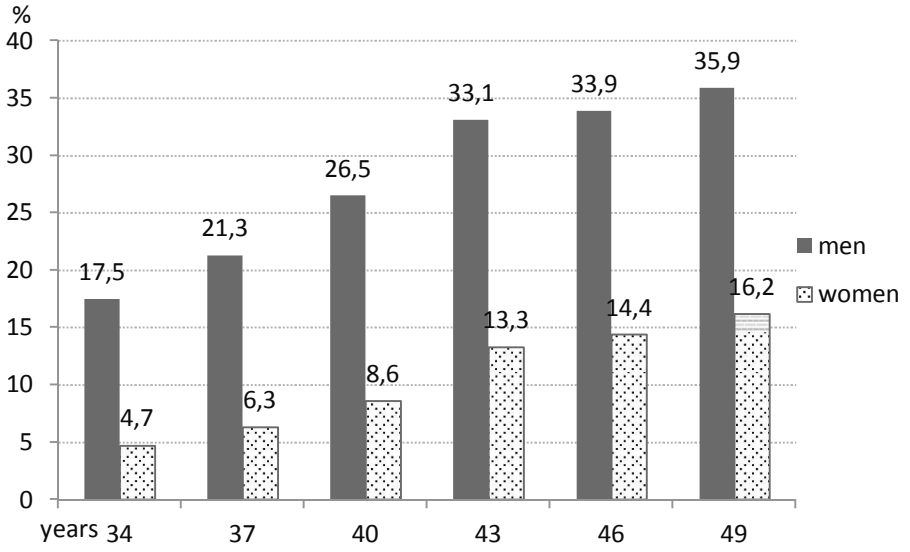


Figure 2b. Age and sex stratified prevalence of fatty liver in the Cardiovascular Risk in Young Finns Study population aged 34-49 in 2011.

Table 4. Multivariable standardized odds ratios and 95% confidence intervals for fatty liver in the whole population and stratified by weight status in participants of the Cardiovascular Risk in Young Finns Study. Due to a strong correlation, BMI and waist circumference were analyzed in separate models. Variables significant in age- and sex-adjusted univariate logistic regression were selected in the models.

	All participants OR (95% CI)	Normal weight OR (95% CI)	Overweight OR (95% CI)
No.	1,998	863	1,135
Male sex	1.80 (1.20-2.71)	2.07 (0.87-4.94)	1.74 (1.16-2.60)
Age (years)	1.03 (0.99-1.07)	0.98 (0.91-1.05)	1.05 (1.02-1.09)
BMI (kg/m2)	2.34 (1.91-2.85)	2.74 (0.76-9.89)	2.00 (1.60-2.49)
Waist (cm)	2.80 (1.73-4.52)		3.78 (1.47-9.68)
Apolipoprotein A1 (mmol/L)		1.34 (0.96-1.88)	
Apolipoprotein B (mmol/L)	1.36 (1.11-1.67)		1.19 (0.96-1.49)
Triglycerides (mmol/L)	1.10 (0.96-1.25)	1.11 (0.95-1.28)	1.44 (1.09-1.89)
Insulin (mU/L)	1.24 (1.05-1.47)		1.23 (1.05-1.45)
Systolic blood pressure (mmHg)	1.32 (1.09-1.61)	1.54 (1.05-2.25)	1.31 (1.09-1.59)
Alanine aminotransferase (U/L)	2.41 (1.80-3.21)	3.05 (1.73-5.40)	2.11 (1.64-2.72)
C reactive protein (mmol/L)			1.13 (0.94-1.36)
Daily smoking (yes/no)		2.56 (1.18-5.52)	
Alcohol consumption (drinks per day)	1.28 (1.10-1.50)	1.41 (1.09-1.83)	1.17 (1.00-1.38)
Physical activity index	0.76 (0.63-0.91)		0.76 (0.63-0.90)
Protein (% of energy)	1.09 (0.91-1.30)		

Table 5. Associations between factors associated with fatty liver in overweight or obese and normal weight participants.

Factor	Overweight/obese (N=1,012)		Normal weight (N=821)		Interaction Term
	OR	P Value	OR	P Value	P Value
Triglycerides (mmol/l)	1.45	0.009	1.09	0.3	<0.0001
Daily smoking (yes/no)	1.00	1	2.48	0.02	0.009
Alcohol intake (drinks per day)	1.14	0.1	1.41	0.01	0.02

5.3 Genetic variants and childhood predictors of adult fatty liver

5.3.1 Associations between genetic variants and fatty liver

The distribution of selected genotypes according to the liver status in the Cardiovascular Risk in Young Finns Study population is shown in **Table 6**. Forty percent of participants with a normal liver are carriers of the rs738409 minor allele near the *PNPLA3* gene whereas 46 percent of the participants with fatty liver are minor allele carriers (P-value 0.003). Ten percent of the participants with a normal liver carry the minor allele in rs58542926 near the *TM6SF2* gene while 18 percent of participants with fatty liver are minor allele carriers (P-value 0.002). 58 percent of the participants with a normal liver and 62 percent of the participants with a fatty liver are carriers of the rs780094 minor allele near the *GCKR* gene (P-value 0.004). In participants with a normal liver, 47 percent are carriers of the rs12137855 minor allele near the gene *LYPLAL1* and in participants with a fatty liver, fifty percent are carriers of the minor allele (P-value 0.1).

Table6. Distribution of *PNPLA3* (rs738409 C>G), *TM6SF2* (rs58542926 C>T), *GCKR* (rs780094 C>T) and *LYPLAL1* (rs12137855 C>T) genotypes in the participants with a normal liver and with a fatty liver in the Cardiovascular Risk in Young Finns Study.

Genetic variant	Normal liver	Fatty liver	P Value
PNPLA3 (CC/CG/GG) (%)	60/36/4	54/38/8	0.003
TM6SF2 (CC/CT/TT) (%)	90/10/0	82/17/1	0.002
GCKR (CC/CT/TT) (%)	42/46/12	38/43/19	0.004
LYPLAL1 (CC/CT/TT) (%)	53/41/6	50/41/9	0.1

5.3.2 Childhood risk factors for adult fatty liver

Age-, sex- and BMI-adjusted ORs and confidence intervals for adult fatty liver according to childhood variables are shown in **Table 7**. Male sex, preterm birth, small for gestational age, *TM6SF2*, *PNPLA3*, age- and sex-adjusted childhood BMI and insulin levels, low birth weight and low birth height showed significant ORs for adult fatty liver.

Multivariable logistic regression models were constructed to examine the independent contributions of childhood risk variables to the development of the adult fatty liver. Significant variables from **Table 7** were selected in the model: age, sex, insulin, BMI, birth weight, *TM6SF2* and *PNPLA3*. In this multivariable logistic regression model, the independent predictors of adult fatty liver included male sex, variant in *PNPLA3*, variant in *TM6SF2*, insulin, BMI and birth weight (**Table 8**). In the interaction analyses, there were no statistically significant predictor variable x gene variant interactions. Excluding participants with excess alcohol intake did not change the results.

5.3.3 Multiple childhood risk factors in predicting adult fatty liver

The ability of multiple childhood risk factors to predict adult fatty liver with several models is shown in **Table 9**. Age and variables independently associated with fatty liver were selected in the models. Interest was focused on discovering whether birth weight or genetic variables improve the predictability of fatty liver. A model that included age, sex, BMI, insulin and birth weight (model 2) performed better than a model including only age, sex, BMI, and insulin (model 1) ($p = 0.03$ model 2 versus 1). When both *PNPLA3* and *TM6SF2* were included in the model (model 3), the area under the curve value increased significantly ($p = 0.002$ when compared to model 1, and $p = 0.05$ when compared to model 2). Consistent improvements were also seen for the net reclassification improvement and integrated discrimination index between models 1 and 3. Notably, the net reclassification improvement for model 3 was more than 28% with respect to the model including only age, sex, BMI and insulin. Excluding participants with excess alcohol intake did not change the results.

