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Eija Kellokoski

GHRELIN AND ATHEROSCLEROSIS

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AND CELL CULTURE STUDIES*

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EIJA KELLOKOSKI

GHRELIN AND ATHEROSCLEROSIS

Human, experimental animal and cell culture studies

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Supervised by
Docent Sohvi Hörkkö
Professor Antero Kesäniemi

Reviewed by
Docent Matti Jauhiainen
Docent Pirkko Pussinen

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Faculty of Medicine, Institute of Clinical Medicine, Department of Internal Medicine, Institute of Biomedicine, Department of Pharmacology and Toxicology, Biocenter Oulu, University of Oulu, P.O. Box 5000, FI-90014 University of Oulu, Finland; Clinical Research Center, Oulu University Hospital, P.O. Box 5000, FI-90014 University of Oulu, Finland

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Abstract

Atherosclerosis is the major cause of cardiovascular diseases and the leading cause of death globally. Atherosclerosis is a complex, chronic disease characterized by lipid accumulation and inflammation within the intima layer of vessel wall. Novel biomarkers and therapeutics are still being sought to provide both better diagnosis and treatment. Ghrelin represents an attractive target for studies into atherosclerosis. Ghrelin is a gastric peptide hormone, which has multiple functions, including regulation of appetite and energy metabolism. Emerging evidence suggests that it may also have a role in the cardiovascular and immune systems.

The aim of the present study was to explore the role of ghrelin in atherosclerosis. The specific aims were 1) to investigate the association between the plasma ghrelin level and early atherosclerosis as determined by carotid artery intima media thickness (IMT) in a large (n = 1024) cross-sectional population-based study of middle-aged subjects, 2) to measure the associations between plasma ghrelin levels and already established risk factors of atherosclerosis in human subjects, 3) to assess the effects of ghrelin on atherogenesis *in vitro* by analyzing monocyte adhesion to endothelial cells, oxidized low density lipoprotein (LDL) binding and acetylated LDL uptake using macrophages, and 4) to study the influence of ghrelin on atherosclerosis using ghrelin vaccination in a mouse model of atherosclerosis.

Plasma total ghrelin levels were positively associated with carotid IMT in male subjects. Association studies demonstrated plasma ghrelin levels to be negatively associated with total and LDL cholesterol, and triglyceride concentrations as well as with body mass index (BMI), and positively associated with high density lipoprotein (HDL) cholesterol concentration in postmenopausal women and in a population-based study. In addition, estrogen increased plasma acylated ghrelin levels in postmenopausal women. Cell culture studies demonstrated that ghrelin could increase the binding of oxidized LDL and monocytes to endothelial cells. Interestingly, when endothelial cells were stimulated with tumor necrosis factor α (TNF α), then ghrelin prevented monocyte adhesion. The study with LDL receptor knockout mice, revealed that ghrelin vaccination could increase plasma ghrelin levels but had no effects on the development of atherosclerosis. However, the plasma MCP-1 level decreased in mice immunized with ghrelin vaccine. In conclusion, these studies suggest that ghrelin has modulatory functions in the vascular system and atherogenesis though the effect may not be as dominant as that of the known traditional risk factors. Whether this effect of ghrelin is positive or negative in atherogenesis will be clarified in further studies.

Keywords: atherosclerosis, cell adhesion molecules, endothelial cells, estrogen replacement therapy, ghrelin, macrophages, mouse model of atherosclerosis, obesity, vaccination

Kellokoski, Eija, Greliini ja ateroskleroosi. Tutkimuksia ihmisillä, eläinmallilla ja soluilla

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Tiivistelmä

Sydän- ja verisuonitaudit ovat suurin kuolinsyy niin Suomessa kuin useimmissa länsimaissakin. Näiden sairauksien taustalla on yleensä valtimonkovettumatauti eli ateroskleroosi, joka voi kliinisesti ilmentyä mm. sepelvaltimotautina, aivoveritulppana ja laskimotautina. Ateroskleroosissa tulehdussoluja ja kolesterolia kertyy verisuonen seinämään muodostaen ahtauman eli ateroskleroosin valtimoon. Valtimonkovettumataudin riskitekijäitä tunnetaan jo hyvin, mutta uusia tautia ennustavia merkkiaineita sekä hoitomuotoja tarvitaan yhä. Greliini on mahalaukusta eritettyä peptidihormoni, joka osallistuu elimistössä mm. ruokahalun, energiametabolian, tulehdustekijöiden sekä sydän- ja verenkiertoelimistön toiminnan säätelyyn.

Tämän työn tavoitteena oli tutkia greliinin yhteyttä ihmisen valtimonkovettumatautiin. Tutkimus toteutettiin käyttämällä kahta eri potilasaineistoa, soluviljelykokeita sekä valtimonkovettumataudin hiirimallia. Laajassa väestöpohjaisessa potilasaineistossa tutkittiin veren greliinipitoisuuden yhteyttä kaulavaltimon seinämän paksuuteen, jota pidetään valtimonkovettumista kuvaavana tekijänä. Veren greliinipitoisuuden yhteyttä valtimonkovettumataudin tunnettuihin riskitekijöihin tutkittiin myös laajassa potilasaineistossa sekä vaihdevuosi-ikäisillä naisilla, joille annettiin estrogeenikorvaushoitoa. Solukokeilla selvitettiin greliinin vaikutusta tärkeisiin valtimonkovettumataudin syntyvaiheisiin käyttäen monosyytti-, endoteelisolu- sekä makrofaagisoluviljelmiä. Greliinin vaikutusta ateroskleroosiin *in vivo* selvitettiin rokottamalla LDL-reseptoripuutteiset hiiret greliini-rokotteella.

Tutkimuksessa havaittiin yhteys veren korkean greliinipitoisuuden ja kaulavaltimon seinämän paksuuden välillä miehillä laajassa potilasaineistossa (n = 1024). Tulosta tukivat soluilla tehdyt kokeet, joissa greliini lisäsi hapettuneen LDL:n sitoutumista makrofaageihin sekä monosyyttien tarttumista endoteelisolujen pinnalle. Greliinin vaikutukset monosyyttien tarttumiseen endoteelisolujen pinnalle olivat päinvastaiset silloin, kun endoteelisolut käsiteltiin tulehdusta stimuloivalla tekijällä. Matalat veren greliinipitoisuudet olivat myös yhteydessä korkeisiin LDL-kolesteroli- ja triglyseriditasoihin sekä painoindeksiin ja matalaan HDL-kolesterolitasoon potilasaineistoissa. Estrogeeni nosti veren greliinipitoisuutta vaihdevuosi-ikäisillä naisilla. Greliinirokote ei vaikuttanut ateroskleroosin kehittymiseen hiirimallissa. Tutkimustulosten perusteella greliinillä näyttäisi osallistuvan valtimonkovettumataudin kehitykseen, vaikkakin sen vaikutus on pienempi kuin aiemmin tunnetuilla taudin riskitekijöillä.

Asiasanat: adheesiomolekyylit, ateroskleroosi, endoteelisolut, eläinmalli, estrogeenihoito, greliini, kaulavaltimot, lihavuus, rokote, syöjäsolut

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Espoo, September 15th 2009

Eija Kellokoski

Abbreviations

Ac-LDL	Acetylated LDL
AMPK	Adenosine monophosphate-induced protein kinase
ANOVA	Analysis of variance
ANCOVA	Analysis of covariance
Apo	Apolipoprotein
ACTH	Adrenocorticotrophic hormone
AVP	Argininvasopressin
BBB	Blood-brain barrier
BMI	Body mass index
BSA	Bovine serum albumin
CAD	Coronary artery disease
DBP	Diastolic blood pressure
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's modified eagle medium
ELISA	Enzyme linked immunosorbent assay
ERT	Estrogen replacement therapy
FBS	Fetal bovine serum
G-CSF	Granulocyte colony stimulating factor
GH	Growth hormone
GHRH	Growth hormone releasing hormone
GHRL	Ghrelin gene
GHS	Growth hormone secretagogue
GHS-R	Growth hormone secretagogue receptor
GOAT	Ghrelin O-acyl transferase
GPCR	G protein coupled receptor
GPR39	G protein-coupled receptor 39
HDL	High-density lipoprotein
HFD	High fat diet
HUVEC	Human umbilical vein endothelial cell
ICAM-1	Intercellular adhesion molecule 1
IL	Interleukine
IMT	Intima-media thickness
IR	Infra red
KLH	Keyhole limpet hemocyanin
KO	Knockout

LDL	Low density lipoprotein
LDLR	LDL receptor
LDLR KO	LDL receptor knockout
LPS	Lipopolysaccharide
MCP-1	monocyte chemoattractant protein 1
M-CSF	Macrophage colony stimulating factor
mRNA	Messenger ribonucleic acid
NH ₂	Amino group
NPY	Neuropeptide Y
OPERA	Oulu Project Elucidating Risk of Atherosclerosis
PADRE	Pan DR epitope
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Peroral estrogen
PPAR	Peroxisome proliferator-activated receptor
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RT-PCR	Real time polymerase chain reaction
SBP	Systolic blood pressure
SMC	Smooth muscle cell
TE	Transdermal estrogen
TNF α	Tumor necrosis factor α
VCAM-1	Vascular cell adhesion molecule 1
VLDL	Very low-density lipoprotein

List of original articles

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

- I Pöykkö SM*, Kellokoski E*, Ukkola O, Kauma H, Päivänsalo M, Kesäniemi YA & Hörkkö S (2006) Plasma ghrelin concentrations are positively associated with carotid artery atherosclerosis. *J Intern Med* 260: 43–52.
- II Kellokoski E, Pöykkö SM, Karjalainen AH, Ukkola O, Heikkinen J, Kesäniemi YA & Hörkkö S (2005) Estrogen replacement therapy increases plasma ghrelin levels. *J Clin Endocrinol Metab* 90: 2954–63.
- III Kellokoski E, Kunnari A, Jokela M, Mäkelä S, Kesäniemi YA & Hörkkö S (2009) Ghrelin and obestatin modulate early atherogenic processes on cells: Enhancement of monocyte adhesion and oxidized LDL binding. *Metabolism* (in press).
- IV Kellokoski E, Kummu O, Serpi R, Lehenkari P, Kesäniemi YA & Hörkkö S Ghrelin vaccination does not affect weight gain or atherosclerosis in mice. *Peptides* (in press).

*Equal contribution as first author

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1 Introduction

Cardiovascular diseases, including coronary artery disease (CAD), stroke, and ischemia, are the leading worldwide cause of death. The major pathological feature of these multifactorial diseases is atherosclerotic plaque formation in small and large arteries. Atherosclerosis is described as occlusions of lipids and inflammatory cells within the intimal layer of vessel wall which results in endothelial cell injury and tissue dysfunction, leading to thickening and hardening of the vessel wall. The atherosclerotic changes begin to develop already at childhood long before the first clinical manifestations, which occur at older ages. The major modifiable risk factors for atherosclerosis are well-known and include high blood pressure, abnormal blood lipid profile (high total and low density lipoprotein (LDL) cholesterol and triglyceride concentrations and low high density lipoprotein (HDL) cholesterol concentration), smoking, physical inactivity, obesity, unhealthy diet (low intake of fruits and vegetables) and diabetes mellitus. In addition, the non-modifiable risk factors include advancing age, gender and family history of premature CAD. Importantly, inflammation is now believed to be a novel risk factor. (Mackey & Mensah 2004) The traditional risk factors predict only part of the future cardiovascular risk, and thus, the factors involved in the complex processes during atherosclerosis development are not fully defined. New factors contributing to atherogenesis are needed, either as novel biomarkers to improve the early diagnosis or as new modalities of disease treatment.

Ghrelin, a gastric peptide found in 1999, is a natural ligand for growth hormone secretagogue receptor (GHS-R) (Kojima *et al.* 1999). It has been shown to have multiple different systemic and peripheral functions especially in feeding behavior and energy metabolism (Pusztai *et al.* 2008). Plasma ghrelin levels are decreased in obesity and increased under negative energy balance (Shiyya *et al.* 2002). Accumulating data indicates that ghrelin has also effects on the cardiovascular and immune systems. Interestingly, ghrelin has been claimed to have anti-inflammatory actions that may have an important role in the development of atherosclerosis (Dixit & Taub 2005).

The aim of the present thesis was to investigate the role of ghrelin in atherosclerosis. The influences of ghrelin in atherosclerosis were investigated using two different human populations, cells involved in atherogenesis and a mouse model of atherosclerosis. The Oulu Project Elucidating Risk of Atherosclerosis (OPERA) and the Estrogen Replacement Therapy (ERT) studies provided good opportunities to investigate the associations between plasma

ghrelin levels and early carotid atherosclerosis and also with the already established factors contributing to atherosclerosis. The *in vitro* effects of ghrelin on atherogenesis were demonstrated in detail by studying monocyte adhesion to endothelial cells and also the binding and uptake of modified LDL using macrophages. The effect of ghrelin on atherosclerosis *in vivo* was studied using a ghrelin vaccine in a widely used mouse model of atherosclerosis, the LDL receptor knockout (LDLR KO) mouse.

2 Review of the literature

2.1 Ghrelin

Ghrelin, a 28 amino-acid peptide, was discovered in 1999 from rat stomach and reported to be a natural ligand for the orphan growth hormone secretagogue receptor type 1a (GHS-R1a) (Kojima *et al.* 1999). Prior to this discovery, only synthetic growth hormone secretagogues (GHS) were known to stimulate the release of growth hormone (GH) via this GHS receptor (Howard *et al.* 1996). A unique feature of the ghrelin peptide is the o-n-octanoylation at the serine-3 residue which is essential for receptor binding, stimulation of pituitary GH release, and eliciting its biological activity (Kojima *et al.* 1999, Matsumoto *et al.* 2001). This acylation also modulates ghrelin transport across the blood-brain barrier (BBB) (Banks *et al.* 2002).

Ghrelin homologs have been identified in a variety of mammalian, avian and fish species (Kojima & Kangawa 2005). Human ghrelin was found to differ from rat ghrelin in only two amino acid residues (Kojima *et al.* 1999, Kojima & Kangawa 2005). The amino acid sequences of ghrelins are highly conserved among mammals, in particular the first 10 amino acids with the acyl-modification of the third residue in the amino (NH₂)-terminus, which indicates that this region is crucially important for the activity of the peptide (Kojima & Kangawa 2005).

Soon after Kojima *et al.* had discovered the ghrelin peptide, Tomasetto *et al.* identified a novel gastric peptide hormone (Kojima *et al.* 1999, Tomasetto *et al.* 2000). This peptide was designated as motilin-related peptide and was found to have an identical amino acid sequence as ghrelin. The name 'ghrelin' is based on 'ghre' in Proto-Indo-European languages meaning 'grow' in reference to its ability to stimulate growth hormone.

2.1.1 Ghrelin gene and transcripts including obestatin

Ghrelin is the major active product of ghrelin gene (GHRL), which is located on chromosome 3p26-25 in humans (Kanamoto *et al.* 2004). The detailed gene structure is still not fully characterized.

GHRL has been very recently found to consist of six exons (Seim *et al.* 2007) as illustrated in Figure 1. Exon -1 and Exon 0 are present in the non-coding region upstream from exon 1, and exons 1 to 4 in coding region described also in

earlier studies in humans (Wajnrajch *et al.* 2000, Kanamoto *et al.* 2004, Nakai *et al.* 2004, Seim *et al.* 2007). The ghrelin gene has been found to consist of exon 0 and exons 1–4 in mice (Tanaka *et al.* 2001). Figure 1 describes the genomic structure and processing of human ghrelin gene (GHRL) into the active ghrelin peptide and obestatin. Mature ghrelin mRNA is encoded by exons 1 to 4 and is translated into a precursor peptide (preproghrelin) (Kojima *et al.* 1999, Jeffery *et al.* 2005). The 28 amino acid ghrelin and a 23 amino acid obestatin peptides are cleaved by proteases. Ghrelin peptide is esterified at the serine-3 residue by an octanoic acid ($\text{CH}_3(\text{CH}_2)_6\text{COOH}$) and obestatin is amidated at the C-terminal end (Kojima *et al.* 1999, Zhang *et al.* 2005).

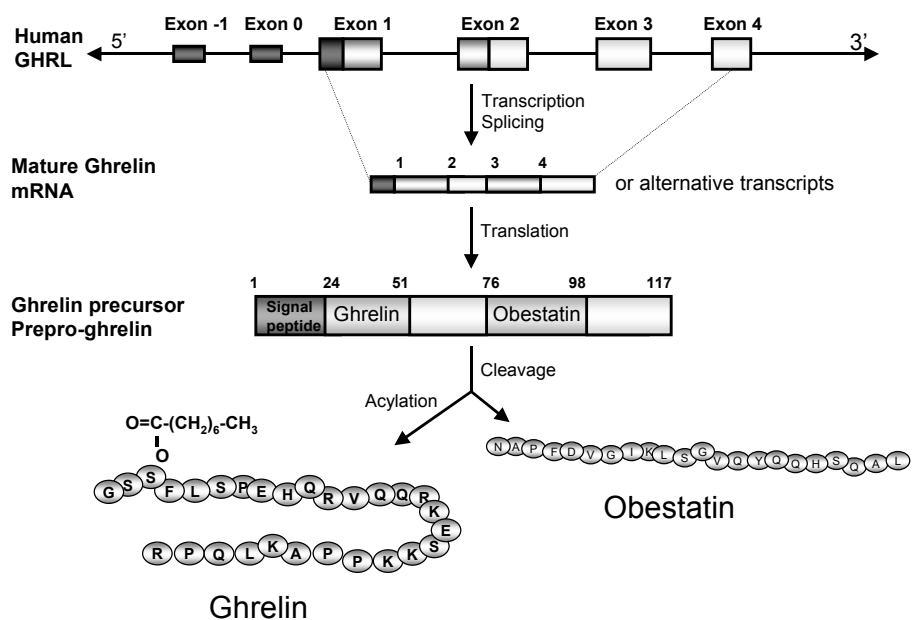


Fig. 1. The processing of active ghrelin and obestatin peptides from human ghrelin gene. Adapted from Seim *et al.* 2007.

Basal transcription activity of the human ghrelin promoter appears to require an essential sequence downstream from the distal initiation site (the noncoding exon (Exon 0) region and proximal region of intron 1) (Wei *et al.* 2005). In addition, numerous putative binding sites for transcription factors have been identified including activator protein-2 (AP-2), basic helix-loop-helix, PEA-3, NF-IL-6 and

NF- κ B and half-sites for estrogen and glucocorticoid response elements (Kishimoto *et al.* 2003, Kanamoto *et al.* 2004). The noncoding exons (Exon -1 and Exon 0) have been identified to contain several transcriptional starting sites, and thus, multiple alternative transcripts can be transcribed and spliced in different ways depending on the tissue (Seim *et al.* 2007). In addition, antisense transcripts for the ghrelin gene have been identified; they may also function in the regulation of the ghrelin preproprotein expression (Seim *et al.* 2008). Interestingly, genetic variations in the ghrelin gene have been associated with BMI, waist circumference and variability of body weight in a human population based study and in a twin study (Vartiainen *et al.* 2006, Leskela *et al.* 2009).

Ghrelin peptide

Mature human ghrelin mRNA codes for preproghrelin, which is a 117 amino acid long peptide (Figure 1) consisting of a 23 amino-acid signal peptide and a 94 amino-acid peptide called pro-ghrelin (1-94). Pro-ghrelin contains the 28 amino acid mature ghrelin (1-28) and a 66 amino acid tail (29-94) (Kojima *et al.* 1999, Jeffery *et al.* 2005). The enzymes which cleave the preprohormone to the final products, are not fully known. However, recent findings indicate that proghrelin (1-94) can be cleaved by prohormone convertase 1/3 (PC1/3) in mouse stomach tissue to generate natural ghrelin *in vivo* (Zhu *et al.* 2006). The Gly1 residue is the first amino acid residue in ghrelin peptide (1-28), which is cleaved after the Agr28 residue. The newly identified enzyme ghrelin O-acyl transferase (GOAT), modifies Ser3 residue with an O-linked octanoyl side group (Yang *et al.* 2008). GOAT is localized in the same cells in stomach fundus with ghrelin (Sakata *et al.* 2009). Genetic disruption of the GOAT gene in mice leads to complete absence of acylated ghrelin in the circulation suggesting strongly that this enzyme is responsible for octanoylation of ghrelin (Gutierrez *et al.* 2008). This octanylated 28 amino acid peptide is the most commonly studied form of ghrelin and it was used in the experiments of this study.

