Meiju Äijälä

STUDIES ABOUT CONTRIBUTION OF LEPTIN RECEPTOR IN CARDIOVASCULAR RISK
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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 18 December 2013, at 12 noon

UNIVERSITY OF OULU, OULU 2013
Abstract

Leptin is a hormone secreted by adipose tissue. It is involved in the regulation of appetite and energy expenditure. Leptin binds to its receptor (LEPR) that is expressed in the central nervous system as well as in other tissues including adipocytes and endothelial cells. Plasma leptin level reflects the amount of adipose tissue and previously, it has been shown to be associated with the risk for coronary artery disease. Two LEPR polymorphisms, Lys109Arg and Gln223Arg, have been extensively studied and they have been associated with several risk factors of atherosclerosis. Earlier studies have also shown that the risk for developing atherosclerosis and various other diseases might already be determined during the fetal period or immediately after birth. It seems that intrauterine undernourishment might cause changes on epigenetic level and result in alterations in gene expression. It has been suggested that the impaired fetal growth could affect plasma leptin level and leptin messenger RNA expression from adipose tissue. Long-term fructose consumption has also been shown to result in leptin resistance. Recently, leptin has been observed to be associated with autophagy. Autophagy has been demonstrated to act in several interesting processes such as fat storage in adipocytes and liver. Autophagy and the leptin system might also regulate each other.

The aim of this thesis was to investigate the association of LEPR polymorphisms with thickness of the wall of carotid artery as well as with fatal and nonfatal cardiovascular disease events. In addition, we aimed to clarify the effects of fetal undernourishment and fructose consumption on the leptin system and autophagy. We were also interested in studying the role of the leptin system and autophagy in elevated triglycerides and liver fat accumulation seen as a result of high-fructose diet. In our studies, we observed that LEPR polymorphisms, Lys109Arg and Gln223Arg, are associated with intima-media thickness of carotid artery. Moreover, 19-year follow-up study showed that 109Arg homozygotes display lower incidence of cardiovascular events and lower total mortality. In our animal experiments, we were able to detect that fructose diet affects both LEPR isoform and autophagy gene expression. It seems that these changes might partly explain the mechanism behind the rise in blood triglyceride levels and liver fat accumulation caused by fructose diet.

In conclusion, the results of this study clarify the role of leptin receptor in cardiovascular diseases. In addition, they offer new information especially about the effects of fructose diet on the leptin system, the dysfunction of which might predispose to the development of diseases.

Keywords: atherosclerosis, autophagy, fructose, leptin receptor


Tutkimuksen tulokset selvitävät leptinireseptorin roolin sydän- ja verisuonitautien taustalla. Lisäksi ne tarjoavat uutta tietoa erityisesti fruktoosinkulutuksen vaikutuksesta leptinijärjestelmään. Tutkimuksessa havaittiin, että puolestaan on osoitettu, että sikiöaikainen aliravitsemus voi vaikuttaa erityisesti fruktoosinkulutuksen vaikutuksesta leptinijärjestelmään, jonka häiriöt altistavat sairauksisten kehittymiselle.
To my family
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Oulu, October 29th 2013

Meiju Äijälä
**Abbreviations**

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>18S rRNA</td>
<td>18S ribosomal ribonucleic acid</td>
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<tr>
<td>α-MSH</td>
<td>α-melanocyte stimulating hormone</td>
</tr>
<tr>
<td>ACC</td>
<td>acetyl-CoA carboxylase</td>
</tr>
<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
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<tr>
<td>AgRP</td>
<td>agouti-related protein</td>
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<tr>
<td>AKT</td>
<td>protein kinase B</td>
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<tr>
<td>AMPK</td>
<td>5′-AMP-activated protein kinase</td>
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<td>ANCOVA</td>
<td>analysis of covariance</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>Arg</td>
<td>arginine</td>
</tr>
<tr>
<td>Atg7</td>
<td>autophagy related gene 7</td>
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<tr>
<td>BAT</td>
<td>brown adipose tissue</td>
</tr>
<tr>
<td>BBB</td>
<td>blood-brain barrier</td>
</tr>
<tr>
<td>BIF</td>
<td>bifurcation enlargement</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>CAD</td>
<td>coronary artery disease</td>
</tr>
<tr>
<td>CCA</td>
<td>common carotid artery</td>
</tr>
<tr>
<td>CHD</td>
<td>coronary heart disease</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
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<tr>
<td>CRH</td>
<td>corticotropin-releasing hormone</td>
</tr>
<tr>
<td>CRH</td>
<td>cytokine receptor homologous</td>
</tr>
<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
</tr>
<tr>
<td>db/db</td>
<td>diabetes/diabetes mouse</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FFA</td>
<td>free fatty acid</td>
</tr>
<tr>
<td>FOXO1</td>
<td>forkhead box O1</td>
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<tr>
<td>FSH</td>
<td>follicle-stimulating hormone</td>
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<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
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<tr>
<td>GH</td>
<td>growth hormone</td>
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<tr>
<td>GHRH</td>
<td>growth hormone-releasing hormone</td>
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<tr>
<td>Gln</td>
<td>glutamine</td>
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<tr>
<td>GnRH</td>
<td>gonadotropin-releasing hormone</td>
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<tr>
<td>GWAS</td>
<td>genome-wide association study</td>
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<tr>
<td>HDL</td>
<td>high-density lipoprotein</td>
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HPRT  hypoxanthine guanine phosphoribosyl transferase
HR    hazard ratio
ICA   internal carotid artery
ICD   International Classification of Diseases
IMT   intima-media thickness
IRS   insulin receptor substrate
IUGR  intrauterine growth restriction
JAK   Janus kinase
kDa   kilodalton
LAMP2 lysosome-associated membrane protein 2
LD    linkage disequilibrium
LDL   low-density lipoprotein
Lep   leptin gene
LEPR  leptin receptor
LH    luteinizing hormone
Lys   lysine
MRI   magnetic resonance imaging
mRNA  messenger ribonucleic acid
mTOR  mammalian target of rapamycin
NPY   neuropeptide Y
ob/ob obese/obese mouse
OPERA Oulu Project Elucidating Risk of Atherosclerosis
PCR   polymerase chain reaction
PI3K  phosphatidylinositol 3-kinase
POMC  proopiomelanocortin
PTP1B a negative regulator non-receptor phospho-tyrosine protein phosphatase
qRT-PCR quantitative real-time polymerase chain reaction
RFLP restriction fragment length polymorphism
RIA   radioimmunoassay
SBP   systolic blood pressure
SH2B1 SH2B adapter protein 1
SNP   single nucleotide polymorphism
SOCS3 suppressor of cytokine signaling 3
STAT  signal transducer and activator of transcription
TG    triglyceride
TRH   thyrotropin-releasing hormone
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<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>TSH</td>
<td>thyroid-stimulating hormone</td>
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<td>Tyr</td>
<td>tyrosine</td>
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<tr>
<td>UBC</td>
<td>ubiquitin C</td>
</tr>
<tr>
<td>VLDL</td>
<td>very low-density lipoprotein</td>
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<tr>
<td>WAT</td>
<td>white adipose tissue</td>
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List of original articles

The thesis is based on the following original articles, which are referred to in the text by Roman numerals:


*Saukko is the maiden name of the author Äijälä
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1 Introduction

Cardiovascular disease (CVD) is an extensive problem of public health and economy. Atherosclerosis is the most frequent underlying cause of coronary artery disease, carotid artery disease, and peripheral arterial disease (reviewed by Falk 2006). The major risk factors for atherosclerosis are cigarette smoking, elevated blood pressure, serum total and low-density lipoprotein cholesterol, low high-density lipoprotein cholesterol, diabetes mellitus and advanced age, as well as, obesity (Grundy et al. 1999). Obesity has become a global issue and worldwide its prevalence is constantly increasing. Obesity is often defined as body mass index (BMI) and high BMI has been demonstrated to increase the risk for diseases such as CVD (Willett et al. 1995) and type 2 diabetes (Colditz et al. 1995).

Adipose tissue produces several hormones, of which leptin has proven to be extremely interesting. Leptin reduces appetite and decreases body weight. Plasma leptin correlates with the mass of adipose tissue and is enhanced in obesity. Mutations in the leptin encoding gene, Lep, and its receptor, LEPR, lead to marked obesity. However, these mutations are rare and the majority of obese subjects have high leptin levels, but leptin fails to signal because of leptin resistance (reviewed by Friedman 2011).

There are six isoforms of LEPR, with LEPR-b considered to be the most important. It is believed to be the isoform which mediates the intracellular signaling of leptin. The function of other isoforms is as yet less well known. It is well characterized that LEPR is expressed in hypothalamus where its central role is to regulate appetite. However, LEPR is also expressed in other tissues although the function is less well known (reviewed by Friedman 2011).

In the LEPR gene, there are various common variations, non-synonymous single nucleotide polymorphisms, resulting in amino acid change on the protein level. The association of these polymorphisms on cardiovascular risk factors has been investigated in several studies. For example, the Lys109Arg variant in LEPR has been associated with obesity (Tabassum et al. 2012), blood pressure (Rosmond et al. 2000), serum lipids (Okada et al. 2010) and type 2 diabetes (Salopuro et al. 2005).

In addition to genetic factors, unhealthy life style is seen behind the epidemics of obesity and adverse metabolic changes. Parallel to the dramatically increasing prevalence of obesity also consumption of fructose-sweetened beverages has become more common. Metabolic syndrome is a cluster of risk
factors that raise the risk for CVD. There are various criteria of defining it, but basically they all agree on the four criteria: obesity, dyslipidemia, insulin resistance and hypertension (Vinluan et al. 2012). Fructose has been associated with several components of the metabolic syndrome such as insulin resistance, elevated waist circumference, dyslipidemia as well as with leptin resistance (reviewed by Dekker et al. 2010). Furthermore, it has been proposed to promote liver fat accumulation (Le et al. 2009, Maersk et al. 2012) predisposing to the development of non-alcoholic fatty liver disease.

Leptin signaling might also be affected by fetal environment (Łukaszewski et al. 2011). Nutrition in early life can act to program the fetal metabolism and this can be the basis for diseases encountered in adult life (reviewed by McMillen & Robinson 2005). Changes in the expression of leptin and LEPR may lead into dysfunction of the leptin system, disturbances in energy balance, weight gain and risk for developing type 2 diabetes or atherosclerosis.

Research on autophagy has recently become a hot topic and it has been associated with obesity and adipogenesis (Singh et al. 2009b, Zhang et al. 2009) as well as with leptin (Malik et al. 2011). Autophagy is a mechanism used by cells to get rid of dysfunctional proteins and to survive during starvation (Choi et al. 2013). Autophagy has been reported to be up-regulated in the adipose tissue of obese people and animal (Jansen et al. 2012). In addition, autophagy has been associated with liver diseases (Hagiwara et al. 2010, Rautou et al. 2008) and atherosclerosis (Ouimet et al. 2011).

The association between common leptin receptor polymorphisms and cardiovascular risk factors is known, but the association with cardiovascular diseases themselves has not been studied previously. Also the effects of fructose and intrauterine environment have been established, but their impact on leptin system, not to mention the impact on autophagy, has not been investigated in detail. The aim of this thesis was to clarify the role of the leptin system in the development of cardiovascular diseases and the objective was to understand better the underlying mechanisms.
2 Review of the literature

2.1 Leptin

2.1.1 History of leptin

The obese/obese (ob/ob) mouse was first discovered in 1950. The ob mutation was known to locate on chromosome 6 and it produced a phenotype with marked obesity, hyperphagia and mild diabetes (Ingalls et al. 1950). In 1961 Zucker and Zucker discovered fatty/fatty (fa/fa) mutation in rat, which also resulted in hyperphagic and obese phenotype, but was suggested to be caused by a mutation in a different gene than ob/ob (Zucker & Zucker 1961). A mouse model, diabetes/diabetes (db/db), with a similar phenotype to the previous ones was characterized in 1966 and it was shown that this mutation was localized on mouse chromosome 4 (Hummel et al. 1966).

The ob/ob and db/db mouse models were intensively studied at the Jackson Laboratory by Douglas Coleman. He used a series of parabiosis experiments in which the blood circulation of two mice is crossed by surgically joining the skin-to-skin anastomosis from the shoulder to the pelvis. Pairing ob/ob and db/db mice with each other and with normal mice, he started to gain a theory that db/db mice produce a satiety factor which is also produced in normal mice, only in smaller amounts. However, db/db mice are unable to respond to this factor whereas the ob/ob mutant responded to it but could not produce it. He also suggested that the satiety factor could be produced by adipose tissue and acts on a receptor in the hypothalamus (reviewed by Coleman 2010). Later, these theories were also proved to be true.

In 1994, Jeffrey Friedman and colleagues managed to clone the ob gene (Zhang et al. 1994) and one year later the product of the ob gene was discovered. This hormone produced by adipocytes was named leptin, derived from the Greek leptos, which means thin (Halaas et al. 1995). In 1995, Louis Tartaglia and co-workers succeeded in identifying the leptin receptor and showing that it is expressed not only in the hypothalamus, as Coleman suggested but also in various other tissues (Tartaglia et al. 1995). After the discovery of these genes, the research in this field has exploded and nearly 24,000 papers have been published about leptin.
2.1.2 Structure and expression of leptin

Leptin is a 16kDa protein encoded by the \textit{Lep} gene (Halaas \textit{et al.} 1995) and in human this gene is located in chromosome 7q31.3 (Green \textit{et al.} 1995). The crystal structure of leptin has revealed four \(\alpha\)-helices and leptin resembles other type I \(\alpha\)-helical cytokine family members (Zhang \textit{et al.} 1997). It is related to hormones such as growth hormone (GH), prolactin and interleukins. There is a substantial divergence in the amino acid sequence of leptin among vertebrates. However, the secondary and tertiary structures as well as the key amino acids required for the biological activity are evolutionarily highly conserved (reviewed by Denver \textit{et al.} 2011).

In mammals the main site for leptin production is adipose tissue, but it is also expressed at low levels in other tissues such as stomach, bone marrow, placenta and ovary (reviewed by Zhou & Rui 2013).

2.1.3 Effects of leptin

The circulating leptin level correlates positively with body fat mass and is considered a key adiposity signal in long time range. Leptin levels also fluctuate according to nutrition and they also function as a short-term indicator of nutritional status (reviewed by Zhou & Rui 2013).

Leptin acts on the brain and regulates food intake and energy expenditure. The main site for leptin action is in the hypothalamus, especially on proopiomelanocortin (POMC) and agouti-related protein (AgRP) neurons. In POMC neurons leptin stimulates the synthesis of anorexigenic (appetite-suppressing) neuropeptide POMC that acts as a precursor for \(\alpha\)-melanocyte stimulating hormone (\(\alpha\)-MSH). \(\alpha\)-MSH reduces body weight by binding to its receptor. The AgRP neurons, instead, produce the orexigenic (appetite-stimulating) neuropeptide Y (NPY) and AgRP, and leptin inhibits both neuronal activity of AgRP neurons and the production of NPY and AgRP (reviewed by Zhou & Rui 2013). Furthermore, leptin affects the neurons that synthesize thyrotropin-releasing hormone (TRH) and corticotropin-releasing hormone (CRH) and apart from inhibiting food intake, also increases thermogenesis and pituitary hormone secretion (reviewed by Denver \textit{et al.} 2011). The main effects of leptin are reviewed in Figure 1.
Fig. 1. The main effects of leptin. Leptin has both central effects (black arrows) and peripheral effects (grey arrows) on target organs. In the hypothalamus leptin induces the expression of proopiomelanocortin (POMC) and inhibits agouti-related protein (AgRP) and neuropeptide Y (NPY). These changes affect the hormone secretion of hypothalamus by increasing gonadotropin-releasing hormone (GnRH), growth-hormone releasing hormone (GHRH) and thyrotropin-releasing hormone (TRH) release. As a response thyroid-stimulating hormone (TSH), gonadotropins (follicle-stimulating hormone, FSH and luteinizing hormone, LH) and growth hormone (GH) are secreted from pituitary. Leptin decreases corticotropin-releasing hormone (CRH) and thus adrenocorticotropic hormone (ACTH) release and inhibits the insulin secretion from pancreatic β-cells.