Table 7. Age-, sex- and BMI-adjusted odds ratios and 95% confidence intervals for adult fatty liver for each of the youth variables (N=2,027). In continuous variables OR indicates a 1-SD increase. In categorical variables, OR indicates a one class increase, for example per one risk allele.

Variable	OR	95% CI	*P-value*
Male sex (age- and BMI-adjusted)	3.33	2.62-4.23	<0.0001
Preterm birth (yes/no)	2.41	1.37-4.25	0.002
Small for gestational age (yes/no)	1.75	1.16-2.63	0.008
Variant in *TM6SF2* (per T allele)	1.71	1.23-1.37	0.002
Variant in *PNPLA3* (per G allele)	1.38	1.12-1.69	0.003
BMI (age- and sex-adjusted) (kg/m^2)	1.34	1.15-1.55	0.0001
Insulin (mU/L)	1.29	1.12-1.47	0.0003
Parental hypertension (yes/no)	1.25	0.89-1.75	0.2
Breastfeeding (yes/no)	1.29	0.80-2.07	0.3
Variant in *LYPLAL1* (per T allele)	1.15	0.95-1.41	0.2
Diastolic blood pressure (mmHg)	1.06	0.94-1.21	0.4
Daily smoking (yes/no)	1.04	0.78-1.40	0.8
Age (sex- and BMI-adjusted) (years)	1.03	1.00-1.07	0.06
Mother´s BMI (kg/m^2)	1.03	1.00-1.06	0.08
Systolic blood pressure (mmHg)	1.03	0.89-1.19	0.7
Father´s BMI (kg/m^2)	1.01	0.97-1.06	0.5
C reactive protein (mg/L)	1.00	0.96-1.04	1
HDL cholesterol (mmol/L)	0.98	0.87-1.10	0.7
Triglycerides (mmol/L)	0.98	0.87-1.10	0.7
Physical activity index	0.97	0.79-1.19	0.8
Total cholesterol (mmol/L)	0.90	0.80-1.02	0.09
LDL cholesterol (mmol/L)	0.89	0.79-1.01	0.06
Large for gestational age (yes/no)	0.87	0.53-1.41	0.6
Parental occupational status*	0.85	0.71-1.02	0.07
Variant in *GCKR* (per C allele)	0.83	0.70-1.00	0.05
Birth height (cm)	0.82	0.72-0.94	0.003
Age at menarche (years)	0.80	0.63-1.01	0.06
Birth weight (grams)	0.77	0.68-0.88	<0.0001
Appropriate for gestational age (yes/no)	0.76	0.55-1.06	0.1

* Parental occupational status was divided into 3 categories: manual, lower-grade non- manual, and higher-grade non-manual.

Table 8. Multivariable odds ratios of childhood risk factors for adult fatty liver (N=1,456).

Variable	OR	95% CI	*P-value*
Male sex	3.33	2.41-4.61	<0.0001
Variant in *PNPLA3* (per G allele)	1.63	1.29-2.07	<0.0001
Variant in *TM6SF2* (per T allele)	1.57	1.08-2.30	0.02
BMI (kg/m^2)	1.30	1.07-1.59	0.009
Insulin (mU/L)	1.25	1.05-1.49	0.01
Age (years)	1.02	0.98-1.06	0.4
Birth weight (grams)	0.81	0.70-0.93	0.004

Table 9. Comparison of models for the prediction of adult fatty liver in 1,456 individuals of the Cardiovascular Risk in Young Finns Study.

	AUC Value	*P*	NRI, %	*P*	IDI, %	*P*	H-L	*P*	Model Used for Comparison
Model 1									
Age, sex, BMI, insulin	0.725						2.1	1	
Model 2									
Model 1 + birth weight	0.736	0.03	12.7	0.06	6.5	0.03	6.8	0.6	1
Model 3									
Model 1 + birth weight, *PNPLA3* and *TM6SF2*	0.749	0.002	28.6	<0.0001	23.5	<0.0001	11.9	0.2	1
		0.05	24.6	0.0003	16.0	<0.0001			2

AUC-value gives the probability that the predicted risk is higher for a participant with fatty liver than for a participant with normal liver. AUC-value varies from 0.5 to 1.0 where 1.0 means perfect discrimination. NRI attempts to quantify how well a new model reclassifies subjects compared to an old model. IDI compares the mean differences in predicted risk between fatty liver and normal liver participants for the models with and without the new risk factor. Calibration is a measure of model´s goodness of fit, and it is tested with H-L (Hosmer-Lemeshow) test, P<0.05 generally indicating poor calibration.

5.4 Fatty liver and serum metabolomics

5.4.1 Cross-sectional associations of serum metabolite measures with fatty liver

The cross-sectional odds ratios for fatty liver for each metabolite measure are shown in **Figure 3**. Age-adjusted and sex-adjusted associations are indicated by blue bars. Overall, 60 out of the 68 metabolite measures were associated with fatty liver in cross-sectional settings ($P < 0.0007$). When adjusted for alcohol consumption, waist circumference, physical activity index, and smoking, 41 measures remained significant; these are indicated with red bars ($P < 0.0007$). High concentrations of large VLDL particles and the triglyceride concentration in extremely large VLDL particles were most strongly associated with the fatty liver. Higher concentrations of large HDL particles were strongly inversely associated with fatty liver, whereas higher concentrations of small HDL particles were directly associated with fatty liver. Several measures of lipoprotein particle size were also strongly associated with the fatty liver, with inverse associations observed for both LDL and HDL particle diameters. Triglyceride-related lipid measurements were more strongly associated with fatty liver than the corresponding cholesterol measures within the same lipoprotein subfractions.

5.4.2 Prospective associations of metabolite measures with fatty liver

The 10-year prospective associations with the metabolites measured in 2001 and the fatty liver diagnosed in 2011 are shown in **Figure 4**. Fifty-four participants whose alanine aminotransferase were more than 30 U/L were excluded from the analysis. In **Figure 4**, age and sex adjusted associations are shown in blue. The prospective association pattern was quite similar and only modestly weaker than the cross-sectional associations. Variations in lipoprotein metabolism and fatty acid balance remained significant for the 10-year risk for fatty liver also after adjusting for baseline waist circumference, smoking, physical activity, and alcohol intake. Disturbances in the circulating concentrations of amino acids and various glycolysis precursors also preceded the onset of fatty liver. Further validation of the prospective biomarker associations with fatty liver risk based on the metabolite concentrations quantified from serum samples drawn in 2007 demonstrated coherent results.