Des-acyl ghrelin

Des-acyl (named alternatively as unacylated, non-acylated or non-octanoylated) ghrelin lacks the unique fatty acid chain at the serine 3 residue. However, this form of ghrelin is the major (> 90%) circulating form of the molecule. (Hosoda *et al.* 2003, Patterson *et al.* 2005, Bang *et al.* 2007) It is not clear how the des-acyl

ghrelin is produced. Des-acyl ghrelin is believed to result from either incomplete acylation of the peptide or from deacylation of ghrelin (De Vriese *et al.* 2007). Initially, des-acyl ghrelin was regarded to be an inactive peptide, since it has been found to lack the ability to bind to the GHS-R1a and showed no GH-releasing activity in rats or humans (Kojima *et al.* 1999, Muccioli *et al.* 2001, Broglio *et al.* 2003a). However, recent data have demonstrated that des-acyl ghrelin has many physiological effects that are independent of the GHS-R1a, including actions in glucose metabolism, inhibition of lipolysis, promotion of adipogenesis, inhibition of cell apoptosis, beneficial cardiotropic effects and vasodilatation and stimulation of food intake (Baldanzi *et al.* 2002, Bedendi *et al.* 2003, Muccioli *et al.* 2004, Thompson *et al.* 2004, Gauna *et al.* 2005, Gauna *et al.* 2006, Granata *et al.* 2006, Kleinz *et al.* 2006, Toshinai *et al.* 2006, Gauna *et al.* 2007, Granata *et al.* 2007). Thus, des-acyl ghrelin may be considered as a metabolically active peptide.

The production of the naturally existing splicing variant Des-Gln14-ghrelin (Hosoda *et al.* 2000b), exon-3 deleted ghrelin (Yeh *et al.* 2005) and other ghrelin gene derived products C-ghrelin (Pemberton *et al.* 2004, Bang *et al.* 2007) and ghrelin (1-27) (Hosoda *et al.* 2003) are shown in Figure 2. The acylation on Ser3 of these ghrelin products is most often done with an octanoyl group (C8:0) but it may also be achieved with the decanoyl group (C10:0) and possibly with a decenoyl group (C10:1)(Hosoda *et al.* 2003). Ingestion of either medium-chain fatty acids or medium-chain triacylglycerols has been reported to increase the extent of acylation of ghrelin (Nishi *et al.* 2005).

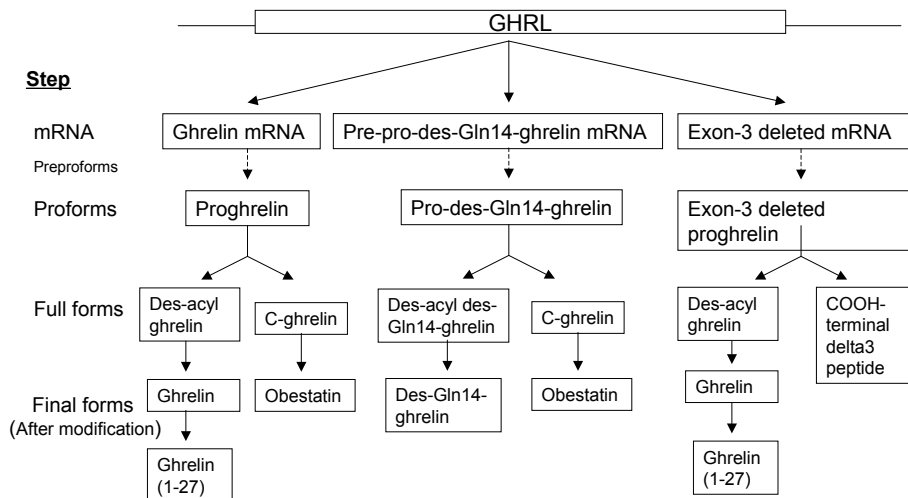


Fig. 2. Ghrelin gene derived products at different steps of production. (Modified from Soares & Leite-Moreira 2008)

Obestatin

The existence of a novel putative peptide hormone derived from the C terminus of proghrelin (76-98) was hypothesized by comparative genomic analysis in 2005 (Zhang *et al.* 2005). This new 23-amino-acid peptide was named obestatin based on its appetite-suppressing potential and it was reported to be the endogenous ligand for the orphan receptor G protein-coupled receptor 39 (GPR39) (Zhang *et al.* 2005, Green *et al.* 2007, Lagaud *et al.* 2007). However, recent reports have emphasized that obestatin is unlikely to be endogenous ligand for GPR39 and regulate satiety signalling and upper GI motility as initially believed (Chartrel *et al.* 2007, Gourcerol *et al.* 2007, Holst *et al.* 2007, Nogueiras *et al.* 2007). Further studies have explored different physiological influences for obestatin.

Obestatin has been found in rodents and humans (Zhang *et al.* 2005, Dun *et al.* 2006). Immunological methods have revealed the presence of obestatin in rodent large and small intestine, stomach, colon, pancreas, spleen and cerebral

cortex (Zhang *et al.* 2005, Chanoine *et al.* 2006). Obestatin has also been detected in cells of the gastric mucosa, myenteric plexus, and in the Leydig cells of the testis in mice and rats (Dun *et al.* 2006). In human tissues, obestatin has been found to have a similar distribution pattern as ghrelin throughout the gastrointestinal tract and in pancreas but also in ductal epithelium in mammary glands (Grönberg *et al.* 2008).

2.2 Tissue distribution of ghrelin

Ghrelin is expressed in various organs and cells. However, the main source of ghrelin is stomach.

2.2.1 Stomach and gastrointestinal organs

Ghrelin was initially isolated from the stomach (Kojima *et al.* 1999). Later distinct ghrelin-containing cells were localized to the oxyntic mucosa of the stomach fundus being X/A-like cells in rat and in P/D₁-cells in human (Date *et al.* 2000, Rindi *et al.* 2002). These endocrine cells account for approximately 20–30% of the oxyntic endocrine cell population in human stomach (Date *et al.* 2000). The cells are most abundant in fundus of stomach where ghrelin expression is highest and the expression decreases towards the distal part of the gastrointestinal (GI) tract such that no (human) or rare (rat) ghrelin expressing cells are observed in the large intestine (Date *et al.* 2000, Tomasetto *et al.* 2000, Papotti *et al.* 2001, Rindi *et al.* 2002, Sakata *et al.* 2002). In human stomach, the ratio of the des-acyl:acylated ghrelin was found to be ~ 2:1 but in circulation, 90% of ghrelin is in non-acylated form (Hosoda *et al.* 2003, Bang *et al.* 2007). Two thirds of the circulating ghrelin in plasma is derived from the stomach with the rest being mainly from the small intestine (Date *et al.* 2000).

Plasma ghrelin levels have been shown to decline by approximately 70% after gastrectomy, thus, supporting the idea that gastric ghrelin is the major source of circulating ghrelin in plasma (Ariyasu *et al.* 2001, Cummings *et al.* 2002). However, this kind of decrease has not been found in all studies and, in addition, plasma ghrelin levels progressively increase again after total gastrectomy suggesting that other cellular sources of ghrelin compensate for stomach-derived ghrelin (Faraj *et al.* 2003, Hosoda *et al.* 2003, Hanusch-Enserer *et al.* 2004, Korner *et al.* 2005).

Stomach is not the major source of ghrelin in fetus. Increasing evidence suggests that the pancreas and the lung are alternatives sources of ghrelin in the fetus. Ghrelin is expressed in pancreas starting from midgestation and the ghrelin levels are six to seven times higher in fetal pancreas than in fetal stomach. (Chanoine & Wong 2004)

2.2.2 Other tissues

Ghrelin has been detected in the heart, cardiomyocytes and endothelial cells (Papotti *et al.* 2000, Gnanapavan *et al.* 2002, Iglesias *et al.* 2004, Kleinz *et al.* 2006). Human ghrelin is expressed at mRNA level in T-lymphocytes, B-lymphocytes, and neutrophils from venous blood of healthy volunteers with a wide individual variation (Hattori *et al.* 2001, Dixit *et al.* 2004). Ghrelin is also expressed in mouse monocyte/macrophages at both mRNA and protein levels (Waseem *et al.* 2008).

Ghrelin is also expressed in various other tissues such as hypothalamic arcuate nucleus, pituitary, pancreatic A-cells, kidney, adrenal gland, thyroid, placenta, ovaries, testis, liver, gall bladder, fat tissue, lung, skeletal muscle, myocardium and skin (Kojima *et al.* 1999, Mori *et al.* 2000, Gualillo *et al.* 2001, Kanamoto *et al.* 2001, Katugampola *et al.* 2001, Korbonits *et al.* 2001, Gnanapavan *et al.* 2002, Lu *et al.* 2002, Tena-Sempere *et al.* 2002, Gaytan *et al.* 2003, Tortorella *et al.* 2003, Kageyama *et al.* 2005, Mondal *et al.* 2005, Knerr *et al.* 2006, Ghelardoni *et al.* 2006). In addition, ghrelin is present in different tumors including neuroendocrine tumors, pituitary adenomas, endocrine tumors of pancreas, breast tumors, and thyroid and medullary thyroid carcinomas (Leontiou *et al.* 2007).

2.2.3 Circulating ghrelin

Two major forms of ghrelin are found in plasma: n-octanoyl-modified and des-acyl ghrelin (Hosoda *et al.* 2006). The majority (more than 90%) of plasma ghrelin is believed to be in des-acyl form (Patterson *et al.* 2005). This is likely due to the shorter half-life of acylated ghrelin than that of des-acyl ghrelin, as plasma acylated ghrelin rapidly disappears from the circulation due to its binding to the GHS-R in the tissues (Akamizu *et al.* 2005). Deacylation of ghrelin to des-acyl ghrelin, which rapidly occurs in the plasma, is also responsible for the reduced half-life of ghrelin (Hosoda *et al.* 2004). To prevention the deacylation

and cleavage of ghrelin in blood samples, EDTA, aprotinin and HCl (1N) have been reported to be used in the handling of plasma samples (Hosoda *et al.* 2000a, 2004).

The majority of circulating acylated ghrelin is bound to larger molecules, mostly lipoproteins (Beaumont *et al.* 2003, Patterson *et al.* 2005, De Vriese *et al.* 2007). Plasma ghrelin has been found to be associated with triglyceride-rich lipoproteins, HDL, very high-density lipoproteins (density > 1.21g/ml), and to some extent with LDL (De Vriese *et al.* 2007). The cause of the binding to lipoproteins in blood has been suggested to be due to the increased hydrophobicity of the acyl side chain of active ghrelin (De Vriese *et al.* 2007).

2.3 Ghrelin receptor (GHS-R)

GHS-R is a G-protein-coupled receptor (GPCR) first cloned from human pituitary in 1996, when its endogenous ligand (ghrelin) was still unknown (Howard *et al.* 1996). GHS-R has two variants GHS-R type 1a (GHS-R1a) and type 1b (GHS-R1b) produced by alternative splicing. Both receptors have a widespread expression. It seems that GHS-R1a mediates biological actions of GHS and ghrelin, but it is unclear whether GHS-R1b is a functional receptor.

Similarly to other GPCRs GHS-R1a consists of 7 transmembrane domains and is able to activate several intracellular second messenger systems, e.g. an increase in the cytosolic calcium concentration (Malagon *et al.* 2003, Carreira *et al.* 2004). GHS-R1a is strongly expressed in central neuroendocrine tissues, such as hypothalamus and pituitary consistent with the observations that ghrelin has actions which are also mediated through the pituitary (Howard *et al.* 1996). GHS-R1a expression has also been reported in multiple peripheral organs, including heart, lung, liver, kidney, pancreas, stomach, small and large intestines, testis, adipose tissue, vascular endothelium, myocardium, and immune cells, such as monocytes, as well as in several endocrine and endocrine tumors and cell lines (Guan *et al.* 1997, Hattori *et al.* 2001, Katugampola *et al.* 2001, Kojima *et al.* 2001, Baldanzi *et al.* 2002, Gnanapavan *et al.* 2002, Volante *et al.* 2002, Gaytan *et al.* 2003, Dixit *et al.* 2004, Cassoni *et al.* 2006). Studies in GHS-R1a knockout mice revealed that this receptor was essential for the stimulatory effects of ghrelin on GH (Sun *et al.* 2004). In a human population, genetic variation in GHS-R1a gene was associated with parameters in insulin metabolism (Vartiainen *et al.* 2004).

GHS-R1b is a C-terminal-truncated form of GHS-R1a containing 5 transmembrane domains (Howard *et al.* 1996). It has been widely detected in both endocrine and non-endocrine human tissues (Gnanapavan *et al.* 2002).

All acyl-modified ghrelin analogs seem to have the same potency to induce an increase in Ca^{2+} concentrations and to stimulate GH release (Hosoda *et al.* 2003). The first four to five residues were found to functionally activate GHS-R as efficiently as the full-length ghrelin (Bednarek *et al.* 2000). It has been hypothesized in many studies using des-acyl ghrelin that a novel alternative GHS-R type binding for both acylated and des-acyl ghrelin may exist (Reviewed by Muccioli *et al.* 2007).

2.4 Physiological functions of ghrelin

Ghrelin has multiple physiological functions in the body. Circulating ghrelin can act centrally by entering directly into brain areas not sheltered by the BBB or by passing through the BBB transport system (Banks *et al.* 2008). Peripheral signals of ghrelin can be mediated also via the vagus afferent nerve to the brain (Date *et al.* 2002a, Williams *et al.* 2003). Some of the main physiological functions of ghrelin are presented here.

2.4.1 Growth hormone secretion

Ghrelin possesses a strong and dose-dependent GH-releasing effect, both *in vitro* and *in vivo*, in humans and animals (Malagon *et al.* 2003). In addition, it has been reported that ghrelin can synergistically stimulate GHRH-dependent secretion of GH (Hataya *et al.* 2001). The GH-releasing effect of ghrelin appears to result from binding of ghrelin to GHS-R1a on somatotrophic cells in the pituitary, ghrelin induced activation of GHRH-neurons and inhibition of somatostatin neurons at the hypothalamus and activation of vagal afferents (Malagon *et al.* 2003, Popovic *et al.* 2003, Burdyga *et al.* 2006). The action of ghrelin is not completely specific for GH, because it also stimulates both lactotrophic and corticotrophic systems (Peino *et al.* 2000, Takaya *et al.* 2000, Arvat *et al.* 2001)

Normally des-acyl ghrelin does not affect GH secretion, since it cannot bind GHS-R1a. However, the role of des-acyl ghrelin in the regulation of GH was demonstrated in a study with transgenic mice overexpressing des-acyl ghrelin: the mice showed a small phenotype (Ariyasu *et al.* 2005).

Neither intravenous nor intracerebroventricular administration of obestatin has been shown to influence the secretion of GH in rats (Zhang *et al.* 2005, Bresciani *et al.* 2006, Nogueiras *et al.* 2007, Samson *et al.* 2007, Yamamoto *et al.* 2007). However, a recent study demonstrated that, under some conditions, obestatin could inhibit exogenous ghrelin action on GH release (Zizzari *et al.* 2007).

2.4.2 Food intake and regulation of energy homeostasis

Numerous studies have implicated ghrelin in both short- and long-term control of food intake and energy balance. Short-term regulation of food intake is concerned mainly with preventing overeating at each meal, and long-term regulation is primarily associated with maintaining the normal amounts of body fuel in the form of fat (Konturek *et al.* 2005). A process of energy homeostasis regulates body weight by controlling total energy intake and expenditure so that they are strictly matched over long periods of time (Cummings & Schwartz 2003). Ghrelin is one of the hormones that seems to play an important role in the regulation of food intake and body weight (Klok *et al.* 2007).

Meal initiation and short-term energy balance

Ghrelin may take part in meal initiation. Ghrelin administration to rodents stimulates short-term spontaneous food intake by encouraging animals to eat, even when they normally would not (Asakawa *et al.* 2001, Nakazato *et al.* 2001, Shintani *et al.* 2001, Lawrence *et al.* 2002). In humans, ghrelin administration also increases appetite and food intake (Wren *et al.* 2001, Levin *et al.* 2006). In addition, plasma ghrelin levels increase before a meal and decrease postprandially suggesting that ghrelin plays a role as a physiological meal controller (Cummings *et al.* 2001, Tschöp *et al.* 2001a). The actual reason for the preprandial rise of plasma ghrelin level in the meal initiation is not clear. In one study, the plasma ghrelin levels increased preprandially in humans without any clues related to time or food (Cummings *et al.* 2004). A very recent study with humans also demonstrated that the timing of plasma ghrelin peaks is related to habitual meal patterns and may rise in anticipation of eating rather than simply eliciting feeding (Frecka & Mattes 2008). However, in rats, both anticipation and feeding/fasting status have been shown to influence pre- and postprandial plasma ghrelin levels suggesting a role for ghrelin in the regulation of anticipatory processes involved in food intake and nutrient deposition (Drazen *et al.* 2006). The orexigenic

effects of ghrelin are mediated either through the vascular system or by activating vagal afferent neurons (Hewson & Dickson 2000, Williams *et al.* 2003).

Long-term energy balance

Ghrelin appears to be part of the body's mechanism for controlling the long-term energy balance. Daily peripheral administrations of ghrelin evoke a weight gain by decreasing the use of fat as a metabolic fuel in mice and rats, whereas intracerebroventricular administration causes a dose-dependent increase in food intake and body weight (Tschöp *et al.* 2000). Ghrelin has been shown to increase feeding also in GH-deficient rats, suggesting that ghrelin alters energy homeostasis independently of its GH-releasing action (Nakazato *et al.* 2001).

In humans, plasma ghrelin levels are negatively associated to body weight and percentage of body fat (Tschöp *et al.* 2001b, Park *et al.* 2005). This is thought to be physiologically important both in times of starvation and lack of food, when the elevated plasma ghrelin levels cause the body to be more energy-efficient and conversely in times of food abundance and positive energy balance, when low plasma ghrelin levels may induce the individual to eat less and to store less fat (Tschöp *et al.* 2000, Wortley *et al.* 2004).

2.4.3 Metabolism

Glucose metabolism

Ghrelin has been implicated in the regulation of both insulin action and glucose homeostasis. In human studies, ghrelin has been shown to inhibit insulin secretion and levels as well as increase glucose levels and reduce its disposal (Broglia *et al.* 2001, Broglia *et al.* 2003c, Broglia *et al.* 2004, Gauna *et al.* 2004, Lucidi *et al.* 2005, Damjanovic *et al.* 2006, Vestergaard *et al.* 2007, Vestergaard *et al.* 2008a). Des-acyl ghrelin has not been shown to display any endocrine effects, even though it was able to antagonize the effects of acylated ghrelin on insulin secretion and glucose levels in normal human subjects (Broglia *et al.* 2003c, Broglia *et al.* 2004). Simultaneous administration of des-acyl and acylated ghrelin has been shown to improve insulin sensitivity (Gauna *et al.* 2004).

Earlier studies with rats indicated that ghrelin stimulates the release of insulin in isolated pancreatic islets and increases its serum level (Date *et al.* 2002b, Lee *et*

al. 2002). However, subsequent studies have demonstrated that exogenous ghrelin inhibits insulin secretion in several species pointing to an inhibitory role of ghrelin in insulin secretion (Egido *et al.* 2002, Reimer *et al.* 2003, Salehi *et al.* 2004). Differences in the actions of ghrelin in insulin secretion may reflect the differences between species and cells used.

The hyperglycemic effect of ghrelin might result from the activation of glycogenolysis either indirectly by stimulation of catecholamine release or directly by affecting hepatocytes to modulate glycogen synthesis and gluconeogenesis (Murata *et al.* 2002, Broglio *et al.* 2003a). There are both clinical and *in vitro* data to suggest that ghrelin directly suppresses glucose-induced insulin secretion from the β -cells (Kageyama *et al.* 2005) and primary hepatocytes, while treatment with a ghrelin antagonist and des-acyl ghrelin has an inhibitory effect and it can counteract the stimulatory effect of acylated ghrelin on glucose release (Gauna *et al.* 2005, Dezaki *et al.* 2006). On the contrary, both des-acyl and acylated ghrelin have been shown to stimulate insulin secretion in rat insulinoma cells (Gauna *et al.* 2006). Recent human studies have revealed that ghrelin infusion decreased both basal and insulin-stimulated glucose disposal, and induced peripheral insulin resistance but did not affect hepatic glucose production (Vestergaard *et al.* 2008a). These effects were demonstrated to be independent of GH and cortisol actions (Vestergaard *et al.* 2008b).

Lipid metabolism

Ghrelin can also affect lipid metabolism. In the liver, ghrelin induces the expression of genes involved in lipogenic pathways and increases triglycerides content, whereas the activity of the stimulator of fatty acid oxidation, adenosine monophosphate-induced protein kinase (AMPK) is reduced (Barazzoni *et al.* 2005). In the muscle, ghrelin reduces the triglyceride content, increases the activity of mitochondrial oxidative enzyme activities and mRNA encoding uncoupling protein 2 (Barazzoni *et al.* 2005). Peroxisome proliferator-activated receptor γ (PPAR γ), which reduces the muscle fat content when activated, is also increased selectively in skeletal muscle (Barazzoni *et al.* 2005). Thus, ghrelin favors triglyceride deposition in the liver over the skeletal muscle. In addition, ghrelin acts directly on adipocytes. It stimulates lipogenesis in differentiated adipocytes *in vitro* and *in vivo* by increasing the levels of PPAR- α and the insulin-induced uptake of glucose, antagonizes lipolysis reducing the isoproterenol-stimulated lipolysis *in vitro* and stimulates the proliferation and differentiation of

preadipocytes (Kim *et al.* 2004, Muccioli *et al.* 2004, Thompson *et al.* 2004, Patel *et al.* 2006). In human subjects, ghrelin treatment increases concentrations of serum free fatty acid, and thus, lipolysis in skeletal muscle and subcutaneous fat (Vestergaard *et al.* 2008a, Vestergaard *et al.* 2008b).