Hypothalamus-pituitary-gonadal axis

In humans, fertility is negatively affected by anorexia and excessive exercise as well as by obesity. Hypothalamus plays a crucial role in controlling fertility by producing gonadotropin-releasing hormone (GnRH), and GnRH stimulates the production of luteinizing hormone (LH) from the pituitary. Leptin has been associated with fertility, but GnRH-producing neurons do not express LEPRs and
it is believed that leptin regulates reproduction indirectly. For example, POMC and AgRP neurons are in close contact with GnRH neurons and might link the energy balance and reproductive functions. Normal leptin levels are also necessary for a normal progression of puberty (reviewed by Hill et al. 2008).

**Hypothalamus-pituitary-thyroid axis**

Leptin stimulates the POMC neurons and suppresses the AgRP neurons in the hypothalamus. As melanocortins stimulate and AgRP inhibits the thyroid axis, leptin induces the production of TRH resulting in enhanced thyroid-stimulating hormone (TSH) secretion from the pituitary and accelerates the metabolic rate (reviewed by Mantzoros et al. 2011).

**Hypothalamus-pituitary-growth hormone axis**

In rodents, leptin enhances the growth hormone-releasing hormone (GHRH) induced growth hormone (GH) secretion and leptin deficiency has been shown to negatively affect growth. However, in humans, children with leptin deficiency have normal growth curves and leptin has been suggested not to regulate the secretion of GH but to modulate the effect of GH (reviewed by Mantzoros et al. 2011).

**Hypothalamus-pituitary-adrenal axis**

It has been shown in mice that leptin inhibits CRH release from the hypothalamus, resulting in decreased adrenocorticotropin hormone (ACTH) secretion from the pituitary and, thus, lower corticosterone levels. In addition, glucocorticoids are capable of stimulating leptin expression and secretion, and circulating leptin restrains the stress response by inhibiting CRH release (Heiman et al. 1997). Moreover, leptin has been found to act directly on the adrenal gland and reduce cortisol secretion (Bornstein et al. 1997).

**Leptin and insulin**

Like leptin, insulin secreted by pancreatic β-cells in response to increased blood glucose is a signal of positive energy balance. The effect of insulin is anorexigenic as is the effect of leptin. Both insulin and leptin affect the POMC
and AgRP neurons of the hypothalamus in a similar way via their own receptors, LEPR and insulin receptor (InsR). In addition, their signaling pathways converge and they both activate the phosphatidylinositol 3-kinases (PI3K) signaling pathway (reviewed by Varela & Horvath 2012). It has also been shown that over-activation of the leptin signaling pathway, especially signal transducer and activator of transcription 3 (STAT3) and suppressor of cytokine signaling 3 (SOCS3), in POMC neurons leads to both leptin and insulin resistance (Ernst et al. 2009).

Leptin directly modifies liver glucose metabolism. This is believed to occur via interactions between leptin and insulin signalling pathways and to be mediated by PI3K. Leptin may also alter the action of insulin in adipocytes and it is generally accepted that leptin reduces insulin release from pancreatic β-cells (reviewed by Mantzoros et al. 2011).

**Leptin and immune system**

Leptin has also been shown to affect innate and adaptive immunity by increasing the phagocytic activity and pro-inflammatory cytokine production in macrophages, acting as a potent chemoattractant for monocytes and macrophages, and influencing the natural killer cell development, activation and cytotoxicity. Furthermore, leptin is suggested to play a role in B-cell development and cytokine production as in balance between the T helper 1-cells and regulatory T-cells (reviewed by Procaccini et al. 2012).

Interestingly, it has also been noticed that autoimmune diseases are on the increase in developed countries, whereas in less developed countries they are rare and secondary immunodeficiency caused by malnutrition is common. This notion is associated with increased prevalence of obesity in developed countries, accompanied with high leptin levels. Indeed, studies suggest that leptin deficiency could lead to immunosuppression, whereas high leptin concentrations might contribute to the induction of autoimmune disease in genetically predisposed individuals (reviewed by Procaccini et al. 2012).

**Leptin and bone metabolism**

Leptin has been suggested to play a role in bone mass control. Most of the literature appears to support the idea that leptin suppresses bone mass via its effects on the hypothalamus and sympathetic signaling. However, controversial
results also exist proposing that leptin could actually promote osteoblast proliferation and differentiation, and increase bone formation. Consensus has not been found and the role of leptin in bone homeostasis still remains unknown (reviewed by Motyl & Rosen 2012).

**Leptin and angiogenesis**

Adipogenesis and angiogenesis are very much correlated and leptin has been associated with the growth of vasculature as well. This was demonstrated by stimulating endothelial cells with leptin, which resulted in increased cell proliferation and enhanced angiogenesis (Bouloumie *et al.* 1998).

**Leptin and endothelium**

Leptin has been associated with endothelial dysfunction. LEPRs are expressed in the endothelial cells of coronary arteries and it has been reported that hyperleptinemia might cause endothelial dysfunction as assessed by response to acetylcholine. On the other hand, in pharmacological high concentrations leptin was demonstrated to result in nitric oxide-mediated vasodilation (Knudson *et al.* 2005). In a recent Swedish study by Gonzalez and colleagues, leptin was observed to associate with reduced vasodilation and hypertension in the elderly (Gonzalez *et al.* 2013). Leptin has also been shown to induce proatherogenic C-reactive protein (CRP) production in cultured coronary artery endothelial cells (Singh *et al.* 2007) and production of cyclooxygenase 2 (COX2) and intercellular adhesion molecule 1 (ICAM1) in cultivated murine aorta tissue suggesting involvement in inflammatory response (Manuel-Apolinar *et al.* 2013).

**Leptin and liver**

Leptin has been shown to have both central nervous-mediated and direct effects on the liver. In rat, leptin infusion has been shown to decrease liver triglycerides, and very low-density lipoprotein (VLDL) secretion rate and to increase fatty acid oxidation. It was concluded that liver leptin resistance might contribute to the development of hypertriglyceridemia and increased VLDL particle size in obesity (Huang *et al.* 2009). Indeed, a liver-specific loss of leptin signaling has been shown to increase lipid accumulation in the liver but also to enhance liver insulin sensitivity (Huynh *et al.* 2010). On the other hand, it was reported that chronic
central leptin infusion might result in increased hepatic insulin efficiency (Berthou et al. 2011) and that central impairment in leptin signaling could manifest as hepatic steatosis in mice (Warne et al. 2011), indicating distinct central effects of leptin on the liver.

**Paracrine effect of leptin on adipose tissue**

Leptin also has an autocrine/paracrine effect on adipose tissue. LEPRs are expressed in adipose tissue and peripheral administration of leptin has been shown to enhance lipolysis in white adipose tissue (WAT) and insulin-stimulated glucose utilization in brown adipose tissue (BAT) (Siegrist-Kaiser et al. 1997), and adipocyte-specific reduction of LEPRs has been shown to result in increased adiposity and metabolic alterations (Huan et al. 2003). Later, it was also reported that the ability of WAT to store fat requires blockade of the paracrine activity of leptin (Wang et al. 2005).

**2.1.4 Leptin deficiency**

The most common reasons for insufficient leptin levels are mutations in the leptin gene and absence of adipose tissue. Mutations of the leptin gene are very rare autosomal recessive conditions accompanied by obesity due to hyperphagia, decreased GnRH secretion leading to hypogonadism and failure to reach puberty. Hyperinsulinemia and dyslipidemia are also seen in these patients (reviewed by Mantzoros et al. 2011).

Lipodystrophy may be caused by autosomal recessive mutation or more commonly, due to human immunodeficiency virus infection. These patients suffer from dyslipidemia, insulin resistance, increased hepatic gluconeogenesis and fat content as well as impaired reproductive functions that can be treated with leptin therapy. Another typical condition is hypothalamic amenorrhea caused by intensive exercise and low body fat resulting in hypoleptinemia. In this condition, leptin treatment has been shown to normalize menstrual cycle and ovulation in most of the subjects (reviewed by Mantzoros et al. 2011).

**2.2 Leptin receptor**

As already discussed, the leptin receptor (LEPR) was discovered in 1995 by Louis Tartaglia and co-workers and was found to be expressed in hypothalamus
as well as in various other tissues (Tartaglia et al. 1995). The next year, Chua and colleagues observed that the similar phenotypes of db/db mouse and fa/fa rat are caused by mutations in the gene encoding LEPR (Chua et al. 1996a). The LEPR gene was later localized on human chromosome 1p31 (Thompson et al. 1997).

2.2.1 Structure and expression of LEPR

LEPR belongs to the class I helical cytokine receptor family which all signal via the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway (reviewed by Denver et al. 2011). In mammals, six alternatively spliced LEPR isoforms (a-f) have been identified, although only five (a-e) have been described in humans in the UniProt database.

Figure 2 is a schematic presentation of the rat LEPR isoforms. LEPRs have a common extracellular domain and a 21-amino-acid-long transmembrane helix, lacking only in LEPR-e, which is soluble. LEPR-b is called a long form and has about a 300-amino-acid-long cytoplasmic domain that mediates intracellular signaling. LEPR-a, -c, -d and -f have a short intracellular domain, about 30-40 amino acids long (reviewed by Denver et al. 2011).
Fig. 2. Schematic presentation of rat LEPR isoforms. Cytokine receptor homolog (CRH), immunoglobulin-like (Ig-like), fibronectin type (FN) domains are located on the extracellular portion of LEPR. The numbers below receptors indicate the length of the receptor isoform in amino acids. (Modified from Wauman & Tavernier 2011).

LEPR-b is the isoform that is believed to mediate the leptin signaling. Its intracellular part contains JAK and STAT binding sites whereas only one JAK binding site is included in the shorter isoform, LEPR-a. The extracellular part of LEPR is comprised of two cytokine receptor homolog (CRH) domains of which CRH1 is not required for leptin binding, while the membrane proximal domain, CRH2, is necessary for ligand binding (Fong et al. 1998). However, it has been shown that CRH1 deletion leads to decreased leptin response (Zabeau et al. 2004). CRH domains resemble the fibronectin type III (FN III) and Ig folds. CRH2
contains a conserved WSXWS motif. CRH domains are connected with an Ig-like domain and followed by two FNIII domains. The Ig-like domain was shown to be crucial as deletion of Ig-like resulted in impaired leptin binding and JAK activation. The cytosolic part contains box 1 and other conserved sequences that are required for intracellular signalling (Zabeau et al. 2004). The human LEPR-b domains and respective amino acids are presented in Figure 3.

Fig. 3. Schematic presentation of human LEPR-b structure. FN: fibronectin type, Ig: immunoglobulin, CRH: cytokine receptor homolog. Tyr-986 and Tyr-1141 are conserved tyrosines that are involved in STAT3 activation.

LEPRs are highly expressed in mammalian hypothalamus and on a lower level in various tissues, such as liver, pancreatic β-cells, immune cells (reviewed by Denver et al. 2011) and adipose tissue (Siegrist-Kaiser et al. 1997).

2.2.2 Leptin signalling via LEPR

Leptin induces multiple signaling pathways. When it binds to LEPR it stimulates receptor dimerization that results in JAK2 activation and autophosphorylation. JAK2 then phosphorylates conserved tyrosines in the LEPR cytosolic domain. Phospho-Tyr-1141 acts as a binding site for STAT3 which is then phosphorylated and activated by JAK2. STAT3 then moves into nucleus and as a transcription factor, regulates gene expression. STAT3 also activates transcription of SOCS3 that binds to JAK2 and Tyr-986 inhibiting leptin signaling and creating a negative feedback. Tyr-1078 is involved in STAT5 phosphorylation. STAT5 acts as a transcription factor as well (reviewed by Morris & Rui 2009, Nanjappa et al. 2011). JAK/STAT signalling is presented in Figure 4.
Fig. 4. Leptin signaling via JAK/STAT pathway. Leptin binding results in LEPR dimerization and JAK2 autophosphorylation. JAK2 then phosphorylates the conserved tyrosines of LEPR. Tyr-986 and Tyr-1141 are involved in STAT3 activation. STAT3 is a transcription factor that activates the transcription of SOCS3 which, in turn, inhibits leptin signaling. Phosphorylation of Tyr-1078 is involved in activation of transcription factor STAT5.

Another important signaling pathway of leptin is the 5'-AMP-activated protein kinase (AMPK) pathway. Activated AMPK inactivates acetyl-CoA carboxylase (ACC) which is a key enzyme in the synthesis of triglycerides (TGs). AMPK also increases catabolic processes such as fatty acid oxidation. AMPK signaling is thought to regulate glucose homeostasis, hepatic gluconeogenesis and lipid metabolism (Nanjappa et al. 2011).
The third, significant module of leptin signaling is the insulin receptor substrate/phosphatidylinositide 3-kinase (IRS/PI3K) pathway. Leptin binding to its receptor leads to phosphorylation of IRS, which in turn activates PI3K. This leads to activation of protein kinase B (AKT) and the downstream mediators of AKT such as mammalian target of rapamycin (mTOR) and transcription factor, forkhead box O1 (FOXO1) (reviewed by Morris & Rui 2009, Nanjappa et al. 2011).

2.2.3 Leptin resistance

There are a few possible mechanisms for leptin resistance.

Firstly, the leptin transport across the blood-brain barrier (BBB) might be impaired. The active transport of leptin across the BBB is shown to be mediated by LEPR-a (Kastin et al. 1999), whereas LEPR-e, a soluble form of LEPR, seems to antagonize the action of LEPR-a and inhibits leptin transport (Tu et al. 2008). Leptin transport in the brain has also been reported to be impaired in obesity (Banks et al. 1999, Caro et al. 1996).

Secondly, leptin resistance might be caused by impaired LEPR-b trafficking to the plasma membrane. LEPR-b trafficking is mediated by Bardet-Biedel Syndrome proteins and deficiency of these proteins is shown to lead to impaired LEPR-b trafficking and leptin signalling (Seo et al. 2009). Moreover, the plasma membrane LEPR-b amount is also regulated by internalization of LEPRs via endocytosis (Belouzard et al. 2004).

Thirdly, impaired leptin signalling is one possible mechanism for leptin resistance. As already discussed, SOCS3 expression is induced by leptin and it acts as a negative regulator of leptin signaling to prevent the over-activation of leptin signaling pathways (Bjorbaek et al. 1999). It was also shown in a transgenic mouse model that overexpression of SOCS3 only in POMC neurons is sufficient to cause leptin resistance and obesity (Reed et al. 2010). Other regulators of leptin sensitivity could also be another negative regulator, non-receptor phospho-tyrosine protein phosphatase (PTP1B), and a positive regulator, SH2B adapter protein 1 (SH2B1), which enhances leptin sensitivity (reviewed by Morris & Rui 2009).
2.2.4 LEPR polymorphisms and mutations

This review concentrates mainly on two single nucleotide polymorphisms (SNPs) of LEPR, Lys109Arg and Gln223Arg, which are located in the CRH1 domain in LEPR, but also other polymorphisms of LEPR have been briefly introduced.