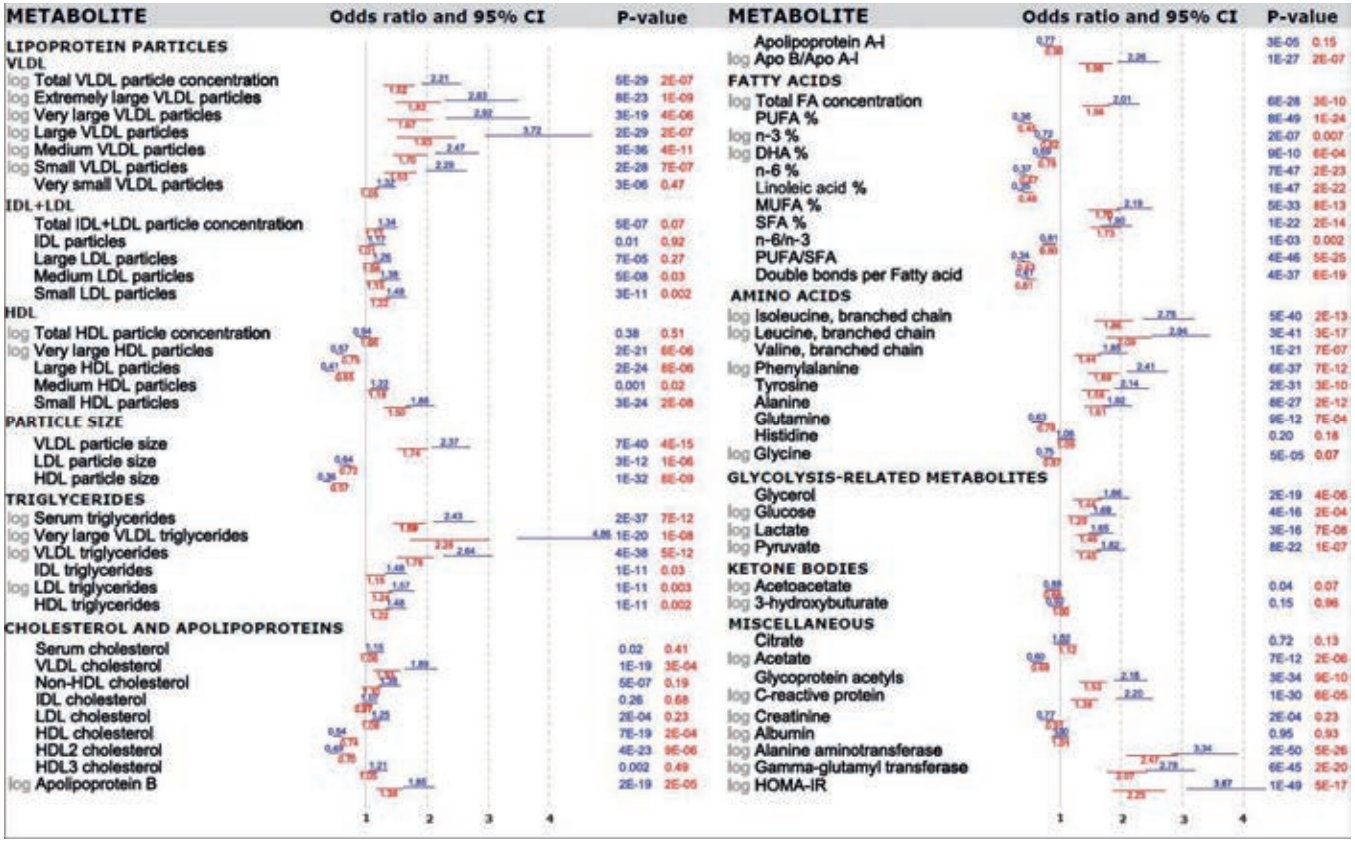


Figure 3. Cross-sectional associations of metabolite measures with fatty liver. OR indicates a 1-SD increase and it is shown adjusted for sex and age (blue) and for sex, age, waist, alcohol intake, leisure-time physical activity and smoking (red). Published in study III (Kaikkonen JE et al. Metabolic profiling of fatty liver in young and middle-aged adults: Cross-sectional and prospective analyses of the Young Finns Study. *Hepatology* 2017;65:2:491-500).

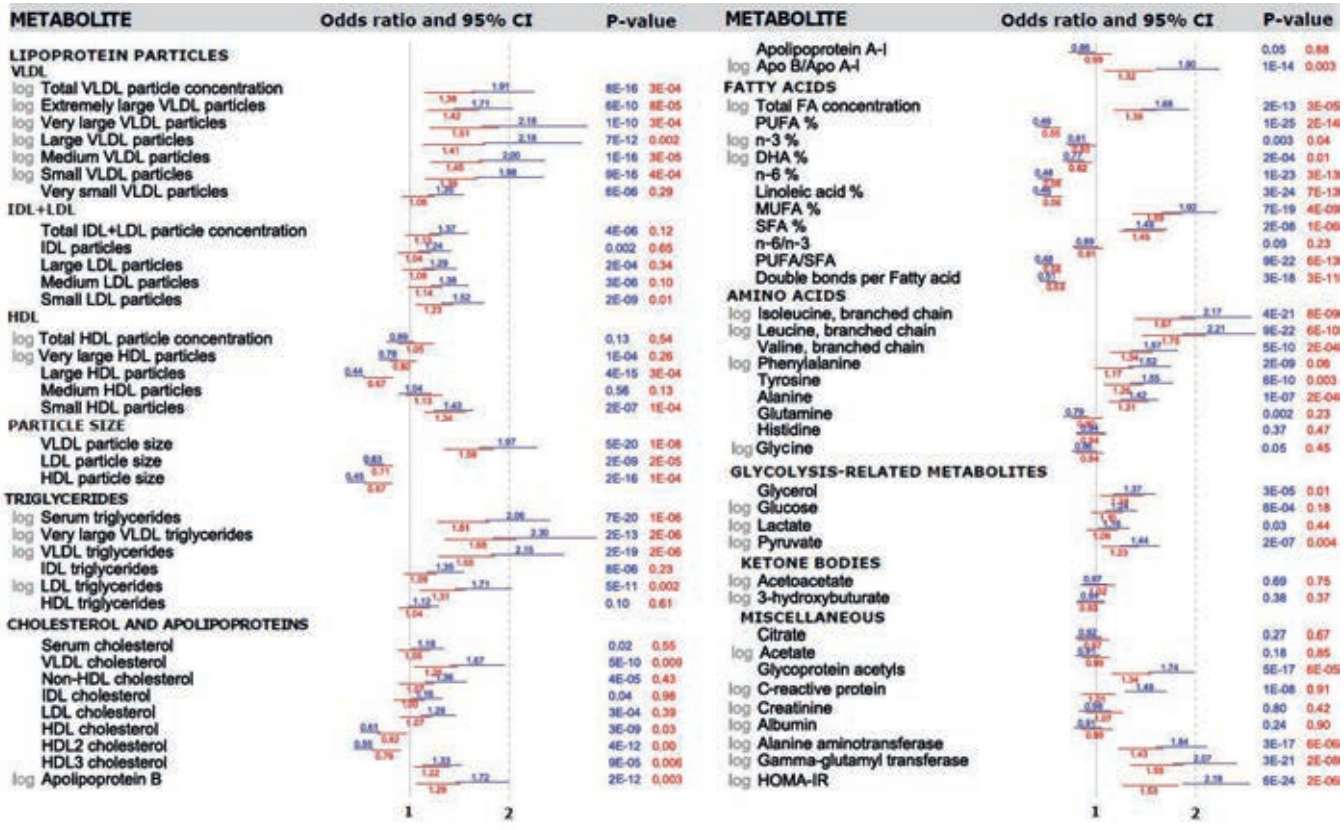


Figure 4. Prospective associations of metabolite measures with a 10-year risk for fatty liver. OR indicates a 1-SD increase and it is shown adjusted for sex and age (blue) and baseline age, sex, waist, alcohol intake, leisure-time physical activity and smoking (red). Published in study III (Kaikkonen JE et al. Metabolic profiling of fatty liver in young and middle-aged adults: Cross-sectional and prospective analyses of the Young Finns Study. *Hepatology* 2017;65:2:491-500).

6 Discussion

6.1 Participants

The participants in this thesis were from the Cardiovascular Risk in Young Finns Study, which is an on-going epidemiological study of cardiovascular risk factors in children and young adults (Raitakari et al., 2008). The study was launched in 1980 when 4,320 participants aged 3, 6, 9, 12, 15 and 18 years were invited to take part in the survey. The participants were randomly selected from different parts of the country, and equally from both sexes in order to represent Finnish children and adolescents as closely as possible. A total of 3,596 subjects, 83% of those invited, participated in the study in 1980 and were considered to be representative of the total random sample (Åkerblom et al., 1985).

Of the original study cohort from 1980, a total of 2,063 individuals (57%) participated in 2011. Lost-to follow-up is a common limitation in longitudinal studies. In our study, participants were similar in all other risk factor levels, but they were older and more often women; the non-participants tended to include more men. Thus, the randomly selected study population represented well the original study population. The sample size of this study is sufficient for statistical analyses and the follow-up bias does not seem to influence the representativeness of the original study population. Therefore, the results in this study may be generalized to populations consisting of white, middle-aged individuals.