There are few studies examining the effects of des-acyl ghrelin on lipid metabolism. Des-acyl ghrelin promotes lipogenesis directly *in vivo* and reduces isoproterenol-induced lipolysis in rat adipocytes by a mechanism independent of GHS-R 1a (Muccioli *et al.* 2004, Thompson *et al.* 2004).

2.4.4 Gastrointestinal effects

Ghrelin stimulates gastric motility and the secretion of gastric acid when administered peripherally or centrally in rodents (Masuda *et al.* 2000, Date *et al.* 2001, Fujino *et al.* 2003, Ariga *et al.* 2008). Ghrelin enhances gastric emptying in mice and rats as well as small bowel and colon transit, and can overcome postoperative ileus in rats (Trudel *et al.* 2002, Dornonville de la Cour *et al.* 2004, Kitazawa *et al.* 2005, Shimizu *et al.* 2006). Ghrelin can also stimulate interdigestive motility in rats (Fujino *et al.* 2003, Edholm *et al.* 2004, Wang *et al.* 2008). The vagal nerve is involved in these actions of ghrelin but also other vagus-independent mechanisms have been reported, such as the enteric nervous system and a direct effect on stomach (Masuda *et al.* 2000, Date *et al.* 2001, Dass *et al.* 2003, Depoortere *et al.* 2005, Xu *et al.* 2005, Levin *et al.* 2006). According to a recent study ghrelin may influence gastric acid secretion in synergy with gastrin (Fukumoto *et al.* 2008). In humans, ghrelin accelerates gastric emptying rate and the passage of food through the small intestine, and also, the motility of the upper GI tract and colon is stimulated by ghrelin (Murray *et al.* 2005, Levin *et al.* 2006). Ghrelin regulates secretion from the exocrine pancreas and protects against ethanol- or stress-induced gastric damage (Li *et al.* 2006b, Konturek *et al.* 2006).

2.4.5 Cardiovascular effects

Numerous studies have reported that ghrelin has a wide array of beneficial cardiovascular properties such as vasodilation (Nagaya *et al.* 2001a, Nagaya *et al.* 2001c, Okumura *et al.* 2002) and beneficial hemodynamic effects in healthy humans (Nagaya *et al.* 2001b, Enomoto *et al.* 2003) as well as improvement of left ventricular dysfunction and cardiac cachexia (Nagaya *et al.* 2001b, Nagaya &

Kangawa 2003a). These effects are most probably GH-independent (Isgaard *et al.* 2008). Similarly beneficial cardiovascular effects of ghrelin have been reported in animals particularly on protection against myocardial injury, hypertrophy and hypertension (Xu *et al.* 2007, Schwenke *et al.* 2008a, 2008b,). In addition, ghrelin reduces apoptosis and cardiotoxicity in cardiomyocytes (Baldanzi *et al.* 2002, Xu *et al.* 2008). However, there are reports with opposite data (Nagaya *et al.* 2001c, Frascarelli *et al.* 2003, Chang *et al.* 2004, Li *et al.* 2006a, Akashi *et al.* 2008, Soeki *et al.* 2008).

2.4.6 Immunomodulation

Ghrelin has been demonstrated to have various immunoregulatory effects in the immune system where GHS-R is widely distributed in different immune cells (Hattori 2009). Plasma ghrelin levels have been shown to be increased in immunological disorders in humans (Granado *et al.* 2005, Maruna *et al.* 2005, Karmiris *et al.* 2006, Zhao *et al.* 2006, Kerem *et al.* 2007). However, a contradictory result described lower plasma ghrelin levels in rheumatoid arthritis in human patients and in rats (Otero *et al.* 2004). In humans, ghrelin has been shown to act as an acute-phase reactant during post-operative stress, as well as in response to bacterial endotoxic shock (Vila *et al.* 2007, Maruna *et al.* 2008).

In animal experiments, lipopolysaccharide (LPS) and TNF α administrations have been shown to decrease fasting plasma ghrelin levels when given a single injection but increase when given repeated injections (Basa *et al.* 2003, Hataya *et al.* 2003, Wang *et al.* 2006, Endo *et al.* 2007). Ghrelin attenuates septic shock and inflammation as well as down-regulating proinflammatory cytokines in animal studies (Chang *et al.* 2003, Wu *et al.* 2005, Wu *et al.* 2007c, Wu *et al.* 2007b, Wu *et al.* 2007c, Dembinski *et al.* 2003, Kasimay *et al.* 2006, Iseri *et al.* 2008, Sibilias *et al.* 2006, Dixit *et al.* 2009). *In vitro*, ghrelin reportedly inhibits the expression of pro-inflammatory cytokines (interleukin 1 β , (IL-1 β), IL-6, TNF α), release the anti-inflammatory cytokine IL-10 and reduce proliferation in activated T cells (Dixit *et al.* 2004, Li *et al.* 2004, Xia *et al.* 2004, Waseem *et al.* 2008). Down-regulation of ghrelin using a RNA interference method has been recently shown to increase Th1 cytokines and IL-17 secretion in primary human T cells (Dixit *et al.* 2009).

Ghrelin has been shown to have numerous other effects e.g. in the reproductive system including gonadal effects, possible participation in the regulation of gonadotropin secretion and the timing of puberty, in bone

physiology, stimulation of prolactin, adenocorticotrophic hormone (ACTH), argininevasopressin (AVP), promotion of slow-wave sleep, memory retention, anxietylike behavior and stimulation of milk production (Garcia *et al.* 2007, Soares & Leite-Moreira 2008). In addition, ghrelin has a role in the regulation of proliferation, apoptosis and differentiation of various normal and neoplastic cell lines, via mechanism dependent and independent of GHS-R1a (Soares & Leite-Moreira 2008).

2.5 Animal models used for studying ghrelin and the ghrelin receptor

The physiological function of ghrelin and its receptor has been studied using transgenic and knock-out mouse models. When ghrelin knock-out (GHRL KO) mice were fed a standard diet, they did not demonstrate any pathophysiological changes (Sun *et al.* 2003, Wortley *et al.* 2004, Zigman *et al.* 2005). However, when on a high fat diet (HFD), these mice proved to be resistant to diet induced obesity (DIO), exhibiting reduced adiposity and increased energy consumption (Wortley *et al.* 2005, Zigman *et al.* 2005). In addition, GHRL KO mice display a phenotype of enhanced insulin release and nearly normal glycemia under HFD conditions (Dezaki *et al.* 2006). Ablation of ghrelin in ob/ob mice increases insulin release and thereby markedly decreases hyperglycemia by reducing the expression of Ucp2 mRNA in the pancreas evidence for an important role for ghrelin in β -cell physiology (Sun *et al.* 2006).

Similarly to GHRL KO mice, GHSR KO mice show no phenotypic differences compared to their wild-type strain littermates (Sun *et al.* 2004). The body weights of adult GHSR KO mice are slightly decreased, which is consistent with the ability of ghrelin to act as an amplifier of GH pulsatility (Sun *et al.* 2004). GHSR KO mice also resisted diet-induced weight gain and had lower adipose weight although, unlike GHRL KO mice, their food intake was lower (Zigman *et al.* 2005). GHSR KO mice have been shown to promote insulin sensitivity and metabolic flexibility and to be protected from several fatty diet-induced features of the metabolic syndrome (Longo *et al.* 2008). This is due to convergent changes in the intake, absorption and utilization of energy (Longo *et al.* 2008).

Transgenic mouse over-expressing des-acyl ghrelin exhibit lower body weight; and length, as well as serum GH and insulin-like growth factor (IGF)-1-levels (Ariyasu *et al.* 2005). Another mouse model over-expressing ghrelin did not show differences in food intake, and, in addition, a very recent study in mice

over-expressing acylated ghrelin showed no changes in body weight but increased hyperphagia, elevated energy consumption and glucose intolerance, as well as, decreased glucose stimulated insulin secretion and leptin sensitivity (Wei *et al.* 2006, Bewick *et al.* 2009).

When both ghrelin and the ghrelin receptor are knocked out, the mice exhibit energy balance changes, which are not observed in single gene-deficient mice on a standard diet (Pfluger *et al.* 2008). Thus, there has been speculation about the existence of additional, as yet unknown, molecular components of the endogenous ghrelin system.

Acquired ghrelin deficiency using a synthetic oligonucleotide that neutralized its effects during adulthood resulted in lower body weight and fat mass in diet-induced obesity (DIO) mice (Shearman *et al.* 2006). Antibody formation against ghrelin using ghrelin vaccination has also been shown to reduce weight gain in rats and pigs (Zorrilla *et al.* 2006, Vizcarra *et al.* 2007). In contrast, antibodies against acylated ghrelin did not affect food intake or body weight gain but did inhibit the orexigenic effect of ghrelin in a mouse model with DIO (Lu *et al.* 2009). Despite the contradictions in the studies of food intake and body weight in transgenic models, the data consistently indicates that ghrelin is important in the control of glucose homeostasis.

2.6 Regulation of plasma ghrelin concentrations

The factors that influence plasma ghrelin concentrations have been investigated in numerous studies. The most important factors are reviewed in this chapter. The factors involved in the regulation of ghrelin secretion are not fully known. Changes in energy intake are acutely reflected in the plasma ghrelin concentrations and feeding is considered to be the most important regulating factor in this respect. Ghrelin levels are affected by prolonged states of metabolic changes. Obese subjects generally have lower plasma ghrelin levels than lean individuals.

Fasting and food intake

Plasma ghrelin levels fluctuate during the day by rising before and then falling after a meal, thus showing a typical secretion pattern (Cummings *et al.* 2001, Shiiya *et al.* 2002). The plasma acylated ghrelin level declined after ingestion of a balanced meal (Lucidi *et al.* 2004, Al Awar *et al.* 2005). Prolonged fasting in

healthy subjects has also been shown to result in a circadian pattern in plasma ghrelin levels (Espelund *et al.* 2005, Natalucci *et al.* 2005).

The volume of the eaten meals does not seem to affect plasma ghrelin levels, since gastric distension e.g. by water, has no influence on plasma ghrelin levels (Tschöp *et al.* 2000, Shiiya *et al.* 2002, Erdmann *et al.* 2003). In addition, cephalic vagal activation did not contribute to the postprandial alterations of plasma ghrelin levels (Erdmann *et al.* 2004). The quantity of calories ingested seems to determine the postprandial suppression of plasma ghrelin (Tschöp *et al.* 2000, Nakazato *et al.* 2001, Callahan *et al.* 2004). Ghrelin secretion has been suggested to be regulated by learned anticipation independent of deprivation, in addition to regulation by the nutrient status (Sugino *et al.* 2002, Drazen *et al.* 2006).

The influence of different macronutrients on postprandial plasma ghrelin levels has been widely studied but results vary between the studies. Plasma total and acylated ghrelin levels appear to be reduced after carbohydrate-rich test meals but return back to the basal levels (Erdmann *et al.* 2003, Monteleone *et al.* 2003, Erdmann *et al.* 2004, Tentolouris *et al.* 2004, Blom *et al.* 2006, Foster-Schubert *et al.* 2008). A high fat meal has been observed not only to induce a decrease in circulating plasma ghrelin but also to increase postprandial plasma ghrelin levels (Erdmann *et al.* 2003, Monteleone *et al.* 2003, Erdmann *et al.* 2004, Greenman *et al.* 2004, Foster-Schubert *et al.* 2008). Plasma acylated ghrelin levels have been observed to decrease or not change after a high fat meal (Tentolouris *et al.* 2004, Al Awar *et al.* 2005, Tannous dit El Khoury *et al.* 2006, Foster-Schubert *et al.* 2008). Protein ingestion has been reported to reduce, increase or have no effects on circulating plasma ghrelin concentrations although a high protein meal has been shown to decrease the amount of acylated plasma ghrelin (Erdmann *et al.* 2003, Erdmann *et al.* 2004, Greenman *et al.* 2004, Al Awar *et al.* 2005, Blom *et al.* 2006, Bowen *et al.* 2006, Foster-Schubert *et al.* 2008, Tannous dit El Khoury *et al.* 2006). In most studies, no associations between postprandial plasma total or acylated ghrelin levels and measurements of satiety have been found (Erdmann *et al.* 2004, Blom *et al.* 2006, Smeets *et al.* 2008).

Glucose and insulin

Insulin decreases plasma total ghrelin levels in healthy normal-weight and overweight persons at pharmacological and pathophysiological concentrations (Lucidi *et al.* 2002, Schaller *et al.* 2003, Leonetti *et al.* 2004, St-Pierre *et al.* 2007). Plasma acylated ghrelin levels were also reduced by insulin among insulin-

sensitive overweight or obese patients but did not have an influence among insulin-resistant obese or healthy subjects (St-Pierre *et al.* 2007, Weickert *et al.* 2008). It has been shown that insulin is needed for prandial suppression of ghrelin (Murdolo *et al.* 2003, Spranger *et al.* 2003). Both oral and intravenous glucose loads diminish ghrelin secretion in humans (Broglia *et al.* 2002, Caixas *et al.* 2002, Nakagawa *et al.* 2002, Shiiya *et al.* 2002).

Estrogen

The effect of estrogen on plasma and tissue ghrelin has been investigated in several human and animal studies. Some of the findings are controversial but the majority of the studies indicate that estrogen increases plasma ghrelin levels. Estrogen replacement therapy for six months has been reported to increase plasma total ghrelin levels in severe undernutrition associated with anorexia nervosa (Grinspoon *et al.* 2004). Furthermore, combined estrogen-progestin therapy in postmenopausal women for two years and in women with polycystic ovary syndrome for three cycles was claimed to elevate plasma total ghrelin concentrations (Di Carlo *et al.* 2007, Sagsoz *et al.* 2009). However, in another study, the plasma total ghrelin concentrations do not differ in eu- and hypoestrogenemic women and estrogen therapy and short-term exposure of estrogen do not alter plasma ghrelin levels in some studies (Purnell *et al.* 2003, Lebenthal *et al.* 2006, Veldhuis *et al.* 2006, Lambrinouadaki *et al.* 2008). In contrast to these findings, both oral and transdermal estrogen therapies for three months decreased the plasma total ghrelin level in women with metabolic syndrome (Chu *et al.* 2006). Transdermal estrogen supplementation enhanced hypothalamus-pituitary sensitivity to ghrelin and increased nighttime plasma concentrations of acylated ghrelin in healthy postmenopausal women (Kok *et al.* 2008, Paulo *et al.* 2008).

Data from animal studies suggest that estrogen can influence the regulation of ghrelin in stomach and in plasma. Aromatase, an estrogen synthetase, has been shown to be expressed in parietal cells of the rat stomach and therefore these cells are capable of producing and secreting a substantial amount of estrogen (Ueyama *et al.* 2002). The levels of plasma ghrelin and gastric ghrelin mRNA and the number of ghrelin producing cells has been found to be transiently increased in female rats after ovariectomy (Matsubara *et al.* 2004). In addition, ghrelin expressing cells were demonstrated to express estrogen receptor alpha (α) (Matsubara *et al.* 2004). Gastric estrogen stimulated directly ghrelin expression

and its production in the rat stomach (Sakata *et al.* 2006). Furthermore, ghrelin producing cells and aromatase mRNA-expressing cells appear to be located close together in the gastric mucosa, suggesting that ghrelin containing cells are exposed to gastric estrogen (Sakata *et al.* 2006). Real time polymerase chain reaction (RT-PCR) studies of female mice trigeminal ganglia demonstrate that ghrelin mRNA is upregulated by over 5-fold at the high estrogen stages compared to its level at the cycle over the low estrogen stage of the cycle (Puri *et al.* 2006). Ovariectomy decreased plasma ghrelin levels similarly with inhibition of orexigenic effects of ghrelin (Clegg *et al.* 2007).

Leptin

Leptin is an adipocyte-derived hormone and a key negative regulator of feeding and energy metabolism (reviewed by (Rasouli & Kern 2008)). Leptin and ghrelin are known to have opposite effects on appetite and activation of neuropeptide Y(NPY)/AgRP neurons, and react in opposite ways to weight gain (Chan *et al.* 2004, Shintani *et al.* 2001, Traebert *et al.* 2002). Leptin has been suggested to be a potential determinant of the circulating ghrelin concentrations (Barazzoni *et al.* 2003, Kempa *et al.* 2007). Very recent studies have demonstrated that leptin suppresses the action of ghrelin in ARC/NPY neurones (Kohno *et al.* 2007, Kohno *et al.* 2008). In rats, hyperleptinemia prevents the increase in plasma ghrelin concentrations stimulated by caloric restriction (Barazzoni *et al.* 2003). Leptin has been shown to increase the ghrelin mRNA expression in rat stomach, though there are two other studies reporting opposite results (Asakawa *et al.* 2001, Toshinai *et al.* 2001, Kamegai *et al.* 2004). A recent study also demonstrated that leptin directly suppresses ghrelin expression in the rat stomach and that the elevation of gastric ghrelin expression level in the fasting state is mediated, at least in part, by the reduced gastric leptin level (Zhao *et al.* 2008). Some human studies have observed negative associations between plasma ghrelin and leptin concentrations (Tschöp *et al.* 2001b, Pöykkö *et al.* 2003b), although not all studies have demonstrated this association (Ikezaki *et al.* 2002, Haqq *et al.* 2003, Purnell *et al.* 2003, Rosicka *et al.* 2003, Chan *et al.* 2004). In addition, no changes in plasma ghrelin levels in response to several days of treatment with physiological or pharmacological doses of exogenous leptin were observed (Chan *et al.* 2004). Due to these conflicting results, the regulatory role of leptin in the ghrelin synthesis/secretion remains unclear.

Other hormones

Several other hormonal factors might affect plasma ghrelin levels. Somatostatin, glucocorticoids, thyroid hormones, urocortin and several gastrointestinal hormones such as pancreatic polypeptide, gastrin and glucagon-like peptide 1 reportedly decrease plasma ghrelin levels (Norrelund *et al.* 2002, Asakawa *et al.* 2003, Davis *et al.* 2004, Lippl *et al.* 2004, Otto *et al.* 2004, Tan *et al.* 2004, Gimenez-Palop *et al.* 2005, Braclik *et al.* 2008,). At present, the ability of melatonin to reduce plasma ghrelin levels is far from clear (Mustonen *et al.* 2004, Canpolat *et al.* 2006, Aydin *et al.* 2008).

In addition to estrogen, other sex hormones (e.g. testosterone) have also been found to have an impact on plasma ghrelin levels (Pagotto *et al.* 2003, Gambineri *et al.* 2005, Lebenthal *et al.* 2006).

Gender and age

Gender differences in plasma ghrelin levels have been described in many studies. Women have reported to have higher plasma total, acylated and des-acyl ghrelin levels than men (Barkan *et al.* 2003, Greenman *et al.* 2004, Akamizu *et al.* 2005, Barazzoni *et al.* 2007, Makovey *et al.* 2007). However, several reports demonstrate no gender differences (Pöykkö *et al.* 2003b, Akamizu *et al.* 2006, Barazzoni *et al.* 2007, Zou *et al.* 2008).

Some studies have reported that both plasma total and acylated ghrelin levels are reduced with increasing age, although no age-related changes have been detected in the plasma des-acyl ghrelin concentrations (Rigamonti *et al.* 2002, Akamizu *et al.* 2006, Schutte *et al.* 2007, Kozakowski *et al.* 2008, Prodam *et al.* 2009). However, most studies have not been able to observe age differences in plasma ghrelin levels, and only one study has described a positive association between the plasma ghrelin concentration and age (Cummings *et al.* 2001, Sturm *et al.* 2003, Langenberg *et al.* 2005, Vilarrasa *et al.* 2005, Bertoli *et al.* 2006, Yukawa *et al.* 2006, Makovey *et al.* 2007, Altinkaynak *et al.* 2008, Gonnelli *et al.* 2008, Zou *et al.* 2008, Tai *et al.* 2009). Obesity might influence the association between the plasma ghrelin concentration and age in young women but also other modifying factors such as menopause are believed to be involved (Schutte *et al.* 2007, Sowers *et al.* 2008).

Bariatric surgery

Bariatric surgery has been successfully used for obesity treatment for morbid obese patients (Brolin 2002). Bariatric surgery includes radical changes in the GI anatomy and physiology. The influence of bariatric surgery on circulating ghrelin levels has been intensively studied but the findings are controversial. It seems that different surgical techniques can cause quite different effects.