Coding polymorphisms

Lys109Arg of LEPR (rs1137100) has been shown to associate with several risk factors for cardiovascular disease (CVD). It has been reported to associate with body mass index (BMI) and systolic blood pressure (SBP) in men (Rosmond et al. 2000), BMI in Korean population (Park et al. 2006) and with the physical activity induced change in SBP in overweight Finnish individuals with impaired glucose tolerance (Kilpelainen et al. 2008). An association was also found between Lys109Arg and leptin levels in overweight and obese postmenopausal women (Wauters et al. 2001a), fasting insulin and glucose metabolism in women with impaired glucose homeostasis (Wauters et al. 2001b), the conversion to type 2 diabetes in high-risk individuals with impaired glucose tolerance (Salopuro et al. 2005) and childhood obesity in Indian children (Tabassum et al. 2012). Lys109Arg association with serum lipids in Japanese obese children has been described as well (Okada et al. 2010). Interestingly, a strong association between Lys109Arg and birth weight has also been observed (Souren et al. 2008). Recently, it was reported that Lys109Arg is associated with non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (Zain et al. 2013).

LEPR Gln223Arg (rs1137101) has been associated with SBP in men (Rosmond et al. 2000), insulin response for glucose in women with impaired glucose tolerance (Wauters et al. 2001b), body composition in overweight and obese women (Wauters et al. 2001a), body weight and BMI in Pacific Islanders (Furusawa et al. 2010), BMI and change in BMI over time (Gallicchio et al. 2009) as well as with birth weight (Souren et al. 2008). Interestingly, a study with Gln223Arg mutation created in mouse revealed no effect between this mutation and body weight, composition or energy expenditure (Stratigopoulos et al. 2009). Furthermore, an association of Gln223Arg with total cholesterol, high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) levels in Japanese men (Takahashi-Yasuno et al. 2003), as well as with NAFLD and non-alcoholic steatohepatitis has been reported (Zain et al. 2013).
Besides these most widely studied LEPR SNPs, some studies have also been published about LEPR Lys656Asn (rs1805094) polymorphism: it was associated with insulin and leptin levels in non-diabetic obese men (de Luis et al. 2008), with fasting insulin and glucose as well as with response to the oral glucose load in women with impaired glucose tolerance (Wauters et al. 2001b), and with diet and exercise induced weight loss (de Luis Roman et al. 2006).

Although various studies have suggested an association between LEPR SNPs and body weight (Gallicchio et al. 2009, Okada et al. 2010, Park et al. 2006, Rosmond et al. 2000, Wauters et al. 2001a), contradicting results exist and quite a recent systematic review showed no association between LEPR Lys109Arg, Gln223Arg, Lys656Asn and obesity (Bender et al. 2011).

Non-coding polymorphisms

Associations have also been found with non-coding SNPs of LEPR. These SNPs are located on the introns or on 5’- and 3’-untranslated regions. Although they do not affect the protein sequence, they may have a regulatory role on expression. A study by Wheeler and colleagues observed an association between LEPR SNP rs11208659 and obesity and it was suggested that this association might be mediated by reduction in the levels of LEPR expression (Wheeler et al. 2013). It has also been reported that LEPR rs1045895 is associated with change in BMI over time (Gallicchio et al. 2009) and another LEPR SNP, rs10889553, with SBP (Sober et al. 2009). Also a noncoding variation, rs2025804, in LEPR was associated with BMI and energy expenditure in Pima Indians (Traurig et al. 2012). In addition, an association between age at death and a LEPR SNPs rs1171278 and rs3790426 was observed (Lunetta et al. 2007).

Functional studies about LEPR mutations

Besides db/db mice, which have a general LEPR dysfunction resulting in hyperphagia, obesity, reduced energy expenditure, growth hormone deficiency and infertility as well as in insulin resistance and diabetic phenotype, there are also other animal models of LEPR deficiency. The s/s mouse carries a mutation of serine residue replacing the Tyr1138 that is essential for STAT3 activation. As a result, these mice are hyperphagic and obese but fertile. However, they develop insulin resistance similar to db/db mice, particularly in the liver (reviewed by Lutz & Woods 2012).
In rats, two animal models with mutations in LEPR have been identified. Koletsky rats have mutations leading to undetectable LEPR mRNA levels, whereas fatty/fatty (fa/fa) rats have a mutation resulting in reduced transportation of LEPRs on cell surface. Both of these rats develop hyperphagia, reduced energy expenditure and morbid obesity as well as impaired glucose tolerance, growth deficiency, hypothyroidism and reduced fertility. In addition, Koletsky rat displays a phenotype with hypertension and more severe insulin resistance than fa/fa rats (reviewed by Lutz & Woods 2012).

It has been suggested that the CRH1 domain might not be involved in leptin binding, but instead CRH2 is essential for that (Fong et al. 1998). However, as a deletion of CRH1 results in a lower leptin response, it could be suggested that CRH1 somehow contributes to the optimal LEPR activation (Zabeau et al. 2004). Both Lys109Arg and Gln223Arg polymorphisms are located in the CRH1 domain in LEPR and a mutation that causes the obese phenotype of the fa/fa rat is also located in this same domain (Chua et al. 1996b, Iida et al. 1996). However, when the Gln223Arg mutation was created in mouse, no effect on leptin signaling measured by activation of STAT3 was observed (Stratigopoulos et al. 2009).

FNIII domains are not involved in ligand binding, but are essential for signaling as mutations in two conserved cysteines on positions 672 and 751 completely disrupt the leptin signaling (Zabeau et al. 2005).

**Leptin receptor-related monogenic obesity**

Even though LEPR mutations are rare, it has been estimated that up to 3% of the severely obese patients might have a loss of function mutation in the LEPR gene (Farooqi et al. 2007).

Individuals with homozygous mutation resulting in abnormal splicing of LEPR transcripts have been described. The mutation in exon 16 creates a truncated protein of 831 amino acids lacking both transmembrane and intracellular domains. The birth weight of the patients was normal but they developed severe obesity rapidly within the first months of life characterized by abnormal eating behavior and aggressive behavior when denied food. They also displayed high serum leptin levels that might be explained by mutated form binding to the circulating leptin and prolonging its half-life. They experienced no puberty and had hypogonadotrophic hypogonadism, decreased growth hormone secretion as well as insufficient thyreotropic secretion (Clement et al. 1998).
Farooqi and colleagues described four mutations in LEPR with three of them (Ala409Glu, Trp664Arg and His684Pro) resulting in complete loss of signaling as measured with phosphorylation of STAT3. The fourth mutation (Arg612His) led to impaired signaling but some ability to phosphorylate STAT3 in response to leptin still remained. Ala409Glu is located in Ig-like domain whereas the rest are in FNIII domains. The patients homozygous for these mutations were obese, displayed a lower lean mass than equally obese subjects without mutations in LEPR, had hyperphagia, hyperinsulinemia, hypogonadotrophic hypogonadism and suffered from infections in childhood (Farooqi et al. 2007).

Yet another known mutation described in LEPR is a homozygous missense mutation Pro316Thr resulting in rapid weight gain in the first few months of life with severe hyperphagia and patients suffering from recurrent upper respiratory tract infections. As these patients were in prepubertal stage, the effect on reproductive system could not be assessed (Mazen et al. 2011). The Pro316Thr mutation is located in the FNIII domain in CRH1 of LEPR.

2.3 Autophagy

Autophagy was initially discovered more than 40 years ago as several scientists started to observe vesicles containing lysosomal enzymes and semi-digested cell organelles in mouse kidneys and rat hepatocytes. In 1963 at CIBA Foundation symposium on lysosomes, Christian de Duve, a Nobel Prize winner in Physiology or Medicine, launched the term autophagy to describe these vesicles and four years later, in 1967, he showed that apart from starvation, also glucagon is a powerful inducer of autophagy in liver (Deter et al. 1967). Ten years later, it was shown that insulin and the autophagy end product, amino acids, inhibit autophagy (Mortimore & Schworer 1977, Pfeifer 1977). Today, it is known that autophagy is induced by AMPK and, conversely, inhibited by the mTOR that is regulated by the PI3K/AKT pathway (Choi et al. 2013).

The word autophagy comes from the Greek words “auto” “self” and “phagein” “to eat”. Autophagy is defined as a catabolic pathway involving degradation of cellular components through the lysosomal machinery. The major subtype of autophagy is macroautophagy, hereafter referred to as autophagy. It involves the sequestration of cytoplasmic components into double-membrane autophagosomes which fuse with lysosomes and the cargo is degraded and recycled (Choi et al. 2013) as is presented in Figure 5. The autophagic process is coordinated by protein products of 32 genes, of which two important key
molecules, autophagy related gene 7 (ATG7) and microtubule-associated protein 1 light chain 3 beta (MAP1LC3β, LC3), have been added to the Figure 5. In the process of autophagy, LC3 propeptide is first cleaved to form cytosolic LC3-I. It then goes through a conjugation with phosphatidylethanolamine to form LC3-II, which is present in autophagosomes. This conjugation process is mediated by ATG7 (Choi et al. 2013).

Fig. 5. Phases of autophagy. Microtubule-associated protein 1 light chain 3 (LC3) propeptide is cleaved to LC3-I, which is then conjugated in ATG7 mediated process with phosphatidylethanolamine to form LC3-II. LC3-II is present in autophagosomes. (Modified from Choi et al. 2013).

2.3.1 Lipophagy

Lipophagy is a selective targeting of lipids to autophagosomes for degradation through the autophagy pathway. In cells lipid is stored in cell organelles called lipid droplets. TGs degraded by lipophagy supply free fatty acids (FFAs) for the energy demands of the cell and the intracellular concentrations of FFAs and cholesterol regulate autophagy (reviewed by Liu & Czaja 2013). The mechanism on how autophagy targets lipid droplets is not known. One candidate is LC3 that apart from being bound on autophagosomal membrane also associates with lipid droplets (Shibata et al. 2009) and might initiate the autophagic process (Singh et al. 2009a).

2.3.2 Autophagy in adipose tissue

In adipocytes, autophagy has been proposed to play a role in adipogenesis (Singh et al. 2009b, Zhang et al. 2009).

The ATG7 gene encodes an enzyme that is essential for autophagosome formation and ATG7 adipocyte-specific knockout mice have been shown to
exhibit impaired autophagy which leads into decreased lipid storage in WAT (Singh et al. 2009b, Zhang et al. 2009) and lower leptin secretion from adipocytes (Zhang et al. 2009). Also ATG5 deletion in mouse embryonic fibroblasts resulted in marked reduction in adipogenesis (Baerga et al. 2009).

Moreover, autophagy has been shown to be upregulated in the adipose tissue of obese human subjects and inhibition of autophagy results in increased production of proinflammatory cytokines although leptin levels are unchanged (Jansen et al. 2012).

2.3.3 Autophagy in liver

In the liver, the role of autophagy is controversial. It has been suggested to be involved in both lipid droplet formation (Shibata et al. 2009) and lipophagy (Singh et al. 2009a).

In cultured hepatocytes it was shown that essential autophagy gene, ATG5, knockdown resulted in increased number and size of lipid droplets (Singh et al. 2009a). The same was true in vivo in liver-specific ATG7 knockout mice that showed an accelerated development of fatty liver compared to control animals (Singh et al. 2009a). On the other hand, contradicting results exist as ATG7 deficiency in mouse liver was reported to lead into decreased lipid droplet formation (Shibata et al. 2009).

Interestingly, in hepatic stellate cells increased autophagy resulted in loss of lipid stores and transdifferentiation to myofibroblasts leading to hepatic fibrosis (Thoen et al. 2011) and loss of autophagic function in cultured mouse stellate cells and in vivo in mice reduced fibrogenesis (Hernandez-Gea et al. 2012).

Impaired autophagy has often been related to non-alcoholic fatty liver disease (NAFLD) (reviewed by Amir & Czaja 2011) but also hyperactive autophagy has been reported in pathological conditions such as liver inflammation in a rat model of diabetes mellitus (Hagiwara et al. 2010) and as a possible cause of hepatocyte death in anorexia nervosa patients (Rautou et al. 2008) making the role of autophagy controversial in liver diseases.

2.3.4 Autophagy and diseases

Autophagy has been associated with several diseases. Autophagy inhibition in AgRP neurons has been shown to reduce AgRP expression and result in lean phenotype in mice (Kaushik et al. 2011), whereas autophagy deficiency in POMC
neurons leads to increased adiposity, higher liver fat content, impaired glucose tolerance and decreased lipolysis, which results in loss of lean mass under starving conditions. Furthermore, aging reduces POMC neuron autophagy, which might explain the metabolic disturbances related to aging (Kaushik et al. 2012).

Genetic X-linked deficiency in lysosome-associated membrane protein 2 (LAMP2) resulting in enhanced number of autophagosomes causes cardiomyopathy known as Danon’s disease (Nishino et al. 2000). Autophagy may also have a protective role in atherosclerosis by enhancing macrophage cholesterol efflux, preventing cytosolic lipid accumulation and suppressing macrophage foam cell formation (Ouimet et al. 2011).

2.3.5 Leptin system and autophagy

It has been demonstrated that leptin induces autophagy in peripheral tissues including liver, muscle, heart and kidney possibly by activating AMPK signalling (Malik et al. 2011). However, leptin has also been shown to inhibit AMPK and activate mTOR, the inhibitor of autophagy, in some cell types such as hypothalamic neurons (Gao et al. 2007) and hepatic stellate cells (Aleffi et al. 2011).

Furthermore, mice lacking autophagy gene ATG7 in POMC neurons develop obesity and show leptin resistance related to the inability of leptin to activate STAT3 (Quan et al. 2012).

2.4 Developmental origins of disease

Previous studies have indicated that the individual risk of developing metabolic syndrome and related disorders could already be determined during the fetal development. Particularly nutrition in early life can act to program the fetal metabolism and this can form the basis for diseases encountered in adult life (reviewed by McMillen & Robinson 2005).

It was already reported in 1976 that men exposed to the Dutch famine (1944–1945) in utero during the first half of pregnancy displayed significantly higher rates of obesity at the age of 19 years (Ravelli et al. 1976). In 1986 Barker and Osmond observed that certain areas in England and Wales with high infant mortality in 1921–1925, suffered from high ischemic heart disease mortality some 50 years later. This geographical association suggested that poor nutrition in early life promotes the effects of generous diet later in life (Barker & Osmond 1986).
The “developmental origins of disease” hypothesis is thus broadly recognized as Barker’s hypothesis. Later, fetal growth restriction has been associated with the development of the features of the metabolic syndrome, including dyslipidemia, visceral obesity and impaired glucose tolerance (reviewed by Briana & Malamitsi-Puchner 2009).

Conversely, the studies of the Leningrad famine (1941–1944) showed that intrauterine malnutrition was not associated with glucose intolerance, dyslipidemia, hypertension or cardiovascular disease in adulthood (Stanner et al. 1997). Already in 1977 Forsdahl had discovered that poverty in childhood followed by prosperity in adulthood is a risk factor for atherosclerosis (Forsdahl 1977). As the Leningrad famine occurred in a previously malnourished population that remained poorly nourished also after the famine, they developed a “thrifty phenotype”, a fetal adaptation that was still advantageous after birth. However, the Dutch famine started suddenly, lasted for a relatively short period and ended abruptly, resulting in a rapid “catch-up growth” after birth as their intrauterine environment was badly matched to the extrauterine environment (reviewed by Hales & Barker 2001). Thus, low birth weight that is followed by exponential childhood growth increases the risk for metabolic syndrome and its features such as obesity, insulin resistance, dyslipidemia and hypertension.

Intrauterine nutrition might affect the offspring by epigenetic mechanisms such as DNA methylation or impaired organ growth and altered structure of organs. For example, the function of the pancreas might be compromised in subjects born small for gestational age and might result in defects in insulin secretion. DNA methylation affects the transcription activity of the genes and altered methylation may lead to metabolic changes caused by gene expression changes (reviewed by Calkins & Devaskar 2011).