6.2 Methods

Results of this study are quite reliable because the methods used for the laboratory measurements are well standardized. All assays were carried out as simultaneously as possible in 1980, 1983 and 1986 in the laboratory of the Rehabilitation Centre of the Social Insurance Institution (Turku, Finland), in the laboratory of the Research and Development Unit of the Social Insurance Institution (Turku, Finland) in 2001, and in the laboratory for Population Research of the National Institute for Health and Welfare (Turku, Finland) in 2007 and 2011. The following methods of the laboratory were also accredited by the Finnish Accreditation Service according to standard ISO / IEC17025: total cholesterol, HDL cholesterol, triglycerides and glucose. The Laboratory of the Rehabilitation Centre of the Social Insurance

Institution continuously checks the cholesterol determinants with the World Health Organization laboratory in Prague. Subsequently, performance of the laboratory methods has been externally evaluated both by Labquality´s external quality assessment program and by using quality assessment testing provided by National Institute of Standards and Technology. The methods for measuring the risk factors for fatty liver such as HDL and LDL cholesterol levels, blood pressure, BMI and glucose levels are well-standardized and are therefore reliable assessments (Raitakari et al., 2008). Self-reported questionnaires for alcohol use, smoking, and physical activity are valid measurements for studying these health factors although these questionnaires may also involve limitations (Raitakari et al., 2008).

In this study, we used ultrasound for detecting fatty liver. However, liver biopsy is at present the gold standard for diagnosing fatty liver, because it is the only way to detect inflammation or fibrosis (Bhatia et al., 2012). Nonetheless, it is an invasive procedure with potential risk of bleeding, and it is not suitable for large-scale population studies of fatty liver (Al Knawy & Shiffman, 2007; Ratziu et al., 2005). Ultrasound imaging has certain limitations: while the test shows high specificity, it has low sensitivity. It has been estimated that conventional ultrasound imaging can only detect steatosis when more than 30% of the liver is affected (Saadeh et al., 2002). However, ultrasound imaging is non-invasive, widely used, and cost-effective. Therefore, it is a reasonable choice for population-based studies into the etiogenesis of fatty liver.

6.3 Results

6.3.1 Cross-sectional risk factors for fatty liver

In the Cardiovascular Risk in Young Finns Study population, the prevalence of fatty liver was 19%, and it was more common in men than in women: 28% of the men had fatty liver versus 11% of the women. Furthermore, the prevalence increased with age. Among the 34-year-old participants, the prevalence was 11% whereas in the oldest age-group, who were 49 years old at the time of survey, the prevalence was 25%. The global prevalence of fatty liver is estimated to be 25% in adults (Younossi et al., 2016). The participants in the Cardiovascular Risk in Young Finns Study were relatively young adults (mean age 41 years in 2011) and this might explain why the prevalence of fatty liver is lower than the global prevalence. However, the prevalence of fatty liver in the oldest age-group is exactly same as the global prevalence. The prevalence of fatty liver is known to

increase with age (Browning et al., 2004), but in elderly people the prevalence declines (Koehler et al., 2012). A higher prevalence of fatty liver in men is in line with other population based studies (Browning et al., 2004; Clark, Brancati, & Diehl, 2003; Ioannou, Boyko, & Lee, 2006; Lazo et al., 2013; Ruhl & Everhart, 2003; Schneider, Lazo, Selvin, & Clark, 2014; Younossi et al., 2012). Alcohol use might be one possible explanation for the higher prevalence in fatty liver among men (Browning et al., 2004; Schneider et al., 2014; Weston et al., 2005). However, our data did not support this, as the sex effect on fatty liver remained virtually unchanged when adjusted for alcohol intake.

In this thesis, the following variables were independently associated with fatty liver: waist circumference, BMI, male sex, systolic blood pressure, alanine aminotransferase, apolipoprotein B, insulin, alcohol consumption, and physical activity with inverse association. In addition, smoking correlated independently to fatty liver in normal weight participants and triglycerides in overweight participants.

The liver enzyme alanine aminotransferase was independently associated with fatty liver in the study population. This was expected because elevated alanine aminotransferase levels are part of fatty liver prediction scores (Weiss et al., 2014; Yki-Järvinen, 2016). Alanine aminotransferase is an enzyme which normally functions in the cytoplasm of liver cells, and in the case of liver damage it is released into the circulation. Alanine aminotransferase is normally less than 20 U/l in women and less than 30 U/l in men (Yki-Järvinen, 2016). In our study population, alanine aminotransferase was 12 U/l in women with a normal liver and 27 U/l in women with a fatty liver. In men with a normal liver, alanine aminotransferase levels were 20 U/l and in men with a fatty liver, 34 U/l.

Systolic blood pressure was independently associated with fatty liver. This finding is in line with several other studies (Browning et al., 2004; Hu et al., 2012; Lau et al., 2010; Lopez-Suarez et al., 2011; Vasunta, Kesäniemi, Ylitalo, & Ukkola, 2012). In a cross-sectional case-control study of 890 Finnish adults, systolic blood pressure was independently associated with ultrasonographic fatty liver (Vasunta et al., 2012). In a recent review, the conclusion was that fatty liver and high blood pressure are associated independently from cardiovascular risk factors (Oikonomou et al., 2018). This independent association might possibly be explained as due to insulin resistance, raised activation of renin-angiotensin-aldosterone system and increased arterial stiffness (Oikonomou et al., 2018). Donati et al. also suggest that insulin resistance is one of the links between fatty liver and hypertension (G. Donati et al., 2004). Insulin resistance might be associated with high blood

pressure because it causes renal sodium retention (Soleimani, 2015) and increased activation of the sympathetic nervous system (Reaven & Hoffman, 1987). In contrast, insulin resistance increases hepatic fat accumulation, and fatty liver might lead to increased insulin resistance through inflammation, increased fatty acids and endoplasmic reticulum stress (Asrih & Jornayvaz, 2015). This may lead to higher blood pressure levels. A component of renin-angiotensin-aldosterone system called angiotensin II has been shown to increase insulin resistance (Olivares-Reyes, Arellano-Plancarte, & Castillo-Hernandez, 2009), which as mentioned earlier affects both liver fat content and blood pressure. Fatty liver is also shown to be associated with arterial stiffness (Chung et al., 2015; Li et al., 2015), and arterial stiffness in contrast might lead to higher blood pressure levels (Mitchell, 2014). In practice, it might be useful to pay extra attention to patients with both fatty liver and hypertension in order to reduce cardiovascular risks and also to consider the possibility of fatty liver in hypertensive patients.

In this thesis, physical activity is inversely associated with fatty liver. Physical activity was measured by a physical activity index where participants reported the quantity and quality of their habitual activity (Telama et al., 2005). This finding is in line with other studies (Gerber et al., 2012; Hattar, Wilson, Tabotabo, Smith, & Abrams, 2011; Zelber-Sagi et al., 2008). The mechanism between physical activity and reduced risk of fatty liver is not fully understood (Golabi et al., 2016). Exercise induces oxidative metabolism and improves oxygen intake and circulation. This guarantees fuel supply to muscles which in turn stimulates fatty acid uptake and oxidation and increases insulin sensitivity (van der Heijden, Toffolo, Manesso, Sauer, & Sunehag, 2009). Furthermore, exercise reduces oxidative stress in the liver (Mauriz et al., 2000; Ordonez, Carbajo-Pescador, Mauriz, & Gonzalez-Gallego, 2015).