Circulating plasma ghrelin levels are most often reduced after the Roux-en-Y gastric bypass operation (Cummings *et al.* 2002, Geloneze *et al.* 2003, Lin *et al.* 2004, Frühbeck *et al.* 2004). After vertical banded gastroplasty or adjustable gastric banding, plasma ghrelin levels appear in some cases to be increased but in others to be unchanged (Frühbeck *et al.* 2004, Hanusch-Enserer *et al.* 2004, Stoeckli *et al.* 2004, Mariani *et al.* 2005, Shak *et al.* 2008). The increase in plasma ghrelin levels has also been observed after biliopancreatic diversion (Adami *et al.* 2004, Frühbeck *et al.* 2004, Garcia-Unzueta *et al.* 2005, Valera Mora *et al.* 2007).

2.6.1 Circulating ghrelin in different pathophysiological conditions

Circulating plasma ghrelin levels have been assessed in numerous different pathophysiological conditions in humans. Plasma ghrelin levels have been found to be elevated in Pader-Willi Syndrome, anorexia and bulimia nervosa, lean type 2 diabetics, patients, with chronic liver disease, renal failure and in diseases with cachexia such as chronic heart failure and lung cancer (DelParigi *et al.* 2002, Shiiya *et al.* 2002, Tanaka *et al.* 2002, Yoshimoto *et al.* 2002, Haqq *et al.* 2003, Nagaya & Kangawa 2003b, Scacchi *et al.* 2003, Shimizu *et al.* 2003, Tacke *et al.* 2003).

The plasma ghrelin level is reduced in human obesity, type 1 diabetes, type 2 diabetes, hypertension, insulin resistance, non-alcoholic liver disease and hyperthyroidism (Tschöp *et al.* 2001b, Ravussin *et al.* 2001, Shiiya *et al.* 2002, Marchesini *et al.* 2003, Pöykkö *et al.* 2003b, Riis *et al.* 2003, Soriano-Guillen *et al.* 2004).

Compared to healthy controls similar or lower plasma ghrelin concentrations have been observed in other pathophysiological conditions such as acromegaly and growth hormone deficiency (Cappiello *et al.* 2002, Kopchick *et al.* 2002, Freda *et al.* 2003, Stewart 2003, Giavoli *et al.* 2004, Malik *et al.* 2004, Jarkovska *et al.* 2006).

2.7 Ghrelin and atherosclerosis

2.7.1 Atherosclerosis

Atherosclerosis is a complex and multifactorial chronic inflammatory disease caused by the accumulation of lipids and fibrous elements in large or medium-sized arteries leading to thickening and hardening of the vessel wall (Ross 1999). Atherosclerosis develops progressively over decades and the lesion formation in the intima layer of the vessel wall can lead to ischemia of the heart, brain, or extremities resulting in infarction (Fig. 3) reviewed by (Libby 2002). The factors involved in atherosclerosis have been intensively studied during the last decades. Based on the experimental and clinical relationship between hypercholesterolemia and atheroma, atherosclerosis was earlier considered mostly as a lipid disease. Currently inflammation is now considered to be an important factor in the progression of the disease (Libby 2002).

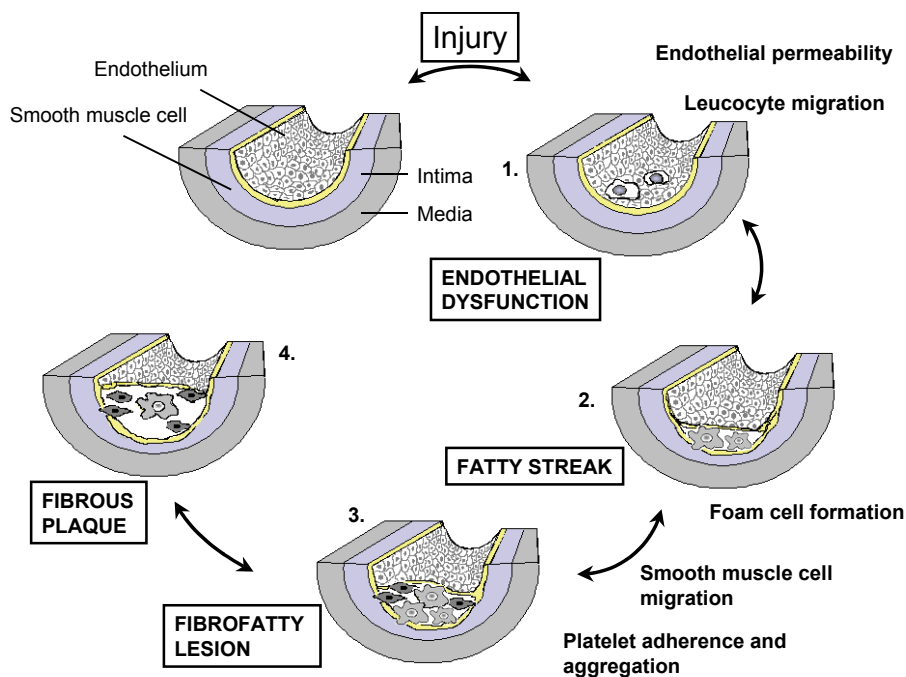


Fig. 3. The progression of atherosclerotic lesion formation according to the response-to-injury hypothesis of atherosclerosis. 1) The earliest changes occur in endothelium including increased endothelial permeability to lipoproteins, the up-regulation of endothelial adhesion molecules and migration of leukocytes into the artery wall e.g. mediated by Ox-LDL, MCP-1, IL-8 and MCS-F. 2) Fatty streaks consisting initially of cholesterol-laden monocytes, macrophages (foam cells) and T lymphocytes are formed. Later, various factors mediate migration of smooth muscle cells, activation of T-cells, formation of foam cells and adherence and aggregation of platelets. 3) As fatty streaks progress to become intermediate and advanced lesions, a fibrous cap is formed covering a mixture of leukocytes, lipid, and debris, which form a necrotic core. These lesions expand their shoulders. 4) Rupture of fibrous cap or ulceration of the fibrous plaque can rapidly lead to thrombosis and usually occurs at a site of thinning of the fibrous cap that covers the advanced lesion. (Modified from Ross 1999)

Atherosclerosis is initiated by endothelial dysfunction according to the response-to-injury hypothesis (Ross 1999) (Fig. 3). A complex endothelial dysfunction is induced by elevated and modified LDL, free radicals, hypertension, infectious microorganisms, shear stress, toxins from cigarette smoke or other factors causing a proinflammatory stimulus (Ross 1999, Packard & Libby 2008). The dysfunctional endothelium produces adhesion and vasoactive molecules,

cytokines and growth factors and is more permeable to lipoproteins and other plasma components as well as having reduced anticoagulant properties (Ross 1999). This leads to adhesion of leukocytes to vascular endothelial cells and infiltration of the cells into the intima layer of arterial wall (Ross 1999). The hallmark of early atherosclerotic lesions is the formation of fatty streaks composed of cholesterol-laden macrophages called foam cells and T lymphocytes with extracellular lipids (Figure 4).

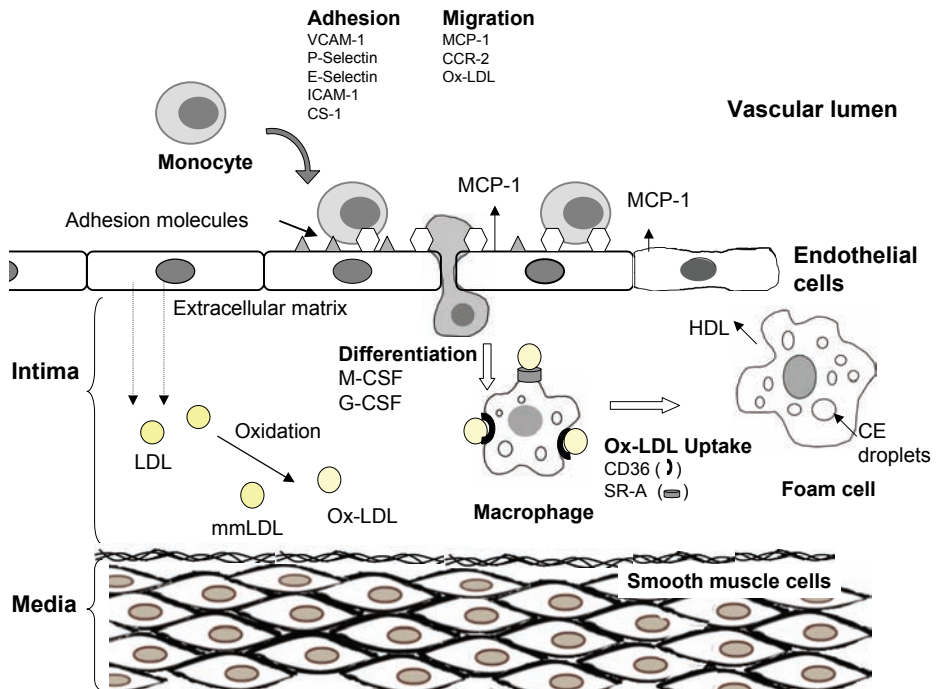


Fig. 4. Initiation events in the development of a fatty streak lesion. LDL is modified by oxidation in the subendothelial space into minimally oxidized LDL (mmLDL) and further into extensively oxidized LDL (Ox-LDL). Monocytes become attached to endothelial cells that have been induced to express cell adhesion molecules by modified LDL and inflammatory cytokines. Adherent monocytes migrate into the subendothelial space and differentiate into macrophages. Uptake of Ox-LDL via scavenger receptors leads to foam cell formation. Ox-LDL derived cholesterol is stored in lipid droplets in the form of cholesteryl fattyacylestes or exported to extracellular HDL acceptors. (Modified from Glass & Witztum 2001)

Accumulation of white blood cells (especially monocytes) into the intima is an essential part of the progression of atherosclerosis (Figure3). Adhesion molecules, P-selectin and vascular cell adhesion molecule-1 (VCAM-1), mediate the attachment of circulating monocytes and lymphocytes. (Packard & Libby 2008) Chemoattractant factors particularly MCP-1 produced by the vascular wall cells respond to modified lipoproteins and recruit leukocytes through the CCR2 receptor on their surface (Charo & Taubman 2004, Packard & Libby 2008). Within the intima, monocytes mature into macrophages under the influence of monocyte colony stimulating factor (M-CSF) and granulocyte colony stimulating (G-CSF). M-CSF also increases the expression of scavenger receptors to take up modified lipoproteins through endocytosis in macrophages. Accumulation of cholesterol esters in the cytoplasmic droplets converts macrophages into foam cells i.e., cholesterol-laden macrophages, characteristic for early-stage atherosclerosis. Simultaneously, macrophages trigger an inflammatory response via secretion of numerous growth factors and cytokines such as TNF α and IL-1 β . The inflammatory activation inside the arterial wall stimulates the migration and proliferation of smooth muscle cells (SMC) inside the intima. SMCs produce extracellular matrix proteins forming a fibrous cap on top of the lesion. The rupture of the vulnerable fibrous cap causes the various and serious clinical manifestations of atherosclerosis. (Glass & Witztum 2001, Libby 2002).

2.7.2 Ghrelin in atherosclerosis

Only a few studies have explored the role of ghrelin in atherosclerosis. Current experimental studies suggest beneficial cardiovascular, anti-inflammatory and anti-apoptotic effects of ghrelin in the cardiovascular system. These findings tend to suggest that ghrelin might have a beneficial role in atherosclerosis but some contradictory data also exist. Only a few studies are available which have demonstrated the direct effects of ghrelin on atherosclerosis or the association between plasma ghrelin levels and an atherosclerotic measurement, i.e. intima-media thickness, in human patients.

The influences of ghrelin on factors contributing to atherosclerosis have been demonstrated in only a few studies on endothelial cells, mononuclear cells, macrophages and smooth muscle cells. Monocyte adhesion to endothelial cells has been studied in two studies. Ghrelin decreased monocyte adhesion in the study of Li *et al.* but in another study by Skilton *et al.*, ghrelin did not affect monocyte adhesion and increased the expression of adhesion molecules in human

vascular endothelial cells (HUVECs) (Li *et al.* 2004, Skilton *et al.* 2005). Ghrelin inhibits chemotactic cytokine production (MCP-1, IL-8) in HUVECs and (TNF α) in LPS-stimulated mouse macrophage, the production of proinflammatory cytokines IL-1 β , IL-6 and TNF α in stimulated peripheral blood mononuclear cells and T-cells and increases IL-10 production in macrophages (Dixit *et al.* 2004, Li *et al.* 2004, Waseem *et al.* 2008). Ghrelin inhibited also CD40 expression in HUVECs pointing to further anti-inflammatory properties of ghrelin (Zhang *et al.* 2007). Studies with human blood mononuclear cells have demonstrated that ghrelin expression appears to associate with the expressions of TNF α , IL-1b and IL-6 (Mager *et al.* 2008). Ghrelin has been also shown to prevent the endotoxin-induced release of IL-6 from peritoneal macrophages isolated from adjuvant-induced arthritic rats (Granado *et al.* 2005).

Ghrelin has been suggested to modulate vasoactive factors *in vitro*. Ghrelin inhibited angiotensin II-induced contraction and proliferation in human aortic smooth muscle cells (Rossi *et al.* 2009). In contrast, ghrelin increased the proliferation in H9c2 cardiomyocytes (Pettersson *et al.* 2002). Since apoptosis is one of the proatherogenic events in atherogenesis, ghrelin has been reported to inhibit apoptosis in different cells in numerous studies (Baldanzi *et al.* 2002, Kim *et al.* 2004, Kim *et al.* 2005, Granata *et al.* 2006, Zhao *et al.* 2007, Xu *et al.* 2008, Zhan *et al.* 2008, Kui *et al.* 2009).

The ghrelin receptor density has been demonstrated to increase in atherosclerotic carotid arteries and saphenous vein grafts (Katugampola *et al.* 2001). Carotid IMT was negatively associated with serum ghrelin levels in a population of older subjects with the metabolic syndrome and in elderly hypertensives and no association was observed between IMT and plasma ghrelin levels in a population of kidney transplant patients (Kotani *et al.* 2006, Genis *et al.* 2007, Yano *et al.* 2008). In addition, some studies have examined the relationship between a low plasma ghrelin concentration and risk factors of atherosclerosis such as high blood pressure, obesity and insulin resistance in cross-sectional population-based studies and also increased oxidative stress in obese subjects (Pöykkö *et al.* 2003b, Suematsu *et al.* 2005, Zwiriska-Korczała *et al.* 2007).

3 Aims of the present study

The aim of this study was to elucidate the role of ghrelin in atherosclerosis. The specific aims were:

1. To study the association of plasma total ghrelin level and early atherosclerosis. This aim was carried out by determining the association between plasma total ghrelin level and intima-media thickness of the carotid arteries within middle-aged subjects in a cross-sectional population-based study.
2. To study the associations between plasma ghrelin levels and established risk factors of atherosclerosis. The association between plasma ghrelin and lipid levels and parameters of lipid metabolism were assessed in human subjects and the effect of estrogen on plasma ghrelin level in postmenopausal women.
3. To study the effect of ghrelin in cell cultures. This aim was carried out by studying the effects of ghrelin on monocyte adhesion to endothelial cells, oxidized LDL binding and acetylated LDL uptake in macrophages. The cellular mRNA expressions of adhesion molecules and MCP-1 were also investigated.
4. To demonstrate the effect of ghrelin on atherogenesis using an animal model of atherosclerosis.

4 Subjects and Methods

4.1 Subjects

4.1.1 Human Subjects (I, II)

The major clinical characteristics of subjects in the OPERA cohort study (Study I) and in the ERT study (Study II) are summarized in Table 1. The studies were carried out according to the principles of the Declaration of Helsinki. The Ethical Committees of the Faculty of Medicine in the University of Oulu (Study I, II) and Oulu Deaconess Institute (Study II) approved the studies.

Table 1. Basic clinical characteristics of study subjects.

Variable	OPERA Study (I)	ERT Study (II)
Total (n)	1037	64
Females (n)	519	64
Males (n)	518	0
Age (years)	51.3 ± 6.0	54.4 ± 3.1
BMI (kg/m ²)	27.7 ± 4.6	26.4 ± 2.5
Serum Lipids (mmol/l)		
Cholesterol		
Total	5.7 ± 1.1	6.42 ± 0.88
LDL	3.5 ± 0.9	4.21 ± 0.81
HDL	1.35 ± 0.38	1.60 ± 0.38
Triglycerides	1.6 ± 1.0	1.36 ± 0.56
Blood glucose (mmol/l)	4.7 ± 1.5	4.44 ± 0.42
Blood pressure (mmHg)		
Systolic	148 ± 22	141 ± 19
Diastolic	89 ± 12	85 ± 8
Current smoking (%)	28.6	26.3

OPERA study (I)

OPERA is a population-based study focused on the risk factors and disease end-points of atherosclerotic cardiovascular diseases. The study population and selection criteria have been described earlier in detail (Kauma *et al.* 1996, Rantala *et al.* 1999). Briefly, the study population consisted of 600 hypertensive (300 men

and 300 women) and 600 control subjects (300 men and 300 women) living in the city of Oulu. The hypertensive subjects were randomly selected by age stratification from the Social Insurance Institute register for reimbursement of antihypertensive medication. According to the register, the subjects were allowed to receive a special refund (higher reimbursement class) of antihypertensive medication endorsed later than August 1980. The criteria for the special refund in 1990 were as follows: diastolic blood pressure 105mmHg or more during a few months' follow-up, unless the patient demonstrated signs of target organ damage (left ventricular hypertrophy, coronary artery disease, heart failure, cerebrovascular disease, renal insufficiency or hypertensive retinopathy), in which case the diastolic blood pressure (DBP) limit was 100mmHg. If the patient was young (men below 50 and women below 40 years), had a family history of cardiovascular disease or sudden death at an early age, had diabetes or severe dyslipidemia or had systolic blood pressure (SBP) above 180mmHg (above 200mmHg in subjects older than 50 years), he or she was eligible for the higher reimbursement even when subject's DBP level during the follow-up was 100–104mmHg. In the case of nephropathy, a DBP level of 95mmHG was sufficient for entitlement to the specific refund. For each hypertensive subject, an age- and sex-matched control was randomly selected from the Finnish National Health Register (including all inhabitants), excluding the subjects with a right to reimbursement for antihypertensive medication. The original study consisted of 1045 subjects (518 hypertensive and 530 controls), who were collected during 1991–1993. A total of 1040 plasma samples were available for ghrelin measurements in the present study.

Estrogen replacement therapy study (II)

Samples were collected during 1993–1994 and were originally designed to investigate the effects of ERT on the regulation of LDL cholesterol, carbohydrate metabolism and blood pressure in postmenopausal women. Seventy-nine postmenopausal women seeking for hormone substitution therapy for climacteric symptoms volunteered to participate in the study. Since the study was focused on the effects of estrogen alone (not progesterone), hysterectomized women were chosen to avoid the need for providing progesterone for endometrium protection. The women were randomized to receive either oral estrogen (PE) (n = 39) or transdermal estrogen (TE) (n = 40) for 6 months as described in detail earlier

(Karjalainen *et al.* 2000a, Karjalainen *et al.* 2000b). Plasma samples from 64 participants were available (29 for PE and 35 for TE) for ghrelin measurement.

4.1.2 Experimental cells and animals (III, IV)

The experimental animals and cells used are summarized in Table 2. The Animal Ethics Committee of the Provincial State Office of Southern Finland approved the animal studies.

Table 2. Animals and cells used in Studies III and IV.

Animals and cells	Source	Used in Study
Mice		
C57BL/6	Experimental Animal Core Facility barrier of the University of Oulu	IV
LDLR KO mice (female)	The Jackson Laboratory	IV
Cells/Cell lines		
EA.hy 926; Human endothelial cells	Kindly provided by Dr. Cora-Jean S. Edgell, The Carolina Cardiovascular Biology Center, North Carolina, USA	III
THP-1; Human monocytic cells	American Type Tissue Culture	III
J774.A1; Mouse monocyte/ macrophages	American Type Tissue Culture	III
Thioglycollate elicited mouse (C57BL/6) peripheral macrophages	Experimental Animal Core Facility barrier of the University of Oulu	III

Study design (IV)

Two different studies using mice were performed. The first study was a preliminary one conducted to assess the responses of different vaccines on antibodies formed against ghrelin and plasma ghrelin levels in C57BL/6 mice. The second study aimed to test the effect of ghrelin vaccination and altered ghrelin levels on atherosclerosis in LDLR KO mice. The study protocols, study groups with vaccines and diets are illustrated in Figure 5.

