Pauliina Pisto

FAT ACCUMULATION IN LIVER AND MUSCLE

ASSOCIATION WITH ADIPOKINES AND RISK OF CARDIOVASCULAR EVENTS
PAULIINA PISTO

FAT ACCUMULATION IN LIVER AND MUSCLE
Association with adipokines and risk of cardiovascular events

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 101A of the Doctoral Training Committee of Health and Biosciences (Aapistie 5 A), on 7 June 2013, at 12 noon
Pisto, Pauliina, Fat accumulation in liver and muscle. Association with adipokines and risk of cardiovascular events
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Oulu, Finland

Abstract
The prevalence of obesity is dramatically on the rise in the Western world. Obesity is associated with several chronic diseases, including diabetes and cardiovascular disease (CVD). Non-alcoholic fatty liver disease occurs when fat is ectopically stored in the liver. It is closely associated with serious metabolic abnormalities. Non-alcoholic fatty liver disease ranges from simple hepatic steatosis with no inflammation to hepatic steatosis with a necroinflammatory component, which may lead to cirrhosis and liver failure. Adiponectin is an adipokine that is solely secreted by adipocytes and has anti-inflammatory, antiatherogenic and insulin-sensitizing properties. Adipose tissue inflammation contributes to reduced plasma adiponectin levels in obesity leading to further metabolic complications. Adiponectin may be a mediator between obesity and fat accumulation in the liver and skeletal muscle. Fatty liver may play a role in the pathogenesis of CVD. Mortality data show that CVD as the cause of death accounts for almost half of all deaths in Finland. Traditional risk factors for CVD are age, gender, smoking, high low-density lipoprotein level, high blood pressure and diabetes.

The aim of the thesis was to investigate the mediators of fat accumulation in the liver and skeletal muscle as well as the role of fatty liver in the future risk for CVD. If one considers the peptide hormones, then adiponectin turned out to be the strongest independent indicator of the brightness of the liver. In addition, an association between a low adiponectin concentration and large muscle fiber size was observed, and this was not dependent on the amount of total fatness. Furthermore, severe fatty liver increased the risk for cardiovascular events, predicted the risk for death from all causes and death from CVD in a long follow-up. Insulin sensitivity seemed to play a more dominant role in developing cardiovascular events.

In conclusion, this study demonstrates that adiponectin may have an important effect on fat accumulation in the liver and skeletal muscle. Adiponectin could be a target when considering the treatment and prevention of ectopic fat accumulation. Fatty liver seems to play a significant role in developing cardiovascular event and mortality to CVD.

Keywords: adipokines, coronary disease, fatty liver, insulin resistance, lipid metabolism, risk factors, skeletal muscle fiber, stroke
**Tiivistelmä**


Acknowledgements

This thesis work was carried out in the Department of Internal Medicine, Institute of Clinical Medicine, Biocenter Oulu, and Clinical research Center, Oulu University Hospital. I wish to acknowledge the institutes for providing excellent research facilities.

I would like to express my warmest gratitude to Docent Olavi Ukkola, MD, PhD, and Professor Antero Kesäniemi, MD, PhD, for professional guidance and for encouraging me in the field of science throughout the whole thesis work. Olavi and Antero have provided outstanding working facilities and resources for my thesis.

My third supervisor, Doctor Merja Santaniemi, PhD, deserves special thanks for the indispensable support, never-ending patience and friendship. Merja has always been available for scientific questions and conversations about everyday life.

I express my warm thanks to my collaborators and co-authors. I am grateful for Juha-Pekka Turpeinen, MD, MSc, and statistician Risto Bloigu, MSc, for friendly collaboration. The official referees of this thesis, Professor Jussi Pihlajamäki, MD, PhD, and Professor Antti Reunanen, MD, PhD, deserve gratitude for their constructive criticism and inspiring comments. I wish to thank Anna Vuolteenaho, MA, for the excellent revision of the language.

I am highly grateful to all the workmates in our research group and in CRC. I wish to thank Meiju Saukko, MSc, and Elina Malo, MSc, for sharing thoughts about science and life. Ms Heidi Häikiö and Ms Saija Kortetjärvi deserve thanks for their skillful work in the laboratory. I warmly thank Secretary Marita Koistinen for her help with several matters. Docent Tiina Hurskainen, PhD, is thanked for providing good laboratory working facilities. Additionally, Anna-Maria, Antti, Justiina, Tiia, Tuire, Malla, Saara and Sari deserve thanks for the help and conversations. I wish to thank Tuija for sharing the dilemmas in the last stage of the thesis.

I owe sincere gratitude to all my friends near and far. Elisa, Antti, Laura, Sakke, Roosa, Maria, Kukka, Eeva, Hanna, Jonna and all my friends and relatives who are not mentioned here: Thank you for the friendship and all the memorable moments and discussions outside of science. I also thank my colleagues in medical school for sharing their thoughts about medicine and everyday life during these years. Markus deserves thanks for the statistical help.
My deepest gratitude goes to my lovely family. Dad, thank you for giving me a solid basis for my life and for everything you have done for me. Tommi, Leena-Maija, Enni, Eero, Aapo, Hugo, Tuulia, Inka, Saila and Ilari, you mean the world to me. It has been a privilege growing up with you. Mama, I miss you so much. From the bottom of my heart, deepest thanks for the love and care. Kari, I wish to thank you for the endless love, understanding and encouraging support during these years. You gave me strength to complete this thesis.

I wish to acknowledge all the financial support given to this work by the Research Council for Health of the Academy of Finland, the Finnish Foundation for Cardiovascular Research and the Sigrid Juselius Foundation.

Oulu, April 10th 2013

Pauliina Pisto
### Abbreviations

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AdipoR</td>
<td>adiponectin receptor</td>
</tr>
<tr>
<td>AMPK</td>
<td>adenosine monophosphate-activated protein kinase</td>
</tr>
<tr>
<td>ANCOVA</td>
<td>analysis of covariance</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ApoB</td>
<td>apolipoprotein B</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
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<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
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<tr>
<td>CETP</td>
<td>cholesterol ester transfer protein</td>
</tr>
<tr>
<td>CHD</td>
<td>coronary heart disease</td>
</tr>
<tr>
<td>CI</td>
<td>confidence interval</td>
</tr>
<tr>
<td>CM</td>
<td>chylomicrone</td>
</tr>
<tr>
<td>CV</td>
<td>coefficient of variation</td>
</tr>
<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
</tr>
<tr>
<td>DNL</td>
<td>de novo lipogenesis</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>FFA</td>
<td>free fatty acid</td>
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<tr>
<td>GLUT4</td>
<td>glucose transporter type 4</td>
</tr>
<tr>
<td>HDL</td>
<td>high-density lipoprotein</td>
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<tr>
<td>HMW</td>
<td>high-molecular weight</td>
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<tr>
<td>hs-CRP</td>
<td>high-sensitivity C-reactive protein</td>
</tr>
<tr>
<td>ICD</td>
<td>international classification of the diseases</td>
</tr>
<tr>
<td>IL-6</td>
<td>interleukin-6</td>
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<tr>
<td>IMCL</td>
<td>intramyocellular lipid</td>
</tr>
<tr>
<td>IMT</td>
<td>intima-media thickness</td>
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<tr>
<td>IR</td>
<td>insulin resistance</td>
</tr>
<tr>
<td>LDL</td>
<td>low-density lipoprotein</td>
</tr>
<tr>
<td>LDLR</td>
<td>low density lipoprotein receptor</td>
</tr>
<tr>
<td>LMW</td>
<td>low-molecular weight</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MMW</td>
<td>middle-molecular weight</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>NAFLD</td>
<td>non-alcoholic fatty liver disease</td>
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<tr>
<td>NASH</td>
<td>non-alcoholic steatohepatitis</td>
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<tr>
<td>NF-κB</td>
<td>nuclear factor-kappa B</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>OGGT</td>
<td>oral glucose tolerance test</td>
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<tr>
<td>OPERA</td>
<td>Oulu Project Eludicating Risk of Atherosclerosis</td>
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<tr>
<td>OR</td>
<td>odds ratio</td>
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<tr>
<td>PAI-1</td>
<td>plasminogen activator inhibitor 1</td>
</tr>
<tr>
<td>PPARα</td>
<td>peroxisome proliferator-activated receptor alpha</td>
</tr>
<tr>
<td>PPARγ</td>
<td>peroxisome proliferator-activated receptor gamma</td>
</tr>
<tr>
<td>QUICKI</td>
<td>quantitative insulin sensitivity check index</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>RBP4</td>
<td>retinol-binding protein 4</td>
</tr>
<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>SAH</td>
<td>subarachnoid hemorrhage</td>
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<tr>
<td>sdLDL</td>
<td>small, dense low-density lipoprotein</td>
</tr>
<tr>
<td>T2DM</td>
<td>type 2 diabetes mellitus</td>
</tr>
<tr>
<td>TG</td>
<td>triglyceride</td>
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<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-alpha</td>
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<tr>
<td>VLDL</td>
<td>very low-density lipoprotein</td>
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List of original articles

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1 Introduction

The prevalence of obesity is increasing dramatically in the Western world, taking on the characteristics of an epidemic. Obesity is associated with a low-grade inflammation and increases the risk for metabolic abnormalities, such as insulin resistance, type 2 diabetes mellitus (T2DM) and dyslipidemia. Obesity can lead to metabolic syndrome, which is a name for a group of risk factors that increase the risk for coronary artery disease, stroke, and T2DM (Despres & Lemieux 2006).

Non-alcoholic fatty liver disease (NAFLD) is a hepatic manifestation of the metabolic syndrome. NAFLD is the most common liver disease and it refers to a spectrum of liver disorders resulting from abnormal fat deposition in the liver, which ranges in severity from simple hepatic steatosis with no inflammation, to non-alcoholic steatohepatitis (NASH) which can progress to liver cirrhosis. NAFLD patients display a larger prevalence of metabolic abnormalities, and the prevalence of metabolic syndrome is more than doubled (Angulo 2002).

Obesity and insulin resistance are regarded to be key mechanisms leading to disordered accumulation of triglycerides (TGs) in the liver and skeletal muscle. In insulin-resistant conditions, such as T2DM and obesity, there is increased lipolysis in the peripheral adipose tissue and excess free fatty acid (FFA) flux to the liver and muscle cells (Van Gaal et al. 2006). Furthermore, there is also perturbation in mitochondrial fatty acid metabolism (Jornayvaz et al. 2010, Smith & Adams 2011a). Metabolic disturbances and impaired energy metabolism lead to fat accumulation within muscle cells and hepatocytes (Lara-Castro & Garvey 2008).

The mechanistic links between obesity, ectopic fat and cardiovascular disease (CVD) are still poorly understood. The mediators between obesity and CVD could be adipocytokines (Van Gaal et al. 2006). Visceral fat is regarded as an endocrine organ and it secretes several hormones called adipocytokines (Tadokoro et al. 2010). Adiponectin is an adipokine, which is solely secreted by adipocytes and has anti-inflammatory, antiatherogenic and insulin-sensitizing properties. Adipose tissue inflammation contributes to reduced plasma adiponectin levels in obesity and its levels are known to be lower in patients with the metabolic syndrome (Galic et al. 2010). Adiponectin is an important hormone, which may associate obesity with fat accumulation in liver and muscle cells. Another important hormone is leptin, the levels of which are elevated in obesity (Rasouli & Kern 2008). Leptin is considered to be antisteatotic hormone that
protects non-adipose tissue such as liver from fat accumulation and lipotoxicity (Lee et al. 2001).

CVD is a growing concern in Western countries (Roger et al. 2011). Obesity is a key factor leading to hypertension, dyslipidemia and insulin resistance, which in turn promote the development of CVD (Van Gaal et al. 2006). It is suggested that the connection between obesity and CVD is indirect and dependent on other metabolic abnormalities. It has been suspected that there is a strong association between NAFLD and CVD, and according to recent studies CVD is the single most important cause of mortality in this patient population (Targher et al. 2010).

The aim of this thesis is to investigate the role of adipocytokines in fat accumulation in liver and muscle as well as the role of hepatic TG content in the future risk of CVD.
2 Review of the literature

2.1 The liver

2.1.1 Healthy liver in lipogenesis and glucose metabolism

The liver is a central organ in lipogenesis, gluconeogenesis and cholesterol metabolism. Under fasting conditions, hepatic fatty acids derive mainly from adipose tissue lipolysis and less than 5% of intrahepatocellular TGs originate from de novo lipogenesis in the hepatocytes (Barrows & Parks 2006). Very low-density lipoprotein (VLDL) FFA spillover from peripheral tissue lipolysis is also a source of intrahepatocellular TGs (Donnelly et al. 2005, Goldberg & Ginsberg 2006). During the fasting state, fatty acid β-oxidation is the predominant source of energy (Bechmann et al. 2012).

In the postprandial state, insulin suppresses hormone-sensitive lipase and the adipose tissue lipolysis (Barrows & Parks 2006). Dietary lipids are packed into lipoprotein particles called chylomicrons. Postprandially, insulin stimulates lipoprotein lipase, the key enzyme in the intravascular catabolism of triglyceride-rich lipoproteins (Hanyu et al. 2004). Chylomicrons deliver triglycerides and free fatty acids to adipocytes. Chylomicron remnants are taken up by hepatocytes. Free fatty acids derived from de novo lipogenesis are either stored in lipid droplets or packed into VLDL and exported into the blood stream (Bechmann et al. 2012).

During the fasting state, the liver produces glucose mainly by glycogenolysis and gluconeogenesis, and these are important pathways that maintain normal plasma glucose levels during starvation (Bechmann et al. 2012). In response to a physiological rise in glucagon, hepatic glucose production is rapidly stimulated. Glucagon is an important regulator of hepatic glucose production during fasting, exercise and hypoglycemia. Hyperglucagonemia mainly has effects on glycogenolysis (Ramnanan et al. 2011) and glucocorticoids are positive regulators of gluconeogenesis.

