Aki Käräjämäki

NON-ALCOHOLIC FATTY LIVER DISEASE (NAFLD) – PERSPECTIVES TO ETIOLOGY, COMPLICATIONS AND LIPID METABOLISM
AKI KÄRÄJÄMÄKI

NON-ALCOHOLIC FATTY LIVER DISEASE (NAFLD) – PERSPECTIVES TO ETIOLOGY, COMPLICATIONS AND LIPID METABOLISM

Academic dissertation to be presented with the assent of the Doctoral Training Committee of Health and Biosciences of the University of Oulu for public defence in Auditorium 10 of Oulu University Hospital, on 8 December 2017, at 12 noon

UNIVERSITY OF OULU, OULU 2017
Abstract

Obesity, insulin resistance, type 2 diabetes (T2D) and non-alcoholic fatty liver disease (NAFLD) form a dangerous quartet which threatens human health all over the world. About 25% of adults around the world have NAFLD, which poses risks for cardiovascular and metabolic well-being and may develop into liver cirrhosis and hepatocellular carcinoma. Apart from lifestyle modification, treatment options for NAFLD are scarce.

This thesis presents atrial fibrillation (AF) as a new complication of NAFLD among general population of 958 individuals aged 40-60 years participating in the OPERA study. Even after multiple-adjustments for confounding factors, ultrasound-based NAFLD predicted the development of AF during about 16 years of follow-up. Moreover, the association between AF and liver fibrosis in 76 individuals aged 64-82 years in a cross-sectional setting is presented.

The thesis also shows that individuals with metabolic syndrome (MetS), with or without NAFLD, are at increased risk of cardiovascular events, T2D and the increase of left ventricular mass index in a study population of 958 individuals aged 40-60 years during a 20-year follow-up. In other words, NAFLD without MetS does not seem to expose to these three cardiometabolic complications.

The thesis also shows that rifampicin-activated pregnane X receptor (PXR), a member of the nuclear receptor superfamily of ligand-activated transcription factors with several endobiotic and xenobiotic activators, increases serum levels of cholesterol, phospholipids and certain fatty acids, assessed by nuclear magnetic resonance metabolomics technique, in a randomized, open, placebo-controlled trial among 34 young and healthy individuals. These serum lipids are considered toxic lipids and capable of transforming hepatosteatosis into steatohepatitis and even more severe forms of NAFLD. Moreover, rifampicin-activated PXR has no effect on serum triglycerides, that are non-toxic lipids, or triglyceride accumulation in the liver, assessed by magnetic resonance imaging, in 15 young and healthy individuals.

In conclusion, this thesis advances the knowledge in the pathogenesis, lipid metabolism, complications and heterogeneous nature of NAFLD. These may have implications for patient care and follow-up.

Keywords: atrial fibrillation, cardiovascular diseases, liver fibrosis, non-alcoholic fatty liver disease, pregnane X receptor, type 2 diabetes
Käräjämäki, Aki, Alkoholin käyttöön liittymättömän rasvamaksan syyt, seuraukset ja rasva-aineenvaihdunta.
Oulun yliopiston tutkijakoulu; Oulun yliopisto, Lääketieteellinen tiedekunta; Medical Research Center; Oulun yliopistolainen sairaala

Acta Univ. Oul. D 1438, 2017
Oulun yliopisto, PL 8000, 90014 Oulun yliopisto

Tiivistelmä

Maailmanlaajuisesti noin 25% täysi-ikäisistä sairastaa alkoholinkäyttöön liittymättömän rasvamaksaa. Sen tiedetään altistavan sydän- ja verisuonisairauksille, aineenvaihduntahäiriöille, maksakirroosille ja jopa maksasyövälle, mutta elämäntapahoidon toa lukuun ottamatta hoitomahdollisuudet ovat toistaiseksi vähäisiä.

Tässä väitöskirjassa osoitetaan ensimmäistä kertaa alkoholinkäyttöön liittymättömän rasvamaksan ennustavan itsenäisesti eteisvärynä ilmaantuvuutta noin 16 vuoden seurannan aikana 958 tavallisen keski-ikäisen ihmisen aineistossa osana OPERA-tutkimusta. Lisäksi väitöskirjassa osoitetaan maksan sidekudosmuodostuksen ja eteisvärinä välillä olevan yhteys poikkileikkausastelmassa 76 iäkkään ihmisen muodostamassa aineistossa.

Väitöstutkimuksessa havaittiin myös, että metabolista oireyhtymää sairastavilla henkilöillä on suurentunut tyypin 2 diabeteksen, sydän- ja verisuonisairauksien sekä vasemman kammion koon suurentumisen riski noin 20 vuoden seurannan aikana 958 tutkittavan henkilön aineistossa riippumatta siitä, onko heillä alkoholinkäyttöön liittymättömän rasvamaksaa. Toisin sanoen alkoholin käyttöön liittymätön rasvamaksan ilman metabolista oireyhtymää ei lisää edellä mainittujen kolmen sairauden riskiä.

Väitöstutkimuksessa esitetään lisäksi, että rifampisiinilla aikaansaatu maksan pregnane D -reseptorin aktivaatio johtaa seerumin fosfolipidien, tiettyjen rasvahappojen sekä usean eri kolesterolityypin lisääntymiseen 34 terveen nuoren henkilön aineistossa. Kirjallisuudessa näiden seerumien rasva-aineiden on esitetty aiheuttavan alkoholin käyttöön liittymätön maksatulehdusta ja jopa rasvamaksan vakavimpia muotoja. Toisaalta rifampisiin ei lisännyt seerumien triglyseridipitoisuutta eikä aiheuttanut magneettitutkimuksella mitattuna triglyseridien kertymisää maksaa 15 terveen nuoren henkilön aineistossa.

Tämä väitöstutkimus antaa lisätietoa rasvamaksan kehittymisestä, rasva-aineenvaihdunnasta ja komplikaatioista sekä korostaa rasvamaksan monimuotoista luonnetta. Nämä löydökset saatavat parantaa rasvamaksaa sairastavien henkilöiden hoitoa ja seurantaa.

Asiasanat: alkoholin käyttöön liittymätön rasvamaksaa, eteisväriinä, maksafibroosi, pregnaaani X reseptori, sydän- ja verisuonisairaudet, tyypin 2 diabetes
To my family
Acknowledgements

I am deeply grateful to my principal supervisor, Professor Olavi Ukkola, MD, PhD, for taking me under his guidance. Your educational and supportive attitude encouraged me to take the first steps into the world of medical science. Likewise, I am deeply grateful to my second supervisor, Docent Janne Hukkanen, MD, PhD, for supporting me and teaching me scientific thinking. You have both made me see how fun it is to make discoveries. I feel privileged to have had such committed and easily approachable supervisors.

I also wish to thank warmly the official reviewers of this thesis, Professor Markus Juonala, MD, PhD, and Professor Timo Lakka, MD, PhD, for their fruitful and constructive comments, which improved the thesis and made it more fluent. I also want to express my gratitude to the chair of my follow-up group, Docent Tapani Ebeling, MD, PhD, and its members, Timo Lauri, MD, PhD, and Riitta-Liisa Vasunta, MD, PhD, for their support during this process.

Professor Antero Kesäniemi, MD, PhD, Professor Heikki Huikuri, MD, PhD, Professor Markku Savolainen, MD, PhD, and Docent Heikki Kauma, MD, PhD, are sincerely thanked for developing excellent research facilities. I also appreciate Professor Mika Ala-Korpela, MSc, PhD, for NMR support, statistician Risto Bloigu, MSc, for statistic help, Anna Vuolteenaho, MA, for expert revision of the language and Secretary Marita Koistinen for helping me with things I did not even know were things. Furthermore, I wish to thank all my co-authors, laboratory staff and research nurses at the Clinical Research Center, Oulu University Hospital.

I am thankful to all my colleagues working in the Abdominal Center of Oulu University Hospital and the Clinics of Internal Medicine of Vaasa Central Hospital. Thanks to you, I am excited to go to work (almost) every morning. In particular, I wish to thank Kaj Lahti, MD, for lightning the fire of enthusiasm towards internal medicine during my very early career, and Alpo Hirvioja, MD, for inflaming this fire later on and guiding me to the world of gastroenterology. I am also grateful to Olli-Pekka Koivurova, MD, for comprehensive support and for enabling me to combine family life, research and clinical practice during the most rushed years of my life. You have my full respect.

My parents are deeply thanked for their never-ending care and support. I hope I manage to be as good a parent as you have been. I also wish to thank my sisters and brothers-in-law for always being close – no matter how physically distant we sometimes are. My relatives and friends are also thanked. You are all league class.
Additionally, Taija Lahtinen, MD, PhD, is thanked for her expertise and for tips on how to proceed and prepare oneself for the public examination day.

Finally, I praise my colleague, best friend, wife and the mother of my children, Annemari. You have given me the really big things in my life. It is also your sacrifices, flexibility, support and intelligence to cope with several matters that, after all, have enabled my specializations and the accomplishment of this thesis. Furthermore, I want to thank our children, Onni, Aarre and Lilja. You have taught me that no matter how busy you think you are, there is always time for playing. Being your dad is the greatest title and duty of honor I can ever have.

This thesis was accomplished in the Abdominal Center of Oulu University Hospital, University of Oulu Graduate School and Medical Research Center of Oulu University Hospital. Funding for this thesis was provided by the Research Council for Health of the Academy of Finland, the Finnish Foundation for Cardiovascular Research, the Sigrid Juselius Foundation, Yrjö Jahnsson Foundation and the Medical Association of Vaasa. All these institutes are acknowledged.

Vaasa, 30. September 2017

Aki Kärälämäki
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1H-MRS</td>
<td>proton magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>AF</td>
<td>atrial fibrillation</td>
</tr>
<tr>
<td>ALT</td>
<td>alanine aminotransferase</td>
</tr>
<tr>
<td>ANCOVA</td>
<td>analysis of covariates</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AST</td>
<td>aspartate aminotransferase</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>CAP</td>
<td>controlled attenuation parameter</td>
</tr>
<tr>
<td>CHD</td>
<td>coronary heart disease</td>
</tr>
<tr>
<td>CYP</td>
<td>cytochrome P450</td>
</tr>
<tr>
<td>CT</td>
<td>computer tomography</td>
</tr>
<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
</tr>
<tr>
<td>ECG</td>
<td>electrocardiogram</td>
</tr>
<tr>
<td>FDR</td>
<td>false discovery rate</td>
</tr>
<tr>
<td>FFA</td>
<td>free fatty acid</td>
</tr>
<tr>
<td>FIB-4</td>
<td>Fibrosis 4 score</td>
</tr>
<tr>
<td>GGT</td>
<td>gamma-glutamyl transpeptidase</td>
</tr>
<tr>
<td>GWAS</td>
<td>genome-wide study of genetic variants associated with the disease</td>
</tr>
<tr>
<td>HCC</td>
<td>hepatocellular carcinoma</td>
</tr>
<tr>
<td>HDL</td>
<td>high-density lipoprotein</td>
</tr>
<tr>
<td>HR</td>
<td>hazard ratio</td>
</tr>
<tr>
<td>hs-CRP</td>
<td>high-sensitive C-reactive protein</td>
</tr>
<tr>
<td>IBD</td>
<td>inflammatory bowel disease</td>
</tr>
<tr>
<td>IDF</td>
<td>international diabetes federation</td>
</tr>
<tr>
<td>IDL</td>
<td>intermediate-density lipoprotein</td>
</tr>
<tr>
<td>LDL</td>
<td>low-density lipoprotein</td>
</tr>
<tr>
<td>LVMI</td>
<td>left ventricular mass index</td>
</tr>
<tr>
<td>LMWM</td>
<td>low-molecular weight molecules</td>
</tr>
<tr>
<td>MBOAT7</td>
<td>membrane bound O-acyltransferase domain containing 7</td>
</tr>
<tr>
<td>MetS</td>
<td>metabolic syndrome</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>NAFL</td>
<td>non-alcoholic fatty liver</td>
</tr>
<tr>
<td>NAFLD</td>
<td>non-alcoholic fatty liver disease</td>
</tr>
<tr>
<td>NAS</td>
<td>NASH Activity Score</td>
</tr>
<tr>
<td>NASH</td>
<td>non-alcoholic steatohepatitis</td>
</tr>
</tbody>
</table>
NFS  NAFLD fibrosis score  
NMR  nuclear magnetic resonance spectroscopy  
OGTT  oral glucose tolerance test  
OPERA  Oulu Project Elucidating the Risk of Atherosclerosis  
PCSSK9  protein convertase subtilisin/kexin type 9  
PDFF  proton density fat fraction  
PNPLA3  Patatin-like phospholipase domain-containing protein 3  
PXR  pregnane X receptor  
QUICKI  Quick index  
SREBP  sterol regulatory element binding protein  
T2D  type 2 diabetes  
TM6SF2  transmembrane 6 superfamily member 2  
TNF-α  tumor necrosis factor α  
VLDL  very-low-density lipoprotein
List of original articles

This thesis is based on the following publications, which are referred throughout the text by their Roman numerals:


## Contents

Abstract
Tiivistelmä
Acknowledgements 9
Abbreviations 11
List of original articles 13
1 Introduction 19
2 Review of the literature 21
   2.1 Definition, pathogenesis and spectrum of NAFLD .................................. 21
   2.2 Epidemiology of NAFLD ................................................................. 26
   2.3 Diagnosis of NAFLD ...................................................................... 27
      2.3.1 The histological diagnosis of NAFLD spectrum ....................... 27
      2.3.2 The noninvasive diagnosis of hepatosteatosis ......................... 31
      2.3.3 The non-invasive diagnosis of NASH ...................................... 33
      2.3.4 The non-invasive diagnosis of liver fibrosis in NAFLD .......... 34
   2.4 Lipid metabolism in NAFLD .......................................................... 38
      2.4.1 Introduction to lipid metabolism in NAFLD ......................... 38
      2.4.2 Lipid metabolism in obese/metabolic NAFLD ....................... 39
   2.5 Glucose metabolism in NAFLD ...................................................... 45
   2.6 Environmental and genetic risk factors of NAFLD ....................... 47
      2.6.1 Gut microbiota ....................................................................... 47
      2.6.2 Alcohol consumption ............................................................ 50
      2.6.3 Toxic compounds, drugs and NAFLD .................................... 51
      2.6.4 Genetic factors predisposing for NAFLD .............................. 56
   2.7 Hepatic complications of NAFLD .................................................. 62
   2.8 NAFLD, metabolic syndrome and type 2 diabetes ...................... 64
      2.8.1 Definitions of MetS and T2D .................................................. 64
      2.8.2 Association between NAFLD and MetS .............................. 66
      2.8.3 Association between NAFLD and T2D .............................. 67
   2.9 Cardiovascular complications of NAFLD ..................................... 68
      2.9.1 Association between NAFLD and AF .................................. 72
   2.10 NAFLD and other comorbidities ................................................. 75
   2.11 Management of NAFLD .............................................................. 75
      2.11.1 Lifestyle modification ......................................................... 75
      2.11.2 Pharmacotherapy ............................................................... 78
2.11.3 Potential future drugs ............................................... 80
2.11.4 Bariatric surgery ...................................................... 81
2.11.5 Liver transplantation .................................................. 82

3 Aims of the study .............................................................. 83

4 Methods ........................................................................ 85
4.1 Study participants .......................................................... 85
   4.1.1 OPERA cohort (Studies I-III) ...................................... 85
   4.1.2 Rifa-Stea (Study IV) ................................................ 86
   4.1.3 Rifa-BP and Rifa-1 (Study IV) ..................................... 87
4.2 Clinical and radiological methods .................................. 87
   4.2.1 Assessment of hepatosteatosis .................................. 87
   4.2.2 Assessment of liver stiffness .................................... 87
   4.2.3 Echocardiographic assessment of the heart ............... 88
   4.2.4 Ascertainment of outcome events ............................. 88
4.3 Laboratory and other methods ....................................... 90
   4.3.1 Genotyping of the I148M polymorphism of the PNPLA3 gene .................................................. 91
   4.3.2 Proton nuclear magnetic resonance spectroscopy .......... 91
   4.3.3 Other methods ......................................................... 92
4.4 Statistical methods ........................................................ 92
4.5 Ethical considerations .................................................... 93

5 Results ............................................................................ 95
5.1 NAFLD with and without MetS as a predictor of cardiovascular diseases, type 2 diabetes and increase of left ventricular mass (Study II) ....................................................... 95
5.2 Non-alcoholic fatty liver disease and atrial fibrillation ............ 97
   5.2.1 NAFLD as a predictor of atrial fibrillation in middle-aged population (Study I) ................................................. 97
   5.2.2 The association of atrial fibrillation and liver stiffness (Study III) ................................................................. 100
5.3 The effect of Pregnane X receptor on hepatic fat accumulation and lipid metabolism (Study IV) .............................................. 101

6 Discussion ....................................................................... 103
6.1 Methods ........................................................................ 103
   6.1.1 Study participants and study designs ......................... 103
   6.1.2 Clinical, radiological and laboratory methods ............. 105
   6.1.3 Statistical methods ................................................... 107
6.2 NAFLD with and without MetS as a predictor of cardiovascular diseases, type 2 diabetes and increase of left ventricular mass
(Study II) .................................................................................................................. 107
6.3 Non-alcoholic fatty liver disease and atrial fibrillation .................. 111
  6.3.1 Non-alcoholic fatty liver disease as a predictor of atrial fibrillation in middle-aged population (OPERA Study)
    (Study I) ............................................................................................................. 111
  6.3.2 The association of atrial fibrillation and liver stiffness
    (Study III) ............................................................................................................ 113
6.4 The effect of Pregnane X receptor on hepatic fat accumulation and lipid metabolism (Study IV) ........................................ 115
6.5 Future perspectives .......................................................................................... 119

7 Conclusions ........................................................................................................... 121
8 References ............................................................................................................ 123
9 Original articles ..................................................................................................... 173
1 Introduction

Non-alcoholic fatty liver disease (NAFLD) is present if at least 5% of liver weight is fat without excess alcohol consumption or secondary causes of fat accumulation in the background (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). Approximately 25% of adults around the world have NAFLD (Rinella & Charlton, 2016; Younossi, Koenig et al., 2016) and the prevalence is still increasing (Seyda Seydel et al., 2016; Younossi, Koenig et al., 2016). NAFLD parallels with its associates, obesity, metabolic syndrome (MetS) and type 2 diabetes (T2D) (Anstee, Targher, & Day, 2013; Seyda Seydel et al., 2016; Younossi, Koenig et al., 2016) and it entails a considerable risk for metabolic disorders (MetS, T2D, dyslipidemia) and cardiovascular diseases (CVDs) (Anstee et al., 2013; Byrne & Targher, 2015; European Association for the Study of the Liver (EASL) et al., 2016). Moreover, some patients with NAFLD will develop progressive NAFLD, a continuum which has cirrhosis and hepatocellular carcinoma (HCC) in the latter end (European Association for the Study of the Liver (EASL) et al., 2016). Thus, the total mortality is increased, mostly due to cardiovascular reasons (Angulo et al., 2015; Ekstedt et al., 2015). The increase is, however, strongly associated with progressive NAFLD forms and may not be expanded to all NAFLD patients (Angulo et al., 2015; Ekstedt et al., 2015; D. Kim, Kim, Kim, & Therneau, 2013). Additionally, the risk factors and risk profile of MetS and NAFLD are similar (Yki-Jarvinen, 2014), leading to lack of knowledge of the precise impact of each of these conditions on the mutual co-morbidity. The global disease burden and the costs of NAFLD are enormous. For instance, in the United States only the direct medical costs of NAFLD are about $100 billion ($1,600 per patient) annually. In Europe, the costs seem to be somewhat lower (Younossi et al., 2016).

NAFLD is a heterogeneous disease. A great majority of subjects with NAFLD will not have a shortened life time or any hepatic event (D. Kim et al., 2013; Rinella & Charlton, 2016). On the other hand, those with progressive NAFLD are at risk of increased hepatic, extra-hepatic and overall morbidity and mortality (Angulo et al., 2015; Ekstedt et al., 2015). In recent years, a growing body of evidence has shown that the risk, clinical picture, and the disease burden of NAFLD is modified by genetic and epigenetic variations, many life-style and environmental factors, inflammatory status, the well-being of the gut microbiota and hormonal balance (Buzzetti, Pinzani, & Tsochatzis, 2016; Petaja & Yki-Jarvinen, 2016; Yki-Jarvinen
& Luukkonen, 2015). To date, there are no good predicting factors for who will develop progressive NAFLD and are at risk of the increased health risks. In addition, the understanding of the pathophysiology of progressive NAFLD is far from complete limiting the possibilities for intervention. For instance, there are signs that different xenobiotic mechanisms, with pregnane X receptor (PXR), a ligand-activated nuclear receptor, in the frontline, may impact on the pathogenesis and progression of NAFLD (Arciello et al., 2013; Hakkola, Rysa, & Hukkanen, 2016; Naik, Belic, Zanger, & Rozman, 2013). However, these have been surprisingly little investigated.

Altogether, given the high prevalence and the enormous disease burden but the heterogeneous nature of the condition, there is an urgent need for profound understanding of the pathophysiology of progressive NAFLD, markers enabling individual follow-up and prognosis setting and further knowledge of the complications of NAFLD. Thus, this thesis aims to find new perspectives to etiological factors, complications and lipid metabolism of NAFLD by presenting three epidemiological studies based on the OPERA study and one randomized, open, placebo-controlled crossover trial on the effects of PXR agonism on hepatic triglyceride fat accumulation and serum metabolomic signature.
2 Review of the literature

2.1 Definition, pathogenesis and spectrum of NAFLD

Non-alcoholic fatty liver disease (NAFLD) is characterized by excessive fat accumulation on the liver as defined by the presence of steatosis > 5% of liver weight according to histological analysis or by a proton density fat fraction (PDFF) > 5.6% in proton magnetic resonance spectroscopy (1H-MRS) or magnetic resonance imaging (MRI) (European Association for the Study of the Liver (EASL) et al., 2016). Moreover, the definite diagnosis of NAFLD requires the exclusion of excess alcohol consumption (≥ 30g a day in men or ≥ 20g a day in women) (European Association for the Study of the Liver (EASL) et al., 2016; Nascimbeni et al., 2013). Notably, the methods used to exclude excess alcohol consumption (anamnesis, biochemical markers) are not fully reliable. Additionally, there is no threshold for harmful alcohol consumption as the alcohol-related hepatic injuries appear to increase uniformly with the amount of alcohol consumed (European Association for the Study of Liver, 2012; Nascimbeni et al., 2013). Thus, the diagnostic thresholds are more or less arbitrary (Nascimbeni et al., 2013). The secondary causes to NAFLD, such as hepatotoxic medical history during the past six months, viral hepatitis, hemochromatosis and chronic autoimmune liver diseases, also need to be excluded (Nascimbeni et al., 2013). Of pharmacological agents, methotrexate, glucocorticoids, isoniazid, amiodarone and tamoxifen are known inducers of fatty liver (Williamson, Price et al., 2011).

NAFLD is an umbrella term covering simple non-alcoholic fatty liver (NAFL), in which there is pure hepatosteatosis only (or steatosis with either mild inflammation or ballooning but not both), and non-alcoholic steatohepatitis (NASH). The co-existence of all these three histopathological features, i.e., steatosis, inflammation and ballooning (swollen hepatocytes), is required for NASH, which also covers the most progressive forms of NAFLD: fibrosis, cirrhosis and HCC (European Association for the Study of the Liver (EASL) et al., 2016). The spectrum of NAFLD is illustrated in Figure 1.
NAFLD progresses slowly (European Association for the Study of the Liver (EASL) et al., 2016). Traditionally, NAFL has been thought to be a benign disease, but an accumulating body of evidence has shown that NAFL may progress to NASH (Buzzetti et al., 2016; Calzadilla Bertot & Adams, 2016; European Association for the Study of the Liver (EASL) et al., 2016; Schuppan & Schattenberg, 2013). It is estimated that over time about 30–40% of subjects with NAFL and elevated liver enzymes will develop NASH and 40–50% of those with NASH will have fibrosis (Ekstedt et al., 2006); in all NAFLD subjects, these numbers are thought to be about 10–15% and 25%, respectively (Figure 1) (Schuppan & Schattenberg, 2013). NAFL may even leap directly to fibrosis (Pais et al., 2013), in which there may still exist mild inflammation but without other mandatory NASH criteria (Singh et al., 2015), or prior NASH may have been missed. In addition, NASH and even NAFL may progress to non-cirrhotic HCC (Buzzetti et al., 2016; Torres & Harrison, 2015) and, thus, cirrhosis is not a mandatory step in the malignant disease progression as was previously thought. Especially male diabetics are at risk of non-cirrhotic HCC (Calzadilla Bertot & Adams, 2016).

According to a meta-analysis by Singh et al., 34% of all NAFLD subjects show NAFLD progression, 43% have stable disease and in 22% of NAFLD subjects, the disease improves over time. Moreover, the rate of fibrosis progression in subjects with NASH is doubled in comparison to the rate in NAFL subjects (the mean annual
fibrosis progression rate is 0.07 in NAFL versus 0.14 in NASH, corresponding to an average progression by at least one fibrosis stage (from F0 up to F4) over 14.3 years in NAFL versus 7.1 years in NASH, respectively), although it should be noted that the variability in the rate is broad (Singh et al., 2015). Indeed, the rate of the disease progression is characteristically slow but very varying depending on, for example, age (reflecting the cumulative sum of metabolic exposures and longer disease duration), genetic variants, hormonal factors (males and postmenopausal women at risk of fibrosis), ethnicity (Asians and Hispanics at the greatest risk), presence of diabetes and obesity and the degree of steatosis (Calzadilla Bertot & Adams, 2016). The overall risk of cirrhosis in subjects with simple steatosis is under 4% in 20 years of follow-up whereas in NASH subjects it is 25% in nine years (Calzadilla Bertot & Adams, 2016).

The pathogenesis of simple steatosis and the consequences it has for the lipid metabolism are depicted in detail in chapter Lipid metabolism in NAFLD. Briefly, sedentary lifestyle accompanied by excess caloric intake leads to accumulating visceral adipose tissue, insulin resistance and the release of proinflammatory factors. These promote the lipolysis of free fatty acids (FFA) from visceral adipose tissue to the liver (Asrih & Jornayvaz, 2015). Simple hepatosteatosis is formed when hepatic FFA input is greater than output, both of which are altered in subjects with NAFLD (Fabbrini, Sullivan, & Klein, 2010; Musso, Gambino, & Cassader, 2009).

The progression of NAFL to NASH and fibrosis requires a complex and multifactorial interplay of genetic, epigenetic, environmental, inflammatory, adipose tissue-derived factors and intrinsic microbial factors (Baran & Akyuz, 2014; Buzzetti et al., 2016; Singh et al., 2015; Tilg & Moschen, 2010) as illustrated in Figure 2. The high levels of FFAs and many other lipid metabolites in a hepatocyte are lipotoxic. As a consequence, mitochondrial dysfunction with oxidative stress and production of reactive oxygen species and endoplasmic reticulum stress-associated mechanisms are activated. Moreover, the proinflammatory factors from visceral adipose tissue are present in the liver (Buzzetti et al., 2016; Cusi, 2009). The inflamed environment leads to chronic hepatic inflammation, which is further amplified by unfavorable genetic and epigenetic modifications and alterations in the gut flora (Buzzetti et al., 2016). The alterations in the gut flora are derived from an unbalanced diet (high fat, high sugar/fructose, low fiber, nutrient/vitamin deficiency), which causes gut microbiota dysbiosis, mild inflammation and alteration in gut barrier function leading to increased translocation of microbial components into splanchnic veins which takes part in the formation of NAFLD progression.
Once NASH and the chronic inflammation have developed, hepatocyte necrosis and apoptosis is promoted. Apoptotic bodies from the damaged hepatocytes can activate hepatic stellate cells and Kupffer cells, which drive the formation of liver fibrosis by inflammatory and fibrogenic responses. Thereby, transformation of hepatic stellate cells into myofibroblasts takes place (Czaja, 2014). This results in the accumulation of collagen, proteoglycans and glycoproteins and, thereby, changes in the extracellular matrix composition (Liang, Kisseleva, & Brenner, 2016; Sanchez-Valle, Chavez-Tapia, Uribe, & Mendez-Sanchez, 2012). Activated hepatic stellate cells also enhance the pro-inflammatory responses and the formation of the vicious circle between inflammation and the profibrotic processes (Czaja, 2014). The transition between hepatic stellate cells to myofibroblasts involves signaling pathways, which in NASH-driven fibrosis seems to be dominated by Hedgehog signaling (Czaja, 2014; S. L. Friedman, 2013).