Intrauterine growth restriction (IUGR) has been shown to affect the leptin system as well. It has been reported that serum leptin concentrations correlate positively with weight in newborns, with smaller children having lower leptin levels after birth (Matsuda et al. 1997), whereas children born with IUGR had significantly higher leptin levels onwards from the end of the first year of life, and it was suggested that they develop leptin resistance to increase their energy balance and enable catch-up growth (Jaquet et al. 1999).
2.5 Atherosclerosis

Atherosclerosis is a complex disease of medium- and large-sized arteries that progresses over many years. Atherosclerosis is responsible for a great number of CVDs and may lead in to life-threatening events such as myocardial infarction and stroke (reviewed by Falk 2006).

A recent report from the American Heart Association revealed that CVD was listed as an underlying cause of death in 32.3% of all deaths in 2009 in the United States. In addition, more than one in every three American adults has CVD. It is estimated that by the year 2030, 40.8% of the US population have some form of CVD (Go et al. 2013). In the late 1960s, CHD mortality of Finnish men was the highest in the world. In 1972, an intervention program to reduce the CHD morbidity and mortality was launched. Since that the CVD mortality rates have reduced markedly (Vartiainen et al. 2010).

2.5.1 Pathogenesis of atherosclerosis

The normal artery contains three layers: tunica intima, media and adventitia. Tunica intima is lined by endothelial cells which are in contact with blood and attached to basement membrane positioned between the intima and media layers. The intima also contains some smooth muscle cells that are present in larger amounts in tunica media.

The atherosclerosis progression is initiated as endothelial cells are irritated by dyslipidemia, hypertension or pro-inflammatory mediators and start to express adhesion molecules that capture leukocytes on their surface. The endothelial permeability increases and LDL particles immigrate to the arterial wall. As a result, monocytes are attracted by chemotactic factors, infiltrate into tunica intima and become macrophages that are able to intake lipids by endocytosis. These lipid-laden macrophages then become foam cells. Plaque formation also involves smooth muscle cells that migrate and proliferate in tunica intima and synthesize extracellular matrix such as collagen to form a fibrous cap for the plaque. Infiltrated macrophages and smooth muscle cells may die in plaque and form a necrotic core filled with lipids. A serious complication of atherosclerosis is thrombosis, in which the fibrous cap of the plaque fractures resulting in blood clotting that may prevent blood flow. Unstable plaques that rupture typically have thin, collagen-poor fibrous caps (reviewed by Libby et al. 2011).
Leptin has been associated with atherosclerosis via various mechanisms, including monocyte recruitment to intima, foam cell transformation, smooth muscle cell proliferation as well as secretion of proatherogenic cytokines, platelet aggregation and endothelial dysfunction (reviewed by Sweeney 2010).

### 2.5.2 Cardiovascular risk factors

Age, gender and family history are classical non-modifiable cardiovascular risk factors. The strongest predictor is age, but also gender is important as the incidence rate of cardiovascular mortality is comparable between women and men ten years younger. Parental and sibling family history is also significant and likely measures the shared genetic factors. The classic modifiable risk factors include hypertension, cholesterol abnormalities (especially high LDL cholesterol), diabetes mellitus and behavioural factors such as smoking, obesity, unhealthy diet and physical inactivity. Recently also inflammatory markers, such as CRP, have been considered as risk factors (reviewed by Payne 2012).

### 2.5.3 Genetics of atherosclerosis

Most cases of coronary artery disease (CAD) and stroke are complex and involve both genetic and environmental factors. It is estimated that genetic factors account for 30–60% of the variation in the risk of CAD between the individuals (reviewed by Lusis 2012). The effect of genetics is more pronounced at younger ages as the effect decreases at older age (Marenberg et al. 1994).

The unit of genetic variation is SNP. They are often used as markers of genomic region and the majority of them have minimal or no effect on biology. However, some extremely rare genetic variants, often called mutations, that have an impact on protein structure and function cause monogenic diseases. Since the effect of the genetic variant is so strong, these disease genes may be found by genotyping the affected families using a collection of genetic markers around the genome and examining the segregation of these markers with the disease. This is called linkage analysis (reviewed by Bush & Moore 2012). Monogenic diseases are also referred to as Mendelian diseases since they are inherited according to the Mendel’s laws. The most well-known mutation affecting the risk for atherosclerosis is a deletion identified in the LDL receptor gene causing familial hypercholesterolemia (Lehrman et al. 1985). Other famous mutations increasing the risk for Mendelian form of CAD are mutations in apolipoprotein E (APOE)
and proprotein convertase subtilisin/kexin type 9 (PCSK9). Both of these genes are involved in the clearance of circulating lipoproteins (reviewed by Welch 2012).

Common variants are present with a high allele frequency in a population and only alter the risk for disease by a small amount. Thus, the prevalence of the disease and the allele frequency of the influential allele are only slightly correlated. Complex diseases are influenced by multiple common alleles and their recombination (reviewed by Bush & Moore 2012).

**Linkage disequilibrium**

Linkage disequilibrium (LD) is a linkage between the markers on a population scale. It describes the degree to which an allele of a SNP is inherited with another allele of another SNP in a population. In addition to population size and the number of generations the population has existed, LD is dependent on the distance between the two loci as the recombination events happen to occur more frequently when the distance is larger and the LD diminishes. The two commonly used measures of LD are $D'$ and $r^2$. $D'$ is scaled between 0 and 1, with 0 indicating complete linkage equilibrium, and the two markers are independently inherited. $D'$ of 1 indicates complete linkage disequilibrium as no recombination has occurred between the markers. In genetic analyses, LD is often reported as $r^2$, a measure of correlation. High $r^2$ indicates that the two SNPs denote the same information as they are often observed together (reviewed by Bush & Moore 2012).

**Genetic studies**

Linkage studies are powerful in identifying genetic loci in single gene disorders but in multifactorial diseases the less clear segregation of the genetic variants and the modest contribution of them to the disease make linkage study difficult and association studies are more useful. Linkage studies are comprised of pedigrees, while association studies include non-related individuals (reviewed by Cardon & Bell 2001). In an association study, a SNP genotyped might be directly associated with the studied trait and is in this case in direct association. However, due to LD another possibility is that the SNP genotyped and associated to the phenotype is not actually the causal SNP but in a high LD with the real influential SNP (reviewed by Bush & Moore 2012).
Before genome-wide association studies (GWAS), candidate gene approach was often used in genetics (reviewed by Lusis 2012). It allows a researcher to investigate the validity of an “educated guess”. Candidate gene selection is often based on their assumed role in the disease. Currently, more than 35 loci associated with CAD have been identified in GWAS and replicated at least in one independent study. The majority of these associations exhibit only a modest effect on relative risk of CAD (reviewed by Welch 2012). The known loci identified in GWAS are thought to explain approximately 10% of the genetic variation of CAD (Schunkert et al. 2011). Because of linkage disequilibrium it is, however, difficult to identify the causal genes at associated loci and the SNP showing the association peak might actually situate kilobases away from the causal gene (reviewed by Lusis 2012). Some of the candidate genes of the CAD loci identified in GWAS have been involved in well-known pathways affecting the traditional risk factors, but there are also a number of SNPs acting through unidentified pathways (Schunkert et al. 2011). Interestingly, in various GWAS, LEPR locus has also been shown to associate with CRP levels (Dehghan et al. 2011, Elliott et al. 2009, Ridker et al. 2008) and with the risk of coronary heart disease (CHD) (Elliott et al. 2009).

**Evolutionary genetics of cardiovascular disease**

Natural selection occurs as a consequence of different fitness of individuals. It is based on the higher reproductive rate of the individuals with advantageous genes. Positive selection may increase the prevalence of these genes whereas purifying selection acts to select against harmful genes (reviewed by Ding & Kullo 2009).

CVD itself has probably not been under selective pressures as it is likely to manifest later in life and unlikely to have an effect on reproductive success. However, the susceptibility alleles for CVD may have been affected by selection. For example, high pro-inflammatory cytokine response may have adverse effect on atherosclerosis later in life, but may have increased the reproductive rate by protecting from fatal infectious diseases (Van Den Biggelaar et al. 2004).

As the environment and lifestyle have changed greatly over time, the selective pressures have also changed, and as we live in a markedly different environment than our ancestors, complex diseases might also result from a mismatch between the ancestral alleles and current lifestyle. One of these is the CVD associated allele of APOE that may have been advantageous in the past as higher serum cholesterol may have been valuable for its role in steroid hormone production.
synthesis during the rapid increase in human size (reviewed by Ding & Kullo 2009).

The “thrifty genotype” hypothesis suggests that certain genetic variants in humans have evolved to maximize the storage of energy and food searching behaviour and have been beneficial in times of food deprivation (Neel 1962). However, today these variants predispose their carriers to the diseases such as obesity and other traits of metabolic syndrome.

2.5.4 Obesity and related disorders behind the risk for cardiovascular disease

Obesity is often defined using BMI, skin fold measurement or waist circumstance. All of these measures represent increased amount of white adipose tissue caused by adipocyte hypertrophy or hyperplasia (reviewed by Henry et al. 2012).

Obesity is a global problem and its prevalence is constantly in increasing worldwide. In the United States, the prevalence of obesity, defined as BMI 30 or greater, was 35.5% among adult men and 35.8% among adult women in 2009–2010 (Flegal et al. 2012). In Finland, the prevalence of obesity (BMI 30 or more) was estimated to be 21% in 2007 with the mean BMI for men being 27.2kg/m² and for women 26.5kg/m² (Vartiainen et al. 2010).

There is a J-shaped relationship between BMI and morbidity (Manson et al. 1995, Whitlock et al. 2009). A very low BMI is associated with increased mortality, whereas obesity increases the risk for CVD (Willett et al. 1995), type 2 diabetes (Colditz et al. 1995) and cancers (Calle et al. 2003).

Previously, it was supposed that the only function of the adipose tissue was to store lipids but today it is known that adipose tissue is a large and very active endocrine organ secreting a large number of adipokines including leptin, adiponectin, tumor necrosis factor α (TNFα) and interleukin 6 (IL-6). Adiponectin, which is reduced in obesity has an anti-inflammatory role and acts protective against type 2 diabetes, NAFLD and atherosclerosis, whereas TNFα and IL-6 are inflammatory cytokines and their secretion is increased in obesity (reviewed by Henry et al. 2012).
Pathogenesis of obesity

Obesity is characterized by chronic low-grade inflammation of adipose tissue. As adipose tissue expands in obesity, the demand for oxygen may not be satisfied and the resulting hypoxia stimulates the expression of proinflammatory cytokines that activate immune cells. The inflamed adipose tissue is also filled with infiltrated macrophages that produce large amounts of proinflammatory cytokines further promoting the chronic inflammation. In obesity the adipocytes also produce increased amounts of proinflammatory leptin as the production of anti-inflammatory adiponectin is downregulated. This results in the activation of the cells of the immune system (reviewed by Suganami & Ogawa 2010).

In addition to the endocrine functions, adipocytes store free fatty acids and TGs from blood in lipid droplets in the form of TGs and under a negative energy balance, lipids are released using lipolysis. Lipid metabolism in adipose tissue is regulated by sympathetic nervous system and hormones. For example, catecholamines (epinephrine, norepinephrine and dopamine) induce lipolysis in a fasted state to provide free fatty acids for the energy requirements of other organs. Instead, in the fed state insulin suppresses lipolysis and promotes lipogenesis (reviewed by Suganami et al. 2012).

As a result of continuous excess nutrition, adipocytes increase in their size (hypertrophy) and number (hyperplasia). As the adipose tissue cannot meet the demand to store more energy, lipids start to accumulate in non-adipose tissue as ectopic fat. This accumulation of fat in the liver and skeletal muscle may lead to insulin resistance and in the pancreas ectopic fat may impair insulin secretion (reviewed by Suganami et al. 2012). Recent studies have also shown overproduction of extracellular matrix in obesity. The increased fibrosis limits the expansion of adipose tissue and causes impaired ability of adipose tissue to store lipids (Khan et al. 2009) which may contribute to the accumulation of ectopic fat.

Insulin resistance

Obesity is often, although not always, accompanied by insulin resistance. Both visceral adipose tissue and subcutaneous fat have been shown to associate with insulin resistance, visceral adipose tissue displaying a stronger correlation (Preis et al. 2010). It has also been proposed that if the capacity of the subcutaneous adipose tissue to store fat is great, the risk for developing insulin resistance is smaller, whereas the large quantity of abdominal fat predisposes to insulin
resistance (McLaughlin et al. 2011). Visceral adipose tissue produces inflammatory cytokines such as IL-6 that are transported directly to the liver via hepatic portal vein and may promote the development of insulin resistance (Rytka et al. 2011). On the other hand, abdominal adipose tissue releases large amounts of free fatty acids that may accumulate in the liver as ectopic fat resulting in insulin resistance (reviewed by Hardy et al. 2012).

In adipose tissue, insulin resistance is manifested as impaired glucose transportation to the adipocyte and reduced inhibition of lipolysis. In liver, insulin resistance leads into impaired suppression of gluconeogenesis and enhanced fatty acid synthesis (reviewed by Hardy et al. 2012).

Non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) is characterized by ectopic fat accumulation and low-grade chronic inflammation. It is defined as fat infiltration affecting over 5% of hepatocytes with a presence of less than 20 grams of alcohol consumption per day. It is a slowly progressive disease and varies from steatosis (fat accumulation in the liver) to steatohepatitis (steatosis and inflammation with variable grades of fibrosis) and eventually leading to cirrhosis that is accompanied by an increased risk of hepatocellular carcinoma (Bhatia et al. 2012b).

NAFLD is strongly associated with insulin resistance and obesity especially defined as increased waist circumference. The hepatic triglycerides that accumulate in fatty liver are derived from dietary sources, adipose tissue and from de novo lipogenesis. NAFLD is believed to promote the progression of atherosclerosis by resulting in pathophysiological processes such as abnormal glucose and lipid metabolism, increased oxidative stress, altered secretion of adipokines, overproduction of coagulation factors and endothelial dysfunction (Bhatia et al. 2012b, Kotronen & Yki-Jarvinen 2008). Indeed, NAFLD patients have in several studies been shown to have increased incidence of adverse cardiovascular outcomes and cardiovascular mortality has even been shown to represent the most common cause of premature death in NAFLD (Bhatia et al. 2012a, Bhatia et al. 2012b).
2.6 Fructose consumption and risk for atherosclerosis

Dietary exposure to fructose has recently increased and there is a concern that excessive fructose consumption in humans may be one of the factors behind the rising incidence of obesity and features of metabolic syndrome worldwide. Fructose is a highly lipogenic sugar that has been associated with several components of the metabolic syndrome such as insulin resistance, elevated waist circumference, dyslipidemia and hypertension (reviewed by Dekker et al. 2010).

Fructose promotes a weight gain similar to glucose but results in different body composition. As subjects consuming either glucose or fructose for 10 weeks were compared, only fructose consumption had led to a significant increase in visceral adipose tissue volume as glucose consumption results primarily in an increase in subcutaneous adipose tissue (Stanhope et al. 2009).

It has been suggested that NAFLD could be a hepatic manifestation of metabolic syndrome and its prevalence has been on the increase in parallel with the rise in fructose consumption. In studies in humans, fructose consumption has been shown to increase the amount of liver fat (Le et al. 2009, Maersk et al. 2012) and liver fibrosis in NAFLD patients (Abdelmalek et al. 2010).

A high-fructose diet has also been shown to induce leptin resistance (reviewed by Dekker et al. 2010). Acutely, fructose feeding decreases the leptin levels in humans that could lead into increased appetite and energy intake (reviewed by Stanhope 2012). However, a long-term fructose feeding induces hyperleptinemia in humans and in rats, insulin resistance, hepatic steatosis and increased TGs. The mechanism for fructose-induced leptin resistance is proposed to be the upregulation of SOCS3 that has been shown to result in increased expression of FOXO1 (reviewed by Dekker et al. 2010). Interestingly, FOXO1 is also known to be a regulator of key autophagy genes (Liu et al. 2009).
3 Aims of the present study

Increasing obesity and accompanying cardiovascular diseases are a tremendous problem of public health and economics. Obesity is often accompanied by failure of leptin to signal via its receptor and the leptin system might also directly contribute to the development of atherosclerosis.