In the postprandial state, carbohydrates are either oxidized or stored as glycogen in the liver and muscle, or converted to fat via de novo lipogenesis in the liver (Jornayvaz et al. 2010). Furthermore, insulin inhibits glycogenolysis and gluconeogenesis as well as stimulates glycolysis and the synthesis of glycogen in the liver (Saltiel & Kahn 2001).
2.1.2 Non-alcoholic fatty liver disease (NAFLD)

NAFLD refers to lipid accumulation in the liver with an alcohol consumption of less than 20–30 g/day (Scaglioni et al. 2011) or any other causes of liver disease (for example viral, toxic or autoimmune liver disease and storage diseases) (Neuschwander-Tetri & Caldwell 2003). NAFLD is closely associated with serious cardiometabolic abnormalities, including T2DM, metabolic syndrome, and coronary heart disease (Fabbrini et al. 2010, Krawczyk et al. 2010). The overall prevalence of NAFLD is reported to be 10–35% in the United States (US) population and from 6% to 35% in the rest of the world (Vernon et al. 2011). According to recent data, the prevalence of NAFLD in a random sample of Finnish population was 21%, which is 3-fold higher than the prevalence of alcoholic fatty liver disease (Kotronen et al. 2010). The prevalence of ultrasound-diagnosed fatty liver has been reported to range from 40% to 70% in patients with T2DM (Vernon et al. 2011). Histologically NAFLD ranges from simple hepatic steatosis with no inflammation to hepatic steatosis with a necroinflammatory component (NASH), which may lead to cirrhosis and liver failure (Ekstedt et al. 2006). Simple steatosis is considered to be benign with excellent long term prognosis, whereas 5–8% of NASH patients may develop liver cirrhosis in five years (Cortez-Pinto et al. 2003), and especially these patients are at risk of developing further liver complications (Adams et al. 2005). Even after adjustment for total adiposity, fatty liver is reported to be associated with diabetes, hypertension, impaired fasting glucose, metabolic syndrome, low high-density lipoprotein (HDL) concentration, high TG concentration and low adiponectin levels (Speliotes et al. 2010). Furthermore, NAFLD has become the most common reason for elevated liver enzymes in several developed countries (Ekstedt et al. 2006).

2.1.3 Metabolic syndrome and NAFLD

Obesity, which is usually present in the metabolic syndrome, is reaching the characteristic of an epidemic. In 2008 the estimated prevalence of overweight and obesity was 67.3% among US adults. In total, 33.7% of US adults are obese (body mass index [BMI] ≥30 kg/m²) (Roger et al. 2011). In Finland in 2007, the prevalence of obesity was 21% (Vartiainen et al. 2010). Obesity can lead to metabolic syndrome, which is a cluster of risk factors suspected of increasing the risk of CVD and T2DM more than the individual components (Paschos & Paletas
Although several different definitions for metabolic syndrome have been proposed, a harmonized definition for metabolic syndrome has recently been suggested to work best. By this definition, metabolic syndrome is diagnosed when three or more of the following five risk factors are present: fasting plasma glucose ≥ 100 mg/dL (5.6 mmol/L) or undergoing drug treatment for elevated glucose, HDL < 1 mmol/L in men or < 1.3 mmol/L in women or undergoing drug treatment for reduced HDL cholesterol, TG ≥ 1.7 mmol/L or undergoing drug treatment for elevated triglycerides, waist circumference ≥ 102 cm in men or ≥ 88 cm in women in Europe, blood pressure ≥ 130 mm Hg systolic or ≥ 85 mm Hg diastolic or undergoing drug treatment for hypertension (Alberti et al. 2009).

Several mechanisms contribute to metabolic syndrome and insulin resistance, including nutrition (positive energy balance), lifestyle and genetic factors (Tilg & Moschen 2008). In an insulin-resistant state, there are several impaired cellular signaling pathways (Utzschneider & Kahn 2006) which may lead to 1) decreased glucose uptake and decreased glycogen synthesis in skeletal muscle, 2) increased lipolysis and impaired glucose uptake and triglyceride synthesis in adipose tissue, 3) increased invasion of mononuclear cells in white adipose tissue, 4) liver dysfunction, 5) pancreatic β-cell dysfunction, 6) impaired vasodilatation, 7) impaired energy metabolism in cardiomyocytes, 8) failure to suppress appetite in the brain and 9) increased CVD risk via several mechanisms. These actions, which are at least partly mediated by insulin resistance, lead to metabolic status which is usually present in metabolic syndrome (Rask-Madsen & Kahn 2012).

NAFLD is regarded as a manifestation of metabolic syndrome. Liver fat accumulation seems to play a major role in the pathogenesis of insulin resistance (D'Adamo et al. 2010) and metabolic syndrome (Kotronen & Yki-Jarvinen 2008). Liver fat content was significantly increased in subjects with the metabolic syndrome compared to subjects without the syndrome independently of age, gender and BMI. Furthermore, liver fat has been shown to be highly significantly and linearly correlated with all components of metabolic syndrome (Kotronen et al. 2007). Increased fatty acid flux into the liver impairs liver metabolism (Despres & Lemieux 2006) and fatty liver overproduces components of the metabolic syndrome, such as glucose (Magkos et al. 2012) and lipids (Bugianesi et al. 2005). Hepatic insulin resistance is a major cause of fasting hyperglycemia in metabolic syndrome because insulin is not able to inhibit hepatic gluconeogenesis and glycogenolysis (Rask-Madsen & Kahn 2012).

Fatty liver overproduces VLDL (Fabbrini et al. 2009) and downregulates low density lipoprotein receptor (LDLR) (Rask-Madsen & Kahn 2012), which lead to
increased proportion of small, dense low-density lipoprotein (sdLDL) cholesterol particles (Chatrath et al. 2012), increased peripheral TG concentrations as well as decreased HDL cholesterol level (Kotronen et al. 2007) (Figure 1). These are hallmarks of diabetic dyslipidemia which are present in metabolic syndrome (Savage & Semple 2010).

**Fig. 1. Glucose and fat metabolism in insulin-resistant state and fatty liver disease.** Increased lipolysis in adipose tissue results in elevated levels of peripheral FFAs, which predispose to IMCL and FFA oversupply in the liver. Hyperinsulinemia leads to increased DNL in the liver. Fatty liver overproduces VLDL and downregulates LDLR, resulting in increased proportion of sdLDL cholesterol particles, decreased HDL cholesterol level and elevated peripheral TG concentrations. Insulin is not able to inhibit hepatic gluconeogenesis and glycogenolysis in fatty liver, which leads to increased blood glucose concentration. CM, chylomicron, DNL, de novo lipogenesis, FFA, free fatty acid, GLUT4, glucose transporter type 4, HDL, high-density lipoprotein, IR, insulin resistance, IMCL, intramyocellular lipid, LDLR, low density lipoprotein receptor, NASH, non-alcoholic steatohepatitis, sdLDL, small dense low-density lipoprotein, TG, triglyceride, VLDL, very low-density lipoprotein. (Modified from Lewis et al. 2002, Zivkovic et al. 2007).
2.1.4 Pathogenesis of fat accumulation in the liver

Obesity and insulin resistance are regarded to be key mechanisms leading to accumulation of TGs in the liver (Tilg & Moschen 2008). It is postulated that fatty liver is a result rather than a cause of peripheral insulin resistance in obesity (Liu et al. 2010). The association between visceral fat, insulin resistance and hepatic steatosis has been reported several times (Cnop et al. 2002, Leite et al. 2009). Noteworthy, NAFLD patients have been reported to be insulin resistant at the level of the adipose tissue, liver, and muscle (Ortiz-Lopez et al. 2012) and hepatic insulin resistance plays role in whole body insulin resistance (Magkos et al. 2012). Visceral obesity in rodents is associated with alterations in insulin signaling, insulin resistance, decreased peroxisome proliferator-activated receptor alpha expression (PPARα) and hepatic steatosis. These alterations may lead to hepatic oxidative stress, necroinflammatory liver injury, cell apoptosis, and collagen deposition (Svegliati-Baroni et al. 2006).

There may be two major mechanisms leading to liver fat accumulation: adipose tissue lipolysis and peripheral hyperinsulinemia (Angulo 2002, Mehta et al. 2012). Furthermore, impaired fatty acid oxidation and increased hepatic de novo lipogenesis play a key role in the pathogenesis of liver fat accumulation (Smith & Adams 2011b). In obesity and insulin resistance, hypertrophied intra-abdominal adipocytes are resistant to the antilipolytic effect of insulin (Despres & Lemieux 2006). This leads to increased lipolysis in the peripheral adipose tissue and excess FFA flux to the liver (Bugianesi et al. 2005). In hepatocytes, hyperinsulinemia increases de novo synthesis of fatty acids (Angulo 2002, Bugianesi et al. 2005). Furthermore, hyperinsulinemia may decrease synthesis of apolipoprotein B-100 (apoB-100), which leads to hepatic lipid accumulation because of decreased VLDL synthesis (Angulo 2002, Smith & Adams 2011b). Noteworthy, increased VLDL-apoB-100 concentrations have been reported in subjects with fatty liver (Chan et al. 2010). Increased fatty acid uptake and lipogenesis by hepatocytes together lead to mitochondrial β-oxidation overload and inadequate compensatory fat oxidation (Rolo et al. 2012).

There is evidence that free fatty acids themselves may not be sufficient to induce fatty liver disease (Parekh & Anania 2007). NASH pathogenesis is widely recognized as a two-hit model. The “first hit” consists of metabolic disturbances that increase the inflow of FFAs into the liver and de novo lipogenesis, leading to hepatic steatosis. Increased levels of intrahepatic fatty acids are a source of oxidative stress. The “second hit” includes oxidative stress from mitochondria.
and cytochrome P-450 system, which is characterized by excessive production of reactive oxygen species (ROS), decreased hepatic adenosine triphosphate (ATP) production, and increased expression and induction of inflammatory cytokines. These factors may trigger necroinflammation leading from steatosis to the progression of steatohepatitis (Rolo et al. 2012).

2.1.5 The role of adipocytokines and other obesity-related hormones in the pathogenesis of NAFLD

Chronic low-grade inflammation is associated with obesity (Despres & Lemieux 2006). Adipose tissue is considered a metabolically active endocrine organ which produces inflammatory molecules that play a major role in developing NAFLD, especially NASH (Utzschneider & Kahn 2006). Adipocytes and the stromal vascular fraction secrete several hormones, complement factors, cytokines (for example tumor necrosis factor-alpha [TNF-α], interleukins), chemokines (for example plasminogen activator inhibitor 1 [PAI-1]), enzymes and peptide hormones called adipocytokines (Rolo et al. 2012).

Adipokines in NAFLD

Adipokines constitute a wide spectrum of factors that regulate body weight, insulin sensitivity, glucose and lipid metabolism and inflammation (Rasouli & Kern 2008). Adiponectin, an adipokine secreted mainly by adipose tissue, is extensively reviewed in chapter 2.3.

Leptin is a 167-amino acid hormone, secreted largely by adipose tissue and considered to control food intake and expenditure, and its levels are elevated in obesity (Rasouli & Kern 2008). Leptin is considered to be an antisteatotic hormone which protects non-adipose tissue such as liver from fat accumulation and lipotoxicity (Lee et al. 2001). In animals, leptin is remarkably effective in improving hyperglycemia in some models of T2DM (Coppari & Bjorbaek 2012). According to human studies, in insulin resistant states, such as obesity, the protective role of leptin is limited, mainly because of leptin resistance. Hyperleptinemia is suspected to have unfavorable effects. It may promote insulin resistance, hepatic steatosis, inflammation, fibrosis and oncogenesis (Polyzos et al. 2011a). Furthermore, serum leptin is an independent predictor of NAFLD, and its levels are reported to be increased in NAFLD compared with healthy controls (Wong et al. 2006). It is positively correlated with the severity of hepatic
steatosis, inflammation and fibrosis, but predicts independently only steatosis (Chitturi et al. 2002).

Resistin is a 108-amino acid peptide hormone, which is expressed by adipocytes in mice and by macrophages in humans (Jamaluddin et al. 2012). Several rodent studies suggest that the major target of resistin is the liver where it induces hepatic insulin resistance leading to additional effects on skeletal muscle and adipose tissue (Tschatzis et al. 2009). Serum resistin concentration has been reported to be elevated in NAFLD and it correlates with the severity of the disease but not with the extent of insulin resistance or obesity (Pagano et al. 2006). Furthermore, there is evidence that resistin has a proinflammatory action by stimulating a nuclear factor-kappa B (NF-κB) pathway. Resistin has a direct detrimental impact on human hepatic lipid and lipoprotein regulation (Costandi et al. 2011).

**Obesity-related hormones and cytokines in NAFLD**

Ghrelin is a 28-amino acid peptide hormone that is primarily expressed by the stomach fundus and pancreas, but also to a lesser extent by the hypothalamus. Ghrelin is a potent appetite stimulant and its levels have been shown to correlate negatively with insulin levels and insulin resistance (Delhanty & van der Lely 2011). Serum ghrelin concentrations have been reported to be decreased in NAFLD (Gutierrez-Grobe et al. 2010), but contrary results also exist (Estep et al. 2011).

High-sensitivity C-reactive protein (hs-CRP) has widely been associated with NAFLD and NASH (Targher et al. 2008a). It is a major human acute phase protein, which is mainly produced by hepatocytes in consequence of inflammatory stimuli. Hs-CRP is highly dependent on BMI although it predicts hepatic steatosis also after adjustment for BMI in obese patients. Noteworthy, the data concerning the role of CRP in the severity of NAFLD is controversial (Targher et al. 2008a, Zimmermann et al. 2011). Fatty liver overproduces CRP and it may cause further metabolic disturbances, such as CVD (Zimmermann et al. 2011).