This theory of the progression of NAFLD is called the multiple-hits theory and it has replaced the outdated two-hits theory (Buzzetti et al., 2016) and distinct hit theory (Yilmaz, 2012). However, it is known that the development of progressive NAFLD occurs over such a long time course that progressive histological follow-up is difficult. To date, no biochemical marker specific for NASH has been found that could point to the distinct pathophysiological routes.
Fig. 2. Multiple hit hypothesis for the development of NAFLD. Abbreviations: FFAs, free fatty acids; DNL, de novo lipogenesis; VLDL, very low density lipoproteins; CH, cholesterol; TNF-α, tumor necrosis factor alpha; IL-6, interleukin 6; TG, triglycerides; ROS, reactive oxygen species; ER, endoplasmic reticulum; UPR, unfolded protein response; LPS, lipopolysaccharide; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis. Dietary and environmental factors, together with obesity, lead to raised serum levels of fatty acids (FFAs) and cholesterol (CH), development of insulin resistance, adipocyte proliferation and dysfunction, and changes in the intestinal microbiome. Insulin resistance acts on adipose tissue worsening adipocyte dysfunction, and induces lipolysis and release of adipokines and proinflammatory cytokines such as TNF-α and IL-6, which also contribute to maintain the insulin resistance state. In the liver, insulin resistance amplifies DNL. The increased hepatic FFAs flux which derives from the above processes and from an altered activity of the gut microbiome leads to two different situations: synthesis and accumulation of triglycerides (TG) and ‘toxic’ levels of fatty acids, free cholesterol and other lipid metabolites which cause mitochondrial dysfunction with oxidative stress and production of ROS and endoplasmic reticulum (ER) stress with activation of UPR, all leading to hepatic inflammation. Also, small bowel permeability can be enhanced with consequent raised circulating levels of molecules which contribute to the activation of inflammasome and ER stress, such as LPS, and to the release of pro-inflammatory cytokines. Genetic factors or epigenetic modifications affect hepatocyte fat content, enzymatic processes and liver inflammatory environment, thus influencing the risk of progression to inflammation and fibrosis (NASH) or persistence in a stable stage of the disease (NAFLD) (Buzzetti, Pinzani, & Tsochatzis, 2016, published by permission of Elsevier).
2.2 Epidemiology of NAFLD

According to a meta-analysis of 86 studies with more than 8,500,000 adults from 22 countries in the years 1989–2015, the global prevalence of NAFLD is about 25% (Younossi, Koenig et al., 2016). By continent, the prevalence was 13.5% in Africa, 23.7% in Europe, 24.1% in North America, 27.4% in Asia, 30.5% in South America and 31.8% in the Middle East (Rinella & Charlton, 2016; Younossi, Koenig et al., 2016). In Finland, the prevalence of NAFLD is reported to be around 20% with male gender, physical inactivity, MetS and its components as risk factors (Kotronen et al., 2010; Suomela et al., 2015). Today, the annual population-based NAFLD incidence is thought to be around 3–5% (European Association for the Study of the Liver (EASL) et al., 2016). The pooled overall NASH prevalence estimate among biopsied NAFLD patients was 59%, and 7–30% among all NAFLD patients without indication for liver biopsy (European Association for the Study of the Liver (EASL) et al., 2016). The estimated population-based prevalence of NASH in the USA and Finland is about 3–5% (Rinella & Charlton, 2016; Seyda Seydel et al., 2016) while obesity, type 2 diabetes (T2D) and male gender are risk factors for NASH (Seyda Seydel et al., 2016). However, due to the requirement of histological analysis to confirm NASH, the true prevalence of NASH in different populations is unknown. It is also noteworthy that the progression of NAFLD is very slow and may take decades (Anstee et al., 2013; European Association for the Study of the Liver (EASL) et al., 2016). Thus, noting the mean age of the NAFLD subjects at the time of diagnosis, progression to cirrhosis would in many cases take well beyond the life expectancy (Younossi, Koenig et al., 2016). In NAFLD subjects, the estimates for the cirrhosis-related deaths over time are 1–4% (Byrne & Targher, 2015; Rinella & Charlton, 2016; Younossi, Koenig et al., 2016). HCC occurs at the frequency of 0.44/1,000 person-years (Younossi, Koenig et al., 2016).

The global prevalence of obesity among adult population is about 37% (Ekstedt et al., 2015). Obesity is a strong risk factor for NAFLD (Angulo et al., 2015; D. Kim et al., 2013; Yki-Jarvinen, 2014), but from the epidemiological point of view, it is not the only predictor of NAFLD as the pooled overall obesity prevalence estimates among NAFLD patients and among NASH patients were 51% and 82%, respectively (European Association for the Study of the Liver (EASL) et al., 2016). Indeed, in addition to obesity and excess caloric intake, genetic susceptibility, cultural phenomena and environmental factors such as dietary composition, physical exercise, environmental chemicals and intestinal microbiota are supposed to affect the NAFLD prevalence and severity (Younossi et al., 2016; Younossi, Koenig et al., 2016).
2016). Also MetS and separately, all components of MetS, parallel the prevalence of NAFLD and increase the risk of NASH, especially with advancing age (Petaja & Yki-Jarvinen, 2016; Yki-Jarvinen & Luukkonen, 2015). Noteworthy, about 70–90% of T2D patients have NAFLD, 20% have NASH, and 5–7% have advanced fibrosis (≥ F3 by the Kleiner classification, see chapter Diagnosis of NAFLD) (Buzzetti et al., 2016).

Due to the silent nature of the disease, the exact prevalence trends are not known. However, given the pandemics of obesity and T2D and the increasing proportional number of liver transplantations for the NAFLD/NASH subjects, the prevalence of NAFLD and NASH is thought to have increased during the recent decades and is still expected to grow (European Association for the Study of the Liver (EASL) et al., 2016; Petaja & Yki-Jarvinen, 2016).

2.3 Diagnosis of NAFLD

Early diagnosis and treatment of NAFL can prevent the development into more progressive NAFLD, such as NASH, liver fibrosis, cirrhosis and HCC (European Association for the Study of the Liver (EASL) et al., 2016; Farrell & Larter, 2006). While liver biopsy is the reference method to diagnose hepatic steatosis and its progressive stages, there are several non-invasive methods to use in the clinical practice (Chalasani et al., 2012; European Association for the Study of the Liver (EASL) et al., 2016).

2.3.1 The histological diagnosis of NAFLD spectrum

The histologic characterization of NAFLD spectrum by definition includes the description of hepatosteatosis and cell injury in addition to inflammation and fibrosis (Kleiner & Brunt, 2012). Hepatosteatosis is graded on its severity: normal (grade 0) < 5%, mild (grade 1) 5–33%, moderate (grade 2) 34–66% and severe (grade 3) > 66% (Kleiner et al., 2005; Petaja & Yki-Jarvinen, 2016). It should be noted, however, that these percentages are different than the percentages used in the qualification of steatosis by magnet resonance-based imaging modalities (Petaja & Yki-Jarvinen, 2016). As stated above, the NAFLD spectrum consists of two clinically and histologically different conditions with different prognosis: NAFL and NASH, the latter covering the more progressive forms including fibrosis, cirrhosis and HCC (European Association for the Study of the Liver (EASL) et al., 2016). In NAFL, there is pure steatosis only or steatosis with mild lobular/portal...
inflammation at maximum but without hepatocellular ballooning or steatosis with hepatocellular ballooning but without inflammation, whereas in NASH there is presence of steatosis, ballooning and lobular inflammation (European Association for the Study of the Liver (EASL) et al., 2016; Kleiner & Brunt, 2012). Ballooning predicts more progressive disease and is also associated with fibrosis (Caldwell et al., 2010). NASH is called early NASH if there is no fibrosis (F0) or mild fibrosis (F1), fibrotic NASH when significant fibrosis (≥ F2) or advanced fibrosis (≥ F3) is present, or cirrhotic NASH when cirrhotic-stage fibrosis (F4) exists, as originally classified by Kleiner (Kleiner et al., 2005). Other features that can be seen in NASH but are not necessary for the diagnosis are, for instance, megamitochondria, microvacuolar steatosis, portal inflammation, Mallory-Denk bodies, apoptotic bodies and polymorphonuclear infiltrates (European Association for the Study of the Liver (EASL) et al., 2016). The histological activity and severity of NAFLD and NASH can be graded with NASH Activity Score (NAS), which consists of 14 histological features, each giving scores, thus being an unweighted sum of steatosis, lobular inflammation and hepatocellular ballooning scores. Score < 3 correlates with non-NASH and score ≥ 5 correlates with NASH (Kleiner et al., 2005; Kleiner & Brunt, 2012). However, in the long-term follow-up NAS seems to have low prognostic value (Ekstedt et al., 2015). It has been criticized for the fact that steatosis has a disproportionate impact on the score. As a consequence, NAS does not adequately detect NASH and thus does not predict prognosis, either (Buzzetti et al., 2016). The Steatosis, Activity and Fibrotic score (Bedossa & FLIP Pathology Consortium, 2014) is an alternative NAFLD and NAS (European Association for the Study of the Liver (EASL) et al., 2016) although it does not seem to correlate with long-term prognosis either (Hagstrom et al., 2017). Fibrosis staging is based on the Kleiner classification, as explained above (European Association for the Study of the Liver (EASL) et al., 2016; Kleiner et al., 2005). This is discussed further in the chapter NAFLD and hepatic complications and NAFLD and cardiovascular morbidity.

Although histology is the golden standard in the diagnosis of NAFLD in its all forms and many other acute or chronic liver diseases, there are some weaknesses and limitations in this diagnostic method. First, the risk of major complications in percutaneous liver biopsy requiring hospital admission (i.e., hemorrhagic complications, pneumothorax, biliary peritonitis) is around 4–8% in blind biopsies and 0.5–2% in ultrasound-guided biopsy, of which about two-thirds are discovered within two hours and nearly all cases within 24 hours after the procedure (Piccinino, Sagnelli, Pasquale, & Giusti, 1986; Sporea, Popescu, & Sirli, 2008). The death rate
has been reported to be 0.009–0.11% with malignancy as a risk factor (McGill, Rakela, Zinsmeister, & Ott, 1990; Piccinino et al., 1986). Second, the size of the liver biopsy specimen which should contain at least 6–8 portal triads, represents about 1:50,000 of the total liver mass (Sporea et al., 2008); as a result, only local analysis of fat accumulation, inflammation, ballooning and fibrosis is available, none of which are always evenly distributed. Moreover, only semiquantitative grading of steatosis is possible (Arun, Jhala, Lazenby, Clements, & Abrams, 2007; Boursier & Cales, 2012). Third, the determination of the steatosis grade is highly subjective and dependent on each pathologist’s individual opinion (Boursier & Cales, 2012) and fourth, due to NAFLD and NASH being so common diseases, it is clear that not everybody with suspected NAFLD or NASH can undergo a liver biopsy. Figure 3 shows the diagnostic flow-chart to assess and monitor NAFLD by European liver, diabetes and obesity associations (European Association for the Study of the Liver (EASL) et al., 2016).
Fig. 3. Diagnostic flow-chart to assess and monitor disease severity in the presence of suspected NAFLD and metabolic risk factors. aSteatosis biomarkers: Fatty Liver Index, SteatoTest, NAFLD Fat score. bLiver tests: ALT AST, γ-glutamyltransferase (GGT). cAny increase in ALT, AST or γ-glutamyltransferase (GGT). dSerum fibrosis markers: NAFLD Fibrosis Score, FIB-4, Commercial tests (FibroTest, FibroMeter, ELF). eLow risk: indicative of no/mild fibrosis; Medium/high risk: indicative of significant fibrosis or cirrhosis (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, published by permission of Springer).
2.3.2 The noninvasive diagnosis of hepatosteatosis

Due to low cost and high availability without radiation exposure, ultrasound is commonly used as a first-line imaging method in the clinical practice. The increased liver-kidney contrast showing an echogenic (bright) liver is a widely accepted criterion to set the diagnosis of hepatosteatosis (Ballestri, Romagnoli, Nascimbeni, Francica, & Lonardo, 2015; Saverymuttu, Joseph, & Maxwell, 1986; Tobari, Hashimoto, Yatsuji, Torii, & Shiratori, 2009). The nature of ultrasound in the assessment of hepatosteatosis is more or less qualitative, but the use of hepatorenal sonographic index may achieve more quantitative assessment (Webb et al., 2009). However, the intraobserver and interobserver repeatability in grading the hepatosteatosis with ultrasound is shown to be highly limited (Cengiz, Senturk, Cetin, Bayrak, & Bilek, 2014). According to a large meta-analysis, the sensitivity and specificity of ultrasound to detect hepatosteatosis depending on the grade of steatosis is 73.3–95.5% and 69.6–85.2%, respectively (Bohte, van Werven, Bipat, & Stoker, 2011). Thus, the drawback with ultrasound is the relatively low sensitivity, especially when steatosis less than 20% is present or with individuals with very high body mass index (BMI, >40 kg/m²) (European Association for the Study of the Liver (EASL) et al., 2016). Magnetic resonance imaging (MRI) and ¹H-MRS are the best non-invasive tools to diagnose hepatosteatosis. According to the meta-analysis of 46 studies, the sensitivity and specificity of MRI, depending on the grade of steatosis, was 82.0–97.4% and 76.1–95.3%, respectively (Bohte et al., 2011). Unlike ultrasound and computed tomography (CT), MRI measures the quantity of steatosis directly by differentiating protons in fat from those in water. This enables the accurate quantification of hepatic steatosis in an objective. Thereby, the intra- and interobserver repeatability is considered excellent (Bohte et al., 2011). Repeatability may even be better than in histological analysis (S. S. Lee & Park, 2014), and Noureddin et al. thus presented that MRI could replace histology as the reference standard in clinical trials due to its higher sensitivity to quantify the increases and decreases in the liver fat content (Noureddin et al., 2013). The sensitivity and specificity for CT in the diagnosis of hepatosteatosis are 46.1–72.0% and 88.1–94.6%, respectively (Bohte et al., 2011). Hepatosteatosis can also be detected by CT by comparing the attenuation of the liver parenchyma to that of the spleen (Petaja & Yki-Jarvinen, 2016; Schwenzer et al., 2009).

While ultrasound and CT provide more or less qualitative information about hepatosteatosis, methods based on MRI and ¹H-MRS are capable to determine more quantitative information as they are able to detect even small amounts
of hepatic fat accurately (Springer, Machann, Claussen, Schick, & Schwenzer, 2010). 1H-MRS is the most accurate method to measure hepatosteatosis, having sensitivity of 72.7% to 88.5% and specificity of 92.0% to 95.7% depending on the grade of steatosis (Bohte et al., 2011). Moreover, it is highly reproducible (Szczepaniak et al., 1999). However, it is expensive and requires specific expertise (Petaja & Yki-Jarvinen, 2016).

Controlled attenuation parameter (CAP) is an innovative technology which measures ultrasound attenuation in the liver parenchyma at the standardized frequency of 3.5MHz (Berzigotti, 2014; Sasso et al., 2010). The greater the hepatic fat content, the more the ultrasound beam attenuates (Boursier & Cales, 2012; de Ledinghen et al., 2014). CAP was first introduced in 2010 (Sasso et al., 2010). CAP is measured simultaneously on the same volume and on the same ultrasound beam as the liver stiffness measurement by transient elastography (FibroScan©) and the results are expressed as decibels per meter (dB/m) (Boursier & Cales, 2012). The final result is the median value of 10 valid measurements (Berzigotti, 2014; Sasso et al., 2010). It is non-invasive, inexpensive, easy to perform, provides immediate results and has a good interobserver reproducibility (Boursier & Cales, 2012; de Ledinghen et al., 2014; Ferraioli et al., 2014). Moreover, it is not affected by fibrosis or cirrhosis (Berzigotti, 2014) and, when comparing to histology-based diagnosis, it is less affected by sampling error as it explores about 100 times greater liver volume than liver biopsy (de Ledinghen et al., 2014). However, high CAP value affects the transient elastography value, which must be taken into account to avoid overestimations of liver fibrosis assessed by transient elastography (Petta et al., 2017). The quantifying of steatosis has not been well studied since histology as the reference method is itself highly subjective as the evaluation of the percentage of hepatocytes containing lipid vesicles depends on the pathologist (Boursier & Cales, 2012). For a sensitivity ≥ 90%, cut-off values for CAP were 215dB/m, 252 dB/m and 296dB/m for steatosis grades > 10%, > 33% and > 66%, respectively (de Ledinghen, Vergniol, Foucher, Merrouche, & le Bail, 2012). Thus, CAP is a very promising tool for the non-invasive diagnosis of hepatosteatosis > 10% as the AUROC values for the diagnosis of steatosis grades S1 (steatosis 11–33%) to S3 (steatosis ≥ 67%) were 0.84 to 0.93. Furthermore, steatosis is the only histological parameter significantly related to CAP (de Ledinghen et al., 2012). However, CAP still needs to be studied with larger samples of subjects with hepatosteatosis without other liver morbidity and directly compared to the most accurate steatosis quantification methods (MRI and 1H-MRS) before it can be routinely applied in clinical setting (Boursier & Cales, 2012).
Fatty Liver Index is a scoring system that predicts hepatosteatosis non-invasively. FLI is an algorithm that is calculated by triglycerides, gamma-glutamyl transpeptidase (GGT), BMI and waist circumference and the score varies between 0 and 100. It has been validated versus ultrasound only. FLI < 30 can be used to rule out (sensitivity 87%; negative likelihood ratio 0.2) and FLI ≥ 60 to rule in hepatic steatosis (specificity 86%; positive likelihood ratio 4.3) (Bedogni et al., 2006). SteatoTest© is a continuous linear biochemical assessment of steatosis grade that consists of the 6 variables in the FibroTest© panel (Ratziu et al., 2006) plus body mass index, serum cholesterol, triglycerides, and glucose adjusted for age and gender. The score varies between 0.00 and 1.00 (Poynard et al., 2005). However, neither scoring system seems to take into account other than obese/metabolic NAFLD type, which may partly explain why in the biopsy-validated study both the Fatty Liver Index and SteatoTest© had clearly lower accuracy than CAP for the diagnosis of hepatosteatosis of every grade (de Ledinghen et al., 2012). There are also other scoring systems that predict hepatosteatosis, such as NAFLD Liver Fat Score, Hepatic Steatosis Index, Visceral Adiposity Index and Triglyceride x Glucose Index. According to European guidelines, the best externally validated scores are Fatty Liver Index, SteatoTest© and NAFLD Liver Fat Score (European Association for the Study of the Liver (EASL) et al., 2016), but the weakness of these and other scores is that they predict reliably only the presence of steatosis, not its severity (European Association for the Study of the Liver (EASL) et al., 2016; Fedchuk et al., 2014).

### 2.3.3 The non-invasive diagnosis of NASH

The European guidelines state very clearly that NASH must be diagnosed only by a liver biopsy showing steatosis, hepatocyte ballooning and lobular inflammation (European Association for the Study of the Liver (EASL) et al., 2016). Some biochemical measures (such as cytokeratin-18), imaging studies or scoring systems have been proposed to diagnose NASH or distinguish NASH from simple steatosis (Feldstein et al., 2009; Hyysalo et al., 2014; Machado & Cortez-Pinto, 2013; Pearce, Thosani, & Pan, 2013), but to date, none of them have been proved accurate enough or externally validated to the degree that they would be generally accepted (Cusi et al., 2014; European Association for the Study of the Liver (EASL) et al., 2016; Machado & Cortez-Pinto, 2013; Pearce et al., 2013; Poynard et al., 2012).
2.3.4 The non-invasive diagnosis of liver fibrosis in NAFLD

For the diagnosis of fibrosis and its severity, a liver biopsy is the golden standard. However, its usefulness is limited due to costs and risk of complications. Thus, it is unrealistic to perform liver biopsy for all subjects with NAFLD, the global prevalence of which is about 25% (Younossi, Koenig et al., 2016).

Ultrasound-based shear wave elastography can be used to assess fibrosis in subjects with NAFLD. Shear waves are generated when a directional force is applied to a tissue, causing shear deformation. Shear waves are attenuated by tissues and the greater the stiffness of the tissue, the greater the speed. They travel at much slower rate (1–10 m/s) than ultrasound (speed of sound, i.e., 330m/s) and are thus easily detected by ultrasound waves. To date, most of the data published on shear wave elastography are based on transient elastography (FibroScan©), an acoustic radiation force impulse, or 2D shear wave elastography using the Aixplorer (Dietrich & Dong, 2016), of which the latter two are developed from transient elastography (Piscaglia, Salvatore, Mulazzani, Cantisani, & Schiavone, 2016).

In transient elastography, a low frequency ultrasound (50Hz) is transmitted from a transducer probe to the liver. This ultrasound results in an elastic shear wave that penetrates throughout the liver tissue. The transducer then measures the velocity of the shear wave, which correlates with fibrosis due to the altered mechanical properties of fibrotic tissue (Wilder & Patel, 2014). The range of transient elastography values is from 1.5kPa to 75kPa (Mikolasevic et al., 2016). The actual volume measured by transient elastography is 100 times greater than the average liver biopsy specimen (de Ledinghen et al., 2014). There is no widely accepted consensus for cut-offs of different fibrosis stages, but perhaps the most common cut-offs for clinically relevant fibrosis and cirrhosis are ≥ 8.0kPa and > 13kPa, respectively (Koehler et al., 2016). The cut-offs are based on the high positive predictive values of clinically relevant fibrosis and cirrhosis (Castera, Forns, & Alberti, 2008; Roulot et al., 2011; V. W. Wong et al., 2010). To have a reliable transient elastography result, 10 successful measurements with < 30% median interquartile range are needed (Alkhouri & Feldstein, 2016). The final result is the median value of the successful measurements (Foucher et al., 2006). Transient elastography is validated measurement of the liver stiffness in various different underlying etiologies (Jeong, Lim, Lee, Jo, & Kim, 2014). The pros are clear: it is the most widely used and validated non-invasive technique, it has high reproducibility, it gives an immediate result after a rapid and painless examination and it is easy to learn (Jeong et al., 2014; Kwok et al., 2014). In
addition, transient elastography is shown to be cost-effective (Jeong et al., 2014; van Katwyk et al., 2016). Still, there are some cons. The most prominent limitation is obesity as liver stiffness may be falsely increased in obese individuals (BMI ≥ 28–30 kg/m²) (Mikolasevic et al., 2016). To overcome this limitation, a new probe using lower shear wave frequency, increased amplitude, deeper focal length and a greater depth of measurement was introduced. The probe was named the XL probe (Mikolasevic et al., 2016). The cut-off values for the XL probe, however, still need better validation as it may offer lower values for advanced fibrosis than the conventional M probe (Alkhouri & Feldstein, 2016). Moreover, there has been discussion of whether hepatosteatosis and hepatic inflammation affect the transient elastography values – and the liver stiffness values based on other ultrasound modalities – resulting in false liver stiffness results. According to this speculation, hepatosteatosis may falsely decrease and inflammation may falsely increase the liver stiffness values (Yoshioka, Hashimoto, & Kawabe, 2015). Other limitations for transient elastography measurements are acute hepatitis, chronic hepatitis with transaminases greater than 5 times the upper normal limit, extrahepatic cholestasis, congestive heart failure, all of which tend to increase transient elastography values and ascites which prohibits the travel of the shear waves (Mikolasevic et al., 2016).

According to a meta-analysis and its overall pooled estimate of the diagnostic accuracy, transient elastography is excellent to detect advanced fibrosis (≥ F3; 85% sensitivity, 85% specificity) and cirrhotic-stage fibrosis (F4; 92% sensitivity and 92% specificity) and moderately accurate to detect significant fibrosis (≥ F2; 79% sensitivity, 75% specificity) in NAFLD subjects. The cut-offs in the studies included in the meta-analysis varied from 6.7kPa to 7.7kPa for F2, from 8.0kPa to 10.4kPa for F3, and from 10.3kPa to 17.5 kPa for F4 (Kwok et al., 2014). At present, transient elastography is accepted to exclude cirrhotic-stage fibrosis (F4) in NAFLD subjects in the European guidelines (European Association for the Study of the Liver (EASL) et al., 2016).

Acoustic radiation force impulse is another ultrasound-based modality to assess liver stiffness. It is a recently developed technique which projects data generated by a conventional ultrasound scanner, thus providing liver stiffness measurements during routine ultrasonography (Alkhouri & Feldstein, 2016; Bruno, Minniti, Bucci, & Pozzi Mucelli, 2016). Acoustic radiation force impulse seems to have similar diagnostic performance as transient elastography (Bota et al., 2013; Ebinuma et al., 2011; Friedrich-Rust et al., 2012) but the rate of successful measurements is even higher with it, mainly because of the concurrent use of conventional ultrasound imaging and thus taking advantage of choosing the position (Bota et al., 2013;
Bruno et al., 2016) and because acoustic radiation force impulse is less sensitive to obesity and ascites (Bruno et al., 2016). With newer acoustic radiation force impulse techniques and in experienced hands, the repeatability and reproducibility rates are shown to be very good at least in phantom models (Dillman et al., 2015) but, as with transient elastography, there is no consensus for the cut-offs to diagnose different fibrotic stages.

Supersonic Imagine by Aixplorer© provides a real-time bidimensional assessment of liver stiffness (Piscaglia et al., 2016). The tissue elasticity is translated into colors. Apparently, there are no studies of Supersonic Imagine on NAFLD subjects, but studies on children (Franchi-Abella et al., 2016) and adults with alcoholic liver disease (Thiele et al., 2016) have given promising results comparable to transient elastography.

Magnetic resonance elastography is an additional hardware to standard MR imaging systems located outside the magnetic room that generates shear waves with a driver device positioned over the liver. It images the shear waves with a modified phase-contrast MR sequence and modifies them to a quantitative image of shear stiffness (elastogram) (Alkhouri & Feldstein, 2016; Yin et al., 2007). This method is not widely available and it is expensive, with a limited number of published studies (Alkhouri & Feldstein, 2016; Kwok et al., 2014), but on the other hand it detects even the early stages of fibrosis, is accurate also in obese subjects or in subjects with ascites, and it examines the whole liver (Alkhouri & Feldstein, 2016; J. Chen, Yin, Glaser, Talwalkar, & Ehman, 2013).

FibroTest© is a commercial panel of biochemical markers that includes age, α2-macroglobulin, apolipoprotein A1, haptoglobin, total bilirubin, and GGT. It was developed for a quantitative assessment of fibrosis. ActiTest for the prediction of inflammation activity grading in NASH and SteatoTest for the prediction of steatosis are based on FibroTest© (Munteanu et al., 2016; Ratziu et al., 2006). The range of FibroTest© (and ActiTest© and SteatoTest©) is 0.00 to 1.00, a higher value predicting higher probability of fibrosis stage (Ratziu et al., 2006). In addition to FibroTest©, Fibrometer© is another commercial blood test panel used to diagnose fibrosis. Originally, it was designed for staging fibrosis of chronic hepatitis C, but today it also covers fibrosis related to NAFLD. In a comparison study of different non-invasive diagnostic tools to detect fibrosis in 235 patients with biopsy-proven fibrosis and NAFLD, FibroTest© showed the highest accuracy to predict significant fibrosis making biopsy avoidable in 97% of cases (Cales et al., 2009).

As obesity, diabetes, older age, platelet count ≤ 200×10⁹/L, and aspartate aminotransferase/alanine aminotransferase (AST/ALT) ratio ≥ 0.8 are risk factors
of advanced fibrosis (≥ F3) in patients with NAFLD (Cichoz-Lach et al., 2012), there are several scoring systems developed that predict fibrosis, mostly based on these variables. Of these, NAFLD Fibrosis Score (NFS; variables: age, BMI, presence of impaired fasting glucose or diabetes, AST, ALT, platelet count and albumin) (Angulo et al., 2007) and Fibrosis 4 Calculator (FIB-4; variables: age, AST, ALT and platelet count) (McPherson, Stewart, Henderson, Burt, & Day, 2010) are the best externally validated scoring systems as they have been validated more than once and in ethnically different NAFLD populations with consistent results (European Association for Study of Liver & Asociacion Latinoamericana para el Estudio del Higado, 2015; European Association for the Study of the Liver (EASL) et al., 2016). There are freely available calculators on the Internet for both scores. The tests work best to distinguish advanced fibrosis (≥ F3) from non-advanced fibrosis (Guha et al., 2008), for which purpose they can be confidently used in the first-line risk stratification (European Association for the Study of the Liver (EASL) et al., 2016). NFS < -1.455 and FIB-4 < 1.30 rule out advanced fibrosis with high accuracy (negative predicting values in the study by McPherson et al. were 92% and 95%, respectively) (McPherson et al., 2010). Moreover, both NFS and FIB-4 are inexpensive (Crossan et al., 2015). Other widely used scoring systems are for instance BARD Score (Harrison, Oliver, Arnold, Gogia, & Neuschwander-Tetri, 2008), AST-to-platelet ratio index (Wai et al., 2003), AST/ALT ratio (Williams & Hoofnagle, 1988), BAAT Score (Ratziu et al., 2000) and Enhanced Liver Fibrosis Score (Wahl et al., 2012). In the comparisons of several noninvasive scoring systems to diagnose advanced fibrosis, FIB-4 seems to work best, followed by NFS (McPherson et al., 2010; Shah et al., 2009).

The combination of transient elastography and NFS offers one, validated method to accurately diagnose or exclude the presence of advanced fibrosis in NAFLD, which also reduces the number of diagnostic liver biopsies in the clinical practice by 50–60% (Petta et al., 2015). One alternative flow-chart of this method is presented in Figure 4.
Figure 4. Algorithm to diagnose advanced fibrosis in NAFLD. The algorithm is based on using the combination of liver stiffness measurement (LSM) by vibration-controlled transient elastography (VCTE) plus the NAFLD fibrosis score (NFS). Having concordant low values for both LSM and NFS indicates the absence of advanced fibrosis and both tests can be repeated in 2–3 years. Having concordant high values for both tests indicates the presence of advanced fibrosis and the need to screen for cirrhosis complications including hepatocellular carcinoma and varices by gastroduodenal enteroscopy (EGD). Having discordant results indicates the need for liver biopsy to determine the fibrosis stage (Alkhouri & Feldstein, 2016, published by permission of Elsevier).