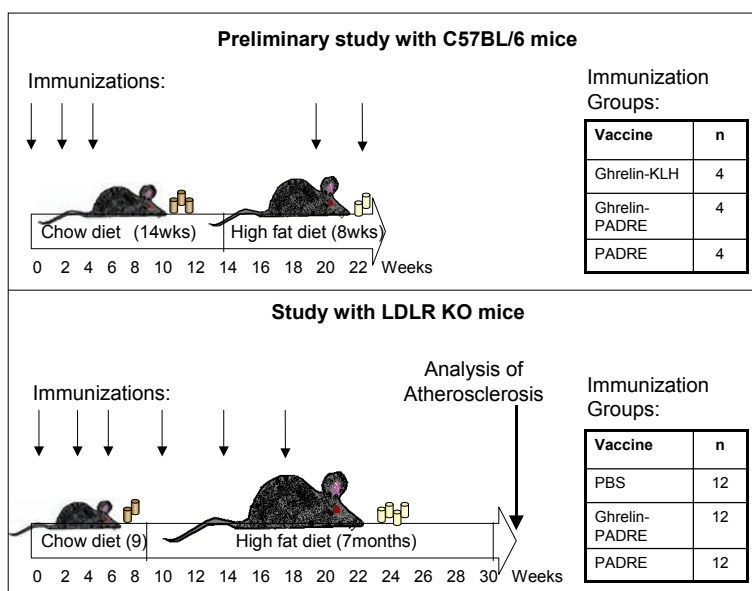


Fig. 5. Study protocols for C57BL/6 and LDLR KO mice. Times of immunizations are indicated with small arrows. The study groups and the durations of diets: chow diet (T.2018.12, Harlan Teklad) and high fat diet (TD88137, Harlan Teklad) are presented.

Ghrelin-PADRE vaccine was a synthetic peptide comprising of first pan DR epitope (PADRE) and mouse octanoylated ghrelin (NeoMPS, Strasbourg, France). In the creation of the ghrelin-keyhole limpet hemocyanin (KLH) vaccine, mouse ghrelin (octanoylated from Phoenix Pharmaceuticals) was coupled to KLH using a NHS coupling procedure (Pierce Chemicals, Inc.).

Mice were subjected to a primary and then booster immunizations as shown by the arrows in Figure 4. In the primary immunization, a single dose of antigen (50 μ g) emulsified in Freund's complete adjuvant 1:1 (v:v) was given subcutaneously and in the booster immunizations, antigen (25 μ g) emulsified in Freund's incomplete adjuvant was given intraperitoneally. Plasma or serum samples (2 hour fasting) were taken from the hind leg before and 5 weeks after the first immunization and then monthly. At the end of the experiments, EDTA-plasma from *vena cava* and tissue samples were collected. Weight gain and food intake were followed during the studies. Atherosclerosis was determined at the end using LDLR KO mice.

4.2 Methods

4.2.1 Laboratory analyses

The main biochemical laboratory analyses used are listed in Table 3.

Table 3. Biochemical laboratory analysis (I, II, IV).

Clinical parameter	Method	Source/Laboratory	Study no.
Plasma total cholesterol	Enzymatic colorimetric method	Boehringer Diagnostica, Roche	I, II IV
Plasma triglycerides	Enzymatic colorimetric method	Boehringer Diagnostica, Roche	I, II IV
VLDL, IDL and LDL cholesterol	Enzymatic colorimetric method from fraction isolated by ultracentrifugation.	Boehringer Diagnostica, Roche	I, II
LDL cholesterol	Calculated by subtracting cholesterol in HDL from the cholesterol concentration of the VLDL-free fraction.		II
HDL cholesterol	Enzymatic colorimetric method from supernatant after the precipitation of lower density lipoproteins from the fraction formed during ultracentrifugation or from EDTA-plasma.		I, II IV
Blood glucose	Glucose dehydrogenase method	Merck	I, II
Plasma glucose	Glucose Assay kit	Cayman Chemicals	IV
Serum estradiol	Radioimmunoassay (RIA)	Orion Diagnostica	II
Plasma IL-1 β , -2, -4, MCP-1, TNF- α	5-plex mouse cytokine assay (MCYTO-70K-05)	Millipore	IV

4.2.2 Ghrelin measurements

Ghrelin was measured from the fasting EDTA-plasma samples of OPERA study (Study I) using a commercial total ghrelin RIA (#RK-031-30, Phoenix Pharmaceuticals Inc., Belmont, CA). The antibody used in the assay detects both acylated and des-acylated ghrelin. The sensitivity of the assay was 1.2 pg/tube, the intra-assay CV 4.0% and the interassay CV 7.5% as reported by the manufacturer. Interassay CV in the analyses of this study was 11.2%.

Plasma acylated ghrelin levels were determined from fasting EDTA-plasma samples of the ERT study (Study II) as duplicates using a commercial ghrelin (active) RIA kit (GHRA-88HK, Linco Research Inc.), which utilizes an antibody

specific for ghrelin with the octanoyl group on Serine 3. The basic principal and protocol for the assay are similar to those of total ghrelin RIA (by Phoenix Pharmaceuticals Inc.).

Total ghrelin was determined also from fasting (at least 2 hours) mouse EDTA-plasma samples (Study IV) as duplicate using a commercial total ghrelin RIA kit (GHRT-89K, Linco Research Inc.), which utilizes an antibody specific for both acylated and des-acyl ghrelin similar to RIA in Study I (by Phoenix Pharmaceuticals Inc.). Acylated ghrelin was determined from mouse EDTA-plasma samples containing also 1mM p-hydroxymercuribenzoic acid using a commercial enzyme immunoassay (A05117, SPI-BIO, Montigny le Bretonneux, France). This assay is based on the double-antibody sandwich technique in which a monoclonal antibody specific to the C-terminal part of ghrelin is immobilized on a plate and binds to any ghrelin introduced in the wells. The detecting Fab' part of antibody conjugated to an acetylcholinesterase enzyme (AChE) recognizes the N-terminal part of acylated ghrelin.

4.2.3 Measurement of carotid IMT (I)

The intima-media thickness of the carotid arteries was measured with the carotid ultrasound procedure as previously described (Päiväsalo *et al.* 1996). Briefly, IMT defines the distance between the media-adventitia interface and the lumen-intima interface. IMT was analyzed from the near and far walls of both sides of the carotid artery. The ultrasound measurements were made on different sites of the carotid artery: the internal carotid artery, the bifurcation enlargement, and three sites of the common carotid artery. The mean value of the 20 measurements (mean IMT), the mean of the far wall measurement of the common carotid artery, the bifurcation enlargement, and the inner carotid artery were used for analysis.

4.2.4 Analysis of atherosclerosis in mice

Atherosclerosis in mice was analyzed from *en face* preparations of the entire aorta and cross sections through the aortic origin as previously described (Tangirala *et al.* 1995) and schematically illustrated in Figure 6.

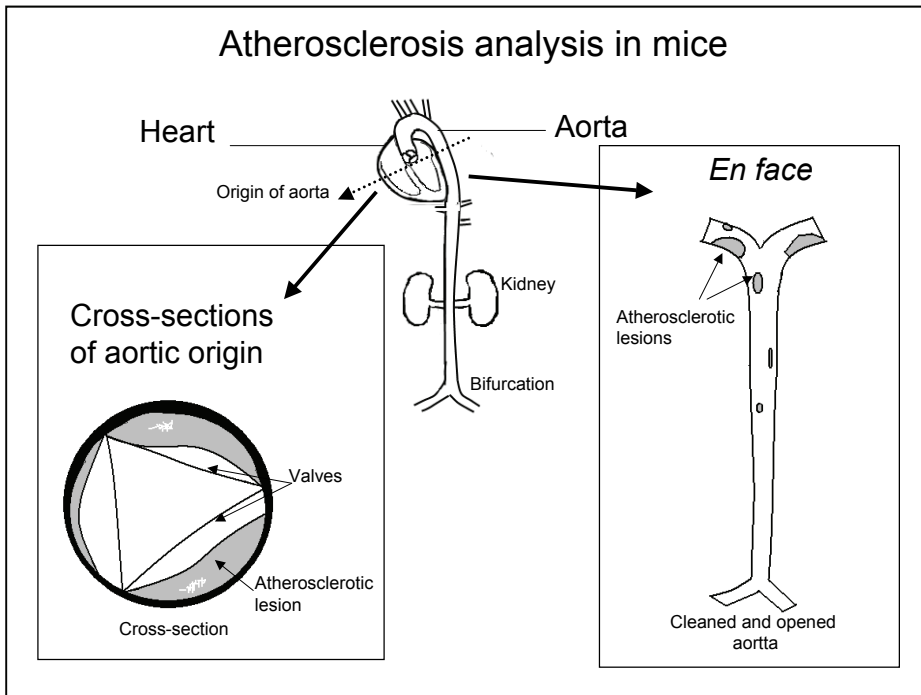


Fig. 6. Methods used for analysis of atherosclerosis in mice. The atherosclerotic lesion area can be determined from the cross-sections of aortic origin where the lesions are formed under the three valves. Lesions areas are analyzed from a prepared and Sudan IV dyed aorta as a percentage of total aortic area.

In brief, in the *en face* method, a mouse aorta was longitudinally opened and cleaned under a stereoscopic microscope and stained with Sudan IV dye. The atherosclerotic lesion areas were analyzed as traditionally from the photographic image and, in addition, from infrared (IR) scanned images of entire aortas using Image J analysis software. As in the traditional *en face* analysis, the lesion area was calculated as a percentage of the total surface area of the aorta. The lesion area was also analyzed as the IR intensities of the aortic lesion area.

Cross sections (6- μm) of paraffin embedded hearts were cut from the aortic origin with a microtome. The mounted slides were stained with hematoxylin-eosin. The area of the atheroma from the sections was analyzed from the digital image using image analysis software. The average surface area of each three valves was determined from five sections.

4.2.5 Immunohistochemical analysis (IV)

Ghrelin was analysed immunohistochemically from mice stomach samples as described in detail in the original paper (IV). Immunohistochemical staining was performed using HistoMousePlus staining kit (Zymed Laboratories, San Francisco, CA.). Ghrelin was detected using serum from ghrelin-PADRE immunized mice giving high IgG titers against ghrelin and serum from ghrelin-PADRE mice before immunization was used as a negative control. Ghrelin-positive cells in gastric mucosa were calculated with a digital image analysis system MCID M4 (Image Research Inc., St. Catharines, Canada) using a Nikon Optiphot II microscope and a Sony DXC-930P color camera.

4.2.6 Cell culture studies (III)

Schematic illustrations of cell culture experiments are shown in Figure 7.

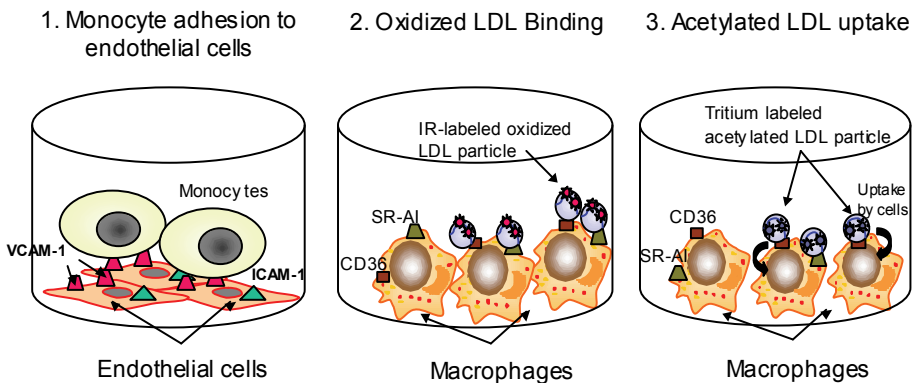


Fig. 7. Cell culture experiments: 1) monocyte adhesion to endothelial cells, 2) oxidized LDL binding on macrophages and 3) acetylated LDL uptake by macrophages.

Monocyte adhesion to endothelial cells

To evaluate monocyte adhesion, human EA.hy 926 endothelial cells were grown in a 96-well plate and exposed to 10 ng/ml (2,96 nmol/l) and 100 ng/ml (29,6nmol/l) ghrelin or 10 and 100 ng/ml obestatin for 4 hour for basal adhesion studies and for 1 hour before 4 hours of incubation with 10 ng/ml and 100 ng/ml TNF α . The media was removed and fluorescently-labeled (calcein-AM, Molecular Probes, Eugene, OR) THP-1 monocytes were added to wells and

incubated for 30 minutes at 37 °C. The wells were inverted and spun at 200g. Medium was added into the wells, the fluorescence of the plate was measured in a plate reader. The results were normalized to control values.

Binding of oxidized LDL

Elicited mouse macrophages were harvested from male C57BL/6 mice by peritoneal lavage with phosphate buffered saline (PBS) 3 days after an intraperitoneal injection of 2ml of thioglycollate medium. The macrophages were plated on 96-well plates at a density of 150000 cells per well in Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS). To remove nonadherent cells, the plates were washed after 3h plating and incubated at 37°C overnight. The cells were washed with PBS and exposed to acylated ghrelin or obestatin in serum-free medium at 37°C for one hour. The plates were placed on ice for 20 minutes before incubation with IR800-dye-labeled (Licor Biosciences, Lincoln, NE) oxidized LDL (15µg/ml) at + 4°C for 3 hours. After three washes with PBS, the plate was allowed to dry and it was scanned with Odyssey IR-scanner at 800nm (Licor Biosciences Lincoln, NE).

Uptake of acetylated LDL

J774.A1 cells were plated on 96-well culture plates at a density of 50000 cells per well in DMEM media containing 10% FBS. After two days, the cultured cells were washed twice with PBS and preincubated with ghrelin and obestatin (100 ng/ml) in serum free DMEM for 1 hour before tritium labeled Ac-LDL was added on the cells at 37 °C for 3 hours. The cells were washed two times with PBS before 100 µl of 0.1 M NaOH was added to the wells which were transferred to a plate shaker to lyse the cells. Cell lysate (25 µl) and 200 µl Optiphase Supermix were added to the plates and the plates were sealed and mixed for 30 minutes. After 30 minutes' incubation at room temperature, radioactivity was counted with a beta-counter (Microbeta, Wallac, Turku, Finland). The protein concentrations of the cell lysates were determined using modified Lowry protein assay (Bio-Rad). Ac-LDL uptake was calculated as counts per second in cell/µg of cell protein.

Cell culture studies for mRNA expressions of various genes

EA.hy 926 cells were exposed to human ghrelin (100 ng/ml = 29.6nM) or obestatin (100 ng/ml = 39.3 nmol/l) in the presence or absence of TNF α (10 ng/ml) in serum-free media for 1 hour (intra cellular adhesion molecule 1 (ICAM-1), VCAM-1) or 3 hours (MCP-1). The mRNA expression of adhesion molecules VCAM, ICAM and MCP-1 was determined in EA.hy 926 cells and the mRNA expression of the scavenger receptors CD36 and SR-A was assessed in elicited mouse macrophages treated with ghrelin or obestatin (100ng/ml) for 1, 3, or 6 hours.

4.2.7 Real-time PCR (III, IV)

RT-PCR was used to examine expression levels of several genes. For these experiments, total RNA was extracted from tissue and cell samples. mRNAs were reverse transcribed to deoxyribonucleic acid (DNA) as described in the original publications (III, IV). Quantitative RT-PCR was performed with Syber Green chemistry (BioRad). The expression of the target gene in various samples was normalized with the expression of the housekeeping gene (β -actin or GAPDH) in the samples. The RT-PCR results are presented as fold changes compared to the control sample.

4.2.8 Statistical Analyses

Data analyses were performed with the software package SPSS for Windows (© SPSS Inc., Chicago, USA). The results for different variables are presented as mean \pm SD, unless stated otherwise. P-value < 0.05 was regarded as statistically significant. For clinical continuous variables, logarithmic transformation was applied when necessary to normalize the distribution. Table 4 shows the main statistical tests used in this thesis.

Table 4. Statistical tests used.

Statistical test	Used for	Study
Student's <i>t</i> -test	Normally distributed data	Studies I, II, III, IV
Wilcoxon-Mann-Whitney U	Not normally distributed data	Studies III, IV
Repeated measurements ANOVA	Comparing treatment groups	Study II
Paired-sample <i>t</i> test	Comparing changes from baseline to 6 months	Study II
Linear regression	Association between ghrelin and other determinants	Studies I, II
Ancova	Association between ghrelin and cIMT	Study I
Multivariate analysis	Risk factors	Study I
Pearson correlation coefficient	Normally distributed data	Studies I, II, III, IV
Spearman correlation coefficient	Not normally distributed data	Studies I, II, III, IV

In Study I, the results of the multivariate analyses were adjusted for the traditional risk factors of atherosclerosis (age, sex, SBP, LDL-cholesterol, smoking, and BMI) (Vogel & Benitez 2000). χ^2 -test was carried out to assess the frequency differences in the ratio of the genders between the ghrelin quartiles. Post hoc analyses were performed if there was a statistically significant interaction between ghrelin and the other explanatory variables (sex, sex-specific BMI tertile) as a predictor of carotid IMT by analysis of covariance (ANCOVA).

5 Results

5.1 Plasma ghrelin levels and carotid IMT (I)

The association between plasma total ghrelin levels and early atherosclerosis measured as carotid intima media thickness was assessed in the large population-based OPERA cohort. The mean IMT of carotid artery within the whole study population was positively correlated with plasma ghrelin levels ($P = 0.028$), however, statistical significance disappeared ($P = 0.052$) after adjustments for the known risk factors of atherosclerosis (age, sex, SBP, LDL cholesterol, smoking and BMI). Univariate analysis revealed a statistically significant interaction between plasma ghrelin concentration and gender. Therefore, analysis was performed also separately in both genders. Figure 8 demonstrates the positive associations of plasma total ghrelin level and IMT in common carotid artery ($P = 0.006$) after adjustments in males but no association was observed in females ($P = 0.960$). Similar correlation was also observed between plasma total ghrelin level and IMT measured in bifurcation enlargement area. Males had higher IMT (Figure 7) and the number of atherosclerotic plaques present ($n = 286$, 56.2%) compared with women ($n = 181$, 35.1%, χ^2 -test, $P < 0.001$).

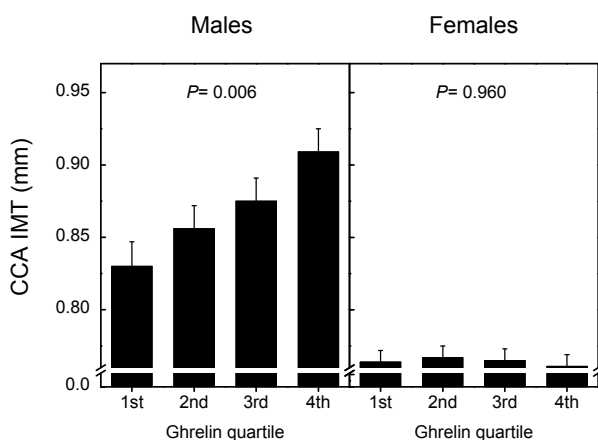


Fig. 8. The association of adjusted common carotid artery IMT and ghrelin quartiles in males ($n = 509$) and females ($n = 515$). P -values for the overall effect of ghrelin (P for trend) were obtained from ANCOVA. Adjustments were made for age, SBP, LDL cholesterol, smoking and BMI.

The association between plasma ghrelin levels and carotid IMT was examined also using multivariate linear regression models, including the traditional risk factors of atherosclerosis. As expected, age, SBP, smoking and LDL cholesterol were positively related with the mean IMT. Positive associations were found between the ghrelin concentrations and the mean IMT within the whole study cohort ($P = 0.007$) and especially in men ($P = 0.001$). Ghrelin levels were not associated with mean IMT in women ($P = 0.941$). In addition, the univariate regression model analysis demonstrated that ghrelin alone explained 1.1% of the variation in IMT values of men (adjusted R^2), while the corresponding values for the commonly accepted major risk factors were 10.7% for age, 5.5% for LDL cholesterol, 4.8% for smoking, 2.6% for SBP and 0.4% for BMI.

5.2 Associations between plasma ghrelin level and the known factors influencing atherogenesis (I, II)

The associations between ghrelin and some of the established cardiovascular risk factors such as lipids, BMI and estrogen were determined in the ERT study (Study II) and in the OPERA study (Study I).

5.2.1 Plasma ghrelin levels and lipids (I, II)

Associations between plasma ghrelin, cholesterol and triglyceride levels in Study I

Plasma ghrelin levels were associated positively with plasma HDL cholesterol ($P = 0.011$) within the whole OPERA study population after adjustment for age, gender, BMI and smoking (Figure 9). No statistically significant associations were seen between plasma ghrelin level and plasma HDL cholesterol when the analysis was performed separately in females ($P = 0.105$) and in males ($P = 0.084$). The plasma ghrelin level did not correlate with plasma total, LDL, VLDL cholesterol and triglyceride levels within the whole study population.