Inflammatory cells infiltrating visceral adipose tissue as well as adipose tissue secrete cytokines, such as TNF-α, which is a key factor in insulin resistance and NAFLD (Rasouli & Kern 2008). Multiple TNF-α-induced mechanisms may be involved in insulin resistance and liver fibrosis. In humans, the serum level of TNF-α is significantly higher in patients with NASH compared to simple steatosis.
and in NAFLD compared to obese controls (Jarrar et al. 2008) and it is highly expressed in the metabolic syndrome. In the liver, TNF-α induces de novo lipogenesis in hepatocytes leading to liver fat accumulation. TNF-α also activates NF-κB leading to transcription of many other proinflammatory cytokines (Wree et al. 2011).

Other adipocytokines which have been reported to have a role in metabolic complications of obesity are retinol-binding protein 4 (RBP4), visfatin and interleukin-6 (IL-6). IL-6 is secreted by adipocytes, immune and endothelial cells. The role of IL-6 in the pathogenesis of NAFLD is controversial. IL-6 is reported to have hepatoprotective action in fatty liver by suppressing oxidative stress and preventing mitochondrial dysfunction, but in the long term it may sensitize the liver to injury and apoptotic cell death (Tsochatzis et al. 2009). IL-6 and TNF-α are the main inducers of CRP secretion in the liver (Van Gaal et al. 2006). RBP4 is predominantly expressed in visceral adipose tissue and its concentration is elevated in insulin resistant state (Rasouli & Kern 2008, Tsochatzis et al. 2009) (Figure 2). Visfatin is secreted by many cells and tissues. Visfatin may have a protective role in NAFLD and its levels have been reported to be lower in NASH patients than in obese controls and in patients with simple steatosis (Jarrar et al. 2008).
2.2 Skeletal muscle

Skeletal muscle is a key metabolic tissue, which is responsible for about 80% of total glucose disposal under normal insulin-stimulated conditions (Taube et al. 2009). Plasma insulin concentration restrains lipolysis in adipocytes and stimulates glucose uptake in skeletal muscle. When fasting, muscle glucose uptake is low and the plasma FFA concentration is elevated (Abdul-Ghani & DeFronzo 2010). Under normal conditions, skeletal muscle contains the majority of the glycogen stores and a small amount of intramyocellular TG. Skeletal muscle has a capacity to utilize either lipid or carbohydrate as a source of energy and effectively transit between these fuel sources. Small amounts of intracellular lipids are important energy sources for skeletal muscle during low glucose supply (Kelley et al. 2002).
2.2.1 Pathogenesis of skeletal muscle fat accumulation

Increased lipolysis in peripheral adipose tissue leads to lipid oversupply and storage of available FFAs in muscle cells when they can no longer be accomplished by adipose tissue (Taube et al. 2009). Increased intramyocellular lipid (IMCL) has been linked to obesity and decreased whole body insulin sensitivity (Kotronen et al. 2008) and skeletal muscle triglyceride content is significantly increased in T2DM (He & Kelley 2004). Furthermore, it is hypothesized that insulin resistance in skeletal muscle decaeses non-oxidative storage of ingested carbohydrates, which are then converted into liver for hepatic de novo lipogenesis resulting in peripheral hypertriglyceridemia (Jornayvaz et al. 2010).

Although NAFLD seems to be central to the pathogenesis of T2DM (D'Adamo et al. 2010), IMCL have also been reported to be associated with impaired insulin-induced glucose metabolism (Hirabara et al. 2010). There is also evidence that skeletal muscle mitochondrial dysfunction is a reason for insulin resistance and T2DM rather than triglyceride content per se (Schrauwen-Hinderling et al. 2007), which means that IMCL is not independently responsible for the development of T2DM. Intramyocellular lipid accumulation has also been associated with decreased peripheral insulin sensitivity in healthy individuals (Salgin et al. 2009), suggesting that IMCL may be an early step in the development of T2DM.

2.2.2 Role of obesity-related peptide hormones in intramyocellular lipid accumulation

Adipocytokines play a role in the pathogenesis of IMCL and insulin resistance. The role of adiponectin is reviewed in chapter 2.3.4. Leptin has a range of important effects in tissues active in energy metabolism, mainly skeletal muscle (Galic et al. 2010). These effects include increased fatty acid oxidation in an adenosine monophosphate-activated protein kinase (AMPK) dependent manner (Minokoshi et al. 2002), increased muscle TG breakdown and decreased TG esterification (Kelley et al. 2002). In obesity, the development of leptin resistance in the skeletal muscle is characterized by suppressed rates of leptin-stimulated AMPK signaling, which may contribute to the aberrant regulation of fatty acid metabolism (Galic et al. 2010).
The role of resistin in skeletal muscle homeostasis is not clear. It is supposed that resistin impairs insulin-stimulated glucose uptake by muscle cells, this observation being somehow dependent on the AMPK pathway. Furthermore, resistin may indirectly diminish muscle cell fatty acid oxidation via AMPK signaling (Dzamko & Steinberg 2009).

Ghrelin may have direct effects on skeletal muscle energy control. The results of one study suggest that ghrelin may stimulate lipolysis directly in skeletal muscle (Vestergaard et al. 2011). Several other adipocytokines have been reported to regulate skeletal muscle glucose and lipid metabolism via the AMPK pathway but ghrelin may have no effect on AMPK (Dzamko & Steinberg 2009).

CRP is mainly produced by the liver. In rats, high CRP levels promote disturbances in insulin and glucose metabolism, including hyperinsulinemia and impaired insulin-stimulated glucose incorporation into skeletal muscle glycogen. Furthermore, CRP is supposed to induce oxidative stress in peripheral tissues, leading to further metabolic disturbances (Pravenec et al. 2011).

2.2.3 Muscle fiber characteristics in metabolic disturbances

In human skeletal muscle, there are two major classes of fiber types, type I (slow) and type II (fast). Based on biochemical staining, type II fibers are divided into two groups. The more oxidative fibers are termed IIa and the more glycolytic ones IIb. Slow-twitch (type I) myofibers contain a high concentration of mitochondria and this fiber type uses oxidative metabolism as its main energy source. Type II fibers, called fast-twitch myofibers, generate ATP mainly through glycolysis (Scott et al. 2001). Muscles rich in type II fibers are more susceptible to fatigue mainly because their glycolytic metabolism causes acidification (Moro et al. 2008).

Skeletal muscle characteristics of healthy individuals differ from the characteristics of obese and insulin-resistant individuals. For instance, the proportion of slow-twitch (type I) fibers is decreased in insulin-resistant subjects (Coen et al. 2010). The number of insulin-stimulated glucose transporters is increased in skeletal muscle enriched with type I muscle fibers (Daugaard et al. 2000). Muscle glycogen has been reported to be reduced in T2DM and there is ~20% reduction in muscle glycogen in T2DM compared with muscle from lean non-diabetics (He & Kelley 2004). In insulin-resistant state, skeletal muscle has been shown to express fewer genes active in energy metabolism leading to mitochondrial dysfunction and impaired β-oxidation (Patti et al. 2003).
Muscle fiber characteristics are related to obesity (Zierath & Hawley 2004). Obese women have been reported to have less type I and more type IIb fibers than lean women (Tanner et al. 2002). In obese individuals, there are increased amounts of fatty acid transporters at the plasma membrane of skeletal muscle leading to enhanced transport of free fatty acids into skeletal muscle and intramyocellular lipid accumulation (Eckardt et al. 2011).

2.3 Adiponectin

Adiponectin, a 244-amino acid collagen-like protein that is solely secreted by adipocytes, has anti-inflammatory, antiatherogenic and insulin-sensitizing properties (Li et al. 2009b). Circulating concentrations of adiponectin are mainly determined by genetic factors, nutrition, exercise and abdominal obesity (Polyzos et al. 2011b). Adiponectin exists in the plasma in a wide range of multimer complexes but there are three major oligomeric forms: a low-molecular weight (LMW) trimer, a middle-molecular weight (MMW) hexamer, and high-molecular weight (HMW) 12- to 18-mer. HMW is regarded to be the more active form of the protein and to play a major role in insulin sensitivity and in protecting against diabetes (Kadowaki et al. 2006).

Adiponectin may reduce the risk of T2DM via several mechanisms. Adiponectin activates fatty acid oxidation and improves insulin sensitivity and glucose uptake in skeletal muscle and liver. Animal experiments have shown that adiponectin has effects on both body weight and insulin sensitivity in the liver and muscle but does not seem to have a major role in long-term body weight control (Lu et al. 2008). Therefore, decreased adiponectin levels may be a consequence rather than a cause of obesity as well as insulin resistance in adipose tissue.

Adiponectin mediates its effects via adiponectin receptors (AdipoR). AdipoR1 is mainly produced in skeletal muscle and to some degree in the liver, whereas AdipoR2 is mainly expressed in the liver and little in skeletal muscle (Dyck et al. 2006). In mice, insulin resistance decreases both plasma adiponectin levels and AdipoR1/AdipoR2 expression leading to reduced fatty acid oxidation and glucose uptake in muscle cells. Furthermore, obesity decreases not only plasma adiponectin levels but also AdipoR1/R2 expression, leading to decreased adiponectin sensitivity and insulin resistance, which in turn aggravates hyperinsulinemia (Tsuchida et al. 2004). The metabolic effects of adiponectin are at least partially mediated by signaling pathways for AMPK and PPARα (Dzamko
Furthermore, adiponectin stimulates AMPK in the hypothalamus and increases food intake (Kubota et al. 2007).

### 2.3.1 Role of adiponectin under normal conditions

**Actions of adiponectin in the liver**

In the normal liver, adiponectin is considered to have insulin-sensitizing, antifibrogenic, and anti-inflammatory properties by having influence on hepatocytes, hepatic stellate cells, and hepatic macrophages (Kupffer cells). Adiponectin binds to AdipoR2 on the hepatic cell membrane. The AMPK pathway seems to play an important role in the insulin-sensitizing action of adiponectin in the liver and adiponectin also increases fatty-acid combustion and energy consumption by activating the PPARα pathway. As a consequence, adiponectin diminishes gluconeogenesis in the liver (Polyzos et al. 2010) and prevents fatty acid accumulation by increasing β-oxidation of FFAs (Yamauchi et al. 2002) and by decreasing de novo lipogenesis within hepatocytes (Awazawa et al. 2009). These actions have been reported to decrease triglyceride content in the liver, and thereby increase insulin sensitivity. Apart from the role as a metabolic mediator, adiponectin is supposed to have antifibrotic action in the liver (Polyzos et al. 2010), mainly by suppressing proinflammatory cytokines (Kern et al. 2003), by inducing anti-inflammatory cytokines (Luo et al. 2011), through down-regulating the expression of aldehyde-oxidase (Neumeier et al. 2006) and by transforming connective tissue growth factor (Walter et al. 2011).

**Role of adiponectin in skeletal muscle**

Under normal conditions, adiponectin has several beneficial effects on skeletal muscle. Adiponectin interacts mainly with AdipoR1 and less with AdipoR2 in skeletal muscle. In myocytes, adiponectin has been reported to stimulate PPARα, AMPK and p38 mitogen-activated protein kinase (MAPK) activation (Yoon et al. 2006a). According to previous data, adiponectin significantly activated PPARα ligand which led to stimulated fatty acid oxidation. AdipoR1 activated AMPK and p38 MAPK resulting in increased fatty acid oxidation and glucose uptake. Thus it is obvious that PPARα activation plays a major role in adiponectin-activated fatty-acid oxidation; other actions of adiponectin seem to be mediated partly via
phosphorylation of AMPK and p38 MAPK (Figure 3). In addition, there may be unsolved molecular pathways (Yamauchi et al. 2003). Furthermore, adiponectin increases skeletal muscle glucose uptake due to enhanced glucose transporter type 4 (GLUT4) translocation; however, it is noteworthy that adiponectin has this effect via the AMPK pathway whereas insulin has a similar effect via the insulin signaling cascade (Dyck et al. 2006, Dzamko & Steinberg 2009).

![Fig. 3. Actions of adiponectin in the liver and skeletal muscle. AdipoR1/2, adiponectin receptor 1/2, AMPK, adenosine monophosphate-activated protein kinase, GLUT4, glucose transporter type 4, PPARα, peroxisome proliferator-activated receptor alpha, DNL, de novo lipogenesis. (Modified from Yamauchi et al. 2002, Yamauchi et al. 2003, Yoon et al. 2006b).]

### 2.3.2 Adiponectin and metabolic disturbances

Several studies have linked decreased adiponectin levels to diabetes, hypertension, atherosclerosis and endothelial dysfunction (Rasouli & Kern 2008). Adiponectin secretion is paradoxically decreased in obesity (Kovacova et al. 2012) and independently associated with a reduced risk of T2DM in apparently healthy individuals (Spranger et al. 2003). Hypo-adiponectinemia seems to mediate the effect of obesity and adipose tissue insulin resistance in other
peripheral tissue, leading to further insulin resistance for example in liver and muscle. In adipose cell culture studies, insulin has a direct stimulatory effect on adiponectin gene expression (Blumer et al. 2008). Expression of adiponectin may be inhibited by obesity-related insulin resistance and inflammation (through factors such as TNF-α, IL-6) in adipose tissue (Lu et al. 2008).

The peroxisome proliferator-activated receptor gamma (PPARγ) pathway is known to be important in insulin sensitizing actions, and activating this pathway increases adiponectin biosynthesis in rodents (Amin et al. 2010). Furthermore, the actions of PPARγ agonists are at least partly mediated by their effect on muscle insulin-stimulated glucose transport (GLUT4) and by their direct influence on the adiponectin and adiponectin receptor expression (Pita et al. 2012). In insulin-resistant state, improving adipose-specific insulin sensitivity by activating PPARγ in adipose tissue, adiponectin gene expression increases independently of changes in total adiposity (Kadowaki et al. 2006).

