GHRELIN, OBESITY AND TYPE 2 DIABETES

GENETIC, METABOLIC AND EPIDEMIOLOGICAL STUDIES
JOHANNA VARTIAINEN

GHRELIN, OBESITY AND TYPE 2 DIABETES
Genetic, metabolic and epidemiological studies

Academic dissertation to be presented with the assent of the Faculty of Medicine of the University of Oulu for public defence in Auditorium 8 of Oulu University Hospital, on 3 April 2009, at 12 noon

OULUN YLIOPISTO, OULU 2009
Ghrelin is a peptide hormone with anabolic functions. It increases growth hormone secretion and appetite, decreases fat utilisation as a metabolic fuel and increases fat storage in the adipose tissue. In addition, ghrelin exerts effects on glucose metabolism, heart function and inflammatory processes. Due to these characteristics ghrelin has become a hot topic for research focusing on obesity and its co-morbidities such as hypertension, type 2 diabetes and atherosclerosis.

The aims of this study were to detect new sequential variations in the genes coding for the ghrelin receptor and ghrelin and to study whether these variations associate with obesity and metabolic risk factors for atherosclerosis. The roles of genetic and environmental factors in the determination of plasma ghrelin levels were also examined. In addition, the association of plasma ghrelin concentrations with disordered glucose regulation and type 2 diabetes was assessed in a longitudinal study.

Five single nucleotide polymorphisms (SNPs) in the exons of the ghrelin receptor and 11 SNPs in the ghrelin gene 5’ flanking area were found. The SNPs in the ghrelin receptor gene did not associate with plasma IGF-1 concentrations, but two of them did reveal an association with insulin and/or lipid metabolism related parameters. The SNPs found in the ghrelin gene 5’ flanking area did not associate with plasma ghrelin levels, but one of them associated with body mass index (BMI). In monozygotic twins discordant for obesity, ghrelin levels were higher in the lean compared with the obese co-twins. Serum ghrelin levels at baseline did not differ between those who maintained normal glucose tolerance and those who developed impaired glucose regulation or type 2 diabetes during the follow up.

In conclusion, the results suggest that two variations in the ghrelin receptor gene might be associated with glucose and lipid metabolism but not with IGF-1 levels. One SNP in the ghrelin gene might be associated with obesity. The results also indicate that plasma ghrelin levels are influenced by acquired obesity rather than genetic determinants. Finally, fasting serum ghrelin concentrations do not seem to have a significant predictive value on the incidence of impaired glucose tolerance and type 2 diabetes.

**Keywords:** diabetes, ghrelin, obesity
Vartiainen, Johanna, Greliini, lihavuus ja tyyppi 2 diabetes. Geneettisiä, aineenvaihdunnallisia ja epidemiologisia tutkimuksia
Lääketieteellinen tiedekunta, Kliinisen lääketieteen laitos, Sisäaudit, Oulun yliopisto, PL 5000, 90014 Oulun yliopisto; Biocenter Oulu, Oulun yliopisto, PL 5000, 90014 Oulun yliopisto; Kliinisen tutkimuksen keskus, Oulun yliopistollinen sairaala, PL 5000, 90014 Oulun yliopisto

Oulu

Tiivistelmä
Greliini on peptidihormoni, jolla on osoitettu olevan anabolisia vaikutuksia. Se voimistaa kasvuhormonin erityystä, toimii ruokahalua ja ravinnonottoa lisäävänä signaalinä, vähentää rasvahapojen käyttöä energianlähteenä ja lisää rasvakudoksen määrää. Lisäksi greliinilla on osoitettu olevan vaikutuksia sokeriaineenvaihduntaan, sydämen toimintaan ja tulehduksellisiin prosesseihin. Näiden ominaisuuksien ansiosta greliiniä on tullut tärkeänä tutkimuskohde lihavuuteen ja sen liitännäissairauksiin, kuten verenpainetaukiin, tyyppiin 2 diabeteskeeseen ja ateroskleroosiin liittyvässä tutkimuksessa.

Tässä väitöskirjatutkimuksessa olivat tavoitteina etsiä uusia geneettisiä poikkeamia greliiniä ja greliinireseptoria koodaavista geneistä ja tutkia löytyneiden poikkeamien kytkymistä lihavuuteen sekä ateroskleroosin aineenvaihdunnallisiin riskitekijöihin. Tavoitteineita oli myös selvittää geneettisten- ja ympäristötekijöiden roolia greliinipitoisuksien säätelyssä. Lisäksi grelinipitoisuksien yhteyttä tyyppiin 2 diabeteskeeseen ja sokeriaineenvaihdunnan häiriöihin tutkittiin seurantatutkimuksessa.


Asiasanat: diabetes, greliini, lihavuus
Acknowledgements

This thesis study was carried out at the Institute of Clinical Medicine, Department of Internal Medicine, Biocenter Oulu, Clinical research Center Oulu, University of Oulu. I appreciate the facilities available in these institutes.

I am deeply grateful to my supervisor Professor Antero Kesäniemi M.D., Ph.D., for giving me the opportunity to enter to the world of medical science as a member of his group. His strong and encouraging character and above all, his vast expertise in many fields of metabolic research have provided me extremely valuable support to carry out these studies.

I also wish to express my most sincere and deep gratitude to my supervisor Olavi Ukkola M.D., Ph.D., who guided me through my work with his expert advice and knowledge on obesity, diabetology and genetics. His support and patience helped me at every step of the way. I am grateful for all his time and effort that he has put into my work.

In addition, my special thanks belong to Seppo Pöykkö, M.Sc., M.D., Ph.D., for not only sharing me his knowledge of statistics but also for being always so positive company as a colleague. Special thanks for statistical support belong also to Pentti Nieminen M.Sc., Ph.D., Risto Bloigu M.Sc., Ph.D., and Jari Jokelainen M.Sc.

I want to acknowledge the official referees Professor Veikko Salomaa M.D., Ph.D., and Adjunct Professor Matti Jauhiainen M.Sc., Ph.D., for their careful review of the manuscript of this thesis, and Ewen MacDonald for the careful revision of the language.

Everyone working in the research laboratory of the Department of internal medicine, University of Oulu, in the Clinical Research Center is thanked for helping me with the practical aspects during my laboratory work. It was nice to work in such a warm and friendly atmosphere. Also the moments shared around the coffee table during the breaks will stay as warm feelings in my memories for the rest of my life. Special thanks belong to Eija Kellokoski for the fruitful conversations on ghrelin. In addition all the other post graduate student colleagues, especially Anne Kunnari M.Sc., Mirella Hietaniemi M.Sc., Elina Malo M.Sc., Merja Santaniemi M.Sc., Outi Kummu M.Sc., Maritta Sämpi M.Sc., Piia Leskelä M.D., Tiia Kangasmäki M.Sc., Antti Nissinen M.Sc., Sanna Mäkelä M.Sc., and Tuija Räisänen M.Sc., are thanked for sharing their thoughts on the research work as well as personal issues, for creating such a good spirit and thereby increasing the motivation for the work. Heidi Häikiö, Saija Kortetjärvi and Sirpa Rannikko.
are thanked for the technical support for the laboratory procedures. Sakari Kakko M.D., Ph.D., and Marja-Leena Kytökangas are thanked for the assistance with the ABI 377 sequencer. Marita Koistinen, Anne Salovaara and Irene Tuomela-Törmänen are warmly thanked for the advice with the paper work during my studies.

During the years that I carried out my thesis studies, I was also a medical student. I am grateful for the friendships I made at the medical school. The support from friends has been of great value and I hope the bond between us will last for the rest of our lives.

I want to thank my mother Pirjo and father Jouko for giving me a safe and supporting childhood and also for encouraging me to reach my goals in life. My sisters Anna-Kaisa and Eeva-Maria are thanked for being two of the dearest friends that I have. The support I receive from them is of great value.

Finally, I wish to thank Sami, the love of my life. With his belief in me I managed to finally finish this book. I thank him for his love and support. I am grateful for his special kind of balance and optimism towards life. These things make the atmosphere in our home peaceful and harmonious. I feel that with him, my skills in setting and achieving my goals have developed. Most importantly, with him I enjoy every moment of my life more than ever before.

This study was supported by grants from the Oulu University Scholarship Foundation, The Finnish Medical Foundation, the Research Council for Health of the Academy of Finland and the Finnish Foundation for Cardiovascular Research. The support has been of great value.

Espoo, March 4th 2009

Johanna Vartiainen
Abbreviations

ACTH  Adrenocorticotrophic hormone
ADP  Adenosine diphosphate
AgRP  Agouti related protein
ANOVA Analysis of variance
ANCOVA Analysis of covariance
ARC  Arcuate nucleus
ASF  Abdominal subcutaneous fat
ATP  Adenosine triphosphate
AUC  Area under the curve
AVF  Abdominal visceral fat
bp  Base pair
BMI  Body mass index
cAMP  Cyclic adenosine monophosphate
CI  Confidence interval
CV  Coefficient of variation
DNA  Deoxyribonucleic acid
DM2  Type 2 diabetes
FSH  Follicle stimulating hormone
GH  Growth hormone
GHS  Growth hormone secretagogue
GHS-R  Growth hormone secretagogue receptor
GHRH  Growth hormone releasing hormone
GIP-1  Gastric inhibitory peptide
GLP-1  Glucagon like peptide-1
GOAT  Ghrelin-O-acyltransferase
GPCR  G-protein coupled receptor
GTP  Guanosine triphosphate
GWAS  Genome wide association study
HDL  High-density lipoprotein
HRT  Hormone replacement therapy
IFG  Impaired fasting glucose
IGF-1  Insulin-like growth factor 1
IGFBP  Insulin-like growth factor binding protein
IGR  Impaired glucose regulation
IGT  Impaired glucose tolerance
IP3  Inositol triphosphate  
i.m.  Intramuscular  
i.v.  Intravenous  
kDa  Kilo Dalton  
LDL  Low density lipoprotein  
LH  Luteinizing hormone  
mRNA  Messenger ribonucleic acid  
NPY  Neuropeptide Y  
ns  Not statistically significant  
OGTT  Oral glucose tolerance test  
OPERA  Oulu Project Elucidating Risk of Atherosclerosis  
PCR  Polymerase chain reaction  
POMC  Pro-opiomelanocortin  
PP  Pancreatic polypeptide  
PYY  Peptide YY  
PRL  Prolactin  
RFLP  Restriction fragment length polymorphism  
RIA  Radioimmunoassay  
S.c.  Subcutaneous  
SNP  Single nucleotide polymorphism  
SS  Somatostatin  
TSH  Thyroid stimulating hormone  
VLDL  Very low-density lipoprotein  
WHO  World Health Organization  
QUICKI  Quantitative insulin sensitivity check index  
QTL  Quantitative trait loci
List of original articles


## Contents

Abstract

Tiivistelmä

Acknowledgements

Abbreviations

List of original articles

Contents

1 Introduction

2 Review of the literature

2.1 Ghrelin – discovery, structure and characteristics

2.1.1 Localisation of ghrelin expression

2.1.2 Ghrelin gene codes for two hormones

2.1.3 Molecular characteristics of circulating ghrelin molecules

2.2 Ghrelin receptor GHS-R1a

2.2.1 Structure and function of GHS-R1a

2.2.2 Localization of GHS-R1a

2.3 Biological effects of ghrelin

2.3.1 Effects of ghrelin on the endocrine system

2.3.2 Effects of ghrelin on appetite and food intake

2.3.3 Effects of ghrelin on long term energy balance in humans

2.3.4 Animal studies addressing the role of ghrelin in energy metabolism

2.3.5 What can be learned from human tumors over-producing ghrelin?

2.3.6 Effects on the gastrointestinal tract

2.3.7 Ghrelin and cardiovascular effects

2.3.8 Other effects of ghrelin

2.4 Regulation of ghrelin plasma concentrations

2.4.1 Diurnal regulation of ghrelin concentrations by hormonal and neuronal factors

2.4.2 Diurnal regulation of ghrelin concentrations by nutritional factors

2.4.3 Long term regulation of ghrelin concentrations

2.5 Variants in genes coding for ghrelin and ghrelin receptor

2.5.1 Ghrelin receptor gene polymorphisms

2.5.2 Ghrelin gene polymorphisms
2.6 Ghrelin, insulin resistance and type 2 diabetes ................................. 46

3 Aims 49

4 Subjects and methods 51
4.1 OPERA study population .................................................................. 51
   4.1.1 Description of the study population of study I ......................... 52
   4.1.2 Description of the study population of study II ....................... 52
4.2 Description of the study population of study III .............................. 53
   4.2.1 Control populations for obesity discordant monozygotic twins ......................................................... 55
4.3 Description of the study population of study IV ............................... 56
4.4 Clinical methods .............................................................................. 57
   4.4.1 Sequencing analyses ................................................................ 59
   4.4.2 Genotyping of the ghrelin gene variants .................................. 60
4.5 Measurements of the ghrelin concentrations .................................... 63
4.6 Statistical methods .......................................................................... 63

5 Results 65
5.1 Sequencing analysis of GHSR exons (study I) ................................. 65
5.2 Sequencing analysis of the GHRL 5’ flanking area (Study II) .......... 68
5.3 Ghrelin concentrations in obesity discordant twins (Study III) ......... 69
5.4 Ghrelin gene polymorphisms in twin-populations (study III) .......... 70
5.5 Ghrelin and the incidence of impaired glucose regulation and type 2 diabetes (Study IV) ................................................................. 71

6 Discussion 75
6.1 Methodological considerations ....................................................... 75
   6.1.1 Study populations and design .................................................. 75
   6.1.2 Ghrelin concentrations measured in this study ......................... 77
6.2 Genetic association studies ............................................................... 78
   6.2.1 Ghrelin receptor variants ......................................................... 79
   6.2.2 Ghrelin gene variants and obesity ............................................ 80
6.3 Leu72Met variation in the ghrelin gene ............................................. 81
6.4 Regulation of ghrelin concentrations ................................................. 82
6.5 Ghrelin and type 2 diabetes ............................................................... 84
6.6 Aspects for future studies ................................................................. 87

7 Conclusions 89
References 91
Original articles 109
1 Introduction

The prevalence of overweight and obesity is increasing rapidly worldwide and now represents a major health crisis. Obesity determined by body mass index (BMI) >30 kg/m² is strongly associated with several co-morbidities such as type 2 diabetes, hypertension, dyslipidaemia, atherosclerosis, stroke, certain types of cancer, gall bladder disease and sleep apnoea and furthermore, it is connected with increased rate of mortality. The major factors contributing to the epidemic of obesity are believed to be the sedentary lifestyle; decreased physical activity at work and leisure, and the popularity of energy rich foods. However, some individuals gain weight more easily and are more vulnerable to the obesogenic environment of today’s world than others. These differences are probably due to their genetic or biological predisposition towards obesity. (World Health Organization 2000.) The heritability of obesity has been estimated in family, twin and adoption studies with estimates ranging from 25% up to 90% having been proposed (Barsh et al. 2000). Therefore it is of considerable importance to identify the metabolic and genetic factors contributing to the risk for obesity and its co-morbidities.

Ghrelin is a multi-functional gut-derived peptide hormone. Administered ghrelin has been shown to increase appetite and food intake and stimulate the secretion of growth hormone in rodents and in humans. Low plasma concentrations of ghrelin are associated with obesity, type 2 diabetes and hypertension. Ghrelin has been demonstrated to have effects on glucose metabolism, blood pressure, heart function and also on inflammatory processes. Based on these findings ghrelin has been proposed to be involved in the physiology and/or pathophysiology of obesity and its associated co-morbidities, such as type 2 diabetes and atherosclerotic cardiovascular diseases. A deeper understanding on this interesting pleiotropic hormone could be anticipated to open new insights into the metabolic pathways causing a predisposition for obesity and its co-morbidities.

In the present thesis, the aims were to screen the genes coding for ghrelin and its receptor for new mutations and study their association with certain obesity related parameters and risk factors for cardiovascular disease. In addition, the regulation of ghrelin concentrations and the association of ghrelin levels with type 2 diabetes were topics of interest. The studies were performed by examining three human study populations.
2 Review of the literature

2.1 Ghrelin – discovery, structure and characteristics

Ghrelin is a peptide hormone that consists of 28 amino acids with a molecular weight of about 3.3kDa. It was identified in 1999 in a study which was designed to search for an endogenous ligand for an orphan receptor, the type 1a growth hormone secretagogue receptor (GHS-R1a). Ghrelin, which was derived from rat stomach extracts, was found to be able to activate this receptor and to stimulate growth hormone release from the pituitary in a dose dependent manner. The name ghrelin comes from this physiological effect: Ghre is the Proto-Indo-European root of the word “grow” (Kojima et al. 1999). Subsequently ghrelin was demonstrated to have other biological activities, such as increasing appetite and food intake, and it has been given the nickname, the “hunger hormone”.

2.1.1 Localisation of ghrelin expression

Ghrelin mRNA expression has been detected in at least 29 different human tissues, i.e. almost all parts of the human body (Gnanapavan et al. 2002). At the protein level, the main source of ghrelin synthesis in all vertebrate species has been shown to be the stomach (Ariyasu et al. 2001). Ghrelin synthesis has been localized in the submucosa of the stomach, in endocrine X/A-like cells in rats and P/D, like cells in humans (Date et al. 2000, Rindi et al. 2002). Lower levels of ghrelin synthesis have been detected in the gastrointestinal tract, such as in duodenum, jejunum, ileum, and colon (Lee et al. 2002). In addition, this hormone has been detected in the central nervous system, e.g. in arcuate nucleus and hypothalamus (Lu et al. 2002), and interestingly in pancreas (Date et al. 2002, Volante et al. 2002, Wierup et al. 2002). In addition, some cells of the immune system, such as peripheral blood mononuclear cells (lymphocytes and monocytes) produce ghrelin (Mager et al. 2008). Furthermore, human ovaries and testes also are able to secrete this peptide hormone (Garcia et al. 2007). In conclusion, several cells of the human body are able to produce ghrelin, though the physiological relevance of this is unknown. It could be postulated, that ghrelin might have not only endocrine but also autocrine and paracrine functions.
2.1.2 Ghrelin gene codes for two hormones

The ghrelin gene (GHRL, GeneID 51738) is located in the chromosome 3p26–25. It contains four exons and three introns (Wajnrajch et al. 2000). Ghrelin is synthesized as a precursor protein containing 117 amino acids, and is called prepro-ghrelin. Prepro-ghrelin is modified by the action of protein convertase enzymes into mature ghrelin, consisting of 28 amino acids and another peptide hormone, obestatin, that consists of 23 amino acids (Zhang et al. 2005). The name obestatin comes from the Latin word obedere, which means to devour, and statin, denoting suppression. The nomenclature resembles the biological activities of this molecule that are opposite to those of ghrelin: hence obestatin decreases appetite and inhibits weight gain.

After post-translational modifications, a fatty acyl group is attached to the third amino acid, serine (Ser3) of the mature ghrelin via an esteric-bond. The most common acyl group found attached on the circulating ghrelin molecules is an octanoyl group (Figure 1). The enzyme that catalyses the reaction in which the octanoate is attached to the hydroxyl group of the Ser3 was identified recently and is called ghrelin-O-acyltransferase (GOAT)(Yang et al. 2008). The other product of ghrelin gene, obestatin, also goes through a post translational modification; the C-terminal glycine residue is amidated. The amidation is believed to be required for the bioactivity of obestatin. (Zhang et al. 2005)
Fig. 1. Ghrelin gene consists of four exons and three introns. The DNA code is transcribed into mRNA, which contains untranslated 5’ and 3’ regions and coding regions. The coding regions are subsequently translated to form a 117 amino-acid chain, prepro-ghrelin. Mature ghrelin peptide is cleaved from the prepro-ghrelin and an octanoyl group is attached to its third amino acid serine. Another peptide hormone, obestatin, is also derived from the prepro-ghrelin molecule. Amino acids are shown as one letter codes.