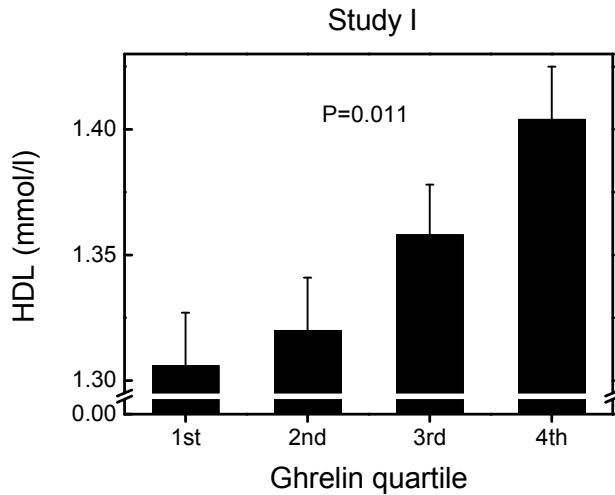


Fig. 9. The association between HDL cholesterol concentration and ghrelin quartiles within whole study population ($n = 1037$). P -value was analyzed by ANCOVA. Adjustments were made for age, sex, BMI and smoking.

Associations between plasma ghrelin, cholesterol, and triglyceride levels in Study II

Plasma acylated ghrelin concentrations were negatively associated with plasma total cholesterol ($r = -0.265$, $P = 0.034$), LDL cholesterol ($r = -0.248$, $P = 0.048$) as well as total triglyceride levels ($r = -0.307$, $P = 0.013$) in women ($n = 64$) after 6 months of therapy (Figure 10). At the baseline, the inverse correlation was statistically significant only between plasma ghrelin and plasma total triglycerides levels ($r = -0.345$, $P = 0.005$). Significant negative correlations were also seen between plasma ghrelin and VLDL cholesterol levels ($r = -0.303$, $P = 0.007$) and VLDL triglyceride levels ($r = -0.372$, $P = 0.002$) at baseline among all the subjects. HDL cholesterol levels increased from 1.60 ± 0.37 to 1.81 ± 0.38 mmol/l ($P < 0.001$) after oral estrogen treatment. No changes in HDL levels were seen after TE therapy. HDL cholesterol concentration showed no association with plasma ghrelin levels among the study subjects.

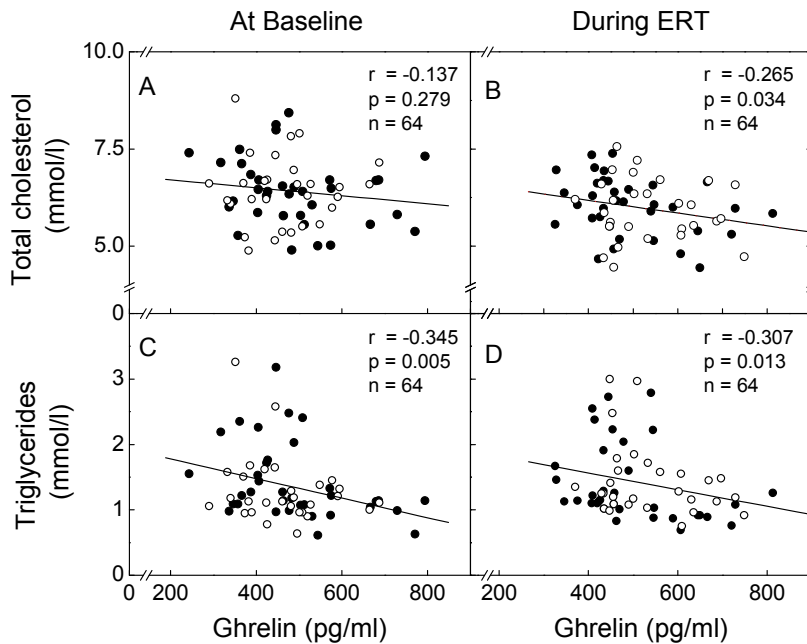


Fig. 10. The associations between acylated ghrelin, total cholesterol level and triglycerides in women at baseline and after 6 months of estrogen therapy. Pearson correlation was used for the analysis. The open circles represent subjects receiving estrogen treatment orally and filled circles transdermally.

Absorption of dietary cholesterol (II)

The associations between plasma ghrelin level and parameters affecting in LDL cholesterol metabolism were assessed. Absolute absorption of dietary cholesterol decreased from 1.96 ± 1.09 to 1.53 ± 0.96 mg/kg-d ($P = 0.019$) during PE therapy and from 2.03 ± 0.86 to 1.71 ± 0.72 mg/kg-d ($P = 0.035$) during TE therapy. Ghrelin levels did not associate with the absolute absorption of dietary cholesterol. No correlation was observed between the changes in plasma ghrelin levels and cholesterol absorption.

LDL clearance rate (II)

LDL clearance rates were increased from 0.295 ± 0.037 to 0.343 ± 0.044 pools per day ($P < 0.001$) in women during PE therapy. No changes in LDL clearance

were observed in women on TE. Ghrelin levels did not associate with LDL clearance rates. Apolipoprotein B (ApoB)-100 levels were negatively associated with ghrelin levels at baseline ($r = -0.262$, $P = 0.036$) and during estrogen therapies ($r = -0.334$, $P = 0.007$).

5.2.2 Effects of estrogen on plasma ghrelin levels (II)

Estrogen replacement therapy increased plasma acylated ghrelin level from 479 ± 118 pg/ml to 521 ± 123 pg/ml ($P = 0.002$) among all study subjects. Oral estrogen therapy increased plasma ghrelin levels from 465 ± 99 pg/ml to 536 ± 104 pg/ml ($P = 0.001$) but TE therapy did not significantly increase plasma ghrelin levels (from 491 ± 132 pg/ml to 509 ± 138 pg/ml; $P = 0.33$). Figure 11 displays the positive correlation between the changes in estradiol concentrations and the changes in acylated ghrelin levels among women receiving estrogen therapy (PE and TE women together).

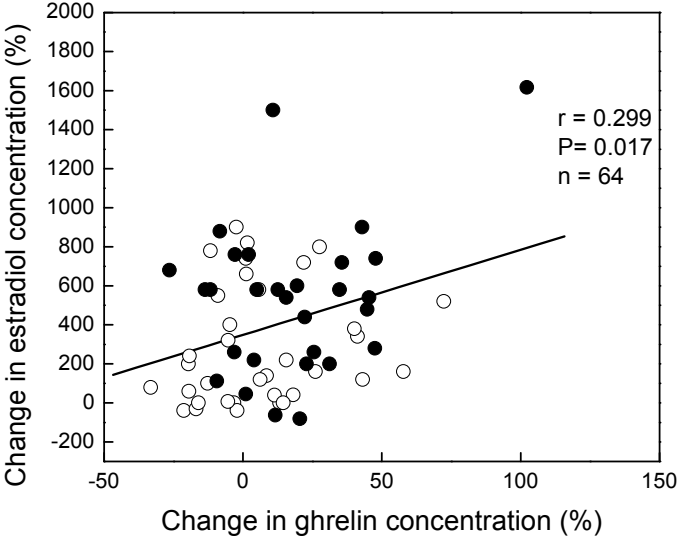


Fig. 11. Association between the changes in plasma acylated ghrelin and plasma estradiol concentrations during estrogen replacement therapies. Statistical significance is assessed by Pearson correlation. The open circles represent subjects receiving estrogen treatment orally and filled circles transdermally.

5.2.3 Plasma ghrelin levels and BMI (I, II)

Figure 12A shows an inverse association between plasma total ghrelin levels and BMI in females and in males in the OPERA (Study I) and (Figure 12B) between plasma acylated ghrelin and BMI in the ERT study (Study II) at baseline ($r = -0.353$, $P = 0.004$, $n = 64$). The associations between the plasma total ghrelin level and BMI were statistically significant within the whole study cohort and in females ($P = 0.002$, $n = 519$) but not in males ($P = 0.197$, $n = 518$). An inverse association between BMI and plasma acylated ghrelin levels was also observed in postmenopausal women on TE ($r = -0.419$, $P = 0.012$) and PE therapies ($r = -0.368$, $P = 0.050$).

BMI in study populations

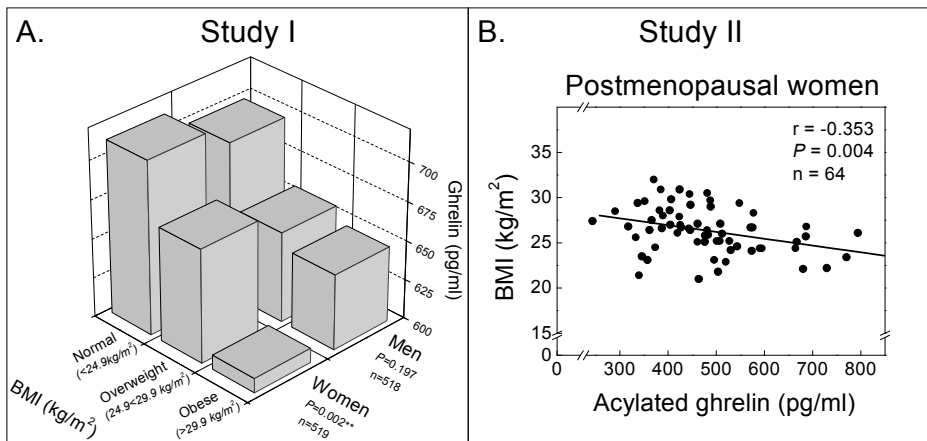


Fig. 12. Associations between plasma total ghrelin levels and BMI in Study I (Panel A) and between plasma acylated ghrelin levels and BMI in Study II (Panel B). Adjustments were made for age, SBP and smoking.

5.3 Ghrelin in cell culture models of atherosclerosis (III)

The effects of ghrelin were investigated using cells that participate in atherosclerotic plaque formation.

5.3.1 THP-1 monocyte adhesion to EA.hy 926 endothelial cells

Effects of ghrelin on monocyte adhesion to endothelial cells without stimulation

Monocyte adhesion was studied first without stimulation by treating EA.hy 926 cells with ghrelin or obestatin alone for 4 hours. Figure 13A shows that ghrelin increased monocyte adhesion to endothelial cells by 48% and 61% when incubated in the presence of 10 ng/ml and 100 ng/ml of ghrelin. Incubation with obestatin did not influence the monocyte adhesion.

Effects of ghrelin on adhesion molecules and MCP-1 expression

In order to assess the possible mechanism behind the monocyte adhesion, the effect of ghrelin and obestatin on relative mRNA expression of adhesion molecules and MCP-1 was determined by quantitative RT-PCR. Ghrelin increased ICAM-1 expression in endothelial cells (Figure 13C), a result consistent with the monocyte adhesion experiment. Ghrelin did not alter MCP-1 mRNA expression. Obestatin treatment did not affect either ICAM-1 (Figure 13C) or MCP1 mRNA levels. The mRNA level of VCAM-1 was too low to be assessed without TNF α stimulation, an observation that has also been described earlier (Thornhill *et al.* 1993).

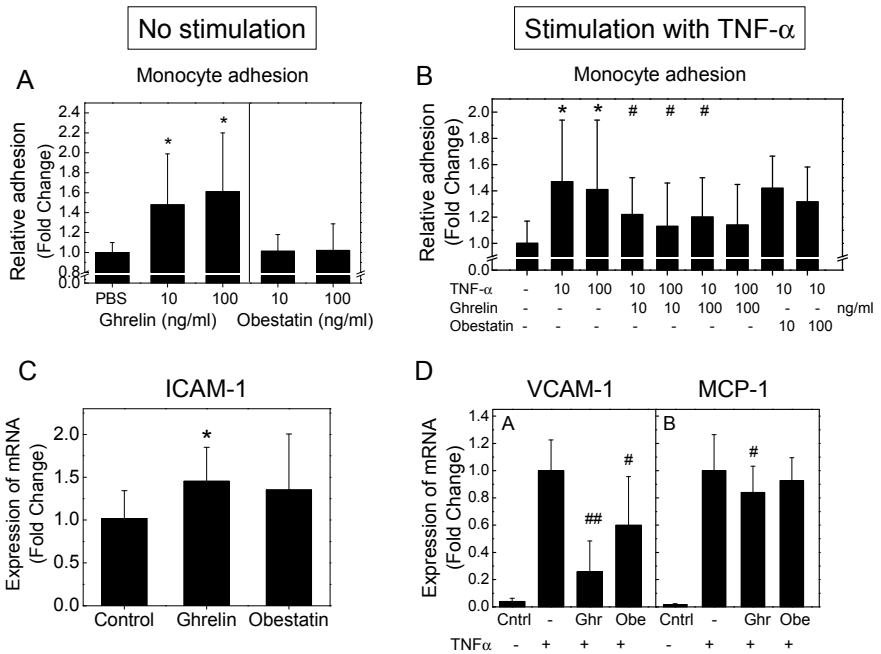


Fig. 13. Monocyte adhesion to endothelial cells and mRNA expression of adhesion molecules and MCP-1. Monocyte adhesion assessed without TNF α stimulation (Panel A) and with TNF α stimulation. EA.hy 926 cells were treated with 100ng/ml of ghrelin or obestatin to determine (Panel B) ICAM-1 expression on endothelial cells without stimulation (Panel C) and VCAM-1 and MCP-1 expression on endothelial cells stimulated with TNF α (Panel D). * $P < 0.05$ indicates statistical significance compared to PBS control, # $P < 0.05$ compared to TNF α control and ## $P < 0.01$ compared to TNF α control. Bars represent mean \pm SD. Cntrl, control; Ghr, ghrelin; Obe, obestatin.

Effects of ghrelin on monocyte adhesion to endothelial cells stimulated with TNF α

The cells were stimulated with a pro-inflammatory cytokine TNF α after ghrelin and obestatin pretreatment to mimic an inflammatory stage in the endothelium. TNF α (10ng/ml) treatment increased monocyte adhesion by 47% compared to PBS control (Figure 13B). Ghrelin pretreatments (10ng/ml and 100ng/ml) before TNF α stimulation decreased monocyte adhesion to endothelial cells by 25% and 20%, whereas obestatin pretreatments had no effect on adhesion (Figure 13B).

Effects of ghrelin on the expression of adhesion molecules and MCP-1 in endothelial cells stimulated with TNF α

The relative mRNA expression of the adhesion molecules and MCP-1 were also assessed in EA.hy 926 cells stimulated with TNF α by RT-PCR. When the endothelial cells were treated with TNF- α (10 ng/ml) alone, the expression of adhesion molecule ICAM-1 increased 35-fold, VCAM-1 1500-fold and MCP-1 58-fold. When the cells were incubated with ghrelin together with TNF α (10 ng/ml), no significant changes in the ICAM-1 expression but reductions in both VCAM-1 and MCP-1 expressions (Figure 13D) were observed compared to TNF α treatment alone. Obestatin treatment together with TNF α decreased also VCAM-1 expression (Figure 13D) but did not alter ICAM-1 and MCP-1 expressions compared to the response obtained with TNF α alone.

5.3.2 The effects of ghrelin on oxidized LDL binding to mouse macrophages

The effects of ghrelin and obestatin on Ox-LDL binding to stimulated mouse peritoneal macrophages were explored. Ghrelin dose dependently increased the binding of oxidized LDL to macrophages (Figure 14). Obestatin treatment also increased binding of Ox-LDL to macrophages. However, no changes were seen in mRNA expression of CD36 or SR-A after ghrelin or obestatin treatment.

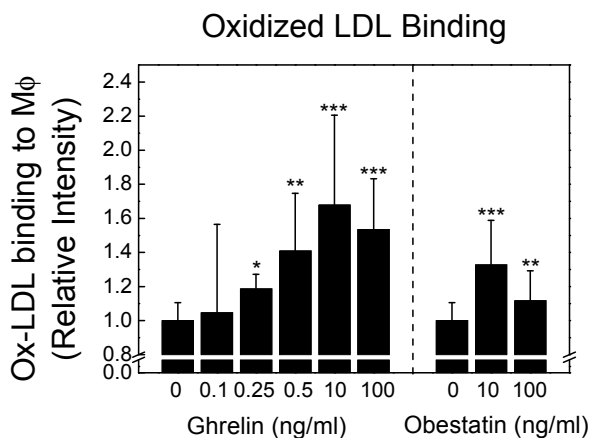


Fig. 14. Ghrelin and obestatin increase binding of Ox-LDL to mouse peritoneal macrophages. * $P < 0.05$, ** $P < 0.01$ and * $P < 0.001$ indicate statistical significance compared to control (0).**

In another sets of experiments where Ac-LDL was used, ghrelin or obestatin did not affect the uptake of Ac-LDL by J774.A1 macrophages suggesting that SR-A function has not changed.

5.4 Effects of ghrelin on atherosclerosis in LDLR KO mice (IV)

In order to assess the role of ghrelin in the development of atherosclerosis *in vivo*, ghrelin vaccination was used to alter functional ghrelin levels in mice sera.

5.4.1 Ghrelin vaccine responses

A pilot study using C57/BL6 mice was first conducted to compare the immune responses and plasma ghrelin levels induced by different ghrelin vaccines.

Vaccine responses in C57BL/6 mice

The C57/BL6 mice were vaccinated with mouse ghrelin linked to PADRE peptide (ghrelin-PADRE, $n = 4$), PADRE peptide alone ($n = 4$) or with traditional technique using KLH as a carrier molecule linked to ghrelin (ghrelin-KLH, $n = 4$). Plasma IgG antibodies against ghrelin were highly increased in mice immunized

with ghrelin-PADRE and ghrelin-KLH. Mice immunized with ghrelin-PADRE demonstrated less inter-individual variability compared to the mice immunized with ghrelin-KLH. The specificity of the IgG derived from ghrelin-PADRE mice was demonstrated in a competition enzyme-linked immunosorbent assay (ELISA) emphasizing the antibody responses were highly specific and that the binding to immobilized ghrelin was blocked by soluble ghrelin (over 80%) and soluble ghrelin-PADRE (about 50%).

Effect of vaccination on ghrelin levels in LDLR KO mice

A pilot study with C57/BL6 mice demonstrated that ghrelin-PADRE vaccine generated a homogeneous and strong immune response to ghrelin. Therefore, this vaccine was selected for the study conducted with LDLR KO mice to determine the effects of ghrelin vaccine on atherosclerosis formation. Three groups of LDLR KO mice were immunized with PBS (n = 12), PADRE peptide alone (n = 12) or ghrelin-PADRE (mouse) (n = 12). Both acylated and total ghrelin levels were significantly higher ($P < 0.0001$) in ghrelin-PADRE immunized mice compared to the PBS and the PADRE groups (Figure 15A).

Ghrelin mRNA and protein expression were determined in stomach cells to assess possible mechanism behind the increased ghrelin levels in mice immunized with ghrelin-PADRE. Ghrelin-PADRE mice tended to have higher ghrelin mRNA levels, but these differences were not statistically significant ($P = 0.092$). No significant differences in the number of ghrelin-immunopositive cells determined immunohistochemically were seen between the groups.

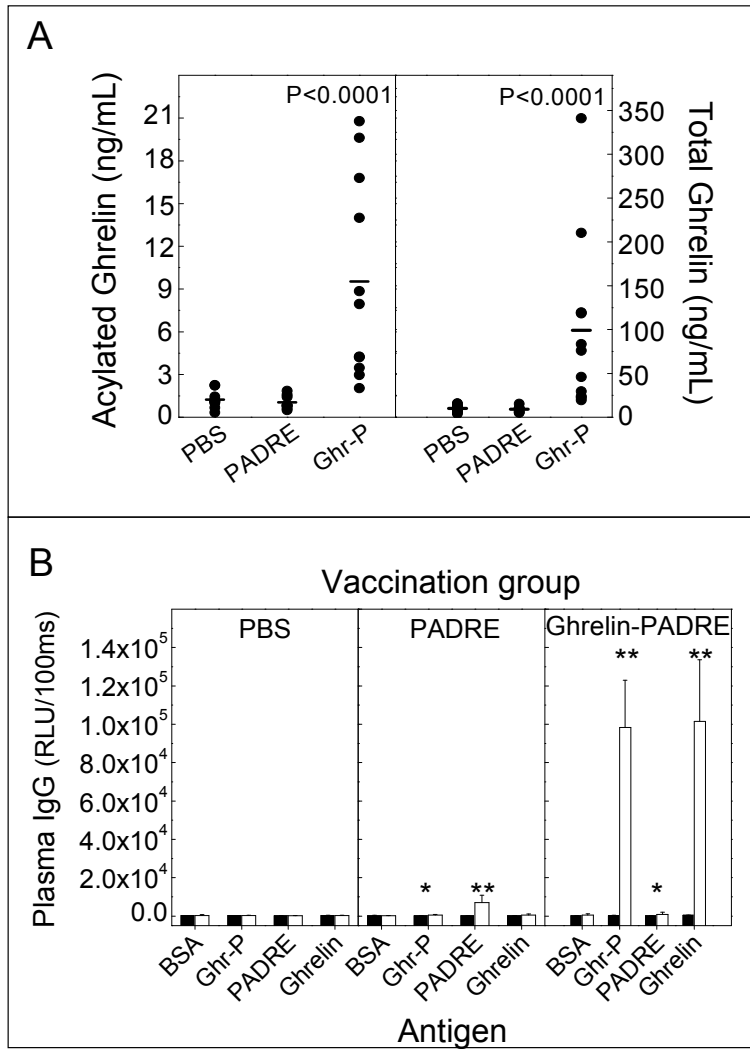


Fig. 15. Vaccine responses in LDLR KO mice. Plasma total and acylated ghrelin levels (Panel A) and IgG antibodies formed against control and ghrelin antigens in LDLRKO mice immunized with different vaccines (Panel B). * $P < 0.05$ and ** $P < 0.001$ indicate statistical significance compared to PBS control mice.