2.3.3 Adiponectin in NAFLD

In NAFLD, adiponectin levels are reported to be decreased (Fabbrini et al. 2009) and its levels predict the severity of liver disease (Jarrar et al. 2008, Targher et al. 2006). NASH patients have been reported to have reduced adiponectin levels compared to matched controls and subjects with simple steatosis without any relation to insulin resistance or waist-hip ratio (Hui et al. 2004). Adiponectin is linked to other adipocytokines and cytokines in a complicated communication network (Polyzos et al. 2011b). Reduced adiponectin level in insulin resistance and obesity can partly be regarded as a marker of inflammatory state and it may establish an individual’s susceptibility to lipotoxicity and risk for developing NASH and advanced hepatic fibrosis (Wree et al. 2011). For example, adiponectin and TNF-α actions are antagonistic. Adiponectin inhibits the expression, secretion and action of TNF-α (Kern et al. 2003), thereby ameliorating insulin sensitivity, whereas TNF-α suppresses adiponectin transcription, secretion and action, enhancing insulin resistance (Li et al. 2009a).

Liver expression of adiponectin is reported to be decreased in morbidly obese patients and patients with NASH compared to simple steatosis. Whether the differences in AdipoR expression play a role in the pathogenesis of NAFLD remains controversial (Polyzos et al. 2010). According to recent data, adiponectin and AdipoR2 messenger ribonucleic acid (mRNA) expression were significantly reduced in liver biopsies of patients with NASH compared with simple steatosis
(Kaser et al. 2005a). In AdipoR knockout mice, expression of either AdipoR1 or AdipoR2 in the liver could improve insulin resistance. Decreased AdipoR expression in the liver may result in increased tissue triglyceride content, inflammation and oxidative stress leading to insulin resistance and marked glucose intolerance (Yamauchi et al. 2007). Interestingly, serum adiponectin levels have been reported to be increased in liver cirrhosis. There are two hypotheses explaining this paradox: hepatic clearance of adiponectin may be diminished in cirrhotic state (Tietge et al. 2004) or adiponectin production may be a compensatory act against the overwhelming production of cytokines by the cirrhotic liver (Kaser et al. 2005b).

### 2.3.4 Adiponectin and impaired skeletal muscle energy homeostasis

Skeletal muscle fiber characteristics are associated with adiponectin levels (Ingelsson et al. 2009). It is postulated that adiponectin could be a partial mediator between skeletal muscle morphology and insulin sensitivity. Adiponectin concentrations have been reported to be increased in subjects with higher skeletal muscle capillary density and with higher proportion of type 1 (slow) muscle fibers, which use oxidative metabolism as a main energy source (Ingelsson et al. 2009). In insulin resistant state and obesity, plasma adiponectin levels are significantly decreased. There also appears to be an impaired response to adiponectin in skeletal muscle (Dyck et al. 2006).

Skeletal muscle adiponectin receptor expression in subjects with family history of T2DM has been reported to be decreased (Civitarese et al. 2004) and hyperinsulinemia might be a partial cause for this phenomenon (Kadowaki et al. 2006). It is noteworthy that exercise/diet intervention, leading to improvement in insulin sensitivity, have been reported to increase skeletal muscle AdipoR expression in insulin-resistant adults (O'Leary et al. 2007). In obese individuals, activation of AMPK in skeletal muscle is diminished (Bruce et al. 2005). Recently, high saturated fat feeding in rats caused a rapid loss of adiponectins stimulatory effect on fatty acid oxidation. This phenomenon was not dependent on skeletal muscle AdipoR1 expression (Mullen et al. 2009). Furthermore, blunted activation of AMPK in skeletal muscle of obese and obese insulin-resistant individuals was not dependent on AdipoR expression (Chen et al. 2005).

Plasma adiponectin levels have been reported to be inversely associated with intramyocellular lipid accumulation in obese women (Perseghin et al. 2007) and HMW-adiponectin has been reported to correlate inversely with IMCL.
Interestingly, paradoxical upregulation of adiponectin has been reported in the muscle of obese and diabetic mice; this phenomenon may result from lipotoxicity and related oxidative stress (Delaigle et al. 2006).

2.4 Cardiovascular disease (CVD)

CVD is a growing concern in Western countries. In 2008, the prevalence of cardiovascular diseases in adults (≥ 20 years) in the US was 36.2%. Mortality data show that CVD as the underlying cause of death accounted for 33.6% of all deaths in the US in 2007 (Roger et al. 2011). Every year, 4.3 million subjects die from CVD in Europe causing nearly half of the all deaths (48%) (Allender et al. 2008). So-called traditional risk factors for CVD are age, gender, smoking, high LDL-cholesterol, hypertension and diabetes (Despres & Lemieux 2006). According to one large study, participants with an optimal risk-factor profile (optimal cholesterol level and blood pressure, non-smoking and non-diabetic status) had substantially lower risks of death from CVD than participants with two or more major risk factors (Berry et al. 2012). Furthermore, the metabolic syndrome is associated with cardiovascular morbidity and mortality (Bonora 2006).

2.4.1 Mechanisms linking obesity, insulin resistance and CVD

Obesity is a key factor leading to inflammation and insulin resistance, which in turn promote the development of CVD (Rader 2007). It is suggested that the connection between obesity and CVD is indirect and dependent on insulin-resistant state, hypertension and dyslipidemia, although there is also some evidence of a direct connection. The direct mediators between obesity and CVD could be adipocytokines, which are mainly produced by adipocytes. These biologically active molecules include adiponectin, leptin, resistin, PAI-1, TNF-α and IL-6 (Galic et al. 2010, Marinou et al. 2010).

Obesity leads to insulin resistance and inflammation via several mechanisms. In obesity, peripheral triglyceride concentrations are usually elevated. Chronic exposure of the pancreas to FFAs may lead to decreased glucose-sensing capacity of β-cells and increased risk for T2DM (Lewis et al. 2002). Furthermore, adipose tissue also produces adipocytokines which are important mediators, but in obesity their functions are unbalanced. FFAs might bind toll-like receptor 4, which is
present in adipose tissue and macrophages, leading to activation of innate immunity (Rocha & Libby 2009).

Diabetes is associated with increased CVD morbidity and mortality (Bonora 2006). Insulin resistance leads to glucose intolerance and increased production of advanced glycation end products, which directly promote atherosclerosis (Prasad et al. 2012). Impaired insulin signaling causes diminished vascular production of nitric oxide, which is important in normal vasodilatory response (Ritchie & Connell 2007). Insulin resistance also leads to hypertension, mainly because of impaired actions of insulin in vessel wall (Cleland et al. 2000). Insulin resistance causes impaired thrombolysis, which predicts CVD (Trost et al. 2006).

Cigarette smoking is known to be a risk factor for CVD and it may add several mechanisms linking obesity to CVD. Smoking may increase cytokine, CRP and IL-6 production, it downregulates adiponectin and HDL level, may increase TNF-α concentration and enhances the oxidative stress leading to insulin resistance and endothelial dysfunction (Van Gaal et al. 2006).

2.4.2 CVD and NAFLD

NAFLD is reported to be associated with increased risk of CVD and NASH patients have a higher risk than patients with simple steatosis (Ekstedt et al. 2006). In humans, NASH can predict a more atherogenic risk profile in a manner that is partly independent of the visceral adiposity (Targher et al. 2008a). Recent data indicate that NASH patients have a more atherogenic lipoprotein profile and greater potential for developing CVD compared to patients with simple steatosis (Musso et al. 2012). Fat accumulation in skeletal muscle and liver result in impaired insulin signaling in these tissues, aggravating peripheral insulin resistance and predisposing to cardiometabolic dysfunction (Fabbrini et al. 2009). Therefore, biological effects of lipid overload occur in several organ systems, such as arterial wall, liver and muscle (Loria et al. 2008).

Several studies have suggested that NAFLD may not be only a marker of CVD but may also play a role in its pathogenesis (Figure 4). Chronic low inflammation and oxidative stress state are present in NAFLD (Alkhouri et al. 2012) and macrophage infiltration in the liver is characteristic in NASH (Kudo et al. 2009). Inflammation, such as increased accumulation of macrophages in adipose tissue and recruitment of inflammatory cells in the intima of arterial wall, is also an important player in the pathogenesis of atherosclerosis (Loria et al. 2008). Hepatocellular damage and fat-derived factors activate the NF-κB pathway.
and lead to increased intrahepatic cytokine (such as IL-6 and TNF-α) expression (Targher et al. 2010). Fatty liver also produces and releases inflammatory markers (such as CRP) and pro-atherogenic factors (such as fibrinogen and PAI-1) (Targher et al. 2008b). These liver-secreted mediators in fatty liver disease are likely to play a role in the pathogenesis of systemic inflammation and atherosclerosis (Bhatia et al. 2012).

Fatty liver also contributes to atherogenic dyslipidemia (Speliotes et al. 2010). In normal liver, insulin has the ability to inhibit the production of VLDL. In fatty liver, the inability of insulin to diminish VLDL production leads to overexpression of VLDL particles (Semple et al. 2009). It is noteworthy that VLDL production is also a compensatory mechanism that protects the liver from triglyceride accumulation but also increases peripheral TG concentrations. In insulin-resistant states, mainly because of increased cholesteryl ester transfer protein (CETP) and hepatic lipase activity, VLDL ends up in the generation of sdLDL particles. SdLDL is regarded as an atherogenic form of LDL because it can move through endothelial fenestrations to the subendothelial space where inflammation and plaque formation occur (Van Gaal et al. 2006). Patients with severe steatosis have been reported to have significantly increased sdLDL particle concentrations (Toledo et al. 2006). Furthermore, low HDL concentrations are associated with increased VLDL production and CETP activity contributing to CVD (Meshkani & Adeli 2009).

Hypertension is a well-known risk factor for CVD (Berry et al. 2012). An independent association between endothelium-dependent flow-mediated dilation and fatty liver has been reported, suggesting that NAFLD is associated with vessel wall endothelial dysfunction (Villanova et al. 2005). Carotid intima-media thickness (IMT) is a marker of early arterial wall change and it is markedly increased in patients with NAFLD (Sookoian & Pirola 2008), and NAFLD histology predicts increased IMT independently of other metabolic risk factors, such as insulin resistance and features of metabolic syndrome (Targher et al. 2006). Furthermore, intrahepatic fat content has been reported to be associated with impaired left ventricular energy metabolism and the association was independent of usual CVD risk factors (Perseghin et al. 2008). Excess FFA flux into cardiac myocytes leads to lipotoxicity and impaired cardiomyocyte oxidative capacity causing increased oxidative stress and eventually cardiac apoptosis and dysfunction (Bhatia et al. 2012).
Fig. 4. Diagram of the pathophysiological processes which increase cardiovascular risk in obesity. Expanded visceral fat mass leads to increased production of inflammatory cytokines, increased insulin resistance and elevated free fatty acid concentrations. These actions predispose to liver fat accumulation and steatohepatitis. Impaired liver functions result in increased production of inflammatory proteins predisposing to chronic inflammation. The diseased liver overproduces coagulation factors leading to hypercoagulation and hypofibrinolysis. Hepatic dysfunction results in development of atherogenic dyslipidemia, dysglycemia and hepatic insulin resistance. IR, insulin resistance, NASH, non-alcoholic steatohepatitis (Modified from Bhatia et al. 2012, Targer et al. 2010).
3 Purpose of the study

The specific aims of the studies are the following:

1. To explore the important independent indicators of increased liver brightness in a large randomized population-based cohort (OPERA).
2. To test the hypothesis that muscle fiber characteristics are associated with overweight and components of metabolic syndrome and whether adipokines/other hormones associate with fiber characteristics.
3. To investigate if the differences in liver fat accumulation predict the risk for development of fatal or nonfatal atherosclerotic endpoints such as coronary heart disease and stroke.
4 Subjects and methods

4.1 Study subjects

The basic details of the study groups used in this thesis are defined in Table 1.

Table 1. Details of the study groups used in the studies.

<table>
<thead>
<tr>
<th>Study</th>
<th>Name of the study</th>
<th>Reason for collecting the data</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>OPERA (Oulu Project Eludicating Risk of Atherosclerosis)</td>
<td>to investigate the risk factors of atherosclerosis</td>
<td>1015</td>
</tr>
<tr>
<td>II</td>
<td>OPERA subgroup study</td>
<td>to investigate if muscle fiber characteristics are associated with cardiometabolic fitness and status</td>
<td>54</td>
</tr>
<tr>
<td>III</td>
<td>OPERA follow-up study</td>
<td>to investigate if metabolic abnormalities predict the risk of cardiovascular events</td>
<td>988</td>
</tr>
</tbody>
</table>

In short, the Oulu Project Eludicating Risk of Atherosclerosis (OPERA) was established to investigate risk factors and end points of atherosclerosis. Out of the defined population of the city of Oulu, treated male and female hypertensives aged 40–59 years and age- and sex-matched controls for them were recruited. The treated hypertensives were randomly selected from the Social Insurance Institute register for the reimbursement of hypertension medication. The controls were randomly selected from the national health register covering the whole population of the city of Oulu. Altogether, 520 men and 525 women participated (n = 1,045): 259 control men, 261 hypertensive men, 267 control women and 258 hypertensive women. Both the hypertensive and the control men were recruited during December 1990 to May 1992 and the women approximately one year later. The participating study subjects visited the research laboratory of the Department of Internal Medicine, University of Oulu. According to the criteria used in 1989, hypertensive subjects were eligible for the refund if they had diastolic blood pressure above 105 mmHg during a few months’ follow-up. The limit was lower if the patient already showed signs of target-organ damage caused by hypertension or if the patient was young, had a family history of CVD or sudden death at an early age, had diabetes or severe hyperlipidemia or had a systolic blood pressure above 180 mmHg (Kiema et al. 1996).