6.3.1.1 Modest alcohol consumption and fatty liver

In this thesis, alcohol consumption was independently associated with fatty liver. Participants with normal liver drank on an average 0.7 drinks per day and participants with fatty liver 1.3 drinks per day. The association of fatty liver with alcohol intake was stronger in the normal weight participants, suggesting that fatty liver in the normal weight participants might be partly caused by a lifestyle characterized by a substantial alcohol intake. A daily alcohol intake higher than 20-30 grams is a known risk factor of alcoholic liver disease (Bellentani et al., 1997; Lefton, Rosa, & Cohen, 2009; Lu et al., 2004). In the normal weight study

participants with fatty liver, the daily alcohol intake was 1.8 standard drinks per day, corresponding to 21 grams ethanol per day. Using the 20 grams of ethanol per day, corresponding to 1.7 standard drinks per day, as the limit of moderate alcohol consumption, the prevalence of alcoholic fatty liver disease in the current study population may be estimated to be 5% and NAFLD to be 13%. The effects of alcohol consumption were further analyzed to study whether the effect of moderate alcohol consumption would be different in the normal weight and the overweight or obese individuals. The OR for fatty liver of moderate alcohol consumption was 1.47 in normal weight participants, and 1.20 in the overweight or obese participants. Thus, among the participants in this study, it was seen that apparently modest alcohol consumption may also markedly increase the risk of fatty liver regardless of weight status. In a Finnish Opera study of 958 participants aged 40-59 years, the average alcohol consumption was 37 grams per week in participants with a normal liver and 55 grams per week in participants with fatty liver (Käräjämäki et al., 2015). Men consuming 210 grams or more of alcohol a week and women 140 grams or more a week were excluded from the study. In a Finnish FIN-D2D study with 2,766 participants aged 45-74 years, participants were stratified into low alcohol consumption and high alcohol consumption groups according to more than 20 grams of alcohol per day in men and more than 10 grams in women (Kotronen et al., 2010). In the low alcohol consumption group, the average alcohol consumption was 4 grams per day. In the high alcohol consumption group, participants with normal liver enzyme levels consumed 26 grams of alcohol per day and participants with elevated liver enzyme levels 32 grams per day. In a Finnish population-based FinDrink study, 42-year-old men (N=306) drank on an average 9.0 units alcohol per week corresponding 1.3 drinks per day (Ilomäki et al., 2010). Our study population is therefore quite representative of the average levels of Finnish alcohol consumption.

The results concerning moderate alcohol consumption and its effect on fatty liver are inconsistent. In a meta-analysis of 43,175 adult individuals, moderate alcohol consumption defined as drinking less than 40 grams of alcohol per day was associated with a significant protective effect on the risk of having fatty liver when compared to non-drinkers (Sookoian, Castano, & Pirola, 2014). In a meta-analysis that included 76,608 participants, light to moderate alcohol consumption was associated with a 23% reduction in risk of fatty liver when compared to non-drinkers (Cao et al., 2016). The results indicated that a greater protective role for fatty liver was found in the light drinkers (less than 20 grams per day) compared with the moderate drinkers (>20–40 g/day). The protective effect of low to moderate alcohol consumption seemed to be greater in female drinkers and

drinkers with a BMI 25 kg/m^2 or more when compared with male drinkers and drinkers with BMI of less than 25 kg/m^2 (Cao et al., 2016). However, in a study of 922 adult Chinese participants, modest drinkers had a significantly higher prevalence of fatty liver than non-drinkers (Wong et al., 2012). After adjustment for age, sex and metabolic syndrome, modest alcohol consumption was no longer associated with fatty liver, and it did not increase the risk of advanced fibrosis (Wong et al., 2012).

As mentioned earlier, the prevalence of alcoholic fatty liver disease in the current study population may be estimated to be 5% and NAFLD to be 13%. Alcoholic fatty liver disease and NAFLD are the most frequent liver diseases worldwide, and both conditions overlap in a significant number of patients (Mahli & Hellerbrand, 2016). In a Finnish FIN-D2D survey with 2,766 participants, the prevalence of NAFLD was 21% and prevalence of alcoholic fatty liver disease 7% (Kotronen et al., 2010). Metabolic syndrome and type 2 diabetes were prevalent both in NAFLD (70% and 25%) and alcoholic fatty liver disease (73% and 24%) (Kotronen et al., 2010). In a Finnish study of 6,732 participants, 49% of alcohol risk users had also metabolic syndrome (Åberg et al., 2018). There is evidence that alcohol and components of metabolic syndrome exhibit combined effects on the development and progression of fatty liver, which are in part additive and to some extent even synergistic (Mahli & Hellerbrand, 2016). Thus, people at risk for NAFLD are also at risk for alcoholic fatty liver based on overlapping genetic factors that contribute to NAFLD and alcohol liver disease pathogenesis. A genetic variant in *PNPLA3* is associated with increased risk of steatosis, fibrosis, and hepatocellular carcinoma in both NAFLD and alcoholic fatty liver (Romeo et al., 2008; Sookoian & Pirola, 2011; Tian, Stokowski, Kershenobich, Ballinger, & Hinds, 2010; Trepo et al., 2011), and a coding variant in *TM6SF2* is associated with increased risk of steatosis and fibrosis both in NAFLD (Kozlitina et al., 2014; Y. L. Liu et al., 2014) and alcohol liver disease (Buch et al., 2015). Epigenetic changes in DNA methylation and expression of microRNA also overlap in NAFLD and alcohol liver disease (Ajmera, Terrault, & Harrison, 2017).

6.3.1.2 Smoking and fatty liver

In this thesis, smoking correlated independently to fatty liver in normal weight participants. Smoking´s effect on fatty liver is in line with other studies as most studies have shown that smoking increases the risk of fatty liver. In a large cohort study with 1,091 participants, smoking was associated with severe fibrosis (Zein et

al., 2011), and long-term smoking was significantly associated with the presence of advanced fibrosis, suggesting that cigarette smoking may accelerate disease progression (Zein et al., 2011). In a recent Dutch cross-sectional cohort study of 56 patients with fatty liver, patients with advanced fibrosis had more pack years than patients with no or early fibrosis (Munsterman et al., 2017). In a community-based cross-sectional survey of 8,580 Chinese participants, a positive association between heavy active smoking and fatty liver was observed (Y. Liu et al., 2013). In addition, tobacco smoking and BMI had a synergistic effect on fatty liver prevalence (Y. Liu et al., 2013). In a follow-up study over a 10-year period of 2,029 subjects, cigarette smoking was an independent risk factor for fatty liver, in addition to age, obesity, dyslipidemia, and the total number of metabolic syndrome risk factors (Hamabe et al., 2011). In obese rats, exposing to cigarette smoking increased the histological severity of fatty liver, favoring the development of nonalcoholic steatohepatitis by worsening the lobular inflammation and hepatocellular ballooning (Azzalini et al., 2010). The underlying mechanisms seem to be induction of hepatocellular apoptosis, oxidative stress and modulation of several signaling pathways (Azzalini et al., 2010). However, some studies have not seen the association between smoking and fatty liver. In a cross-sectional study of 933 Mexican subjects, there were no differences in fatty liver prevalence between current smokers and nonsmokers, and furthermore, no differences were observed in heavy smokers (more than 20 packs per year) (Chavez-Tapia, Lizardi-Cervera, Perez-Bautista, Ramos-Ostos, & Uribe, 2006).

Smoking may trigger the progression of fatty liver (Sinha-Hikim, Sinha-Hikim, & Friedman, 2017) due to inflammation, oxidative stress, and apoptosis (Buzzetti, Pinzani, & Tsochatzis, 2016; Mantena, King, Andringa, Eccleston, & Bailey, 2008; Trauner, Arrese, & Wagner, 2010). It promotes the production of reactive oxygen species, which enhances oxidative stress and lipid peroxidation due to impaired antioxidative action (Avti, Kumar, Pathak, Vaiphei, & Khanduja, 2006; Muriel, 2009). Yuan et al. (H. Yuan, Shyy, & Martins-Green, 2009) reported that passive cigarette smoking increases lipid accumulation in hepatocytes by modulating the activity of 5'-AMP-activated protein kinase and sterol response element binding protein-1. These changes of activity lead to accumulation of triglycerides in hepatocytes.

6.3.2 Childhood and Genetic Risk Factors for Adulthood Fatty Liver

In this study, low birth weight and high childhood BMI and insulin levels were associated with the risk of adulthood fatty liver. These results may help early identification of children with higher risk of fatty liver in adulthood and may help to concentrate early intervention for these children in child welfare clinics and school health care. However, early life exposure for example to elevated systolic blood pressure and low physical activity did not predict adult fatty liver. This emphasizes the role of childhood BMI as a modifiable risk factor for adult fatty liver.