2.1.3 Molecular characteristics of circulating ghrelin molecules

Other molecular forms of ghrelin in addition to the Ser3 octanoylated ghrelin have been identified. In the rat stomach, a ghrelin lacking the 14th amino acid glutamine, des-Gln14-ghrelin, has been detected (Hosoda et al. 2000). This variant is suggested to be a result of alternative splicing. In addition, several other ghrelin derived molecules in the human circulation and in lysed human stomach tissue have been found. These differ from each other on one hand by their amino acid sequence length and on the other hand by the characteristics of the acyl side chains attached to Ser3. There are 28 and 27 acids long ghrelin molecules; In the 27 amino acid long ghrelin molecule the last amino acid (Arginine 28) is cleaved, probably by alternative splicing. The acyl groups that are attached to Ser3, are
octanoyl (eight carbon atoms and no double bonds), decanoyl (ten carbon atoms and no double bonds) and decenoyl (ten carbon atoms and one double bond) side chains. (Hosoda et al. 2003). In addition, ghrelin molecules without an acyl group (des-acyl ghrelin) have been detected in the circulation (Hosoda et al. 2000).

It was shown that des-acyl ghrelin is not able to bind and activate the known ghrelin receptor GHS-R1a, and in this way it differs from the 28 amino acids long Ser3 octanoylated ghrelin. Thus, it was proposed initially that only the acylated ghrelin would possess endocrine and appetite stimulating activity (Kojima et al. 1999). Subsequently this suggestion was proven wrong; des-acyl ghrelin has been shown to have biological activities too. For example, it decreased food intake when administered centrally or when over-expressed in transgenic mice. This finding suggests that it has an opposing action to that of acylated ghrelin (Asakawa et al. 2005). Opposing effects of des-acyl ghrelin and acylated ghrelin have also been observed in the regulation of insulin secretion in pancreas (Qader et al. 2008) and in the regulation of glucose secretion in the liver hepatocytes (Gauna et al. 2005). In addition to these opposing effects of des-acyl ghrelin and acylated ghrelin, in some circumstances these two molecules can display similar biological effects. For example, they both have been observed to protect cardiomyocytes from injury (Li et al. 2006). In summary, there is no clear consensus about the results concerning the biological effects of des-acyl ghrelin. Furthermore, a receptor specifically binding and mediating the activities of des-acyl ghrelin has been claimed to exist, but it remains elusive and unidentified. Even though it has been previously shown that des-acyl ghrelin is not capable of binding and activating the only known ghrelin receptor GHS-R1a, there are also one study with a contrary result. One recent study claimed that des-acyl ghrelin might after all be a potent agonist of the known ghrelin receptor GHS-R1a, at least in vitro (Gauna et al. 2007).

The des-acyl ghrelin is the most abundant form of ghrelin in the circulation. The molar ratio of acylated and des-acylated forms of ghrelin in the circulation is on the average 1:5 (Espelund et al. 2003), though other researchers have reported even smaller ratios such as 1:10 (Hosoda et al. 2004). These differences are probably due to different methods in sample collection, handling and measurement protocols. It was recently shown in vitro, that acylated ghrelin can lose its acyl side chain in a hydrolysis reaction to form des-acyl ghrelin and thus the amount of acylated ghrelin decreased and the level of des-acylated ghrelin increased as a function of time in a plasma sample in vitro. Subsequently, des-acyl ghrelin is degraded into small peptide fragments. The half life ($T_{1/2}$) for
hydrolysis is 45 minutes and that for degradation was 204 min, indicating, that des-acyl ghrelin is more stable than acylated ghrelin (Rauh et al. 2007). Earlier, the T$_{1/2}$ of ghrelin has been reported to be much shorter, namely 10 minutes (Nagaya et al. 2001). The difference might be due to the much improved methodology using mass spectrophotometry, used in the study of Rauh et al. (2007), which can be considered to be much more reliable, compared to the antibody based method used by Nagaya et al. (2001).

After its synthesis, ghrelin is stored in secretory vesicles inside the cell and released subsequently into the bloodstream where it circulates presumably as a free molecule. One study has suggested that ghrelin might be able to interact with high density lipoprotein (HDL) particles in the circulation (Beaumont et al. 2003). Furthermore, another study showed that ghrelin might bind to other lipoproteins in plasma and that acylated and des-acyl ghrelin might be carried by different lipoprotein fractions (De Vriese et al. 2007). No other studies have addressed this question so far. It must be noted that several small peptides in the circulation are bound to plasma proteins e.g. to albumin and some hormones even have their own carrier molecule.

Ghrelin is an unstable molecule in a blood or plasma sample. If the sample is not acidified and treated with a proteinase inhibitor, about one third is degraded in one hour at 37°C. Freezing and thawing cycles also affect ghrelin molecules, e.g. after 3 cycles, up to 40 per cent of the ghrelin molecules might be lost, according to Hosoda et al. (2004). However, in most of the studies on ghrelin the samples have not been treated with a proteinase inhibitos or with acid and they have been exposed to freezing and thawing and the obtained concentrations might be lower than the real physiological concentrations.

Ghrelin can be measured with commercial radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) kits from several biological aliquots, such as serum, plasma, saliva, milk and urine as well as from cell culture supernatant samples. There are several products available. It is possible to detect acylated and des-acyl ghrelin molecules separately by both RIA and ELISA kits. Alternatively, there are also RIA and ELISA kits which do not recognize whether the ghrelin molecule has the acyl side chain or not, hence they measure the level of total ghrelin. These total ghrelin kits are the oldest and most widely used commercial products for assessing ghrelin concentration. It must be noted, that the ghrelin concentrations measured by different kits can vary by as much as 10-fold, and therefore the hormone levels obtained in different studies are not comparable (Gröschl et al. 2004). The most recent means for ghrelin
measurement is a method in which des-acylated and acylated ghrelin molecules can be measured with a validated liquid chromatography-tandem mass spectrometry. This method enables a simultaneous and rapid measurement of the different ghrelin molecules present in one sample aliquot (Rauh et al. 2007).

2.2 Ghrelin receptor GHS-R1a

One receptor for ghrelin has been identified. This receptor, growth hormone secretagogue receptor type 1a (GHS-R1a) was found actually 3 years before ghrelin itself. It was originally identified as a target for growth hormone secretagogues, synthetic molecules that stimulate growth hormone secretion from the pituitary. The discovery of this receptor led immediately to the presumption that a natural ligand for this orphan receptor must exist (Howard et al. 1996). In 1999 ghrelin was shown to bind and activate this receptor and to stimulate growth hormone secretion (Kojima et al. 1999).

2.2.1 Structure and function of GHS-R1a

The gene coding for ghrelin receptor is mapped at the chromosomal location 3q26.3 and it spans approximately 4100bp. It is highly conserved throughout different animal, bird, fish and non-vertebrate species indicating that the gene product might have an important physiological function. The mRNA product of the gene can be processed to code for two different receptor proteins, GHS-R1a and -R1b, by alternative splicing. (Howard et al. 1996, Petersenn et al. 2001.)

GHS-R1a is a member of the G-protein coupled receptor superfamily, and possesses the typical molecular structure of these receptors. It consists of seven trans-membrane domains, an amino terminal end on the extracellular side and a carboxy terminal end on the intracellular side of the cell membrane. There are also loops connecting the transmembrane domains on both sides of the cell membranes. The extracellular parts of the receptor are thought to be involved in ligand binding and the intracellular parts participate in the activation of the signal transduction cascades in the cytosol. This receptor consists of 366 amino acids and its molecular weight is approximately 41kDa. (Howard et al. 1996.) Type 1b receptor in turn consists of 289 amino acids, and proteomic prediction shows 5 potential transmembrane regions. The type 1b receptor has been expressed in vitro at the protein level in oocytes, and it was shown to be devoid of any activation after exposure to growth hormone secretagogues (Howard et al. 1996) GHS-R1b
was thought to be without a ligand and any biological activity, until recently. It was found to modulate the activation of black seabream GHS-R1a receptor when it was co-expressed with GHS-R1a in HEK293 cells (Chan & Cheng 2004). However, this has not been repeated with mammalian GHS-R1b, or in other mammalian cells and furthermore, the existence of this type 1 b receptor has not been confirmed in non-transfected cells. The structure of human GHS-R1a is illustrated in Figure 2.

GHS-R1a can be activated by ghrelin as well as by synthetic growth hormone secretagogues (Howard et al. 1996, Kojima et al. 1999). The activation of the receptor causes the stimulation of the G-protein subunit Gα11, a molecule attached to the receptor in the intra-cellular space (Howard et al. 1996). This leads
to the activation of intracellular signalling cascades via the phospholipase C (PLC) pathway. Subsequently, the production of inositol triphosphate (IP3) is increased, which in turn leads to release of Ca\(^{2+}\) from intracellular stores to the cytosol. Phospholipase C can alternatively lead to reactions that phosphorylate and inhibit voltage gated potassium channels on the cell membrane. Depolarization of the membrane together with the elevation in the intracellular level of Ca\(^{2+}\) lead to the release of growth hormone. (Smith et al. 1996.) GHS-1a has been shown to mediate also biological functions other than growth hormone release, and to trigger the activation of different intracellular cascades depending on the target cell. For example, its effects on cell proliferation are mediated via pathways that involve protein kinase C / mitogen activated protein kinase and tyrosine kinase molecules (Nanzer et al. 2004). Adding to the complexity of the receptor function, GHS-R1a has been shown to possess high constitutive activity in cells over-expressing this receptor. In another words, the receptor has basal activity, even when not activated by ghrelin or any other agonist. In addition to the effect of ghrelin and synthetic GHS which function as agonists of this receptor, a full antagonist of GHS-R1a has been identified, namely D-Arg\(^1\)-D-Phe\(^5\)-D-Trp\(^7\)-Leu\(^{10}\)-substance P, which is a synthetic molecule capable of blocking the constitutive activity of GHS-R1a. (Holst et al. 2003.) Furthermore, the activity of ghrelin receptor is thought to be modulated or fine-tuned further by ago-allosteric modulation, heterodimerization and receptor internalization (Muccioli et al. 2007).

### 2.2.2 Localization of GHS-R1a

The GHS-R1a has been shown to be expressed at the mRNA level mainly in the hypothalamus and pituitary, where it was first detected and was shown to take part in the release of GH and regulation of appetite (Howard et al. 1996, Kojima et al. 1999). However, several other tissues such as stomach, pancreas, adipocytes, heart and lung, thyroid gland, adrenal gland express this receptor, indicating that ghrelin might have also activities in many more locations and not be exclusive to the central nervous system (Gnanapavan et al. 2002, Nagaya et al. 2001). GHS-R1a has been found in several lymphoid tissues as well as in T- and B-cell lines, and mononuclear cells indicating that ghrelin might have functions in immunomodulation (Dixit et al. 2004, Mager et al. 2008). Furthermore, the expression of the ghrelin receptor in human testis and ovaries as well as in several tumors was viewed as evidence that ghrelin might be involved in the control of cell proliferation as well as in reproduction (García et al. 2007).
2.3 Biological effects of ghrelin

As soon as ghrelin was discovered, its potent stimulatory effect on GH release was observed (Kojima et al. 1999). In the subsequent ten years, various other biological activities have been demonstrated and it seems that ghrelin is a hormone with a plethora of functions. The effects of ghrelin have been intensively studied in vitro and in vivo and in different vertebrate and non-vertebrate species. For clarity, the observations obtained in humans will be emphasized. Cell culture and animal studies will be mentioned only when the findings are of special importance.

2.3.1 Effects of ghrelin on the endocrine system

Exogenous ghrelin has been shown to increase GH secretion dose-dependently both in rodents (Kojima et al. 1999) and in humans (Peino et al. 2000). The stimulatory effect of administered ghrelin on GH release was subsequently observed in several studies, as shown in Table 1. The actions of GH secretion occur in hypothalamus and anterior pituitary gland, where the GH producing cells, somatotrophs, as well as ghrelin receptors are located. Before the time of the discovery of ghrelin, GH release was thought to be under the control of two hormones: GH release stimulating growth hormone releasing hormone (GHRH) and inhibiting somatostatin (SS). (Frohman & Jansson 1986.) The discovery of ghrelin revealed a third player which operates in these regulatory circuits. The effect of co-administration of GHRH and ghrelin on GH release is synergistical (Arvat et al. 2001) and the action of ghrelin on GH release can not be totally inhibited by somatostatin (Di Vito et al. 2002). These findings suggest that the effects of these three hormones are mediated by different receptors. The regulation of GH secretion by ghrelin, SS and GHRH is illustrated in Figure 3.
Fig. 3. Growth hormone (GH) is secreted from the somatotroph cells of the pituitary gland. The secretion is inhibited by somatostatin (SS), and stimulated by growth hormone releasing hormone (GHRH) and ghrelin. Binding of SS and GHRH to their receptors (SS-R and GHRH-R) on the cell surface leads to activation of G-proteins that inhibit (G0 and Gi) and stimulate (Gs) adenylate cyclase (AC). The activation of AC increases the concentration of cyclic AMP (cAMP) in the cytosol, this in turn stimulates protein kinase A (PKA). Activated PKA phosphorylates voltage-gated calcium channels, leading to influx of calcium ion (Ca\(^{2+}\)) into the cell. The increase in the Ca\(^{2+}\) concentrations induces the secretion of GH from the intracellular secretory granules. Ghrelin stimulates the secretion of GH via binding to its receptor GHS-R1a, which activates a G-protein \(G_{\alpha 11}\), and this activated G-protein then stimulates phospholipase C. The action of this lipase increases the intracellular concentration of inositol triphosphate (IP3), which causes the release of Ca\(^{2+}\) from intracellular stores. The action of PLC can alternatively lead to the stimulation of voltage-gated calcium channels, and increase the intracellular Ca\(^{2+}\) concentration in that way. It is the increase in the intracellular Ca\(^{2+}\) that leads to the release of GH.

In humans the effect of ghrelin in GH release has been shown to be two to three fold greater than the effect of GHRH (Arvat et al. 2001, Di Vito et al. 2002). Exogenous ghrelin is probably the most effective stimulant for GH secretion in
humans. However, the role of endogenous ghrelin on GH secretion is not clear. The diurnal secretion patterns of these hormones show different pulsatility compared to each other and no studies addressing the correlation between ghrelin and GH plasma concentrations have been published so far.

In order to achieve a full effect on GH release by ghrelin administration, normally functioning hypothalamus-pituitary connections are required (Maghnied et al. 2007). However, some studies in rodents have suggested, that the vagus nerve might mediate part of the actions of ghrelin on GH release (Date et al. 2002).

In addition to these GH releasing effects, exogenous ghrelin increases the plasma concentrations of several other hormones in humans. Increases of prolactin (PRL), adrenocorticotropic hormone (ACTH), and cortisol concentrations after ghrelin injection have been reported in several studies. In contrast, the secretions of luteinizing hormone (LH) and thyroid stimulating hormone (THS) were not affected by ghrelin administration. The secretion of follicle stimulating hormone (FSH) was noted to decline in one but not all studies (see Table 1 for references). The physiological relevance of these findings remains unknown. Treatment with ghrelin does not affect the secretion of leptin, one of the most potent satiety hormones (Fusco et al. 2007). Though interestingly, ghrelin has been shown to increase the secretion of aldosterone (Arvat et al. 2001).

One of the most interesting endocrine actions of ghrelin is its putative role in the regulation of insulin. Ghrelin administration has been shown to decrease insulin levels in humans in several studies (Table 1). It must be noted, that insulin in turn might have down-regulatory effects on ghrelin levels. Furthermore, ghrelin administration seems also to increase glucose levels though it is not known whether this is due to a decrease of insulin secretion or whether it is a direct effect (stimulation of glycogenolysis and gluconeogenesis). This complex interplay between ghrelin, insulin and glucose will be discussed later in more detail in chapter 2.5.3 Ghrelin, insulin resistance and type 2 diabetes.

In summary, administered ghrelin has a potent effect on the regulation of GH, and to a minor extent of ACTH, prolactin and cortisol secretion and probably no effect on other pituitary hormones or on leptin levels in humans. The endocrinological effects of administered ghrelin are mediated via GHS-R1a and in part, also by vagal afferents. However, the role of endogenous ghrelin remains unknown.
<table>
<thead>
<tr>
<th>Reference</th>
<th>Subjects (n)</th>
<th>Amount of ghrelin</th>
<th>GH</th>
<th>PRL</th>
<th>ACTH</th>
<th>Cortisol</th>
<th>Insulin</th>
<th>LH, FSH, TSH</th>
<th>Other</th>
<th>B-gluc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peino et al. 2000</td>
<td>12</td>
<td>0, 0.25, 0.5, 1, 3.3, 6.6µg/kg</td>
<td>↑</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Takaya et al. 2000</td>
<td>4</td>
<td>0.2, 1 and 5µg/kg</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td></td>
<td></td>
<td></td>
<td>↔</td>
</tr>
<tr>
<td>Arvat et al. 2001</td>
<td>7</td>
<td>1µg/kg</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Broglio et al. 2001</td>
<td>11</td>
<td>1µg/kg</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↑</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hataya et al. 2001</td>
<td>4</td>
<td>0.08, 0.2, 1µg/kg</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td></td>
<td></td>
<td></td>
<td>↔</td>
</tr>
<tr>
<td>Nagaya et al. 2001</td>
<td>6</td>
<td>0 and 10µg/kg</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td></td>
<td></td>
<td></td>
<td>↔</td>
</tr>
<tr>
<td>Di Vito et al. 2002</td>
<td>7</td>
<td>1µg/kg</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aimaretti et al. 2002</td>
<td>13</td>
<td>1µg/kg</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arosio et al. 2003</td>
<td>8</td>
<td>3.3µg/kg</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>SS↑, PP↑, Gastrin↔</td>
<td>↑</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Broglio et al. 2003</td>
<td>7</td>
<td>1µg/kg</td>
<td>↑</td>
<td>↓</td>
<td>↑</td>
<td>↑</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enomoto et al. 2003</td>
<td>6</td>
<td>1, 5 and 10µg/kg</td>
<td>↑</td>
<td>↑</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cremonini et al. 2006</td>
<td>38</td>
<td>3.3µg/kg</td>
<td>↑</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Guido et al. 2007</td>
<td>35</td>
<td>1µg/kg</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↑</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maghnie et al. 2007</td>
<td>26</td>
<td>1µg/kg</td>
<td>↑</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fusco et al. 2007</td>
<td>21</td>
<td>1µg/kg</td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
<td>TSH ↔</td>
<td>Leptin↔</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kluge et al. 2008</td>
<td>10</td>
<td>0 and 50µg</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Ghrelin was administered intravenously except in the study of Enomoto et al. where it was injected subcutaneously. GH = growth hormone, PRL = Prolactin, ACTH = Adrenocorticotropic hormone, LH = Luteinizing hormone, FSH = Follicle stimulating hormone, TSH = Thyroid stimulating hormone, IGF-1 = Insulin-like growth factor-1, SS = Somatostatin, PP = Pancreatic polypeptide. B-gluc = blood glucose. ↑ = increase, ↓ = decrease, ↔ = no effect.
2.3.2 Effects of ghrelin on appetite and food intake

Administered i.e. exogenous ghrelin has been shown to increase appetite and food intake in rodents (Nakazato et al. 2001) as well as in humans (Wren et al. 2001). Furthermore, endogenous ghrelin has been suggested to be involved in the initiation of food intake, since plasma levels of ghrelin increase markedly before and decrease shortly after food intake (Cummings et al. 2001). Based on these findings, it is not surprising that ghrelin is often called the “hunger hormone”. The putative role for ghrelin as a stimulant for appetite has been supported by recent findings in rodents, which revealed, that when endogenous ghrelin was abolished from the circulation with neutralizing antibodies, a decrease in appetite and food intake was observed (Zorrilla et al. 2006). Ghrelin is currently the only known molecule with food intake increasing, i.e. orexigenic activity.