In study I, participants with exceptional adiponectin concentration were excluded (adiponectin outliers, n = 13) as were subjects who did not participate in liver brightness measurement (n = 17).

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In study II, OPERA data were used but this study was based on non-smoking, normotensive men with normal oral glucose tolerance test (OGTT). Some of the control men were recruited to undergo cardiorespiratory fitness, physical activity and body composition measurements 4–6 years after baseline examinations. In total, 91 subjects out of the 135 selected volunteered, but two of them had to be excluded because of problems in the VO$_2$$_{max}$ test which provided unreliable data in their cases. All the other individuals (n = 89) who participated in the exercise test were invited to provide a muscle biopsy. Sixty-three of them volunteered, and finally 54 muscle specimens of adequate quality could be obtained. No swelling or dissolving of muscle fibers was accepted.

In study III, original OPERA data were used. In this study, subjects with previous myocardial infarction or stroke (n = 41) at baseline were excluded. Only subjects with hospital-diagnosed myocardial infarction or stroke were excluded. Finally, 988 subjects with baseline ultrasound measurements available participated in the study.

All patients were interviewed. The interviews were conducted using standard forms and questionnaires. Special attention was given to the medical history, symptoms of dyspnea or chest pain, current medication, family history of CVD and all other diseases, smoking history and physical activity (Kauma et al. 1998). The estimation of alcohol consumption was based on a detailed and extensive interview concerning drinking frequency and the usual amount of beer, wine and strong alcoholic beverages consumed per occasion (Khavari & Faber 1978). The results were based on the interview by a trained physician with special competence in internal medicine.

In the OPERA dataset, there were 3 subjects with type 1 diabetes at the baseline. Subjects with type 2 diabetes were taken into account by adjusting all important models with QUICKI.

### 4.2 Determination of liver fat

The determination of liver adiposity (OPERA) was based on liver-kidney contrast measured with ultrasonography by one trained radiologist with extensive experience in abdominal ultrasound examinations. The ultrasound examination was carried out using a Toshiba SSA 270 ultrasound system (Toshiba Corp., Tokyo, Japan) with a scanning frequency of 5 Mhz. The entire scanning procedure was captured on video with a Super-VHS video-cassette recorder (Panasonic Corp., Osaka, Japan) and the videotapes were analyzed later. The
severity of liver adiposity was based according to the brightness of the liver estimated as a numerical value ranging from 0 to 2 (0 = normal brightness, indicating a non-fatty liver, 1 = medium brightness, moderate lipid accumulation and 2 = intensely bright, severe lipid accumulation and fatty liver).

4.3 Laboratory methods

BMI was calculated as [mass (kg)] / {\([\text{height (m)}]^2\)}$. Waist circumference was measured to the nearest 0.5 cm with a tape-measure midway between the lower rib margin and the iliac crest in light expirium. Blood pressure was measured according to the recommendations of the American Society of Hypertension in a sitting position from the right arm with an oscillometric device (Dinamap† model 18465X, Criticon Ltd, Ascot, UK) after an overnight fast and after a 10 to 15-minute rest. Three measurements were made at 1-minute intervals, and the means of the last two were used in the analyses.

Body fat percentage was estimated from volunteer control men, who participated in the exercise test. It was measured from four skinfolds with a caliper. Skinfold caliper is four spot formula by Durnin and Womersley and the calculation of body fat percent involves measuring four skinfold sites, subscapular, triceps brachii, biceps brachii and crista iliaca, and substituting the log of their sum into equation (Durnin & Womersley 1974).

Plasma adiponectin concentrations were measured with an enzyme-linked immunosorbent assay (ELISA) technique devised in our laboratory (Santaniemi et al. 2006). Monoclonal antihuman adiponectin antibody (R&D-systems, Cat. MAB10651) was used as the capture antibody and biotinylated monoclonal antihuman adiponectin antibody (R&D-systems, Cat. BAM1065) as the detection antibody. Both antibodies were used in a concentration of 2 µg/mL. To detect biotin-labeled detection antibody, alkaline phosphatase labeled NeutrAvidin was used and diluted in a ratio of 1:18,000 (Pierce Cat. 31 002) and 30% Lumiphos530 (Lumigen, Cat. P-501). The standard curve from 1.56 to 100ng/mL was prepared from human recombinant adiponectin (Biovendor, Cat. RD172023100). Plasma samples were diluted in the ratio of 1:500 and the concentrations were measured in duplicate. In this method, the intra-assay variation was 13.9% whereas inter-assay variation was 15.9% before and 6.5% after correction.

Fasting plasma leptin concentrations were measured using a commercial double antibody radioimmunoassay (RIA) (Human Leptin RIA Kit; Linco
Research, Inc., St. Charles, MO) with an intra-assay coefficient of variation of 3.4–8.3% and an interassay coefficient of variation of 3.0–6.2%. Fasting ghrelin concentrations were measured from plasma samples stored at -20°C using a commercial RIA kit (Phoenix Pharmaceuticals, Belmont, CA), which recognizes both acylated and desacylated ghrelin and uses 125I-labeled bioactive ghrelin as a tracer molecule and a polyclonal antibody raised in rabbits against full-length octanoylated human ghrelin. The intra- and interassay coefficient of variation (CV), as given by the manufacturer, were 4.0% and 7.5%. All assays included three control samples from one fresh plasma sample that was aliquoted and frozen at the beginning of the study and used to normalize each test for interassay variability. Interassay CV in the analyses of this study was 11.2%. Hs-CRP was measured using commercially available enzyme-linked immunoassay (ELISA) kits with a detection limit of 0.31 ng/mL (Diagnostic Systems Laboratories, Texas, US).

Plasma resistin levels (OPERA) were measured from control men samples (n = 525) in duplicate using a commercially available ELISA (Linco Research Inc., USA; intra- and interassay coefficients of variation 4.5 and 7.4%, respectively) according to the manufacturer’s instructions. We determined the interassay coefficient of variation to be 5.2% in our measurements.

After fasting blood had been drawn, the subjects were given a 75-g glucose load. Both 1-hour and 2-hour glucose and insulin concentrations were determined, except for previously known insulin-treated diabetics. The venous blood glucose concentrations were measured with the glucose dehydrogenase method (Diagnostica, Merck, Darmstadt, Germany). The plasma insulin levels were measured using a two-site immunoenzymometric assay (AIA-PACK IRI, Tosoh Corp., Tokyo, Japan). Insulin sensitivity was assessed via fasting insulin concentrations and a quantitative insulin sensitivity check index (QUICKI) \( \{\text{QUICKI} = \frac{1}{\log(\text{fasting insulin}) + \log(\text{fasting glucose})}\} \) (Katz et al. 2000).

VLDL (d < 1.006 g/ml) was isolated by centrifuging plasma in a Kontron TFT 45.6 rotor at 105,000 g and 15°C for 18 h. Half a milliliter 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 min, aliquots of the supernatant were taken for analysis of HDL concentration. LDL concentration was then calculated by subtracting the cholesterol concentration in HDL from that in the VLDL-free fraction. The concentrations of total cholesterol and triglycerides in the plasma and lipoprotein fractions were determined by enzymatic colorimetric methods (kits of Boehringer Diagnostica, Mannheim 42
GmbH, Germany, catalog nos. 236691 and 701912), respectively, using a Kone Specific, Selective Chemistry Analyzer (Kone Instruments, Espoo, Finland). The coefficients of variation for the determination of plasma total cholesterol, HDL cholesterol and triglycerides were 2.1%, 5.5% and 5.3%.

4.4 Muscle biopsy

Skeletal muscle biopsies were obtained from musculus vastus lateralis from the middle of the line between spina iliaca anterior superior and the upper outer corner of the patella by using Bergström needle (Unimed, Lausanne, Switzerland) (5mm) and by taking the tissue sample via a 5–8 mm incision through the skin and fascia (at a depth of 1.5–2.0 cm from the fascia). An average 318 muscle fibers per subject were examined in the morphometric analysis. The total surface area was 1,879,137 µm² and a single muscle fiber area was 6,545 µm². Muscle specimen for the morphometric analysis was placed in isotonic NaCl compression for transportation and stored (2–3 h) at +4°C until examined under a microscope. After muscle fiber orientation, the specimen was frozen in 2-methylbutane (99% p.a.) for cutting tissue slices in a cryostat (-20°C) for muscle fiber types I and II (myosin ATPase activity at pH 4.6 and pH 9.4 according to Dubowitz & Brooke) (Dubowitz et al. 1973). The analyses of the stained sections were performed by magnification (x 4 for muscle cell typing) under a microscope (Nikon, Tokyo, Japan) and by transferring (Camera MTI/CCD 72, Image Research Inc., St Catherine, Ontario, Canada) the artifact-free image to a computer screen for morphometric analysis (Image analysis program M1 5.0 Rev. 1.5, Image Research, Ontario, Canada). The intrareader variability and correlation coefficient were 1.0% and 0.97 for the muscle cell count and 16.1% and 0.94 for the muscle cell area. The interreader variability and correlation were 4.6% and 0.94 for the muscle cell count and 10.5% and 0.96 for the muscle cell area.

Quantitative reverse transcription polymerase chain reaction

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to measure the expression levels of AdipoR1 and AdipoR2 gene. Total RNA was separated from muscle cell samples using RNeasy Mini Kit (Qiagen, Valencia CA). Complementary deoxyribonucleic acid (cDNA) synthesis was performed with RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, Helsinki, Finland). QRT-PCR employed SYBR Green Supermix and the iQ5 real-time PCR
detection system (Bio-Rad, Hercules, CA). GAPDH was used as a reference gene to assess the overall cDNA content in the tested samples with calculations done with the iQ5 Optical System software 2.0 (Bio-Rad, Hercules, CA).

4.5 Mortality and CVD follow-up

Both the hypertensive and the control men were recruited between December 1990 and May 1992 and the women approximately one year later (n = 1,045). In total, 1023 subjects had liver ultrasound result available at baseline. Hospital events were collected from the registry of the National Institute for Health and Welfare (Hilmo register) and mortality data were collected from the National Death Registry. The follow-up time ended on December 31 2009 or whenever the first event occurred. Only the first events were counted. Cardiovascular events included fatal and non-fatal endpoints. Subjects with previous myocardial infarction or stroke (n = 41) at the baseline were excluded. Finally, 988 subjects participated in the study.

CVD included a major coronary heart disease (CHD) event and stroke (excluding subarachnoid hemorrhage [SAH]) - whichever of these happened first. The evidence of CHD was based on the following diagnosis: I20.0, I21, I22 (International Statistical Classification of Diseases [ICD-10] and Related Health Problems) / 410, 4110 (ICD-8/9) as the main diagnosis (symptom or cause) and I21, I22 (ICD-10) / 410 (ICD-8/9) as a first side diagnosis (symptom or cause) or second side diagnosis (symptom or cause) and third side diagnosis (ICD-8/9 only) or if a subject had experienced a coronary artery bypass graft surgery or angioplasty. CHD as a cause of death included I20–I25, I46, R96, R98 (ICD-10) / 410–414, 798 (not 7980A) (ICD-8/9) as underlying cause of death or immediate causes of death and I21 or I22 (ICD-10) / 410 (ICD-8/9) as first to third contributing cause of death. Stroke (excluding SAH) 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 main diagnosis (symptom or cause) or as first or second side diagnosis (symptom or cause) or as third side diagnosis (ICD-8/9 only) or as an underlying cause of death or immediate cause of death or as first to third contributing cause of death.
4.6 Statistical analysis

In studies I and II, statistical analysis was performed by using SPSS for Windows, Version 16 (Chicago: SPSS Inc). In study III, IBM SPSS Statistics for Windows, Version 20.0 (Armonk, NY: IBM Corp.) was used. Analysis of variance (ANOVA) was used to compare the means of the variables measured. Post hoc tests were performed using Tukey’s method. Statistical significances between percentages were measured by chi-square test. If covariates were added into the model, the analysis of covariance (ANCOVA) was used. In multivariate analysis, pairwise comparisons were done with no adjustments. Continuous variables were examined for skewness and curtosis. If skewness and/or kurtosis was +/- 2, the variable was logarithmically transferred. After this, if the variable did not reach normal distribution (+/- 2 or visually not normally distributed), it was examined by Kruskall-Wallis test. Multiple comparisons were performed using Mann-Whitney test corrected with Benjamini Hochberg method. Normally distributed correlations were studied with Pearson correlation coefficient and otherwise with Spearman rank correlation. In study I, logistic regression analysis was performed to investigate the associations of the different variables associated with liver brightness (fat). In study III, cumulative survival rates were estimated using the Kaplan-Meier method. Cox regression analysis was performed to investigate if liver brightness (fat) predicts the future risk for total mortality, cardiovascular death or cardiovascular events. P-value < 0.05 was regarded as significant. OriginPro 8.5 was used in visualizing the results.

In studies I and III, the study populations were categorized into three groups according to liver brightness status. However, in logistic regression analysis in study I, subjects were divided into two groups according to the result of liver brightness measurement. In study II, study population was divided into tertiles according to muscle fiber size.
5 Results

5.1 Liver fat accumulation is associated with metabolic abnormalities

According to laboratory analyses at baseline, subjects with severe liver fat accumulation (n = 146) tended to have more metabolic abnormalities compared to subjects with moderate fat accumulation (n = 135) or subjects with no fat in the liver (n = 747) (Table 2).