High childhood insulin level was an independent predictor of adult fatty liver in this study. A potential explanation is that liver fat accumulation occurs when hyperinsulinemia and insulin resistance lead to hepatic accumulation of triglycerides (Petta et al., 2016). This process usually results from an imbalance between increased free fatty acid flux from adipose tissue to the liver, increased caloric intake, and increased lipogenesis in the liver and the liver's handling and export of the extra fat (Petta et al., 2016). The free fatty acids are usually either oxidized in the mitochondria or esterified to triglycerides, which in turn are either packaged as VLDL for export or are used to produce lipids (J. C. Cohen, Horton, & Hobbs, 2011).

In our study, low birth weight was an independent predictor of adult fatty liver. This is in line with a study of 2,003 Finnish adults, where a significant association between adulthood liver fat score and birth weight was seen in women (Sandboge et al., 2013). One explanation is the shared genetic effects with early growth phenotypes and adult cardiometabolic disease (Horikoshi et al., 2016). In a genome-wide association study with 153,781 individuals, 60 loci were identified where fetal genotype was associated with birth weight (Horikoshi et al., 2016). Strong inverse genetic correlations were found between birth weight and systolic blood pressure and coronary artery disease, and the study demonstrated that genetic factors were the major contributor to the negative covariance between birth weight and future cardiometabolic risk (Horikoshi et al., 2016). Another possible explanation of the association between low birth weight and adult fatty liver is weight catch-up growth (Breij, Kerkhof, & Hokken-Koelega, 2014; Faienza et al., 2013). Catch-up growth is recognized as a risk factor for later development of insulin-related complications and chronic diseases like abdominal obesity, type 2 diabetes and cardiovascular disease (Dulloo, 2006). In a longitudinal study of 51 Spanish children, small for gestational age children gained progressively more body fat and abdominal fat mass than appropriate for gestational age children between ages 2 and 4 years (Ibanez, Ong, Dunger, & de Zegher, 2006). These

differences occurred despite the small for gestational age children having already completed their catch-up growth and weight gain by the age of 2 years (Ibanez et al., 2006). In this study low birth weight was associated with adult fatty liver even when adjusted with childhood BMI and insulin levels, suggesting that this association cannot be simply explained by catch-up growth.

In our population, *PNPLA3* and *TM6SF2* were independently associated with fatty liver. Furthermore, prediction of adulthood fatty liver was significantly improved using single nucleotide polymorphisms in the *PNPLA3* and *TM6SF2* genes compared with prediction models consisting of only age, sex, and childhood BMI and insulin levels. These genetic variants in *PNPLA3* and *TM6SF2* genes are generally recognized to be involved in determining the risk of fatty liver (Dongiovanni et al., 2015; Eslam et al., 2018; Marzuillo, Del Giudice, & Santoro, 2014). In *PNPLA3* gene, rs738409 is a C/G single nucleotide variation on human chromosome 22. A cytosine to guanine DNA substitution (rs738409) encodes an isoleucine to methionine loss-of-function substitution at the amino acidic residue 148 of the *PNPLA3* protein (Pingitore et al., 2014). Mutated *PNPLA3* variant is attached on the surface of lipid droplets reducing triglyceride breakdown leading to lipid retention in the hepatocyte lipid droplet (Dongiovanni et al., 2013; Dongiovanni et al., 2015). Alternatively, it may stimulate the hepatic triglycerides synthesis (B. Donati et al., 2016; Pirazzi et al., 2012). I148M *PNPLA3* reduces hepatic VLDL secretion, possibly by reducing the mobilization of triglycerides stored in lipid droplets (Pirazzi et al., 2012). The variant increases the risk of fatty liver but is not associated with increased risk of type 2 diabetes or cardiovascular disease (Yki-Järvinen, 2014). However, as the carriers of the variant allele are at a high risk of developing advanced liver disease, they may be an important target group for lifestyle intervention (Yki-Järvinen & Luukkonen, 2015). The low-frequency rs58542926 polymorphism of *TM6SF2* encodes the loss-of-function E167K variant that has been linked with fatty liver and lower serum lipoproteins (Dongiovanni et al., 2015). The mechanism is related to reduced secretion of VLDL resulting in intrahepatic retention of triglycerides (Dongiovanni et al., 2015).

These results highlight the need to reduce overweight and insulin resistance early in life in order to prevent later fatty liver. This study also highlights the important role of non-modifiable factors such as male sex, *PNPLA3* and *TM6SF2* variants and birth weight. NAFLD is a progressive disease with substantial interpatient variation. This variation can be at least partially attributed to differences in genetic variants (Eslam et al., 2018). It is suggested that approximately half of hepatic fat

content variability is explained by genetic factors (Eslam et al., 2018). Genotyping might be a useful way to target lifestyle and diet interventions to the population at risk of fatty liver. Genotyping of *PNPLA3* and *TM6SF2* is recommended for selected patients and clinical studies in the European Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease by the European Association for the Study of the Liver, European Association for the Study of Diabetes and European Association for the Study of Obesity; however, it is not recommended routinely (European Association for the Study of the Liver (EASL), European Association for the Study of Diabetes (EASD), & European Association for the Study of Obesity (EASO), 2016). Genotyping might possibly be useful for example among preterm infants to identify a higher risk of fatty liver. Major genetic variants affecting fatty liver, *PNPLA3* and *TM6SF2*, would possibly lead to the development of new drug targets and individualization and improvement the quality of care for patients (Eslam et al., 2018). Besides that clinically useful gene-based risk scores might perhaps progress (Eslam et al., 2018).

6.3.3 Fatty Liver and Serum Metabolomics

In our study, several circulating biomarkers were associated with a fatty liver and with a future risk of fatty liver. Metabolic biomarkers for the risk of fatty liver were observed among lipids and lipoprotein subclasses, fatty acid composition, and several amino acids and glycolysis-related metabolites. The metabolic biomarkers were associated with fatty liver diagnosed 10 years later. This suggests that the diverse alterations in the metabolome can already be seen before a fatty liver diagnosis is made.

The strongest individual biomarkers for fatty liver and its future risk were VLDL particles and the related triglyceride concentrations. These results are in line with several reviews (J. C. Cohen et al., 2011; Fon Tacer & Rozman, 2011; Haas, Francque, & Staels, 2016). In a study of dyslipidemic patients, elevated triglycerides were those most strongly associated with fatty liver (Assy et al., 2000). Hepatic triglyceride accumulation has an important role in the pathogenesis of fatty liver (J. C. Cohen et al., 2011). Thus, our study´s findings of elevated levels of circulating triglycerides are in line with this. Low large HDL particle concentration and low HDL size also had strong associations with fatty liver and furthermore small LDL particle size was strongly predictive of fatty liver. In the study of Speliotes et al., fatty liver associated dyslipidemia was characterized by

increased plasma concentrations of VLDL triglycerides and decreased HDL cholesterol (Speliotes et al., 2010).

Insulin resistance is associated with fatty liver, and in insulin resistance the secretion of VLDL particles is increased. Insulin resistance leads to increased lipolysis within white adipose tissue, with increased delivery of free fatty acids to the liver and increased expression of hepatic fatty acid transport proteins (Kawano & Cohen, 2013). At the same time, hyperinsulinemia leads to increased lipogenesis (Choi & Ginsberg, 2011; Kawano & Cohen, 2013; Xu, So, Park, & Lee, 2013) and, furthermore, hyperglycemia leads to lipogenesis by the activation of a carbohydrate response element binding protein (Xu et al., 2013). Thus, the net effects of these changes increase the supply of hepatic triglycerides due to a greater uptake of plasma free fatty acids and to higher rates of de novo fatty acid synthesis (Choi & Ginsberg, 2011; Kawano & Cohen, 2013). However, the number of VLDL particles cannot increase sufficiently to ease the triglyceride accumulation in the liver and prevent the development of fatty liver (D. E. Cohen & Fisher, 2013).