The site of action for ghrelin on appetite and food intake as well as stimulation of GH secretion is the central nervous system. Peripheral circulating ghrelin might have direct actions on the anterior pituitary gland, which is not protected by the blood brain barrier (BBB)(Horvath et al. 2001). Ghrelin is also capable of passing through BBB and thus it can have direct effects in such areas of the brain that are protected by the BBB (Banks et al. 2002). Furthermore, peripheral ghrelin might modulate neuronal activity in the central nervous system via its action on the vagus nerve. A recent study showed that treatment with ghrelin was unable to increase appetite or food intake in humans who had undergone surgery during which the vagus nerve had been severed. However, the stimulatory effect of ghrelin on GH release remained normal in these individuals, despite the non-functioning vagus nerve. This result indicates that the effects of ghrelin on appetite and food intake are mainly mediated via the vagal afferents whereas the vagus is not necessary for ghrelin to stimulate GH secretion, suggesting that its effects on GH secretion are probably a result of the direct effects of ghrelin on the pituitary somatotrophs. (le Roux et al. 2005.) In addition to peripheral ghrelin, there are also observations, that ghrelin might be produced in the central nervous system, e.g. in hypothalamic nuclei and in the pituitary. In these areas, ghrelin expression has been detected at the mRNA level as well as in protein level in rodents and in humans (Cowley et al. 2003, Kojima et al. 1999, Korbonits et al. 2001) These findings support the assumption that in the central nervous system, ghrelin might exert at least part of its effects on GH release and food intake in a paracrine/autocrine manner.
Ghrelin stimulates food intake by affecting certain areas of the central nervous system that are involved in controlling hunger and satiety. One important area of the central nervous system involved in the regulation of energy balance is the arcuate nucleus (the ARC). Ghrelin is able to stimulate the neurons in the ARC and in addition, other areas of the central nervous system, for example nucleus paraventricularis, dorsomedial parts of hypothalamus, and areas in the brain stem nucleus tractus solitarius and the area postrema, which all take part in the modulation of appetite control. (Lawrence et al. 2002, Wilding 2002.) In the ARC, ghrelin administration has been shown to activate neuropeptide Y (NPY) and agouti related protein (AgRP) containing neurons. NPY and AgRP are molecules that are released as neurotransmitters into the synapse and they decrease the production and release of alpha melanocyte stimulating hormone (α-MSH) from the post-synaptic pro-opiomelanocortin (POMC) neurons. The effect of ghrelin on food intake is abolished if the effects of NPY and AgRP are removed by antagonists or by neutralizing antibodies. (Nakazato et al. 2001.)

Leptin, a peripheral satiety hormone, has been shown to act on the same neurons in the ARC as ghrelin but it has an opposite effect: it decreases the signalling of NPY and AgRP neurons, increases α-MSH release and thereby promotes satiety and decreases food intake. Ghrelin and leptin are not the only molecules that are able to affect the activity of the NPY/AgRP neurons in the ARC but ghrelin is the only known peripheral signal that has appetite stimulating and food intake increasing, i.e. orexigenic, effects mediated by these neurons. However, in addition to leptin, anorexigenic effects on these neurons are mediated by several other metabolic signals such as glucose, insulin, cholecystokinin, pancreatic polypeptide (PP) and peptide YY (PYY) and leptin. (Abizaid & Horvath 2008, Traebert et al. 2002, Tung et al. 2001.)

### 2.3.3 Effects of ghrelin on long term energy balance in humans

Some suggestions have been made that ghrelin might have a role in the regulation of long term energy balance. This is supported by the findings showing that ghrelin concentrations are inversely correlated with certain obesity related parameters such as BMI, waist circumference and percentage of body fat. Obesity is characterized by decreased ghrelin levels whereas lean and anorectic patients have increased ghrelin concentrations as compared with normal weight controls. (Shiiya et al. 2002, Tschöp et al. 2001.) The only exception to this inverse correlation of ghrelin and BMI is Prader-Willi syndrome, the most common form
of human syndromic obesity, in which the ghrelin levels are high even though the subjects with this syndrome are typically over-weight or obese. Other features of this syndrome are GH deficiency, hypotonia, cognitive impairments and hyperphagia. Hyperghrelinemia in Prader-willi syndrome is believed to play a role in the development of hyperphagia. GH deficiency might in turn be a result of an over-stimulation of somatotrophs by the extremely high ghrelin concentrations. (Cummings et al. 2002)

Fasting ghrelin concentrations are altered if the amount of stored energy in the body changes, and it could be proposed that the ghrelin concentration at a given time reflects the prevailing energy balance. The high ghrelin concentrations of anorectic patients decrease as the individuals gain weight (Otto et al. 2001) and diet induced weight loss in turn elevates the low levels of ghrelin in obese subjects (Hansen et al. 2002). It has been shown, that low ghrelin levels have no value for predicting future weight gain in humans (Langenberg et al. 2005). In addition, the magnitude of weight gain induced by overfeeding for 100 days is not dependent on the ghrelin levels at baseline (Ravussin et al. 2001). These findings indicate that ghrelin does not play a causative role in the development of obesity and that changes in ghrelin levels are probably secondary effects that occur in response to the metabolic changes.

It has been suggested that the increased ghrelin levels might be contributing to the relapse in eating habits that many individuals encounter during weight maintenance after weight loss. In obesity in turn, low levels of ghrelin are claimed to be caused by a feedback mechanism to reduce hunger in a chronic positive energy balance. (Tschöp et al. 2001.) In summary, the molecular mechanisms linking ghrelin to energy metabolism are not yet totally clarified.

2.3.4 Animal studies addressing the role of ghrelin in energy metabolism

In rodents, ghrelin administration increases feeding and stimulates GH secretion in a dose-dependent manner. Chronic administration of ghrelin in rodents has been shown to have other anabolic functions such as reducing the utilisation of fat as a metabolic fuel and favouring the storage of fat, indicating that ghrelin might be involved in the regulation of long term energy balance (Tschöp et al. 2000). This is supported by findings that in vitro, ghrelin has been shown to stimulate the differentiation of preadipocytes and to antagonize lipolysis from the adipocytes dose dependently (Choi et al. 2003). Furthermore, in vivo, ghrelin (and des-acyl
ghrelin) administration has been shown to induce adipogenesis in the bone marrow of rodents, probably by a mechanism that is not mediated via GHS-R1a (Thompson et al. 2004). However, it is not known, whether ghrelin possesses these effects on energy storage in humans and if so, do they occur physiological concentrations.

In 2003 a ghrelin knock-out mouse model was characterized. Surprisingly, ghrelin-null mice displayed normal growth and appetite compared to the wild type control mice. They also gained weight at the same rate as the controls in studies focusing on diet-induced obesity. In addition, ghrelin deficient mice exhibited no differences in insulin or leptin levels compared with controls. These results were a blow to the high expectations which had been placed on research into ghrelin after its discovery. These results cast serious doubts on its role, i.e: endogenous peripheral or central ghrelin might not play such a crucial role in the regulation of appetite, growth or energy balance in normal physiology after all. (Sun et al. 2003.)

In 2004, ghrelin knock-out mice were observed to respond in a different manner to a high-fat diet (45% fat) when compared with control mice. The ghrelin-null mice had normal spontaneous food intake, but the respiratory quotient (RQ; ratio of VCO₂/VO₂) was significantly decreased in the ghrelin knock-out mice. The decline in the RQ is evidence that these animals had used fat as an energy substrate more than carbohydrates. In another words, ghrelin deficiency in mice had changed the metabolic fuel preference in a direction towards overse of fat as a substrate. This suggests that endogenous ghrelin might play a role in the regulation of energy metabolism by affecting the determination of the type of metabolic substrate (carbohydrate v.s. fat) when exposed to a high-fat diet. (Wortley et al. 2004.)

The third important ghrelin knock-out study was reported in 2005. It showed that young ghrelin deficient mice gained weight less effectively after being exposed to a high-fat diet compared with their wild type control mice. Ghrelin-null mice had less adiposity and they displayed more physical activity and greater energy expenditure compared with the controls. These results suggest that ghrelin might be used in the adaptation of different nutrient availability. (Wortley et al. 2005.)

In 2006, ghrelin knock out mice were seen to be prevented from suffering hyperglycaemia after being exposed to high-fat –diet, in contrary to their wild type littermates. Furthermore, insulin secretion was markedly increased in knock
out mice in a glucose tolerance test. These findings indicate that ghrelin might be involved in the regulation of glucose and insulin metabolism. (Dezaki et al. 2006.)

Interesting results were obtained in 2006, when rats were given a vaccination against endogenous ghrelin. Vaccinated rats had significantly reduced ghrelin plasma concentrations and they gained less weight than the control rats with normal ghrelin levels. Vaccinated rats also had less adipose tissue and an increased amount of lean mass. This result supports the findings which suggest that endogenous ghrelin has a role in the regulation of energy-metabolism. (Zorrilla et al. 2006.)

Interesting observations have been also obtained in a few studies where the biological activities of ghrelin were disturbed by manipulating its receptor, GHS-R1a. In one rat model, GHS-R antisense mRNA was expressed in a tissue specific manner in the arcuate nucleus resulting in attenuated production of ghrelin receptor. These rats had lower body weight, less adipose tissue, reduced food intake and they did not react to GHS administration by initiating feeding as the normal wild type rats. (Shuto et al. 2002.) In addition, GHS-R knock-out mouse models have shown, that mice lacking the ghrelin receptor have lower body weight and IGF-1 concentrations in the circulation compared with wild type control mice (Sun et al. 2003). Additionally, GHS-R knock-out mice responded to western type of high-fat diet by gaining less weight and fat mass compared with their wild type littermates. With normal chow diet, no differences in body weights and compositions were observed. Interestingly, if they consumed normal chow diet, GHS-R knock-out mice displayed lower blood glucose levels than the control mice but their insulin levels were not altered. These findings suggest that ghrelin might be linked in some unknown way to the regulation of glucose homeostasis. (Zigman et al. 2005.)

Interesting findings on the central effects of ghrelin were obtained recently on studies with Spiegelmers. Spiegelmers are non-natural mirror-molecules (Spiegel = German: a mirror), synthetic polyethylene glycol-modified L-RNA oligonucleotides that specifically bind and inactivate their target molecules. Administration of the spiegelmer for n-octanoylated ghrelin was shown to abolish the stimulatory effect of administered ghrelin on GH secretion and feeding, as well as inhibiting the activation of neurons in the ARC (Helmling et al. 2004, Kobelt et al. 2006). On the other hand, fasting was able to evoke an activation of orexigenic signals in the ARC, even though the effects of ghrelin were inhibited with a Spiegelmer administration. This result suggests that in the fasting condition, endogenous ghrelin does not play a major role in activating the neurons in the
central nervous system which induce appetite and feeding. Rather, it is one participant in the complex network of several central and peripheral signals. (Becskei et al. 2008.)

On the contrary to these mouse models of whether there has been almost complete or total abolition of the effects of ghrelin or ghrelin receptor, ghrelin over-expressing animal models have also been reported. Over-expression of mouse des-acyl ghrelin in mice led to a phenotype with a lower body weight and body length accompanied with diminished levels of circulating GH and IGF-1 (Ariyasu et al. 2005). Another study showed that mice over-expressing the human ghrelin gene were normal sized. In chronic hyperghrelinemia in these animals, there was a diminished GH response to exogenous ghrelin, indicating that ghrelin might have lost its stimulating role for GH release when its production exceeds the normal physiological range. (Wei et al. 2006.)

In conclusion, these animal models where there is either lack of ghrelin or its receptor as well as models with over expression of ghrelin indicate that alterations in ghrelin signalling have significant, though not dramatic, effects on energy metabolism. The effects are modulating and they become apparent mainly when the animals are exposed to an abnormal energy supply. However, the exact mechanisms by which ghrelin participates in the complex network of growth, feeding and energy metabolism remain still to be clarified.

2.3.5 What can be learned from human tumors over-producing ghrelin?

Ghrelin expressing cells have been found in several gastrointestinal and pancreatic endocrine tumors. However, only a small minority of these tumors lead to over-production of ghrelin and subsequently to increased ghrelin concentrations in the circulation, i.e. hyperghrelinemia, as shown previously (Corbetta et al. 2003). One recent study described a malignant gastric endocrine tumor that secreted ghrelin as a major hormone (Tsolakis et al. 2004). Even though ghrelin levels were elevated in subjects with a ghrelin producing tumors in the studies of Corbetta et al. (2003) and Tsolakis et al. (2004), GH and IGF-1 levels were within the normal range. In addition there were no phenotypic signs of acromegalia. These findings would seem to indicate, that endogenous ghrelin might not be such a potent GH stimulant as originally speculated, even though ghrelin treatment has been shown to stimulate GH secretion in several studies. On the other hand, it is not known whether the pulsatility of ghrelin secretion is
conserved in subjects with ghrelinoma. Thus, it is possible that the tumor keeps ghrelin levels constantly elevated and that somatotrophs can not respond to this constant stimuli in terms of GH secretion. In another words, it could be postulated, that a pulsatility in the ghrelin secretion might be in order that GH release is stimulated.

2.3.6 Effects on the gastrointestinal tract

Intravenously administered ghrelin increases gastric acid secretion and stimulates gastric motility and emptying in rats in a dose-dependent manner. The effects on gastric acid secretion are believed to be mediated via the vagus nerve, since vagotomy and atropine treatment could abolish these actions of ghrelin (Masuda et al. 2000). Gastric acid secretion has been observed to be increased also after intracerebro-ventricular (i.c.v.) ghrelin administration (Date et al. 2001). However, in mice, a stimulation of gastric motility but no effect on gastric acid secretion after ghrelin administration was observed (Dornonville de la Cour et al. 2004). The discrepancy in the results concerning the role of ghrelin in gastric functions might be due to differences between species or study protocols.

Ghrelin administration increased the gastric emptying rate in normal weight healthy humans in a recent double-blinded, randomized cross-over study (Levin et al. 2006). The same observation was noted in subjects with a diabetic and idiopathic gastroparesis (Murray et al. 2005; Tack et al. 2005). Furthermore, plasma concentrations of endogenous acylated ghrelin have been shown to correlate negatively with gastric emptying half-time. In lean subjects ghrelin was shown to be an independent determinant of gastric emptying. Interestingly this association was not observed in obese subjects indicating that the actions of ghrelin might be altered according to the level of obesity. (Valera Mora et al. 2005.) It must be noted that in one study, administered ghrelin (0.33µg/kg i.v.) had non-significant changes in gastric volumes before and after meals and there was no effect on gastric emptying either in obese or in normal weighed subjects (Cremonini et al. 2006). The discrepancy in these results might be due to differences in the study protocols and the amount of administered ghrelin. In conclusion, the majority of the published studies addressing the role of ghrelin in the regulation of motility of the GI-tract indicate that ghrelin increases gastric motility and gastric acid secretion. However, the effects of ghrelin may vary in obese compared with subjects with normal body weight.
2.3.7 Ghrelin and cardiovascular effects

There is a growing body of evidence indicating that ghrelin might have beneficial effects on heart function and hemodynamics. The mechanisms might be mediated indirectly via the actions of GH/IGF-1 and/or directly via GH-independent mechanisms. The ghrelin receptor GHS-R1a has been detected to be synthesized in the heart and vessels in rodents and in humans (Nagaya et al. 2001). Ghrelin is synthesized and secreted by human and mouse cardiomyocytes, and it has been shown to protect the cells from apoptosis, probably via paracrine/autocrine actions (Iglesias et al. 2004). Interestingly, also des-acyl ghrelin possesses beneficial cardiovascular properties. It has been shown that acylated ghrelin as well as des-acyl ghrelin can protect cardiomyocytes from isoprenaline-induced injury in rodents. However, the cardioprotective effects of des-acyl ghrelin were noted to be weaker than those of acylated ghrelin (Li et al. 2006). Furthermore, at least in rabbits, ghrelin was claimed to might modulate cardiac function and blood pressure by decreasing the activity of the sympathetic nervous system (Matsumura et al. 2002). In summary, there are several possible mechanisms of action by which ghrelin and des-acyl ghrelin might modulate the function of the cardiovascular system.

Ghrelin has been shown to evoke vasodilatation in isolated human vessels in vitro (Wiley & Davenport 2002). In vivo, ghrelin administration was observed to increase blood flow in human forearm arteries dose-dependently, as evaluated by plethysmography (Okumura et al. 2002). In addition, intravenous injection of ghrelin not only decreased arterial pressure, but also increased cardiac output in healthy humans (Nagaya et al. 2001). There are also promising preliminary human studies examining whether ghrelin might improve heart function in patients with chronic heart failure. Administration of ghrelin (2µg/kg, twice a day, i.v.) for three weeks significantly improved left ventricular function, exercise capacity and muscle wasting in a small study (n = 10) (Nagaya et al. 2004). Thus, administration of ghrelin might represent a new therapeutic tool to treat chronic heart failure or cachexia that is often accompanied with a long-lasting chronic heart failure. However, these preliminary promising results will need to be confirmed in more studies.
2.3.8 Other effects of ghrelin

Several cell culture and animal studies have provided evidence that ghrelin (acylated and des-acyl ghrelin) might have anti-apoptotic properties and other effects on cell proliferation and differentiation. The mechanisms are suggested to be mediated through GHS-R1a and also via pathways independent of this receptor. Ghrelin and its receptor have been found to be present in several human cell tumors and tumor cell lines, where they have demonstrated to possess both anti-proliferative and proliferative effects, depending on the cell line. In addition, acylated ghrelin might be involved in the functions of the immune system. Interestingly, the production of pro-inflammatory cytokines has been shown to be diminished by ghrelin in some studies. Ghrelin might also be involved in bone formation by stimulating osteoblast proliferation and differentiation as well as by increasing the mineral density of bone. Effects on sleep, memory and behaviour have been described. In summary, ghrelin seems to be a molecule potentially modulating several biological functions as reviewed recently (Soares & Leite-Moreira 2008). A summary of the non-endocrine biological effects of administered ghrelin in humans is shown in Table 2.

Table 2. Non-endocrine functions of administered ghrelin in humans.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Subjects (n)</th>
<th>Amount of ghrelin administered</th>
<th>Hunger / food intake</th>
<th>Gastric emptying</th>
<th>Cardiovascular / hemodynamic effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wren et al. 2001</td>
<td>9</td>
<td>5pmol/kg/min</td>
<td>↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nagaya et al. 2001</td>
<td>6</td>
<td>0 and 10µg/kg</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enomoto et al. 2003</td>
<td>6</td>
<td>1, 5 and 10 µg/kg</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tack et al. 2005</td>
<td>6</td>
<td>40µg/30min</td>
<td>↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Murray et al. 2005</td>
<td>10</td>
<td>5pmol/kg/min</td>
<td>↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Binn et al. 2006</td>
<td>6</td>
<td>1-4µg/kg</td>
<td>↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cremonini et al. 2006</td>
<td>38</td>
<td>3,3µg/kg</td>
<td>↔</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Levin et al. 2006</td>
<td>14</td>
<td>10pmol/kg/min, 180min</td>
<td>↑, ↑</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Ghrelin was administered intravenously except in the study of Enomoto et al. where it was given subcutaneously. ↑ = increase, ↓ = decrease, ↔ = no effect, + = beneficial effects such as an increase in left ventricular ejection fraction and or stroke volume, decrease in blood pressure.
2.4 Regulation of ghrelin plasma concentrations

Peptide hormones, such as ghrelin, are synthesized within the endocrine cells and they are stored in secretory granules and released into the bloodstream after a specific stimulus. In general, there are a variety of signals that can act as such a stimulus. For example, the cells may react to the presence of certain some substances in the blood. On the other hand, endocrine cells can secrete hormones in response to other circulating hormones. Furthermore, the endocrine cells may be regulated by the direct action of nerves. (Pocock & Richards 1999.)

The concentration of a certain hormone in the circulation at a given time depends not only to the rate of its secretion but also to its stability in the circulation and possibly to the rate of its excretion. Several peptide hormones, such as ghrelin, are subjected to proteolytic degradation in the blood by proteinase enzymes, and in addition, because of their small molecular weight, they are believed to be excreted from the circulation by the kidneys. (Pocock & Richards 1999.) The possible excretion of ghrelin by the kidneys is supported by findings that this peptide can be detected in the urine (Aydin et al. 2008) and furthermore, in renal failure there are elevated ghrelin plasma concentrations (Jarkovská et al. 2005). The stability and degradation of ghrelin was discussed earlier (in 2.1.3 Molecular characteristics of circulating ghrelin molecules). The regulatory signals that affect ghrelin secretion will be discussed in more detail in this chapter.