5.2 Peptide hormones and liver brightness

In study I, plasma adiponectin (p < 0.001) and ghrelin (p < 0.01) were negatively associated whereas leptin (p < 0.001) and hs-CRP concentrations were positively associated with liver brightness before adjustments (Table 2). After adjustments for age, sex and BMI, the association between plasma adiponectin levels and liver brightness remained strongly statistically significant (p < 0.001). Associations between plasma leptin (p < 0.01) and hs-CRP (p < 0.001) levels with liver brightness remained statistically significant as well. After further adjustments for waist circumference, QUICKI, alcohol consumption, smoking, ghrelin, leptin and hs-CRP-concentration, adiponectin levels still remained statistically significant related to liver brightness (p < 0.05) (Figure 5).

Logistic regression analysis was performed to identify independent indicators of the liver brightness. Patients were divided into two groups (no fat in the liver vs. fatty liver). The following covariates were added into the model: age, sex, BMI, waist circumference, adiponectin, ghrelin, leptin, hs-CRP, QUICKI, smoking, and alcohol consumption. Nagelkerke R square value for the whole model was 0.43 and adiponectin (odds ratio [OR] 0.96, confidence interval [CI] 0.93–0.99, p < 0.05), QUICKI (OR 0.22, CI 0.15–0.31, p < 0.001) and alcohol consumption (OR 1.21, CI 1.06–1.38, p < 0.01) remained statistically significant risk factors for liver brightness. When these variables were added into the logistic regression analysis independently, adiponectin reached Nagelkerke R square value 0.09, QUICKI 0.37 and alcohol consumption 0.01.
Table 2. The main characteristics of the OPERA study group as means (standard deviations) or percentages (numbers of subjects) grouped by liver brightness.

<table>
<thead>
<tr>
<th>Grade of liver brightness</th>
<th>0 (n = 747)</th>
<th>1 (n = 135)</th>
<th>2 (n = 146)</th>
<th>P</th>
<th>aP</th>
<th>bP</th>
<th>cP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>51.0</td>
<td>52.1</td>
<td>51.6</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>(6.0)</td>
<td>(6.1)</td>
<td>(5.5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>45.0%</td>
<td>65.9%</td>
<td>56.8%</td>
<td>&lt; 0.001</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(n = 336)</td>
<td>(n = 89)</td>
<td>(n = 83)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking (pack years)</td>
<td>11.0</td>
<td>14.3</td>
<td>13.9</td>
<td>&lt; 0.01</td>
<td>&lt; 0.05</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>(13.6)</td>
<td>(15.0)</td>
<td>(14.5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alcohol consumption (g/week)</td>
<td>51.0</td>
<td>91.0</td>
<td>82.1</td>
<td>&lt; 0.05</td>
<td>&lt; 0.05</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>(82.4)</td>
<td>(115.3)</td>
<td>(104.5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.5</td>
<td>30.0</td>
<td>31.8</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.01</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>(3.9)</td>
<td>(4.9)</td>
<td>(4.9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>86.9</td>
<td>98.0</td>
<td>102.1</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.05</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>(11.9)</td>
<td>(11.7)</td>
<td>(11.8)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertensives</td>
<td>42.2%</td>
<td>66.7%</td>
<td>71.9%</td>
<td>&lt; 0.001</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(n = 315)</td>
<td>(n = 90)</td>
<td>(n = 105)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>5.7</td>
<td>5.9</td>
<td>5.8</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>(1.0)</td>
<td>(1.1)</td>
<td>(1.1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/L)</td>
<td>1.4</td>
<td>1.2</td>
<td>1.2</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>NS</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>(0.4)</td>
<td>(0.3)</td>
<td>(0.4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.4</td>
<td>1.9</td>
<td>2.2</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>NS</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>(0.8)</td>
<td>(1.0)</td>
<td>(1.4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting insulin (mmol/L)</td>
<td>10.8</td>
<td>18.4</td>
<td>23.7</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>(7.6)</td>
<td>(10.6)</td>
<td>(17.6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>QUICKI</td>
<td>0.6</td>
<td>0.5</td>
<td>0.5</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>(0.1)</td>
<td>(0.1)</td>
<td>(0.1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adiponectin (µg/mL)</td>
<td>16.5</td>
<td>14.1</td>
<td>12.2</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.05</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>(6.3)</td>
<td>(5.4)</td>
<td>(5.3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leptin (ng/mL)</td>
<td>9.8</td>
<td>12.0</td>
<td>13.6</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.05</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>(7.5)</td>
<td>(9.7)</td>
<td>(9.2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ghrelin (pg/mL)</td>
<td>682.3</td>
<td>632.7</td>
<td>622.5</td>
<td>&lt; 0.01</td>
<td>NS</td>
<td>NS</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>(240.1)</td>
<td>(226.5)</td>
<td>(260.3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hs-CRP (mg/L)</td>
<td>3.2</td>
<td>4.6</td>
<td>6.1</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.01</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>(6.8)</td>
<td>(10.4)</td>
<td>(6.6)</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Data are as means (standard deviations) or percentages (numbers of subjects). Differences between groups were assessed by the ANOVA-test. Post hoc tests were performed using Tukey’s method. Statistical significances between percentages were measured by using chi-square test. BMI, body mass index, HDL, high-density-lipoprotein, hs-CRP, high-sensitivity C-reactive protein, QUICKI, quantitative insulin sensitivity check index. P values for the difference between whole group. aP values for differences between groups 0 and 1, bP values for differences between 1 and 2, cP values for the differences between 0 and 2.
5.3 Muscle fiber characteristics and association with metabolic disturbances

The baseline characteristics of the study group are shown in Table 3.
Table 3. The main characteristics of the study group and concentrations of peptide hormones and high-sensitivity C-reactive protein of the study group as means (standard deviations) grouped by muscle cell size divided into tertiles. N = number of subjects.

<table>
<thead>
<tr>
<th>Muscle fiber size divided into tertiles</th>
<th>1 ( (n = 18) )</th>
<th>2 ( (n = 18) )</th>
<th>3 ( (n = 18) )</th>
<th>( P )</th>
<th>( aP )</th>
<th>( bP )</th>
<th>( cP )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle fiber size (µm²)</td>
<td>4661.1</td>
<td>6256.2</td>
<td>8718.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(519.2)</td>
<td>(582.5)</td>
<td>(977.1)</td>
<td>( aP )</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>53.5</td>
<td>54.9</td>
<td>55.6</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>(4.9)</td>
<td>(7.3)</td>
<td>(5.0)</td>
<td>( bP )</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.1</td>
<td>25.5</td>
<td>28.1</td>
<td>&lt; 0.05</td>
<td>NS</td>
<td>&lt; 0.05</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>(2.6)</td>
<td>(3.0)</td>
<td>(2.9)</td>
<td>( cP )</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amount of fat (%)</td>
<td>23.8</td>
<td>23.9</td>
<td>26.6</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>(5.4)</td>
<td>(5.1)</td>
<td>(4.9)</td>
<td>( ‡ )</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>QUICKI</td>
<td>0.6</td>
<td>0.6</td>
<td>0.6</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>(0.1)</td>
<td>(0.1)</td>
<td>(0.1)</td>
<td>( ‡ )</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adiponectin (µg/mL)</td>
<td>16.7</td>
<td>16.5</td>
<td>12.7</td>
<td>&lt; 0.05</td>
<td>NS</td>
<td>&lt; 0.05</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>(6.0)</td>
<td>(4.0)</td>
<td>(4.4)</td>
<td>( † ) &lt; 0.05</td>
<td>NS</td>
<td>&lt; 0.05</td>
<td>&lt; 0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>Leptin (ng/mL)</td>
<td>3.8 (2.3)</td>
<td>3.4 (1.6)</td>
<td>5.5 (2.9)</td>
<td>&lt; 0.05</td>
<td>NS</td>
<td>&lt; 0.05</td>
<td>NS</td>
</tr>
<tr>
<td>( n = 13 )</td>
<td>( n = 16 )</td>
<td>( n = 16 )</td>
<td>( † )</td>
<td>NS</td>
<td>&lt; 0.05</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Hs-CRP (mg/L)</td>
<td>1.0 (1.1)</td>
<td>1.3 (2.6)</td>
<td>3.6 (6.0)</td>
<td>&lt; 0.05</td>
<td>NS</td>
<td>&lt; 0.05</td>
<td>NS</td>
</tr>
<tr>
<td>( n = 17 )</td>
<td>( n = 18 )</td>
<td>( n = 18 )</td>
<td>( † )</td>
<td>NS</td>
<td>&lt; 0.05</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Differences between groups were assessed by the ANOVA-test. Post hoc tests were performed using Tukey’s method. \(^\dagger\) Differences between groups were assessed by the Kruskall-Wallis and Mann-Whitney tests. Hs-CRP, high-sensitivity C-reactive protein, QUICKI, quantitative insulin sensitivity check index. \( P \) values for the trend. \( aP \) values for differences between groups 1 and 2, \( bP \) values for differences between 2 and 3, \( cP \) values for the differences between 1 and 3. \( ‡ \) (whole row) \( P \) values after adjustments for age and total adiposity.

5.3.1 Peptide hormones and muscle fiber size

In the second study, concentrations of adiponectin \( (p < 0.05) \), leptin \( (p < 0.05) \) and hs-CRP \( (p < 0.05) \), but not resistin or ghrelin, displayed associations with the muscle fiber size before adjustments. Adiponectin levels were the lowest while leptin and hs-CRP levels were the highest among subjects with large muscle fibers. Significant differences were observed between groups with medium-sized and largest muscle fibers \( (p < 0.05) \) and between those with small and large muscle fiber tertiles \( (p < 0.05) \) (Table 3). However, after adjustments for age and
total fatness, only adiponectin levels were significantly higher in the subjects with the smallest muscle fibers ($p < 0.05$) (Figure 6).

![Figure 6](https://example.com/figure6)

**Fig. 6.** Plasma adiponectin level and muscle fiber size tertiles in bars. Boxes represent median and middle quarters of adiponectin concentration and whiskers represent lowest and highest quarters. $o =$ means before adjustments (tertile 1 = 16.7, tertile 2 = 16.5, tertile 3 = 12.7). $*$ = means after adjustment for age and body fat percentage (tertile 1 = 16.6, tertile 2 = 16.4, tertile 3 = 12.9). $**P$ (before adjustments) values significant ($P < 0.05$) between middle-sized and largest and between small and large fiber area groups. $***P$ (after adjustments) values significant ($P < 0.05$) between medium-sized and large as well as between small and large-sized groups.

### 5.3.2 Muscle fiber type and adipocytokines

No significant differences were found between the tertiles of percentage of fiber types I or II or any of the adipocytokines (data not shown). Lipid oxidation in the basal state did not associate with muscle fiber size or adiponectin plasma concentration (data not shown).
5.3.3 The expression of adiponectin receptors

No associations were observed between the expression of AdipoR1 and AdipoR2 and features of metabolic syndrome such as increased waist circumference or insulin resistance. Adiponectin receptor expression was not associated with muscle fiber characteristics (data not shown).

5.4 Fatty liver and cardiovascular risk

In the third study, 13.5% of the subjects with no fat in the liver (97/720), 24.2% (30/124) of subjects having moderate fat accumulation and 29.2% (42/144) of the subjects having severe fat accumulation experienced a CVD event during the follow-up time (p < 0.001). CVD was the cause of death in 3.6% of the subjects with non-fatty liver and 8.1% of the subjects with moderate fat accumulation, while 12.5% of the subjects with severe fatty liver had CVD as the cause of death (p < 0.001). Severe fat accumulation predicted the risk for future risk of cardiovascular event when adjusted for age, gender and study group (OR 1.92, CI 1.32–2.80, p < 0.01). When further adjustments for smoking, alcohol consumption, LDL-cholesterol, BMI and systolic blood pressure were made (OR 1.75, CI 1.16–2.63), the risk still remained statistically significant (p < 0.01). Statistical significance disappeared when further adjustment for QUICKI was made (OR 1.49, CI 0.97–2.30, p = 0.07). In CVD event sensitivity analyses all covariates were added one by one, investigating whether the odds ratios changed or remained stable when further adjustment with one covariate was made. After adjusting for statistical significant variables (age, gender, smoking, systolic blood pressure and QUICKI) in sensitivity analyses, the association between severe fatty liver was not significant (OR 1.43, CI 0.93–2.18, NS). When QUICKI was removed from the former model, severe fatty liver predicted the risk for future risk of CVD event (OR 1.76, CI 1.21–2.56, p < 0.001).

The future risk of death from CVD in participants with severe fat accumulation was significant when age, gender and study group were added as covariates (OR 2.95, CI 1.58–5.51, p < 0.01). After further adjustments with other conventional risk factors (smoking, alcohol consumption, LDL-cholesterol, BMI and systolic blood pressure) were made statistical significance remained (OR 1.99, CI 1.01–3.93) (p < 0.05). When QUICKI was added as a covariate, significance disappeared (OR 1.63, CI 0.79–3.38, NS) (Figure 7).
In total, 11.9% of the participants not having fatty liver, 18.5% of the subjects having moderate fatty liver and 22.2% of the subjects with severe fatty liver died from all causes (p < 0.01). Severe fat accumulation predicted the risk for mortality from all causes when age, gender and study group were added as covariates (OR 1.60, CI 1.05–2.43, p < 0.05). The significance disappeared when further adjustments with BMI were made.