2.4 Lipid metabolism in NAFLD

2.4.1 Introduction to lipid metabolism in NAFLD

Under physiological conditions, dietary fatty acids are absorbed from the small intestine, aggregated into triglycerides and merged into chylomicrons. After entering the lymphatics, about two-thirds are delivered to adipose tissue and one-third to the liver. There is considerable traffic between adipose tissue and the liver during eating and fasting. For instance, under fasting conditions fatty acids are released from adipose tissue to the liver where they are oxidized in situ by mitochondria or aggregated to triglycerides and VLDL particles and secreted to the plasma.
Moreover, excess carbohydrates are also synthetized to fatty acids in hepatic de novo lipogenesis (Cohen, Horton, & Hobbs, 2011). Thus, under physiological conditions the liver is more a processor of fatty acids than a storage depot (Kawano & Cohen, 2013). It is also notable that while in physical activity, the uptake of FFAs (and carbohydrates) to the skeletal muscles is enhanced resulting in reduced hepatic influx and, thus, accumulation of triglycerides.

When the hepatic FFA availability (influx and de novo synthesis with esterification to triglycerides) is greater than FFA disposal (oxidation and secretion), hepatosteatosis develops (Fabbrini et al., 2010; Kawano & Cohen, 2013; Musso et al., 2009; Valenti, Bugianesi, Pajvani, & Targher, 2016). In the Western world, the most common background for this is higher energy intake as compared to energy expenditure: lipids (mainly free fatty acids from adipose tissue) begin to accumulate in tissues and organs not designed to store fat, such as the liver or the omentum. This phenomenon is called ectopic fat accumulation (Byrne & Targher, 2015; Shulman, 2014; Valenti et al., 2016) and hepatosteatosis is thus an example of it. Each of the variables (influx, de novo synthesis, oxidation and secretion) is altered in NAFLD (Musso et al., 2009). In hepatosteatosis, the lipids are mainly triglycerides (Alkhouri, Dixon, & Feldstein, 2009), the primary source of energy storage and transport (Browning & Horton, 2004), but other lipid metabolites such as different FFAs, diacylglycerols, free cholesterol, cholesterol esters, ceramides, and phospholipids are also present (Alkhouri et al., 2009; Cheung & Sanyal, 2008).

Straightforwardly, there are two types of NAFLD: obese/metabolic NAFLD, resulting from energy surplus, and genetic NAFLD due to genetic alterations in lipid metabolism in the liver – although combinations of these are also commonly met (Petaja & Yki-Jarvinen, 2016; Yki-Jarvinen & Luukkonen, 2015). Lipid metabolism in genetic NAFLD is presented in chapter Environmental factors and genetic risk factors of NAFLD.

2.4.2 Lipid metabolism in obese/metabolic NAFLD

Higher energy intake than energy expenditure either due to sedentary lifestyle, excess energy intake or both causes energy imbalance that leads to the storage of excess energy as fat in white adipose tissue (Asrih & Jornayvaz, 2015). The accumulating visceral adipose tissue leads to local hypoxia, predisposes to the death of adipocytes, the infiltration of macrophages surrounding the dead adipocytes and the release of proinflammatory cytokines such as NK-κB, tumor necrosis factor α (TNF-α), IL-1β, IL-6, IL-8 and transforming growth factor-β (Asrih & Jornayvaz, 2015).
The inflamed adipose tissue is insulin resistant, that promotes the release of FFAs from visceral adipose tissue to the liver (Asrih & Jornayvaz, 2015; Yki-Jarvinen, 2014). Visceral fat has been suggested to be more harmful compared to subcutaneous fat because it may release more pro-inflammatory cytokines than subcutaneous fat, the rate of lipolysis is higher in visceral fat and FFAs from visceral fat are released directly into the portal vein (Tchernof & Despres, 2013; Yki-Jarvinen, 2014). All these are amplified by the decrease of plasma adiponectin secretion from the inflamed visceral adipose tissue (Yki-Jarvinen, 2014). Thus, increased release of FFAs from visceral adipose tissue results in increased hepatic FFA influx (Fabbrini et al., 2010). The FFA influx is further increased and redirected to the liver by the increased transcription of FFA translocase CD36 in hepatocellular cell membranes and skeletal muscle whereas the expression is decreased in adipose tissue (Fabbrini et al., 2010). Other fatty acid transport proteins in the hepatocellular membrane are fatty acid transport proteins 2 and 5 (Kawano & Cohen, 2013).

Soon after entering the hepatocellular cytoplasm, FFAs are rapidly converted to fatty Acyl-CoAs. The molecular cascade behind this process is not yet fully understood. However, it may be that fatty acid transport protein possesses fatty acyl-CoA synthetase activity or that it may activate the long chain acyl-CoA synthetases resulting in the formation of Acyl-CoAs (Kawano & Cohen, 2013). Acyl-CoAs are the key players in hepatic fatty acid metabolism. They can be a) oxidized in mitochondria, which results in the formation of Acetyl-CoAs and energy, b) synthesized to triglycerides and stored in the liver (Browning & Horton, 2004) or, c) secreted as VLDL particles to the Disse space outside the hepatic cells, or d) are precursors in de novo lipogenesis (Kawano & Cohen, 2013).

In de novo lipogenesis, Acetyl-CoA is converted through various cycles of metabolic reactions to palmitic acid. Acetyl-CoA itself is derived either from Acyl-CoA (oxidation in mitochondria is described above) or glucose (after glycolysis and the oxidation of pyruvate, which is then converted to Acetyl-CoA) (Fabbrini et al., 2010; Kawano & Cohen, 2013). Palmitic acid is elongated and desaturated by long chain fatty acid elongase 6 and stearoyl-CoA desaturase 1 to monounsaturated fatty acids. Next, glycerol-3-phosphate from glycolysis is esterified with newly synthesized fatty acid to generate lysophosphatidic acids by glycerol-3-phosphate acyltransferase (Coleman & Mashek, 2011; Kawano & Cohen, 2013). Finally, after the catalyzing reactions by 1-acylglycerol-3-phosphate acyltransferase, lipin 1 and acyl-CoA:diacylglycerol acyltransferase, triglycerides are formed (Kawano & Cohen, 2013).
Hepatic \textit{de novo} lipogenesis is primarily controlled by sterol regulatory element binding proteins 1c (SREBP-1c) (Ferre & Foufelle, 2010) and carbohydrate responsive element binding protein (H. Yamashita 	extit{et al.}, 2001). SREBP-1c is controlled by insulin and carbohydrate responsive element binding protein by glucose, and these genes activate the expression of several lipogenic genes active in \textit{de novo} lipogenesis (Fabbrini 	extit{et al.}, 2010). Once the liver gets fatty, the ability of insulin to suppress glucose production in the liver is repressed but somehow it preserves its key regulator role in SREBP-1c induction (Cook, Langlet, Kido, & Accili, 2015; Kawano & Cohen, 2013). Thus, in hyperinsulinemia, i.e., insulin resistance, SREBP-1c is induced – and lipogenesis is upregulated (Cook 	extit{et al.}, 2015). The mechanisms behind this so-called ‘selective insulin resistance’ are not yet completely elucidated, but it may be explained by the simultaneous regulation of gluconeogenesis (through FoxO1) and \textit{de novo} lipogenesis (through SREBP-1c). However, this selective insulin resistance is a key player in the pathophysiology of type 2 diabetes development in a subject with NAFLD (Cook 	extit{et al.}, 2015; Kawano & Cohen, 2013). Moreover, an \textit{in vivo} study of postprandial glucose metabolism revealed that increased \textit{de novo} lipogenesis is a predictor of the development of NAFLD (Fabbrini 	extit{et al.}, 2010; Petersen 	extit{et al.}, 2007). Altogether, \textit{de novo} lipogenesis is increased by 2- to 3-fold in individuals with NAFLD as compared to individuals without NAFLD, indicating a substantial role in the NAFLD pathogenesis (Diraison, Moulin, & Beylot, 2003; Donnelly 	extit{et al.}, 2005; Fabbrini 	extit{et al.}, 2010; Lambert, Ramos-Roman, Browning, & Parks, 2014).

FFA oxidation occurs primarily in the mitochondria and to a lesser extent in peroxisomes and endoplasmic reticulum (Berlanga, Guiu-Jurado, Porras, & Auguet, 2014). Carnitine palmitoyltransferase (CPT) 1 and 2 translocate fatty Acyl-CoAs from cytosol across the mitochondrial membrane. Within the mitochondria, Acyl-CoAs are consumed step by step by the β-oxidation cycle into acetyl-CoAs, which are further oxidized by the mitochondrial tricarboxylic acid cycle to generate energy (Kawano & Cohen, 2013). Deficiencies in mitochondrial FFA oxidation lead to hepatosteatosis (Fabbrini 	extit{et al.}, 2010). Because there are no reliable methods to measure hepatic FFA oxidation rate directly, it is controversial to which direction fatty acid oxidation is altered in NAFLD (Cortez-Pinto 	extit{et al.}, 1999; Fabbrini 	extit{et al.}, 2010; Kawano & Cohen, 2013; Schmid 	extit{et al.}, 2011; Sunny, Parks, Browning, & Burgess, 2011). It is evident, however, that fatty acid oxidation is not increased to the same extent as \textit{de novo} lipogenesis and/or FFA influx are increased, and, thus, the FFA input and output are not in balance (Kawano & Cohen, 2013). It is of interest that mitochondrial dysfunction in FA oxidation may amplify the oxidative
stress and thereby contribute to the development of NASH lesions (Pessayre & Fromenty, 2005).

Very low-density lipoproteins (VLDL) are lipoprotein particles that are produced in endoplasmic reticulum and further processed in Golgi apparatus. Their production and secretion is a complex and tightly controlled process. During the process, water-insoluble triglycerides are translated to water-soluble form so that they can be exported from the liver to the circulation. High VLDL levels in circulation are often converted to atherogenic low-density lipoprotein (LDL) particles (Fabbrini et al., 2010; Tiwari & Siddiqi, 2012). Insulin resistance enhances the synthesis and secretion of VLDL (Choi & Ginsberg, 2011; Sundaram & Yao, 2010). In hepatosteatosis, the ability of insulin to repress the production and secretion of VLDL is impaired, which contributes to hypertriglyceridemia and low high-density lipoprotein (HDL) level (Adiels et al., 2007; Petaja & Yki-Jarvinen, 2016; Seppala-Lindroos et al., 2002; Yki-Jarvinen, 2014). However, although the VLDL secretion rate is increased in NAFLD, it is not able to adequately compensate the fatty acid input. This is mainly for two reasons: first, due to the limited capacity of apoB100 secretion, which is an essential structural component of VLDL (Tiwari & Siddiqi, 2012), VLDL secretion reaches a plateau phase in NAFLD whereas in BMI- and body fat-matched controls the secretion is linearly increased (Fabbrini et al., 2008; Fabbrini et al., 2010). Moreover, due to the limited capacity to enhance the apoB100 production, triglyceride content in each VLDL particle is increased; as a result, VLDL particles cannot penetrate the sinusoidal endothelial pores to get out of the liver (Fabbrini et al., 2010; Horton, Shimano, Hamilton, Brown, & Goldstein, 1999). Second, fatty acids from non-systemic sources (intrahepatic or visceral fat lipolysis or de novo lipogenesis) are responsible for the stimulation of VLDL secretion, whereas increased lipolytic rate from systemic sources (primarily from subcutaneous adipose tissue) is unable to stimulate secretion rates sufficiently (Fabbrini et al., 2008).

In total, there are three sources of hepatic triglycerides. Donnelly et al. reported that in their study 59% of hepatic triglycerides accumulation is from serum FFAs, 26% from de novo lipogenesis and 15% from diet (Donnelly et al., 2005).

In summary, hepatosteatosis is formed when VLDL secretion and Acyl-CoA oxidation are overwhelmed by enhanced FFA influx and de novo lipogenesis. The lipid accumulation in the liver is mainly formed of triglycerides (Alkhouri et al., 2009), which are not lipotoxic nor induce insulin resistance to the same extent as many other lipids do (Takamura, Misu, Ota, & Kaneko, 2012). Indeed, converting FFAs to triglycerides may be seen as the liver protecting itself from the toxic effects
of the excess FFAs, cholesterols, diacylglycerols, phospholipids and its components (ceramides, sphingolipids, lysophosphatidyl choline) in the surrounding milieu leading to oxidative stress, insulin resistance and more severe NAFLD (Kikuchi & Takamura, 2016; Musso, Gambino, & Cassader, 2013; Neuschwander-Tetri, 2010; Takamura et al., 2012). The intrahepatic lipid metabolism in NAFLD is illustrated in detail in Figure 5.
Fig. 5. Mechanisms of hepatocellular lipid metabolism and their dysregulation in non-alcoholic fatty liver disease (NAFLD). Fatty acid uptake: fatty acid transport protein (FATP) 2, FATP5 and CD36 mediate transport of non-esterified fatty acids (NEFA) across the plasma membrane. Once taken up into cytosol, fatty acids are activated to form acyl-CoAs by the activity of FATPs or fatty acyl-CoA synthetases (ACSs). De novo lipogenesis: palmitic acid is newly synthesized from glucose. Acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) catalyze the rate-limiting and final steps, respectively. After ACS-mediated activation, palmitoyl-CoA is elongated by long chain fatty acid elongase 6 (ELOVL6) and desaturated by stearoyl-CoA desaturase 1 (SCD1). Acyl-CoAs are esterified by glycerol-3-phosphate (G-3-P) acyltransferase (GPAT) to form lysophosphatidic acid (LPA) and by 1-acylglycerol-3-phosphate acyltransferase (AGPAT) to form phosphatidic acid (PA). PA is dephosphorylated by lipin 1 to form diacylglycerol (DAG), which is esterified to another acyl-CoA molecule to form triglyceride (TG) by acyl-CoA: diacylglycerol acyltransferase (DGAT). Fatty acid oxidation: acyl-CoAs are transported into mitochondria across the outer mitochondrial membrane (OMM) and inner mitochondrial membrane (IMM) by the activities of carnitine palmitoyl transferase (CPT) 1, CPT2 and carnitine acylcarnitine translocase (CACT). Within mitochondria, acyl-CoAs are oxidized to form acetyl-CoA. Very low density lipoprotein (VLDL) synthesis: TGs are packaged together with apoB 100 into VLDL in the endoplasmic reticulum (ER) by the activity of microsomal triglyceride transfer protein (MTP) and secreted into space of Disse. Pink arrows denote the increases and decreases that occur in NAFLD and are described in the text. In NAFLD patients, enhanced acquisition of fatty acids through uptake and rates of de novo lipogenesis are not compensated by possible increases in rates of fatty acid oxidation or higher production rates of VLDL particles (Kawano & Cohen, 2013, published by permission of Springer).
2.5 Glucose metabolism in NAFLD

Insulin is the key regulator of glucose metabolism. It is the only hormone that induces glucose influx from the circulation to the liver, muscles and adipose tissue. Additionally, it suppresses hepatic gluconeogenesis during fasting (Yki-Jarvinen, 2014). NAFLD is associated with insulin resistance (Jornayvaz & Shulman, 2012). Thus, individuals with NAFLD have higher rates of gluconeogenesis (Sunny et al., 2011), which leads to mild hyperglycemia (Yki-Jarvinen, 2014) and a growing need of insulin to hold the blood glucose in the non-diabetic range. Over time, pancreatic β cells may be unable to secrete sufficient amounts of insulin, which provokes T2D. Of note, the amount of intrahepatic fat is shown to correlate with multiorgan insulin sensitivity (Fabbrini et al., 2010; Kotronen, Westerbacka, Bergholm, Pietilainen, & Yki-Jarvinen, 2007; Seppala-Lindroos et al., 2002).

The pathogenesis of insulin resistance in NAFLD is manifold (Begriche, Igoudjil, Pessayre, & Fromenty, 2006). If mitochondria cannot handle the increased lipid influx, mitochondrial dysfunction, activation of pro-inflammatory c-Jun NH2-terminal kinase-1, burst of oxidative stress and endoplasmic reticulum stress develop (Begriche et al., 2006; Buzzetti et al., 2016; Cusi, 2009). Especially, saturated fatty acids, ceramides, sphingolipids and free cholesterol are toxic lipids that provoke liver cell injury and insulin resistance through these mechanisms (Mota, Banini, Cazanave, & Sanyal, 2016; Tarantino & Caputi, 2011).

The endoplasmic reticulum is involved in the production and processing of several membrane lipids and their intermediates (Pagliassotti, Kim, Estrada, Stewart, & Gentile, 2016). When there is a lack of adenotriphosphate or imbalance between the production or processing needs and the ability of the endoplasmic reticulum to process this load, endoplasmic reticulum stress is triggered (Buzzetti et al., 2016; Pagliassotti et al., 2016). Endoplasmic reticulum stress, in turn, leads to unfolded protein response, which is an adaptive response to the overload aiming at diminishing the workload and boosting the process capacity of endoplasmic reticulum (Buzzetti et al., 2016). However, unfolded protein response also results in the activation of c-Jun NH2-terminal kinase-1 and SCREBP-1c, which maintains and deepens the inflammatory responses, intrahepatic fatty acid accumulation and endoplasmic reticulum stress (Buzzetti et al., 2016). c-Jun NH2-terminal kinase-1 also disrupts insulin signaling through serine phosphorylation of insulin receptor substrates 1 and 2 (Aguirre, Uchida, Yenush, Davis, & White, 2000). Additionally, if mitochondria are unable to handle the increased lipid processing requirements, respiratory oxidation may collapse, followed by accumulation of lipid-derived
toxic metabolites and oxidative stress (Buzzetti et al., 2016). The pro-inflammatory state and oxidative stress, in subjects with NASH in particular, further interfere with the insulin signaling cascade (Buzzetti et al., 2016). Excess of fatty acids, especially saturated, is associated with peroxisome proliferator-activated receptor α activation leading to the impairment of insulin signaling. (Mota et al., 2016). Moreover, c-Jun NH2-terminal kinase-1 -dependent cell death is triggered by saturated fatty acids and lysophosphatidyl choline, the production of which is stimulated by increased fatty acid levels (Mota et al., 2016; Ricchi et al., 2009). Cell death stimulates the pro-inflammatory environment that further worsens insulin’s actions in the liver. Ceramides and their derivatives such as sphingolipids are also involved in deepening insulin resistance, oxidative stress, pro-inflammatory state and endoplasmic reticulum stress (P. K. Luukkonen, Zhou, Sadevirta et al., 2016; Mota et al., 2016). Also the abundance of intrahepatic free cholesterol is associated with activation of inflammatory factors and endoplasmic reticulum stress (Mota et al., 2016). In subjects with NAFLD the gut microbiota is often dysbiotic (X. He, Ji, Jia, & Li, 2016), which further leads to increased gut permeability, fatty acid absorption, bacteria-derived toxins (e.g. lipopolysaccharides) and activation of inflammatory pathways, for example, the release of proinflammatory cytokines such as IL-6 and TNF-α from hepatic Kupffer cells (Buzzetti et al., 2016; Kirpich, Marsano, & McClain, 2015; Stefan, Kantartzis, & Haring, 2008). The low level of adiponectin, which is anti-inflammatory and TNF-α-neutralizing adipokine, also deepens the pro-inflammatory activity. All these mechanisms lead to chronic hepatic inflammation, which induces insulin resistance by interfering with the intra-cellular insulin signaling (Malbon, 2004; Pirola, Johnston, & Van Obberghen, 2004; Tilg & Hotamisligil, 2006).

Moreover, there is a vicious cycle between insulin resistance and intrahepatic fatty acid content: in insulin resistant states the potent action of insulin to suppress adipose tissue lipolysis is impaired (Buzzetti et al., 2016) and increased efflux of fatty acids into the liver results in worsening insulin resistance. In addition to being a direct substrate for de novo lipogenesis, glucose and the glycolytic product, pyruvate, increase the production of Acetyl-CoA and its conversion to malonyl-CoA for de novo lipogenesis (Buzzetti et al., 2016; Hazlehurst, Woods, Marjot, Cobbold, & Tomlinson, 2016; Wei, Rector, Thyfault, & Ibdah, 2008). β-oxidation of fatty acids is also diminished in the insulin resistant liver (Buzzetti et al., 2016). Furthermore, hepatosteatosis itself provokes a subacute inflammatory response in the liver through activation of nuclear factor-κB pathways that amplifies insulin resistance locally in the liver but also systemically (Anstee et al., 2013).
There is also evidence of systemic release of hepatokines (organokines of the liver that affect the energy metabolism through autocrine, paracrine and endocrine activity, analogous to adipokines of adipose tissue) and proinflamatory biomarkers affecting glucose metabolism and insulin action (Byrne & Targher, 2015; T. W. Jung, Yoo, & Choi, 2016; Targher, Marchesini, & Byrne, 2016). Of hepatokines, the most important are fetuin-A and fibroblast growth factor 21 (Ix & Sharma, 2010; T. W. Jung et al., 2016; Targher & Byrne, 2013; J. Zhang & Li, 2015) and of proinflamatory biomarkers, C-reactive protein, TNF-α and IL-6 (Byrne & Targher, 2015; Targher & Byrne, 2013).

It remains unclear whether NAFLD is only a cause or also a consequence of insulin resistance (Asrih & Jornayvaz, 2015). Because of complex and multi-directional relationships between NAFLD, insulin resistance and chronic hyperglycemia, it is extremely challenging to dissociate the causes and the consequences of these conditions (Valenti et al., 2016). The present paradigm is, however, that the association is two-way (Yki-Jarvinen, 2014).

2.6 Environmental and genetic risk factors of NAFLD

Excess energy intake and consequent overweight is in the core of the development of NAFLD (Yki-Jarvinen, 2014). Also the sources of extra-calories affect the amount of liver fat and its composition. These and other aspects of the impact of diet and physical activity on the pathogenesis and treatment of NAFLD are discussed earlier (Lipid metabolism in NAFLD and Glucose metabolism in NAFLD) and later (Management of NAFLD). However, there are several other environmental factors that impact on NAFLD development.

2.6.1 Gut microbiota

The core of the gut microbiota is established in the early years of life and is determined, for instance, by birth/perinatal colonization, breast versus formula feeding diet, possible antibiotic treatment and the microbiome of close family members (Ussar, Fujisaka, & Kahn, 2016). Although the majority of the microbiota remains stable during adulthood, the composition is somewhat altered in response to alterations in the environment such as diet, antibiotics or other drugs and host susceptibility (Ussar et al., 2016). There are tens of thousands of different microbial species in the gastrointestinal tract (Sartor, 2008). In addition to bacteria, the gut microbiota consists of fungi and bacteriophages, among others, the latter
maximizing the ability of the bacteria to adapt to the changing gut environment, such as changing diet or antibiotic use (S. R. Modi, Lee, Spina, & Collins, 2013). Of bacteria, there are two major phyla (divisions), Firmicutes (66% of gut microbiota composition) and Bacterioides (16%), other 15 detected phylas comprising the rest (Ley et al., 2008). Thus, each gut has its own individual microbiota and is affected by host age, gender, ethnicity, diet and geographic location.

When the gut microbiota is in equilibrium and harmony, the condition is called eubiosis (eu = good, biosis = life). In a eubiotic gut microbiota, Bacterioides, for instance, are generally considered as ‘the good bacteria’ which control ‘the bad bacteria’ (Iebba et al., 2016). In eubiosis, the host and the microbiota live in mutually beneficial co-operation (Iebba et al., 2016) as the gut microbiota is involved in, for instance, the formation of the anatomo-microbiological gut barrier, the defense against infectious pathogens, immune-modulation and different metabolic functions (Iebba et al., 2016; Kamada, Seo, Chen, & Nunez, 2013; Kitamoto, Nagao-Kitamoto, Kuffa, & Kamada, 2016). In lean humans, the ratio of Bacterioides versus Firmicutes is shown to be greater than in their obese counterparts. Moreover, after weight loss in obese humans, this ratio is increased irrespective of the diet type used (Ley, Turnbaugh, Klein, & Gordon, 2006).

If the equilibrium of the eubiosis is destabilized, quali-quantitative alterations in the gut microbiota take place. This condition in which ‘the good bacteria’ are no longer able to control ‘the bad bacteria’ is called dysbiosis (Iebba et al., 2016). Dysbiosis is related to various gastrointestinal diseases, such as inflammatory bowel disease, colon carcinoma, celiac disease and irritable bowel disease (Nagao-Kitamoto, Kitamoto, Kuffa, & Kamada, 2016) – and NAFLD, which has been demonstrated by correlative studies and by transplant of microbiota from obese mice or humans into germ-free mice (Arslan, 2014; Gonzalez, Jiang, & Patterson, 2016; Machado & Cortez-Pinto, 2016). The dysbiotic gut induces hepatosteatosis by resulting in increased intestinal permeability and thus in increased energy availability and inflammatory cytokines and portal endotoxemia in the liver (Haque & Barritt, 2016; Machado & Cortez-Pinto, 2016). Moreover, dysbiotic gut modulates the bile acid metabolism via farnesoid X receptor, a crucial modulator of bile acid metabolism which is known to have anti-inflammatory effects on the liver (Zhu, Liu, Zhang, & Guo, 2016). Bile acids have a crucial role in the lipid absorption in the intestine and potent antimicrobial properties (Haque & Barritt, 2016; Machado & Cortez-Pinto, 2016). Dysbiotic gut microbiota in NAFLD subjects is associated with the depletion of choline, a water-soluble substance needed, for instance, in the VLDL secretion from the liver (Haque & Barritt, 2016; Spencer et al., 2011).
Certain gut microbiota can further promote choline depletion by metabolizing it to \textit{trimethylamine}, which is further metabolized into a toxic compound, \textit{trimethylamine N-oxide}, by the liver (Machado & Cortez-Pinto, 2016). Additionally, leaky gut and small intestine bacterial overgrowth are associated with NAFLD (Farhadi et al., 2008; Miele et al., 2009; V. W. Wong \textit{et al.}, 2015) and endotoxemia (a burst of endotoxins or lipopolysaccharides, a major component of the cell wall of Gram-negative bacteria in the blood) is shown to correlate with the existence of NAFLD (Kitabatake \textit{et al.}, 2017; Thuy \textit{et al.}, 2008), NAFLD disease severity (Harte \textit{et al.}, 2010; Pang \textit{et al.}, 2017) and insulin resistance (Lassenius \textit{et al.}, 2011; Pussinen, Havulinna, Lehto, Sundvall, & Salomaa, 2011). Thus, dysbiotic gut is part of the ‘multiple hit’ theory explicating the NAFLD pathogenesis (Buzzetti \textit{et al.}, 2016).

There are some small studies evaluating the dysbiotic microbiota in NAFLD subjects (Haque & Barritt, 2016; Machado & Cortez-Pinto, 2016), but without unambiguous reports (Boursier \textit{et al.}, 2016; Del Chierico \textit{et al.}, 2017; Famouri, Shariat, Hashemipour, Keikha, & Kelishadi, 2017; Ferolla \textit{et al.}, 2016; Haque & Barritt, 2016; Jiang \textit{et al.}, 2015; Machado & Cortez-Pinto, 2016; Mouzaki \textit{et al.}, 2013; Raman \textit{et al.}, 2013). The reasons for these inconsistent findings are speculative: for example, there may not be any single group of organisms driving NASH development, different study populations may have different microbial populations with different microbiome interplay, or a single snapshot of the intestinal microbiome does not reflect the alterations in the microbiome over time. It is also of note that the number of different microbes vastly outweighs the number of samples and may lead to spurious suppositions (Haslam, 2017). However, there is a relatively solid consensus that decreased microbial diversity is present in obesity and NAFLD (Haslam, 2017).

Microbial alterations may be used in the cure of NAFLD as well. Human trials of fecal microbiota transplantation are scarce, but the preliminary results are promising. For instance, in the Dutch-Finnish trial of duodenal transplant of allogenic gut microbiota infusion, insulin resistance in obese subjects with metabolic syndrome was alleviated as controlled six weeks after the procedure (Vrieze \textit{et al.}, 2012). There are at least two ongoing trials on the potential role of fecal transplantation in the management of NAFLD (Clinicaltrials.gov). Moreover, there are some reports that probiotic use could have a potential therapeutic role in NAFLD improvement (Haque & Barritt, 2016).
2.6.2 Alcohol consumption

According to the definition of NAFLD, alcohol consumption more than 30g a day in men or 20g a day in women is an exclusion criterion for the NAFLD diagnosis (European Association for the Study of the Liver (EASL) et al., 2016). If these limits are exceeded, the condition is called alcoholic fatty liver disease. However, even moderate alcohol consumption below these limits may predispose to NAFLD depending on many factors, such as drinking patterns and individual or genetic susceptibility as, for example, heavy episodic alcohol intake may be associated with fibrosis progression (Ajmera, Terrault, & Harrison, 2017; European Association for the Study of the Liver (EASL) et al., 2016). Especially, those at metabolic risk tend to have NAFLD even with moderate alcohol consumption (European Association for the Study of the Liver (EASL) et al., 2016). Indeed, from the liver point of view, there are no precise safety limits for alcohol use (European Association for the Study of Liver, 2012). Thereby, the consumption limits are more or less arbitrary and designed to be used in the disease definitions. On the other hand, there are epidemiological data showing moderate alcohol consumption to be beneficial in the prevention of NAFLD development (Dunn et al., 2012; European Association for the Study of the Liver (EASL) et al., 2016; H. K. Kwon, Greenson, & Conjeevaram, 2014; Liangpunsakul & Chalasani, 2012) and in the protection of cardiovascular diseases in the general population when compared to total abstinence (Ajmera et al., 2017). However, this J-curve theory is widely opposed. For instance, a large meta-analysis of 56 studies with over 260,000 European subjects was not able to show the cardioprotective role of moderate alcohol consumption (Holmes et al., 2014). Instead, the subjects who drank less were more often carriers of the A- allele of alcohol dehydrogenase 1B gene (rs1229984), and had a reduced risk of coronary heart disease at all levels of alcohol consumption. Simultaneously, further subdivision of the subjects into light, moderate and heavy drinkers did not show a cardioprotective effect of the allele within the group as compared to the non-allele carriers. Thus, the study concluded that a reduction in alcohol drinking, even for the light or moderate drinkers, is beneficial for cardiovascular health (Holmes et al., 2014). Moreover, due to the methodological problems related to the studies of alcohol consumption, such as imperfect adjustment for confounding factors, selection bias and failure to measure the lifetime use and patterns of alcohol consumption, it is impossible to set recommendations of moderate alcohol drinking (Ajmera et al., 2017; Holmes et al., 2014). In any case, it is clear that at least subjects with cirrhosis should avoid any alcohol consumption (European Association for the Study of the Liver (EASL) et al., 2016).
2.6.3 Toxic compounds, drugs and NAFLD

The toxic compounds, whether from the air, water, soil or food, may induce systemic inflammation and, thus, activate Kupffer cells and several different pro-inflammatory pathways in the liver (Arciello et al., 2013). Of note, there are also reports from rodents and humans that different pollutants are capable of worsening obesity, insulin resistance, NAFLD and even HCC (Arciello et al., 2013; Naik et al., 2013), but hepatosteatosis may be induced by xenobiotics, such as anabolic-androgenic steroids, even without the induction of insulin resistance (Schwingel et al., 2011). Methotrexate, amiodarone, valproate, glucocorticoids, synthetic estrogens, tetracycline and tamoxifen are other known steatogenic medication (Arkkila, 2009). Additionally, some chemicals, specifically synthetic ones, may mimic endogenous hormones, leading to disruption of the endocrine homeostasis and development of hepatosteatosis (Arciello et al., 2013). Some environmental heavy metals (e.g. mercury, lead, cadmium, arsenic) and polychlorinated compounds also seem have direct toxic effects on NAFLD and insulin resistance through oxidative stress (Arciello et al., 2013; Cave et al., 2010).