2.4.1 Diurnal regulation of ghrelin concentrations by hormonal and neuronal factors

Ghrelin concentrations vary diurnally in a pulsatile fashion: hormone levels increase before and decrease after food intake (Cummings et al. 2001). The regulation of this pre- and postprandial oscillation has been extensively studied but it is still a matter of debate. Several hormonal, metabolic and neuronal factors have been shown to decrease ghrelin levels postprandially. On the contrary, there are few factors that evoke a preprandial increase in ghrelin levels are few.

The preprandial increase in ghrelin concentrations has not been shown to be attributable to any hormone and it has been suggested to be regulated mainly by neuronal factors. The neurotransmitter, acetylcholine, was shown to increase ghrelin secretion in humans via its muscarinic receptors, indicating that ghrelin secretion might be under cholinergic control. Based on this finding, one can
propose that the parasympathetic nervous system, or more specifically, the vagus nerve might take part in the regulation of ghrelin secretion (Broglio et al. 2004).

In rodents, ghrelin secretion was shown to be under the control of both the cholinergic and adrenergic components of the autonomic nervous system. Removal of the action of the vagus nerve by vagotomy caused an initial decrease in the ghrelin levels in the short term but increased the secretion over the long term (Hosoda & Kangawa 2008).

The postprandial decrease has been shown to be mediated by several hormonal and nutritional factors. It has been claimed in several studies that insulin might be one of the most potent down-regulatory factors for ghrelin (Table 3). One study recently showed that insulin might even be an essential factor for the postprandial decrease of ghrelin (Murdolo et al. 2003). On the other hand, there are some studies that seem to indicate that insulin does not play a significant role in the regulation of ghrelin plasma levels. For example, in subjects with type 1 diabetes who do not produce any endogenous insulin, ghrelin levels decreased in the same manner as in the non-diabetic control subjects after food intake. In addition, injection of insulin did not have any effect on the ghrelin decrease in these subjects with type 1 diabetes. (Spranger et al. 2003.) It has also been stated that it is food intake and not insulin that is responsible for the postprandial decreases in ghrelin secretion (Caixás et al. 2002).

There is some data showing that the incretin hormones, glucagon like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP), might modulate the postprandial decrease in ghrelin concentrations, but further studies need to be performed since the effects of these hormones might be mediated by the actions of insulin or by the action of these hormones in modifying the gastric emptying rate (Blom et al. 2006, Hagemann et al. 2007). The sex hormones, testosterone and oestrogen, have been shown to increase fasting plasma ghrelin levels over the long term in several studies. However, the mechanisms by which these hormones are able to alter the plasma concentrations of ghrelin are not known. The studies addressing the hormonal regulation of ghrelin levels are described in Table 3.
Table 3. Hormonal regulation of ghrelin concentrations in humans.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Subjects</th>
<th>Hormone</th>
<th>Administration</th>
<th>Effect on ghrelin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caixás et al. 2002</td>
<td>17</td>
<td>Insulin</td>
<td>s.c.</td>
<td>↔</td>
</tr>
<tr>
<td>Saad et al. 2002</td>
<td>8</td>
<td>Insulin</td>
<td>i.v.</td>
<td>↓</td>
</tr>
<tr>
<td>Flanagan et al. 2003</td>
<td>11</td>
<td>Insulin</td>
<td>i.v.</td>
<td>↓</td>
</tr>
<tr>
<td>Spranger et al. 2003</td>
<td>2</td>
<td>Insulin</td>
<td>s.c.</td>
<td>↔</td>
</tr>
<tr>
<td>Murdolo et al. 2003</td>
<td>12</td>
<td>Insulin</td>
<td>i.v. and s.c.</td>
<td>↓</td>
</tr>
<tr>
<td>Schaller et al. 2003</td>
<td>9</td>
<td>Insulin</td>
<td>i.v.</td>
<td>↓</td>
</tr>
<tr>
<td>Tan et al. 2004</td>
<td>4</td>
<td>Somatostatin</td>
<td>i.v.</td>
<td>↓</td>
</tr>
<tr>
<td>Broglio et al. 2002</td>
<td>6</td>
<td>Somatostatin</td>
<td>i.v.</td>
<td>↓</td>
</tr>
<tr>
<td>Pagotto et al. 2003</td>
<td>26</td>
<td>Testosterone</td>
<td>HRT</td>
<td>↑</td>
</tr>
<tr>
<td>Otto et al. 2004</td>
<td>13</td>
<td>Prednisolone</td>
<td>p.o. (30mg/day)</td>
<td>↓</td>
</tr>
<tr>
<td>Chan et al. 2004</td>
<td>5</td>
<td>Leptin</td>
<td>i.v. 0.01 and 0.1 mg/kg</td>
<td>↔</td>
</tr>
<tr>
<td>Grinspoon et al. 2004</td>
<td>78</td>
<td>Estrogen</td>
<td>contraceptives</td>
<td>↑</td>
</tr>
<tr>
<td>Kellokoski et al. 2005</td>
<td>64</td>
<td>Estrogen</td>
<td>HRT</td>
<td>↑</td>
</tr>
<tr>
<td>Blom et al. 2006</td>
<td>15</td>
<td>GIP</td>
<td>i.v.</td>
<td>↓</td>
</tr>
<tr>
<td>Hagemann et al. 2007</td>
<td>14</td>
<td>GLP-1</td>
<td>1,2pmol/kg/min</td>
<td>↓</td>
</tr>
</tbody>
</table>

Hormone was given intravenously (i.v.) as a bolus or continuously in clamp studies, subcutaneously (s.c.) or per os (p.o.). In Hormone replacement therapies (HRT) the hormone was administered transdermally, or p.o. Contraceptives were given p.o. GIP = Gastric inhibitory peptide, GLP-1 = Glucagon like peptide -1.

2.4.2 Diurnal regulation of ghrelin concentrations by nutritional factors

The role of macronutrients in the regulation of postprandial decline in ghrelin levels is an interesting topic of research. It has been shown that drinking water does not suppress ghrelin levels whereas this can be achieved by i.v. or per oral glucose load. This would seem to indicate that the down-regulation of ghrelin levels might dependent on the glucose concentration in the blood (Shiiya et al. 2002). This was supported also by another study: when 5g glucose was administered intravenously in subjects with type 2 diabetes whose early insulin response was abolished, ghrelin levels decreased significantly after the glucose load. This is evidence that insulin itself might not be the crucial factor down regulating ghrelin levels but instead, it is the blood glucose concentration, that might be more important. (Briatore et al. 2003.)

A meal rich in carbohydrates evoked a greater decrease in plasma ghrelin levels than a high-fat meal in 14 non-obese healthy women (Monteleone et al.
The role of carbohydrates in the down-regulation of ghrelin levels received support from the finding that glucose and fructose caused an increase in the insulin levels and a decrease in circulating ghrelin levels, when administered either enterally or parenterally (Prodam et al. 2006). In some other studies, proteins and lipids have had a suppressing effect on ghrelin levels: The postprandial decrease in (acylated) ghrelin levels was highest after a carbohydrate rich meal, intermediate after protein rich meal and lowest after a high-fat meal. Interestingly, ghrelin levels remained significantly lower after a protein rich meal compared with the other two meals when measured 180min after the meal (Tannous dit El Khoury et al. 2006). A similar result has been obtained in a second study (Al Awar et al. 2005). These results indicate that the proteins in food might promote longer-term postprandial ghrelin suppression and satiety. On the contrary, one study showed that the postprandial decrease of a similar magnitude in ghrelin levels was achieved irrespective of the macronutrient content of the test meal, and the declines were similar in obese and lean subjects (Marzullo et al. 2006). The most recent study addressing the role of macronutrients in the down-regulation of (acylated and total) ghrelin levels showed that the postprandial decrease is greater after carbohydrate and protein beverage than after a lipid containing isocaloric beverage. Furthermore, three hours after the beverage had been consumed, there was a rapid increase in ghrelin levels that resulted in much higher ghrelin concentrations than measured at baseline. This rebound effect was not seen after consumption of protein or lipid beverages. These results suggest that macronutrient composition of the consumed food might differentially affect signals of satiety and hunger and that this should be taken into account when designing weight reducing diets. (Foster-Schubert et al. 2008.)

In conclusion, there is a growing body of evidence that the macronutrient composition of the food might affect the magnitude of postprandial suppression of ghrelin and indicates that the feelings of satiety obtained postprandially might be manipulated by designing diets that affect to the gastrointestinal satiety and hunger hormones in a beneficial way.

2.4.3 Long term regulation of ghrelin concentrations

Ghrelin levels are low in obese and high in lean individuals as compared with normal weight subjects (Shiiya et al. 2002). In obesity not only the fasting concentrations but also the magnitude of the pulsatility in ghrelin levels has been shown to be diminished (English et al. 2002). Weight loss, induced either by
caloric-restriction or by increased amount of exercise, increases and conversely weight gain decreases ghrelin concentrations (Cummings et al. 2002, Foster-Schubert et al. 2005, Hansen et al. 2002, Otto et al. 2001, Robertson et al. 2004). It seems that the amount of the adipose tissue present in the body might be involved in the regulation of ghrelin levels by some unknown mechanism. However, in one weight loss study, ghrelin levels increased in parallel with the weight loss but surprisingly decreased towards their baseline concentrations during weight maintenance in a study with a longer follow up time (Garcia et al. 2006). This indicates that factors other than the level of obesity might interfere with the regulation of ghrelin levels over the long term. An overfeeding study in monozygotic twins suggested that the basal ghrelin concentrations might be a familial trait, hence, it is possible, that the regulation of ghrelin levels might be partly genetic (Ravussin et al. 2001).

At present, gastric bypass surgery is the most effective therapeutic method to achieve a weight reduction in morbid obesity (BMI > 40 kg/m²). Subjects undergoing this kind of operation lose weight dramatically and their appetite diminishes markedly (Maggard et al. 2005). The most commonly used gastric bypass method nowadays is the Roux-en Y bypass. In this procedure, the stomach is made smaller by creating a small pouch at the proximal parts of the stomach (antrum and fundus) by using surgical staples or a plastic band. The smaller stomach is connected directly to the middle portion of the small intestine (jejunum), bypassing the rest of the stomach and the proximal parts of the small intestine (duodenum). A weight loss after a Roux-en Y gastric bypass has resulted in markedly decreased ghrelin levels, and this was suggested to contribute to the dramatic post-operative decrease of appetite (Cummings et al. 2002). The ghrelin decrease after a gastric bypass operation has been found in other studies (Geloneze et al. 2003). However, there are also some observations that ghrelin levels remain unaltered or even become elevated along with the marked weight loss after a gastric bypass (Garcia-Fuentes et al. 2008, Karamanakos et al. 2008). Differences in the surgical techniques and handling of the vagus nerve during the operation have been proposed to be responsible for the discrepancies between the studies. However, these findings provide evidence that it is probably not only the level of obesity that determines the ghrelin levels in the long term, at least after bariatric surgery.

Some studies indicate that ghrelin levels might decrease with age. One study which compared children at their different developmental stages showed that ghrelin concentrations are at their highest during early postnatal life, at times
when changes in food intake, GH secretion and growth occur (Soriano-Guillén et al. 2004). Ghrelin concentrations are lower in the elderly in comparison to the levels in young healthy men (Kozakowski et al. 2008). However, there are only a few longitudinal studies on ghrelin. Caution must excersiced in interpreting the results of cross-sectional studies concerning ghrelin and age.

2.5 Variants in genes coding for ghrelin and ghrelin receptor

Several whole genome scans have suggested that certain areas of chromosome 3, the same chromosome where ghrelin and the ghrelin receptor genes are located, might be linked with obesity or the metabolic syndrome (Kissebah et al. 2000, Wu et al. 2003). These findings, in addition to the relevant biological functions of these proteins, increase the potential for the genes coding for ghrelin and its receptor to be considered as candidate genes in the genetic studies focusing on obesity and obesity related co-morbidities.

2.5.1 Ghrelin receptor gene polymorphisms

The ghrelin receptor has been screened for genetic variants recently. Seven variants were found, five of which were single nucleotide polymorphisms (SNPs) which did not change the amino acid code of the protein. These five mutations did not associate with obesity or height. However, two genetic variants which caused amino acid changes, namely Ala204Glu and Phe279Leu, were found. The first of these was found in one obese child and the latter in one child with a short stature. Ala204Glu was subsequently transfected into HEK-3 cells, resulting in a reduction in the constitutive activity of the receptor. Furthermore, the ghrelin receptor with this missense allele was expressed to a lower extent. (Pantel et al. 2006, Wang et al. 2004.) Phe279Leu variation was also proposed to influence the constitutive activity of the receptor. Amino acid 279 is located within the ligand binding pocket of the receptor and it has been demonstrated to play a crucial role in the constitutive activity of the receptor, i.e. it may keep the ghrelin receptor in an active conformation (Holst et al. 2004). Other functional variants in the ghrelin receptor gene have not been identified.
2.5.2 Ghrelin gene polymorphisms

The ghrelin gene has been a candidate gene in several studies focusing on the genetic variations underlying obesity and growth. Several variations have been found to be associated with obesity related parameters. The genetic variation 152G>A in the preproghrelin gene, which is responsible for an amino acid change Arg51Gln of prepro-ghrelin (that is Arg28Gln of mature ghrelin), has been shown to be associated with obesity. Six subjects who were heterozygous for this variation were found to be obese and the mutation was not detected in normal weight subjects (Ukkola et al. 2001). However, also contrary reports have been published, i.e. the Gln51 allele has been shown to be equally distributed in obese and normal weight subjects (Pöykkö et al. 2003, Vivenza et al. 2004). Thus, there is no clear consensus about its association with obesity. Interestingly, subjects with the Gln51 allele were shown to be at a higher risk for type 2 diabetes and hypertension in Finnish middle-aged subjects (Pöykkö et al. 2003). The variation changes the last amino acid of the mature peptide, but it is not known how this change might be linked to obesity at the molecular level. It is possible, that this amino acid change might disturb the action of proteases and disrupt the post-translational cleavage. This might in turn lead to longer ghrelin molecules and change the configuration or even the activity of the peptide. However, these hypothetical changes have still not been demonstrated. The Arg51Gln variation has been reported to be quite rare in several studies, with the allelic distribution of the 51Gln being around 0–2%, depending on the study population (Ukkola et al. 2002).

Another variation of prepro-ghrelin gene, 214C>A, changes amino acid leucine72 to methionine (Leu72Met). This amino acid 72 is not present in the mature ghrelin peptide. The allelic frequency of this variant has been shown to vary around 1–9% in different studies (Ukkola et al. 2002). The mechanism by which a variant outside the region coding for the mature peptide might be linked with certain phenotypes is unknown, but there has been some speculation that the variant might alter the stability of the mRNA or interfere with the splicing of the prepro-hormone. The 72Met allele has been associated with obesity (Korbonits et al. 2002), earlier onset of obesity (Miraglia del Giudice et al. 2004, Ukkola et al. 2001) and a positive family history of obesity (Vivenza et al. 2004) and metabolic syndrome (Xu et al. 2008). This variation has been shown to be associated with a higher BMI in Japanese middle-aged men but not in women or older subjects (Kuzuya et al. 2006). However, the association of this variant with obesity and
related parameters has not been confirmed in all studies. For example, in the Finnish diabetes prevention study, Leu72Met polymorphism was not associated with BMI or waist circumference. Interestingly, in this study, the subjects with the 72Met allele were shown to have an increased risk for the development of type 2 diabetes (Mager et al. 2006). Subjects with the 72Met allele had decreased first-phase insulin secretion during OGTT, and it has been suggested, that this variation might contribute to defects in insulin and glucose metabolism (Korbonits et al. 2002). Furthermore, this variant was shown to be associated with a higher prevalence of the metabolic syndrome, higher fasting glucose and triglyceride levels as well as lower HDL-cholesterol in subjects of the Amish Family diabetes study (Steinle et al. 2005). On the contrary, in a large study of Danish subjects, no associations between Leu72Met variation and the BMI, waist circumference or parameters of glucose and insulin metabolism were observed (Bing et al. 2005). In addition, no linkage of Leu72Met polymorphism and type 2 diabetes was observed among Koreans (Choi et al. 2006). In summary, there are many discrepancies in the results of different genetic association studies addressing the role of this polymorphism.

A mutation 269A>T, which causes the Gln90Leu change in the prepro-ghrelin has been described. The 90Leu allele has been shown to be significantly more frequently detected among extremely obese individuals than in normal weight subjects but when this was examined in a larger study population, the association disappeared (Hinney et al. 2002). Hinney et al. (2002) found also a frameshift mutation in the ghrelin gene, a 2bp deletion at codon 34, but this variant was not observed to have any effect on phenotype.

Some other variants in the ghrelin gene have been found, but they have been studied less extensively. In Japanese women, an intron variant T3065C showing a strong linkage disequilibrium with Leu72Met, was found and the variant allele associated with higher body weight, BMI and waist circumference and lower serum HDL concentrations, whereas Leu72Met variants showed no association with these parameters. The authors suggested that this intron variant might be even more important with respect to obesity than Leu72Met (Ando et al. 2007).

In conclusion, there is a growing body of evidence, that variations in the ghrelin gene might associate with obesity and its co-morbidities but there are discrepancies between the different studies conducted in different populations.
2.6 Ghrelin, insulin resistance and type 2 diabetes

Pöykkö et al. (2003) showed that fasting plasma concentrations of total ghrelin were lower among subjects with type 2 diabetes compared to those without the disease. The difference remained significant even when age, sex and BMI were taken into account in multivariable models. In addition, an inverse correlation was observed between ghrelin and insulin concentrations as well as insulin sensitivity, measured by quantitative insulin sensitivity check –index (QUICK). Low ghrelin concentrations were shown to associate independently with type 2 diabetes and insulin resistance. (Pöykkö et al. 2003.) Subsequently, ghrelin levels were observed to be significantly lower in subjects with type 2 diabetes or hyperinsulinemia compared with normal weighted normoinsulinemic subjects (Erdmann et al. 2005). A strong inverse correlation between ghrelin concentrations and insulin resistance measured by the homeostasis model of insulin resistance (HOMA-IR) or/and insulin concentrations has been observed also in other studies, for example among women with polycystic ovary syndrome (Schöfl et al. 2002), in obese children (Zou et al. 2009), obese adolescents (Stylianou et al. 2007) and with obese middle-aged subjects (McLaughlin et al. 2004). A recent study aimed to clarify the relationships of total, acylated and des-acyl ghrelin molecules with insulin resistance and indicated that these molecules might be involved in glucose and insulin metabolism in different ways. Total and acylated ghrelin were negatively associated with insulin resistance but instead, the correlation between acylated ghrelin and HOMA-IR was shown to be positive. In addition, the study showed that the ratio of acylated/des-acyl ghrelin was significantly higher in obese subjects compared to non-obese subjects (Barazzoni et al. 2007).

On the other hand, contrary results i.e. no association between ghrelin and pathologies of glucose metabolism have been published. In healthy middle-aged men in a euglycaemic hyperinsulinemic clamp study, no association between insulin sensitivity and ghrelin concentrations was observed (Fagerberg et al. 2003). In addition, ghrelin concentrations of subjects with type 2 diabetes were not different from those of normal weight controls and ghrelin concentrations seemed to vary according to the BMI and not by diabetic status. Obese subjects with type 2 diabetes had lower and lean subjects with type 2 diabetes had higher ghrelin concentrations indicating that ghrelin did not appear to be associated with glucose or insulin metabolism (Shiiya et al. 2002). However, the majority of the publications addressing the relationship between ghrelin and insulin resistance
and/or diabetic states suggest that a correlation between ghrelin and insulin resistance and/or diabetes might exist.

Glucose and insulin metabolism is closely involved with the metabolic events taking place in the liver. In fasted states, the liver produces glucose from glycogen stores and from proteins by gluconeogenesis and then the glucose is released into the circulation. Ghrelin has been shown to participate in these metabolic events, at least in cell culture studies. It has been shown to modulate insulin signalling cascades in the hepatocytes and to up-regulate genes that are involved in gluconeogenesis (Murata et al. 2002). Acylated ghrelin stimulated the production of glucose in hepatocytes in a dose-dependent manner even though the cells did not express the ghrelin receptor. However, des-acyl ghrelin exhibited effects opposite to those of acylated ghrelin. These findings point to a role for ghrelin in hepatic glucose metabolism, and also, indicate that there might be another, still unidentified receptor for ghrelin in hepatocytes (Gauna et al. 2005).