Fig. 7. Kaplan Meier cumulative survival rates censored for cardiovascular death in subjects with no fat in the liver, moderate fat accumulation and severe fat accumulation. Cox regression analysis is used for adjustments. M1 (Model 1): adjusted for study group, age and gender. M2 (Model 2): further adjustments for smoking, alcohol consumption, systolic blood pressure, LDL-cholesterol level and body mass index. M3 (Model 3): further adjustment for QUICKI. CI, confidence interval, CVD, cardiovascular disease, LDL, low-density lipoprotein, OR, odds ratio, QUICKI, quantitative insulin sensitivity check index. **P < 0.01, *P < 0.05.
6 Discussion

6.1 Methodological aspects

6.1.1 Study populations

The strength of the study is the OPERA data, which is a large and clinically well-defined population. Out of the defined population of the city of Oulu, treated male and female hypertensives aged 40–59 years and age- and sex-matched controls for them were recruited. Noteworthy, people are not equally healthy in Finland and age-adjusted morbidity index in the Oulu city area is higher than in Finland in general (Vaarama et al. 2010). It was a population-based, epidemiological cross-sectional cohort study. Hypertensives were a random sample of medically-treated hypertensives from the Social Insurance Institute register for the reimbursement of hypertension medication. Age- and sex-matched controls were recruited from the same register. There was some sampling bias because the OPERA study group was not a random sample of the whole population. Furthermore, the response rate was 86.5% in the hypertensive cohort and 87.7% in the control group, which caused some nonresponse bias.

In general, people in Finland had high blood pressures at the time when OPERA data was recruited. Furthermore, blood pressure limits were different (Kiema et al. 1996) compared to current limits. Although only half of the study population were medically-treated hypertensives, several control subjects were hypertensives according to the current limits (systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg) (Kastarinen et al. 2009). Originally, the OPERA study was a case-control study and this may cause some selection bias in the cohort study. Among OPERA subjects, there were more hypertensives than in the Finnish population in general. This should be noted when interpreting the results of this thesis because hypertension is known to be a risk factor for metabolic complications such as CVD (Bonora 2006).

In the second study a subgroup of OPERA data was used including non-smoking control men with normal OGTT. Muscle fiber characteristics are widely investigated in obesity and T2DM. Whether muscle fiber characteristics are associated with overweight is not well known. Therefore, middle-aged men with normal glucose metabolism were investigated. Nevertheless, it is also one limitation of the study. Despite the random recruitment of the control men, the
data may not cover the whole population and there may be some selection bias. Furthermore, the recruitment of non-smoking control men with normal OGTT caused sampling bias. It should be noted that 135 people were invited to participate in the study but only 54 muscle biopsies were included in the analyses, which can be regarded as a nonresponse bias. The subjects of this study may be healthier than the Finnish population in general, and this should be noted in the interpretation of the results.

6.1.2 Statistical tests

In multiple statistical tests, there is always a possibility of obtaining false positive result. A p-value of 0.05 means that there is a 5% chance of obtaining a false result. This is called α error. In ANOVA Tukey’s method was used because it is generally recommended and takes into account the dispersion of the whole data. In multivariate analysis, no adjustments were used in multiple comparisons since several tests were not performed. It should also be noted that the possibility to get a false positive result by coincide increases when numerous tests are performed.

6.1.3 Measurement of fatty liver

In studies I and III, the grade of liver brightness was measured by ultrasound. Previous studies have used biochemical, radiological and histological methodology for NAFLD diagnosis and staging (Bhatia et al. 2012). It is known that ultrasound is not the most reliable imaging method but it has many advantages. Ultrasound has been reported to have high specificity but low sensitivity. Compared to other imaging techniques, ultrasound is the cheapest, safest and most patient-friendly imaging technique and is a reasonable alternative to liver biopsy in many circumstances, for example when it is not necessary to distinguish between simple steatosis and steatohepatitis (Joy et al. 2003). Real-time ultrasound using a combination of sonographic findings has a high specificity, but the problem is that it underestimates the prevalence of hepatic steatosis when there is < 20% fat (Dasarathy et al. 2009). A previous study of 235 ultrasound-diagnosed NAFLD patients found a high specificity of 97% but a low sensitivity of 64%. Noteworthy, when patients with more than 30% hepatic steatosis were excluded from the study, specificity increased to 100% and sensitivity to 89.7% (Palmentieri et al. 2006). Furthermore, a study of 94 subjects found a specificity of 100% and sensitivity of 91.7% for subjects with more than
10% hepatic steatosis. In total, 7.4–8.8% of the cases with NAFLD could not be detected with ultrasound and among these patients, the area of steatosis ranged from 15 to 40%. The authors concluded that mild to moderate steatosis can be misdiagnosed, but the presence of fibrosis is not related to misdiagnosis (Hamaguchi et al. 2007a). It is known that the accuracy of ultrasound decreases when BMI increases. In a previous study of morbidly obese subjects, the sensitivity of ultrasound was 49.1% and specificity 75% (Mottin et al. 2004). MRI has been reported to have high accuracy, but using this method is limited because it is not suitable for subjects with iron overload (Charatcharoenwitthaya & Lindor 2007). Today, magnetic resonance spectroscopy is regarded as the “golden standard” for the quantification of liver fat, but this method is limited due to its availability (Szczepaniak et al. 2005). In the OPERA study, using MRI or MRI method was not possible. Furthermore, it is obvious that taking liver biopsies from large groups of symptomless subjects is ethically unjustifiable.

According to a recent study, the NAFLD liver fat score and liver fat equation turned out to be simple and noninvasive tools to predict NAFLD and liver fat content. Data on the metabolic syndrome, T2DM and serum insulin, aspartate aminotransferase and alanine aminotransferase concentrations reached prediction of NAFLD with a specificity of 71% and sensitivity of 86% (Kotronen et al. 2009).

Among OPERA study subjects, the prevalence of moderate liver fat accumulation was 13.1% and that of severe fat accumulation 14.2%, which is in line with the overall prevalence of NAFLD in the Western world (Vernon et al. 2011). Because ultrasound has been reported to have high specificity but low sensitivity it is possible that there are several subjects in the non-fatty liver group with true mild steatosis. Furthermore, 33 subjects out of 77 morbidly obese subjects had severe liver brightness, but because accuracy of ultrasound decreases when BMI increases, there may be some misdiagnosis among these morbidly obese subjects.

6.1.4 Muscle fiber characteristics

Altogether 54 high-quality biopsies were available for the analysis. It would have been important to quantify fat infiltration in the muscle fibers of our biopsies. However, taking into account the previous data, BMI correlates strongly with intramyocellular lipid accumulation (Thomas et al. 2012). Furthermore, obese individuals had larger muscle fibers and more intramyocellular lipid.

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accumulation. More central distribution of lipid droplets was also observed in the muscle fibers of obese compared to lean subjects (Malenfant et al. 2001).

6.1.5 Measurement of plasma adiponectin

Plasma adiponectin concentrations were measured with an ELISA technique devised in our laboratory (Santaniemi et al. 2006). Total adiponectin concentration was measured. According to previous data, globular adiponectin exists as a trimer and full-length adiponectin as multimers (LMW-trimer, MMW-hexamer, HMW-multimer) (Kadowaki et al. 2006). Today, measuring all isoforms of adiponectin is recommended (Kaser et al. 2008), but this was not a standard procedure at the time when our ELISA technique was devised.

It would be beneficial to measure all adiponectin isoforms because they are reported to have different affinities in adiponectin receptors, for example in muscle and liver cells (Yamauchi et al. 2003), leading to different actions of adiponectin in different tissues. According to recent data, AMPK and PPARα in skeletal muscle are stimulated by globular and full-length adiponectin while AMPK and PPARα activation in the liver is stimulated by full-length adiponectin only (Yamauchi et al. 2002, Yamauchi et al. 2003).

According to previous data, HMW adiponectin is regarded as a more active form of adiponectin and has an important role in protecting against diabetes (Kadowaki et al. 2006). Both total adiponectin level (Kovacova et al. 2012) and HMW adiponectin levels are decreased in obesity (Yamauchi & Kadowaki 2008), and total adiponectin levels seems to be decreased due to reduced levels of HMW adiponectin (Kaser et al. 2008). It seems that HMW-adiponectin concentration correlates with total adiponectin level and therefore, measuring total adiponectin level is also reasonable.

6.1.6 CVD follow-up

In study III, approximately 19-year follow-up time was used. This study may be the first follow-up study with a large population-based study group with such a long follow-up time and ultrasound-diagnosed fatty liver. The diagnosis of cardiovascular events was based on the registry of the National Institute for Health and Welfare (Hilmo register) and mortality data were obtained from the National Death Registry, using earlier verified FINRISK classification (Pajunen et al. 2011). It is known that there are several trustworthy and exceptional official
registers in Finland. For instance, the coverage of the cancer registry in solid
tumors is almost 100% (Teppo et al. 1994). The Treatment Declaration Register
(Hilmo) is statutory, mandatory, extensive and based on the Finnish identification
system (Sund 2012).

In the OPERA follow-up study the reliability of event diagnosis data is quite
accurate and the classification systematic. Furthermore, the exact time interval
between the baseline and the event/death was available. All subjects who had
myocardial infarction or stroke before baseline were excluded because history of
myocardial infarction is known to increase the risk of recurrent myocardial
infarction or cardiovascular death (Beckman et al. 2002) and medication as well
as lifestyle secondary prevention strategies are intensive (Joseph & Teo 2011).

6.2 Plasma adiponectin and association to fatty liver (Study I)

In study I, plasma adiponectin was an independent indicator of liver fat
accumulation even after adjustment for several metabolic risk factors including
age, sex, obesity, QUICKI, smoking and alcohol consumption. Also other
adipocytokines and obesity-related peptide hormones were added into the same
model, including leptin, ghrelin and hs-CRP. Plasma adiponectin has been
associated with metabolic disturbances in several studies. When it comes to
peptide hormones, this study is the first large study supporting the independent
role of adiponectin in liver fat accumulation.

Study I was cross-sectional and it was therefore not possible to investigate
whether adiponectin was a cause or a consequence of fatty liver. According to one
follow-up study, the level of adiponectin at the baseline was significantly lower in
patients who developed NAFLD in 7 years’ follow-up (Zelber-Sagi et al. 2012),
suggesting that low adiponectin may play a role in developing NAFLD. It is
known that adiponectin has an active role in regulating lipid and glucose
metabolism and controlling inflammation in the liver (Tilg & Hotamisligil 2006).
Adiponectin may protect hepatocytes from triglyceride accumulation (Bredella et
al. 2011) increasing β-oxidation and (Dobrzyn et al. 2004) decreasing de novo
fatty acid synthesis in the liver (Da Silva Morais et al. 2009). In addition,
adiponectin decreases hepatic glucose production (Berg et al. 2001).

Although low adiponectin levels are associated with obesity (Kern et al.
2003), decreased serum adiponectin levels have been reported also in non-obese
individuals with low insulin sensitivity (Hammarstedt et al. 2012). Insulin
resistance, which is usually linked to genetic (Manning et al. 2012), nutritional
and lifestyle factors (Sese et al. 2012), leads to imbalance of adipokines/cytokines, resulting in worsening of insulin resistance and NAFLD and the progression from fatty liver to NASH (Polyzos et al. 2010). Furthermore, NAFLD predicts the risk for future T2DM (Sung & Kim 2011).

In study I, hyperinsulinemia was present in subjects with fatty liver and decreased adiponectin. When adiponectin level, liver fat, QUICKI and other metabolic and lifestyle markers were added into the same logistic regression model, only adiponectin, QUICKI and alcohol consumption remained statistically significant. QUICKI was the strongest independent indicator of liver brightness. It is a very important fact that adiponectin also tended to be an independent predictor of liver fat accumulation. Although insulin resistance seems to be an important mechanism linking adipose tissue to liver fat accumulation, decreased adiponectin may not only be a marker of fatty liver but may also play an active role in the pathogenesis of fatty liver. This speculation must be confirmed in further studies in this field.

It is known that fatty liver overproduces CRP (Zimmermann et al. 2011). According to study I, hs-CRP levels were significantly higher in patients with severe fatty liver and low adiponectin levels, although hs-CRP was not an independent predictor for liver brightness. There is evidence that inflammatory cytokines play an active role in NASH pathogenesis (Rolo et al. 2012) and adiponectin predicts mainly the severity of fatty liver disease (Savvidou et al. 2009). It can be speculated that adiponectin may be able to diminish the production of CRP in the liver indirectly by suppressing TNF-α. In the current study, the possible inflammatory processes and severity of liver disease remained unsolved because the ultrasound method was used. Biopsies are needed to investigate the histological severity of fatty liver disease (Dasarathy et al. 2009) and in vitro studies could offer more information about molecular mechanisms between adiponectin and fatty liver disease.

Adiponectin expression may be genetically determined and variation in adiponectin gene is associated with insulin resistance and T2DM (Li et al. 2009b). Adiponectin concentrations are also affected by age, the levels being higher in the elderly (Cnop et al. 2003). In OPERA study subjects, adiponectin levels were higher in women than in men; this observation is in line with previous data (Cnop et al. 2003). Adiponectin is also physiologically affected by ethnicity (Sohara et al. 2005). This is not a concern in the current study. Furthermore, several studies have reported increased serum adiponectin levels in liver cirrhosis, mainly because of decreased hepatic clearance of adiponectin and compensatory
adiponectin production against overwhelming cytokines in cirrhosis (Sohara et al. 2005). Among OPERA study subjects there were men and women with high adiponectin levels and severe bright liver, but it was not possible to detect subjects with cirrhosis. Furthermore, only total adiponectin concentration was measured although HMW adiponectin is reported to associate strongly with fatty liver (Kantartzis et al. 2009). It should be noted that liver fat accumulation can be caused by other factors such as malnutrition, medication (cortisone, methotrexate), toxins and diseases (inflammatory bowel disease) (Allard 2002). Therefore, it is important to identify all the factors that may have an effect on adiponectin concentrations and liver fat accumulation in order to avoid false positive associations. In OPERA, these factors were taken into account as well as it was possible. Validated questionnaires were used and all patients were interviewed. Special attention was given to the medical history and current medication, history of all diseases, smoking habits, alcohol consumption and physical activity (Kauma et al. 1998).