Moreover, the toxic and detrimental effects of xenobiotic chemicals are also supposed to be mediated by drug-metabolizing enzymes, such as phase 1 enzymes, i.e., cytochrome P450s (CYPs) that catalyze hydroxylation reactions, and phase 2 enzymes involved in conjugation reactions (Naik et al., 2013). There are several targets for the drug metabolizing enzymes to mediate the toxic and detrimental effects of xenobiotic chemicals such as PXR, constitutive androstane receptor, farnesoid X receptor, liver X receptor and peroxisome proliferator-activated receptor (Naik et al., 2013).

PXR was first identified in 1998 as a xenobiotic-sensing receptor that induces CYP genes (Kliewer et al., 1998). PXR belongs to the nuclear receptor superfamily of ligand-activated transcription factors (Kliewer et al., 1998) and it is highly expressed in the liver, the intestine and the kidneys while lower expression levels are found in other tissues (Ihunnah, Jiang, & Xie, 2011). PXR has many endobiotic and xenobiotic ligand-activators and is thus involved in many pathophysiological conditions (Hakkola et al., 2016; Ihunnah et al., 2011). PXR is an exceptional nuclear receptor as it can accept a wide variety of ligands with significant structural differences (Hakkola et al., 2016). It regulates the overall metabolic functions in ways that are not yet fully understood (Hakkola et al., 2016). The broad spectrum of PXR-activating drugs and the harmful metabolic effects have raised a question whether the long-term use of PXR activators is involved in different kinds of metabolic diseases (Hakkola et al., 2016).
Originally, PXR was thought to regulate enzymes involved in drug-metabolism and to be involved in several drug-drug-interactions, but the expanding knowledge has revealed its broad functions in energy, bile acid and steroid hormone metabolism as well (Hakkola et al., 2016; Ihunnah et al., 2011). For instance in humans, PXR activation has been shown to induce the transcription of CYP3A4, CYP2B9, CYP2C8 and CYP2C9 (Ferguson, Chen, LeCluyse, Negishi, & Goldstein, 2005; Ihunnah et al., 2011). It also has many xenobiotic agonists, including rifampicin, lovastatin, dexamethasone and spironolactone, to name a few (Kliewer et al., 1998). Additionally, many endobiotic ligands such as many steroid-like ligands (pregnanes, progesterones, corticosterones, estrogens, testosterone and bile acids) are shown to activate it (Ihunnah et al., 2011; Xue et al., 2007). PXR is also involved in bile acid homeostasis and detoxification of toxic bile salts (G. L. Guo et al., 2003; Rezen, Rozman, Pascussi, & Monostory, 2011; Sonoda et al., 2002). It is noteworthy that there are many similarities but also some species-specific PXR actions in humans and rodents (Hakkola et al., 2016; Jonker, Liddle, & Downes, 2012). The summary of the transcriptional circuits and metabolic effects PXR has are shown in Figure 6 (Ihunnah et al., 2011).
There is solid evidence from human cell model and mice studies that PXR regulates glucose and lipid metabolism (Hakkola et al., 2016). For instance, it affects the risk of hyperglycemia and diabetes, obesity, dyslipidemia, atherosclerosis and hepatosteatosis (Gao & Xie, 2012; Hakkola et al., 2016; Hakkola et al., 2016). However, only PXR-induced hyperglycemia is shown to occur in humans in vivo (Hakkola et al., 2016; Rysa et al., 2013; Stage et al., 2016; Takasu et al., 1982): In a randomized, open, placebo-controlled crossover trial, rifampicin, a prototypic PXR agonist (J. Chen & Raymond, 2006), impaired postprandial glucose tolerance and caused postprandial hyperinsulinemia whereas fasting glucose and insulin were not affected (Rysa et al., 2013). This finding is in line with the prior in vivo studies of known PXR agonists. Indeed, St John’s
wort induced hyperglycemia, which was detectable even 6 weeks after the last dose intake (Stage et al., 2016). St John’s wort also reduced insulin secretion. Moreover, although with discordant reports (Hakkola et al., 2016), rifampicin, but not other tuberculosis medicines, has been shown to cause hyperglycemia in oral glucose tolerance test in patients with pulmonary tuberculosis (Takasu et al., 1982). As many statins are weak PXR agonists, it has been speculated that the slight but widely known association of statins and hyperglycemia is linked via PXR activation (Hakkola et al., 2016). There is also strong evidence from rodent studies and human cell models that PXR activation has a causal association with hyperglycemia (Hakkola et al., 2016).

The mechanisms by which PXR agonism induces hyperglycemia in humans is not yet fully understood. It is interesting that one of the species-specific differences in the PXR action is the difference on modification of gluconeogenesis by activated PXR: in human cell models PXR seems to directly and indirectly induce the gluconeogenic genes phosphoenolpyruvate carboxykinase and glucose-6-phosphatase whereas in rodents PXR downregulates them (Hakkola et al., 2016). Thus, this results in stimulated gluconeogenesis in human primary hepatocytes but repression in rodents (Hakkola et al., 2016). Additionally, at least in rodents, PXR represses the transcription of glucose transporter 2 and glucokinase, genes involved in hepatic glucose uptake (Hakkola et al., 2016).

In addition to hyperglycemia, PXR activation has induced hepatosteatosis in rodents and in humans in vitro (Hakkola et al., 2016). In human primary hepatocytes, activated PXR up-regulates SREBP-1a directly (Hakkola et al., 2016) and SREBP-1c indirectly through hyperinsulinemia (S. Y. Kim et al., 2004; Rysa et al., 2013; Xie et al., 2009). SREBP-1a and SREBP-1c are splice variants of gene SREBF1 (Xie et al., 2009). SREBP-1a induces the transcription of several genes needed in fatty acid synthesis such as acetyl-CoA carboxylase 1, fatty acid synthase, elongation of long-chain fatty acids family member 6 and stearoyl-CoA desaturase 1 (Amemiya-Kudo et al., 2002; Hakkola et al., 2016; Xie et al., 2009). SREBP-1a also activates the genes for cholesterol synthesis (Musso et al., 2013; Xie et al., 2009) while SREBP-1c activates genes involved in fatty acid synthesis only (Xie et al., 2009). Moreover, PXR activation leads to enhanced transcription of thyroid hormone-responsive spot 14 protein, which, in turn, activates transcription of fatty acid synthase and adenosine triphosphate citrate lyase, which is also an enzyme needed in the fatty acid synthesis (Hakkola et al., 2016). Intrahepatic fatty acid content may be increased also due to downregulation of β-oxidation (Hakkola et al., 2016; Moreau et al., 2009) after the suppression of carnitine palmitoyltransferase 1 and
acetyl-CoA acyltransferase 2 transcription (Hakkola et al., 2016). An *in vitro* study by Moreau et al. showed direct stimulation of fatty acid synthesis after the PXR activation (Moreau et al., 2009). Notably, human *in vivo* studies of the possible PXR-induced hepatosteatosis do not exist.

Additionally, PXR is speculated to activate cholesterol synthesis as well (Xie et al., 2009). In mice and human cell model studies PXR activation exposes to hypercholesterolemia by repressing the CYP7A1 and CYP8B1, CYPs needed for the bile acid synthesis from cholesterol. This is achieved through suppression of hepatocyte nuclear factor 4 (Bhalla, Ozalp, Fang, Xiang, & Kemper, 2004; Jonker et al., 2012; T. Li & Chiang, 2005; Russell, 1999; M. Zhang & Chiang, 2001). However, in humans *in vivo* this seems not to be the case as PXR induced CYP3A4 but CYP7A1 and CYP8B1 remained unaltered (Marschall et al., 2005). However, hyperinsulinemia induces the transcription of SREBP-2 (Musso et al., 2013; Xie et al., 2009), which up-regulates the genes that favor cholesterol synthesis over triglycerides and phospholipids (Weber, Boll, & Stampfl, 2004; Xie et al., 2009). There are also many human *in vivo* studies confirming the stimulating effect of PXR on cholesterol and/or bile acid synthesis (Coyne, Bonorris, Goldstein, & Schoenfield, 1976; Lutjohann et al., 2004; Marschall et al., 2005; von Bergmann, Fierer, Mok, & Grundy, 1981). The mechanisms behind the stimulated cholesterol synthesis are speculative. Human cell model studies have shown that PXR enhances the activities of SREBP-1a (Bitter et al., 2015) and citrate uptake to hepatocytes (L. Li et al., 2015) whereas mice studies have revealed the induction of insulin-induced gene 1 and lipin-1 after PXR activation (J. He et al., 2013; Roth et al., 2008). There is also indirect evidence of the stimulative effect of rifampicin induced PXR activation on phospholipid metabolism in forms of sphingomyelin synthesis and phospholipid degradation (Block et al., 1997). All these alterations on lipid metabolism after PXR activation are of interest as fatty acids, cholesterol and phospholipids are seen as toxic lipids able to produce endoreticulum stress, inflammation, apoptosis and necrosis resulting in NAFLD progression (Mota et al., 2016; Musso et al., 2013; Neuschwander-Tetri, 2010) to NASH and fibrosis. Of interest, Sookoian, et al. evaluated certain PXR genotypes of 290 subjects in a case-control association study (Sookoian et al., 2010). They concluded that PXR polymorphisms may contribute to disease severity in NAFLD (Sookoian et al., 2010).

In conclusion, PXR activation has various impacts on liver energy metabolism. In human studies with healthy volunteers, PXR activation causes postprandial hyperglycemia. The mechanisms of hyperglycemia may include the repression of
2.6.4 Genetic factors predisposing for NAFLD

There is a great variability in the prevalence of NAFLD around the world (Rinella & Charlton, 2016; Younossi, Koenig et al., 2016). Furthermore, in the USA the prevalence of hepatosteatosis varies significantly by ethnicity (Hispanics, whites, blacks, respectively) irrespective of body mass index, insulin resistance, alcohol consumption, or medication use (Browning et al., 2004; Kahali, Halligan, & Speliotes, 2015). The majority of the variability between the ancestries is explained by genetic differences (Kahali et al., 2015; Palmer et al., 2013; Romeo et al., 2008). The heritable component of NAFLD through ancestries is estimated to be 22–38% (Kahali et al., 2015). In recent years, the study of the genetic factors predisposing for NAFLD has been intense. Straightforwardly, there are two main strategies to do the genetic studies: genome-wide association studies (GWAS, genome-wide study of genetic variants associated with the disease) and candidate gene studies (study of the selected candidate gene) (Macaluso, Maida, & Petta, 2015). To date, GWAS has introduced the three best verified genetic variants affecting NAFLD, patatin-like phospholipase domain-containing 3 (PNPLA3) transmembrane 6 superfamily member 2 E167K variant (TM6SF2) (Macaluso et al., 2015) and membrane bound O-acyltransferase domain containing 7 (MBOAT7) (Macaluso et al., 2015; Mancina et al., 2016). All these genetic NAFLD phenotypes dissociate from metabolic syndrome.

An association between increased hepatic fat level, inflammation and I148M (rs738409) variant in PNPLA3 gene, encoding for the isoleucine-to-methionine substitution, was first introduced by Romeo et al. in 2008 (Romeo et al., 2008). The PNPLA3 gene is localized on human chromosome 22 (Xu, Tao, Zhang, Deng, & Chen, 2015). Adiponutrin, the PNPLA3 protein, is expressed in the hepatocytes and adipocytes (Xu et al., 2015) and has hydrolytic activity on triglycerides and glycerolipids (Macaluso et al., 2015; Sookoian & Pirola, 2012). Moreover, the I148M variant may also induce the synthesis of phosphatidic acid (Kumari et al., 2012). The I148M variant of PNPLA3 gene loses the activity which PNPLA3 normally has (S. He et al., 2010; Pingitore et al., 2014; Pirazzi et al., 2014). This, in turn, leads to alterations in impairment of lipid catabolism, VLDL secretion and lipid droplet remodeling (Dongiovanni, Romeo, & Valenti, 2015). Furthermore,
Luukkonen et al. have shown that the increase of liver fat content in the I148M variant of PNPLA3 gene is due to polyunsaturated triacylglycerols while other lipids were unchanged (P. K. Luukkonen, Zhou, Sadevirta et al., 2016). The polyunsaturated triacylglycerols are shown not to expose to insulin resistance because they are not lipotoxic (E. H. Kim, Bae, Hahm, & Cha, 2012; Perez-Martinez, Perez-Jimenez, & Lopez-Miranda, 2010; Valenzuela et al., 2012). In the absence of adipose tissue inflammation and more favorable hepatic lipid composition (P. K. Luukkonen, Zhou, Sadevirta et al., 2016; Petaja & Yki-Jarvinen, 2016), PNPLA3 I148M does not induce insulin resistance, dyslipidemia or obesity or their features, such as cardiovascular morbidity (Figure 7) (Petaja & Yki-Jarvinen, 2016; Sookoian & Pirola, 2011). There is a dose-effect risk with heterozygote risk intermediate between CC and GG alleles (Valenti et al., 2010). This finding has been confirmed in many ethnic populations (Kahali et al., 2015; Petaja & Yki-Jarvinen, 2016). According to a meta-analysis, the prevalence of the heterozygous PNPLA3 I148M gene variant is around 30–40% and that of the homozygous variant about 5% in West-Eurasian populations (Kollerits et al., 2009). An association exists between the variant and elevated ALT levels, imaging-based hepatosteatosis and histologic NAFLD, including NASH, fibrosis and cirrhosis (Kahali et al., 2015). A meta-analysis revealed that homozygous carriers of PNPLA3 I148M have 73% higher hepatic fat content, 3.4-fold greater risk of developing NASH and 3.2-fold greater risk of fibrosis than non-carriers (Sookoian & Pirola, 2011; Sookoian & Pirola, 2012). The same figures for heterozygous carriers were 10%, 2.7-fold and 2.4-fold, respectively (Sookoian & Pirola, 2011). The risk of cirrhosis is 1.7-fold in heterozygous genotype and 3.4-fold in homozygous genotype as compared to non-carriers (J. H. Shen et al., 2015). The HCC risk is reported to be 1.8-fold per I148M allele (Trepo et al., 2014).

TM6SF2 is located on chromosome 19 and is expressed in the intestine and the liver (Mahdessian et al., 2014). Although the specific molecular functions of the TM6SF2 gene are still unclear, functional studies have shown that its activity is crucial in VLDL secretion (Mahdessian et al., 2014). Gene variant rs58542926 leads to substitution of glutamate to lysine at residue 167 (E167K) resulting in the decrease of gene function as compared to the wild-type TM6SF2 gene. This contributes to NAFLD phenotype in mice and human hepatocytes (Dongiovanni, Romeo et al., 2015; Kozlitina et al., 2014). About 7% of Europeans have this gene variant (Kozlitina et al., 2014). Heterozygous carriers have 1.7-fold and homozygous carriers 4.8-fold risk of ultrasonographically detected NAFLD whereas each allele of the TM6SF2 rs58542926 was associated with 1.4-fold risk.
of histologically verified hepatosteatosis (Y. L. Liu *et al.*, 2014). In a combined Italian-Finnish cohort, the carriers of E167K (either heterozygous or homozygous), even after adjusting for several confounding variables, were at 1.8-fold risk of NASH, 2.1-fold risk of advanced fibrosis and overall more progressive NAFLD (Dongiovanni *et al.*, 2015). In the carriers, the risk of having NAFLD-related HCC is about 1.9-fold compared to non-carriers (Y. L. Liu *et al.*, 2014). However, due to decrease in plasma cholesterol and triglyceride levels and unaltered insulin sensitivity, TM6SF2 E167K offers some protection from cardiovascular events and development of carotid plaques (Figure 7) (Dongiovanni *et al.*, 2015; Kozlitina *et al.*, 2014; Macaluso *et al.*, 2015; Petaja & Yki-Jarvinen, 2016).

MBOAT7, an enzyme needed in the phospholipid acyl-chain remodeling, is highly expressed in endoplasmic reticulum, mitochondria and lipid droplets of the liver (Mancina *et al.*, 2016). A variant in MBOAT7 at rs641738 has been associated with down-regulation of the MBOAT7 (Mancina *et al.*, 2016). First this variant was shown to increase the risk of alcoholic-related cirrhosis (Buch *et al.*, 2015) but very recently rs641738 has also been associated with increased risk of entire NAFLD spectrum as each rs641738 C>T allele increased the odds of hepatosteatosis (1.42, 95% CI 1.07–1.91), NASH (1.18, 1.00–1.40) and clinically significant fibrosis (≥ F2) (1.30, 1.06–1.70) (Mancina *et al.*, 2016). Similar finding has been reported by other studies (Krawczyk *et al.*, 2017; P. K. Luukkonen, Zhou, Hyotylainen *et al.*, 2016; Viitasalo *et al.*, 2016; Viitasalo *et al.*, 2016), some of them from Finland (P. K. Luukkonen, Zhou, Hyotylainen *et al.*, 2016; Viitasalo *et al.*, 2016). MBOAT7 at rs641738 may also predispose to HCC even after adjustments for age, sex, obesity, T2D and presence of advanced fibrosis (OR 1.65, 1.08- 2.55) (Donati *et al.*, 2017). Interestingly, MBOAT7 at rs641738 has not been associated with insulin resistance either (Mancina *et al.*, 2016). The prevalence of this variant, however, has not been reported in community-based studies.

There are also some other suggested genes exposing for NAFLD identified by GWAS. These genes, PNPLA3, TM6SF2 and MBOAT7 are introduced in Table 1.
Table 1. Genes suggested for promoting NAFLD identified by GWAS. The table is based on (Macaluso, Maida, & Petta, 2015, from open access journal) if not otherwise stated.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene function (wild-type)</th>
<th>SNP</th>
<th>Association with</th>
<th>Odds ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNPLA3 (Patatin-like phospholipase domain-containing 3)</td>
<td>hydrolytic activity on triglycerides and glycerolipids (references above)</td>
<td>rs738409</td>
<td>throughout NAFLD spectrum</td>
<td>3.3 (steatosis), 2.7 (NASH), 2.4 (fibrosis) (Sookoian &amp; Pirola, 2011; Speliotes et al., 2011)</td>
</tr>
<tr>
<td>TM6SF2 (transmembrane 6 superfamily member 2 E167K variant)</td>
<td>secretion of triglyceride-rich lipoproteins (references above)</td>
<td>rs58542926</td>
<td>throughout NAFLD spectrum</td>
<td>1.7 (NAFLD), 1.8 (NASH), 2.1 (fibrosis) (Dongiovanni et al., 2015; Y. L. Liu et al., 2014)</td>
</tr>
<tr>
<td>MBOAT7 (membrane bound O-acyltransferase domain containing 7)</td>
<td>reduces the acyl remodeling of phosphatidylinositol in mitochondria and endoplasmic reticulum (Mancina et al., 2016)</td>
<td>rs641738</td>
<td>throughout NAFLD spectrum</td>
<td>1.4 (steatosis), 1.2 (NASH), 1.3 (fibrosis) (Mancina et al., 2016)</td>
</tr>
<tr>
<td>NCAN (Neurocan)</td>
<td>cell adhesion and lipoprotein metabolism</td>
<td>rs2228603</td>
<td>steatosis</td>
<td>1.65 (steatosis) (Speliotes et al., 2011)</td>
</tr>
<tr>
<td>PPP1R3B (protein phosphatase 1 regulatory subunit 3b)</td>
<td>promotes glycogen synthesis, inhibits glycogen breakdown (Kahali et al., 2015)</td>
<td>rs4240624</td>
<td>steatosis</td>
<td>1.28 (steatosis) (Hernaez et al., 2013)</td>
</tr>
<tr>
<td>GCKR (glucokinase regulatory protein)</td>
<td>increased activity of hepatic glucokinase</td>
<td>rs780094</td>
<td>steatosis, severity of fibrosis</td>
<td>2.00 (NAFLD) (Lin, Chang, Chang, &amp; Ni, 2014)</td>
</tr>
<tr>
<td>LPLAL1 (lysophospholipase-like 1)</td>
<td>complementary to the PNPLA3 protein in triglyceride catabolism</td>
<td>rs2137855</td>
<td>steatosis</td>
<td>1.37 (steatosis) (Speliotes et al., 2011)</td>
</tr>
<tr>
<td>FDFT1 (farnesyl dipiphosphate farnesyl transferase)</td>
<td>increase of cholesterol synthesis</td>
<td>rs2645424</td>
<td>Fibrosis (Ballestri, Day, &amp; Daly, 2011)</td>
<td>1.57 (moderate/severe fibrosis) (Ballestri et al., 2011)</td>
</tr>
<tr>
<td>Gene</td>
<td>Gene function (wild-type)</td>
<td>SNP</td>
<td>Association with</td>
<td>Odds ratio</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>-------------------------------------------------------------------------------------------</td>
<td>-----------</td>
<td>---------------------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>PDGFA (platelet-derived growth factor alpha)</td>
<td>activates hepatic stellate cells (Sharma et al., 2015)</td>
<td>rs343062</td>
<td>severity of fibrosis</td>
<td>not available</td>
</tr>
<tr>
<td>COL13A1 (collagen type XIII alpha1)</td>
<td>modifies the inflammatory response genes (Chalasani et al., 2010)</td>
<td>rs1227756</td>
<td>lobular inflammation</td>
<td>not available</td>
</tr>
<tr>
<td>LTBP3 (latent transforming growth factor-beta-protein 3)</td>
<td>increased TGFβ signaling (Zilberberg et al., 2015)</td>
<td>rs6591182</td>
<td>lobular inflammation</td>
<td>not available</td>
</tr>
<tr>
<td>EFCAB4B (EF-hand calcium binding domain 4B)</td>
<td>induced cell death, highly expressed in T cells (Srikanth et al., 2010)</td>
<td>rs887304</td>
<td>lobular inflammation</td>
<td>not available</td>
</tr>
</tbody>
</table>
Fig. 7. Schematic representation of the causes and consequences of Obese/metabolic NAFLD (top) and TM6SF2 NAFLD and PNPLA3 NAFLD (bottom). Abbreviations: BMI, body mass index; CHD, coronary heart disease; DM, diabetes mellitus; FFA, free fatty acids; fs, fasting serum; HCC, hepatocellular carcinoma; HDL, high density lipoprotein; MCP-1, monocyte chemoattractant protein-1; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; LDL, low density lipoprotein; P, plasma; PNPLA3, patatin-like phospholipase domain-containing 3; S, serum; TM6SF2, transmembrane 6 superfamily member 2; TNF-α, tumor necrosis factor-α (Petaja & Yki-Jarvinen, 2016, from open access journal).
2.7 Hepatic complications of NAFLD

The average risk of cirrhosis in subjects with simple steatosis is under 4% in 20 years, but in NASH subjects the risk reaches up to 25% in nine years (Calzadilla Bertot & Adams, 2016). Moreover, the amount of NAFLD-related cirrhosis is likely to be underestimated because the histological signs of steatohepatitis may no longer exist at the cirrhotic stage, leading the cirrhosis to be labeled as ‘cryptogenic’, and because there is a lack of systematic evaluation in community-based studies (Calzadilla Bertot & Adams, 2016). Indeed, given the high prevalence, NAFLD is the leading cause of cryptogenic cirrhosis and the second or third most common cause of liver transplantation (Calzadilla Bertot & Adams, 2016; European Association for the Study of the Liver (EASL) et al., 2016; R. J. Wong et al., 2015). NAFLD is predicted to be the most common cause of liver transplantation in the near future (Calzadilla Bertot & Adams, 2016; Charlton et al., 2011). Looking back, there has been a 5-fold increase in the transplantations for NASH during this millennium (Agopian et al., 2012).

Liver-related deaths (cirrhosis or HCC) accounted for up to 9–28% of all deaths in three long-term longitudinal studies of biopsied NAFLD patients (Angulo et al., 2015; Ekstedt et al., 2015; Younossi et al., 2011), although CVDs remain the most common cause of death in NAFLD subjects (Ekstedt et al., 2015; European Association for the Study of the Liver (EASL) et al., 2016). Liver death is the third most common cause of death after CVDs and non-gastrointestinal malignancies among biopsied NAFLD patients (Angulo et al., 2015; Ekstedt et al., 2015). Among all NAFLD subjects, the liver-related mortality is estimated to be around 2% worldwide (Figure 8) (Rinella & Charlton, 2016; Younossi, Koenig et al., 2016). However, as the global prevalence of NAFLD is reported to be 25%, which equals about 1 billion adult NAFLD subjects, this will lead to 20,000,000 liver-deaths among patients with NAFLD who are currently alive (Rinella & Charlton, 2016). This exceeds hepatitis C as a cause of liver-related death (Rinella & Charlton, 2016). Nonetheless, only the presence of fibrosis seems to predict the liver-related mortality, along with CVD-related and all-cause mortalities, and the more severe the fibrosis stage is, the greater the risk (Angulo et al., 2015; Dulai et al., 2017; Ekstedt et al., 2015).

Once at cirrhosis stage, the overall prognosis is still relatively good as 10-year survival has been reported to be around 81–84% in all NASH-related cirrhotics (Hui et al., 2003; Sanyal et al., 2006). However, nearly half of Child-Pugh A cirrhotics (45%) develop decompensated cirrhosis during 10 years
which is, nonetheless, a lower proportion than in hepatitis C-related cirrhosis (60%) (Sanyal et al., 2006). After decompensation, there are no differences in mortality between NASH- or hepatitis C-related cirrhotics and the survival is reduced as nearly every subject with Child-Pugh B or C dies within 2–3 years (Sanyal et al., 2006). As compared to cirrhotics from hepatitis C after ten-year follow-up, NASH-cirrhotics seem to develop less ascites (ascites-free subjects at baseline: 41% vs. 14%, respectively) and HCC (17% vs. 7%, respectively) while no difference in variceal hemorrhage (10% for NASH-cirrhosis without variceal hemorrhage at baseline) or encephalopathy (15% for NASH-cirrhosis subjects free from encephalopathy at entry) is detected (Sanyal et al., 2006).

HCC is the sixth most prevalent cancer and third most common etiology (over 9%) of cancer-related deaths worldwide accounting for nearly 700,000 deaths annually (Ferlay et al., 2010). Its incidence is growing primarily due to the growing pandemic of diabetes, obesity and NAFLD, which are all risk factors of HCC (Bruix, Gores, & Mazzaferro, 2014; European Association For The Study Of The Liver & European Organisation For Research And Treatment Of Cancer, 2012; Forner, Llovet, & Bruix, 2012; White, Thrift, Kanwal, Davila, & El-Serag, 2017; Zoller & Tilg, 2016). Other risk factors for NAFLD-related HCC are pro-inflammatory state, high age, alcohol consumption and iron load (Zoller & Tilg, 2016). Of these, insulin resistance and inflammation provide sustained proliferative signaling enhancing HCC growth (Zoller & Tilg, 2016). Nevertheless, according to a meta-analysis with follow-up periods of up to 20 years, HCC remains an uncommon complication of NAFLD as the cumulative mortality related to HCC in non-cirrhotic NAFLD subjects was 0–3% (White, Kanwal, & El-Serag, 2012; Zoller & Tilg, 2016). Not surprisingly, the risk of HCC development rises steeply by advancing fibrosis stage (Calzadilla Bertot & Adams, 2016; Ekstedt et al., 2015; Kawamura et al., 2012; Zoller & Tilg, 2016); for example, advanced fibrosis has 17-to 25-fold risk of HCC development as compared to all NAFLD subjects (Ekstedt et al., 2015; Kawamura et al., 2012). It is noteworthy that HCC development on a non-cirrhotic basis has been retrospectively reported in 6–46% of NAFLD-related HCC cases around the world (Ertle et al., 2011; Guzman et al., 2008; Leung et al., 2015; Piscaglia et al., 2016). Especially those with MetS are at risk (Guzman et al., 2008). This is interesting, because, for instance, nearly all hepatitis C-related HCC have developed on a cirrhosis basis (Piscaglia et al., 2016). The overall one-year mortality in NAFLD related HCC is around 60%, which implies slightly poorer prognosis than HCV/HBV-related HCC, but is similar to the prognosis
of alcoholic-HCC and autoimmune-HCC (Piscaglia et al., 2016; Younossi et al., 2015). Treatment of HCC is described elsewhere (European Association For The Study Of The Liver & European Organisation For Research And Treatment Of Cancer, 2012; Zoller & Tilg, 2016).