The most important organ that is involved in the glucose and insulin metabolism is the pancreas, i.e. it is the islet cells that secrete insulin, glucagon, somatostatin and pancreatic polypeptide, in another words the hormones that regulate glucose metabolism. At the cellular level, ghrelin and the ghrelin receptor have been shown to be expressed in pancreatic islets, suggesting a paracrine/autocrine activity for ghrelin in pancreas (Date et al. 2002, Gnanapavan et al. 2002, Volante et al. 2002). In addition, ghrelin has been measured to be present in pancreatic veins in concentrations much higher than in pancreatic arteries. This indicates that ghrelin produced in the pancreas is also secreted into the bloodstream, hence the pancreas produces ghrelin that has not only an autocrine/paracrine effects but also endocrine activity (Dezaki et al. 2006). In rodents, insulin secretion can be diminished by ghrelin administration both in vitro and in vivo (Reimer et al. 2003, Salehi et al. 2004). Furthermore, in pancreatic islets isolated from ghrelin gene knock-out mice an enhanced glucose-induced insulin release was detected, whereas the density, size and the insulin contents of the islets were unaltered compared to islets obtained from wild type littermates. This study demonstrated that ghrelin produced in pancreatic islets might have a physiological role in suppressing or in modulating insulin secretion in response to glucose induced insulin release (Dezaki et al. 2006). Ghrelin was shown to decrease glucose induced insulin secretion of isolated pancreatic islets when administered at low concentrations in vitro. However, supraphysiological ghrelin concentrations had an opposite effect on insulin secretion (Salehi et al. 2004). These results suggest that under physiological conditions, ghrelin might
negatively modulate insulin secretion. In a more recent study, the roles of acylated ghrelin, des-acyl ghrelin and obestatin in the regulation of pancreatic hormone secretion were examined in rodent pancreatic islets. Acylated ghrelin was shown to be inhibitory in a dose-dependent manner whereas the roles of des-acyl ghrelin and obestatin were stimulatory in the regulation of insulin secretion. It is possible that the inhibitory effect of acylated ghrelin on insulin secretion might be modulated by obestatin and blunted by des-acyl ghrelin under physiological conditions (Qader et al. 2008).

These findings from in vitro studies on acylated and des-acyl ghrelin have been supported by in vivo studies. Administration of des-acyl ghrelin blocked the hyperglycaemic effect of the exogenous acylated ghrelin in humans (Broglio et al. 2004). It seems that acylated and des-acyl ghrelin might be involved in the energy metabolism by modulating the release of one of its most crucial hormones, insulin and that acylated and des-acyl ghrelin might have different, even opposite, effects, despite the fact that contradictory effects of ghrelin on insulin release on pancreatic cells have also been published (Date et al. 2002), in conclusion can be said that the majority of studies have provided evidence indicative of a suppressive role of ghrelin in the release of insulin from the pancreatic islets. The modulation of insulin secretion by ghrelin provides a new insight into the physiological control of insulin and glucose metabolism and pathological conditions related to pancreatic function.
3 Aims

The aim of this thesis project was to search for undiscovered variations in the genes coding for ghrelin and its receptor which might affect the ghrelin signalling or which could be associated with obesity or with some metabolic risk factors for atherosclerosis. Other aims were to elucidate the role of genes in the regulation of ghrelin levels and to study the relationship between ghrelin and type 2 diabetes with a prospective study design.

The following specific questions were addressed:

1. Are there unidentified functional variations in the ghrelin receptor gene which might affect the mediation of the physiological effects of ghrelin? The hypothesis was that ghrelin receptor variants might affect the function of the receptor, and in that way change the downstream signalling cascades and modulate the GH / IGF-1 –axis. In addition, the variants might affect energy metabolism related clinical parameters, such as plasma glucose, insulin, and lipid levels.

2. Are there unidentified functional variations in the 5´flanking region of the ghrelin gene, which might play a role in the determination of the fasting plasma concentration of ghrelin in humans? The aim was to investigate putative regulatory areas upstream of the ghrelin gene utilizing human plasma samples with high and low plasma ghrelin concentrations.

3. What is the role of genetic background in the regulation of fasting plasma ghrelin levels? The main interest was to measure ghrelin levels in a special twin population of monzygous twins discordant for obesity. In addition, the gene-environment interaction was studied by genotyping two ghrelin gene polymorphisms in three sets of monzygotic twins: obesity discordant, obesity concordant and lean concordant twins.

4. Do fasting plasma ghrelin levels have a predictive value with respect to the incidence of type 2 diabetes and impaired glucose regulation? To answer this question, a prospective study was carried out in a middle-aged Finnish population.
4 Subjects and methods

In this thesis, three different human study populations were examined. In studies I and II, sub-populations derived from a large population based cohort study, Oulu Project Elucidating Risk for Atherosclerosis (OPERA) were used to screen the genes coding for ghrelin and its receptor for new variants. In study II, also the whole OPERA population was examined in genetic association studies. Study III was carried out in monozygotic twins. The study IV was conducted in a population based follow-up study population of middle-aged Finns.

4.1 OPERA study population

The OPERA study population was collected in the Department of Internal Medicine, University of Oulu. It consists of 600 hypertensive subjects (300 men and 300 women) and 600 control subjects (300 men and 300 women). Hypertensive subjects were chosen from the Social Insurance Institute register of individuals receiving reimbursement of antihypertensive medication. According to the register, the study subjects belonged to a higher reimbursement class and were entitled to a special refund for antihypertensive medication. For each hypertensive subject, an age and sex matched control was randomly selected from the national health register. Subjects receiving a reimbursement for antihypertensive medication were excluded from the control population. Both hypertensive and control subjects were living in Oulu, and were between 40–59 years old on September 1st, in 1990, when the selection of subjects to the study was performed. The study subject visited the research laboratory of the Department of Internal Medicine for laboratory tests, a physical examination and a detailed interview covering the past medical history, current and former medication use and physical activity, alcohol consumption and smoking. The study subjects volunteered to take part in the study and informed consent was obtained from each patient. The study was approved by the Ethical Committee of the Faculty of Medicine, University of Oulu, and it was carried out according to the principles of the Declaration of Helsinki. This study has been described in more detail previously (Kauma et al. 1996, Kiema et al. 1996).
4.1.1 Description of the study population of study I

In study I, associations between the possible genetic variations in GHS-R1a gene and the GH/IGF-1 –axis were investigated. For this approach, patients with the highest (n = 96) and the lowest (n = 96) IGF-1 plasma concentrations of the hypertensive group of the OPERA were selected. The groups were stratified for gender by choosing 48 men and 48 women into both of the groups. In addition, the physiological age-dependent reduction in IGF-I levels was taken into account by age-stratifying both of the study groups. The IGFBP-1 plasma concentrations did not differ between the groups. The study population is characterized in detail in Table 4.

Table 4. Characteristics of the study population in study I.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Low IGF-1</th>
<th>High IGF-1</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (men/women)</td>
<td>96 (48/48)</td>
<td>96 (48/48)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>51.7 (50.1-52.8)</td>
<td>51.1 (49.9-52.4)</td>
<td>0.505</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>29.6 (28.6-30.7)</td>
<td>28.0 (27.2-28.8)</td>
<td>0.012</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>95.5 (92.8-98.2)</td>
<td>91.7 (89.2-94.1)</td>
<td>0.039</td>
</tr>
<tr>
<td>IGF-1 (ng/ml)</td>
<td>30.2 (27.0-33.4)</td>
<td>136.6 (127.6-145.6)</td>
<td>≤0.000</td>
</tr>
<tr>
<td>IGFBP-1 (ng/ml)</td>
<td>2.3 (1.3-4.8)</td>
<td>2.3 (1.3-3.6)</td>
<td>0.517</td>
</tr>
<tr>
<td>FP-glucose (mmol/l)</td>
<td>4.7 (4.2-5.5)</td>
<td>4.5 (4.1-5.0)</td>
<td>0.027</td>
</tr>
<tr>
<td>FP-insulin (mU/l)</td>
<td>12.9 (9.1-22.8)</td>
<td>12.7 (9.1-20.4)</td>
<td>0.742</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>5.9 (5.6-6.1)</td>
<td>5.8 (5.6-6.0)</td>
<td>0.468</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>1.3 (1.2-1.4)</td>
<td>1.3 (1.2-1.3)</td>
<td>0.607</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>3.6 (3.4-3.8)</td>
<td>3.5 (3.3-3.7)</td>
<td>0.619</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.7 (1.2-2.4)</td>
<td>1.5 (1.1-2.1)</td>
<td>0.227</td>
</tr>
<tr>
<td>VLDL cholesterol (mmol/l)</td>
<td>0.57 (0.49-0.66)</td>
<td>0.54 (0.45-0.63)</td>
<td>0.563</td>
</tr>
<tr>
<td>Ghrelin (ng/ml)</td>
<td>770 (723-817)</td>
<td>619 (566-674)</td>
<td>≤0.000</td>
</tr>
</tbody>
</table>

Values shown are means (95%CI) for normally distributed variables and medians (25th-75th percentiles) for non-normally distributed variables (IGFBP-1, FP-glucose, FP-insulin, triglycerides). Logarithmic transformations were used for non-normally distributed variables to meet the criteria for parametric tests. P-values are from t-test. BMI = body mass index, IGF-1 = Insulin like growth factor -1, IGFBP-1 = insulin like growth factor binding protein -1, FP = fasting plasma, LDL = low density lipoprotein, HDL = high density lipoprotein, VLDL = very low density lipoprotein, ns = non-significant.

4.1.2 Description of the study population of study II

The subjects were also chosen as a sub-sample from the OPERA study. As the main interest was to determine whether some genetic variants affecting the determination of the ghrelin plasma concentrations existed in the gene coding for
ghrelin, patients having the highest (n = 50) and the lowest (n = 50) fasting ghrelin plasma concentration were chosen from the whole OPERA study population (hypertensive and control group). The number of both men and women was 25 in both study groups. There were no differences in the ages, BMIs, waist circumferences or in the concentrations of obesity related adipokines, namely leptin and adiponectin between the groups, as described in more detail in the original publication II and in Table 5.

Table 5. Characteristics of the study population of study II.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Low ghrelin group</th>
<th>High ghrelin group</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (men/women)</td>
<td>25/25</td>
<td>25/25</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>53.9 (52.3-55.5)</td>
<td>51.8 (50.6-53.1)</td>
<td>0.04</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>28.9 (27.5-30.4)</td>
<td>27.4 (26.1-28.7)</td>
<td>0.117</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>94.1 (90.298.1)</td>
<td>90.2 (85.8-94.7)</td>
<td>0.189</td>
</tr>
<tr>
<td>FP-leptin (pg/ml)</td>
<td>11.4 (9.9-15.1)</td>
<td>7.2 (7.8-12.7)</td>
<td>0.108</td>
</tr>
<tr>
<td>FP-adiponectin (mg/L)</td>
<td>15.3 (13.6-17.0)</td>
<td>15.4 (13.6-17.2)</td>
<td>0.976</td>
</tr>
<tr>
<td>FP-glucose (mmol/l)</td>
<td>4.4 (4.1-5.1)</td>
<td>4.3 (4.0-5.9)</td>
<td>0.109</td>
</tr>
<tr>
<td>FP-insulin (mU/l)</td>
<td>16.9 (8.5-11.6)</td>
<td>11.4 (6.3-14.3)</td>
<td>0.004</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>5.6 (5.3-5.9)</td>
<td>5.7 (5.3-6.0)</td>
<td>0.855</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>1.3 (1.2-1.4)</td>
<td>1.4 (1.3-1.5)</td>
<td>0.136</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>3.4 (3.2-3.7)</td>
<td>3.4 (3.2-3.7)</td>
<td>0.818</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.4 (1.1-1.9)</td>
<td>1.3 (1.0-2.0)</td>
<td>0.442</td>
</tr>
<tr>
<td>FP-ghrelin (ng/ml)</td>
<td>253 (244-261)</td>
<td>1243 (1219-1267)</td>
<td>≤ 0.000</td>
</tr>
</tbody>
</table>

The values shown are means (95% CIs) for normally distributed variables and medians and 25th-75th percentiles for non-normally distributed variables (FP-glucose, FP-insulin and triglycerides). FP = fasting plasma, HDL = high density lipoprotein, LDL = low density lipoprotein. P-values are obtained from t-test, non-normally distributed variables were normalized with logarithmic transformation prior to the analysis.

4.2 Description of the study population of study III.

The third study was based on a sample of 23 monozygotic twin pairs with a unique phenotypic feature: discordance for obesity. In other words, even though the co-twins share identical genes, the other co-twin is over-weight or obese, whereas his or her co-twin is normal-weight or lean. These discordant twin pairs were selected from the Finnish Twin Cohort (Kaprio et al. 1978) that includes all Finnish twins (4307 monozygous, 9581 like-sexed dizygous pairs) born before 1958 and alive in 1975.

The criteria for the selection of the obesity discordant twins from the cohort was that the intra-pair BMI difference between the lean and the obese co-twin had
to be more than 4 kg/m\(^2\), with the lean co-twin having a BMI less than 25 kg/m\(^2\) and the obese co-twin having a BMI more than 27 kg/m\(^2\). In addition, twins had to be born between 1932 and 1957. Furthermore, those subjects who had diseases (thyroid disorders, psychiatric diseases, diabetes or major musculoskeletal problems) or who were taking any continuous medication (e.g. beta blockers or diuretics) with a possible influence on lipid or glucose metabolism were excluded. Twenty eight twin pairs fulfilled the criteria and they were clinically examined in 1992.

In the clinical examinations, pairs with an intrapair difference in BMI of more than 3 kg/m\(^2\) were included in the present study, and with this criteria, two of the examined pairs were excluded. One pair was excluded because of the obese subject was found to have a previously undiagnosed type 2 diabetes.

The monozygosity was primarily determined by a questionnaire concerning the similarities of the twins during school ages and it was validated by 11 blood group markers (Sarna et al. 1978). The monozygosity of the twins that were selected to this study was assured by dermatoglyphic analysis of the fingertip prints (Smith & Penrose 1955). All except 6 pairs were confirmed to be monozygous. These 6 pairs were further studied for their zygosity by genotyping some markers from six different polymorphic gene loci (DIS80, APOB, D17S30, COL2A1, VWA and HUMTH). Four of these pairs were determined to be monozygous and the two remaining dizygous pairs were excluded. Thus, the study population of monozygote twins discordant for obesity consisted of 23 twin pairs (9 male and 14 female pairs) with more than 3 kg/m\(^2\) intrapair difference in BMI and a mean intrapair difference in body weight of more than 18 kg. The subjects were healthy and were not using any continuous medication. The study subjects had provided informed consent to participate in studies of the genetics of obesity and related disorders. Ethics committees of the Turku University Hospital and Helsinki University Hospital approved the data collection protocols. The obesity discordant monozygotic twins are described in detail in Table 6.
Table 6. Description of the study population of study III, 23 pairs of monozygotic twins discordant for obesity.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Male twin pairs (9 pairs)</th>
<th>Female twin pairs (14 pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Obese</td>
<td>Lean</td>
</tr>
<tr>
<td>Age (years)</td>
<td>44 (7)</td>
<td>44 (7)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>28.8 (1.6)</td>
<td>23.6 (0.9)</td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>25.7 (3.0)</td>
<td>21.8 (2.7)</td>
</tr>
<tr>
<td>WHR</td>
<td>0.97 (0.07)</td>
<td>0.90 (0.05)</td>
</tr>
<tr>
<td>AVF (cm³)</td>
<td>128.0 (67.0)</td>
<td>56.6 (25.1)</td>
</tr>
<tr>
<td>ASF (cm³)</td>
<td>238.1 (61.1)</td>
<td>172.4 (54.9)</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>127 (9)</td>
<td>119 (12)</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>83 (8)</td>
<td>76 (9)</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>6.1 (1.1)</td>
<td>5.8 (1.3)</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>4.8 (1.0)</td>
<td>4.3 (1.1)</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>1.2 (0.1)</td>
<td>1.4 (0.2)</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.8 (0.8)</td>
<td>1.4 (0.2)</td>
</tr>
<tr>
<td>FP-glucose (mmol/l)</td>
<td>5.20 (0.16)</td>
<td>4.95 (0.15)</td>
</tr>
</tbody>
</table>

Values shown are means and standard deviations (SD). Intra-pair differences were analysed with paired samples t-test and logarithmic transformations were used to normalize the distribution of non-normally distributed variables (triglycerides, fasting glucose, AVF, triglycerides, HDL cholesterol, systolic and diastolic blood pressure). BMI = body mass index, WHR = waist - hip ratio, AVF = abdominal visceral fat, ASF = abdominal subcutaneous fat, SBP = systolic blood pressure, DBP = Diastolic blood pressure, LDL = low density lipoprotein, HDL = high density lipoprotein, FP = fasting plasma, ns = non-significant.

4.2.1 Control populations for obesity discordant monozygotic twins

The control populations consisted of age- and sex-matched monozygotic twin pairs, also from the Finnish Twin Cohort. One control group consisted of 43 monozygotic concordant twin pairs with a normal body mass index (BMI < 25 kg/m²) and it was referred to as the lean concordant group. The other group consisted of 46 monozygotic twin pairs with a BMI > 30 kg/m² and this group was referred to as the obese concordant group. In addition to sex, age and BMI data, DNA was available for these twin pairs.
The subjects of these control groups had also provided informed consent and approvals for the protocols were obtained from the ethics committees of the Turku University Hospital and Helsinki University Hospital.

4.3 Description of the study population of study IV

In the fourth study, the study population consisted of 201 subjects (66 men and 135 women), who belonged to a larger population based survey (n = 1008) that was collected for assessing the incidence and risk factors of type 2 diabetes and impaired glucose tolerance. The study subjects of this cohort study were clinically examined at the beginning of the study in 1990–1992, eight hundred thirty-one subjects attended the first phase. After a mean follow up time of 5.1 years, in 1996–1998 the subjects were re-examined. The study was originally designed and carried out in the Department of Public Health and General Practice, University of Oulu, Finland. All subjects provided informed consents and the study protocols were approved by the Ethical Committee of the Faculty of Medicine, University of Oulu. The study subjects were born in 1935 and were living in Oulu, Finland, on October 1st 1990. The inclusion criteria for this present study were that at the beginning of the study the subjects had to have normal glucose tolerance according to the most recent WHO criteria (World Health Organization 1999) and in addition, there had to be data available describing the glucose tolerance status at the follow-up. In the whole cohort, 451 subjects were normoglycaemic at the beginning of the study and there were 347 subjects whose glucose tolerance was measured at the end of the follow-up study. A serum sample for the ghrelin measurements was available in 2007 from 201 of those individuals and they formed the final study population. There was no significant differences in clinical parameters between those whose ghrelin levels were measured compared to those 347 subjects who fulfilled the inclusion criteria but whose serum was not available, as described in more detail previously (Jalovaara et al. 2008). The study population at baseline is described in Table 7. There were significant differences in 0h and 2h blood glucose after 75g glucose intake in OGTT, in serum total- and HDL-cholesterol, systolic and diastolic blood pressure values between men and women (Table 7).
Table 7. Description of the study population of study IV at the beginning of the follow-up study.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Men</th>
<th>Women</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>66</td>
<td>135</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.0 (25.5-26.6)</td>
<td>26.0 (25.1-26.7)</td>
<td>0.766</td>
</tr>
<tr>
<td>FP-glucose (mmol/l)</td>
<td>4.7 (4.5-4.8)</td>
<td>4.5 (4.3-4.6)</td>
<td>0.009</td>
</tr>
<tr>
<td>2-h CB glucose (mmol/l)</td>
<td>5.7 (5.4-6.0)</td>
<td>6.0 (5.9-6.3)</td>
<td>0.029</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>5.4 (5.1-5.7)</td>
<td>5.9 (5.6-6.1)</td>
<td>0.024</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>1.2 (1.1-1.3)</td>
<td>1.5 (1.4-1.6)</td>
<td>≤ 0.000</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>3.7 (3.4-3.9)</td>
<td>3.9 (3.7-4.1)</td>
<td>0.247</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.0 (0.7-1.5)</td>
<td>0.8 (0.6-1.1)</td>
<td>0.061</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>144 (140-148)</td>
<td>134 (131-137)</td>
<td>≤ 0.000</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>92 (90-95)</td>
<td>86 (84-88)</td>
<td></td>
</tr>
</tbody>
</table>

Values shown are means and 95% confidence intervals, except for triglycerides, which was a parameter showing a skewed distribution. The values shown for triglycerides are medians and 25th – 75th percentiles. P-values are obtained from t-test, the non-normally distributed variable was normalized with logarithmic transformation prior to the statistical analyses. BMI = body mass index, FP = fasting plasma, CB = capillary blood, HDL = high density lipoprotein, LDL = low density lipoprotein, SBP = systolic blood pressure, DBP = Diastolic blood pressure.