The principal aims of the present study were:

1. to investigate the role of LEPR Lys109Arg and Gln223Arg polymorphisms in the development of early atherosclerosis by exploring the IMT and traditional risk factors for atherosclerosis between genotypes in a population-based cohort.
2. to assess if LEPR Lys109Arg and Gln223Arg polymorphisms have a predictive role in cardiovascular events and death.
3. to study the effect of IUGR and a postnatal fructose diet on the leptin system and autophagy gene expression and investigate if these changes could explain the metabolic changes encountered in IUGR and as a result of fructose consumption.
4 Subjects, animals and methods

The study designs and methods used in this study are described below in outline and presented in more detail in the original articles (I-III).

4.1 Study subjects

In studies I and II the population-based OPERA (Oulu Project Elucidating Risk of Atherosclerosis) population was used. OPERA is an epidemiological study designed to address the risk factors and disease end points of atherosclerotic cardiovascular diseases. The original reasons for recruiting this study population and the selection criteria have been previously described in detail (Rantala et al. 1999). In short, subjects were randomly selected, middle-aged drug-treated hypertensives and their age- and sex-matched control subjects recruited to the OPERA study between 1990 and 1993 in the area of Oulu, Finland. The participants were interviewed, examined, and tested in our research laboratory. Altogether 1,045 subjects were included in the study. The study was conducted according to the principles of the Declaration of Helsinki, and approved by the Ethical Committee of the Faculty of Medicine, University of Oulu. Informed consent was obtained from each participant. In study I, we examined the control cohort of the OPERA project consisting of 526 subjects. In study II, mortality and hospital events of altogether 1,045 subjects from OPERA were followed up until 2009.

4.2 Experimental animals

In study III, Sprague-Dawley rats were obtained from the Center of Experimental Animals at the University of Oulu, Finland, and the experimental design was approved by the Animal Care and Use Committee of the University of Oulu, Finland. Nine-week-old first-time pregnant rats were randomly assigned into two dietary treatment groups on day four of gestation. A group of control dams was fed ad libitum with standard laboratory chow throughout the pregnancy. A group of food-restricted dams received 50% of ad libitum food intake, which had been determined earlier (Hietaniemi et al. 2009). All dams were caged individually in 12h/12h light/dark cycles and had free access to water. After delivery, all dams and offspring received food ad libitum.
The day of parturition was defined as postnatal day 0. On postnatal day 1, the offspring were weighed and the litter size was adjusted to eight pups per litter. Half of the pups delivered by food-restricted dams (R) were randomly cross-fostered to control dams (C) while the rest of the pups were nursed by their own dams. Thus the following study groups were assigned: *ad libitum* fed dams with their own pups (CC), *ad libitum* fed dams with pups born from food-restricted dams (RC), and food-restricted dams with their own pups (RR).

From 1 month of age onwards, half of the litters of each study group received a fructose-rich diet (fru) (Harlan Teklad TD89247 60% fructose diet, energy density 3.6 kcal/g, 20.2% of calories from protein, 12.9% from fat and 66.8% from carbohydrate) while the other half continued receiving the standard diet (st) (Lactamin R36; energy content 3.1 kcal/g, 18.5% protein and 4.0% fat). The study design is shown in Figure 6. At the age of six months six standard diet fed litters from groups CC and RC and seven litters of RR as well as six fructose diet fed litters from each group were studied as described in the following. Two male offspring from each litter were fasted for 12h, anesthetized by isoflurane inhalation and killed by decapitation. Blood was collected by a cardiac puncture into heparinized vacutainers and centrifuged. Plasma was removed and stored at -70 °C for later use. The visceral adipose tissue and liver samples were collected, immediately flash-frozen in liquid nitrogen and stored at -70 °C.
4.3 Methods

4.3.1 Clinical methods

In studies I and II, the blood pressure measurements were recorded with an automatic blood pressure recorder. The resting blood pressure was measured three times at 1-min intervals on the right arm after the patient had been seated at least 5 min and after that two times at 1-min intervals with the patient standing. The mean value of the second and the third blood pressure measurement from the sitting patient was used in the analysis. Smoking history was obtained by a questionnaire. The lifetime smoking burden was calculated as pack-years (1 pack-year = 20 cigarettes smoked/day in 1 year). Height was measured to the nearest centimeter without shoes. Weight was measured to the nearest 0.1 kg on a lever balance with the subject wearing only light underwear without shoes. BMI was
calculated as weight (kg) divided by height squared (m$^2$). All of the measurements were performed by the same specially trained nurses.

### 4.3.2 Genotyping

Genotypes used in studies I and II, were determined by the restriction fragment length polymorphism (RFLP) method. Polymerase chain reaction (PCR) was performed and the PCR products of Lys109Arg were digested over night with HaeIII and of Gln223Arg with MspI restriction enzyme. The digested samples were then separated on an agarose gel. The following primers were used to amplify the regions of the LEPR. There was a mismatch (italic) in the Lys109Arg reverse primer to create an artificial restriction site. Lys109Arg F: 5’-CTGTTGAACCTAAGTTTAATTCAAG-3’; Lys109Arg R: 5’-GCCATTGTGTCAACAGTAATTTCAAG-3’ and Gln223Arg F, 5’-AGCTAGCAAATATTTTGTAAGCAAT-3’; Gln223Arg R, 5’-AAATACTGGATAGGCTTTTAAAGCAGGA-3’. The genotyping was successfully performed for 1,028 subjects in the OPERA study.

### 4.3.3 Carotid ultrasonography

The intima-media thickness (IMT) used in study I, was measured by one single trained radiologist without knowledge of the clinical data. IMT, defined as the distance between the media–adventitia interface and the lumen–intima interface, was measured using a duplex ultrasound system with a 7.5-MHz scanning frequency in B-mode, pulsed Doppler mode, and color mode. The far-wall IMT was measured on both sides at three different locations: One measurement from the internal carotid artery (ICA), one measurement from the bifurcation enlargement (BIF), and three measurements from the common carotid artery (CCA) (proximal, middle, and distal from the bifurcation). The mean IMT (IBC) was defined as the mean of ICA, BIF, and the three highest CCA measurements.

### 4.3.4 Outcome classification

In study II, the information on causes of death and events leading to hospitalization was obtained from the Finnish Causes-of-Death Register and the Hospital Discharge Register. The diagnoses were classified according to the International Classification of Diseases, Eight Revision (ICD-8) or Ninth
Revision (ICD-9) before 1994 and the Tenth Revision (ICD-10) thereafter. CHD was defined as I20, I21, I22 [ICD-10] and 410, 4110 [ICD-8/9], coronary artery bypass graft or coronary angioplasty or as I20-I25, I46, R96, R98 [ICD-10] and 410-414, 798 (not 7980A) [ICD-8/9] as causes of death. CVD was defined as CHD or stroke that included I61, I63 (not I636), I64 [ICD-10] and 431, 4330A, 4331A, 4339A, 4340A, 4341A, 4349A, 436 [ICD-9] or 431 (excluding 43101, 43191), 433, 434, 436 [ICD-8] according to FINRISK criteria (Pajunen et al. 2011).

4.3.5 Microscopy

In the study III, the size of adipocytes was microscopically determined from rat visceral adipose tissue samples. The samples were prepared as paraffin blocks and stained with hematoxylin and eosin. The slides were photographed at 20x magnification using Leitz Aristoplasn microscope connected to MicroPublisher 5.0 RTV digital camera and computer. We used MCID-Image analysis software to objectively analyze the adipocyte sizes. From every individual there were two slides of which two fields were photographed. From every field, the five largest adipocytes were selected and analyzed with MCID Core software (version 7.0). The maximal adipocytes were selected to minimize the variation due to different cut levels of the cells: by selection of maximal diameter we wanted to avoid the cells which were cut at the edges. The mean of the twenty adipocyte maximal area per rat was calculated.

The liver samples of rats in study III were prepared as paraffin blocks and stained with hematoxylin and eosin. The slides were studied at 63x magnification using Leica DM3000 microscope connected to digital camera Leica DEC420 and computer and photographed with LAS V4.1.0 software. From every rat there was 1 slide of which 20 fields were examined. The matrix of 100 points was used for every field and for each point it was defined if it was in cytoplasm or lipid vacuole. The ratio was calculated by dividing the vacuole points with the sum of cytoplasmic and vacuole points. All the microscopic analyses were performed double-blinded as the person preparing the slides had no information about the study design, and at this point the slides were given new identifier numbers for microscopy: the person performing microscopy was thus uninformed about the study groups (III).
4.3.6 Blood sample assays

The lipids in studies I and II were measured as follows. The very low-density lipoprotein (VLDL) fraction was separated from plasma by ultracentrifugation. The plasma HDL concentration was measured by mixing 0.5ml of the VLDL-free fraction with 25 ml of 2.8% (wt/vol) heparin and 25 ml of 2M manganese chloride and measuring the cholesterol concentration in the supernatant after centrifugation at 1,000×g and 4 °C for 30 min. The LDL concentration was calculated by subtracting the cholesterol concentration in HDL from that in the VLDL-free fraction.

In study I, the concentrations of total cholesterol and triglycerides in the plasma were determined by enzymatic colorimetric methods. C-reactive protein was analyzed by using commercially available enzyme-linked immunosorbent assay (ELISA) kits. The glucose concentrations were measured with the glucose dehydrogenase method.

Fasting plasma leptin concentrations in studies I and II were measured using a commercial double antibody radioimmunoassay (RIA) (Human Leptin RIA Kit; Linco Research, Inc., St. Charles, MO).

In study III, leptin was measured from rat plasma samples with a commercial Rat Leptin ELISA Kit (Millipore).

4.3.7 RNA extraction and qRT-PCR

The total RNA in study III was extracted from the tissues collected from the pups at the age of one month and six months. The tissues were homogenized with TissueLyser LT (Qiagen) according to the custom protocol defined in the manufacturer’s instructions. The RNA was extracted from adipose tissue with RNeasy Lipid Tissue Mini Kit (Qiagen) and from liver with RNeasy Mini Kit (Qiagen). The RNA concentration and purity were determined spectrophotometrically at wavelengths 260 and 280 nm. The mRNA was then converted to cDNA with RevertAid First Strand cDNA Synthesis Kit (Fermentas). Prior to the synthesis, the genomic DNA was removed from the RNA preparation with DNase treatment (Fermentas).

The gene expression was studied by using quantitative real-time PCR (qRT-PCR) (III). The measurements were done with SYBR Green chemistry on an iQ5 real-time PCR detection system (Bio-Rad). The primers for the genes and isoforms and the annealing temperatures in study III are presented in Table 1.
Table 1. Primers and the annealing temperatures used for studying the gene expression in rat

<table>
<thead>
<tr>
<th>Gene/isoform</th>
<th>Primer 5’-3’</th>
<th>T_m (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lep</td>
<td>f-GAGACCTCCTCCATCCTGCTG</td>
<td>58.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>r-CTCAGAGCCACCACCTGCTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LEPR</td>
<td>f-GAGAGGCTGCTGAAATCTGCT</td>
<td>58.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>r-CTCCAGACTCCTGAGCCATC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LEPR-a</td>
<td>f-CACCTGTAATTTCACACCAGAG</td>
<td>58.9</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>r-GTCATTCAAACCATTGTTAGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LEPR-b</td>
<td>f-GCATGCAGAATCGATGATTTGG</td>
<td>61.4</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>r-CAGGCTGATCGACACTGATTTTCTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LEPR-c</td>
<td>f-TGCTCGGAACACTGTTAAT</td>
<td>58.9</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>r-ATAGAGCTATCTAACCCTGACCTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LEPR-d</td>
<td>f-TGCTGGACACTGTCACCTAA</td>
<td>58.9</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>r-ATCAGGATTTGCAATTTACA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LEPR-f</td>
<td>f-GCTGCTCGGAACACTGTTAAT</td>
<td>58.9</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>r-ACGGCATCCACCTCTATATCCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOCS3</td>
<td>f-GCCAGTGCCCCGCTTTGACT</td>
<td>64.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>r-GAGGAAGGGTTCGCTGCGGGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACC</td>
<td>f-GCCTCTTCCTGACAAACGAG</td>
<td>61.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>r-TCCATACGCCTGAAAACATGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATG7</td>
<td>f-GTGGCTCCTCTGCTCAAAAC</td>
<td>63.3</td>
<td></td>
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<tr>
<td></td>
<td>r-CAGGAGTGGCTGGATTAGGTTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAP1LC3β</td>
<td>f-CGTCTGGACAGAACCAACTG</td>
<td>61.4</td>
<td>b</td>
</tr>
<tr>
<td></td>
<td>r-AGTGCTCTCCGAGAGGACTC</td>
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<tr>
<td>LAMP2</td>
<td>f-AAGACTTTATGGGCGATTCAC</td>
<td>61.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>r-GGTTGGAATATGGGGCTGTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>f-ATGACTCTACCCAGGCGAAG</td>
<td>58.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>r-GGAAAGATGGATGGGTCCTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPRT</td>
<td>f-TCCCAAGCTGCTGATTAGGTA</td>
<td>58.9</td>
<td>c</td>
</tr>
<tr>
<td></td>
<td>r-CTCTGATGACATCCTGAGAAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UBC</td>
<td>f-ATCTAGAAAGACCCCTTCTTGTCG</td>
<td>58.9</td>
<td>c</td>
</tr>
<tr>
<td></td>
<td>r-ACACCTCCCCATCAAACCC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a (da Silveira Cavalcante et al. 2009), b (Sengupta et al. 2009) and c (Martinez-Beamonte et al. 2011). Primers with no reference are self-designed.

The expression was normalized with the gene expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18S ribosomal ribonucleic acid (18S rRNA) in WAT or hypoxanthine guanine phosphoribosyl transferase (HPRT) and ubiquitin C (UBC) in liver measured from the same samples. To minimize the possible effect of study design on the reference gene expression, the reference
genes were selected from a few candidates with NormFinder software (Andersen et al. 2004).

4.3.8 Protein extraction and western blot

In study III, the samples from WAT were homogenized by TissueLyser (Qiagen) for 2min in 35MHz in lysis buffer (20mM Tris, 100mM NaCl, 0.4% SDS 0.2% Triton-X) with protease inhibitor and centrifuged at 13,200 rpm at 4 °C for 10min. The liver samples were treated as adipose tissue samples except that no SDS was included. Supernatants were quantified for protein with DC Protein Assay (Bio-Rad), measured with 650nm and frozen in -70 °C for future use.

The samples were treated with β-mercaptoethanol and boiled in 95 °C for 10min. 50mg of total protein from liver or 10mg of WAT was loaded onto 15% gels for studying LC3. For studying p62, 30mg of total protein of WAT was loaded onto 12% gels. Samples were separated by SDS-PAGE first with 80V 10min and then 100V, transferred to PVDF membrane 30V for 90min and blocked with 10% non-fat milk for 1h. Membranes were incubated with primary antibody anti-LC3 (LC3-5F10; Nanotools Antikoerpertechnik) dilution of 1:200, anti-p62 (ab56416; Abcam) with a dilution of 1:667 or anti-β-actin (A5441, Sigma Aldrich) with a dilution of 1:3000 overnight at 4 °C and then with secondary antibody anti-mouse (NA931V; GE Healthcare Life Sciences) dilution 1:1500 or 1:3000 for 1h at room temperature. The secondary antibody was detected by ECL method. Fluorescent densities were determined using Quantity One software (Bio-Rad). The intensity of LC3-II and LC3-II/LC3-I ratio was calculated. Positive control cell lysate (0911/Neuro2A/Co; Nanotools Antikoerpertechnik) was used for normalizing the differences between the LC3 blots and a control sample from a rat of the CCst group as a control for p62 blots.