Fig. 8. The natural history of NAFLD. The natural history of NAFLD is depicted, with the lifetime frequency of clinically relevant progression of fibrosis and development of liver-related and non-liver-related deaths (Rinella & Charlton, 2016, published by permission of John Wiley and Sons).

2.8 NAFLD, metabolic syndrome and type 2 diabetes

2.8.1 Definitions of MetS and T2D

MetS is a cluster of metabolic abnormalities, typically associated with central obesity. There are several definitions and criteria for MetS (Alberti et al., 2009; Kassi, Pervanidou, Kaltsas, & Chrousos, 2011). In the focus of all these criteria is insulin resistance, which is frequently associated with obesity (Asrih & Jornayvaz, 2015; P. K. Chugh & Sharma, 2012; McKenney & Short, 2011).

T2D is characterized by chronically high plasma glucose, which increases the risk of several acute and chronic complications that have a hazardous impact on quality of life and prognosis. Of note, a subject with T2D is often obese and has dyslipidemia and, thus, fills the criteria of MetS (Alberti & Zimmet, 1998; Laakso et al., 2016). Vice versa, MetS is a leading risk factor of cardiovascular and T2D-
related mortality and morbidity (Gami et al., 2007; Hanley et al., 2005; Lorenzo et al., 2003; Simons, Simons, Friedlander, & McCallum, 2011).

Due to the still growing worldwide pandemic of obesity, the prevalence of MetS (today about 20–40%), T2D (15%) and NAFLD (25%) are increasing (Alberti et al., 2009; Kassi et al., 2011; Onat, 2011; Seyda Seydel et al., 2016; Younossi, Koenig et al., 2016).

The pathophysiological mechanisms between NAFLD and MetS as well as NAFLD and T2D are described in Figure 9 and elsewhere in this thesis (see chapter Glucose metabolism in NAFLD).

Fig. 9. The central role of insulin resistance between NAFLD and metabolic syndrome. Insulin resistance is the highway between NAFLD and metabolic syndrome. However, there are also some other mechanisms linking metabolic syndrome to progressive NAFLD and NAFLD to increased CVD risk. Abbreviations: CVD, cardiovascular disease; IR, insulin resistance; NAFLD, non-alcoholic fatty liver disease; MetS, metabolic syndrome.
2.8.2 Association between NAFLD and MetS

The association between NAFLD and MetS is evident. For instance, in the Rotterdam study with nearly 3,000 elderly participants, every component of MetS, namely, waist circumference, fasting glucose, hypertension and hypertriglyceridemia, increased the risk of NAFLD (by 4.9-fold, 2.1-fold, 1.8-fold and 1.6-fold, respectively) while the total prevalence of MetS was 35% (Koehler et al., 2012; Yki-Jarvinen, 2014).

In a recent meta-analysis of 53,000 subjects from 7 studies, Ballestri et al. reported that serum liver enzyme-based NAFLD was associated with 2-fold risk of incident MetS and over 3-fold risk if the diagnosis was ultrasonography-based (Ballestri et al., 2016). The European DIONYSOS study cohort with 3,000 participants showed the strong association between NAFLD and obesity, a central component of MetS and insulin resistance. NAFLD was present in 25% of study subjects with normal weight (BMI 20.0–24.9 kg/m²), 67% of overweight participants (BMI 25.0–29.9 kg/m²) and 94% of obese participants (BMI over 30 kg/m²) (Anstee et al., 2013).

This linear association between BMI and the prevalence of NAFLD is confirmed by another global study (Lazo & Clark, 2008). Overall, cross-sectional studies universally confirm the NAFLD-MetS association (Lonardo, Ballestri, Marchesini, Angulo, & Loria, 2015). Moreover, Kotronen et al. have showed the linear relation between the hepatic fat accumulation, measured by 1H-MRS, and the presence of every MetS component (negative correlation with HDL-cholesterol, positive correlation with all other components) (Kotronen et al., 2007). In conclusion, Mets and NAFLD are very strongly associated with each other.

Whether NAFLD is a predictor of MetS or vice versa is not that clear. Prospective studies on this topic are few, and the results of these studies are conflicting. Adams et al. diagnosed NAFLD by increased ALT without secondary liver disease and found that NAFLD did not predict MetS in Australian individuals followed for 11 years (Adams, Waters, Knuiman, Elliott, & Olynyk, 2009). In contrast, ultrasonographically verified NAFLD has been shown to predict MetS independently in men in two Asian prospective studies (Ryoo et al., 2013; T. Zhang et al., 2014). Additionally, in China the presence of NAFLD was a risk factor of each component of metabolic syndrome. It is noteworthy that the increased risk remained even in non-obese NAFLD subjects in comparison to subjects without NAFLD or obesity (Fan et al., 2007). Altogether, Lonardo et al. have summarized the longitudinal studies (3 retrospective and 16 prospective) that support that NAFLD is a precursor of the metabolic syndrome (Lonardo et al., 2015). However, of the 16 prospective studies, only one was based on ultrasonographically verified
NAFLD (Ryoo et al., 2013), whereas in others NAFLD was diagnosed by liver enzyme surrogates (Lonardo et al., 2015). Simultaneously, there are several studies indicating that MetS precedes NAFLD (Feng et al., 2014; Pais et al., 2013; Sorrentino et al., 2010; Zeb et al., 2013; T. Zhang et al., 2015). For instance, subjects with MetS have a 2-fold greater risk of developing NAFLD than subjects without MetS, and the more MetS components there are at the baseline, the greater the risk of NAFLD (T. Zhang et al., 2015). Moreover, MetS components are also linked to the NAFLD progression (Pais et al., 2013; Sorrentino et al., 2010). Thereby, the causal association between MetS and NAFLD is likely to be reciprocal (Wainwright & Byrne, 2016; Y. Zhang et al., 2015). According to the Bayesian network analysis, the total effect of MetS on NAFLD seems to overwhelm the effect of NAFLD on MetS (Y. Zhang et al., 2015). However, European guidelines recommend that all subjects with NAFLD should be screened for MetS and vice versa (European Association for the Study of the Liver (EASL) et al., 2016).

### 2.8.3 Association between NAFLD and T2D

T2D and NAFLD often co-exist (Hazlehurst et al., 2016; Valenti et al., 2016). According to the longitudinal studies, NAFLD increases the risk of T2D about 2- to 5-fold depending on the population studied, duration of follow-up, methodology used to diagnose NAFLD and NAFLD severity (Byrne & Targher, 2015; Hazlehurst et al., 2016; Valenti et al., 2016). Moreover, the risk of incident T2D increases with the severity of NAFLD (Valenti et al., 2016). However, the great majority of the longitudinal studies are from Asian cohorts with only few from Europe, which raises a need of further evidence from non-Asian populations given that the anthropometric phenotypes and diets are different in Asia and Western countries (Byrne & Targher, 2015; Valenti et al., 2016). There are also caveats in many studies of the associations of NAFLD and T2D as the potential confounding variables, such as family history of T2D, physical activity level or waist circumference, are not always segregated or the 2-hour oral glucose tolerance test is not used to set the diagnosis of T2D (Valenti et al., 2016). Nonetheless, T2D has the potential to promote the progression of NAFLD (Anstee et al., 2013; Byrne & Targher, 2015). For instance, T2D increases the standardized mortality ratio for cirrhosis about 2.5-fold (de Marco et al., 1999) and diabetic individuals have about 3-fold risk of dying of chronic liver disease, mainly associated with NAFLD (Zoppini et al., 2014). It is illustrative that up to 70% of subjects with T2D have NAFLD, 20% of them have NASH, and 5–7% of them have advanced fibrosis (Hazlehurst et al., 2016).
Reciprocally, NAFLD worsens the prognosis of T2D subjects: it doubles the all-cause mortality (Wild SH, Byrne CD, for the Scottish Diabetes Research Network Epidemiology Group, 2017) as well as almost doubles the risk of macro- (CVD events and chronic kidney disease) and microvascular complications (retinopathy) (Targher et al., 2007; Targher et al., 2008).

2.9 Cardiovascular complications of NAFLD

Over the past decade NAFLD has been shown to affect extra-hepatic organs. For example, in addition to the hepatic morbidity related to NAFLD, i.e., NASH, fibrosis, cirrhosis and HCC, NAFLD increases the risk of CVDs, T2D and chronic kidney disease. Although the relative risk of hepatic-related death increases the most in NAFLD subjects, the greatest absolute risk of death among them is attributable of CVDs (Angulo et al., 2015; Byrne & Targher, 2015; Ekstedt et al., 2015; Lonardo, Sookoian, Pirola, & Targher, 2016).

There is solid epidemiological evidence of a causative association between NAFLD and CVDs. In a Medline search for CVD events, i.e coronary heart disease (CHD) and ischemic stroke, in prospective studies of subjects with imaging- or histology-based NAFLD and after exclusions of dual studies from the same database, 10 studies were found (Table 2) (Ekstedt et al., 2015; El Azeem et al., 2013; Fracanzani et al., 2016; Hamaguchi et al., 2007; Haring et al., 2009; Stepanova & Younossi, 2012; Targher et al., 2007; Valbusa et al., 2016; V. W. Wong et al., 2016; Y. J. Zhou, Li, Nie, Huang, & Cao, 2012). Moreover, a meta-analysis of 25,800 subjects from six prospective studies about the association of imaging-based NAFLD and CVD events was published very recently (Mahfood Haddad, Hamdeh, Kanmanthareddy, & Alla, 2016). The overall clinical CVD risk in NAFLD subjects was 1.8-fold, risk of CHD 2.3-fold, risk of stroke 2.1-fold and risk of cardiovascular mortality 1.5-fold in comparison to individuals without NAFLD (Mahfood Haddad et al., 2016). There are also numerous cross-sectional and longitudinal studies that confirm the role of NAFLD in the CVD development. NAFLD is also shown to predict left ventricular hypertrophy, diastolic dysfunction, atrial fibrillation (AF; in diabetic population) and aortic valve sclerosis (Bhatia, Curzen, Calder, & Byrne, 2012; Byrne & Targher, 2015; H. Liu & Lu, 2014; Targher, Day, & Bonora, 2010; Targher et al., 2013). Additionally, NAFLD induces hypertension (Ma et al., 2017; Ryoo et al., 2014) and is associated with carotid intermedia thickness and carotid atherosclerotic plaques (Sookoian & Pirola, 2008). A recently published study by Ma et al. showed that the causative association between NAFLD and CVD risk
factors is dual-way, resulting in a vicious circle between these two (Ma et al., 2017). These links between artery disease risk factors and NAFLD may also explain why NAFLD associates with and predicts chronic kidney disease (Musso et al., 2014).

Table 2. Prospective studies of NAFLD as a risk factor for cardiovascular diseases. Only imaging- or histology-based NAFLD are included.

<table>
<thead>
<tr>
<th>Author</th>
<th>Diagnosis of NAFLD</th>
<th>Study population</th>
<th>Length of follow-up (years)</th>
<th>Main results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Targher, et al.</td>
<td>ultrasound</td>
<td>Middle-aged DM2 outpatient cohort, n = 2,103 (Italian)</td>
<td>6.5 (mean)</td>
<td>1.9-fold risk of fatal or non-fatal CVD event with NAFLD after multiple adjustment</td>
</tr>
<tr>
<td>Haring, et al.</td>
<td>ultrasound + GGT</td>
<td>Middle-aged, with elevated GGT, n = 4,160 (German)</td>
<td>7.3 (median)</td>
<td>2.4-fold risk of fatal CVD event among men after multiple adjustment (no statistical difference in women)</td>
</tr>
<tr>
<td>Hamaguchi, et al.</td>
<td>ultrasound</td>
<td>Middle-aged, community-based healthy cohort, n = 1,637 (Japanese)</td>
<td>5.8 (mean)</td>
<td>4.1-fold risk of non-fatal CVD events in subjects with NAFLD vs. without NAFLD after multiple adjustment.</td>
</tr>
<tr>
<td>Wong, et al. (V. W. Wong et al., 2016)</td>
<td>ultrasound</td>
<td>Middle-aged subjects and senior citizens requiring coronary angiogram, n = 612 (Hong Kong Philippines)</td>
<td>6 (mean)</td>
<td>No increased risk of mortality or cardiovascular complications in NAFLD subjects</td>
</tr>
<tr>
<td>Valbusa, et al.</td>
<td>ultrasound</td>
<td>Senior citizens, patients hospitalized for acute HF, n = 107 (Italian)</td>
<td>1 (not specified)</td>
<td>NAFLD independently predicts 5.5-fold risk of 1-year rehospitalization (97% of cardiac etiology) in patients hospitalized for acute HF</td>
</tr>
<tr>
<td>Zhou, et al. (Y. J. Zhou et al., 2012)</td>
<td>ultrasound</td>
<td>Middle-aged, adolescents included, community-based, n = 624 (Chinese)</td>
<td>4 (median)</td>
<td>Annual CVD mortality rate is about 3-fold higher in NAFLD subjects than non-NAFLD subjects (0.54% vs. 0.17%)</td>
</tr>
</tbody>
</table>
Obese/metabolic NAFLD is an epiphenomenon for insulin resistance, which, in turn, is a traditional risk factor for CVDs. Additionally, NAFLD entails several other risk factors for CVDs (Figure 10) (Lonardo et al., 2016). However, there is growing evidence that NAFLD is also an active player in the pathogenesis of subclinical atherosclerosis independent of traditional CVD risk factors (Lonardo et al., 2016; Oni et al., 2013). For instance, in a meta-analysis of 27 cross-sectional studies, NAFLD was independently, i.e., not related to traditional risk factors or metabolic syndrome, associated with carotid intima media thickness, coronary calcification, endothelial dysfunction and arterial stiffness (Oni et al., 2013). In the ROMICAT II Trial, CT-defined NAFLD was associated with 2.1-fold risk of the presence of high-risk coronary plaque, even after adjusting for most essential traditional risk factors (age, BMI, sex, hypertension, diabetes, dyslipidemia, current

<table>
<thead>
<tr>
<th>Author</th>
<th>Diagnosis of NAFLD</th>
<th>Study population</th>
<th>Length of follow-up (years)</th>
<th>Main results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stepanova, et al. (Stepanova &amp; Younossi, 2012)</td>
<td>ultrasound</td>
<td>Middle-aged, community-based, n = 11,613 (USA)</td>
<td>14.3 (median)</td>
<td>1.23-fold risk of CVD events in NAFLD subjects after multiple adjustment. No difference in CVD mortality.</td>
</tr>
<tr>
<td>Ekstedt, et al. (Ekstedt et al., 2015)</td>
<td>biopsy</td>
<td>Middle-aged, all with elevated transaminases, n = 229 (Sweden), a matched reference population from national registries</td>
<td>26.4 (mean)</td>
<td>1.6-fold risk of CVD events in all NAFLD subjects. In the subanalysis, only subjects with fibrosis grade F3-F4 were at increased CVD risk (4.4-fold) compared to the reference population.</td>
</tr>
<tr>
<td>Fracanzani, et al. (Fracanzani et al., 2016)</td>
<td>ultrasound</td>
<td>Middle-aged, NAFLD subjects in tertiary liver center with matched controls from the same region, n = 375 (Italian)</td>
<td>10 (not specified)</td>
<td>19% of NAFLD subjects vs. 10% of controls developed CVD</td>
</tr>
<tr>
<td>El-Azeem, et al. (El Azeem et al., 2013)</td>
<td>ultrasound</td>
<td>Middle-aged, obese subjects without prior CVD events, n = 747 (Saudi-Arabian)</td>
<td>3 (not specified)</td>
<td>51% of NAFLD subjects vs. 23% of non-NAFLD subjects developed CVD event. No multiregression analysis available.</td>
</tr>
</tbody>
</table>
or former smoking) (Puchner et al., 2015). It is largely accepted that the more progressive the NAFLD, the greater the risk of CVDs (Oni et al., 2013; Sun et al., 2015) The increased risk of CVDs above the traditional risk factors may at least partly be explained by the development of subclinical atherosclerosis, endothelial dysfunction and cardiac dysfunction, which may be mediated by the toxic effects of systemic inflammation and oxidative stress (Lonardo et al., 2016; Oni et al., 2013; Puchner et al., 2015). It is speculated that the co-existing oxidative stress, rather than NAFLD per se, is the reason for the increased CVD risk (Lonardo et al., 2016; Morling et al., 2015). In addition to the traditional risk factors, NAFLD may provoke autonomous dysfunction (Y. C. Liu et al., 2013), hypertension (Byrne & Targher, 2015) and expose to hypercoagulable state as injured hepatocytes release inflammatory and coagulation factors (Byrne & Targher, 2015; Petaja & Yki-Jarvinen, 2016). The pro-coagulative state, however, has recently been associated with metabolic syndrome rather than NAFLD (Lallukka et al., 2017). It is also proposed that only the severe histologic forms of NAFLD expose to CVD events (Ekstedt et al., 2015; Lonardo et al., 2016; Puchner et al., 2015). However, it is important to note that while age is an essential risk factor of atherosclerosis, a long exposure to NAFLD is also needed for the development of its more severe forms (Lonardo et al., 2015; Lonardo et al., 2016; Wainwright & Byrne, 2016).
Fig. 10. The myriad of CVD risk factors in patients with NAFLD. Patients with NAFLD have the typical traits of the metabolic syndrome and have multiple non-traditional risk factors and risk markers for CVD (Lonardo, Soo koian, Pirola, & Targher, 2016, published by permission of Elsevier).

2.9.1 Association between NAFLD and AF

AF is the most common sustained cardiac arrhythmia with an estimated prevalence of 1–4% in the Western world. However, due to a silent and asymptomatic tendency, the true prevalence may be even higher (Rahman, Kwan, & Benjamin, 2014). The estimated life-time risk of AF is about 25% (Heeringa et al., 2006; Lloyd-Jones et al., 2004) and the prevalence nearly doubles with each decade of life (Heeringa et al., 2006; Rahman et al., 2014). Other major risk factors of AF development are male gender, obesity, smoking, hypertension, diabetes, CHD, heart failure, valvular diseases, alcohol overuse and hyperthyroidism (Rahman et al., 2014). There is also a growing body of evidence of the association between AF and inflammatory states (Aviles et al., 2003; Chung et al., 2001; Y. Guo, Lip, & Apostolakis, 2012; Harada, Van Wagoner, & Nattel, 2015; Rahman et al., 2014). AF increases the risk of embolic stroke, heart failure, myocardial infarction, dementia and chronic kidney
disease (Healey et al., 2016; Rahman et al., 2014). The most feared complication among these, embolic stroke, has an average annual risk of 4.4% in subjects with AF but without proper anticoagulation therapy (Gage et al., 2001). These comorbidities are the reasons behind the decrease of quality of life and the increase of total mortality. For instance, the age-adjusted relative risk of death attributable to AF is reported to be 1.6–1.9 (S. S. Chugh et al., 2014; Ohsawa et al., 2007; Suzuki et al., 2011; T. Yamashita, 2013) and according to the Euro Heart Survey, the average annual risk of death in subjects with AF is as high as 5.3%, two-thirds of which are of cardiovascular etiology (Nieuwlaat et al., 2008). Thus, the AF-related human burden and the public health care costs are vast (Rahman et al., 2014).

There are some studies that link NAFLD and AF. Firstly, elevated hepatic transaminase concentrations have been shown to be independently associated with increased incidence of AF in a prospective study (Sinner et al., 2013). Likewise, there has been a linear association between GGT values and the risk of AF in two cross-sectional studies (Alonso et al., 2014; Markus et al., 2016). Although subjects with NAFLD often have normal liver enzymes (Clark, Brancati, & Diehl, 2002; Mofrad et al., 2003), NAFLD, due to its high prevalence in the general population, is the most common etiology of elevated transaminases and GGT (Clark et al., 2002). Thereby these studies give indirect clues of the association of NAFLD and the risk of AF. Moreover, NAFLD has been shown to predict later AF incidence with over 6-fold risk (after multiple adjustments) as compared to non-NAFLD (Targher et al., 2013). Correspondingly, NAFLD has been associated with about 6-fold greater odds (after multiple adjustments) with AF than non-NAFLD (Targher et al., 2013). In both of these studies, NAFLD was ultrasound-verified and the studies were performed in Italian subjects with T2D. Of note, the association between NAFLD and AF may partly explain the risk of stroke that NAFLD is shown to imply.

The possible causative links between these two conditions may be diverse. First, there is an association between inflammation and NAFLD as inflammation is shown to take part in the NAFLD progression (Buzzetti et al., 2016; Tilg & Moschen, 2010) and hepatic fibrosis (Czaja, 2014; S. L. Friedman, 2008; U. E. Lee & Friedman, 2011). Vice versa, NAFLD is associated with the release of systemic inflammatory parameters (Foroughi et al., 2016; Ndumele et al., 2011; Nigam et al., 2013; Oruc et al., 2009; Targher et al., 2008). Simultaneously, AF has been linked to being a cause (Y. Guo et al., 2012; Wijesurendra & Casadei, 2015) and a consequence (Aviles et al., 2003; Chung et al., 2001; Y. Guo et al., 2012; Gutierrez & Van Wagoner, 2015; Harada et al., 2015) of the systemic inflammation. Second, obesity and NAFLD are strongly associated as depicted elsewhere in this thesis. In addition to that, obesity
is also a risk factor of AF (Tsang et al., 2008; T. J. Wang et al., 2004), which may explain the association as well. However, in the study by Targher et al., NAFLD remained as an independent predictor of AF even after adjustment for several known AF risk factors, such as BMI (Targher et al., 2013). Third, NAFLD has a putative role in the development of autonomic dysfunction (Y. C. Liu et al., 2013; Newton, Pairman, Wilton, Jones, & Day, 2009; Sun et al., 2015), which predicts AF (H. W. Park et al., 2012; Perkiomaki et al., 2014; M. J. Shen & Zipes, 2014). Fourth, NAFLD is shown to induce diastolic dysfunction (Bonapace et al., 2012; Fotbolcu et al., 2010; Graner et al., 2014; J. Y. Jung et al., 2017; Mantovani et al., 2015; Petta et al., 2015), which provokes AF (Nagarakanti & Ezekowitz, 2008; Tsang et al., 2002; Uetake et al., 2016). Additionally, the associations between obesity and autonomic dysfunction (Indumathy et al., 2015; Sztajzel et al., 2009), systemic inflammation and autonomic dysfunction (Luttmann-Gibson et al., 2010), obesity and diastolic dysfunction (Lavie et al., 2013) as well as obesity and systemic inflammation (Bleau, Karelis, St-Pierre, & Lamontagne, 2015; Exley, Hand, O’Shea, & Lynch, 2014; Greenberg & Obin, 2006) make the network even more complex. NAFLD is also associated with impaired atrial conduction properties (Ozveren et al., 2016) and structural and metabolic alterations in myocardial tissue potentially providing substrate for arrhythmias (Ballestri et al., 2014). Thus, there are several possible causative paths, some of them bidirectional, between NAFLD and AF (Figure 11).

Fig. 11. The possible mechanistic links between NAFLD and atrial fibrillation (AF).
2.10 NAFLD and other comorbidities

There are some diseases shown to be associated with NAFLD, such as a wide range of extrahepatic cancers (Sanna, Rosso, Marietti, & Bugianesi, 2016), periodontitis (Han, Sun, & Yang, 2016), psoriasis (Mantovani, Gisondi, Lonardo, & Targher, 2016) and celiac disease (Reilly, Lebwohl, Hultcrantz, Green, & Ludvigsson, 2015). Also sleep apnea, osteoporosis and polycystic ovary syndrome have been reported to be associated with NAFLD (Byrne & Targher, 2015). Bearing in mind the leaky gut theory, it is of interest that in a small cross-sectional study the severity or extent of inflammatory bowel diseases (IBDs), whether colitis ulcerosa or Crohn’s disease, were not associated with the severity of NAFLD as assessed by NFS (Carr et al., 2017). However, correlation between IBD disease severity and the existence of metabolic syndrome was noted (Carr et al., 2017). According to another study, IBD patients with older age and longer disease duration (over 20 years) are at greater risk of NAFLD as compared to the IBD patients with younger age and shorter disease duration. Moreover, individuals with IBD seem to have NAFLD with fewer metabolic risk factors than the individuals without IBD while individuals receiving anti-TNF-α therapy for IBD are at a lower risk of NAFLD (Glassner, Malaty, & Abraham, 2017; Sourianarayanane et al., 2013).

2.11 Management of NAFLD

Lifestyle modification with weight reduction in overweight or obese people, physical activity and diet control is at the core of all management of NAFLD. At present, the pharmacotherapeutic agents available for NAFLD are scarce, but some potential new drugs are seen in the horizon. The agents are targeting insulin resistance, weight reduction and fibrotic or inflammatory processes. Bariatric surgery or liver transplantation may be used for selected patients.

2.11.1 Lifestyle modification

There is a strong epidemiologic and pathogenetic relationship between sedentary lifestyle and NAFLD as well as between physical inactivity and more severe NASH, which makes lifestyle modification essential in the treatment of NAFLD (European Association for the Study of the Liver (EASL) et al., 2016; Mahady & George, 2016; Yki-Jarvinen, 2014; Zelber-Sagi, Ratziu, & Oren, 2011). According to European guidelines, moderate-intensity aerobic physical activities
are generally preferred (European Association for the Study of the Liver (EASL) et al., 2016). The guidelines also state that there is a dose-effect relationship between the benefit of physical activity and NAFLD improvement. Both aerobic and resistance activity are beneficial, and high levels of physical inactivity should be avoided (European Association for the Study of the Liver (EASL) et al., 2016; Hashida et al., 2017). Moreover, it is reported that time spent in sedentary activity is an independent predictor of NAFLD irrespective of physical activity levels (Mahady & George, 2016). In a 48-week randomized control trial, weight loss of at least 7% of original body weight in response to a lifestyle intervention improved steatosis, lobular inflammation, ballooning and NAS but not fibrosis (Promrat et al., 2010). Vilar-Gomez et al. showed the dose-effect relationship between lifestyle change-triggered weight loss and histologic improvement in NASH (Vilar-Gomez et al., 2015). These findings were confirmed later (Harrison, Fecht, Brunt, & Neuschwander-Tetri, 2009). Studies of the impact of weight loss for persons with normal or only mildly elevated BMI are lacking (Mahady & George, 2016).

All kinds of hypercaloric diets increase while hypocaloric diets decrease fat accumulation on the liver (Yki-Jarvinen, 2015). However, different fats have different impacts: polyunsaturated and monounsaturated fatty acids are preferable, whereas saturated fats, in addition to other known harmful effects on inflammation, dyslipidemia and cardiovascular disease, also promote hepatic triglyceride accumulation, gluconeogenesis, insulin resistance and ATP concentration. They also induce peripheral insulin resistance in adipose tissue and skeletal muscle (Hernandez et al., 2017; Mahady & George, 2016; Yki-Jarvinen, 2015). According to the study by Luukkonen et al., overfeeding with saturated fat or carbohydrates induces de novo lipogenesis in a way that leads to overproduction of harmful fatty acids (shorter fatty acid chains) as compared to overfeeding with unsaturated fat. Moreover, the fat accumulation is the greatest with diet enriched with saturated fat, followed by overeating carbohydrates and unsaturated fat (nonsignificant difference between the latter two). Furthermore, the sources of the liver fat seem to differ as extra calories from carbohydrates stimulate de novo lipogenesis, while saturated fat induces adipose tissue lipolysis whereas unsaturated fat decreases it (P. Luukkonen et al., 2017). Similar findings are reported elsewhere as well as presented in Figure 12 (Yki-Jarvinen, 2015). Consistently, Mediterranean diet is recommended in the guidelines (European Association for the Study of the Liver (EASL) et al., 2016). Especially people with NAFLD should avoid abundant use of fructose, due to an association between
high fructose intake and NAFLD (Barrera & George, 2014; European Association for the Study of the Liver (EASL) et al., 2016; Mahady & George, 2016). The harmful effects of fructose may be explained by its metabolic and physiological effects. The metabolism of fructose is almost completely constricted to the liver as fructose is efficiently extracted from the portal blood at the first pass and there are signs that other tissues are not capable of metabolizing fructose (Tappy & Le, 2010). Furthermore, fructose does not seem to stimulate satiety to the extent glucose does (Teff et al., 2004) and it induces bacterial overgrowth in the small intestine increasing the endotoxin flow to the portal vein (de Wit, Afman, Mensink, & Muller, 2012). The consumption of nuts, omega 3 supplementation or probiotics may have beneficial roles in NAFLD but cannot be recommended due to insufficient data (Mahady & George, 2016). Of beverages, alcohol worsens liver histology (European Association for the Study of the Liver (EASL) et al., 2016) while coffee, due to its antioxidant, anti-inflammatory and antifibrotic effects (S. Chen, Teoh, Chitturi, & Farrell, 2014) and the evidence gathered from one prospective (Zelber-Sagi et al., 2015) and two cross-sectional studies (A. A. Modi et al., 2010; Molloy et al., 2012), is classified as protective against NAFLD in recently published European NAFLD guidelines, which, however, does not give any specific recommendations on the amounts of coffee use (European Association for the Study of the Liver (EASL) et al., 2016).
2.11.2 Pharmacotherapy

The recommendations of pharmacotherapy in NAFLD guidelines are unestablished. However, there are some interesting agents with potential benefit available and some promising agents are seen in the horizon. Because subjects with NAFLD are at increased risk of cardiometabolic diseases, the use of recommended pharmacotherapies in their management has an essential role (Barb, Portillo-Sanchez, & Cusi, 2016). Liver-specific pharmacotherapy should be assessed only for those with NASH, particularly those with significant fibrosis (≥ F2), or those at high risk of disease progression (presence of MetS or T2D, persistently increased transaminases or necroinflammation) (European Association for the Study of the Liver (EASL) et al., 2016).