4.4 Clinical methods

The clinical methods used in this thesis are summarized briefly in Table 8. The techniques have been described in detail in the original publications.
Table 8. Clinical and biochemical methods used in the original publications.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Method</th>
<th>Used in publication</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI</td>
<td>Mass(kg) / height (m)^2</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td>Waist circumference</td>
<td>Tape measure, at midway between the lower rib margin and the iliac crest</td>
<td>I, II, III</td>
</tr>
<tr>
<td>Body fat %</td>
<td>Four component method</td>
<td>III</td>
</tr>
<tr>
<td>Areas of AVF, ASF</td>
<td>MRI</td>
<td>III</td>
</tr>
<tr>
<td>Blood pressure</td>
<td>According to the guidelines of the American Society of Hypertension 1992</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td>OGTT</td>
<td>According to the guidelines of WHO study group 1985</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td>Blood glucose</td>
<td>Glucose dehydrogenase method</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td>Plasma insulin</td>
<td>Two-site immunoenzymometric assay, Commercial RIA</td>
<td>I, II, III</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>Enzymatic colorimetric method</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>Enzymatic colorimetric method</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td>VLDL cholesterol</td>
<td>Enzymatic colorimetric method from the fraction formed during ultracentrifugation</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>Enzymatic colorimetric method from the supernatant after the precipitation of lower density lipoproteins from the fraction formed during ultracentrifugation</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>Calculated by subtracting cholesterol in HDL from the cholesterol concentration of the VLDL free fraction</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Commercial ELISA</td>
<td>I, II</td>
</tr>
<tr>
<td>IGFBP-1</td>
<td>Commercial immunoenzymometric assay</td>
<td>I, II</td>
</tr>
<tr>
<td>Adiponectin</td>
<td>Non-commercial ELISA</td>
<td>I, II, III</td>
</tr>
<tr>
<td>Leptin</td>
<td>Commercial RIA kit</td>
<td>I, II, III</td>
</tr>
<tr>
<td>Ghrelin</td>
<td>Commercial RIA</td>
<td>I, II, III, IV</td>
</tr>
</tbody>
</table>

BMI = body mass index, AVF = abdominal visceral fat, ASF = abdominal subcutaneous fat, MRI = magnetic resonance imaging, OGTT = oral glucose tolerance test, WHO = World Health Organization, RIA = radioimmunoassay, QUICK = quantitative insulin sensitivity check index, AUC = area under curve, VLDL = very low density lipoprotein, HDL = high density lipoprotein, LDL = low density lipoprotein, IGF-1 = insulin like growth factor-1, IGFBP-1 = IGF binding protein-1, ELISA = enzyme-linked immunosorbent assay.

In study IV, the blood glucose levels were used to classify the subjects according to the 1999 WHO criteria (World Health Organization 1999) into groups with normal glucose tolerance (NGT), impaired glucose regulation (IGR) and type 2 diabetes (DM2). In the WHO criteria, IGR refers to subjects with impaired fasting glucose (IFG) and/or impaired glucose tolerance (IGT). According to the criteria, diabetes was defined as fasting blood glucose (FBG) ≥ 6.1mmol/l, or 2 h capillary blood glucose ≥ 11.1mmol/l. Impaired glucose tolerance was defined as FBG ≤
6.1 and 2h capillary blood glucose of 7.8 to 11.0 mmol/l. Impaired fasting glucose was defined as FBG of 5.6 to 6.0 mmol/l and 2h capillary blood glucose ≤ 7.7 mmol/l. Normoglycaemia was defined as FBG ≤ 5.5 mmol/l, and 2h capillary blood glucose ≤ 7.7 mmol/l.

More detailed descriptions of the OPERA study (studies I and II) and the measurements of the clinical parameters can be found elsewhere (Kiema et al. 1996, Rantala et al. 1999). The detailed information of the methods concerning the above mentioned measurements have been described previously for study III (Rönnermaa et al. 1997, Rönnermaa et al. 1998) and for study IV (Rajala et al. 2000, Rajala et al. 2001, Rajala et al. 2002).

4.4.1 Sequencing analyses

In study I, the exons of the gene coding for GHS-1a, GHSR, were sequenced from the patient DNA samples. At first, two DNA fragments covering the first exon and one DNA fragment covering the second exon were amplified by the polymerase chain reaction (PCR) as described in detail in the original paper (I) and as illustrated in Figure 4. Primers and PCR conditions are also listed in Table 9, which contains the information of all PCR primers and enzymes that were used. The PCR products were enzymatically purified and then sequenced in both directions (5’→3’ and 3’→5’) using a DYenamic ET terminator Cycle sequencing kit (Amersham Biosciences, Bucks, UK) and ABI 377 sequencer (PE Applied Biosystems) equipment. The chromatogram files were analyzed nucleotide by nucleotide with the Chromas 2.23 program (Technelysium Pty Ltd). The sequences were then aligned by using the Clustal W Multiple sequence alignment program, version 1.8 (Thompson et al. 1994) on the internet.

![Fig. 4. Sequencing of the two exons of the GHS-R1a gene was carried out by screening the first exon (Exon 1) by two fragments (1 and 2) and the second exon (Exon 2) by a single fragment (3). The overlapping area between the fragments 1 and 2 was 60 nucleotides. The 2152 bp intronic sequence was not studied.](image-url)
In study II, a total of 1657 base pairs upstream (in the 5´flanking area) and 60 nucleotides downstream from the transcription starting site of the ghrelin gene \( GHRL \) were screened for new sequential variants. This was carried out by sequencing four overlapping DNA fragments, shown in Figure 5. The PCR conditions (the primers, enzymes and annealing temperatures) are described in detail in the original paper II and in Table 9. The sequencing was carried out from the purified PCR products with the same sequencing protocol and the equipment than in study I. In addition, the same programs were used in the analyses of the chromatograms as in study I.

![Fig. 5. Sequencing of the 5´flanking area of the ghrelin gene was carried out from four DNA fragments (1–4) that were amplified by PCR.](image)

### 4.4.2 Genotyping of the ghrelin gene variants

One of the SNPs found in study II, namely -501A>C, was further studied in the entire OPERA study population \((n = 1054)\). It was genotyped by the restriction fragment length polymorphism (RFLP) –technique. The DNA fragment containing the SNP (fragment 2, study II), was amplified with PCR (Table 9) and digested with \( Mwol \) restriction enzyme (New England Biolabs). The restriction reaction conditions and the protocol is described in the original paper II in detail. The restriction fragments were separated by their size by 2% agarose gel (QA agar) electrophoresis and visualized on an ultraviolet transluminator containing 2mg/ml ethidium bromide.

In study III, two previously known variants of ghrelin gene, namely polymorphisms causing Arg51Gln and Leu72Met amino acid changes, were genotyped from the DNA samples with the RFLP –technique. A DNA fragment containing both variants was amplified by PCR as described in Table 9. These variants were genotyped in twins discordant for obesity, obese and lean concordant twins. The Arg51Gln mutation was identified from the 618bp PCR product using the restriction endonuclease SacI, which retains the mutated site.
(guanine replaced by adenine) at base 346 in exon 2 of the prepro-ghrelin gene as being undigested. The Leu72Met polymorphism is caused by a cytosine to adenine transition at base 408 in the exon 2 of the prepro-ghrelin gene, which leads to loss of the BsrI restriction site. Also this polymorphism was identified from the same 618bp PCR product. The digestion reactions were performed at 37°C (SacI) and at 65°C (BsrI) overnight with 5U of the enzyme. The fragments were separated on a 1.5 % agarose gel and visualised under UV light after staining with GelStar nucleic acid gel stain (Biowhittaker Molecular Applications, Rockland, ME, USA).
<table>
<thead>
<tr>
<th>Study / fragment</th>
<th>Left primer</th>
<th>Right primer</th>
<th>Enzyme</th>
<th>T Ann (°C)</th>
<th>Cycles</th>
<th>Fragment size (bp)</th>
<th>S/R</th>
<th>Restriction enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Study I</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fragment 1</td>
<td>5'ctccctacggtggtcagc 3'</td>
<td>5'ggaagcagatggggaagtag 3'</td>
<td>a</td>
<td>56</td>
<td>35</td>
<td>569</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>Fragment 2</td>
<td>5'caggagagagctgacactagc 3'</td>
<td>5'cgaaggacacgggaggtag 3'</td>
<td>b</td>
<td>58</td>
<td>30</td>
<td>491</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>Fragment 3</td>
<td>5'tgcgtgtgctttctactgac 3'</td>
<td>5'ctccctacagatggggaagtag 3'</td>
<td>b</td>
<td>56</td>
<td>30</td>
<td>430</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td><strong>Study II</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fragment 1</td>
<td>5'cccatctcatcctactcagc 3'</td>
<td>5'cgaagctcagccagacagt 3'</td>
<td>a</td>
<td>67</td>
<td>35</td>
<td>502</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>Fragment 2</td>
<td>5'cagctactctrcaagctactac 3'</td>
<td>5'caggagagaggttagagctgta 3'</td>
<td>a</td>
<td>61</td>
<td>35</td>
<td>509</td>
<td>S &amp; R</td>
<td>MwoI</td>
</tr>
<tr>
<td>Fragment 3</td>
<td>5'aggcatctcgcaaaataggc 3'</td>
<td>5'ggaagagctgttgctcaagc 3'</td>
<td>b</td>
<td>60</td>
<td>35</td>
<td>501</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>Fragment 4</td>
<td>5'tgcgtgtgctttactacagc 3'</td>
<td>5'ctccctacagagctgtggc 3'</td>
<td>a</td>
<td>58</td>
<td>35</td>
<td>510</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td><strong>Study III</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fragment 1</td>
<td>5'gctgggctcctacctgagc 3'</td>
<td>5'ggaccctgttcactgccac 3'</td>
<td>b</td>
<td>60</td>
<td>30</td>
<td>618</td>
<td>R</td>
<td>SacI</td>
</tr>
<tr>
<td>Fragment 4</td>
<td>5'gctgggctcctacctgagc 3'</td>
<td>5'ggaccctgttcactgccac 3'</td>
<td>b</td>
<td>60</td>
<td>30</td>
<td>618</td>
<td>R</td>
<td>BsrI</td>
</tr>
</tbody>
</table>

PCR reactions were performed with a) AmpliTaq Gold DNA polymerase (PE Applied Biosystems) b) DyNAzyme (Finnzymes, Espoo Finland). T Ann = Temperature for annealing, S = sequencing, R = Restriction by the restriction fragment length polymorphism -technique.
4.5 Measurements of the ghrelin concentrations

In study III, fasting total ghrelin concentrations from the plasma samples were measured by RIA using a commercial kit (Peptide Radioimmuno Assay Kit, Phoenix Pharmaceuticals, Inc., Belmont, CA, USA). Samples were measured in duplicate and the procedure was carried out following the manufacturer’s instructions. The inter- and intra-assay coefficients of variation (CV), as given by the manufacturer, were 7.5 and 4.0% respectively. Intra-assay CV in the analyses of this study varied between 0.2 – 7.8%, mean CV% was 2.1%. In study III, the samples were measured with one single kit, and therefore there was no need to calculate the inter-assay CV%.

In study IV, total ghrelin concentrations were measured from deep frozen (-74°C) serum samples using a commercially available RIA kit (Linco Research, Catalog # GHRT 89-HK, Missouri, USA). The measurements were done in duplicate following the manufacturer’s instructions. The intra- and inter-assay CV, as given by the manufacturer, were 3.3–10% and 14.0–17.8% respectively. The mean intra-assay CV in the analyses of this study was 5.1% (range: 0.1–15.7%). The manufacturer did not recommend to include any internal standard samples into each kit measured. Instead, the manufacturer provided each kit with two standard samples (“high” and “low” ghrelin concentration) and instructed that when these standard samples give ghrelin concentrations that fall into the range given, then the results obtained from separate kits can be regarded as being comparable with each other, and having the interassay CV% within the range that the manufacturer provided (14–17.8%). In addition, when the standard samples gave ghrelin concentrations that were outside the given range of the correct value, then all the results obtained from that specific kit had to be omitted and the measurements had to be repeated with a new kit. In other words, the inter-assay CV% was not measured in study IV but it can be assumed to be within 14–17.8%.

4.6 Statistical methods

Statistical analyses were performed with the SPSS package (Versions 9.0 and 11.5 SPSS Inc., Chicago, IL, USA) except for the analysis of the genotyping data in study III, which was analysed with Stata 8.2. Logarithmic transformations were used prior to the parametric test for non-normally distributed variables. Non-parametric tests (Mann-Whitney U-test and Kruskall-Wallis test) were used only when necessary.
In studies I and II, the chi-squared test was used to assess the fit of the observed allele frequencies to Hardy-Weinberg equilibrium. Also the distributions of the SNPs between the study groups were studied with the chi-squared test. The associations of the found SNPs with several parameters related to obesity, insulin and glucose metabolism and with some known risk factors for atherosclerosis were studied using the analysis of variance (ANOVA) and the analysis of covariance (ANCOVA).

Paired samples t-test was used when studying the intrapair differences in ghrelin levels and in other variables. Differences between sexes were compared using delta values (difference between obese and lean co-twin) in independent samples t-test. Delta values were also used when the correlation of intrapair differences in ghrelin levels and those of other variables was assessed. Correlations were calculated using Pearson’s correlations and the linear regression model was used to adjust the found correlations for sex. Differences in the genotype frequencies between the groups overall, and between the discordant and concordant groups were tested using Fisher's exact test.

In study IV, subjects with baseline ghrelin concentrations higher than 3 standard deviations over the mean were considered to be outliers and were excluded from the analyses (four subjects). T-test was used to assess the differences in the studied parameters between the study groups. Adjustments for BMI and sex were performed in ANCOVA. A linear regression was used to further examine the association of ghrelin levels with the change in blood glucose levels over time. Correlations between fasting serum ghrelin levels and other clinical parameters were studied with Pearson’s correlations and adjustments for BMI were carried out with partial correlations.
5 Results

5.1 Sequencing analysis of GHSR exons (study I)

Five single nucleotide polymorphisms in the first exon of the GHS-R1a gene were found. All of the SNPs were previously known variants. None of the SNPs changed the amino acid code of the protein (Figure 6). No variations were found in the second exon.

Fig. 6. Genomic screening of the GHS-R1a gene revealed five SNPs in the first exon. The SNPs did not change the amino acid sequence of the protein. Numbers in the names of the SNPs refer to the nucleotide at which the nucleotide change takes place (number 1 corresponds to the first nucleotide at the first codon of exon 1). C = cytosine, T = thymine, G = guanine and A = adenine.

The five SNPs were equally distributed between the study groups with low and high IGF-1 plasma concentrations (data not shown). In addition, if the subjects were grouped by their glycaemic status according to the latest WHO criteria (WHO 1999) into three groups: 123 subjects with normal glucose tolerance, 42 subjects with impaired glucose tolerance and 27 subjects with type 2 diabetes), there were no differences in the genotype distributions between the three groups. However, in the association studies, SNP 171C>T was found to associate with IGFBP-1 concentrations and with the area under the insulin curve (AUCIN) values. The 171C/C wild type homozygotes had the lowest IGFBP-1 levels and highest AUCIN values compared with the other genotypes. The differences were significant in ANOVA (P = 0.009 and 0.020 respectively) and remained significant after adjusted for BMI and sex in ANCOVA (P = 0.038 and 0.038 respectively). The pairwise comparison of AUCIN values showed a difference
between C/C and C/T genotypes \((P = 0.011 \text{ and after Bonferroni correction } P = 0.032)\). Pairwise comparison of IGFBP-1 values between the genotypes revealed a difference between C/C and C/T genotypes \((P = 0.012 \text{ and } 0.037 \text{ after Bonferroni correction})\).

SNP 477G>A was associated with AUCIN values, the wild type 477G/G genotype had the highest AUCIN values \((P = 0.007)\). After adjustment for age, sex and BMI in ANCOVA the difference remained significant \((P = 0.049)\). In pairwise comparisons, a difference was detected between the G/G and G/A genotypes \((P = 0.014 \text{ and after Bonferroni correction } P = 0.032)\). This SNP 477G>A was also associated with VLDL and HDL cholesterol concentrations \((P = 0.032 \text{ and } 0.032 \text{ respectively})\). The variant homozygote 477A/A genotypes had the lowest and the heterozygotes 477G/A displayed the highest HDL cholesterol levels. However, the association between the SNP 477G>A with VLDL-cholesterol was lost after adjusting for sex, age and BMI. The association of the SNP with HDL-cholesterol remained of borderline significance \((P = 0.05)\) after adjustment for age, sex, BMI and alcohol consumption. In the pairwise comparisons, there were no significant differences observed after Bonferroni correction. No associations between the SNPs and other tested variables were observed (Table 10).