4.3.9 Statistical methods

The data were analyzed with SPSS version 16.0 (I) and 19.0 (II, III). Results with a p-value less than 0.05 were considered statistically significant.

In study I, the allele and haplotype frequencies and linkage disequilibrium were calculated with CubeX (Gaunt et al. 2007). The chi-squared test was carried out to determine if the observed frequencies were in Hardy–Weinberg equilibrium. Analysis of covariance (ANCOVA) was used to compare the means of the variables measured. In the multivariate analysis, sex, age, BMI, smoked pack-years, LDL concentration, and SBP were added as explanatory factors.
normalize the skewed distributions, log-transformed values of IMT measurements, BMI, SBP, leptin, total cholesterol, triglyceride, LDL, HDL, and CRP concentrations were used. The Bonferroni test of SPSS was used to distinguish the significance of differences in the traits between each genotype (I).

In study II, the baseline data and the number of events and deaths during the follow-up were tested with Pearson Chi-Square or analysis of variance (ANOVA) when appropriate. To estimate the impact of Lys109Arg on the events and death, logistic regression was performed and time-to-death analyses were performed with Cox regression. In the genetic analyses, the subjects with diagnosed myocardial infarction (n=20) and with a disorder of the cerebral circulation diagnosed in the hospital (n=22) already at baseline were included in the events.

In study III, three-way ANOVA was performed to study the effect of fetal undernourishment (IUGR or normal conditions during pregnancy) and postnatal diet (fructose-rich or standard chow from one month to six month of age). Also suckling period (lactating dam food-restricted during pregnancy or not) was included in the analysis to cover all the different treatments between the study groups. To normalize the skewed distributions logarithmic transformation was applied. A t-test was performed when appropriate. Correlation was tested as Pearson’s correlation. The protein levels were normalized against total protein and the differences between the blots were normalized using the control sample. The relationship between TG levels and gene expression was further studied by linear regression modelling using both one-by-one approach and stepwise modelling of SPSS.
5 Results

The main results of each study are presented below. The additional findings of each study have been described in more detail in the original papers (I-III).

5.1 The association of leptin receptor Lys109Arg and Gln223Arg polymorphisms with early atherosclerosis (I)

The aim of study I was to investigate the role of SNPs Lys109Arg and Gln223Arg as risk indicators for atherosclerosis and the association of polymorphisms with early atherosclerosis measured as intima media thickness (IMT). The study population was the OPERA control cohort consisting of 526 subjects.

The allele frequency of Arg109 was 35.8% and that of Gln223 39.5%, and the genotype frequencies were in Hardy-Weinberg distribution. However, these polymorphisms were found to be in linkage disequilibrium with standardized linkage disequilibrium (D') of 1.0 and r^2 0.3647.

The SNPs studied had associations with traditional risk factors. Lys109Arg was statistically significantly associated with BMI and total cholesterol. Interestingly, Lys109Arg heterozygotes displayed the lowest BMI whereas Arg109 homozygotes exhibited the highest total cholesterol. SBP associated with and was the highest among Gln223Arg heterozygotes. There were no differences in the leptin concentrations between Lys109Arg and Gln223Arg genotypes.

In study I, the degree of atherosclerosis was defined by IMT measurements and it was shown that both SNPs were associated with IMT. Lys109Arg was significantly associated with BIF (p=0.005), ICA (p=0.034) and IBC (the mean of ICA, BIF, and the three highest CCA measurements) (p=0.012) when adjusted only with sex. After additional adjustments for sex, age, BMI, smoked pack-years, LDL, SBP and fasting blood glucose, the association between Lys109Arg and BIF (p=0.018) and IBC (p=0.042) remained significant. When further adjustment of lipid and blood pressure-lowering medication was included in the analysis, association of Lys109Arg with BIF (p=0.017) and IBC (p=0.038) still remained with Arg109Arg homozygotes displaying the lowest IMT.

Gln223Arg was also associated with all the IMT measurements, CCA (p=0.005), BIF (p=0.050), ICA (p=0.001) and IBC (p=0.004) when adjusted with sex. The IMT measurements were significantly lower among Arg223Arg homozygotes. After adjusting for sex, age, BMI, smoked pack-years, LDL, SBP and fasting blood glucose, differences in ICA (p=0.006) and IBC (p=0.041)
between Gln223Arg genotypes remained significant. Even when further adjusting with lipid and blood pressure-lowering medication, association of Gln223Arg with ICA (p=0.006) and IBC (p=0.039) still remained significant. The mean IMT measurements in relation to both SNPs are shown in Figure 7.

Fig. 7. Mean intima media thickness (IMT) values in relation to Lys109Arg (A) and Gln223Arg (B) in 526 subjects. The IMT is presented as logarithmic values. The whiskers range from 5% to 95%.

5.2 The impact of leptin receptor Lys109Arg on the total mortality and cardiovascular disease incidence and death (II)

In study II, the OPERA cohort including both controls and hypertensives was used (n=1045) to investigate the role of Lys109Arg and Gln223Arg in predicting cardiovascular events and death. The allele frequency of Arg109 was 35.5% and of Arg223 59.3% in our whole OPERA study population, as they were 35.8% for Arg109 and 60.5% for Arg223 in control cohort of OPERA in study I. The alleles followed the Hardy-Weinberg distribution but showed linkage disequilibrium with standardized linkage disequilibrium (D’) 1.0 and r2 0.3757.

At the baseline, a total of 20 subjects were already diagnosed with myocardial infarction (MI) and 22 subjects with stroke. The baseline events as well as subjects with history of diabetes were evenly distributed between the genotypes. The Arg109 homozygotes displayed the lowest BMI at the baseline, but the other traditional risk factors were similar across the genotypes. Leptin levels were not affected by Lys109Arg genotype, but varied between Gln223Arg genotype with Gln223 homozygotes displaying the highest leptin levels. The baseline data are presented in Table 2.
Table 2a. Measurements of the risk factors for atherosclerosis at the baseline in relation to LEPR Lys109Arg genotype.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Lys109Lys</th>
<th>Lys109Arg</th>
<th>Arg109Arg</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=427</td>
<td>n=473</td>
<td>n=128</td>
<td></td>
</tr>
<tr>
<td>Males/females (n)</td>
<td>195/232</td>
<td>245/228</td>
<td>65/63</td>
<td>0.171†</td>
</tr>
<tr>
<td>Hypertensive/Control (n)</td>
<td>214/213</td>
<td>233/240</td>
<td>62/66</td>
<td>0.935†</td>
</tr>
<tr>
<td>Age (years)</td>
<td>52 (6)</td>
<td>51 (6)</td>
<td>50 (7)</td>
<td>0.122‡</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>28.1 (4.7)</td>
<td>27.5 (4.7)</td>
<td>27.0 (4.2)</td>
<td>0.033‡</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>149 (23)</td>
<td>149 (22)</td>
<td>144 (21)</td>
<td>0.236‡</td>
</tr>
<tr>
<td>LDL-C (mmol/L)</td>
<td>3.5 (1.0)</td>
<td>3.5 (0.9)</td>
<td>3.6 (0.9)</td>
<td>0.118‡</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>11.4 (8.5)</td>
<td>10.1 (8.1)</td>
<td>10.3 (7.6)</td>
<td>0.349‡</td>
</tr>
<tr>
<td>Smoking (pack-year)</td>
<td>12.5 (14.5)</td>
<td>12.0 (13.9)</td>
<td>10.5 (13.1)</td>
<td>0.406‡</td>
</tr>
<tr>
<td>MI at baseline (n)</td>
<td>8</td>
<td>11</td>
<td>1</td>
<td>0.527†</td>
</tr>
<tr>
<td>Stroke at baseline (n)</td>
<td>9</td>
<td>11</td>
<td>2</td>
<td>0.868†</td>
</tr>
</tbody>
</table>

Values are means and SD. P-values are achieved with Pearson Chi-Square† or ANCOVA‡ adjusted for sex, age and BMI when appropriate. BMI (body mass index), SBP (systolic blood pressure), LDL-C (low density lipoprotein cholesterol), MI (myocardial infarction), ** p<0.05

Table 2b. Measurements of the risk factors for atherosclerosis at the baseline in relation to LEPR Gln223Arg genotype.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Gln223Gln</th>
<th>Gln223Arg</th>
<th>Arg223Arg</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=167</td>
<td>n=507</td>
<td>n=360</td>
<td></td>
</tr>
<tr>
<td>Males/females (n)</td>
<td>84/83</td>
<td>239/268</td>
<td>187/173</td>
<td>0.364†</td>
</tr>
<tr>
<td>Hypertensive/Control (n)</td>
<td>86/81</td>
<td>254/253</td>
<td>171/189</td>
<td>0.634†</td>
</tr>
<tr>
<td>Age (years)</td>
<td>51 (6)</td>
<td>52 (6)</td>
<td>51 (6)</td>
<td>0.369‡</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>28.1 (4.8)</td>
<td>27.9 (4.7)</td>
<td>27.3 (4.5)</td>
<td>0.077‡</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>151 (23)</td>
<td>149 (22)</td>
<td>146 (21)</td>
<td>0.056‡</td>
</tr>
<tr>
<td>LDL-C (mmol/L)</td>
<td>3.5 (0.9)</td>
<td>3.5 (0.9)</td>
<td>3.5 (1.0)</td>
<td>0.849‡</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>11.4 (9.4)</td>
<td>10.8 (6.0)</td>
<td>10.2 (7.9)</td>
<td>0.039‡</td>
</tr>
<tr>
<td>Smoking (pack-year)</td>
<td>11.7 (14.4)</td>
<td>12.7 (14.4)</td>
<td>11.2 (13.4)</td>
<td>0.077‡</td>
</tr>
<tr>
<td>MI at baseline (n)</td>
<td>3</td>
<td>12</td>
<td>5</td>
<td>0.582†</td>
</tr>
<tr>
<td>Stroke at baseline (n)</td>
<td>2</td>
<td>14</td>
<td>6</td>
<td>0.361†</td>
</tr>
</tbody>
</table>

Values are means and SD. P-values are achieved with Pearson Chi-Square† or ANCOVA‡ adjusted for sex, age and BMI when appropriate. BMI (body mass index), SBP (systolic blood pressure), LDL-C (low density lipoprotein cholesterol), MI (myocardial infarction), ** p<0.05

Out of a total 1,028 subjects genotyped for Lys109Arg, 151 coronary heart disease (CHD) and 211 cardiovascular disease (CVD) events or deaths including 58 CHD and 69 CVD deaths were observed during the follow-up, and 165 subjects died. The total mortality was significantly lower among the Arg109 homozygotes (7.8%) compared to Lys109Lys (15.7%) and Lys109Arg (18.6%)
Among 1,045 subjects genotyped for Gln223Arg 152 CHD and 212 CVD events or deaths including 58 CHD and 69 CVD deaths were observed and during the follow-up 166 subjects died. The number of events and deaths did not differ between the Gln223Arg genotypes.

As decreased mortality among Arg109Arg carriers was observed we performed the logistic regression analyses of the cardiovascular event and death by comparing Lys109Lys and Lys109Arg against Arg109Arg genotype. As the baseline events of MI and disorder of the cerebral circulation were similar between the genotypes, the subjects with baseline diagnosis were included in this analysis as well. The logistic regression analysis revealed a significant association between Arg109Arg genotype and lower CHD (p=0.017) and CVD event or death (p=0.030) incidence as well as decreased total mortality (p=0.007) when adjusted with age, sex and study group. After further adjustment with BMI, smoking status, systolic blood pressure and low-density lipoprotein cholesterol, the association of Arg109Arg to decreased incidence of CHD event or death (p=0.031) and total mortality (p=0.024) still remained significant.

The mortality and the major causes of death were also analyzed with Cox regression, which takes the follow-up time into account. When the model was adjusted for sex, age and study group, Arg109Arg genotype protected from total mortality (p=0.007) and CHD death (p=0.038). These results are presented in Table 3 and Kaplan-Meier curves in Figure 8.

**Table 3. The Cox regression analysis of the total mortality and major causes of death in relation to Lys109Arg.**

<table>
<thead>
<tr>
<th>Cause of death</th>
<th>Lys109Lys+Lys109Arg</th>
<th>Arg109Arg</th>
<th>HR (CI 95%)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>All deaths</td>
<td>155 (17.2%)</td>
<td>10 (7.8%)</td>
<td>0.413 (0.218-0.783)</td>
<td>0.007 **</td>
</tr>
<tr>
<td>CHD</td>
<td>56 (6.2%)</td>
<td>2 (1.6%)</td>
<td>0.224 (0.055-0.919)</td>
<td>0.038 **</td>
</tr>
<tr>
<td>CVD</td>
<td>64 (7.1%)</td>
<td>5 (3.9%)</td>
<td>0.493 (0.198-1.225)</td>
<td>0.128</td>
</tr>
<tr>
<td>Cancer</td>
<td>40 (4.4%)</td>
<td>4 (3.1%)</td>
<td>0.639 (0.228-1.790)</td>
<td>0.394</td>
</tr>
</tbody>
</table>

The model is adjusted with sex, age and study group. ** p<0.05
As the total number of deaths in the Arg109Arg group was 10 and there were only two CHD deaths, we decided that the number of CHD deaths was insufficient for further modelling, but that it might still be possible to perform a stepwise modelling for total mortality (Table 4). When sex, age, study group, BMI, SBP and smoking were included in the model, the association of Arg109Arg on lower total mortality remained, suggesting that the polymorphism does indeed have an independent effect on all-cause mortality.
Table 4. The Cox regression modelling of factors predicting total mortality.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Crude</th>
<th>Model 1</th>
<th>Model 2</th>
<th>Model 3</th>
<th>Model 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys109Arg</td>
<td>0.422</td>
<td>0.423</td>
<td>0.428</td>
<td>0.448</td>
<td>0.469</td>
</tr>
<tr>
<td></td>
<td>(0.222-0.799)**</td>
<td>(0.223-0.803)**</td>
<td>(0.226-0.813)*</td>
<td>(0.236-0.850)*</td>
<td>(0.246-0.891)*</td>
</tr>
<tr>
<td>Sex</td>
<td>2.192</td>
<td>2.418</td>
<td>2.330</td>
<td>1.750</td>
<td>1.678</td>
</tr>
<tr>
<td>Age</td>
<td>1.074</td>
<td>1.080</td>
<td>1.077</td>
<td>1.072</td>
<td>1.067</td>
</tr>
<tr>
<td></td>
<td>(1.046-1.103)**</td>
<td>(1.050-1.111)**</td>
<td>(1.046-1.108)**</td>
<td>(1.041-1.103)**</td>
<td>(1.037-1.109)**</td>
</tr>
<tr>
<td>Study group</td>
<td>1.463</td>
<td>1.387</td>
<td>1.307</td>
<td>1.594</td>
<td>1.411</td>
</tr>
<tr>
<td></td>
<td>(1.074-1.992)**</td>
<td>(1.002-1.918)*</td>
<td>(0.937-1.823)</td>
<td>(1.169-2.175)**</td>
<td>(1.011-1.970)*</td>
</tr>
<tr>
<td>BMI</td>
<td>1.045</td>
<td>1.027</td>
<td>1.019</td>
<td>1.012</td>
<td>1.012</td>
</tr>
<tr>
<td></td>
<td>(1.014-1.076)**</td>
<td>(0.992-1.062)</td>
<td>(0.984-1.056)</td>
<td>(0.978-1.048)</td>
<td>(0.978-1.048)</td>
</tr>
<tr>
<td>SBP</td>
<td>1.015</td>
<td>1.006</td>
<td>1.007</td>
<td>1.007</td>
<td>1.007</td>
</tr>
<tr>
<td></td>
<td>(1.008-1.022)**</td>
<td>(0.998-1.014)</td>
<td>(0.999-1.014)</td>
<td>(0.999-1.014)</td>
<td>(0.999-1.014)</td>
</tr>
<tr>
<td>Smoking</td>
<td>1.046</td>
<td>1.037</td>
<td>1.037</td>
<td>1.037</td>
<td>1.037</td>
</tr>
<tr>
<td></td>
<td>(1.036-1.057)**</td>
<td>(1.026-1.048)**</td>
<td>(1.026-1.048)**</td>
<td>(1.026-1.048)**</td>
<td>(1.026-1.048)**</td>
</tr>
</tbody>
</table>

Values are HR (CI 95%). * p<0.05, **p<0.01. BMI (body mass index), SBP (systolic blood pressure)

5.3 The effects of long-term fructose diet and intrauterine growth restriction on leptin system (III)

Study III was performed to investigate the effect of fructose consumption and IUGR on adipose tissue and liver leptin system. Also the role of autophagy was studied.