Therapeutic strategies to upregulate adiponectin in NAFLD have been widely speculated on. A relatively modest weight loss of 5–10% improves both biochemical and histological abnormalities seen in NASH. Increase in adiponectin in patients who lost ≥ 9% of body weight with orlistat was strongly associated with improvement in the severity of fatty liver disease (Harrison et al. 2009). Thiazolidinediones are insulin sensitizers and improve insulin sensitivity. This phenomenon may be at least partly mediated by adiponectin (Berg et al. 2001). Furthermore, PPARγ activation increases the plasma level and gene expression of adiponectin (Sharabi et al. 2007), which may lead to the prevention of NAFLD.

6.3 Association of plasma adiponectin with muscle fiber size (Study II)

Skeletal muscle is an important organ in glucose uptake and plays a major role in glucose homeostasis (Shulman et al. 1990). In study II, reduced plasma adiponectin concentration was an indicator of increased muscle fiber size. The association between low adiponectin and large muscle fiber size was independent of total adiposity. Furthermore, elevated leptin and hs-CRP concentration associated with muscle fiber size. This association was dependent on total adiposity.
According to study II, overweight was associated with enlarged muscle fiber size. Recent data indicate that subjects with high BMI and waist circumference have higher IMCL content (Kotronen et al. 2008) although contrary results also exist (Koska et al. 2008). Adiponectin concentration and IMCL are highly dependent on adiposity (Kotronen et al. 2008), but other studies suggest that adiponectin has an effect on muscle fiber size independent of total body fatness (Koska et al. 2008). Skeletal muscle fat content is also highly dependent on insulin sensitivity (Jacob et al. 1999). Subjects in study II were healthy control men with normal OGTT. It is noteworthy that subjects with larger muscle fibers were overweight. It is known that adipose tissue insulin resistance leads to muscle triglyceride accumulation and reduced muscle insulin sensitivity in the long term (Kelley et al. 2002). It could be speculated that control men already had some extra droplets within muscle fibers although they had not yet produced peripheral insulin-resistant state.

There are at least two ways in which insulin resistance develops in skeletal muscle (Jornayvaz et al. 2010). Increased FFA delivery and/or a defect in lipid oxidation leads to IMCL (Jacob et al. 1999), which predisposes to decreased insulin sensitivity. Studies in young, lean insulin-resistant subjects suggest that insulin resistance is caused by decreased muscle glycogen synthesis because ingested carbohydrates are then converted to hepatic de novo lipogenesis resulting in increased TG synthesis leading to IMCL (Jornayvaz et al. 2010). Under normal conditions, adiponectin has fatty-acid combustion (Yoon et al. 2006b) and insulin-sensitizing properties in skeletal muscle and adiponectin signaling is an important regulator of skeletal muscle mitochondrial oxidative capacity (Patel et al. 2012). In insulin-resistant state and obesity, diminished adiponectin production and impaired adiponectin signaling result in further complications in skeletal muscle.

Study II was cross-sectional and we were not able to investigate if low adiponectin level was causally related to muscle fiber size. Plasma adiponectin level was clearly lower in subjects with large muscle fibers. Recent data suggest that adiponectin may be a partial mediator between skeletal muscle morphology and insulin sensitivity (Ingelsson et al. 2009). Circulating adiponectin levels have been reported to be associated with increasing skeletal muscle capillary density in individuals with higher proportion of type I (slow oxidative) muscle fibers even after adjustment for age, physical activity, fasting glucose and pharmacological treatment for diabetes (Ingelsson et al. 2009). Furthermore, several studies have demonstrated that IMCL correlates with peripheral insulin resistance (Kelley &
Goodpaster 2001). It could be speculated that the control men who participated in the study were too healthy and too homogenous a study group to confirm these results of previous studies.

Subjects with increased IMCL are usually insulin-resistant in spite of some exceptions. Elite endurance-trained athletes are extremely insulin-sensitive although their intramyocellular lipid concentration is high. This is called the athlete’s paradox (Dube et al. 2008). According to the questionnaire, the volunteer control men were equally physically active. Another exception is lipodystrophies, when there is partial or complete loss of adipose tissue and low plasma leptin level promotes hyperphagia and fat is stored ectopically (Simha et al. 2003). Because these participating control men were rather healthy, this issue was not a concern. In general, age also has an effect on muscle fiber size (Barani et al. 2003). The volunteers in study II were about the same age. It is well known that obese individuals have higher lean mass which may correlate with the muscle fiber size (Janssen et al. 2000). Therefore, the adjustments were performed with the body fat percentage rather than BMI or waist circumference.

6.4 Role of fatty liver in the development of CVD (Study III)

Subjects with NAFLD have significant metabolic abnormalities increasing the risk of CVD. Fatty liver is not only a marker of metabolic disturbances but may also play an active role in the pathogenesis of CVD (Bhatia et al. 2012). According to study III, during the 19-year follow-up severe fatty liver predicted the future risk for death from all causes, death from CVD and risk for cardiovascular events. Furthermore, severe fatty liver predicted the risk for future cardiovascular events even after adjustment for several traditional risk factors (age, gender, smoking, systolic blood pressure, LDL-cholesterol). After further adjustment of the model for QUICKI, the statistical significance between severe fatty liver and cardiovascular event disappeared. It means that compared to fatty liver, insulin sensitivity is a stronger predictor for CVD and may have an active role in predicting cardiovascular events.

It is well known that traditional risk factors for CVD are age, gender, smoking, high LDL-cholesterol, hypertension and diabetes (Van Gaal et al. 2006). Smoking, LDL-cholesterol and hypertension are related to CVD in a causal, independent and graded manner. Furthermore, the reduction is associated with a similarly graded benefit (Graham et al. 2012). Subjects with fatty liver had numerous CVD risk factors at the baseline. They were more often hypertensive,
obese subjects with high TG, fasting insulin and glucose levels. They also had significantly higher hs-CRP levels compared to subjects with a healthy liver. At the baseline the subjects with severe fatty liver were more often men but did not differ in age, smoking habit or LDL cholesterol level compared to those subjects with moderate fatty liver or non-fatty liver.

In the 19-year follow-up, age, gender, smoking, systolic blood pressure and QUICKI predicted independently the risk for future cardiovascular event. Although LDL-cholesterol is a well-known risk factor for CVD (Emerging Risk Factors Collaboration 2009), in the OPERA follow-up database severe fatty liver predicted CVD better than LDL-cholesterol and furthermore, LDL-cholesterol did not have an independent role in predicting cardiovascular events. A previous study has reported similar results (Hamaguchi et al. 2007b). In OPERA, LDL may not predict cardiovascular events independently because subjects with high LDL cholesterol levels usually have other metabolic disturbances. It is known that peripheral LDL particles contain 90% of the apoB lipoprotein particles and investigators have postulated that apoB is the most potent marker of CVD risk compared to non-HDL and LDL concentration (Sniderman et al. 2011). Furthermore, LDL particle number is more strongly related to CVD compared to LDL concentration and subjects with low LDL concentration may still have an increased number of LDL particles (Cromwell et al. 2007).

It is noteworthy that at the baseline only 27 subjects had medical treatment for lipids (usually statin treatment). Follow-up data about statin treatment in the OPERA database was not available.

The role of liver fat accumulation in predicting CVD has been investigated (Hamaguchi et al. 2007b) but large follow-up studies with ultrasound-diagnosed fatty liver are needed. Liver dysfunction has been reported to associate with CVD mortality (Dunn et al. 2008, Ruttmann et al. 2005) and CHD event (Treeprasertsuk et al. 2012) in a follow-up study and especially survival of subjects with NASH is reported to be reduced (Ekstedt et al. 2006, Söderberg et al. 2010). According to recent data, subjects with NAFLD died from cardiovascular and liver-related causes or due to malignancy (Adams et al. 2005, Ekstedt et al. 2006). It should be noted that study III was a rather large follow-up study and subjects with severe liver fat accumulation died more often from CVD compared to those without fat in the liver. Furthermore, severe fatty liver was an independent indicator for cardiovascular mortality after adjustment for several traditional risk factors. When QUICKI was added as a covariate, the significance disappeared, which means that insulin resistance may play an important role in
developing cardiovascular death. Noteworthy, severe fatty liver did not independently predict all-cause mortality, which was in line with previous data (Lazo et al. 2011).

Liver brightness was measured with ultrasonography. This method has some limitations because it measures only the severity of liver brightness and may underestimate the amount of fat (Dasarathy et al. 2009). Studies have used biochemical, radiological and histological methodology for NAFLD diagnosis and staging, which leads to challenging interpretation of the previous results. Liver biopsy is regarded as a “golden standard” for diagnosing possible or assumed fatty liver disease, the extent of inflammatory activity and the stage of fibrosis (Tannapfel et al. 2011). For instance, gamma-glutamyltransferase level, which is a potential marker of NAFLD or alcohol consumption, is reported to associate with incident vascular events (Haring et al. 2009, Lee et al. 2006). Alanine aminotransferase is a marker of hepatic steatosis. Whether elevated alanine aminotransferase predicts CVD independently is still under discussion (Schindhelm et al. 2007, Yun et al. 2009).

Inflammation may be an important link between NAFLD and CVD. CRP is regarded to be a moderate predictor of CHD (Danesh et al. 2004), and in multiple prospective epidemiological studies CRP has been reported to predict incident myocardial infarction, stroke, peripheral arterial disease, and sudden cardiac death (Ridker 2003). Although inflammation is known to be central in the pathogenesis of CVD, it is postulated that there are several weak points in including CRP in the risk assessment (Perk et al. 2012). Therefore hs-CRP was not included in the model of study III. It is known that TNF-α, IL-6 and PAI-1 are associated with CVD and play an important role in CVD pathogenesis (Van Gaal et al. 2006), but the levels of several central inflammatory cytokines were not measured at the baseline so it was not possible to investigate whether these cytokines play a role in prediction of cardiovascular events. In addition, only baseline characteristics of the study group were available and the follow-up data included mortality and event data, but no follow-up data about the other characteristics of the subjects exist. Whether fatty liver disease is causally related to CVD remains unsolved. It is known that several metabolic abnormalities and rather a long period of time are needed to develop both CVD and fatty liver.
6.5 Future aspects

This thesis demonstrated that adiponectin is a strong independent indicator of liver fat accumulation. Adiponectin may also play an active role in skeletal muscle fiber characteristics. The exact mechanisms of how adiponectin prevents peripheral tissues, such as liver and skeletal muscle, from fat accumulation should be further investigated. It can be speculated as to whether low adiponectin is a cause or a consequence of fatty liver disease and intramyocellular lipid accumulation, and follow-up studies and in vitro studies in this field are therefore needed. The possible mechanisms linking fatty liver disease to CVD are widely investigated. According to the current knowledge, the best speculation is that an excess of inflamed visceral fat mass leads to increased production of inflammatory cytokines, increased insulin resistance and increased free fatty acid concentrations. These actions lead to impaired liver functions, which result in increased production of inflammatory proteins and coagulation factors. Finally, chronic inflammation and atherogenic dyslipidemia contribute to CVD (Bhatia et al. 2012). It is still poorly understood how active a role a fatty liver plays in the pathogenesis of CVD, or whether fatty liver is only a marker of insulin-resistant state. Further follow-up studies are needed.

In the clinical setting, adiponectin could serve as a potential marker in the diagnosis of liver fat accumulation and its potential therapeutic usefulness for the treatment and prevention of NAFLD should be tested. Adiponectin may offer a potential source for treatment of fatty liver disease and fat accumulation in other peripheral tissues. It is known that adiponectin has several beneficial effects on numerous physiological processes, but the use of adiponectin or its receptors as therapeutic targets may be complex. The presence of different adiponectin isoforms and production sites and multiple receptors with different affinities for adiponectin isoforms as well as cell-type-specific effects in different tissues (Yamauchi et al. 2002) make it extremely challenging to develop new treatment strategies. However, there are already some promising targets. For instance, thiazolidinediones are very interesting PPARγ agonists and insulin-sensitizers which have been reported to increase adiponectin levels (Polyzos et al. 2010). Because decreased adiponectin levels are associated with inflammatory state (Van Gaal et al. 2006), it can be speculated that cytokine inhibitors could at least theoretically offer a target for increasing adiponectin production in peripheral tissues. Promising data already exist. For instance, TNF-α antagonism has been
reported to increase adiponectin levels in some (Stanley et al. 2011), but not in all reports (Ferraz-Amaro et al. 2011). Further studies are needed in this field.
7 Conclusions

The conclusions of the thesis are as follows:

1. Adiponectin is a strong predictor for liver brightness, even after adjustment for numerous other metabolic risk factors, markers of inflammation, and novel obesity-related peptide hormones. Detailed mechanistic studies are needed to unravel the specific metabolic pathways of adiponectin in relation to the pathogenesis of NAFLD.

2. Skeletal muscle fiber characteristics are related to overweight. In addition, an association between a low adiponectin concentration and large muscle fiber size was observed and this was not dependent on total fatness. Further experiments will be needed to understand the specific role of adiponectin in fat accumulation in the muscle fibers.

3. Severe liver fat accumulation increases the risk of future cardiovascular event and mortality to CVD in long-term follow-up but it seems to be dependent on insulin sensitivity. Larger cohorts and follow-up studies are needed to understand the specific role of fatty liver in the development of CVD.
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Original publications are not included in the electronic version of the dissertation.

1187. Laukkanen, Päivi (2012) Occurrence of high risk human papillomaviruses and cervical cancer among fertile-aged women in Finland


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1197. Aro, Elinoura (2013) Prolyl 4-hydroxylases, key enzymes regulating hypoxia response and collagen synthesis: the roles of specific isoenzymes in the control of erythropoiesis and skeletogenesis


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