The other three SNPs, namely 60C>T, 447C>G and 531C>A, had low variant allele frequencies. The associations of these SNPs were tested only for IGF-1, IGFBP-1 and BMI and no associations were detected (data not shown).
<table>
<thead>
<tr>
<th>Variable</th>
<th>171C/C</th>
<th>171C/T</th>
<th>171 T/T</th>
<th>477G/G</th>
<th>477G/A</th>
<th>477A/A</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (m/w)</td>
<td>20 (9/11)</td>
<td>81 (42/39)</td>
<td>91 (45/46)</td>
<td>81 (42/39)</td>
<td>85 (42/43)</td>
<td>26 (12/14)</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m^2)</td>
<td>29.6 (27.8-31.4)</td>
<td>28.4 (27.4-29.5)</td>
<td>29.0 (28.1-30.0)</td>
<td>ns</td>
<td>29.5 (28.6-30.4)</td>
<td>28.1 (27.1-29.1)</td>
<td>29.2 (26.9-31.4)</td>
</tr>
<tr>
<td>IGF-1 (nmol/l)</td>
<td>101 (66-135)</td>
<td>89 (75-103)</td>
<td>74 (62-86)</td>
<td>ns</td>
<td>84 (69-99)</td>
<td>86 (72-100)</td>
<td>72 (52-91)</td>
</tr>
<tr>
<td>IGFBP-1 (ng/ml)</td>
<td>1.5 (1.0-2.3)</td>
<td>2.5 (1.5-2.5)</td>
<td>2.3 (1.2-2.3)</td>
<td>0.009</td>
<td>2.1 (1.2-4.0)</td>
<td>2.4 (1.5-3.8)</td>
<td>2.4 (1.2-5.1)</td>
</tr>
<tr>
<td>F-glucose (mmol/l)</td>
<td>5.7 (4.2-7.3)</td>
<td>5.1 (4.7-5.6)</td>
<td>4.9 (4.6-5.1)</td>
<td>ns</td>
<td>5.1 (2.7-5.5)</td>
<td>5.1 (4.7-5.6)</td>
<td>4.7 (4.4-5.0)</td>
</tr>
<tr>
<td>F-Insulin (mU/l)</td>
<td>16.8 (6.7-23.4)</td>
<td>11.9 (6.3-21.9)</td>
<td>12.9 (6.8-17.9)</td>
<td>ns</td>
<td>13.8 (9.6-23.0)</td>
<td>12.4 (8.4-33.6)</td>
<td>12.2 (9.3-37.3)</td>
</tr>
<tr>
<td>AUCIN (mmol/h)</td>
<td>203 (113-304)</td>
<td>117 (77-185)</td>
<td>143 (79-193)</td>
<td>0.020</td>
<td>164 (89-228)</td>
<td>116 (76.1-172)</td>
<td>127 (70-245)</td>
</tr>
<tr>
<td>QUICKI</td>
<td>0.53 (0.49-0.57)</td>
<td>0.57 (0.55-0.60)</td>
<td>0.57 (0.55-0.59)</td>
<td>ns</td>
<td>0.55 (0.53-0.58)</td>
<td>0.58 (0.55-0.62)</td>
<td>0.57 (0.54-0.62)</td>
</tr>
<tr>
<td>Total chol (mmol/l)</td>
<td>5.8 (5.4-6.2)</td>
<td>5.7 (5.5-6.0)</td>
<td>5.9 (5.7-6.1)</td>
<td>ns</td>
<td>5.7 (5.5-6.0)</td>
<td>5.9 (5.7-6.1)</td>
<td>6.0 (5.6-6.4)</td>
</tr>
<tr>
<td>HDL (nmol/l)</td>
<td>1.3 (1.1-1.5)</td>
<td>1.3 (1.2-1.4)</td>
<td>1.3 (1.2-1.4)</td>
<td>ns</td>
<td>1.3 (1.2-1.3)</td>
<td>1.4 (1.3-1.4)</td>
<td>1.2 (1.1-1.3)</td>
</tr>
<tr>
<td>LDL (mmol/l)</td>
<td>3.5 (3.1-3.8)</td>
<td>3.6 (3.3-3.8)</td>
<td>3.6 (3.4-3.8)</td>
<td>ns</td>
<td>3.4 (3.2-3.6)</td>
<td>3.7 (3.5-3.9)</td>
<td>3.8 (3.3-4.0)</td>
</tr>
<tr>
<td>VLDL (mmol/l)</td>
<td>0.6 (0.3-0.9)</td>
<td>0.4 (0.3-0.6)</td>
<td>0.5 (0.3-0.7)</td>
<td>ns</td>
<td>0.5 (0.3-0.7)</td>
<td>0.4 (0.2-0.6)</td>
<td>0.5 (0.4-0.7)</td>
</tr>
<tr>
<td>Trig (mmol/ml)</td>
<td>2.0 (1.4-2.6)</td>
<td>1.5 (1.1-2.0)</td>
<td>1.6 (1.1-2.3)</td>
<td>ns</td>
<td>1.7 (1.2-2.4)</td>
<td>1.4 (1.1-2.1)</td>
<td>1.7 (1.3-2.8)</td>
</tr>
<tr>
<td>F-ghrelin (pg/ml)</td>
<td>678 (545-810)</td>
<td>670 (613-727)</td>
<td>720 (667-774)</td>
<td>ns</td>
<td>690 (630-749)</td>
<td>700 (644-754)</td>
<td>700 (592-805)</td>
</tr>
</tbody>
</table>

Values shown are means (95% CIs) for normally distributed variables and medians (25th and 75th percentiles) for non-normally distributed variables (IGFBP-1, insulin, AUCIN, VLDL and triglycerides). BMI = body mass index, IGF-1 = insulin-like growth factor-1, IGFBP-1 = insulin-like growth factor binding protein-1, F = fasting, AUCIN = area under the insulin curve in OGTT, QUICKI = quantitative insulin-sensitivity check index. P-values are from ANOVA, Total chol = total cholesterol, HDL = high density lipoprotein, LDL = low density lipoprotein, VLDL = very low density lipoprotein, Trig = triglycerides.
5.2 Sequencing analysis of the GHRL 5’ flanking area (Study II)

We sequenced 1657 bp upstream and 60 bp downstream from the transcription starting site of ghrelin gene for new variations in 100 subjects belonging to the OPERA cohort. We found 11 SNPs, 5 of which were new variants (Figure 7).

![Fig. 7. Eleven SNPs were found in the 5’flanking area of the ghrelin gene by sequencing analysis in one hundred human DNA samples. Five were new variants. The numbers in the names of the SNPs refer to the nucleotide at which the nucleotide change takes place (number -1 corresponds to the first nucleotide upstream of the transcription starting site). C= cytosine, T = thymine, G = guanine and A = adenine.](image)

The found SNPs were shown not to be associated with the fasting plasma ghrelin concentrations. However, the SNP -501A>C was observed to be associated with BMI, with -501A/A wild-type having the highest (mean: 29.1 kg/m²) and -501C/C variant homozygote having the lowest BMI (mean: 24.7 kg/m²; \( P = 0.016 \)). The difference remained significant after adjustment for age and sex (\( P = 0.018 \)). This SNP was studied further in the OPERA study subjects (n=1054). A non-significant trend of subjects with -501A/A genotype having the highest and -501C/C genotype having the lowest BMI was found. The difference was not significant when the three genotypes were compared in analysis of variance. Furthermore, no significant differences were seen in other obesity related parameters between the three genotypes. However, when -501A/A genotypes and -501 A/C genotypes were combined and compared with -501C/C genotypes by using t-test, then a significant difference was observed in BMI (\( P = 0.047 \)). After stratification with age and sex, this difference disappeared. In addition, this SNP was associated with waist circumference (\( P = 0.049 \)) but the difference disappeared after adjustment for age and sex. No other significant associations were found. The results are described in more detail in the original article II.
Ghrelin concentrations in obesity discordant twins (Study III)

Ghrelin concentrations were significantly lower among the obese monozygotic twins, compared with their lean co-twins. In female twins, the difference between obese and lean co-twin was seen more clearly (Figure 8). The result suggests that ghrelin levels are affected by acquired obesity independently of the genetic background.

Fasting ghrelin concentrations correlated negatively with BMI ($r = -0.484; P = 0.001$), abdominal subcutaneous fat ($r = -0.356; P = 0.024$) and abdominal visceral fat ($r = -0.43; P = 0.005$). A borderline significant correlation was found between BMI and waist-hip-ratio ($r = -0.291; P = 0.05$). When adjusted for sex in the linear regression model, BMI, AVF and ASF remained as significant predictors for the variation in the ghrelin concentrations. No correlation between plasma ghrelin and leptin levels was observed in this population. The results are shown in more detail in the original article III.
Fig. 8. Ghrelin levels in obesity discordant monozygotic twin pairs. Co-twins are connected by solid lines. Obese (O) subjects had lower ghrelin levels than their lean (L) co-twins. In female twins, the intrapair difference was significant.

5.4 Ghrelin gene polymorphisms in twin-populations (study III)

Two single nucleotide polymorphisms Leu72Met and Arg51Gln of the ghrelin gene were genotyped in three populations of monozygotic twins: twins discordant for obesity, obese concordant twins and lean concordant twins. The distribution of the Arg51Gln genotypes did not differ between the twin groups. On the contrary, 72Met allele was detected among the obesity discordant twins less frequently than among the other twin groups. The genotype and allelic frequencies of the Leu72Met variation are shown in Figure 9.
Fig. 9. Genotype and allelic frequencies among three groups of monozygotic twin pairs. Genotypes are shown as number of pairs and allelic frequencies as percents. There were significantly less Met carriers among obesity discordant pairs, compared with obese concordant or lean concordant pairs.

5.5 Ghrelin and the incidence of impaired glucose regulation and type 2 diabetes (Study IV)

During the follow-up period of 5.1 years, six subjects (3.0%) developed type 2 diabetes, six (3.0%) developed impaired fasting glucose (IFG) and 35 subjects (17.4%) impaired glucose tolerance (IGT) and the remaining 154 subjects maintained normal glucose tolerance (Figure 9).
Baseline characteristics were compared between the two groups: those who maintained normal glucose tolerance (NGT group) and those who developed either impaired glucose regulation (IGR = IFG + IGT) or were diagnosed as new type 2 diabetics (IGR and DM2 group). At baseline, the subjects who belonged to the NGT group had lower levels of triglycerides and 2h capillary blood glucose than the subjects in the other group. BMIs, blood pressures and the concentrations of fasting plasma glucose, cholesterol and triglycerides did not differ between the two groups (Data shown in original article IV).

In this study, the main finding was that ghrelin concentrations did not differ at baseline between those who maintained NGT and those who developed some degree of impaired glucose regulation. The median fasting serum total ghrelin concentration was 733 pg/ml (25th-75th percentiles: 571–961 pg/ml) among those who maintained NGT, and 661 pg/ml (25th-75th percentiles: 527–878 pg/ml) among those who developed IGR or type 2 diabetes ($P = 0.421$). Ghrelin
concentrations at baseline for those who maintained NGT, as well as for those who developed impaired fasting glucose (IFG), impaired glucose tolerance (IGT) and type 2 diabetes are shown as a boxplot in Figure 11.

Fig. 11. Fasting serum total ghrelin concentrations at the beginning of the study shown as a boxplot. Subjects were grouped according to their glucose status at the end of the study. NGT = normal glucose tolerance, IFG = impaired fasting glucose, IGT = impaired glucose tolerance and DM = type 2 diabetes mellitus. Two subjects from the NGT group and two from the IGT group were excluded from the analysis because of their extremely high ghrelin concentrations (+3SD of the mean).

During the follow-up, there was a mean average decrease of 28.5 pg/ml (95%CI = -66.3 – 10.1 pg/ml) in ghrelin concentrations. BMIs increased slightly (mean BMI change +1.4 kg/m² and 95%CI = 1.1–1.6 kg/m²). A weak negative correlation between the change in ghrelin and the change in BMI was observed (Pearson’s correlation: r = -0.145, P = 0.043). Serum lipid and blood glucose levels increased during the follow-up time (described in detail in the original article). Correlations between change in ghrelin and that of serum total-, LDL- HDL- cholesterol, triglycerides and blood glucose levels were also studied, but no significant correlations were observed (data not shown). In addition, according to the linear regression, the ghrelin concentrations at baseline did not explain the change either in fasting plasma glucose or in 2h capillary blood glucose that was examined during the follow-up.
At the end of the follow-up, the fasting serum total ghrelin concentrations did not differ between those who maintained NGT and those who developed IGR or DM2.

The statistical power to detect a difference between NGT and IGR+DM2 groups was calculated with a programme available on the internet (http://www.dssresearch.com/toolkit/spcalc/power.asp). These calculations showed that the power to detect a difference between the two groups with the observed ghrelin concentrations using a significance level of $P<0.05$ was 13.1%. However, the power to detect a difference of one standard deviation in ghrelin concentrations between these two groups in this study population was 100%, and a difference of 0.5 standard deviation could have been detected with a power of 82.6%.
6 Discussion

6.1 Methodological considerations

6.1.1 Study populations and design

In studies I and II, the OPERA study population or subjects belonging to some subgroups of this project were examined. In study I, the subjects were selected from the hypertensive group by choosing those subjects with the highest and the lowest IGF-1 plasma levels. The number of men and women was equal in both groups, and they did not differ in terms of age. In study II, the subjects were chosen from hypertensive and control subjects according to their ghrelin concentration; i.e. subjects with the highest and the lowest concentrations and also here, the groups were sex stratified. The benefit of examining such subgroups, i.e. extremes of a certain phenotype, is that there is an increased probability of finding functional variants affecting the phenotype of interest. If an association is found, it needs to be further examined in larger populations or in cell culture or animal studies, when appropriate.

In study I, the aim was to find subjects whose ghrelin receptor might function abnormally (extremely effectively or defectively) and to screen the ghrelin receptor gene exons to detect new functional variants from such subjects. The ideal way to choose patients would have been to first assess the GH response to administered ghrelin in the whole study population, and then create the subgroups by choosing the subjects with the highest and the lowest growth hormone secretion response. Since this was not possible, we estimated that if there is a functional defect in GHS-R1a, also the signal transduction mediated by this receptor might be altered leading to an altered GH secretion. In this study population, the GH concentrations were not measured directly, but we used an indirect surrogate measure, the IGF-1 concentration, as a marker for growth hormone secretion.

The rationale for using the IGF-1 concentration as a marker is that IGF-1 has been shown to be a mediator for the anabolic actions of GH and it is known that plasma concentrations of IGF-1 are regulated by GH secretion. For example: in GH deficiency the IGF-1 plasma concentrations are low and in GH hypersecretory conditions, such as in acromegalia, the IGF-1 concentrations are high. The plasma concentrations of IGF-1 are routinely used in the diagnosis and
monitoring for both hypo- and hypersecretory conditions of GH. (Kato et al. 2002.) However, the plasma concentrations of IGF-1 are regulated also by other factors, such as age and sex. These factors were controlled when selecting the subjects for study I. It must be admitted that because of this selection and also the relatively small population size, the results obtained can not be generalized to an unselected middle-aged population. The results are preliminary and need to be replicated elsewhere in a larger and more heterogeneous population.

In study II, we first carried out a sequencing study in a sub-group from the OPERA participants and subsequently examined the most promising finding in a larger population. The results obtained from OPERA can be interpreted to apply quite well to the middle-aged Finnish population.

The third study was conducted in a unique study population of monozygotic twins discordant for obesity. The entire Finnish twin cohort of thousands of MZ pairs was screened in order to find these 23 discordant twin pairs. This study might encounter some criticism for its small population size. However it is not a trivial matter to increase the sample size.

MZ twins discordant for obesity have the same genes and yet can display large differences in body mass (Hakala et al. 1999, Rönnemaa et al. 1997, Rönnemaa et al. 1998). These phenotypic differences between the co-twins are believed to be caused by environmental factors, epigenetic effects or gene-environmental interactions. It can be speculated that discordant pairs carry alleles that are more permissive in the presence of pressures on energy balance, the so-called "variability genes". These are in contrast to level genes, where the level of the traits is determined by the presence or absence of specific alleles, a classic example being the effect of the apoE polymorphism on lipid values (Utermann et al. 1979). The variability genes might contribute to the discrepancy between the estimates of heritability of obesity and they might explain in part why some individuals but not others become obese in the same obesogenic environment. Monozygotic twins discordant for obesity are an interesting study population with which to reveal possible variability genes for obesity.

In the fourth study, we studied the role of ghrelin concentrations with respect to the incidence of impaired glucose regulation and type 2 diabetes with a prospective study design. The samples were obtained from a population based survey. Three hundred and forty seven subjects fulfilled the inclusion criteria. A serum sample was available from 60.5% of them, and these 201 subjects formed the actual study population whose ghrelin concentrations were measured. However, the study subjects whose ghrelin concentrations were measured differed
from those whose serum was not available only in terms of their fasting plasma glucose concentration, which was lower among those whose ghrelin was not measured. In another words, the subjects whose ghrelin levels were measured were not in a less favourable situation with respect to glucose metabolism. It has been suggested that the results obtained can be generalized to apply the whole normoglycaemic population (Jalovaara et al. 2008). This study was the first longitudinal study to assess the possible predictive role of ghrelin on the incidence of type 2 diabetes in Caucasians.

6.1.2 Ghrelin concentrations measured in this study

In studies III and IV, the total ghrelin concentrations were measured from the fasting plasma and serum samples. The antibodies of the kit that measures the total ghrelin are able to bind both the acylated and des-acylated ghrelin molecules, i.e. the value obtained with the total ghrelin kit represents the amount of acylated plus des-acylated ghrelin molecules summated together. On the other hand, by measuring the total ghrelin, one can not obtain information on the separate proportions of the two major forms of ghrelin in the circulation. This procedure might face some criticism since to some respect the different forms of ghrelin can be regarded as different hormones. For example, acylated and des-acyl ghrelin might possess different or even opposite biological effects on insulin secretion, appetite or on adipogenesis as reviewed recently (Soares & Leite-Moreira 2008). It is therefore possible, that the ratio of acylated and des-acylated ghrelin might be a more important determinant for the biological effects of these molecules than their total amount together. In fact, a recent publication showed that subjects with constitutional thinness have a lower percentage of acylated over total ghrelin compared with control subjects and it was suggested that the ratio of these molecules might play a role in the determination of body thinness (Germain et al. 2008). However, it was the regulation of ghrelin concentrations rather than the biological effects of ghrelin that was the main interest of study III. Fasting plasma total ghrelin concentrations were chosen to be measured, since they have been shown to reflect the diurnal secretion profile of ghrelin. Hence fasting plasma total ghrelin was used as a marker for the overall ghrelin secretion efficiency in this study.

In aim of study IV was to determine, whether serum ghrelin concentrations might predict abnormalities in glucose metabolism. The total ghrelin was chosen to be measured, because it was the total ghrelin concentration which was
previously shown to associate with type 2 diabetes in a cross-sectional study (Pöykkö et al. 2003). It must be noted that in this study, measurement of both forms of ghrelin (acylated and des-acylated) separately would have provided more interesting data. Even though our study did not detect any association between baseline total ghrelin concentrations and the development of problems in glucose tolerance in the future, it would be worth repeating the prospective study, but with measurement of the acylated and des-acylated separately.

The measurement of total ghrelin in our studies can be justified by some other arguments. For example, the des-acyl ghrelin has been shown to be a more stable molecule than acylated ghrelin in several conditions (Hosoda et al. 2004, Rauh et al. 2007). Since the plasma samples were not acidified or treated with proteinase inhibitors in these studies and the samples had been exposed to freezing and thawing several times, it could be postulated that a substantial proportion of acylated ghrelin might have undergone hydrolysis to des-acyl ghrelin, and hence the relation between acyl v.s. des-acyl ghrelin molecules may well have been altered in the samples and thus the total ghrelin measurements might give a more reliable result. However, all the samples have undergone the same handling procedures and it can be assumed that the ghrelin molecules might have degraded at about same rate in all the samples. Nonetheless, there might be differences in the concentrations and activities of different proteinase enzymes between the samples, which might affect the degradation rate of ghrelin. The ghrelin concentrations obtained can be assumed to be comparable within the samples of this study, but caution must be used when comparing these results with other studies.

It is worth noting that measuring fasting total ghrelin concentration has its pitfalls. Ghrelin pulsatility is acutely dependent on the rhythm of food intake and fasting (Cummings et al. 2001). However, there is evidence, that ghrelin levels might retain their pulsatile rhythm even during fasting, hence the concentrations may start to decline even when nothing was eaten (Natalucci et al. 2005). Therefore it is possible that when blood samples are drawn in the morning, in some subjects the levels might have passed their peak value. In this study, the blood samples were drawn between 08–10am after an overnight fast of 10–12h.

6.2 Genetic association studies

Much effort has been expended during the past few decades on identifying the genes that contribute to the risk for obesity, type 2 diabetes and cardiovascular
diseases. The methods used have included several candidate gene approaches, mapping of satellite markers and linkage studies. There are now more advanced methods available, for example the genome-wide association studies (GWAS), which have greatly enhanced our understanding of the common and complex traits. There are GWAS chips commercially available which enable the detection of up to one million SNPs simultaneously. (Frayling 2007.) It must be remembered, that even though the genotyping methods have undergone these great advances such as GWAS, there is still a need for the more conservative types of genetic studies. Where GWAS try to find genes or genetic loci that associate with certain complex diseases, genetic association studies with single candidate genes provide a focused view on the complex physiological processes underlying certain complex traits, hence they complement the information obtained from GWAS. (Barsh et al. 2000.)

In the present thesis, genes coding for ghrelin and the ghrelin receptor were chosen as candidate genes. We tried to find new sequential variations which might associate with the GH/IGF-1 axis, ghrelin concentrations, BMI, and with certain metabolic risk factors for cardiovascular disease.

### 6.2.1 Ghrelin receptor variants

In this study, no GHS-R1a gene variants associating with IGF-1 concentrations were found. Nor were these variants associated with height, weight or BMI. These results indicate that the variants found in this study do not play a significant role in the regulation of IGF-1 levels, growth or determination of bodyweight. The results are consistent with the study of Wang et al. (2004), who initially found that there was a significant trend in SNP 171T>C (rs495225) variant to be more abundant among extremely obese children, but that this could not be confirmed in a larger study population within the same study (Wang et al. 2004). Accordingly, the SNPs within the exons of the RHS-R1a gene do not seem to be involved in weight regulation.