5.3.1 Microscopic analyses of adipose tissue and liver

The size of adipocytes was microscopically studied from visceral adipose tissue samples. The size of adipocytes correlated significantly with the weight at the age of six months (r=0.622, p=0.000), but there were no statistical differences between the groups in maximal adipocyte size by IUGR or fructose diet although a trend towards smaller adipocytes in both RCst and RCfru groups was seen.

The liver lipid accumulation was also microscopically determined. The ratio of vacuole points to vacuole and cytoplasmic points varied between standard and fructose diet fed rats (p=0.025) with fructose fed rats displaying more lipid vacuoles in their livers. IUGR, instead, had no impact on liver fat accumulation.
5.3.2 LEPR isoforms in adipose tissue and liver of standard diet fed rats

The expression of the LEPR isoforms a, b and f was found to be on a measurable level in WAT and LEPR-b and -f in liver. Interestingly, LEPR-f was the most abundant in both WAT and liver in the pooled sample of standard diet fed rats.

5.3.3 Effects of long-term fructose diet

Fructose did not seem to affect the plasma leptin levels significantly although Lep mRNA expression was clearly down-regulated by fructose consumption (p=0.000) (Figure 9).

General LEPR expression was significantly reduced in the WAT of fructose fed animals (p=0.000) as shown in Figure 10A. Especially, LEPR-b (p=0.011) and -f (p=0.005) mRNA levels were declined in WAT as a result of fructose consumption together with strongly decreased SOCS3 expression (p=0.000).

The WAT expression of ACC mRNA was also decreased (p=0.006) in fructose diet fed rats and autophagy genes, ATG7 (p<0.001) and LAMP2 (p=0.004), were down-regulated in WAT, as shown in Figures 11A and 11C.
Fig. 10. LEPR mRNA expression in A) WAT and B) liver. The primers used here amplify all the isoforms of LEPR. The whiskers indicate the range from minimum to maximum value. The expression is relative to GAPDH and 18S rRNA expression. LEPR expression was down-regulated in WAT (p<0.001) and up-regulated in liver (p<0.001) in fructose fed rats. IUGR resulted in up-regulation of LEPR in WAT (p<0.001). The abbreviations are explained in Figure 6, n=12-14/group. (Figure from study III).
Fig. 11. Expression of autophagy genes mRNA. A) *ATG7* expression in WAT was
downregulated by the fructose diet (p<0.001) and upregulated by IUGR (p=0.020). B) *ATG7*
expression in liver was increased by the fructose diet (p=0.024) and decreased
by IUGR (p=0.007). C) *LAMP2* expression in WAT was slightly downregulated in
fructose fed rats (p=0.004) and also by IUGR (p=0.031). D) *LAMP2* was upregulated in
liver by the fructose diet (p<0.001). In WAT, the expression is relative to *GAPDH* and
18S rRNA expression and in liver relative to *HPRT* and *UBC* expression. The whiskers
indicate the range from minimum to maximum values. The abbreviations are explained
in Figure 6, n=12-14/group. (Figure from study III).

The expression of *MAP1LC3β* mRNA was likewise decreased (p<0.001) in WAT.
In the western blot, only LC3-II protein band was detected in WAT and no
statistically significant differences were found in the levels of LC3-II (p=0.065).
However, also p62 protein was detected in WAT, and fructose diet fed rats
displayed higher p62 levels than the standard diet fed rats (three-way ANOVA;
p<0.001). The results of p62 are presented in Figure 12.
Fig. 12. p62 western blot results in WAT. A) p62 band was seen in WAT and B) the protein level of p62 was higher in fructose fed animals, suggesting reduced amount of autophagy (p<0.001). The figure also shows β-actin for the blot but as it seems that β-actin as well is affected by fructose diet, the results are analyzed relative to total protein. The whiskers indicate the range from minimum to maximum values. The abbreviations for groups are explained in Fig.6, n=6/group. Plus sign (+) indicates the positive control sample from the CCst group. (Figure from study III).

In the liver, fructose consumption induced the general LEPR expression (p<0.001), as shown in Figure 10B, which was seen also in the increase in LEPR-b (p=0.001) and -f (p<0.001) expression. Fructose also increased liver ACC (p=0.010), ATG7 (p=0.024), MAP1LC3β (p=0.003) and LAMP2 (p<0.001) mRNA expression (Figure 11B and 11D). Both LC3-I and LC3-II bands were visible in the western blot of liver but no differences caused by fructose consumption were detected in the protein levels of LC3-II (p=0.597) or in the ratio of LC3-II/LC3-I (p=0.305).

5.3.4 Effects of intrauterine growth restriction

The effect of fetal undernourishment was evident in the pups of the food-restricted dams. Pups of the food-restricted dams were significantly smaller at birth (p=0.000) compared to the pups of the ad libitum fed dams indicating IUGR.
The litter size was not affected by the food restriction. In addition, at the end of the pregnancy, the food-restricted dams had gained significantly less weight (mean 48.7g; SD 20.2g) than control dams (mean 157.3g; SD 25.1g) (p=0.000) and were also leaner after delivery (mean 240.0g; SD 26.6g) than control dams (mean 299.3g; SD 25.3g) (p=0.002).

Plasma leptin levels were affected by IUGR (p=0.005). This was seen in standard diet fed rats as IUGR induced decrease in leptin. Lep mRNA expression in WAT was also decreased by IUGR (p=0.020). The results of plasma leptin and adipose tissue mRNA levels are shown in Figure 9.

LEPR-a and -f mRNA levels were both increased by IUGR in WAT (p=0.015 and p=0.021, respectively), which was also seen by measuring general WAT LEPR expression that shows a significant up-regulation (p=0.000) especially in standard diet fed rats (Figure 10A). However, the gene expression of SOCS3 in WAT remained unchanged by IUGR. Autophagy gene ATG7 showed some increase in mRNA (p=0.020) contrary to LAMP2, which was somewhat decreased (p=0.031) in IUGR rats WAT (Figure 11A and 11C).

The general LEPR mRNA expression was unchanged in the liver by IUGR (Figure 10B), although the expression of LEPR-b isoform was enhanced in the livers of IUGR rats (p=0.000). However, no change in SOCS3 expression was detected. In addition, ATG7 expression was decreased by IUGR in the liver (p=0.007) (Figure 11B).

5.3.5 The connection between plasma triglycerides, leptin receptors and autophagy

In adipose tissue, LEPR expression correlated with mRNA expression of SOCS3 (r=0.790, p<0.001), ATG7 (r=0.765, p<0.001) and MAP1LC3β (r=0.599, p<0.001). Also in the liver, LEPR expression correlated with ATG7 (r=0.505, p<0.001), LAMP2 (r=0.515, p<0.001), MAP1LC3β (r=0.501, p<0.001) and ACC (r=0.462, p<0.001) expression.

Plasma TG levels correlated with both ATG7 and LEPR expression of WAT. Plasma TG displayed a negative correlation especially with general LEPR expression (r=-0.557, p<0.001), SOCS3 (r=-0.612, p<0.001), ATG7 (r=-0.564, p<0.001) and MAP1LC3β (r=-0.617, p<0.001) in WAT and a less strong correlation with the leptin mRNA (r=-0.380, p=0.001), LEPR-b (r=-0.289, p=0.013) and -f (r=-0.317, p=0.006).
The plasma TG concentrations were also positively correlated with liver gene expression. A strong correlation was seen between TGs and general LEPR expression ($r=0.706$, $p<0.001$), LEPR-f ($r=0.591$, $p<0.001$), LAMP2 ($r=0.591$, $p<0.001$) and MAP1LC3β ($r=0.468$, $p<0.001$) in the liver, and a weaker correlation between TGs and LEPR-b ($r=0.286$, $p=0.013$), ATG7 ($r=0.353$, $p=0.002$) and ACC ($r=0.282$, $p=0.017$).

The stepwise modelling with linear regression showed that general LEPR expression in the liver and MAP1LC3β expression in WAT are the most important determinants of plasma TGs in these male rats. The modeling shows that the liver LEPR expression alone explained 49.1% of the variation in the plasma TG levels ($B=0.863$, SE=0.102, $R^2_{adj}=0.491$, $p<0.001$). If MAP1LC3β expression in WAT was also included, then the model was even better, explaining 57.5% of the variation ($B_{LEPR\text{liver}}=0.646$, SE_{LEPR\text{liver}}=0.109 and $B_{MAP1LC3\text{ WAT}}=-0.222$, SE_{MAP1LC3 WAT}=0.057; $R^2_{adj}=0.575$, $p<0.001$). Weight, plasma leptin, ATG7, LAMP2 and ACC mRNA expression as well as LC3 protein in WAT and liver were also considered but did not explain the TGs. The correlations between TGs and the variables in the model are presented in Figure 13.

![Fig. 13. The relationship between LEPR liver expression, MAP1LC3β WAT expression and the plasma triglyceride level. LEPR mRNA expression in the liver explains 49.1% of the plasma TG levels ($p<0.001$). LEPR mRNA expression in the liver and MAP1LC3β mRNA expression in the WAT together explain 57.5% of the TGs ($p<0.001$). The black squares represent standard diet fed rats (CCst, RCst and RRst) and the grey ones are fructose fed rats (CCfru, RCfru and RRfru). (Figure from study III).](image)
6 Discussion

6.1 LEPR Lys109Arg and Gln223Arg polymorphisms and early atherosclerosis (I)

6.1.1 Study population

For the hypertensive group of OPERA the selection criterion was the reimbursement for antihypertensive medication and the control group used in study I consists of their age- and gender-matched subjects. The control group cannot be considered normotensive, instead, is comparable to the normal population in Finland. Therefore, the control group is appropriate for this kind of study.

6.1.2 IMT as a measure of atherosclerosis

Thickening of carotid IMT is considered to be a preclinical marker of atherosclerosis and it is a strong predictor of cardiovascular disease (Polak et al. 2013). In addition, IMT measurement has been shown to reliably indicate the general atherosclerosis in autopsy analysis (Iwakiri et al. 2012). IMT measured by ultrasonography is also a non-invasive method, requires no exposure to the radiation and is hence convenient and safe to use.

6.1.3 LEPR polymorphisms

In the OPERA study the SNPs (rs1137100 and rs1137101) in the LEPR that lead into Lys109Arg and Gln223Arg variations on a protein level were found to be in linkage disequilibrium. No subjects with genotype Arg109Arg/Gln223Gln were detected although it would have been expected based on the allele frequencies of these SNPs and it remains to be studied if there is some selection pressure involved.

The allele frequencies of Arg109 and Arg223 were 35.5% and 59.3%, respectively, in the whole OPERA study population (n=1,028, study II), and 35.8% and 60.5% in the control cohort of OPERA (n=526, study I). These are very well in line with the LEPR allele frequencies in the Finnish Diabetes Prevention Study ($f_{\text{Arg109}}=36.3\%$ and $f_{\text{Arg223}}=57.4\%$, n=507) (Salopuro et al. 2005)
suggesting that the genotyping is reliable. The frequencies are also parallel with the HERITAGE Family Study (f_{Arg109}=26.8% and f_{Arg223}=45.8%, n=501) and Quebec Family Study (f_{Arg109}=29% and f_{Arg223}=45%), whereas in Pima Indians the allele frequencies are very different (f_{Arg109}=58% and f_{Arg223}=75%) (Chagnon et al. 1999).

Lys109Arg and Gln223Arg polymorphisms are located in the CRH1 domain in LEPR. Some functional studies have suggested that the CRH1 domain is not involved in leptin binding (Fong et al. 1998), but a deletion of CRH1 resulted in a lower leptin response, suggesting that CRH1 contributes to optimal LEPR activation (Zabeau et al. 2004). Furthermore, a mutation Gln269Pro that causes the obese phenotype of the fa/fa rat is located in this same domain (Iida et al. 1996). Fa/fa rats have impaired transportation of both LEPR-a and -b on cell surface and decreased leptin binding affinity (da Silva et al. 1998) and they display hyperphagia, reduced energy expenditure, morbid obesity, impaired glucose tolerance, growth deficiency, hypothyroidism and reduced fertility (reviewed by Lutz & Woods 2012). In addition, human Pro316Thr mutation, which is located in the FNIII domain in CRH1, results in rapid weight gain with severe hyperphagia and the patients are known to suffer from upper respiratory tract infections (Mazen et al. 2011). Thus, it might be suggested that these two polymorphisms, Lys109Arg and Gln223Arg, could affect the function of LEPR, the leptin-mediated signaling and alter the effects of leptin.

6.1.4 The association of polymorphisms on IMT

As far as we are aware, this study is the first to have evaluated these SNPs in the LEPR in relation to IMT measurements in humans. The most significant finding of this study was the lowest IMT values in the subjects homozygous for Arg109 and Arg223 of the LEPR. The association observed here between variations in the LEPR gene and IMT measurements remained significant after adjustment for conventional cardiovascular risk factors. Previously, Arg109 and Arg223 alleles have also been shown to protect from the conversion to type 2 diabetes in high-risk individuals with impaired glucose tolerance (Salopuro et al. 2005).

We also found an association between Lys109Arg and total cholesterol levels. Interestingly, Arg109 homozygotes possessed the highest total cholesterol levels. It must also be pointed out that also HDL tended to be higher among Arg109 carriers and the association of LDL with LEPR Lys109Arg variation was non-significant. Therefore, the total cholesterol-to-HDL ratio, a ratio that has been
used in predicting an individual’s risk of developing atherosclerosis, did not differ between the three genotype groups. The fact that the association observed between the LEPR Lys109Arg polymorphism and IMT measurements remained significant after adjustment for total cholesterol, among other conventional cardiovascular risk factors, indicates that the variation in IMT among LEPR Lys109Arg genotypes is not mediated through lipid abnormalities.

These results suggest that LEPR Lys109Arg and Gln223Arg polymorphisms may have some additional mechanisms for development of early atherosclerosis others than the effect on conventional cardiovascular risk factors although they seem to have some impact on them as well. Thus, these polymorphisms seem to be associated with early atherosclerosis independently of the traditional risk factors with genotypes producing Arg109Arg and Arg223Arg of LEPR displaying lower IMT values.

6.2 LEPR Lys109Arg and total mortality, cardiovascular disease incidence and death (II)

6.2.1 Study population

The strength of study II is the OPERA data which is a well-defined and large population. Also the 19-year follow-up time that was used in the study is exceptionally long.

The major limitation is the number of events among the subjects with Arg109Arg. Since the frequency of the interesting Arg109Arg genotype is quite low (12.5%), the number of deaths and events in this group is not very high. The decrease in all-cause mortality is most probably a result of lower incidence for CHD deaths although the power of the study is not sufficient to discriminate this.