Pioglitazone has profound positive effects on insulin sensitivity, hepatic inflammatory status and lipid metabolism, and upregulates plasma adiponectin
All these are shown to decrease the risk of NAFLD and NASH development (Buzzetti et al., 2016; Polyzos & Mantzoros, 2016; Yki-Jarvinen, 2014). There are two controlled studies which have both proved that pioglitazone improves steatosis, lobular inflammation and ballooning but does not induce fibrosis improvement (Belfort et al., 2006; Sanyal et al., 2010). Another controlled study shows quite the opposite: improvement in fibrosis but no improvement in steatosis or inflammation (Aithal et al., 2008). However, it is debated whether pioglitazone is associated with an increased risk of bladder cancer (Barb et al., 2016) but a recently published meta-analysis did not show this association (Monami, Dicembrini, & Mannucci, 2014). However, pioglitazone has potential adverse effects such as mild to moderate weight gain and exacerbation of congestive heart failure (Barb et al., 2016). It is also reported to be associated with bone loss in women (Barb et al., 2016). According to European guidelines, pioglitazone can be used for selected patients with NASH, particularly in T2D (European Association for the Study of the Liver (EASL) et al., 2016). It should be noted, however, that the use of pioglitazone is registered to T2D only. According to some expert opinions, it is may be advisable to add pioglitazone early on to patients with T2D and NASH (Barb et al., 2016).

Vitamin E is an antioxidant with several targets (Barb et al., 2016). Vitamin E (800 IU/day) has been shown to induce resolution of NASH more often than placebo but without statistical significance in the improvement of fibrosis (Sanyal et al., 2010). Similar finding was reported later among patients with biopsy-proven NASH. The number of diabetic patients was not reported although most patients were insulin resistant (Lavine et al., 2011). Thus, vitamin E has not been unambiguously tested in subjects with diabetes or advanced liver disease (Barb et al., 2016). Moreover, vitamin E may increase the risk of total mortality (Bjelakovic, Nikolova, Gluud, Simonetti, & Gluud, 2007), hemorrhagic stroke and prostate cancer in older males (Barb et al., 2016). The European guideline submits that the use of vitamin E seems to be safe in non-diabetic non-cirrhotic subjects and can be used in these patients with NASH, but further studies are needed (European Association for the Study of the Liver (EASL) et al., 2016).

Metformin and statins do not improve liver histology but both agents can be safely used in other indications in patients with NAFLD (Barb et al., 2016). Although with lack of evidence, orlistat may improve hepatosteatosis and NASH in proportion to weight loss (Barb et al., 2016). However, it does not seem to have direct effects on liver histology (Barb et al., 2016). Omega-3 polyunsaturated fatty acids, angiotensin converting enzyme inhibitors, sodiumglucose co-transporter...
2 inhibitors, dipeptidyl peptidase-4 inhibitors, ursodeoxycholic acid and pentoxifylline have all been proven to have either no impact on liver histology or no reliable evidence of the effect (Barb et al., 2016).

### 2.11.3 Potential future drugs

Several new treatment options for NAFLD and NASH are currently being evaluated and developed, specifically for NASH, targeting insulin resistance, dyslipidemia, hepatic inflammation or fibrosis (Ratziu, 2016). Of the possible future options, those with the most potential are discussed here.

Glucagon-like peptide-1 analogues, especially liraglutide, are promising in NASH treatment due to their potential to induce weight loss and insulin sensitivity, which may have a direct beneficial hepatic effect leading to decreasing hepatocyte triglyceride accumulation and fibrosis (Armstrong et al., 2013; Armstrong et al., 2016; Barb et al., 2016). However, more extensive and longer-term studies are needed until the role of Glucagon-like peptide-1 analogues can be established in the treatment of NASH (European Association for the Study of the Liver (EASL) et al., 2016).

Obeticholic acid is an agonist of farnesoid X receptor (Ijssennagger et al., 2016), which has several beneficial metabolic effects on the liver (Ratziu, Goodman, & Sanyal, 2015). A large multicenter study is now being conducted to assess the long-term safety and efficacy of obeticholic acid (Barb et al., 2016) but the preliminary results are promising even though pruritus and unfavourable cholesterol profile may cast doubts on the long-term safety (Neuschwander-Tetri et al., 2015).

Elafibranor, a dual agonist of peroxisome proliferator-activated receptor α and δ, is being studied as a NASH treatment (Barb et al., 2016; Souza-Mello, 2015). To date, there are conflicting reports on its efficacy but it may have favourable cardiovascular effects (Ratziu et al., 2016).

Cenicriviroc is a selective inhibitor of chemokine-chemokine receptors 2 and 5, which are expressed on the surface of Kupffer cells, macrophages and hepatic stellate cells. Originally, cenicriviroc was developed as an anti-HIV agent but due to its actions in the liver there is a rationale for its use in NASH (Ratziu, 2016) due to antifibrotic and anti-inflammatory effects (Lefebvre, Moyle et al., 2016; Ratziu, 2016). There have not been any safety concerns with cenicriviroc and it has been well tolerated (Lefebvre et al., 2016; Thompson et al., 2016). The large double-blind, randomized, multinational phase 2b CENTAUR trial is currently ongoing. The first interim analysis will be presented at one year (S. Friedman et al., 2016).
Recently, an interesting detail was presented regarding the level of circulating proprotein convertase subtilisin/kexin type 9 (PCSK9). It was shown to associate with increased *de novo* lipogenesis and severity of hepatosteatosis. Furthermore, PCSK9 levels are associated with hepatic fat accumulation (Ruscica *et al.*, 2016). Thereby, it will be interesting to see whether PCSK9 inhibitors, a novel hypercholesterolemia treatment (Stoekenbroek, Kastelein, & Huijgen, 2015), have an effect on fat accumulation on the liver.

### 2.11.4 Bariatric surgery

Bariatric surgery aims to reduce excess body weight, improve quality of life and avoid obesity-related morbidities. Today, bariatric surgery is indicated if BMI is at least 35 kg/m² with co-morbidities or at least 40 kg/m² without co-morbidities and conventional body weight control has not succeeded (Hahl, Peromaa-Haavisto, Tarkiainen, Knutar, & Victorzon, 2016). At present, the most widely used procedures are Roux-en-Y gastric bypass and laparoscopic sleeve gastrectomy (Angrisani *et al.*, 2015; Bachler, le Roux, & Bueter, 2016; Victorzon & Salminen, 2015).

Bariatric surgery induces remission of T2D, improves the quality of life, have beneficial effects on hypertension and plasma lipids and reduces total mortality and cardiovascular mortality when compared to controls (Aguilar-Olivos, Almeda-Valdes, Aguilar-Salinas, Uribe, & Mendez-Sanchez, 2016; Driscoll, Gregory, Fardy, & Twells, 2016; Lindekiilde *et al.*, 2015; Magallares & Schomerus, 2015; Mingrone & Castagneto, 2009; Pontiroli & Morabito, 2011; Ribaric, Buchwald, & McGlennon, 2014).

According to Lassailly *et al.*, hepatosteatosis is improved after all types of procedures. Moreover, Roux-en-Y gastric bypass seemed to have favorable outcomes in all kinds of NAFLD spectrum states. However, the evidence is still scarce, prompting the authors to conclude that ‘additional studies are required to determine whether fibrosis progresses in patients treated with current surgical procedures’ (Lassailly, Caiazzo, Pattou, & Mathurin, 2013; Lassailly *et al.*, 2015). These findings are confirmed elsewhere (Bower *et al.*, 2015; Klebanoff *et al.*, 2017). According to Europeans guidelines from the year 2016, ‘by improving obesity and diabetes, bariatric surgery reduces liver fat and is likely to reduce NASH progression; prospective data have shown an improvement of all histological lesions of NASH, including fibrosis’ (European Association for the Study of the Liver (EASL) *et al.*, 2016). Roux-en-Y gastric bypass and other bariatric surgery procedures containing a malabsorptive component induce greater weight loss and disappearance of NASH than restrictive procedures such as banding or sleeve gastrectomy (Buchwald *et al.*, ...)
The difference may result from that Roux-en-Y gastric bypass acts through several mechanisms (Lassailly et al., 2013; J. V. Li et al., 2011; Scholtz et al., 2014).

### 2.11.5 Liver transplantation

Today, NASH-related cirrhosis is the second or third leading cause of liver transplantation even though some NASH-elicited cirrhosis cases are labeled as cryptogenic cirrhotics (European Association for the Study of the Liver (EASL) et al., 2016; Khan & Newsome, 2016; R. J. Wong et al., 2015), and it is predicted to become the leading cause within the next few years (Charlton et al., 2011). Transplantation can be considered in selected subjects in end-stage liver disease or HCC according to general indications for transplantation (C. M. Ho et al., 2016; Khan & Newsome, 2016; Newsome et al., 2012). After transplantation, the overall one-year survival in NASH patients is 84–91% and three-year survival is about 78–80%, which are similar to other etiologies (Charlton et al., 2011; Khan & Newsome, 2016; X. Wang et al., 2014). However, in the subanalysis, the subjects with NASH had a higher risk of mortality from cardiovascular or infectious causes but a lower risk of graft failure than those without NASH (X. Wang et al., 2014). The transplant failure rate at 10 years is around 10% and at 20 years around 45% (European Association for the Study of the Liver (EASL) et al., 2016). The preoperative evaluation is similar to other indications, but, due to increased risk of cardiovascular mortality, cardiovascular evaluation is essential (Khan & Newsome, 2016).
3  Aims of the study

The specific goals of the thesis are to:
   Explore whether NAFLD independently predicts AF in the long-term follow-up in a middle-aged, community-based population (Study I)
   Evaluate the impact of MetS on the cardiometabolic outcomes of individuals with NAFLD in a middle-aged, community-based population (Study II)
   Investigate the association between AF and liver fibrosis in an elderly Finnish population (Study III)
   Assess the effects of PXR activation on intrahepatic triglyceride fat accumulation and serum metabolomic signature in young healthy volunteers (Study IV)
4 Methods

4.1 Study participants

4.1.1 OPERA cohort (Studies I-III)

This thesis aims at expanding the knowledge of pathophysiology, lipid metabolism and cardiovascular and metabolic complications of NAFLD.

The OPERA (Oulu Project of Elucidating the Risk of Atherosclerosis) study, originally planned to investigate the risk factors of atherosclerotic cardiovascular diseases, was established in the early 1990s (Rantala et al., 1999). The OPERA cohort was based on 600 individuals (300 men, 300 women) with hypertension diagnosis and a verified need for antihypertensive medication along with their 600 age- and sex-matched controls. The individuals with hypertension were randomly selected from the Register of the Social Insurance Institute for the reimbursement of hypertension medication whereas the controls were randomly selected from the National Health Register (all inhabitants included). All men were recruited between December 1990 and May 1992 and the women about one year later. All individuals were initially aged 40–59 years and were living in the city of Oulu, Finland. Of a total of 1,200 individuals, 1,045 participated in the study. Of the participants, 520 were men (261 from the hypertension group, 259 from the control group) and 525 were women (258 from hypertension group, 267 from control group). Thus, the overall participation rate was 87.1% (Rantala et al., 1999). The participants gave a written informed consent for the use of their clinical records in the present study and standardized health questionnaires were completed.

Subjects with heavy alcohol drinking (≥ 210g a week in men or ≥ 140g a week in women) were excluded from the present study (European Association for the Study of the Liver (EASL) et al., 2016). After this, there were 969 subjects left in the study cohort. However, there was no ultrasonography data for 11 participants. The final study sample thus included 958 participants. Of the participants, 472 subjects (49.3%) were from the hypertensive group and 486 (50.7%) were from the control group; of the participants 450 (47.0%) were men and 508 (53%) were women. The mean age of the patients was 51.3 years (range 40.2–62.0 years). The final study sample of 958 participants was used in Studies I and II.

Altogether 600 participants of the OPERA study were alive and available for the scheduled control visit during 2013–2014. Liver transient elastography
was performed for 86 of these 600 participants. After excluding five participants with excess alcohol intake at the baseline or control visit and five participants with unreliable transient elastography measurement due to obesity, 76 participants were available for the Study III. The patient selection criteria are provided in more detail later (Assessment of liver stiffness in Clinical and radiological methods).

4.1.2 Rifa-Stea (Study IV)

Based on power analysis, 16 healthy volunteers aged 18–40 years with BMI 18.5–25.0 kg/m² were recruited for the Rifa-Stea study that is a randomized, open, placebo-controlled crossover trial designed to assess the effect of PXR activation on fat content of the liver. The power analysis was calculated with $\alpha = 0.05$ and $1-\beta = 0.81$ assuming that the true difference in the hepatic fat fraction between treatments is 1.20 percentage points and that the standard deviation of the difference in the response variable is 1.56. A written, informed consent was obtained from each study participant.

The latter MRI fat fraction measurement for the last study subject failed for technical reasons, which went unnoticed for several weeks. A replacement volunteer was not recruited because it would not have changed the result of the study. Thus, there were 15 study subjects with successful MRI measurements.

Medical and psychiatric health was evaluated based on the patients’ medical records, history, physical examination and basic laboratory results. The exclusion criteria were: any regular medication (hormonal intrauterine device was allowed), any major somatic or psychiatric morbidity, systolic blood pressure over 150mmHg, general contraindications for MRI, insensitivity to rifampicin, continuous use of soft contact lenses (rifampicin may color), pregnancy or breast feeding, drug or alcohol abuse, history of difficult venipuncture and participation in any other medical study during the study or the past one month. In total, there were 5 women and 11 men. The means (standard deviation) of age, height, weight and BMI of the 16 participants were 23.2 (2.8) years, 177 (10) cm, 72.4 (11) kg and 23.0 (2.0) kg/m², respectively.
4.1.3 Rifa-BP and Rifa-1 (Study IV)

Althogether 22 participants of the Rifa-BP study and 12 participants of the Rifa-1 study were invited to participate in Study IV in order to analyze the effect of activated PXR on serum metabolomic signature. Originally, the Rifa-BP study (unpublished) and the Rifa-1 study (Rysa et al., 2013) were designed to evaluate effects of the PXR activation on 24-hour blood pressure and glucose metabolism among young and healthy individuals. The inclusion criteria for the Rifa-BP study were identical to those of the Rifa-Stea study except that BMI was 19.0–30.0 kg/m$^2$ and blood pressure was 95–140/< 90mmHg. In Rifa-1 study, there were no BMI and blood pressure limitations. Of the 34 participants, 11 were women and 23 were men. The mean age (standard deviation) of age, height, weight and BMI were 24.3 (4.1) years, 173 (10) cm, 72.1 (11) kg and 23.8 (2.4) kg/m$^2$.

4.2 Clinical and radiological methods

4.2.1 Assessment of hepatosteatosis

In Studies I-III, the presence or absence of hepatosteatosis was determined by the liver-kidney contrast assessed with ultrasound (a Toshiba SSA 270 ultrasound system (Toshiba Corp., Tokyo, Japan), scanning frequency of 5 MHz) (Ballestri et al., 2015). All examinations were performed by one trained radiologist with long experience of abdominal ultrasound procedures. He was also blinded to other OPERA data.

In Study IV, the hepatic fat fraction was measured with 1.5T MRI (GE Healthcare Inc. Chicago, Illinois) shift with a commercially available sequence (IDEAL IQ) (GE Healthcare Inc.) (Meisamy et al., 2011; Yu et al., 2011). A proper axial slice of the liver was selected and three regions (size 1.5 cm$^2$) of normal parenchyma were targeted, avoiding vessels. Mean of the three measurements was calculated for the final hepatic fat fraction.

4.2.2 Assessment of liver stiffness

Liver transient elastography (Echosens Fibroscan 402, Paris, France) was used to assess liver stiffness (Friedrich-Rust et al., 2008; Petta et al., 2011; V. W. Wong et al., 2010). The 76 study subjects were selected evenly from four subgroups: subjects with AF and NAFLD (n = 18), with AF but not NAFLD (n = 18), with NAFLD but
without AF (n = 20) and those without either condition (n = 20), aiming to ensure that the basic characteristics (age, gender, BMI) were as similar as possible. The examinations were performed in 2015 by an experienced gastroenterologist who did not know the other OPERA data. The examinations were performed in the morning after an overnight fast. At least 10 successful measurements with the M probe were required. All measurements were done from the right-sided central axis line.

For subjects with NAFLD, liver stiffness was also estimated with NFS, a proxy surrogate for liver fibrosis in NAFLD (Angulo et al., 2007; European Association for the Study of the Liver (EASL) et al., 2016). The NFS was calculated with a calculator freely available on the Internet (www.nafldscore.com) for each OPERA study subject with NAFLD (n = 129) at the follow-up visit during 2013–2014.

4.2.3 *Echocardiographic assessment of the heart*

At baseline, all echocardiographic M-mode, two-dimensional and Doppler examinations were performed with a Hewlett-Packard 77020A ultrasound color system, Sonos 500 (Hewlett-Packard Company, Massachusetts, USA) and during the follow-up visit, with a GE Healthcare Vivid E 9 VERSION 110. x.x ultrasound (Lang et al., 2015). The examinations were done by two experienced cardiologists, one at the baseline and another during the OPERA follow-up visit. Both cardiologists were blinded to all other OPERA-related data. The formula of Troy (Troy, Pombo, & Rackley, 1972) was applied to calculate the left ventricular mass. Left ventricular mass index (LVMI) was determined by dividing left ventricular mass by body surface area.

4.2.4 *Ascertainment of outcome events*

Study I was a prospective study in which new AF events were followed. Study II was another prospective study in which CVDs (see later in this chapter), new T2D events and the change of the left ventricular mass index were followed.

The data on CVD and AF events were obtained from national healthcare registers, including the National Death Registry, the Hospital Discharge Register and the Care Register for Health Care (HILMO) of the National Institute for Health and Welfare. Even though AF may be silent and paroxysmal, the adequate validity of this method in AF studies has been shown in epidemiological studies (Alonso et al., 2009; Jensen et al., 2012). The follow-up time for men began at the time of the OPERA recruitment study visit between December 1990 and May 1992, and for
women approximately one year later. The follow-up ended on December 31, 2009 or whenever the first event occurred. Thus, the mean follow-up time of CVDs and AF in Studies I and II was more than 16 years.

In Study I, the diagnosis of AF was based on standard 12-lead resting ECG and the AF diagnosis was made if ICD-code I48 (atrial flutter included) was listed in the above mentioned registers during the follow-up time.

In Study II, CVD events included major CHD events and strokes (subarachnoid hemorrhage and transient ischemic attack excluded), whichever occurred first. The evidence of CHD was based on the ICD codes as follows: I20.0, I21, I22 (ICD-10) / 410, 4110 (ICD-8/9) as the primary diagnosis (symptom or cause) and I21, I22 (ICD-10) / 410 (ICD-8/9) as the first or second secondary diagnosis (symptom or cause) and the third secondary diagnosis (ICD-8/9 only) or if coronary artery bypass grafting or coronary angioplasty was done. CHD as the cause of death included I20–I25, I46, R96, R98 (ICD-10) / 410–414, 798 (not 7980A) (ICD-8/9) as the underlying cause of death or the immediate cause of death and I21 or I22 (ICD-10) / 410 (ICD-8/9) as the first to third contributing cause of death. Stroke (excluding subarachnoid hemorrhage and transient ischemic attack) included I61, I63 (not I636), I64 (ICD-10) / 431, 4330A, 4331A, 4339A, 4340A, 4341A, 4349A, 436 (ICD-9) / 431 (except 43101, 43191) 433, 434, 436 (ICD-8) as the primary diagnosis (symptom or cause) or as the first or second secondary diagnosis (symptom or cause) or as the third secondary diagnosis (ICD-8/9 only) or as the underlying cause of death or immediate cause of death or as the first to third contributing cause of death.

T2D status was assessed in all studies according to the World Health Organization criteria (fasting plasma (blood) glucose value repeatedly ≥ 7.0 mmol/L, a 2-h oral glucose tolerance test > 11.0 mmol/L, HbA1C ≥ 48mmol/L (or ≥ 6.5%), or fasting glucose ≥ 11.0 mmol/L with diabetes-related symptoms) (Alberti & Zimmet, 1998; Laakso et al., 2016) and was based on patients’ medical records or a 2-hour oral glucose tolerance test (2h-OGTT) (Alberti & Zimmet, 1998; Laakso et al., 2016; Pisto, Ukkola, Santaniemi, & Kesaniemi, 2011). For the follow-up data in Study II, patients’ medical records were checked, 2h-OGTT was performed and LVMI was measured during the OPERA control visit in 2013–2014. Thus, the mean follow-up time for T2D and LVMI in Study II was about 21 years.

In Study III, the diagnosis of AF was based on the same criteria as in Study I. The diagnosis was made if AF was listed in the patients’ medical records at the follow-up visit during 2013–2014. The diagnosis was checked by a specially trained nurse.
In Study II, the diagnosis of MetS was based on the criteria of the International Diabetes Federation (IDF) definition for MetS (Alberti et al., 2009; Kassi et al., 2011). According to these criteria, central obesity is required for the diagnosis of MetS (waist circumference ≥ 94 cm for men and ≥ 80 cm for women) plus any two of the following factors: triglycerides ≥ 1.7 mmol/L, HDL cholesterol < 1.0 mmol/L (men) or < 1.3 mmol/L (women), blood pressure ≥ 130/ ≥ 85 mmHg and fasting glucose ≥ 5.6 mmol/L. From several MetS criteria, IDF criteria were chosen due to the imperative role of central obesity, which may simplify clinical decision making. In Study II, the presence or absence of MetS was assessed at baseline and during the follow-up visit.

4.3 Laboratory and other methods

All blood samples were taken after an overnight fast by a vein puncture. Blood glucose was measured with the glucose dehydrogenase method (Diagnostica, Merck, Darmstadt, Germany) and plasma insulin using a two-site immunoenzymometric assay (AIAPACK IRI, Tosoh Corp., Tokyo, Japan).

After fasting venous blood had been drawn, 2h-OGTT was carried out by measuring blood glucose concentrations at 0, 60, and 120 min after oral administration of 75 g glucose (Pisto et al., 2011). Quick index (QUICKI) was used as a proxy for insulin sensitivity and was counted with the equation of 1/(log (fasting insulin)+log (fasting glucose)) (Katz et al., 2000).

After centrifuging plasma in a Kontron TFT 45.6 rotor at 105,000 g and 15°C for 18 hours, VLDL (d < 1.006 g/mL) was isolated. Altogether 500 μL of the VLDL-free fraction was mixed with 25 μL of 2.8% (w/v) heparin and 25 μL of 2 M manganese chloride. After centrifugation at 1,000 g and 4°C for 30 minutes, aliquots of the supernatant were analyzed for HDL concentration. After these procedures, LDL content was calculated by subtracting the cholesterol concentration in HDL from the cholesterol concentration in the VLDL-free fraction. The concentrations of total cholesterol and triglycerides in the plasma as well as lipoprotein fractions were measured using enzymatic colorimetric methods (kits of Boehringer Diagnostica, Mannheim GmbH, Germany, catalog nos. 236691 and 701912), respectively, by a Kone Specific, Selective Chemistry Analyzer (Kone Instruments, Espoo, Finland). The variation coefficients for the determination of total cholesterol, HDL cholesterol and triglycerides were 2.1%, 5.5% and 5.3%, respectively.
4.3.1 Genotyping of the I148M polymorphism of the PNPLA3 gene

PNPLA3 I148M (rs738409) polymorphism was reported in Study II. After DNA was extracted from peripheral blood mononuclear cells using the phenol-chloroform method, PNPLA3 I148M was genotyped by a TaqMan assay (assay on demand for rs738409; Applied Biosystems, Foster City, CA).

4.3.2 Proton nuclear magnetic resonance spectroscopy

The circulating concentrations of total lipids (sum of free and esterified cholesterol, triglycerides and phospholipids) and the particle concentration of 14 lipoprotein subclasses were measured for Study IV. Moreover, their five main lipid component (free, esterified and total cholesterol, triglycerides and phospholipids) concentrations were measured and reported (ratios of each lipid component in each subclass were not reported). Also low-molecular weight molecules (LMWM) were measured and reported. The measurements were performed using proton nuclear magnetic resonance spectroscopy (NMR). The lipoprotein subclasses were determined by their particle size. The fasting concentrations were measured from serum samples. Before the NMR measurements, serum and a sodium phosphate buffer 1:1 were mixed and the samples were preheated to 37.5°C due to some heat lost during the sample transfer into NMR inside the magnet (Soininen et al., 2009). A Bruker AVANCE III spectrometer operating at 500.36MHz was used (Soininen et al., 2009; Soininen, Kangas, Wurtz, Suna, & Ala-Korpela, 2015). In NMR spectroscopy, there are three molecular windows: LIPO, LMWM and LIPID (Soininen et al., 2015). LIPO detects macromolecules, such as lipoprotein subclasses, whereas LMWM identifies smaller molecules, e.g. ketone bodies, glycolysis-related metabolites and amino acids (Soininen et al., 2015). LIPO and LMWM windows work for native serum samples. After these measurements, the same samples go through a standardized lipid extraction procedure and the third window, LIPID, measures, for instance, fatty acids from lipid extracts (Soininen et al., 2015). The exact details of the NMR procedure and its validation in metabolomics studies are discussed elsewhere (Soininen et al., 2009; Soininen et al., 2015; Würtz et al., 2017).
4.3.3 Other methods

Blood pressure was measured with an automatic oscillometric blood pressure recorder (Dinamap, Critikon Ltd) from the right arm in a sitting position after an overnight fast and after 10 to 15 minutes’ rest. The means of the last two of three measurements, made at 1-minute intervals, were used in the analyses.

To calculate glomerulus filtration rate, the equation of Glomerular filtration rate by the Chronic Kidney Disease Epidemiology Collaboration was applied (Levey et al., 2009).

BMI (kg/m²) was calculated as weight (kg) in light indoor clothing divided by height squared (m²). Waist circumference was measured with a tape measure midway between the iliac crest and the lower rib margin in light expirium. The result was reported to accuracy of 0.5 cm.

Studies I-III, data on lifestyle factors were obtained from standardized health questionnaires. The questionnaires were completed by two specially trained nurses and the details were checked by a physician later during the same visit. The questionnaires were re-checked during the follow up visit. For instance, smoking habits, level of physical activity, alcohol consumption habits, dietary behavior and marital status were questioned. In Study IV, lifestyle factors were checked in an interview.

4.4 Statistical methods

In all studies, statistical analyses were performed using the IBM SPSS Statistics for Windows software, Version 23.0 (IBM Corp., Armonk, NY, USA). In Study IV, also GraphPad Prism software, version 7.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used to analyze the changes of each variables between the study arms. The normality of the distributions of the continuous variables was tested visually using histograms and the Kolmogorov-Smirnov test. In case of significant skewness (visual or p < 0.05 in Kolmogorov-Smirnov test), the analysis was performed using non-parametric tests such as the Mann-Whitney test or the Kruskal-Wallis test. Associations with a p-value < 0.05 were considered statistically significant.

The Analysis of Variance (ANOVA) and the t-test for independent samples were used to compare continuous variables and the chi-square test was used to compare categorical variables between the groups (Studies I—III). Post-hoc analysis was performed by Tukey’s method (Study II). The Kaplan-Meyer cumulative proportional probability (cumulative hazard or survival) curves and the Log Rank
test were used to evaluate the statistical significance of the separation of the curves (studies I and II). The estimated risk ratios and their 95% confidence intervals were measured with the Omnibus Test (Studies I and II). In Study I, Cox proportional hazards regression models were used to analyze estimated relative risk of AF between the study groups (participants with NAFLD vs. participants without NAFLD). Association was analyzed with forced Cox proportional hazards regression models unadjusted and with adjustment for age and sex (model 1); age, sex, original study group and diabetes status (model 2); and age, sex, original study group, diabetes status, BMI, waist, alcohol consumption, smoking, serum ALT, systolic blood pressure, QUICKI, CHD, atrial natriuretic peptide, LVMI, left atrial diameter and hs-CRP (model 3). In Study III, analysis of Covariance (ANCOVA) was used to compare the differences between a) left atrial diameter among transient elastography tertiles; b) transient elastography among the four subgroups; c) NFS among NAFLD patients with AF and NFS among NAFLD patients without AF with adjustments of BMI, age, sex, alcohol drinking, smoking, QUICKI and systolic blood pressure. In Study IV, the Wilcoxon test was used throughout the metabolomics data to compare the rifampicin arm and the placebo arm. The MRI variables between these study arms were compared with the two-sided t-test. To control the false discovery rate in the metabolomics data, the Benjamini-Hochberg procedure was used. The Hochberg correction was counted by SPSS Syntax with formula $p^*_i = \min(p(i+1), \frac{n}{i} p(i))$, $i = (n-1), \ldots, 1$, where $p(1) \leq p(2) \leq \ldots \leq p(n)$ is the number of variables. Both nominal and corrected p-values were reported, but the corrected p-values were preferred in the interpretation of the results. Associations with a corrected p-value of < 0.05 were considered statistically significant.