A recent study demonstrated that five SNPs in the gene coding for GHS-R, and the estimated haplotypes constructed from these five SNPs, were shown to associate with obesity. Three of the SNPs were in the 5’flanking area, one in the first exon and one in the intronic area (Baessler et al. 2005). In this thesis, however, the intronic and 5’flanking areas of the GHS-R1a gene were not studied.
6.2.2 Ghrelin gene variants and obesity

One of the aims of this thesis was to find new functional variants in the ghrelin gene 5’flanking area by sequencing DNA samples. The main finding was that the SNP -501A>C was shown to associate with BMI and waist circumference. The borderline significant association with this variant and BMI was maintained in the OPERA study population even after adjustments. Interestingly, this SNP was studied further recently in a large population of Central Europeans. There was a non-significant trend of this SNP -501A>C variant to be associated with higher BMI, i.e. results similar to that shown in the population examined in study II, but no significant associations were found (Hubacek et al. 2008). This suggests that the significance of this SNP with BMI or weight regulation is unlikely to be dramatic. However, it must be noted, that single genetic factors contributing to the susceptibility for weight gain are likely to have a very small effect – it is the combined effects of several factors that determine the overall risk. The Finnish population can be regarded as an isolate and it has special features in its genome. It is characterized by a strong founder effect and by the genomic heterogeneity (Kere 2001, Peltonen et al. 1999). These features enable also SNPs with only a minor effects to appear in genomic association studies, whereas the same SNP might be missed in other, more hetrogenic populations, such as the central European population.

Interestingly, more recent analyses of the human ghrelin 5’flanking region have suggested that the ghrelin gene might have more than four exons. It is claimed that one putative non-coding exon consisting of 20 bp is located between nucleotides -502 and -529 (Nakai et al. 2004). In addition, the most recent revision of the genomic structure of the ghrelin gene demonstrated that the ghrelin gene spans over 7200 bp and consists of six exons two of which are located upstream of the previously known first exon. However, the function of these exons is still unresolved (Seim et al. 2007). The newly discovered exons in the 5’flanking region of the ghrelin gene might encode for new, still unknown proteins or the mRNA transcripts might for example function as non-coding regulatory RNA for the production of ghrelin and obestatin. These findings place the variants found in this thesis in the ghrelin 5’flanking region into a different light. The variants described in study II in fact seem to be intronic variants, and the actual promoter areas might be several thousand base pairs further in upstream to those that were analysed in the present study.
6.3 Leu72Met variation in the ghrelin gene

The study with monozygotic twins showed that Leu72Met polymorphism was unequally distributed between the three sets of monozygotic twins. Leu72Leu wild type was the predominant form found in twins discordant for obesity, whereas the Met allele was present in obesity concordant and lean concordant twins at much a greater allelic frequency than in obesity discordant twins. This finding suggests that subjects with Leu allele are more vulnerable to react to environmental factors that promote up- or down-regulation of body weight. This might indicate that the ghrelin gene could be a variability gene for human obesity.

Leu72Met polymorphism of ghrelin gene has been associated with obesity or with BMI in several studies (Korbonits et al. 2002, Miraglia del Giudice et al. 2004, Ukkola et al. 2001, Ukkola et al. 2002). However, several other studies have failed to confirm these associations (Bing et al. 2005, Dossus et al. 2008, Hinney et al. 2002, Larsen et al. 2005, Mager et al. 2006). Ghrelin gene polymorphism Leu72Met has also been associated with the risk for type 2 diabetes (Mager et al. 2006) though in other studies, no such association was found (Larsen et al. 2005). Since the results concerning this SNP are conflicting, nothing conclusive can be said about its role in human physiology. However, these conflicting results may indicate that the Leu72Met polymorphism of this gene does not have a causative role in the regulation of human body adiposity (and glucose metabolism). Instead, they support the proposal made in the study II of this thesis that this SNP might contribute to the variability for the body weight determination as an individual is exposed to different environmental factors. The suggestion is supported by a recent observation in a study addressing gene-diet interactions. Subjects carrying Leu72Leu alleles were affected by fat intake more than other genotypes, and these individuals displayed a more significant association of dietary fat intake with waist circumference and triacylglycerol concentrations (Robitaille et al. 2007). These findings indicate that the ghrelin gene might be a variability gene for obesity. However, the molecular mechanism by which the Leu72Met mutation might affect the susceptibility to react to environmental pressures to gain or lose weight is impossible to define in the light of these results.
6.4 Regulation of ghrelin concentrations

It is a well known phenomenon that fasting plasma ghrelin concentrations are decreased in human obesity. However, the factors that regulate ghrelin levels are not known in detail. It has been suggested that also genetic factors might play a role in the regulation (Ravussin et al. 2001). Fasting total ghrelin concentrations were determined to examine the possible role of genes in the regulation of ghrelin levels in a sample of monozygotic twin pairs discordant for obesity. The rationale is that since MZ twins share identical genes by heredity and yet, some twin pairs exhibit considerable differences in BMI and other obesity markers, would one observe plasma total ghrelin differences between lean and obese co-twins or would a genetic regulation of the hormone level prevail? One of the main findings of this study was that the intrapair difference in total ghrelin concentrations among the obesity discordant twin pairs was significant, indicating that the genetic factors are unlikely to play a major role in the regulation of fasting plasma ghrelin concentrations. The ghrelin levels in this thesis were affected by acquired obesity independent of the genetic background. The obese co-twins had lower ghrelin levels than their lean co-twins. This result is contrary to a recent Hispanic family study that showed rather high heritability ($h^2$) values for the ghrelin concentration ($h^2 = 0.67$) in children (Fisher et al. 2007). Interestingly, heritability for the regulation of ghrelin concentrations has been also detected in other species than in humans. In baboons, there was high heritability for the ghrelin concentration ($h^2 = 0.25$) (Voruganti et al. 2008). It is difficult to explain the discrepancy, but one possibility is that genes might play a different role in the regulation of ghrelin levels during childhood compared with later adult life.

Interestingly, the intrapair difference in ghrelin levels between obese and lean co-twin was seen more clearly among female than male discordant pairs. This might be explained by the larger intrapair difference in BMI, percentage of body fat and ASF among female pairs. However, the finding could also be interpreted to mean that there might be sexual dimorphism in the regulation of ghrelin levels. In addition, in study IV, the ghrelin concentrations of women were 1.2 times higher compared with men and the difference remained significant even after adjustment for BMI (data not shown). Sexual dimorphism in ghrelin levels has been observed in some but not all earlier studies with women having higher ghrelin levels than men (Barkan et al. 2003, Makovey et al. 2007, Pöykkö et al. 2003). Sexual dimorphism has been observed also with other obesity-related hormones, such as leptin (Saad et al. 1997). Sex hormones might affect the
secretion of ghrelin, as indicated in by some studies (Grinspoon et al. 2004, Kellokoski et al. 2005). However, it is not known whether ghrelin concentrations vary in women during the menstrual cycle. This kind of oscillation might, however, contribute to the discrepancy and explain why sexual dimorphism in ghrelin levels is observed in some, but not all, studies.

When the intrapair differences of ghrelin levels in males are examined in detail, there are only two twin pairs in which the obese co-twin displayed a low and the lean twin had a high ghrelin concentration. In five out of nine male twin pairs, there was virtually no intrapair difference (less than 50 pg/ml) in ghrelin levels between the co-twins, indicating that in these pairs the ghrelin concentration was not regulated by acquired obesity. There are also 3 pairs in which the obese co-twin had a higher ghrelin level than his lean co-twin. However, due to the rather small size of the population, one can only speculate about the possible sex-dependent differences in the determination of ghrelin concentrations.

The possible role of genetic factor in the regulation of ghrelin concentrations was also investigated in study II. The putative regulatory areas of the ghrelin gene 5´flanking area were screened to detect variations that might contribute to the regulation of ghrelin levels. Eleven variants were found but none of these were associated with ghrelin levels. These results together with the result from study III provide evidence that ghrelin concentrations are regulated by adiposity of the body and genetic factors might not play a crucial role in the determination of the circulating levels of the peptide.

In the fourth study, also the regulation of ghrelin levels over the long term was examined, even though this was not the main aim of this study. The results indicated that the average change in the fasting ghrelin concentrations exhibited a slight decrease over time, and that showed a borderline significant correlation with the changes occurring in the BMI. This finding is consistent with several previous studies which have shown that weight loss, induced either by caloric-restriction (Cummings et al. 2002, Hansen et al. 2002) or by increased amount of exercise (Foster-Schubert et al. 2005), increases and conversely weight gain decreases (Otto et al. 2001, Robertson et al. 2004) plasma ghrelin levels. These results all support the concept that adiposity of the body might be one of the most important determinants of fasting ghrelin concentrations. However, the mechanism behind this phenomenon remains to be elucidated.

Even though the SNP -501A>C as well as the other found variants in the ghrelin gene 5´flanking area were not associated with fasting plasma ghrelin
concentrations, these findings do not necessarily mean that these SNPs have no effect on ghrelin gene expression. The rate of gene expression might affect the plasma concentrations to some extent, but the plasma concentration of any hormone at one time point is the result of gene expression, combined with the pharmacokinetics of the hormone, i.e. its storage, release and clearance. In fact, it has been recently shown that the SNP -501A>C was associated with ghrelin expression in human peripheral blood mononuclear cells, with the -501C/C genotype having the lowest and -501A/A genotype having the highest ghrelin mRNA expression (Mager et al. 2008). The effect of this variant on the expression of ghrelin gene should be studied also in the ghrelin producing cells of the gastro-intestinal tract. Furthermore, even though this SNP did not affect the fasting ghrelin concentrations, it would be interesting to measure 24h plasma profiles of ghrelin in subjects with this variant.

One additional point to be mentioned is that in study IV it was shown that the change in ghrelin levels during the follow-up correlated negatively with the change in BMI. This supports the concept from study III that acquired obesity is one of the most important factors regulating ghrelin plasma concentrations.

6.5 Ghrelin and type 2 diabetes

The main finding in study IV was that fasting serum total ghrelin levels at baseline did not differ between those who maintained and those who failed to maintain normal glucose tolerance. This is an interesting finding since it has been previously suggested that low circulating ghrelin levels might be associated with type 2 diabetes (Pöykkö et al. 2003) and in addition, several hypothetical links between low ghrelin and the pathogenesis of type 2 diabetes can be drawn from cross-sectional human studies, as well as animal and cell culture experiments. However, the finding of study IV might suggest that it is unlikely that there is a causal relationship between low ghrelin levels and the development of disturbances in glucose metabolism.

The result of study IV is compatible with the data reported by others recently. In one prospective study involving middle-aged Afro-Jamaicans who were normoglycaemic at baseline, the plasma ghrelin concentrations associated with impaired glucose tolerance but when the model was adjusted for adiponectin and insulin resistance, the association was lost. In addition, ghrelin concentrations at baseline did not associate with the incidence of type 2 diabetes. (Bennett et al. 2008) These are the first two prospective studies investigating whether ghrelin
has any predictive value for the incidence of impaired glucose tolerance and type 2 diabetes. They both show a negative result and indicate that there is no causal relationship between ghrelin and type 2 diabetes.

However, it must be noted, that the population sizes in both of these studies were rather small (n = 201 and 393 respectively) and follow-up times were quite short (5.1 and 4.1 years). Therefore it is difficult to draw any clear conclusions. In addition, in study IV of this thesis, the small number of individuals who developed type 2 diabetes did not allow comparison of ghrelin concentrations between the newly diagnosed diabetics as a single group and those without the diagnosis. In other words, the study IV does not give an absolute answer, whether low ghrelin concentrations might predict the incidence of type 2 diabetes. On the other hand, study IV did provide considerable evidence that the baseline ghrelin concentrations might not associate with the incidence of prediabetic conditions IFG and IGR and with type 2 diabetes. In addition, there were no correlations between the baseline ghrelin concentrations and the changes in blood glucose levels over time. Furthermore, the study had adequate power to detect even minor differences (±0.5SD) in ghrelin concentrations between those who maintained NGT and who encountered problems in glucose homeostasis. The results indicate that ghrelin might not be a strong predictor for the incidence of abnormalities in glucose tolerance.

Circulating ghrelin has been shown to have several interesting biological activities which might link it theoretically to the pathogenesis of type 2 diabetes. Ghrelin interacts with several hormones that are involved in the regulation of glucose homeostasis. For example, its strong association with GH secretion represents one important pathway. It could be speculated that low blood ghrelin levels might affect growth hormone (GH) / insulin-like growth factor -1 (IGF-1) - axis which in turn might increase insulin resistance and lead eventually to the development of type 2 diabetes. Low levels of GH and IGF-1 have been shown to associate with several components of the metabolic syndrome (Maison et al. 2007), a cluster of metabolic disturbances that increases the risk for type 2 diabetes. The levels of both GH and IGF-1 have been shown to be low in human obesity and it has been suggested that low IGF-1 levels might predict the development of glucose intolerance (Sandhu et al. 2002). It must be noted that even though GH and ghrelin both are negatively correlated with BMI, no causative link between ghrelin and hyposomatotropism of obesity was observed previously (Lindeman et al. 2002). Therefore the theoretical link between low
ghrelin levels, hyposomatotropism and the pathogenesis of type 2 diabetes remains highly speculative.

Another possible mechanism by which low ghrelin levels could be involved in the pathogenesis of insulin resistance and type 2 diabetes might be related to the effects of ghrelin on insulin release from the pancreatic islet cells. Treatment with ghrelin evoked a decrease in insulin secretion in humans (Broglio et al. 2001). It can be proposed that ghrelin might have an inhibitory effect on insulin secretion. This is supported by the findings from several cell culture studies of rodent pancreatic islet cells, where ghrelin has been shown to affect insulin secretion (Qader et al. 2008, Salehi et al. 2004). However, little is known about whether ghrelin can induce or reduce insulin secretion under physiological conditions. Nonetheless, it could be speculated that if the proposed inhibitory effect of ghrelin on insulin release also occurs in humans with endogenous ghrelin, then low blood ghrelin concentrations might lead to diminished suppression of insulin secretion. Thus insulin secretion from pancreas might then be excessive and lead to hyperinsulinemia, which is a common feature in insulin resistance, and often encountered in the early stages of type 2 diabetes.

The third possible link between low ghrelin, insulin resistance and type 2 diabetes might be the autonomic nervous system. It has been shown that a higher ratio of sympathetic v.s. parasympathetic activity is strongly associated with the amount of visceral adipose tissue and insulin resistance in humans. It has been suggested, that a disequilibrium in the sympathetic / parasympathetic balance might promote the development of insulin resistance and type 2 diabetes (Lindmark et al. 2005). On the other hand, the autonomic nervous system also has been shown to be a factor regulating ghrelin concentrations (Hosoda & Kangawa 2008). Therefore it can be hypothesized, that dysregulation of the autonomic nervous system might evoke the decrease in ghrelin levels as well as being involved in promoting the development of disturbances in glucose metabolism. Hence, this hypothesis suggests that there is no causal relationship between ghrelin level and type 2 diabetes and instead both could be a consequence of the dysregulation of the autonomic nervous system.

In summary, despite all the expectations derived from the early cross-sectional associations between ghrelin and type 2 diabetes and the hypothetical physiological links between ghrelin and type 2 diabetes, this thesis study detected no value for low ghrelin concentrations in the prediction of impaired glucose regulation and type 2 diabetes. This thesis suggests that it seems unlikely that
ghrelin concentrations play any major role in the pathogenesis of impaired glucose regulation or type 2 diabetes.

### 6.6 Aspects for future studies

Ghrelin is a pleiotropic hormone that could be involved in appetite control and energy metabolism. High hopes on studies evaluating ghrelin and its receptor GHS-R1a have been raised during the recent years, in the hope that they will reveal new insights into the pathogenesis of obesity and provide a new target for drugs attempting to restore dysregulation of body weight.

In this study, ghrelin and its receptor were examined at both the genetic and the protein level. The regulation of ghrelin levels and potential association with type 2 diabetes was studied. The results suggest that genetic factors have only a minor if any, effect on the regulation of plasma ghrelin concentration in comparison to the impact of acquired obesity on these levels. The mechanisms regulating ghrelin levels need to be further studied. The preprandial up-regulation of ghrelin levels is not understood in detail. In addition, Little is known about the exact mechanism linking increased adiposity with low ghrelin levels that do not show significant pulsatility. It would be interesting to study in more detail the role of the autonomic nervous system in regulating ghrelin levels. Cell culture and animal studies, as well as studies with larger human populations are needed to address these issues.

In this present study, it was shown that low ghrelin levels do not seem to possess any predictive value with respect to the incidence of impaired glucose regulation and type 2 diabetes. Studies with larger study populations and longer follow-up times are needed to evaluate this potential association in greater detail. In addition, measuring acylated and des-acyl ghrelin separately in a similar study design might provide further information on the role of this multifunctional gut-derived hormone in the regulation of glucose homeostasis.
7 Conclusions

The conclusions to the specific study aims are as follows:

1. Five SNPs in the first exon of the ghrelin receptor gene were found but none of these were associated with IGF-1 plasma concentrations. One of the SNPs, 171C>T associated with an insulin metabolism related parameter, AUCIN as well as with the level of insulin-like growth factor binding protein, but not with any other insulin or glucose metabolism related parameters. Another SNP, 477G>A was associated also with AUCIN as well as with plasma concentrations of HDL-cholesterol and VLDL-cholesterol. These results show that variants in GHS-R1a gene are not associated with IGF-1 levels but might be involved in the insulin/IGFBP-1 physiology.

2. Eleven SNPs in the 5’flanking area of the ghrelin gene were observed, five of which were new variants. None of these variants associated with fasting ghrelin plasma concentrations. One of these variants, -501A>C, was associated with BMI and waist circumference, when the variant homozygotes were compared with A-carriers (-501 A/A and -501A/C genotypes). The variants in the putative regulatory areas of the ghrelin gene do not seem to play an important role in the regulation of ghrelin concentrations, but might be associated with obesity.

3. Obese co-twins had lower ghrelin concentrations than their lean co-twins. Fasting plasma concentrations of ghrelin appear to be regulated by acquired obesity rather than determined by the genetic background. In addition, the ghrelin gene variant that causes the Leu72Met amino acid change was unequally distributed among three sets of monozygotic twins. The frequency of 72Met allele was significantly lower among twins discordant for obesity compared with controls. The ghrelin gene might be a variability gene for obesity.

4. The baseline ghrelin levels did not differ between those who developed and those not developing impaired glucose regulation or type 2 diabetes during the follow-up. Low levels of ghrelin do not seem to be associated with any increased risk for impaired glucose regulation and type 2 diabetes.
Fig. 12. A schematic summary of the associations of circulating ghrelin, ghrelin receptor GHS-R1a and the SNPs of the ghrelin receptor gene (GHSR) and ghrelin gene (GHRL) with atherosclerosis and its known risk factors. The arrows represent the associations. The results of this thesis study are shown inside the stars; minus means that no association was observed and plus means that an association was detected. In this study, associations of GHSR SNPs with insulin and lipid metabolism related parameters and between GHRL SNPs and obesity were found. In addition, fasting ghrelin plasma concentrations were shown to be regulated more by obesity than by genes, providing further evidence for the known strong inverse associations of ghrelin and the measures of obesity. Interestingly, no association of ghrelin plasma concentrations with impaired glucose regulation (IGR) or type 2 diabetes was observed.
References


107


Original articles


The original articles were reproduced with the permission of the publishers BioScientifica (I) and Elsevier (II and III) that hold the copyrights of the articles.

The original articles are not included in the electronic version of the dissertation.
994. Tuomisto, Anne (2008) The role of collagen XIII in B-cell lymphoma development, and characterization of its biosynthesis and tissue distribution
996. Erkko, Hannele (2008) TOPBP1, CLSPN and PALB2 genes in familial breast cancer susceptibility
1000. Lajunen, Taina (2008) Persistent Chlamydia pneumoniae infection, inflammation and innate immunity
1005. Inatssiniemi, Sari (2009) Fall accidents and exercise among a very old home-dwelling population
1006. Westerlund, Tarja (2009) Thermal, circulatory, and neuromuscular responses to whole-body cryotherapy
1008. Kuisma, Mari (2009) Magnetic resonance imaging of lumbar degenerative bone marrow (Modic) changes. Determinants, natural course and association with low back pain
Johanna Vartiainen

GHRELIN, OBESITY AND TYPE 2 DIABETES

GENETIC, METABOLIC AND EPIDEMIOLOGICAL STUDIES