Effect of vaccination on antibody responses in LDLR KO mice

Similarly to preliminary study, ghrelin-PADRE vaccine generated a homogeneous and strong immune response to ghrelin. Figure 15B shows how the mice immunized with ghrelin-PADRE developed high IgG titers to mouse ghrelin and ghrelin-PADRE ($P < 0.0001$ compared to PBS and PADRE alone immunized mice), but only very low IgG titers to PADRE alone ($P < 0.01$ compared to PBS immunized mice). Mice immunized with PBS did not develop IgG titers to ghrelin-PADRE, mouse ghrelin, or PADRE alone and mice immunized with PADRE alone developed only IgG titers only against PADRE and ghrelin-PADRE. IgM antibody titers were low in all groups.

5.4.2 Atherosclerosis analysis in LDLR KO mice

LDLR KO mice immunized with ghrelin-PADRE, PADRE alone and PBS were challenged with high fat diet for 7 months. Atherosclerosis was measured at the aortic root by cross-sectional analysis of mouse heart (Figure 16B), by the *en face* method of the entire aortic trunk (Figures 16C) and by IR scan of Sudan IV stained entire aorta (Figure 16D) at the end of the study. No differences were seen in lesion areas at the aortic root cross-sections or the surface area of lesions or in IR intensities (700nm) in the entire aorta between the groups (Figure 16A).

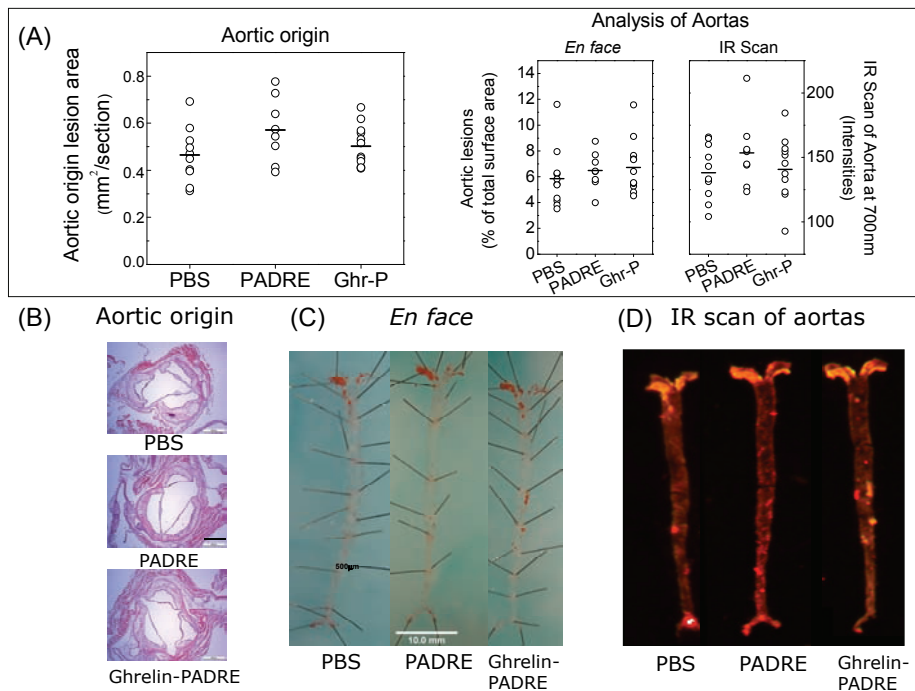


Fig. 16. Atherosclerosis determined from the aortic root and from the aortic trunk by en face and infrared scanning.

5.4.3 Plasma lipids, glucose and insulin in LDLR KO mice

No differences were seen on plasma total and HDL cholesterol, triglycerides, insulin and glucose concentrations between the different vaccination groups, and no associations were observed between ghrelin levels and plasma lipids, glucose and insulin.

5.4.4 Plasma MCP-1 and cytokines in LDLR KO mice

Since ghrelin has been previously shown to exert potent inhibitory effects on the secretion of several pro-inflammatory cytokines, the effect of ghrelin vaccination on plasma MCP-1 levels as well as on several other cytokines (IL-1 β , IL-2, IL-4 and TNF α) was investigated. Figure 17 shows the statistically significant decrease in plasma MCP-1 levels in mice immunized with ghrelin-PADRE compared to

PBS mice ($P < 0.007$). No differences were observed in plasma IL-1 β , IL-2, IL-4 or TNF α levels between the different vaccination groups.

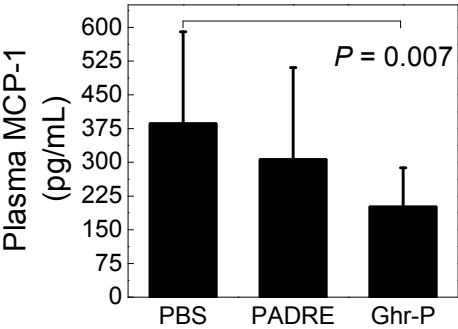


Fig. 17. Plasma MCP-1 levels in different vaccination groups of LDLR KO mice.

5.4.5 Weight gain and food intake in LDLR KO mice

No differences in weights were observed between the different immunization groups fed the two diets. No association was seen between body weight and ghrelin levels in mice. In addition, there were no differences in weekly food intakes during the chow diet (7 weeks) and high fat diet (14 weeks) between the immunization groups.

6 Discussion

Atherosclerosis is a complex and multifactorial disease in which atheromatous plaques are formed in arterial wall. This study was conducted to investigate the role of ghrelin in atherosclerosis. In the beginning of this project, virtually nothing was known about the influence of ghrelin on atherosclerosis. There were some reports that ghrelin has have e.g. beneficial hemodynamic effects and the binding of labeled ghrelin had been shown to increase in both atherosclerotic coronary arteries and saphenous vein grafts with advanced intimal thickening compared with normal vessels (Katugampola *et al.* 2001, Nagaya *et al.* 2001a, Nagaya *et al.* 2001c). The present thesis attempted to elucidate the role of ghrelin on atherosclerosis and factors associating closely associated to atherosclerosis using human population, experimental cell and animal models. First, a positive association between plasma total ghrelin level and the degree of subclinical atherosclerosis measured as IMT of carotid arteries was seen within male subjects in a large cross-sectional population-based study. Studies using cells essential in early key events in atherogenesis demonstrated that ghrelin can have pro-atherogenic effects by increasing Ox-LDL binding and monocyte adhesion on endothelial cells. However, when the endothelial cells were stimulated with an inflammatory cytokine, TNF α , ghrelin was revealed to have anti-atherogenic effects on cells by decreasing monocyte adhesion on endothelial cells subsequently with decreases in adhesion molecule VCAM-1 and MCP-1. In contrast to our initial finding in the carotid IMT, plasma ghrelin levels were inversely associated to plasma total and LDL cholesterol, triglycerides and BMI, and positively to HDL cholesterol, the factors already established to be risk factors for atherosclerosis. In addition, estrogen increased plasma acylated ghrelin levels and the changes in plasma ghrelin levels were associated with the changes in serum estradiol concentrations among postmenopausal women receiving estrogen replacement therapy. Finally, ghrelin vaccination did not affect atherosclerosis in LDLR KO mice. The data of all these studies together indicate that ghrelin is one of the factors contributing to atherosclerosis. Although, it does not play a major role similarly as the traditional risk factors, it has a modulatory role in the disease and the influence of ghrelin can vary between the stages of atherosclerotic arterial disease.

6.1 Ghrelin and atherosclerosis

The finding of a positive association between plasma ghrelin levels and the degree of subclinical atherosclerosis measured as intima media thickness of the carotid artery in the large population-based cohort (n = 1024) among the male subjects was unexpected. Our earlier study had demonstrated that low plasma ghrelin concentrations were associated with increased systolic and diastolic blood pressure, insulin resistance and obesity, the factors contributing atherosclerosis, in the same population-based cohort study (n = 1034) (Pöykkö *et al.* 2003a). In other studies, low plasma ghrelin levels have also been associated with obesity, a known risk factor for cardiovascular diseases, indicating high plasma ghrelin concentrations have anti-atherogenic influences (Shiyya *et al.* 2002, Sowers 2003).

Previous findings from carotid atherosclerosis in humans were not available but later, conflicting findings on the association between plasma ghrelin levels and subclinical atherosclerosis in different study populations have been published (Genis *et al.* 2007, Kotani *et al.* 2006, Yano *et al.* 2008). Kotani *et al.* found an inverse association between serum total ghrelin levels and carotid IMT in a smaller study (n = 101) consisting of subjects with the metabolic syndrome. Yano and co-workers also found a significant inverse correlation between plasma des-acyl ghrelin level and cIMT in elderly hypertensives (n = 263) (Kotani *et al.* 2006, Yano *et al.* 2008). Furthermore Genis *et al.* did not observe an association between the plasma ghrelin level and cIMT in kidney transplantation patients (n = 85) (Genis *et al.* 2007). The study populations are different and, also the analyses used for ghrelin determination have varied between these studies, which partly might explain their differences. However, one of the advantages in the present study is the large study cohort population.

Monocyte adhesion to endothelial cells is a key early step in the development of atherosclerosis and it is known to be modulated by many factors i.e. adhesion molecules, chemokines, pro-inflammatory cytokines such as TNF α , adipokines and also by oxidized lipoproteins (Gerrity 1981, Takahashi *et al.* 2002). In this study, ghrelin treatment increased monocyte adhesion to endothelial cells, elevated the expression of ICAM-1 mRNA in endothelial cells and also promoted the binding of Ox-LDL to mouse peritoneal macrophages. These findings support the proatherogenic role for ghrelin as observed in the association between the plasma ghrelin concentration and cIMT in the population-based cohort study. However, when endothelial cells were stimulated with TNF α , the influence of

ghrelin was reversed, ghrelin decreased monocyte adhesion, and also reduced mRNA expression of adhesion molecule VCAM-1 and MCP-1.

Only a few earlier studies have investigated the *in vitro* effects of ghrelin on endothelial cells. Contrast to monocyte adhesion data of this study, Skilton and co-workers did not see influence of ghrelin on monocyte adhesion using isolated human monocytes and HUVECs (Skilton *et al.* 2005). Similarly to the findings of ICAM-1 expression in the present study, they also observed a dose-dependent increase in both ICAM-1 and VCAM-1 expression at the protein level when the HUVEC cells were treated with ghrelin (Skilton *et al.* 2005). When TNF α stimulation was used, the results of this study were in line with a previous study where ghrelin was shown to decrease MCP-1 expression and monocyte adhesion to HUVEC endothelial cells (Li *et al.* 2004). Similarly to the decrease of VCAM-1 mRNA expression found in the present study, Hu *et al.* showed very recently that ghrelin could inhibit nicotine-induced VCAM-1 expression in HUVEC cells (Hu *et al.* 2009). TNF α stimulation of EA.hy 926 endothelial cells highly increased the expression of adhesion molecules and these cells are similar to HUVECs as earlier described (Bouis *et al.* 2001). The contradictory results between these studies may be attributable to differences in cell lines and conditions. Inflammation plays an important role in atherosclerosis (Ross 1999) and several previous studies have already established that ghrelin exhibits anti-inflammatory properties. Ghrelin has been shown to inhibit expression of pro-inflammatory cytokines (e.g. IL-1 β , IL-2, IL-6 and TNF α) via its action on the GHS-receptors in human peripheral blood mononuclear cells and T-cells (Dixit *et al.* 2004, Xia *et al.* 2004). Also, *in vivo* studies in rats with sepsis have demonstrated down-regulation of pro-inflammatory cytokines TNF α and IL-6 (Wu *et al.* 2007b). These findings are analogous with the present data on the TNF α stimulated EA.hy cells and support the belief the ghrelin has an anti-inflammatory modulatory function.

Studies in macrophages represented another step in the process of atherogenesis. It was noted that ghrelin dose-dependently increased oxidized LDL binding to macrophages. Increase in oxidized LDL uptake by macrophages will lead to lipid accumulation followed foam cell and eventually fatty streak formation in the arterial wall (Glass & Witztum 2001). Although, ghrelin increased Ox-LDL binding, it did not have any effect on Ac-LDL uptake by mouse macrophages. Differences in these binding and uptake results might be due to different pathways and mechanisms behind the lipid uptake of these ligands. Ac-LDL is taken up mostly (~80%) by the SRA-I receptor pathway whereas Ox-

LDL is taken up (as much as 90%) through SR-A and CD36 scavenger receptors (Nicholson *et al.* 1995, Suzuki *et al.* 1997). However, ghrelin did not change the mRNA expression of SR-A and CD36 scavenger receptors in the present study. Previously, a synthetic growth hormone releasing peptide hexarelin, which binds to ghrelin receptor (GHS-R1a) and CD36, has been demonstrated to reduce lipid accumulation in human THP-1 macrophages (Avallone *et al.* 2006). However, the binding properties of ghrelin and hexarelin to GHSR and CD36 receptors differ (Torsello *et al.* 2003). The findings of the present study indicate that ghrelin appears to have some proatherogenic effects. Interestingly, the role of ghrelin differs in altered inflammatory conditions and it displays both pro- and anti-inflammatory influences.

In the present study we found that obestatin did not affect monocyte adhesion to endothelial cells, or ICAM-1 and MCP-1 expression in endothelial cells with or without TNF α treatment. Interestingly, obestatin decreased VCAM-1 expression in endothelial cells when stimulated with TNF α . In addition, obestatin increased Ox-LDL binding to macrophages. Obestatin seemed to have similar or less effective properties than ghrelin in these *in vitro* studies. This is in line with a study where obestatin did not modify metabolism or viability of cardiomyocytes (Iglesias *et al.* 2007). The role of obestatin in atherosclerosis is yet totally unknown and studies are needed to elucidate the effects of obestatin either on its own or combined with ghrelin on atherosclerosis.

The study using an animal model of atherosclerosis i.e. LDLR KO mice demonstrated ghrelin vaccination to induce high plasma ghrelin levels but no effects on atherosclerosis. However, plasma MCP-1 levels were decreased in mice immunized with PADRE-ghrelin known to increase plasma ghrelin levels compared to mice immunized with PBS. This finding agrees with the *in vitro* data where ghrelin decreased MCP-1 (and VCAM-1) mRNA expression and adhesion of monocytes on EA.hy 926 endothelial cells stimulated with TNF α . Since MCP-1 has been demonstrated to have a central role in monocyte recruitment and atherosclerotic lesion formation, the decreased plasma MCP-1 level in ghrelin-PADRE group would have been predicted to decrease the level of atherosclerosis in ghrelin-PADRE mice (Aiello *et al.* 1999, Liehn *et al.* 2006). Nonetheless, no differences in atherosclerosis were seen between the different groups. In the present study, the signs of atherosclerosis were assessed in detail by determining both the atherosclerotic plaques from the aortic tree and the area of lesions at the aortic root area. The discrepancies between decreased MCP-1 levels and degree of atherosclerosis can partly result from the modulatory nature of ghrelin i.e. it

possesses both pro- and anti-inflammatory effects in human and mouse cells (Li *et al.* 2004, Skilton *et al.* 2005, Waseem *et al.* 2008). In addition to inflammatory influences, ghrelin has been demonstrated to have anti-apoptotic actions in cardiomyocytes and endothelial cells (Baldanzi *et al.* 2002, Zhao *et al.* 2007, Zhan *et al.* 2008, Kui *et al.* 2009). Thus, the pro-atherogenic factors can be compensated by the anti-atherogenic factors and the net influence of ghrelin could be less prominent in atherosclerosis *in vivo*. In addition, the presence of obestatin can contribute to the results even though the cell culture studies (in Study III and Iglesias *et al.* 2007) indicated that obestatin had a similar but less efficacious effect on the atherogenic factors.

The study with LDLR KO mice offers additional and important information on the effects of ghrelin on atherosclerosis. This is needed also to further confirm the findings observed in our and other association studies previously. Human and mouse studies concerning the direct influences of ghrelin on the formation of atheroma are virtually lacking. Previously hexarelin (a ghrelin mimetic) has been shown to bind to the CD36 receptor and prevent the development of atherosclerosis in ApoE KO mice (Bodart *et al.* 2002, Avallone *et al.* 2006). However, ghrelin and hexarelin do not share similar binding capacities to the CD36 receptors (Bodart *et al.* 2002) and therefore, this can partly explain the opposite findings between the studies. Studies with animals have shown that ghrelin administration can protect rat myocardium against myocardial injury and reduce the infarct size following ischemia, although reports exist claiming that ghrelin has no beneficial effects (Nagaya *et al.* 2001c, Frascarelli *et al.* 2003, Chang *et al.* 2004, Li *et al.* 2006a, Akashi *et al.* 2008, Soeki *et al.* 2008). Ghrelin evokes vasoconstriction in the rat coronary circulation and intracoronary infusion of ghrelin has been demonstrated to decrease coronary blood flow and cause coronary vasoconstriction in pigs (Pemberton *et al.* 2004, Grossini *et al.* 2007). Thus, both beneficial and detrimental cardiovascular effects have been reported in animal studies and no clear data extrapolation to human context can be performed.

6.2 Ghrelin and the known factors influencing atherosclerosis

Atherosclerosis is a complex disease and several already established factors contribute to the development of atherosclerosis. The major risk factors for atherosclerosis are well-known including elevated serum total and LDL cholesterol and triglycerides levels, low serum HDL cholesterol concentration, gender, overweight, and inflammatory markers (Grundy *et al.* 2001). Plasma LDL

cholesterol concentrations are determined by the production of apo B, apolipoprotein of LDL cholesterol, by the conversion of VLDL to LDL and by LDL receptor mediated clearance. Furthermore, the cholesterol absorption efficiency and enterohepatic cholesterol metabolism modify LDL cholesterol levels in plasma. (reviewed by Dietschy *et al.* 1993) LDL turnover studies *in vivo* using radiolabeled autologous LDL have provided information with regard to a number of factors affecting the clearance and/or secretion of LDL in man (Goodman *et al.* 1980, Packard *et al.* 1983). The LDL turn over method was used also in the ERT study.

In this thesis, plasma ghrelin levels were inversely associated with risk factors for atherosclerosis such as plasma total, LDL and VLDL cholesterol, triglycerides, apoB as well as BMI within postmenopausal women and positively to the HDL cholesterol level within the OPERA cohort study population. Furthermore, estrogen increased plasma acylated ghrelin levels in postmenopausal women receiving estrogen replacement therapy. These findings are contradictory to the positive association found between plasma ghrelin levels and cIMT in the cross-sectional population-based study and point to a more protective role for ghrelin in atherosclerosis.

Ghrelin has been demonstrated to interact with triglyceride-rich lipoproteins, HDL, very high-density lipoproteins, and to some extent with LDL (Beaumont *et al.* 2003, De Vriese *et al.* 2007). In population studies, plasma ghrelin concentrations have also been found to associate positively to HDL cholesterol levels and LDL particle size and negatively with LDL and triglyceride concentrations similarly to the findings in this thesis (Fagerberg *et al.* 2003, Purnell *et al.* 2003, Zwirska-Korczała *et al.* 2007, Lee *et al.* 2008b). Genetic variations in ghrelin and ghrelin receptor gene have been demonstrated to play a role in the determination of plasma levels of HDL-cholesterol in some populations, but this finding has not been duplicated in all populations (Vartiainen *et al.* 2006, Hubacek *et al.* 2007, Li *et al.* 2008, Martin *et al.* 2008). However, no correlation was found between the plasma ghrelin level and the parameters of LDL cholesterol metabolism such as cholesterol absorption or LDL clearance examined in the present thesis. In addition, a contradictory positive association between plasma ghrelin levels and plasma LDL cholesterol has been found (Karapanagiotou *et al.* 2009). Recent investigations have demonstrated that ghrelin administration in rats can induce tissue-specific changes in the expression of genes associated with mitochondrial and lipid metabolism and it can cause triglyceride deposition in the liver in preference to the skeletal muscle (Barazzoni

et al. 2005). These interesting findings suggest that ghrelin indeed has a role in lipid metabolism. However, the observations in this study are merely associations between plasma active or total ghrelin levels and parameters of plasma lipids or LDL metabolism, and the possible underlying mechanisms for these findings have not been elucidated. Further studies investigating the direct effect of ghrelin on lipid metabolism are definitely warranted.