In this study, serum fatty acids composition was strongly associated with the risk of fatty liver. These same fatty acids are also associated with type 2 diabetes (Mahendran et al., 2013) and cardiovascular disease (Wurtz et al., 2015). An increased monounsaturated fatty acid proportion was associated with the risk of fatty liver. These results are in line with other studies (Petit et al., 2012; Puri et al., 2009). The association between fatty acids and fatty liver were similar in the cross-sectional and prospective analyses. One possible explanation between the association of fatty acids and fatty liver is the fatty acids effect on gene expression: long-chain polyunsaturated fatty acids can affect signaling molecules that inhibit lipogenesis and increase beta-oxidation (Nakamura, Cheon, Li, & Nara, 2004).

In this thesis, circulating levels of isoleucine, leucine, valine, phenylalanine, tyrosine and alanine were strongly associated with the presence of fatty liver and also its future risk. This is in line with previous cross-sectional studies (Cheng et al., 2015; Lake et al., 2015; Sunny et al., 2015). Differing levels of these amino acids are thought to be due to dysfunctional mitochondrial energy metabolism, adiposity, and insulin resistance, and are associated with the risk of the development of type 2 diabetes (Stancakova et al., 2012; Sunny et al., 2015; T. J. Wang et al., 2011; Wurtz et al., 2012; Wurtz et al., 2013; Wurtz et al., 2014). Metabolism and transport gene expression changes associated with amino acids and branched chain amino acids including leucine, isoleucine and valine have important roles in sustaining a healthy liver. These roles range from cancer stem cell suppression to inhibition of reactive oxygen species (Lake et al., 2015).

Branched chain amino acid supplementation is also reported to improve insulin resistance and can promote liver regeneration (Miyake et al., 2012; Nagao, Kawaguchi, Ide, & Sata, 2012; Tajiri & Shimizu, 2013; Yoshida et al., 2012). In a study of 45 human liver samples, significant changes in hepatic branched chain amino acid composition were revealed during the progression of NAFLD (Lake et al., 2015). Significant elevations of hepatic branched chain amino acids in nonalcoholic steatohepatitis demonstrate the disorder of systemic amino acid homeostasis that occurs in NAFLD patients. The association between elevated branched chain amino acid levels and fatty liver might possibly be explained by NAFLD associated insulin resistance which is associated with the progressive inability of insulin to suppress plasma branched chain amino acids, which in turn results in their elevated plasma levels (Sunny et al., 2015).

In this thesis, glutamine and glycine levels were inversely associated with present fatty liver. In previous studies, glutamine levels were inversely associated with insulin resistance and diabetes risk (Cheng et al., 2012; Wurtz et al., 2013). Glycine is a non-toxic amino acid and needed for detoxification processes (Bilzer et al., 2002; Weinberg, Davis, Abarzua, & Rajan, 1987; Weinberg, Bienholz, & Venkatachalam, 2016). Via a glycine-gated chloride-channel, which leads to decreased calcium-inflow and subsequent inhibition of Kupffer cell activation, glycine offers reliable protection from hepatic ischemia and improved liver function (Ikejima, Qu, Stachlewitz, & Thurman, 1997). Glycine has been demonstrated to protect from ischemia in both in vitro and in vivo models by inhibition of Kupffer cell activation (Bruns et al., 2011; Ikejima et al., 1997; Schemmer et al., 2001).

Apolipoprotein A1 is the main apolipoprotein of HDL particles and apolipoprotein B is the main apolipoprotein in VLDL, intermediate-density lipoprotein (IDL), and LDL particles. In this thesis, apolipoprotein B was independently associated with fatty liver. Elevated non-HDL lipoprotein levels may lead to the accumulation of lipids within hepatocytes (Adeli, Taghibiglou, Van Iderstine, & Lewis, 2001; Nguyen et al., 2008)(White et al., 1998). In a prospective study of 128 participants, levels of non-HDL lipoprotein were significantly higher in participants with steatohepatitis than participants with fatty liver (Corey et al., 2012). Thus, in practice, apolipoprotein B would be useful to identify individuals with a higher risk to progressive liver disease. The higher levels of apolipoprotein B in fatty liver may partly be explained by insulin resistance related to fatty liver as insulin suppresses secretion of apolipoprotein B (Adeli et al., 2001; Sparks, Sparks, & Adeli, 2012). In this study, association between fatty liver and apolipoprotein B

was diluted but remained significant after adjustment with insulin. By contrast, levels of apolipoprotein A1 were inversely associated with fatty liver. Apolipoprotein A1 is the main component of HDL cholesterol particle, and it functions as a cofactor for the lecithin cholesterolacyltransferase enzyme which makes cholesterol transfer into HDL particles (Välimäki, Sane, & Dunkel, 2009). Low levels of apolipoprotein A1 are associated with a higher risk for coronary artery disease (Walldius & Jungner, 2004). In a study of 8,327 Korean participants, lower serum apolipoprotein A1 levels were associated with a higher prevalence of fatty liver in normal weight participants (Yang, Sung, & Gwak, 2016). The risk of fatty liver increased with increasing apolipoprotein B/A1 ratio in a study of 27,033 Korean subjects (Choe et al., 2013).

In conclusion, serum metabolomics is predictive of the risk for fatty liver 10 years prior to its diagnosis, indicating that many aberrations in the systemic metabolic profile might precede the onset of fatty liver. These results may eventually make it possible to achieve early detection of fatty liver disease and subsequently to target preventive strategies based on the detailed metabolic profile of an individual. This wide range of serum metabolomics might be more stable than person's clinical characteristics and in that way might predict fatty liver better. Understanding serum metabolomics affecting fatty liver may also help to develop patient-specific therapies (Safaei et al., 2016).

Determinants associated with fatty liver in this thesis are shown in **Table 9**.

Table 9. Determinants of fatty liver in this thesis. Determinants with OR >1.5 marked in bold.

Determinants of fatty liver						
Waist circumference	**Alanine aminotransferase**	**BMI**	**Amino acids**	**Very low-density lipoprotein**	**Male sex**	**Serum fasting triglycerides**
Variant in *PNPLA3*	**Variant in *TM6SF2***	**Serum fatty acids**	Glycolysis-related metabolites	Apolipoprotein B	Systolic blood pressure	Childhood BMI
Alcohol consumption	Childhood insulin	Insulin	Low-density lipoprotein	low HDL	Low physical activity index	Low birth weight

6.4 Strengths and limitations

An important strength of this study is the large, randomly selected, and carefully phenotyped cohort of young men and women prospectively followed up for up to 31 years since early childhood. Extensive data were available on several possible childhood physical, environmental, and genetic determinants of fatty liver that could be comprehensively considered in multivariable models. Thus, an important strength of this study is the population-based design of the Cardiovascular Risk in Young Finns Study, and due this there was a sufficient number of participants in the statistical analysis. In addition, all the methods used including laboratory, physical and ultrasound examinations were properly established. However, a common limitation in longitudinal studies is non-participation at a follow-up. The representativeness of the remaining cohort has been examined several times. Early in the study, the participants who were lost-to-follow-up tended to be older, more often male and more often smokers (Porkka et al., 1997). A detailed analysis of the lost-to-follow-up participants was done in 2001. The participation has been dynamic: over half of the subjects lost-to-follow-up early in the study have returned to the study later (Juonala et al., 2004). When the representativeness of the participants in 2001 was tested by comparing their baseline (1980) characteristics to the baseline characteristics of the lost-to-follow-up participants, it was found that the participants were more often women and older than the lost-to-follow-up participants (Juonala et al., 2004). Otherwise, comparing lost-to-follow-up participants and participants using age-adjusted analysis found no significant differences in either men or women in total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, blood pressure, BMI or parents' study years. In addition, there was no difference in physical activity between participants and dropouts. In the 2011 follow-up, participants were older and more often women than the non-participants. Therefore, the rates of adult fatty liver in our cohort might be an underestimation of the actual rates. In a Finnish study of 1,587 participants with a mean age of 62 years and a liver fat assessed with a liver fat score, the prevalence of fatty liver was 43% in men and 23% in women (Sandboge et al., 2013). In another Finnish study, the prevalence of liver enzyme levels assessed NAFLD was 21% in 2,766 participants aged 45-74 years (Kotronen et al., 2010). Nevertheless, because the baseline risk factors levels were mostly similar among participants and non-participants and the study group has been dynamic, the present study population was thought to be quite well representative of the original population.