4.5 Ethical considerations

Studies I-III had been approved by the Ethics Committee of the Medical Department of Oulu University (48/2009). All participants gave a written informed consent for the use of their clinical records.

Study IV was approved by the Regional Ethics Committee of the Northern Ostrobothnia Hospital District, Oulu, Finland (Rifa-1: 78/2009, Rfa-BP: 6/2012, Rifa-Stea: 83/2014) and the Finnish Medicines Agency Fimea. The ethical standards of the Declaration of Helsinki and guidelines on Good Clinical Practice were followed. The study participants were financially compensated for taking part
in the study (120 euros per study subject). The clinical trials of Study IV were registered at ClinicalTrials.gov (Rifa-Stea NCT02329405, Rifa-1 NCT00985270, Rifa-BP NCT01690104). All participants gave a written informed consent for the use of their clinical records.
5 Results

5.1 NAFLD with and without MetS as a predictor of cardiovascular diseases, type 2 diabetes and increase of left ventricular mass (Study II)

Of all 958 participants of the OPERA study, 186 (19%) subjects had NAFLD and MetS, 63 (7%) had NAFLD but not MetS, 164 (17%) subjects had MetS but not NAFLD and 545 (57%) had neither of them. All characteristics of individuals in these four groups are depicted in detail in the original article (Study II). Of note, individuals having NAFLD and MetS had higher fasting insulin (p < 0.001), fasting glucose (p < 0.001) and plasma/serum liver enzyme levels (p < 0.001 for GGT and for ALT) as compared to individuals having NAFLD but not MetS. The I148M variant in PNPLA3 was most prevalent in individuals having NAFLD but not MetS (p = 0.008).

During the 16-years of follow-up, a cardiovascular event occurred in 54 (30%) of 186 individuals having NAFLD and MetS, in 12 (20%) of 63 individuals having NAFLD only, in 35 (22%) of 164 individuals having MetS only and in 63 (12%) of 545 individuals having neither of them (p < 0.001). Converting to HRs, individuals with both conditions had 2.8-fold risk of future CVD events and those with MetS only had 2.1-fold risk of future CVD events. These HRs remained statistically significant after adjustment for age, gender, original study group, LDL cholesterol, smoking and alcohol consumption. NAFLD without MetS, however, was not a statistically significant CVD event predictor (before adjustments 1.7-fold risk, p = 0.068). CVD survivals in all subgroups are presented as a Kaplan-Meyer curve in Figure 13.
Fig. 13. Cumulative survival from cardiovascular events in different study groups by Kaplan-Meier curves. Follow-up began in 1990-1993 and lasted until the end of the year 2009. Abbreviations: NAFLD, non-alcoholic fatty liver disease; MetS, metabolic syndrome

Altogether 566 individuals (59%) of all 958 individuals participated the control visits. Of these 566 individuals 93 (50% from the original subgroup) had NAFLD and MetS, 38 (60%) had NAFLD but not MetS, 92 (56%) had MetS but not NAFLD and 343 (63%) had neither NAFLD nor MetS. Individuals having NAFLD and MetS (44/47%) were more likely to develop T2D than individuals having only NAFLD (9/24%) (p = 0.029 for difference) and individuals having neither NAFLD nor MetS (66/19%) (p < 0.001 for difference) during about 16 years of follow-up. Individuals having only MetS (37/40%) were more likely to develop T2D than individuals having neither NAFLD nor MetS (p < 0.001 for difference).

The mean LVMI increased by 17.7 g/m² in individuals having NAFLD and MetS, by 6.1 g/m² in individuals having only NAFLD, by 15.5 g/m² in individuals having only MetS and by 4.5 g/m² in individuals having neither NAFLD nor MetS at baseline. The mean LVMI increased statistically significantly more in individuals having NAFLD and MetS (p = 0.001 for difference) and in individuals having only MetS (p = 0.006 for difference) than in individuals having neither NAFLD nor MetS.
NAFLD at the baseline predicted later MetS development, because 27 (71%) of individuals having NAFLD but not MetS at the baseline and 166 (48%) of individuals having neither NAFLD nor MetS at baseline had been diagnosed with MetS during the follow-up (p = 0.008).

Of note, there are several alternative criteria for MetS. In the present study, MetS was determined by IDF criteria. However, the main results were re-tested using the consensus criteria from year 2009, but the result remained the same (data not shown).

5.2 Non-alcoholic fatty liver disease and atrial fibrillation

5.2.1 NAFLD as a predictor of atrial fibrillation in middle-aged population (Study I)

The basic characteristics of patients with NAFLD and individuals without it are depicted in detail in the original article (Study I). The most important findings of these baseline data are that the prevalence of NAFLD among all participants of the OPERA study was 249/958 (26.0%) and patients with NAFLD were more obese (p < 0.001), had more abdominal fat (p < 0.001), smoked more (p = 0.002), drank more alcohol (p < 0.001), had higher systolic (p < 0.001) and diastolic (p < 0.001) blood pressure, higher values of GGT (p < 0.001), ALT (p < 0.001), hs-CRP (p < 0.001), total cholesterol (p = 0.022), triglycerides (p < 0.001) and fasting glucose (p < 0.001) but lower HDL cholesterol (p < 0.001) and QUICKI (p < 0.001) than those without NAFLD at the baseline. Moreover, diabetes (p < 0.001) and CHD (p = 0.034) were more prevalent among patients with NAFLD. Also left atrial diameter (p < 0.001) and LVMI (p = 0.001) were greater in patients with NAFLD than in individuals without it.

After the follow-up of about 16 years, AF had been diagnosed for 37 of 249 (14.9%) patients with NAFLD at baseline and for 56 of 709 (7.9%) individuals without it at baseline (p = 0.001). Thus, patients with NAFLD at baseline had about 2-fold greater risk of developing AF during the 16 years of follow-up (hazard ratio (HR) 1.96, 95%CI 1.29–2.97). The cumulative proportional probability (cumulative hazard) of AF in both groups is illustrated in Figure 14.
Patients with NAFLD had about 2-times higher likelihood of developing AF during about 16 years of follow-up adjusted for several known risk factors for AF (Table 3). However, when metabolic syndrome (by IDF criteria) was added to the Cox regression analysis as a confounding factor, NAFLD did not predict AF any more (data not shown).
Table 3. Association between NAFLD and risk of AF during follow-up. Values are expressed as ORs (95% CIs) as assessed by multivariate Cox regression analyses. Independent predictors of AF are highlighted in bold type. Relevant covariates were chosen as potential confounding factors. Both waist and BMI were added to separate abdominal obesity from total obesity. Abbreviations: BMI, body mass index; ALT, alanine aminotransferase; CHD, coronary heart disease; ANP, atrial natriuretic peptide; LVMI, left ventricular mass index; hs-CRP, high-sensitive C-reactive protein.

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Unadjusted model</th>
<th>Adjusted model 1</th>
<th>Adjusted model 2</th>
<th>Adjusted model 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatty liver (yes vs. no)</td>
<td>1.96 (1.29–2.97)</td>
<td>1.79 (1.18–2.71)</td>
<td>1.73 (1.09–2.73)</td>
<td>1.88 (1.03–3.45)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>1.09 (1.05–1.13)</td>
<td>1.09 (1.05–1.13)</td>
<td>1.06 (1.01–1.11)</td>
<td></td>
</tr>
<tr>
<td>Sex (male vs. female)</td>
<td>1.63 (1.07–2.49)</td>
<td>1.63 (1.07–2.49)</td>
<td>0.78 (0.32–1.90)</td>
<td></td>
</tr>
<tr>
<td>Study group (hypertensive vs. control)</td>
<td>1.12 (0.73–1.71)</td>
<td>0.70 (0.40–1.23)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes status (yes vs. no)</td>
<td></td>
<td>1.00 (0.53–1.91)</td>
<td>1.01 (0.48–2.13)</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td></td>
<td>0.91 (0.80–1.03)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Waist (cm)</td>
<td></td>
<td>1.03 (0.98–1.08)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alcohol consumption (grams/week)</td>
<td></td>
<td>1.00 (0.99–1.00)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking (pack-years)</td>
<td></td>
<td>1.00 (0.98–1.02)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum ALT (U/L)</td>
<td></td>
<td>1.00 (0.99–1.01)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td></td>
<td>1.01 (1.00–1.02)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quick index</td>
<td></td>
<td>0.93 (0.06–14.90)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHD (yes vs. no)</td>
<td></td>
<td>1.70 (0.86–3.39)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANP (pmol/L)</td>
<td></td>
<td>1.002 (1.000–1.003)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVMI (g/m²)</td>
<td></td>
<td>1.01 (1.00–1.02)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left atrial diameter (mm)</td>
<td></td>
<td>1.03 (0.97–1.09)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsCRP (mg/L)</td>
<td></td>
<td>1.00 (1.00–1.00)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
5.2.2 The association of atrial fibrillation and liver stiffness (Study III)

Of the 76 study participants there were 61 (80%) men and 15 (20%) women. The means (standard deviation) of the main characteristics of the study participants were: age 73 (5.3) years, BMI 30.1 (4.3) kg/m², alcohol consumption 37 (50) g/week, smoking 11 (19) pack years, systolic blood pressure 136 (21) mmHg and QUICKI 0.500 (0.083) L/mmol. The study participants with AF or without AF had no differences in gender (p = 0.952), age (p = 0.285), BMI (p = 0.964), systolic (p = 0.128) or diastolic (p = 0.346) blood pressure, alcohol consumption (p = 0.964) smoking (p = 0.639) and QUICKI (p = 0.914), whereas the participants with AF had larger left atrial diameter (p < 0.001), greater GGT (p = 0.011), lower albumin (p = 0.030) and LDL cholesterol (p = 0.023). All characteristics of the study participants by the presence of AF are depicted in detail in the original article.

After transient elastography values were converted to tertiles, it was noticed that the greater the tertile, the more prevalent AF was: in the first tertile there were 6 (27%) individuals with AF, in the second tertile 10 (36%) individuals had AF and in the third tertile 20 (77%) individuals had AF (p for trend = 0.001). Furthermore, the size of left atrial diameter, a pathognomonic phenomenon of AF, increased collaterally with growing transient elastography tertile: the mean (standard deviation) left atrial diameter in the first tertile was 39 (7) mm, in the second 45 (7) mm and in the third 49 (8) mm (p for trend < 0.001). The statistical significance prevailed even after multiple adjustments for BMI, age, gender, alcohol intake, smoking, QUICKI and systolic blood pressure (p = 0.012). Moreover, clinically relevant transient elastography value (> 8 kPa, (Koehler et al., 2016)) was present in 18 (50%) in individuals with AF, whereas there were only 5 (13%) individuals with clinically relevant transient elastography value in those without AF (p < 0.001). The mean transient elastography value was 9.3 kPa in individuals with AF and 6.3 kPa in those without AF (p = 0.018). The statistically significant difference prevailed even after multiple adjustments for BMI, age, gender, alcohol intake, smoking, QUICKI and systolic blood pressure (p = 0.005). The mean transient elastography value in both groups was also tested with non-parametric Mann-Whitney U test but the result was unchanged (p = 0.002).

The participants were also divided into four subgroups by the presence or absence of AF and NAFLD and the mean (standard deviation) transient elastography value was measured for the groups. The mean transient elastography was 5.3 (1.8) kPa in individuals who had neither NAFLD nor AF, 7.4 (4.8) kPa in individuals with NAFLD but not AF, 10.8 (9.0) kPa in individuals with both diseases and 7.8
(2.5) kPa for those without NAFLD but with AF (p = 0.019). After adjustments (BMI, age, gender, alcohol intake, smoking, QUICKI and systolic blood pressure), the groups still differed statistically significantly (p = 0.006). These results with descriptive characteristics of the groups are shown in the original article. After testing with non-parametric Kruskal-Wallis test the statistical significance remained (p = 0.004).

In total, there were 566 study participants from the original OPERA study available for control visit during 2013-2014. Of these participants, 129 (23%) individuals had NAFLD. The mean (standard deviation) age of the participants with NAFLD was 73 (5) years and BMI was 31.7 (5.5) kg/m²; 79 (61%) were men. The NFS (Angulo et al., 2007) was counted for those 129 individuals with NAFLD. The participants with AF had higher NFS (0.685) than the participants without AF (0.129) (p = 0.038 and after adjustments for BMI, age, gender, alcohol intake, smoking, QUICKI and systolic blood pressure, p = 0.037).

5.3 The effect of Pregnane X receptor on hepatic fat accumulation and lipid metabolism (Study IV)

The mean hepatic fat fraction was 2.45% (standard deviation 1.73, range 1.0–6.3%) after the rifampicin arm and 2.53% (2.40, 0.7–9.3%) after the placebo arm, the ratio of means of the hepatic fat fractions being 0.97 (p = 0.685).

Serum total cholesterol (+7%, p = 0.010), free cholesterol (+7%, p = 0.010), esterified cholesterol (+7%, p = 0.014), IDL cholesterol (+10%, p = 0.010), and LDL cholesterol (+12%, p = 0.020) as well as sphingomyelins (+7%, p = 0.045) and Apo-A1 (+3%, p = 0.041) increased more after the rifampicin arm than after the placebo arm. Of interest, serum total triglycerides changed decreased after the rifampicin arm as compared to the placebo arm but without statistically significant difference (-6%, false discovery rate (FDR) corrected p = 0.320). Presented p values are FDR corrected.

Of the measured fatty acids, omega-6 fatty acids (+5%, p = 0.033) and 18:2 linoleic acid (+5%, p = 0.045) increased more after the rifampicin arm than after the placebo arm. There were also trends towards larger increases in total fatty acids (+5%, p = 0.064), polyunsaturated fatty acids (+4%, p = 0.074) and 16:1 and 18:1 monounsaturated fatty acids (+6%, p = 0.094) after the rifampicin arm than after the placebo arm. These trends were statistically significant according to nominal p-values but not after FDR correction. Presented p values are FDR corrected.
Rifampicin administration also led to decreased levels of acetate (-16%, p = 0.010) and citrate (-10%, p <0.001) as compared to placebo. Presented p values are FDR corrected.

The original manuscript shows the illustrations of these main findings and the results of all 155 metabolite measurements.
6 Discussion

The main findings of the present thesis are illustrated in Figure 15.

Fig. 15. The main findings of the thesis. NAFLD predicts independently AF, which, in turn, is associated with liver stiffness as measured by transient elastography, a surrogate for liver fibrosis. Additionally, PXR activation may promote development of progressive NAFLD, that is, NASH and fibrosis. Simultaneously, NAFLD predicts CVDs, increase of left ventricular size and T2D only if MetS is also present. In other words, NAFLD without MetS does not predict these events. Abbreviations: AF, atrial fibrillation; CVDs, cardiovascular diseases; LVH, left ventricular hypertrophy; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; MetS, metabolic syndrome; PXR, pregnane X receptor; T2D, type 2 diabetes.

6.1 Methods

6.1.1 Study participants and study designs

One of the strengths of the OPERA study is its large and clinically well-defined cohort. Moreover, all study participants were randomly selected from the Social Insurance Institute register and the National Health Register, which cover all citizens
of Finland. However, half of the participants had hypertension. Additionally, there were some subjects in the control group with occult hypertension verified first at the recruitment study visit. Thus, the overall prevalence of hypertension in the subgroup of OPERA cohort used in Studies I and II (n = 958) was 51% (52% in men and 51% in women). The nation-wide prevalence of hypertension in Finland among middle-aged individuals has been reported to be 47% (Siven, Niiranen, Aromaa, Koskinen, & Jula, 2015), which is close to the prevalence in the cohort used in studies I and II. Thereby, studies I and II can be seen as very close to community-based studies. However, there was a slight preponderance of women (53%) in the current cohort used in Studies I and II, the total response rate in the total OPERA cohort was not complete (87%), and all subjects were living in the city of Oulu, which may cause some selection bias. Notably, people are not of equal health across Finland. For instance, the age-adjusted morbidity index is higher in Oulu than in Finland in general (Sipilä P. et al., 2014), which may affect the outcomes in the present studies. Moreover, socioeconomic status was not determined at the baseline. While it is associated with obesity and cardiometabolic morbidities (Bann, Johnson, Li, Kuh, & Hardy, 2017; Tang, Rashid, Godley, & Ghali, 2016), this must be seen as study limitation.

In the third study, a subgroup of the OPERA cohort (n = 76) was used to study the association between atrial fibrillation and liver stiffness. The participants were selected so that the basic characteristics (BMI, age, gender) would be as similar as possible in the four subgroups based on the presence or absence of AF and NAFLD. As a result, the participants were older and obese Finnish individuals and more often men than women. These factors may cause selection bias and limit the interpretation of the study results.

PXR activation was recently reported to impair glucose tolerance in young, healthy, non-obese study participants (Rysa et al., 2013). Thus, the aim of the fourth study was to investigate whether PXR activation induces hepatic fat accumulation among similar study participants providing an explanation to impaired glucose tolerance. However, interpretation of the study results may be questioned by whether a study with subjects with NAFLD or with sedentary lifestyle and thus with different hepatic metabolism would have resulted in different results. Moreover, the number of study subjects intended to participate the study (n = 16) was based on power analysis. Unfortunately, the MRI of the last study subject failed for technical reasons so that the final number of study subjects was 15, which may raise questions of whether the present study was underpowered. However, it was decided that a replacement volunteer would
not be recruited because it could be seen unethical to recruit a volunteer as the final result would not have changed from the MRI results of the first 15 participants.

6.1.2 Clinical, radiological and laboratory methods

The long-term follow-up of the outcomes using national healthcare registers, including the National Death Register and hospital discharge registry (HILMO), which collect clinical information all over the country, is another strength of the OPERA study. Although it is impossible to diagnose all AF cases because it is often asymptomatic and paroxysmal, the validity of this method to diagnose AF has been shown in epidemiological studies (Alonso et al., 2009; Jensen et al., 2012).

Liver transient elastography was used to assess liver stiffness in Study III. Liver stiffness is a non-invasive proxy surrogate for liver fibrosis. The most widely used cut-off for clinically relevant fibrosis in NAFLD, ≥ 8.0 kPa (Koehler et al., 2016), was used in this study. Transient elastography is validated in different NAFLD populations but also in several other liver diseases with liver biopsy as a reference (European Association for the Study of the Liver (EASL) et al., 2016; Jeong et al., 2014; Kwok et al., 2014). A profound understanding of the measurement techniques and operator experience are, however, needed to get reliable results (Castera et al., 2010). In the present study, the operator had long experience of the transient elastography measurements. Additionally, volume overload may be related to decompensated heart failure and is known to falsely increase transient elastography values (Mikolasevic et al., 2016). Eventhough none of the study participants were clinically decompensated, it is possible that AF may have promoted subclinical hepatic decompensation, which may have affected the transient elastography values. Transient elastography values may also be falsely increased in obese persons (Castera et al., 2010). This was taken into account by abandoning the results considered unreliable from obese subjects (n = 5). The NFS, in turn, has been validated to assess the presence of liver fibrosis in subjects with NAFLD, but not in other underlying etiologies (Angulo et al., 2007; European Association for the Study of the Liver (EASL) et al., 2016; McPherson et al., 2010). Thus, these scores were calculated only for NAFLD subjects available at the OPERA control visit (n = 129).

In this thesis (Studies I-III), hepatosteatosis was diagnosed by liver-kidney contrast on ultrasound. Nonetheless, ultrasound has limited sensitivity to determine hepatosteatosis (Bohte et al., 2011). Especially, the sensitivity is poor for detecting
mild steatosis in obese persons (European Association for the Study of the Liver (EASL) et al., 2016). According to some reports, visual assessment of hepatosteatosis on ultrasound may have substantial observer variability (Cengiz et al., 2014; Strauss, Gavish, Gottlieb, & Katsnelson, 2007), but there are also reports of good interobserver concordance (Palmentieri et al., 2006; Williamson et al., 2011). Thus, even when performed by an experienced radiologist, it is possible that the diagnosis of hepatosteatosis is not unambiguous or that it does not cover all NAFLD cases. Moreover, in order to be of non-alcoholic origin, that is, NAFLD, a patient with hepatosteatosis should not have excess alcohol drinking in the background (≥30 g a day in men or ≥20 g a day in women). However, heavy-drinking people, in particular, tend to underestimate their alcohol consumption (Allen, Wurst, Thon, & Litten, 2013; Del Boca & Darkes, 2003). As there are no good clinical biomarkers to assess the amount of alcohol drinking, it is possible that there were subjects in the NAFLD group who actually have alcoholic fatty liver disease or mixed alcoholic/non-alcoholic fatty liver disease. Additionally, secondary causes of fatty liver should be excluded before NAFLD is diagnosed (European Association for the Study of the Liver (EASL) et al., 2016). From a global perspective, viral hepatitis, in particular, should be excluded. Because the prevalence of hepatitis B and C is so low in Finland (Farkkila, 2003), it is widely accepted that exclusion of viral hepatitis is not mandatory in the Finnish NAFLD studies (Younossi, Koenig et al., 2016). Thus, the presence of hepatitis B or C was not examined in Studies I-III. In Study IV, MRI was used to assess the hepatic fat fraction. MRI has excellent specificity and sensitivity to detect even small amounts of hepatic fat (Bohte et al., 2011; Springer et al., 2010) and may be even better than liver biopsy in the quantification of hepatic fat accumulation (Noureddin et al., 2013).

It is notable that the QUICKI has been shown to be an accurate surrogate for insulin sensitivity (H. Chen, Sullivan, & Quon, 2005; Katz et al., 2000; Malita et al., 2006). However, while the QUICKI is based on fasting state, it should be taken into account that in real life, postprandial state is often present. Accordingly, a combination of OGTT (values at 30–90–120min) and QUICKI could provide the best in vivo assessment of insulin sensitivity (Bastard et al., 2007). Moreover, the QUICKI seems to work best in insulin resistant subjects (Muniyappa, Lee, Chen, & Quon, 2008). However, the QUICKI is still considered an extensively validated, simple, accurate and reproducible method to assess insulin sensitivity (Muniyappa et al., 2008) but its limitations to reflect the real life insulin sensitivity must be taken into account. Likewise, NMR, used for the lipid metabolomics measurements in Study IV, is a validated, reliable and rapid method for the quantification of serum
lipid and lipoproteins (Soininen et al., 2009; Soininen et al., 2015; Würtz et al., 2017). Thus, NMR has been presented as an ideal method for hypothesis-free research approach – such as Study IV.

6.1.3 Statistical methods

The possibility for false positive results is highlighted when performing a very large number of tests. Thus, to control the FDR in the lipid metabolomics in Study IV, the p-values were corrected by Benjamini-Hochberg procedure. Benjamini-Hochberg was adopted because it is most widely used for FDR. It is not seen as too conservative, like the Bonferroni procedure, or as too liberal, like the Rothman procedure. Indeed, Benjamini-Hochberg modifies the smallest p-values the most, and the greatest the least.

In Study II, Tukey’s method was chosen to be used for ANOVA multiple comparisons as it is the most widely used method and considered the best when sample sizes are not equal. It is also considered more powerful than Bonferroni.

6.2 NAFLD with and without MetS as a predictor of cardiovascular diseases, type 2 diabetes and increase of left ventricular mass (Study II)

NAFLD and MetS often overlap (Yki-Jarvinen, 2014) while insulin resistance plays a central role in this association (Alberti et al., 2009; Angelico et al., 2005; Korenblat, Fabbri, Mohammed, & Klein, 2008; Kotronen et al., 2007; H. Liu & Lu, 2014; Seppala-Lindroos et al., 2002; Yki-Jarvinen, 2014). Additionally, these conditions share the same risk profile as both are risk factors for CVDs and T2D (Byrne & Targher, 2015; Laaksonen et al., 2002; Lakka et al., 2002; Yki-Jarvinen, 2014). However, studies where NAFLD patients with MetS and those without it have been explored separately are lacking.

In the present study, the long-term risk of CVDs, T2D and increase of LVMI was assessed in patients with NAFLD and MetS, patients with NAFLD but not MetS, patients with MetS but not NAFLD, and the patients without either of these conditions. Patients with NAFLD but not MetS did not have an increased incidence of T2D, risk of cardiovascular endpoints or increase of LVMI during a long-term follow-up as compared to patients without either of these conditions. Instead, when NAFLD was combined with MetS, the risk for all these events was substantially greater than that of individuals without NAFLD or MetS.
The CVD risks that NAFLD causes are most of all a consequence of insulin resistance and obesity, which are closely associated with MetS (Angelico et al., 2005; Bhatia et al., 2012; Korenblat et al., 2008; Kotronen et al., 2007; Seppala-Lindroos et al., 2002; Yki-Jarvinen, 2014). Thus, according to the present epidemiological study, the CVD risk that NAFLD provides through other pathophysiological routes is not that significant (Figure 9). It is also of note that while NAFLD related to the I148M variant in PNPLA3 is not seen as CVD risk factor (European Association for the Study of the Liver (EASL) et al., 2016; Petaja & Yki-Jarvinen, 2016), this gene variant was the most prevalent in the subjects with plain NAFLD.

An interesting and novel finding was also that in the absence of MetS, subjects with NAFLD are not at increased risk for future T2D when compared to individuals with neither NAFLD nor MetS. However, there were more subjects with plain NAFLD than those without either of these conditions at the baseline who developed MetS during the follow-up. This may indicate that NAFLD precedes MetS and, as more fat accumulates ectopically, MetS may develop and the risk for cardiometabolic diseases will eventually follow. In agreement with this theory, the subjects with both conditions had substantially increased levels of fasting insulin and liver enzyme values, which are known to correlate with the amount of intrahepatic lipid accumulation (Kotronen et al., 2007; Kotronen et al., 2009). If this is so, it gives us a critical time period for intervention and prevention of CVD complications and T2D. This theory is reviewed in detail by Lonardo et al. who suggested that the etiology of hepatosteatosis, such as the I148M variant in PNPLA3 and lipid intra-hepatocytic compartmentation, are the major determinants of whether hepatosteatosis induces insulin resistance or not (Lonardo et al., 2015). Unfortunately, hepatosteatosis status was not examined during the follow-up or at the control visit so that the present study does not depict the risk of future NAFLD in the subjects with plain Mets.

There are some studies showing that patients with NAFLD but without overweight or obesity are at increased risk for development of T2D, but in none of these studies all metabolic risk factors were adjusted for (Chon et al., 2012; Feng et al., 2014; Fukuda et al., 2016; W. D. Li et al., 2015). Additionally, NAFLD has been linked to the components of MetS and hyperuricemia in general, mostly by cross-sectional studies that do not provide any evidence of the causality of the studied entities (H. J. Kim et al., 2004; Y. M. Kwon et al., 2012). Along with insulin resistance, there are some other pathophysiological mechanisms, e.g. disordered lipid metabolism, increased oxidative stress and inflammation that could link NAFLD to T2D (Leite, Villela-Nogueira, Cardoso, & Salles, 2014). According to the present study, the impact of these factors seems to be minor.
Some studies report that NAFLD is associated with greater LVMI in non-diabetic population (Fotbolcu et al., 2010; Goland et al., 2006) whereas others disagree with this finding (Cassidy et al., 2015; Fallo et al., 2009; Hallsworth et al., 2013). There are also divergent reports on this matter in diabetic populations (Bonapace et al., 2012; Mantovani, Zoppini, Targher, Golia, & Bonora, 2012; Mantovani et al., 2015). Apparently, there is only one previous study in which the association of NAFLD and LVMI was studied after adjusting with MetS (Sesti et al., 2014). In the study by Sesti et al. there was an association between hepatic fibrosis and LVMI even after adjustments for metabolic syndrome, age, gender and smoking history (Sesti et al., 2014). However, the study was cross-sectional and the study cohort had also greater co-morbidity as compared to the present study (Sesti et al., 2014). It is thus difficult to conclude how great the contradiction between these two studies actually is. Indeed, the present study is the first long-term assessment of LVMI change in subjects with NAFLD. Moreover, it carefully segregates the NAFLD subtypes by MetS.

The I148M variant in PNPLA3 has been associated with the development and progression of non-obese NAFLD (Honda et al., 2016; J. Shen et al., 2014) and with NAFLD without MetS (J. Shen et al., 2014; Sookoian & Pirola, 2011). This study provides similar data by showing that I148M variant in PNPLA3 was more prevalent in the subjects with NAFLD but without MetS.

It may be possible that in those who have NAFLD but not MetS, NAFLD originates from a genetic background more often than in individuals with NAFLD and with MetS. Meanwhile, those who have both NAFLD and MetS have them due to a Western lifestyle and its consequences, obesity and insulin resistance. The perception that there are different phenotypes of NAFLD and that the major division between the phenotypes is whether the NAFLD is combined with insulin resistance, is not new (Lonardo et al., 2015; J. H. Park et al., 2015; Petaja & Yki-Jarvinen, 2016; J. Shen et al., 2014; Speliotes et al., 2010; Younossi, Otgonsuren, Venkatesan, & Mishra, 2013). The results of the present study are particularly similar to the reports by Younossi et al. (Younossi et al., 2013) and Luukkonen et al. (P. K. Luukkonen, Zhou, Sadevirta et al., 2016). Younossi et al. showed that all-cause mortality and cardiovascular mortality are higher in subjects with both NAFLD and MetS as compared to NAFLD subjects without MetS and that subjects with NAFLD but without MetS do not have increased mortality (all-cause, liver-related nor cardiovascular) compared to individuals without NAFLD (Laaksonen et al., 2002; Lakka et al., 2002; Younossi et al., 2013). Moreover, Luukkonen et al. showed that there are two different phenotypes of NAFLD, ‘metabolic NAFLD’
and ‘PNPLA3 NAFLD’. The ‘metabolic NAFLD’ increases the risk for T2D and cardiovascular diseases whereas ‘PNPLA3 NAFLD’ does not (P. K. Luukkonen, Zhou, Sadevirta et al., 2016).