Most reports suggest a beneficial role for ghrelin in normal and pathological conditions of the cardiovascular system. Both ghrelin and the ghrelin receptor have been shown to be expressed in cardiovascular system (Papotti *et al.* 2000, Gnanapavan *et al.* 2002, Kleinz *et al.* 2006). The hemodynamic effects of ghrelin have been widely investigated. Ghrelin has been shown to have vasodilatory effects in humans and also beneficial hemodynamic actions in rats (Nagaya *et al.* 2001a, Nagaya *et al.* 2001b, Nagaya *et al.* 2001c, Okumura *et al.* 2002, Enomoto *et al.* 2003). In addition, ghrelin treatment has been demonstrated to have beneficial effects in patients with heart failure (Nagaya *et al.* 2001b, Nagaya & Kangawa 2003a). Ghrelin treatment also improved endothelium-dependent vasodilatation in subjects with the metabolic syndrome, suggesting that high ghrelin levels might be beneficial for endothelial function (Tesauro *et al.* 2005). Contradicting data has been reported in the relation between ghrelin and oxidative stress (Suematsu *et al.* 2005, Sakane *et al.* 2008).

6.3 Ghrelin and estrogen

Interestingly, estrogen increased plasma acylated ghrelin levels in hysterectomized postmenopausal women receiving estrogen replacement therapy for six months. This was the first study to examine the effect of estrogen alone on plasma ghrelin levels. Estrogen has been suggested to be cardioprotective, based on the observation that women have a lower incidence of cardiovascular diseases compared to men before menopause, but the risk rises to the same level after menopause (Colditz *et al.* 1987). However, randomized prospective clinical trials were unable to demonstrate any beneficial effects of estrogen or combined hormone replacement therapy on cardioprotective factors (Hulley *et al.* 1998, Grady *et al.* 2002, Rossouw *et al.* 2002, Anderson *et al.* 2004). Nonetheless, estrogen therapy has had beneficial influences on lipoprotein levels as seen in this study and estrogen has also been shown to modify the lipid composition, HDL particle distribution, cholesterol efflux (reverse cholesterol transport) and anti-oxidative properties (Ulloa *et al.* 2002, Hockerstedt *et al.* 2004, Badeau *et al.*

2009). Thus, an increase in plasma ghrelin level would indicate that ghrelin may have favourable anti-atherogenic associations with the lipid profile. This finding did not clarify the drastic difference seen between males and females in the association between plasma ghrelin levels and carotid IMT.

At present, numerous studies have investigated the effects of estrogen or estrogen-progestin on plasma ghrelin levels in humans. Estrogen treatment demonstrated both stimulatory and inhibitory influences on plasma ghrelin levels. Estrogen or combined estrogen-progestin replacement therapy has been shown to increase plasma total ghrelin levels (Grinspoon *et al.* 2004, Di Carlo *et al.* 2007, Sagsoz *et al.* 2009). Transdermal estrogen supplementation enhanced hypothalamus-pituitary sensitivity to ghrelin and also increased night-time concentrations of plasma acylated ghrelin in healthy postmenopausal women (Kok *et al.* 2008, Paulo *et al.* 2008). Plasma total ghrelin concentrations did not differ in normal and hypoestrogenemic women and also, estrogen therapy and short-term exposure of estrogen did not change the plasma ghrelin levels (Purnell *et al.* 2003, Lebenthal *et al.* 2006, Veldhuis *et al.* 2006, Lambrinoudaki *et al.* 2008). There is an opposite claim that estrogen treatment (both oral and transdermal) for three months, decreased plasma total ghrelin level in women with the metabolic syndrome (Chu *et al.* 2006). The contradictory findings may be explained by the differences in the therapies, study populations and assay methods for plasma ghrelin levels.

6.4 Ghrelin, weight gain and obesity

Although weight gain and obesity were not the focus of this present thesis, we assessed the effect of ghrelin on weight gain, because ghrelin is closely associated to energy homeostasis, weight regulation and obesity. Obesity is one of the risk factors for atherosclerosis and it is becoming a more and more serious health problem all around the world. The plasma ghrelin level was inversely associated with BMI in the present study among middle-aged subjects in the OPERA study and also among postmenopausal women in the ERT study. Circulating plasma ghrelin levels are reportedly inversely associated with BMI in humans (Tanaka *et al.* 2002, Paik *et al.* 2004, Cummings 2006, Lee *et al.* 2008a). In humans, ghrelin secretion is decreased in obesity and is normalized following recovery to ideal body weight (Tschöp *et al.* 2001b). Exogenous administration of ghrelin is known to acutely increase food intake and chronic treatment greatly enhances body fat in rodents (Tschöp *et al.* 2000, Hosoda *et al.* 2002). Thus, the associations seen

between plasma ghrelin levels and BMI in this thesis are consistent with the previously reported findings.

Ghrelin-PADRE vaccination did not affect the weight gain in LDLR KO mice compared to mice immunized with control vaccines. This finding is not in agreement with two previous vaccination studies in rats and pigs proposing that ghrelin vaccination would be a therapeutic tool against weight gain (Zorrilla *et al.* 2006, Vizcarra *et al.* 2007). Rats actively immunized with ghrelin epitopes linked to KLH displayed decreased brain to blood ghrelin levels and gained less body weight than the control animals (Zorrilla *et al.* 2006). Pigs immunized with ghrelin-bovine serum albumin (BSA), exhibited a decreased weight gain and increased antibody titers to ghrelin (Vizcarra *et al.* 2007). In these studies, the vaccines contained ghrelin peptides linked to either KLH or BSA as carrier proteins. A similar vaccination approach was used in the present study to alter plasma ghrelin levels but we used a synthetic PADRE peptide linked to ghrelin as the carrier. Food intake and body weight gain were followed throughout the study (both chow and high fat diet periods) but no differences were seen in body weights. The result in weight gain was similar in our pilot study where we used C57/BL6 mice and a similar vaccination protocol to that of Zorrilla and co-workers (KLH-ghrelin) as well as ghrelin-PADRE. One difference between our study and the earlier studies is that we did not use the same adjuvant and this could have influenced the type of immune responses (Zorrilla *et al.* 2006, Vizcarra *et al.* 2007). However, our results are in line with a very recent mouse study using acylated ghrelin neutralization antibody in which no differences were observed in body weight and food intake though these works detected an increase in plasma acylated ghrelin levels compared to controls (Lu *et al.* 2009). The strength of the present study is, however, the larger group size and very homogenous and strong immune responses to ghrelin compared to the previous reports. One possible explanation for the negative effect of ghrelin-PADRE vaccination on weight gain could be that obesity induced by consumption of a high fat diet in mice is known to impair ghrelin secretion and induce ghrelin insensitivity (Perreault *et al.* 2004), and this regulatory function may have been overridden by the increased plasma ghrelin levels achieved via vaccination-induced immune responses. Alternatively, ghrelin's acute central actions and GHSR-ghrelin signaling may be different in the LDLR KO mouse strain (Egecioglu *et al.* 2008). Overall, the conclusion from this study was that PADRE-ghrelin vaccine does not represent a potential target for therapeutic use against weight gain.

6.5 Methodological considerations

6.5.1 Study populations, materials and design

The present thesis was designed to use different experimental approaches such as human subjects, specific cell cultures and animals for investigating the aims set out at the start of this thesis.

Study I consisted of the OPERA study population which is a large ($n = 1040$) cross-sectional study consisting hypertensives and controls. The main characteristics of the control study population are comparable to the general Finnish population (Vartiainen *et al.* 1994), and therefore, it can be expected that the control cohort represents fairly well the general Finnish population. Although the hypertensive cohort represents a population with a higher level of cardiovascular risks compared to the control cohort, the study cohorts were analyzed together. Even though association studies on cross-sectional data are commonly used approaches, it is worth noting that this approach cannot confirm causalities.

Study II consisted of 64 hysterectomized postmenopausal women receiving either oral or transdermal estrogen replacement therapy for 6 months. This study included only hysterectomized women, because it was intended to examine the influence of exogenous estrogen alone. The uterus is removed in hysterectomy and this can decrease dramatically the endogenous sex hormone levels and probably this will have consequences on metabolism (Gallicchio *et al.* 2006). In this study, the metabolic effects of hysterectomy itself were not determined.

Study III utilized the relevant cells participating in atherogenesis. In these *in vitro* studies, THP-1 human monocytic cell line and human endothelial cells, EA.hy 926, were used as a model of monocyte adhesion to aortic endothelium. EA.hy 926 cell line has been generated by fusion of HUVEC with the human lung carcinoma cell line A549 (Lieber *et al.* 1976). These immortalized and well characterized cells express several characteristics typical of endothelial cells, such as von Willebrand factor, integrins and also adhesion molecules (ICAM, VCAM, E-selectin) upon stimulation with TNF α , i.e. they represent a rather good model of large vessel endothelium with which to study adhesion and pro-inflammatory responses (Bouis *et al.* 2001). The influences of ghrelin were studied in both TNF α stimulated and non-stimulated EA.hy 926 cells. Thus, the results obtained in the EA.hy 926 endothelial cells with TNF α stimulation may reflect better the situation in endothelium, but non-stimulated cells might reflect the situation when

there is no inflammation involved, as in the earlier phases of atherogenesis. However, primary endothelial cells, HUVECs, express a wider variety of adhesion molecules constitutively after stimulation and thus, HUVECs could have been a good alternative for studies modelling vascular endothelium.

Thioglycollate-elicited mouse macrophages were isolated from mouse peritoneum and used for the Ox-LDL binding studies. These macrophages are highly active in expressing scavenger receptors and provided a functional binding assay platform in our system. Mouse J774A.1 monocyte/macrophages utilized in this study had been earlier used for the uptake studies of acetylated LDL and these cells have been demonstrated to accumulate cholesterol esters (Lesnik *et al.* 1997, Qin *et al.* 2006).

Study IV was conducted using a mouse model, LDLR KO mouse, which is a good tool with which to study the development of atherosclerosis *in vivo*. The LDLR KO mouse is one of most widely used mouse models of atherosclerosis and has been instrumental in evaluating and testing new atherosclerotic drugs (Zadelaar *et al.* 2007). Normally mice have high levels of HDL cholesterol and they do not develop atherosclerosis. The deficiency of the LDL receptor in these mice decreases the clearance of VLDL and LDL particles which are normally taken up by the liver LDL receptor and therefore, a marked increase is seen in the IDL/LDL fraction and a small increase in the VLDL levels (Ishibashi *et al.* 1994). The LDLR KO mouse displays elevated cholesterol levels and mild to moderate atherosclerotic lesions in the aortic root and coronary arteries when fed an atherogenic, high fat diet (Ishibashi *et al.* 1994). After consumption of a high fat diet for 22 weeks in the present study, the mice developed atherosclerosis. Due to the major differences between humans and mice, however, the findings cannot be extrapolated to humans.

6.5.2 Measurements of ghrelin

Ghrelin was the first bioactive peptide reported to have an essential n-octanoyl modification at the hydroxyl group of serine (Kojima & Kangawa 2005). This acylated ghrelin was first considered as an active form of ghrelin which binds to ghrelin receptor (GHS-R) and can induce the release of growth hormone. However, a ghrelin peptide without the acyl group, des-acyl ghrelin, was found to have many functions and the existence of a still unknown receptor for des-acyl ghrelin has been postulated (Baldanzi *et al.* 2002, Cassoni *et al.* 2004, Muccioli *et al.* 2004, Filigheddu *et al.* 2007). Thus both forms of ghrelin, acylated and des-

acylated, are relevant and they may have different biological functions. Therefore, it would have been desirable to have access to assay techniques for both forms of ghrelin.

In the present work, ghrelin was measured from fasting plasma samples using different commercial ghrelin assays. The first ghrelin measurements were performed from plasma samples of the subjects of OPERA study (Study I) using a total ghrelin RIA kit. This commercial RIA contained an antibody that recognizes both acylated and des-acylated forms. Subsequently commercial assays detecting only acylated ghrelin became available. Active (acylated) ghrelin was measured from the plasma samples of ERT study using a commercial RIA. Both total ghrelin and acylated ghrelin were analyzed from plasma samples of LDLR KO mice using a commercial RIA detecting total ghrelin and a commercial ELISA assay measuring acylated ghrelin.

It does seem reasonable that the total ghrelin concentration was assayed for several reasons. Ghrelin-like immunoreactivity is almost complete ($\approx 90\%$) for des-acyl ghrelin (Patterson *et al.* 2005), as deacylation of ghrelin occurs rapidly in plasma and thus, des-acyl ghrelin is more stable under all conditions (Hosoda *et al.* 2004). Total ghrelin is a good surrogate of acylated ghrelin since their levels are correlated, as also seen in LDLR KO mice, and the ratio of these two forms stays constant under a wide variety of conditions (Ariyasu *et al.* 2002, Beck *et al.* 2003, Marzullo *et al.* 2004). Degradation of ghrelin in plasma samples can be avoided by adding EDTA and aprotinin when collecting the blood specimen and by immediately acidifying the sample before freezing and storage at low temperature (Hosoda *et al.* 2004). The measurement of acylated ghrelin from the EDTA-plasma samples from the ERT study might have resulted in lower concentrations, because these samples had been collected ten years earlier without treatment with acid. Nevertheless the sample collection and storage conditions were identical and it can be assumed that degradation occurs similarly in all samples. Plasma samples from mice for acylated ghrelin measurements were handled according to the manufacturer's instructions using a protease inhibitor.

Ghrelin levels have been shown to fluctuate during the day, increasing before and decreasing after a meal (Cummings *et al.* 2001). The amplitude and the frequency of ghrelin pulses have been shown to be higher in healthy subjects than in morbidly obese subjects (Mingrone *et al.* 2006). However, the periodical increases and decreases in plasma ghrelin concentrations also occur during fasting in the absence of food consumption (Natalucci *et al.* 2005). In the OPERA cohort and ERT study, the majority of the blood samples were drawn in the morning but

the time of collection can vary between some subjects and it is probable that some subjects had passed their peak ghrelin level.

6.5.3 Vaccination

A novel vaccination technique was utilized for the assessment of the role of ghrelin in mouse atherosclerosis. PADRE peptides are used in this technique and combined with antigens. PADRE induces an important “co-stimulatory”-signal which makes it possible to achieve antigen-specific immune responses. This artificial protease resistant 13-mer peptide contains D-alanine, L-cyclo-hexyl-alanine and amino-caproic acid residues and can be synthesized by modern chemical methods. Most human and murine class II and mouse class IA major histocompatibility complex molecules bind this sequence and helper T-cell activity is provided to help antibody responses *in vivo* (Alexander *et al.* 1994, del Guercio *et al.* 1997). PADRE peptides have been successfully used for vaccination studies in human and in experimental animals. In a recent study, murine IL-12 was linked to PADRE and plasma IL-12 levels and the signs of atherosclerosis in LDLR KO mice were decreased (Hauer *et al.* 2005).

In the present thesis, a synthetic ghrelin-PADRE peptide, which consists of PADRE peptide and n-octanoylated mouse ghrelin (1-28), was used to assess the effects of ghrelin on the development of atherosclerosis in LDLR KO mice. The ghrelin-PADRE vaccine generated a strong and homogeneous IgG response against ghrelin in agreement with previous studies using different immunogens (del Guercio *et al.* 1997, Hauer *et al.* 2005). The antibody response induced by PADRE-ghrelin vaccine was more homogenous in comparison with the traditional vaccination technique using KLH as a carrier protein for ghrelin in C57BL/6 mice. Similarly in a vaccination study, a high level of antibodies against ghrelin was not seen in all immunized rats (Zorrilla *et al.* 2006).

Ghrelin-PADRE vaccination increased both plasma total and acylated ghrelin levels in the present study and hence it was used to study the influence of increased plasma ghrelin levels on the development of atherosclerosis. The increase seen in ghrelin plasma levels in the present study was contradictory to the findings seen in the IL-12-PADRE vaccination study where plasma IL-12 levels declined in the LDLR KO mice (Hauer *et al.* 2005). The cells responsible for the most of the secretions of IL-12 and ghrelin are different, and therefore, the mechanisms that regulate the secretion of the products from those cells may also be very different. Ghrelin mRNA and protein expression levels in stomach

were not significantly different between the groups and no explanation to clarify this mechanism could be generated in the present thesis. Interestingly, a recent study using a ghrelin neutralization antibody saw a similar increase (8x) in plasma acylated ghrelin levels and these workers could also show that the antibodies blocked ghrelin signaling at the cellular level and suggested that blocking peripheral ghrelin could induce compensatory increases in the acylated ghrelin level (Lu *et al.* 2009). Increased plasma ghrelin concentrations via ghrelin-PADRE vaccination may have been caused by the formation of ghrelin-IgG immune complexes that are unable to be cleared from the circulation. Even though these immune complexes could not be detected in these experiments, they may possibly have interfered with the *in vivo* clearance of acylated ghrelin. It would have been interesting to test plasma samples of immunized mice with an *in vitro* functionality test in a cell line expressing GHS-R. That kind of experiment could have clarified how the vaccination is affecting ghrelin signaling *in vivo*. Unfortunately, this possibility was not available in this present study due to lack of the samples. The present thesis did not provide any detailed understanding about how the vaccination increased plasma ghrelin levels.

6.5.4 Determination of atherosclerosis

The degree of human atherosclerosis can be analyzed using several different methods such as intravascular ultrasound (Nissen 2001) and cardiac magnetic resonance imaging (Davis *et al.* 1997). In this study, the carotid artery wall thickness was determined by B-mode ultrasound. The benefits of this method are its ease, safety, inexpensiveness, precision and reproducibility (Aminbakhsh & Mancini 1999). Increased arterial wall thickness is clearly linked to the risk of suffering cardiovascular diseases (Burke *et al.* 1995) being an excellent predictor of coronary and cerebrovascular complications (Simon *et al.* 2002).

Atherosclerosis can be quantified from mouse models of atherosclerosis using cross-sections of the aortic root or the *en face* analysis of the entire aortic trunk. Both morphometric methods provide valid and complementary information on the degree and distribution of atherosclerotic signs (Tangirala *et al.* 1995). These traditional methods were used in this study and additionally the aortas stained with Sudan IV were scanned using Odyssey infrared scanner at 700nm. The results between this new quantification method and the *en face* method displayed a good correlation.

6.6 Future aspects

Ghrelin is a multifunctional hormone that may possibly be involved in atherogenesis and cardiovascular disease. Ghrelin has been a topic being very intensively researched over recent years. Since ghrelin can influence a wide array of different physiological functions, there has been speculation about the possibility of its clinical use in a large number of pathological conditions including cardiovascular disease either for diagnosis or even treatment.

In this thesis, the associations between plasma ghrelin levels and carotid atherosclerosis measurements and factors influencing atherogenesis were investigated in human subjects of a large population-based cohort study and among postmenopausal women. In addition, the effects of ghrelin were demonstrated in cells participating in early key events in atherosclerosis and in a mouse model of atherosclerosis. The positive association between carotid IMT and plasma ghrelin levels was contradictory to the associations observed between plasma ghrelin levels and the factors influencing atherosclerosis. The cell culture experiments revealed that ghrelin can have both pro-and anti-inflammatory influences which could be interpreted to mean that ghrelin has a different role in atherosclerosis depending on the stage of the disease. The results suggest that ghrelin is one of the components playing a role in atherogenesis. The modulatory role of ghrelin at different steps in atherogenesis clearly needs to be further elucidated. In addition, further experimental and human studies to assess the role of different forms of ghrelin (e.g. acylated and des-acyl ghrelin and obestatin) in atherogenesis and lipid metabolism are warranted in order to clarify the role and significance of ghrelin in atherogenesis.

As a conclusion, based on the present findings of human association studies of atherosclerosis and factors influencing atherosclerosis as well as *in vitro* and *in vivo* experiments into atherogenesis, it seems likely that ghrelin is one of the components playing a role in atherogenesis. It has modulatory functions on the vascular system and atherogenesis albeit the contribution is not as dominant as that of the better known traditional risk factors.

7 Conclusions

The present study investigated the role of ghrelin in atherosclerosis. The major conclusions can be presented as follows:

1. Plasma total ghrelin levels were associated with carotid artery atherosclerosis measured as cIMT only in males.
2. Plasma ghrelin levels were associated inversely with plasma total and LDL cholesterol and triglyceride levels as well as BMI and positively with HDL cholesterol levels in postmenopausal women and in the population-based cohort study. Treatment with estrogen increased plasma acylated ghrelin level and the changes in plasma acylated ghrelin level and estradiol concentrations were positively associated in hysterectomized women receiving estrogen replacement therapy for 6 months.
3. Ghrelin increased monocyte adhesion to endothelial cells, elevated the expression of ICAM-1 in endothelial cells and prompted oxidized LDL binding to macrophages. These effects were reversed in the presence of TNF α when ghrelin decreased monocyte adhesion and the expression of endothelial VCAM-1 and MCP-1. Ghrelin showed no effects on acetylated LDL uptake by J774.A1 mouse macrophages.
4. Ghrelin vaccination induced high plasma levels of antibodies against ghrelin and increased ghrelin levels. However, ghrelin vaccination did not affect the development of atherosclerosis though plasma MCP-1 levels were reduced in LDLR KO mice.

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