Because our study cohort was a homogeneous ethnic group, the generalizability of our results is limited to Caucasians.

Ultrasound imaging has its limitations for diagnosing fatty liver: while the test shows high specificity, it has low sensitivity (AlShaalan, Aljiffry, Al-Busafi, Metrakos, & Hassanain, 2015). It has been estimated that conventional ultrasound imaging can only detect steatosis when more than 30% of the liver is affected (Saadeh et al., 2002). However, a large meta-analysis (Hernaez et al., 2011) concluded that ultrasonography allows for reliable and accurate detection of moderate-severe fatty liver, compared to histology. Liver ultrasound is an operator-dependent modality with varying results between operators. In the meta-analysis by Hernaez et al., the range of kappa values for intra-rater evaluation was 0.54– 0.92 (Hernaez et al., 2011). In the Cardiovascular Risk in Young Finns Study, all ultrasound images were graded by one trained operator who was masked as regards to the participant's clinical characteristics. Because ultrasound imaging is non-invasive, widely accessible, and cost-effective, it is a reasonable choice for population-based studies into the etiogenesis of fatty liver (Hernaez et al., 2011). As a conclusion, ultrasound imaging is safe, user-friendly and cheap, but does not recognize mild steatosis.

By using data collected from national hospital discharge registries it could be verified that none of the participants had viral or autoimmune causes of fatty liver. Furthermore, all results remained similar after excluding the participants with a history of cancer (N = 4) and psychotic disorders (N = 3), who may potentially have had medications influencing liver fat metabolism. It is also a strength that the fatty liver risk alleles included in the study are important determinants of liver fat content (Anstee & Day, 2013; Kozlitina et al., 2014; Leon-Mimila et al., 2015; Sookoian & Pirola, 2011).

NMR spectroscopy was used to detected serum metabolites. This is cost-effective and safe, and therefore suitable for population-based studies. However, its sensitivity is low in comparison to mass spectrometry-based approaches (Brennan, 2014). Baseline metabolic biomarkers were measured in 2001 and ultrasound imaging of participants' livers was done in 2011. This prevented formal analyses of fatty liver incidence. However, the young age group, long follow-up, and exclusion of individuals with high baseline alanine aminotransferase are all indicators of the minimal prevalence of fatty liver at baseline. A further strength is the consistent findings from detailed metabolic profiling at three time points during the 10 years of follow-up.

The lack of replication in an independent cohort is a limitation. However, in the metabolic data, the consistent results of the prospective analyses at two different time points enhance the validity of the biomarker findings. Additionally, we had no information on body composition of the participants, so there might be individuals with an athletic or muscular body in the group of overweight or obese participants.

6.5 Clinical implications

In total, 19 % of the participants of the Cardiovascular Risk in Young Finns Study showed evidence of fatty liver by ultrasound. Waist circumference, BMI, male sex, systolic blood pressure, alanine aminotransferase, apolipoprotein B, insulin and alcohol consumption were independently associated with fatty liver. A high physical activity index gave protection from fatty liver. Fatty liver may have quite dissimilar determinants in normal weight and overweight individuals, with potentially useful clinical implications: a finding of increased liver fat in a normal weight individual might indicate a lifestyle characterized by substantial alcohol intake and cigarette smoking, whereas a finding of fatty liver in an overweight or obese individual may indicate the presence of a metabolic syndrome. In addition, the presence of these risk factors may help to discover people who are at risk of fatty liver.

The findings of this thesis suggest that a multifactorial approach if implemented would improve early identification of children with a high risk of adult fatty liver. It would be effective to target follow-ups and instructions to those people with a high future risk for this disease. Low birth weight, the SNP rs738409 in the *PNPLA3* gene, the SNP rs58542926 in the *TM6SF2* gene and insulin and BMI levels measured in childhood were independently related to fatty liver detected 31 years later in adulthood. These results highlight the need to already reduce overweight and insulin resistance early in life to prevent later development of fatty liver. Genotyping might be a useful way to target lifestyle and diet interventions to the population at risk of fatty liver. Major genetic variants affecting fatty liver might possibly lead to the development of new drug targets and individualization and improvement the quality of care for patients. Besides that clinically useful gene-based risk scores might progress.

Circulating biomarkers from multiple metabolic pathways are strongly reflective of the presence of fatty liver in young adults both in cross-sectional and prospective settings. These results may eventually make it possible to achieve early detection

of fatty liver disease and subsequently to target preventive strategies based on the detailed metabolic profile of an individual. Person´s metabolomics might be more stable than single clinical sample, e.g. serum fasting insulin, and therefore describe person´s fatty liver risk better. Understanding serum metabolomics may also help to develop patient-specific therapies for fatty liver.

6.6 Future research directions

6.6.1 Ongoing monitoring of fatty liver among Finnish adults

Results from this thesis suggest that about one fifth of the Finnish population has fatty liver. Because fatty liver can be a progressive disease with severe outcomes, these findings highlight the need for ongoing monitoring and intervention as regards the fatty liver risk factor levels among Finnish adults. Because fatty liver was assessed by ultrasound, we were unable to clarify the prevalence of steatohepatitis in the population. In the future, this would be of interest. Studying associations between fatty liver risk factors and endpoints of fatty liver disease was not possible due to the young age of the study participants. As the participants become older, research on the effects of risk factors and fatty liver disease severity will be possible. Additionally, it would be of interest to test whether the susceptibility to fatty liver is hereditary from parent to child.

6.6.2 Genetics of fatty liver

In this thesis, the SNP rs738409 in the *PNPLA3* gene and the SNP rs58542926 in the *TM6SF2* were independently related to fatty liver. Furthermore, genetic variants in *PNPLA3* and *TM6SF2* enhanced ability to predict adult fatty liver. However, more research is needed to explore if genetic analysis has potential in clinical and research settings as well as in the fatty liver risk assessment.

6.6.3 Metabolomics

Results from this thesis suggest that multiple circulating metabolites are associated with ultrasound assessed fatty liver. Further studies are needed to elucidate the biological mechanisms underlying the associations with fatty liver, and to clarify the clinical utility of these biomarkers to identify individuals with a high risk of fatty liver.

7 Summary and conclusions

Firstly, the prevalence of fatty liver was 19% in the Cardiovascular Risk in Young Finns Study population of young and middle-aged adults (**Study I**). Fatty liver was cross-sectionally independently associated with waist circumference, BMI, alanine aminotransferase, male sex, apolipoprotein B, elevated systolic blood pressure, alcohol intake and insulin. Waist circumference and BMI were strong determinants, and fatty liver was detected in nearly one-third of overweight or obese individuals. However, fatty liver also occurred in 5% of the normal weight individuals. In normal weight individuals, fatty liver may indicate an unhealthy lifestyle, whereas a finding of fatty liver in an overweight or obese individual may indicate the presence of metabolic syndrome.

Secondly, low birth weight, the SNP rs738409 in the *PNPLA3* gene, the SNP rs58542926 in the *TM6SF2* gene and insulin and BMI levels measured in childhood were independently related to fatty liver that was detected 31 years later in adulthood (**Study II**). Including information on birth weight, rs738409 and rs58542926 in addition to childhood BMI and insulin levels significantly improved the ability of the statistical model to predict adult fatty liver. These findings suggest that a multifactorial approach would improve early identification of children with a high risk of adult fatty liver.

Thirdly, circulating biomarkers from multiple metabolic pathways are strongly reflective of the presence of fatty liver in young adults (**Study III**). The metabolic biomarkers are also predictive of the risk of fatty liver 10 years prior to its diagnosis, indicating that seemingly many aberrations in the systemic metabolic profile precede the onset of fatty liver.

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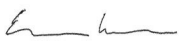

Emmi Lyyra

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