It is obvious that NAFLD is heterogeneous. For example, the two best-known genetic conditions exposing to NAFLD, the I148M variant in PNPLA3 and the E167K variant in TM6SF2, are not accompanied by insulin resistance (Petaja & Yki-Jarvinen, 2016). Considering that both these genetic conditions and MetS are common (Petaja & Yki-Jarvinen, 2016), there must be many subjects with ‘double trouble NAFLD’, that is, subjects who have at least one of these gene variants and ‘obese/metabolic NAFLD’ (Petaja & Yki-Jarvinen, 2016). Obviously, more studies are needed to elucidate the complex interplay of genetic and environmental factors, NAFLD, insulin resistance and MetS behind the individual cardiometabolic prognosis.

One of the strengths of the present study is that the follow-up time was long. For CVD events the follow-up time was more than 16 years and for LVMI and new T2D events more than 20 years. Moreover, the present study has very detailed baseline characteristics.

It is noteworthy, however, that there were only 63 individuals with NAFLD but without MetS that accounts for 6% of all participants. This may limit the interpretation of the results. Assuming that the subjects with NAFLD but without MetS were genetically predisposed to NAFLD, the proportion of NAFLD without MetS may differ around the world. In addition, the IDF criteria from the year 2005 were used to define MetS. Today, the most commonly used criteria in MetS definition are the new consensus criteria from 2009 (Alberti et al., 2009). However, the main results remained similar by using these criteria for MetS.

From a therapeutic point of view, it is of utmost importance to select the NAFLD subjects who are at increased risk for future cardiovascular or metabolic co-morbidities. The present study shows that NAFLD without MetS may be just an earlier stage of the disease spectrum predisposing to MetS and its complications. By providing tools to identify these subjects, this study may help to individualize the prognosis and follow-up and save the scarce resources of the healthcare system. However, the strengths and limitations of the present study must be noted.

In conclusion, the present study provides epidemiological evidence that NAFLD with MetS and NAFLD without MetS are different subtypes. Certain genetic conditions may expose to NAFLD without MetS whereas NAFLD with MetS is more of a consequence of a Western lifestyle. Alternatively, NAFLD without MetS is just an earlier stage of the disease spectrum predisposing to MetS.
and its complications. However, NAFLD without MetS does not seem to increase long-term cardiometabolic co-morbidities. In contrast, NAFLD with MetS causes a broad spectrum of cardiovascular and metabolic co-morbidity and mortality.

6.3 Non-alcoholic fatty liver disease and atrial fibrillation

6.3.1 Non-alcoholic fatty liver disease as a predictor of atrial fibrillation in middle-aged population (OPERA Study) (Study I)

The main finding in this study was that NAFLD predicts atrial fibrillation independently of other AF risk factors in the middle-aged Finnish population in a long-term follow-up.

The finding is in line with the study by Targher et al. who demonstrated that ultrasonographically detected NAFLD is independently associated with increased incidence and prevalence of AF among hospitalized type 2 diabetic patients (Targher et al., 2013; Targher et al., 2013). There are, however, some differences between their prospective study and our study: the duration of the study by Targher et al. was shorter (about 10 years), and it had a smaller number of study subjects, all of whom were diabetic individuals and, on average, older than subjects in this study. They did not have echocardiographic data available at the baseline, either (Targher et al., 2013).

There are also two studies showing that serum levels of liver enzymes correlate with the incidence of AF (Alonso et al., 2014; Sinner et al., 2013). The study by Alonso et al. (Alonso et al., 2014) showed that GGT was linearly associated with the risk of AF irrespective of alcohol drinking habits. Moreover, a study based on the Framingham Heart Study, with over 3,700 patients who were followed for up to 10 years, showed that ALT and AST are significantly associated with a greater risk of incident AF and that the association is not dependent on the main AF risk factors or alcohol consumption (Sinner et al., 2013). Of interest, in the present study GGT and ALT predicted AF as well. In agreement with the present study, all above-mentioned studies suggest that NAFLD is an independent risk factor for AF in both diabetic and non-diabetic population.

It is clear that there are common risk factors and co-morbidities with AF and NAFLD. First, NAFLD induces systemic inflammation by multiple mechanisms (Ndumele et al., 2011; Targher et al., 2008). An increasing state of inflammation in the liver may predispose to systemic inflammation (Furukawa et al., 2004; Machado & Cortez-Pinto, 2014; Tateya, Kim, & Tamori, 2013), which is a potent
trigger of AF (Aviles et al., 2003; Chung et al., 2001; Y. Guo et al., 2012; Harada et al., 2015). Vice versa, AF seems also to produce and sustain a pro-inflammatory environment (Y. Guo et al., 2012). Thus, the cause-and-effect link between NAFLD and AF via systemic inflammation may be bidirectional. Second, NAFLD predicts autonomic dysfunction (Y. C. Liu et al., 2013; Newton et al., 2009; Sun et al., 2015). The variation of sympathovagal activation, in turn, is proarrhythmic in the initiation and maintenance of AF, being a risk factor for it (H. W. Park et al., 2012; Perkiomaki et al., 2014; M. J. Shen & Zipes, 2014). These associations between NAFLD and autonomic dysfunction as well as autonomic dysfunction and AF may provide a link between NAFLD and AF. Third, NAFLD is shown to be associated with cardiac diastolic dysfunction (Fotbolcu et al., 2010; Graner et al., 2014; Petta et al., 2015), which provokes AF through various processes (Nagarakanti & Ezekowitz, 2008; Tsang et al., 2002). There are also reports that this association between NAFLD and diastolic dysfunction may be restricted to the presence of T2D, a frequent co-morbidity of NAFLD (Bonapace et al., 2012; Cassidy et al., 2015; Mantovani et al., 2015). However, in the present study, patients suffering from AF during the follow-up had a higher LVMI at the baseline, and LVMI was a significant and independent predictor of AF in the NAFLD group. Unfortunately, though, left ventricular diastolic dysfunction was not assessed at baseline. Notably, while obesity is shown to be closely associated with NAFLD as reviewed in this thesis, it is also presented as a risk factor for AF (Tsang et al., 2008; T. J. Wang et al., 2004). The possible mechanisms linking NAFLD to AF and the complex interplay between the associates are illustrated in Figure 11.

There are some limitations that may affect the interpretation of the results. A population without CVDs at the baseline would have been the best option to investigate the risk factors of the incidence of AF. Notably, however, the distribution of NAFLD, but not the distribution of AF, differed statistically significantly according to the original OPERA study group (subjects with hypertension medication versus subjects without hypertension medication). In addition, the original OPERA study group was included in the ANCOVA analysis when the association between NAFLD and AF was estimated. Furthermore, the indication for the use of digitalis at the baseline (n = 12) was not documented. Assumingly, these subjects had chronic heart failure, because in the re-checked original OPERA cohort data (n = 1,045) there was only one AF diagnosis at baseline.

In conclusion, the main finding was that NAFLD predicted independently the onset of AF in middle-aged Finnish population in a long-term follow-up study.
6.3.2 The association of atrial fibrillation and liver stiffness (Study III)

The central finding was that there is an association between liver stiffness and the presence of AF and the association increases by the progression of liver stiffness.

The present study is in agreement with the previous reports that NAFLD predicts AF independently (Targher et al., 2013) and the cardiovascular risk increases with the severity of NAFLD (Ekstedt et al., 2015). However, due to the cross-sectional design of the study, it is not possible to determine whether the relationship is causal or temporal. The simplest explanation for the association is that there are only common risk factors and co-morbidities between the entities. However, the adjustments for mutual risk factors did not attenuate the association between AF and liver stiffness.

The association between NAFLD and AF may be mediated, for instance, through systemic inflammation, diastolic dysfunction, obesity and autonomic dysfunction, all of which are risk factors for AF. Moreover, AF and systemic inflammation have a bidirectional relationship (Y. Guo et al., 2012; Joseph et al., 2016; Kallergis et al., 2008; Patel, Dokainish, Tsai, & Lakkis, 2010; Wijesurendra & Casadei, 2015) (Figure 11). Concurrently, chronic inflammation induces liver fibrosis (Buzzetti et al., 2016; Czaja, 2014; S. L. Friedman, 2008; U. E. Lee & Friedman, 2011; Singh et al., 2015). It is thus possible that the pro-inflammatory factors released by AF may provoke the progression of liver stiffness. However, obesity is known to be associated with inflammatory state (U. J. Jung & Choi, 2014; Yki-Jarvinen, 2014) and, simultaneously, progressive NAFLD (Anstee et al., 2013). Thereby, obesity and its associates, such as insulin resistance, must be taken into account as confounding factors.

Growth factors, cytokines, chemokines and free radicals have a key role in the development of advanced liver stiffness. For example, NADPH oxidase 4, induces the production of free radicals, and, thereby, takes part in the pathogenesis of liver fibrosis (Liang et al., 2016). In addition, it is reported to be activated in the heart by AF and to contribute to AF-related cardiac fibrosis (Joseph et al., 2016; J. Zhang et al., 2012). Also galectin-3, which is a multifunctional protein mainly secreted by macrophages, is a central player in cell apoptosis, proliferation, adhesion, migration and differentiation as well as in angiogenesis and inflammatory responses. Galectin-3 is shown to be activated in fibrotic models and increased in fibrotic diseases, for instance in the liver (Bacigalupo, Manzi, Rabinovich, & Troncoso, 2013; Henderson et al., 2006; L. C. Li, Li, & Gao, 2014). Likewise, the evidence of the association between galectin-3 and cardiac fibrotic processes...
in heart failure is solid (L. C. Li et al., 2014). Moreover, there are reports of an association between AF and galectin-3, although galectin-3 is speculated to be just a bystander, with obesity and other co-morbidities as the real promoters of fibrosis, but there are also reports that even ‘lone AF’ is able to increase galectin-3 values (Gurses et al., 2015; J. E. Ho et al., 2014; Kornej et al., 2015; Lippi, Cervellin, & Sanchis-Gomar, 2015). Thus, galectin-3 and NADPH oxidase 4 may participate in the fibrinogenesis of both the heart and the liver.

AF also produces a hypercoagulable state (Fu, Wu, Wu, & Qiu, 2011; Spronk et al., 2017; Watson, Shantsila, & Lip, 2009). There are numerous studies linking hypercoagulable state to liver fibrosis by thrombosis-related ischemia and cell death, which lead to inflammatory processes and fibrinogenesis (Anstee et al., 2008; Anstee, Dhar, & Thursz, 2011; Gonzalez-Reimers et al., 2016; Rullier et al., 2008; Tripodi, Anstee, Sogaard, Primignani, & Valla, 2011). The hypercoagulability is also characterized by the increase of thrombin formation within blood circulation. Fibroblasts and hepatic stellate cells express protease-activated receptors, subdivided into four subtypes, 1 to 4. Thrombin and coagulation factor Xa can activate protease-activated receptor 1, resulting in ‘direct stellate cell activation’, i.e., stellate cell activation, production of extracellular matrix, tissue remodeling and fibrinogenesis (Anstee et al., 2008; Anstee et al., 2011; Gonzalez-Reimers et al., 2016; Rullier et al., 2008; Spronk et al., 2017; Tripodi et al., 2011). Moreover, protease-activated receptor 2 activation is also associated with hepatic fibrinogenesis (Knight, Tchongue, Lourensz, Tipping, & Sievert, 2012). Thus, the activation of protease-activated receptors through the AF-produced hypercoagulable state may be one mechanism between AF and liver fibrosis. Notably, anticoagulants may attenuate the pro-fibrotic responses (Spronk et al., 2017).

There are some study limitations. Being an invasive procedure, biopsy samples cannot be taken without suspected liver morbidity. Thus, liver stiffness surrogates must be used. Of these, the NFS is a validated scoring system for NAFLD patients whereas transient elastography measures liver stiffness irrespective of the etiology. Notably, however, the newfound association between liver stiffness and AF was observed by both these methods, which can be thought to strengthen the observation.

Furthermore, the study subjects selected for the transient elastography measurements were obese while the reliability of transient elastography has been put into doubt in obesity (European Association for the Study of the Liver (EASL) et al., 2016; Petta et al., 2011). However, subjects with unreliable measurement due to obesity were excluded from the study. This and other possible study limitations
from the use of transient elastography as a liver stiffness surrogate are discussed in detail in chapter Clinical, radiological and laboratory methods. Moreover, the number of study subjects available for transient elastography analysis was quite small, they were older individuals and mostly men, which limits the interpretation of the results. However, the results prevailed in the analysis of the NFS with more even gender distribution.

To conclude, the main finding in this study was that atrial fibrillation and liver stiffness, measured by transient elastography, are associated with each other even after adjustment for mutual risk factors.

6.4 The effect of Pregnane X receptor on hepatic fat accumulation and lipid metabolism (Study IV)

Earlier reports from rodents and human cell models have shown that PXR activation induces hepatosteatosis (Bitter et al., 2015; Hakkola et al., 2016; J. He et al., 2013; L. Li et al., 2015; Moreau et al., 2009; Nakamura, Moore, Negishi, & Sueyoshi, 2007; J. Zhou et al., 2006). However, in the present study, rifampicin-activated PXR did not induce hepatosteatosis in humans in vivo as assessed with MRI. Thus, there is a discrepancy between this result and the earlier reports of PXR actions in rodents (J. He et al., 2013; Nakamura et al., 2007; J. Zhou et al., 2006) and human cell models (Bitter et al., 2015; L. Li et al., 2015; Moreau et al., 2009). Notably, there are certain species-specific differences in the actions of PXR and its metabolic effects between rodents and humans (Hakkola et al., 2016; Jonker et al., 2012). Moreover, in vitro studies do not necessarily reflect in vivo circumstances. In the present study, the effects of rifampicin-activated PXR on a spectrum of metabolites as assessed with NMR metabolomics were measured and increased concentrations of IDL and LDL of all sizes, serum total cholesterol, esterified and free cholesterol in the rifampicin study arm as compared to the placebo arm were observed. Moreover, the levels of apolipoprotein A-I, sphingomyelins, 18:2 linoleic acid and omega-6, both of which are polyunsaturated fatty acids, were increased, whereas citrate and acetate concentrations were decreased. Consistently with the results from MRI, which detects only triglycerides but not other lipids (Schwenzer et al., 2009), triglycerides were unaltered.

In mice and human cell model studies, PXR activation suppresses CYP7A1 and CYP8B1, which are crucial in the bile acid synthesis (Bhalla et al., 2004; Jonker et al., 2012; T. Li & Chiang, 2005; Russell, 1999; M. Zhang & Chiang, 2001). Instead, in an elegant study by Marschall et al., otherwise healthy gallstone
patients scheduled for cholecystectomy were randomized to rifampicin (600mg a day for 1 week), ursodeoxycholic acid or placebo (Marschall et al., 2005). The subjects in the rifampicin group showed induction of CYP3A4, as expected, but the expression of CYP7A1 and CYP8B1 remained unchanged. Moreover, total lipid, cholesterol, and primary bile acid concentrations in gallbladder bile were increased, as was a serum marker of bile acid synthesis, 7α-hydroxy-4-cholesten-3-one (Marschall et al., 2005). Also Lütjohann et al. reported of stimulated bile acid synthesis (increase of serum 7α-hydroxy-4-cholesten-3-one) and increased cholesterol synthesis (increase of serum lathosterol to cholesterol ratio, a marker of cholesterol synthesis), whereas serum total cholesterol remained unchanged after 10 male subjects were administered with rifampicin (600mg a day for 6 days) (Lütjohann et al., 2004). Furthermore, Von Bergmann et al. showed enhanced bile acid synthesis in 4 patients treated with rifampicin in the clinical practice (von Bergmann et al., 1981). Moreover, an increase in serum lathosterol concentration in the Rifa-BP study has previously been detected (unpublished) as a sign of stimulated cholesterol synthesis. Phenobarbital, an activator of PXR and constitutive androstane receptor, has been reported to induce the activities of HMG-CoA reductase, a crucial enzyme in cholesterol synthesis, and cholesterol 7α-hydroxylase in vivo in liver biopsy samples of subjects with gallstone disease (Coyne et al., 1976). Thus, the present result of increased serum total, free, esterified, LDL and IDL cholesterol may be a result of increased cholesterol synthesis. As both acetate and citrate are precursors in cholesterol and fatty acid synthesis (Kamphorst, Chung, Fan, & Rabinowitz, 2014; Lemus & Mendivil, 2015), the decreased concentrations of acetate and citrate after the rifampicin administration also support this view. A citrate uptake transporter, SLC13A5, is a direct target of PXR and is induced by rifampicin in human primary hepatocytes (L. Li et al., 2015). The increased citrate uptake by PXR activation secures the availability of citrate for cholesterol and fatty acid synthesis. SREBP-1a, which is induced by rifampicin in human primary hepatocytes (Bitter et al., 2015), and possibly insulin induced gene 1 and lipin-1, regulated by PXR in animal models, may be the links behind the increased cholesterol synthesis by PXR activation (J. He et al., 2013; Roth et al., 2008).

Activated PXR enhances also fatty acid metabolism in many direct and indirect ways. PXR activation induces SREBP-1a which upregulates several lipogenic enzymes participating in several steps of fatty acid synthesis (Amemiya-Kudo et al., 2002; Hakkola et al., 2016; Xie et al., 2009). The production of acetyl-CoA carboxylase 1, fatty acid synthase, elongation of long-chain fatty acids family
member 6, and stearoyl-CoA desaturase 1 is stimulated (Hakkola et al., 2016). In addition, PXR induces the transcription of thyroid hormone-responsive spot 14 protein leading to the increased transcription of FAS and adenosine triphosphate citrate lyase that participates in fatty acid synthesis (Hakkola et al., 2016). Additionally, activated PXR downregulates the mitochondrial β-oxidation by repressing the carnitine palmitoyltransferase 1, a regulator of mitochondrial fatty acid β-oxidation (Hakkola et al., 2016; Moreau et al., 2009). Thereby, the surplus of fatty acids perhaps leads to the intrahepatic accumulation of fatty acids and to the increase of circulating fatty acids as shown in this thesis. The stimulated fatty acid synthesis is supported by Moreau et al. They treated human primary hepatocytes with rifampicin vs. solvent control for 96 hours. They observed increased content of myristic, stearic, oleic, γ-linoleic, palmitic, and palmitoleic acid (Moreau et al., 2009). Thus, unlike in this study, not only polyunsaturated fatty acids were increased as saturated (myristic, stearic, palmitic) were increased as well (Moreau et al., 2009). In contrast to animal experiments (Nakamura et al., 2007), ketone bodies were not suppressed by PXR activation as acetoacetate and 3-hydroxybutyrate tended to increase in the present study.

In addition to cholesterols and fatty acids, sphingomyelins were increased in the present study. Sphingomyelins consist of a phosphocholine or phosphoethanolamine head group, a sphingosine, and a fatty acid, which are both crucial components of plasma membrane. Block et al. concluded that 6 days’ administration of rifampicin in healthy volunteers resulted in elevated ratios of phosphomonoester and phosphodiester to nucleoside triphosphate as assessed with 31P-magnetic resonance spectroscopy (Block et al., 1997). As the phosphomonoester to nucleoside triphosphate ratio reflects phosphocholine and phosphoethanolamine signals, the finding of elevated phosphomonoester could be construed as a sign of induced sphingomyelin synthesis (Block et al., 1997). The phosphodiester signal contains glycerophosphorylcholine and glycerophosphorylethanolamine thought to represent cell membrane degradation products (Solga, Horska, Clark, & Diehl, 2005). Although the hepatic triglyceride content was not increased in the present study as assessed with MRI, rifampicin-activated PXR elevates hepatic phospholipid signals as was shown by Block et al. Of note, most of the studies examining the effect of PXR on hepatic cell and tissue lipid accumulation have used Oil Red O or Nile Red stains, which stain only neutral lipids (triglycerides, diacylglycerols and cholesterol esters), whereas polar lipids (phospholipids, sphingolipids, ceramides, and free cholesterol) are not stained (Mehlem, Hagberg, Muhl, Eriksson, & Falkevall, 2013). Thereby, the intense staining by PXR activation
could be caused by hepatic accumulation of esterified cholesterol, given that an increase in serum cholesterol esters reflects hepatic cholesterol ester content. In addition, the discrepancy between the negative result on hepatic triglyceride content and increased staining in tissue analyses could be that animal \textit{in vivo} and human cell \textit{in vitro} models do not reflect human \textit{in vivo} conditions.

Although hepatosteatosis is mainly formed of the accumulation of triglycerides, cholesterol metabolism is significantly affected in NAFLD (Fabbrini \textit{et al.}, 2010; Kawano \textit{et al.}, 2015; Mannisto \textit{et al.}, 2014; Musso \textit{et al.}, 2013; Simonen \textit{et al.}, 2011) and NASH (Musso \textit{et al.}, 2013). Indeed, hepatic triglyceride accumulation itself may not be toxic but rather inert and serve as a buffer for the lipotoxic precursors of triglycerides (Anderson & Borlak, 2008; Malhi & Gores, 2008; Musso \textit{et al.}, 2013; Neuschwander-Tetri, 2010). Consistently, subjects who are able to store excess fat as neutral cholesterol esters or triglycerides are described as ‘good fat storers’ as they will develop hepatosteatosis but not NASH, whereas those who are unable to synthetize neutral lipids accumulate toxic lipids and are at risk of having NASH (Musso \textit{et al.}, 2013). Toxic lipids lead to endoreticulum stress, inflammation, apoptosis and necrosis, i.e., to lipotoxic liver injury in NASH (Neuschwander-Tetri, 2010). There is solid evidence that the hepatic free cholesterol accumulation is associated with progressive NAFLD, including NASH, fibrosis and cirrhosis (Musso \textit{et al.}, 2013). Free fatty acids, saturated fatty acids in particular, diacylglycerols, phospholipids or their components (ceramides, sphingolipids, lysophosphatidyl choline) are considered as the other central lipotoxic intermediates (Mota \textit{et al.}, 2016; Musso \textit{et al.}, 2013; Neuschwander-Tetri, 2010). Therefore, it is interesting that PXR activation \textit{in vivo} mainly results in the excess of lipotoxic lipids and, thus, possibly exposes to progressive NAFLD forms. It is also of interest that mRNA and activity of CYP3A4, the major PXR-regulated hepatic enzyme, is downregulated in NALFD patients while a negative relationship between severity of steatosis and hepatic CYP3A activity is reported (Kolwankar \textit{et al.}, 2007; Woolsey, Mansell, Kim, Tirona, & Beaton, 2015). Woolsey \textit{et al.} showed recently that fibroblast growth factor 21, which is elevated in NAFLD subjects, reduces nuclear localization of PXR and the activation of CYP3A4 in experimental models (Woolsey \textit{et al.}, 2016). Furthermore, PXR protein expression is lower in patients with NASH as compared to patients without NASH (Bitter \textit{et al.}, 2015). Thus, PXR activation seems to activate cholesterol, fatty acid, and sphingomyelin synthesis which could lead to lipotoxicity while fibroblast growth factor 21 -mediated mechanism can be hypothesized to reduce the notorious effects of activated PXR.
Some potential study limitations must be taken into account. First, the study protocol was planned to be identical to the study in which rifampicin induced post-prandial impaired glucose tolerance (Rysa et al., 2013) to assess if hepatosteatosis could explain the phenomenon. Therefore, all study volunteers were healthy with regular diet as in the mentioned study. It is possible that the effect of rifampicin on hepatic triglyceride content on the subjects with NAFLD or the subjects with sedentary lifestyle could be different. Second, it is also speculative whether longer duration of rifampicin administration, perhaps with high-fat diet, would have induced hepatic triglyceride accumulation. Third, the antibiotic effect of rifampicin may affect the metabolome by modulating gut microbiota or inflammatory state. Fourth, all study volunteers were Caucasian (ethnic Finns), which may limit the interpretation of the study results.

In conclusion, the main finding of the fourth study was that, as opposed to *in vitro* studies and rodent studies, PXR activation by rifampicin does not induce triglyceride accumulation in humans *in vivo*. Instead, it led to increased serum concentrations of several lipid components previously thought of as ‘toxic lipids’ that are reported to be associated with progressive NAFLD disease.

### 6.5 Future perspectives

Non-alcoholic fatty liver disease is widely present in general population. However, it is also very heterogeneous. For instance, only a minority of subjects with NAFLD will have shortened life expectancy, while the major reasons of death are attributable to cardiovascular diseases. Moreover, only a small proportion of NAFLD population will die from hepatic reasons, but, simultaneously, due to its widespread nature, NAFLD will become the main reason for liver transplantation in the coming years. Thus, there is an urgent need for factors and clinical parameters to help individualize the follow-up and prognosis setting. The present thesis offers a new prognostic aspect as the presence of MetS is revealed to determine the cardiometabolic prognosis in NAFLD. However, this requires confirmation from other prospective studies and it would be interesting to see whether this phenomenon is detectable in hepatic prognosis as well.

Likewise, the incidence of AF is expected to rise, for instance, due to ageing population in the Western world. Thus, factors predicting AF are also eagerly desired to determine who is at increased risk of AF. The predicting factors may provide a time period critical for intervention in an early stage of the development of AF. Thus, it is notable that NAFLD is presented as a novel predictor of AF in this thesis.
Currently, the grade of NAFLD can only be detected by liver biopsy. Especially, once hepatosteatosis is detected in the clinical practice, the knowledge of the presence or absence of NASH, fibrosis or cirrhosis, the most ominous grades in the NAFLD spectrum, is of high importance. However, due to potential complications and high cost, liver biopsy cannot be taken without suspected ominous liver disease. To date, there are some advances to rule out severe fibrosis in subjects with NAFLD. However, applications able to reliably rule in these deleterious NAFLD grades are lacking and are thus highly desired.

During the last two decades, epidemiological data of the association of MetS, T2D and NAFLD has been presented. Nonetheless, this data is mainly from cross-sectional studies or from Asian studies. Simultaneously, there is a global pandemic of obesity and its associates MetS, T2D and NAFLD. Thereby, to detect the crucial time point for efficient interventions to halt the detrimental development of CVDs, it would be crucial to deepen the knowledge of the longitudinal relations between these three conditions in different populations.

Although some potential treatment options may become available in the near future, to date, there is no specific pharmacotherapy for NAFLD. Therefore, the management of NAFLD is mainly focused on lifestyle modifications, such as weight loss and nutritional factors. In order to develop new treatment options for NAFLD, more specified knowledge of the pathogenesis of NAFLD and NASH, in particular, is needed.

In summary, further studies are needed on the pathogenesis as well as possible biomarkers and factors predicting the development of progressive NAFLD.
Conclusions

The main conclusions of this thesis are:

NAFLD is an independent risk factor for AF in general middle-aged population (Study I).

A combination of NAFLD and MetS implies a considerable risk for CVDs, T2D and the increase of LVMI, whereas NAFLD without MetS does not (Study II).

There is an association between AF and liver stiffness, which may have multiple explanations and mechanistic links (Study III).

Unlike in human cell models and rodents, administration of PXR activator rifampicin does not lead to increased triglyceride content in the liver in vivo as assessed with MRI. However, rifampicin dosing leads to increased serum total, free, esterified, IDL, and LDL cholesterol, sphingomyelins, Apo-A1, omega-6 fatty acids, and linoleic acid and decreased serum citrate and acetate. As these lipids are considered as ‘toxic lipids’, these findings may have implications for the development of NAFLD and NASH (Study IV).
References


153


Yilmaz, Y. (2012). Review article: Is non-alcoholic fatty liver disease a spectrum, or are steatosis and non-alcoholic steatohepatitis distinct conditions? *Alimentary Pharmacology & Therapeutics, 36*(9), 815–823. doi:10.1111/apt.12046 [doi]


9 Original articles


Studies I and III are published in open acces journal. Study II is reprinted by permission of Elsevier.

Original articles are not included in the electronic version of the thesis.
1423. Suhonen, Noora-Maria (2017) Cognitive and behavioral characteristics of frontotemporal lobar degeneration
1426. Karhu, Toni (2017) Isolation of novel ligands for MAS-related G protein-coupled receptors X1 and X2, and their effect on mast cell degranulation
1427. Mantere, Tuomo (2017) DNA damage response gene mutations and inherited susceptibility to breast cancer
1428. Salokorpi, Nina (2017) Treatment of craniosynostoses
1429. Männikkö, Niko (2017) Problematic gaming behavior among adolescents and young adults: relationship between gaming behavior and health
1431. Lavander, Päivi (2017) Nimikesuojattujen ja laillistettujen ammattihenkilöiden työntekijänmuutoksen muuttuvassa toimintaympäristössä
1434. Hulkko, Anja (2017) The association of lifetime antipsychotic and other psychiatric medications with cognition in schizophrenia: the Northern Finland Birth Cohort 1966 Study
1435. Ramsay, Hugh (2017) Predictors of psychosis risk and neurocognitive deficits
1436. Kuitunen, Hanne (2017) DLBCL, primary and secondary central nervous system involvement, treatment and prophylaxis
1437. Filatova, Svetlana (2017) Incidence of schizophrenia and associations of schizophrenia and schizotypy with early motor developmental milestones

Book orders:
Granum: Virtual book store
http://granum.uta.fi/granum/
Aki Käräjämäki

NON-ALCOHOLIC FATTY LIVER DISEASE (NAFLD) – PERSPECTIVES TO ETIOLOGY, COMPLICATIONS AND LIPID METABOLISM