6.2.2 Outcome classification

Information on causes of death and events leading to hospitalization was obtained from the National Death Register and the Finnish Hospital Discharge Register (Hilmo register). The diagnoses were originally made according to the International Classification of Diseases, Eight Revision (ICD-8) or Ninth Revision (ICD-9) before 1994 and the Tenth Revision (ICD-10) thereafter and classified in our study according to the FINRISK criteria (Pajunen et al. 2011).
The registers in Finland are known to be trustworthy and the Hilmo register is a valuable source of data. The most obvious limitation in the validity of Hilmo is the recording of secondary diagnoses and secondary operations, which, however, does not compromise the relevance of the data (reviewed by Sund 2012).

There were 20 subjects with reliably diagnosed myocardial infarction and 22 with disorder of the cerebral circulation diagnosed in hospital already at the baseline. As they were equally distributed across the genotypes, they were included in the events.

6.2.3 LEPR Arg109Arg and CVD incidence

In study II we have shown that individuals with Arg109Arg genotype have lower incidence for coronary heart disease events and decreased mortality. In addition, they displayed the lowest BMI. This is in line with the results from study I indicating that Arg109Arg homozygotes also have lower intima-media thickness measurements.

Our results are also in concordance with the study of Rosmond and colleagues showing that Arg109 homozygotes had lower systolic and diastolic blood pressure, BMI and abdominal sagittal diameter accompanied by higher levels of HDL cholesterol (Rosmond et al. 2000). Arg109 carriers have also been shown to be protected from conversion to type 2 diabetes (Salopuro et al. 2005). Japanese obese children with Arg109Arg had lower serum LDL and triglyceride levels (Okada et al. 2010) and Indian children with Arg109Arg were less obese measured as BMI and waist and hip circumference (Tabassum et al. 2012).

LEPRs are expressed in endothelial cells (Knudson et al. 2005), and as leptin has been associated with impaired endothelial function (Gonzalez et al. 2013), a plausible mechanism for protective the effect of Arg109Arg would be via its impact on leptin signaling on the endothelium. Apart from the possible impact on vasodilation, leptin may also act on inflammatory response on endothelium (Manuel-Apolinar et al. 2013, Singh et al. 2007). It may be speculated that Arg109Arg genotype might result in decreased leptin signaling which could, in turn, be favorable for endothelial function. Furthermore, LEPRs are expressed in platelets and leptin has been suggested to promote platelet aggregation (Nakata et al. 1999) which might be reduced in Arg109Arg genotype and partly explain the lower incidence of cardiovascular events.

However, it is impossible to say if Lys109Arg itself is actually the functional variation affecting the mortality or whether it is linked to the causative
polymorphism. Indeed, an association between age at death and a LEPR SNPs (rs1171278 and rs3790426) other than that encoding Lys109Arg was identified in the Framingham study (Lunetta et al. 2007). However, it must be noted that in this study, Gln223Arg genotypes displayed no difference in the cardiovascular events or death although Gln223Arg is located rather close to Lys109Arg and these SNPs are in linkage disequilibrium.

Results of study II suggest that Arg109 is beneficial and that the Arg109Arg genotype of LEPR or possibly another polymorphism linked to it seems to protect against cardiovascular events as well as total mortality independently of the traditional risk factors.

6.3 Long-term fructose diet and IUGR effect on leptin system (III)

6.3.1 Animal model and study design

The strength of study III is that the number of rats is high. The total number of rats in the study was 74 animals. This study is excellent especially for studying the long-term fructose consumption, since 38 animals were on standard diet and 36 on fructose diet. For studying IUGR, the group sizes were a bit smaller: n=24 pups in CC and RC groups and n=26 in RR group but considered to be sufficient for IUGR comparisons. However, as the effect of fructose was so evident, it might overrun the effects of IUGR. A major limitation is that the suckling period examination is lacking a group in which the offspring from control dams would have been lactated by dams that were food-restricted during pregnancy.

The rat has often been used in studying IUGR and, thus, the selection of rat was natural to be able to compare the results to previous studies. In addition, the rat is a relatively large animal and it was possible to obtain sufficient tissue samples for multiple studies.

When studying the effects of fructose, it would also have been useful to include a control group on a 60% glucose diet to be able to distinguish the exact effect of fructose consumption from the effect of high carbohydrate consumption. Although the energy content of both chows was quite similar, the amount of fat in standard chow was 4.0%, whereas in the fructose chow it was 12.9%. High-fat diet used in animal experiments, however, contains even 40–60% fat.
6.3.2 Visceral adipose tissue depots

Adipose tissue was harvested from the abdominal cavity from all rats, but the exact spot may vary according to the person performing the preparation. This is a limitation in the study. However, this is a non-systematic error as the all groups were treated similarly and should not affect the results.

6.3.3 Microscopy

The size of adipocytes was microscopically determined from visceral adipose tissue samples. The maximal adipocytes were selected to minimize the variation due to different cut levels of the cells: by selection of maximal diameter we wanted to avoid the cells which were cut at the edges. The maximal adipocyte size correlated with the body weight as expected, suggesting that our method for the size analysis is reliable. However, the size of adipocytes in visceral fat displayed no difference attributable to fetal growth restriction or consumption of a fructose diet. This is consistent with the previous study that showed no difference in the size of adipocytes between the 70% food-restricted rats and the controls (Lukaszewski et al. 2011).

We have reported earlier (Malo et al. 2013) in the same study setting as examined here that the fructose diet fed rats displayed a slightly higher lipid content in the intraperitoneal and retroperitoneal adipose tissue than the controls measured by magnetic resonance imaging (MRI). This has also been found in humans as Maersk et al. performed a trial of six months with subjects consuming sucrose-sweetened soft drinks and detected a marked increase in visceral fat also measured by MRI, but the adipocyte size was not studied (Maersk et al. 2012). As the adipocyte size in our study was unchanged, it might be suggested that lipid accumulation occurred in the macrophages of adipose tissue or in perivascular tissue.

The liver samples were studied microscopically. Our results showed that the lipid content of the liver was higher in fructose diet fed animals whereas IUGR had no impact on liver fatness. The effect of fructose has previously been shown in human as well. Studies show that high fructose or sucrose consumption increases the liver fat significantly (Le et al. 2009, Maersk et al. 2012) and fructose intake is also associated with increased liver fibrosis in patients with NAFLD (Abdelmalek et al. 2010).
6.3.4 Gene expression studies

The gene expression results were normalized with the gene expression of GAPDH and 18S rRNA in WAT or HPRT and UBC in liver measured from the same samples. To minimize the possible effect of IUGR on the reference gene expressions, the reference genes were selected from a few candidates with NormFinder software (Andersen et al. 2004).

6.3.5 Western blots to study autophagy

Measuring autophagy is challenging. The autophagy genes showed marked expression changes in WAT and liver suggesting altered autophagy: this was supported by p62 protein levels in the WAT when the intensity of the p62 band was considered relative to total protein. This might be the most appropriate way of analyzing the results as it seems that the reference protein, β-actin, was also affected by the experimental design and might, thus, not be suitable as reference in western blot.

p62 is a substrate for autophagy and is often used to measure the amount of autophagy (Bjorkoy et al. 2009). Our results suggested that the amount of p62 protein is significantly higher in WAT of fructose fed rats indicating reduction in autophagy and supporting the gene expression results. However, LC3-II protein levels displayed no differences between the standard diet fed and fructose diet fed rats. This might be caused by the post-transcriptional regulation. On the other hand, LC3-II is often used as a measure of autophagosome formation but it is also degraded by autophagy, making the interpretation of the results difficult (Mizushima & Yoshimori 2007).

In the liver, p62 protein could not be detected in the western blot. This is most likely explained by the experimental design as the offspring were fasted 12h before killing and sample collecting. Starvation leads to activation of autophagy in the liver and results in degradation of p62.

6.3.6 Statistical analysis

The changes in the gene expression caused by IUGR needs to be evaluated cautiously as the number of the individuals in these groups might be insufficient for detecting minor changes in the gene expression. In addition, since the suckling period examination lacks a group in which the offspring from control dams would
have been lactated by dams that were food-restricted during pregnancy, the effect was included in the analysis as a confounding variable. Comparison is thus statistically challenging. However, as the investigation of the effects of fructose included 38 rats on a standard and 36 on a fructose diet, it is considered to be sufficient for detecting the changes in a reliable manner.

6.3.7 The fructose induced changes in leptin system and autophagy may predispose to metabolic alterations

Fructose consumption has been suggested to lead to an increase in the amount of liver fat (Le et al. 2009, Maersk et al. 2012) and promote liver fibrosis in NAFLD patients (Abdelmalek et al. 2010). Furthermore, a long-term fructose diet has been shown to induce leptin resistance, hepatic steatosis and increase TGs (reviewed by Dekker et al. 2010).

Our results from the study III suggest that as a result of the long-term fructose diet, the capacity of adipose tissue to store lipids could be compromised and lipids might accumulate as ectopic fat and be directed to the non-adipose tissue, which should be further studied.

In fructose-fed animals, the expression of autophagy genes ATG7, LAMP2 and MAP1LC3β were similarly regulated in the adipose tissue and liver as LEPR expression. Autophagy has been proposed to play a role in lipid storing to adipose tissue (Singh et al. 2009b, Zhang et al. 2009) and as adipocyte hypertrophy would have been expected in fructose-fed animals, the down-regulation of autophagy genes might explain the unchanged adipocyte size. In both adipose tissue and liver the autophagy genes were expressed similarly as LEPR, possibly pointing to a regulatory role of leptin to autophagy. A correlation between LEPR and autophagy gene expression in both adipose tissue and liver could also suggest a role for leptin in autophagy regulation. These results are supported by a previous study (Malik et al. 2011) demonstrating that leptin might regulate autophagy both in cell culture and in mice. It was postulated that leptin may induce autophagy via AMPK signalling and inhibit it through mTOR depending on the cell type (Malik et al. 2011).

Lipid accumulation in the non-adipose tissue organs might lead to increased insulin resistance. It has been claimed that liver fat accumulation is an independent determinant of insulin resistance, also independent of weight (Seppala-Lindroos et al. 2002), and our results suggested that the fat content in the liver was indeed higher in fructose fed rats. The high levels of VLDL
produced in the liver increasing the plasma TG levels are seen in insulin resistance (Sparks et al. 2012), which was also reported previously in our rats (Malo et al. 2013).

**Fig. 14.** A hypothetic mechanism aiming to explain the metabolic changes seen as a result of long-term fructose consumption. As LEPRs are over expressed in liver, it might result in insulin resistance by SOCS3 inhibitory effect and enhanced autophagy activity possibly via AMPK activation. Because of the insulin resistance, VLDL secretion may enhance and the TG levels in blood rise. On the other hand, leptin signalling and autophagy are reduced in the WAT and might result in decreased ability of adipocytes to store fat. High insulin plasma levels are not able to down-regulate autophagy in the liver because of the insulin resistance, but as WAT has remained insulin sensitive, insulin is able to suppress autophagy there.

Insulin has also been proposed to suppress liver autophagy (Ezaki et al. 2011) and over-activation of autophagy in the liver might be evoked by the liver insulin resistance. Extensive liver autophagy results in non-selective catabolization of cytoplasmic components into amino acids and free fatty acids, which are used in gluconeogenesis and beta-oxidation (Komatsu 2012). Autophagy is usually considered a protective mechanism but hyperactive autophagy has also been reported in pathological conditions such as liver inflammation in a rat model of diabetes mellitus (Hagiwara et al. 2010) and as a possible cause of hepatocyte death in anorexia nervosa patients (Rautou et al. 2008), suggesting that both diminished or excessive autophagy could be deleterious. It has also been reported that leptin treatment might have unfavorable consequences via an overactivated leptin system and an SOCS3 inhibitory effect might lead to insulin resistance, hepatic steatosis and liver fibrosis (Polyzos et al. 2011).
The results of this study suggest that a long-term fructose diet disturbs the leptin system by decreasing the expression of LEPRs and autophagy genes in white adipose tissue and enhancing their expression in the liver of male rats. We propose that the LEPR and autophagy gene expression changes in adipose tissue and liver with the resulting alterations in autophagy activity contribute to the metabolic alterations including elevated plasma TG and insulin resistance seen in fructose-fed animals.

6.3.8 IUGR reduces leptin levels

The effects of IUGR were less clear than those of fructose and should be interpreted cautiously. Nevertheless, IUGR seemed to have some effect on the leptin system as Lep mRNA expression and leptin plasma levels were decreased at least in animals on standard diet and LEPR mRNA expression was shown to be slightly up-regulated in both WAT and liver. However, as SOCS3 mRNA expression was not changed and microscopic analyses showed no differences in the histology of WAT and liver, the rise in LEPR expression might be a compensatory mechanism seen as a result of the reduction in leptin levels. Decreased leptin levels may be caused by lower body weight that was seen in IUGR rats still in adulthood (Malo et al. 2013).

Previous studies with rats are controversial to our study as they showed that IUGR either increases leptin plasma levels (Lukaszewski et al. 2011, Vickers et al. 2000) and Lep mRNA expression and decreases LEPR-b mRNA expression in WAT (Lukaszewski et al. 2011) or has no effect on leptin at all (Hietaniemi et al. 2009). However, in the studies by Vickers and Lukaszewski the rats were Wistar rats while we used Sprague-Dawley rats. Also the rats in these studies were examined at the age of four months while our rats were followed until the age of six months (Lukaszewski et al. 2011, Vickers et al. 2000). As was seen in our animal experiment (Malo et al. 2013), also the studies by Lukaszewski and Vickers demonstrated no catch-up growth in IUGR animals (Lukaszewski et al. 2011, Vickers et al. 2000). In the study by Hietaniemi and colleagues that was performed in our laboratory, Sprague-Dawley rats were used and followed up until the age of one month. Interestingly, although the leptin levels were not statistically changed by IUGR, there was a trend towards lower leptin levels in 50% food-restricted pups (Hietaniemi et al. 2009).
6.4 Clinical relevance of the studies

The results of this study manage to assess the role of leptin receptor in the development of atherosclerosis and prediction of cardiovascular disease. Furthermore, the results of the animal study suggest a new possible mechanism of how fructose consumption might affect the metabolism. It seems that the regulation of leptin signaling, performed by altering the expression of leptin receptor, might contribute to the development of insulin resistance in the liver as well as affect the plasma triglyceride levels. In addition, our results propose a link between fructose diet, leptin signalling and autophagy. The alterations in autophagy activity might participate in the metabolic changes seen as a result of fructose consumption.

The study about the mechanisms behind the development of cardiovascular diseases is slow and challenging as the pathogenesis of the disease is very complex. The study of autophagy has recently become a hot topic in the research related to metabolism and cardiovascular diseases and these results contribute some more information about this topic.

The relevance of these results is not unambiguous and they warrant further studies. However, the present results provide interesting clues for future studies.
7 Conclusions

The purpose of this study was to investigate the role of LEPR on developing CVD and on the predisposing risk factors. The main findings of the present study were as follows:

1. Two polymorphisms in LEPR, Lys109Arg and Gln223Arg, associate with IMT measurements independently of conventional risk factors for atherosclerosis. This suggests that these variations in the LEPR gene contribute to the development of early atherosclerosis. Genotypes Arg109Arg and Arg223Arg might be somewhat protective against early atherosclerosis. To our knowledge, this has not been reported previously.

2. Arg109Arg homozygotes display lower incidence of cardiovascular events and mortality as well as total mortality, suggesting that the Arg109Arg genotype might be beneficial.

3. A long-term fructose diet disturbs the leptin system by decreasing the expression of LEPR and autophagy genes in white adipose tissue and enhancing their expression in the liver of male rats. These gene expression changes in adipose tissue and liver may contribute to the metabolic alterations including elevated plasma TG and insulin resistance seen in fructose-fed animals.

4. IUGR seems to have some effect on the leptin system by reducing the leptin plasma levels and mRNA expression. On the other hand, this might be a result of lower body weight. Possibly as a compensatory mechanism for reduced leptin, the LEPR mRNA expression is up-regulated in liver and WAT.
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