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URSULA MAGER

The Role of Ghrelin in Obesity and Insulin Resistance

Doctoral dissertation

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Abstract

Obesity has become a global public health problem and is a major risk factor for type 2 diabetes. Ghrelin is an appetite-stimulating hormone which acts through its receptor on the hypothalamus to regulate energy balance and thus plays a major role in the aetiology of metabolic diseases.

The aims of the studies conducted were 1) to investigate possible associations between single nucleotide polymorphisms (SNPs) in the ghrelin (*GHRL*) and ghrelin receptor (*GHSR*) genes and obesity, type 2 diabetes or related phenotypes in persons with impaired glucose tolerance participating in the Finnish Diabetes Prevention Study, 2) to study the role of circulating ghrelin concentrations in the context of metabolic disturbances and 3) to evaluate the possibility of ghrelin gene expression in peripheral blood mononuclear cells (PBMCs) for use as a surrogate marker for ghrelin metabolism.

SNPs were genotyped using either a restriction fragment length polymorphism technique or TaqMan allelic discrimination assays. The Leu72Met SNP in *GHRL* was associated with type 2 diabetes. A common genotype combination of *GHRL* SNPs was associated with low blood pressure levels and lower risk of hypertension. The rs490683 SNP in the 5'-region of *GHSR* was associated with weight loss and disrupts a putative binding site for the transcription factor nuclear factor-1. Further gelshift experiments conducted with nuclear extract from rat hypothalamus showed higher protein binding to the minor G allele of the rs490683 SNP *in vitro*, which could lead to increased GHSR expression and thus increased food intake in persons with the risk allele. Ghrelin plasma concentrations were not restored by weight loss in persons with the metabolic syndrome; instead, those in the control group showed a further decrease in ghrelin levels. *GHRL* and *GHSR* were expressed in PBMCs but were not altered upon metabolic changes or associated with certain phenotypes. Thus, ghrelin expression in PBMCs seems unsuitable as a surrogate marker for ghrelin metabolism during lifestyle changes. Nevertheless, ghrelin expression correlated with the expression of pro-inflammatory cytokines, suggesting that ghrelin may play a role as an autocrine factor in the immune system.

In conclusion, SNPs in the *GHRL* gene may indicate a risk of hypertension, one of the features of metabolic syndrome; however, variations in *GHRL* seem not to be strong risk factors for type 2 diabetes or obesity. Genetic factors leading to increased GHSR expression and thus potentially to increased ghrelin signalling might ultimately lead to an increase in appetite and weight gain.

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Kuopio, April 2008

Ursula Mager

Abbreviations

αMSH	α melanocyte-stimulating hormone
ACTH	adrenocorticotrophin
AgRP	agouti-related protein
AIR	acute phase insulin response
AMPK	adenosine monophosphate-induced protein kinase
ANOVA	univariate analysis of variance
AP2	activator protein-2
ARC	arcuate nucleus
AU	arbitrary units
AUC	area under the curve
BBB	blood-brain barrier
bHLH	basic helix-loop-helix
BMI	body mass index
bp	base pair
BP	blood pressure
CAD	coronary artery disease
CNS	central nervous system
DPS	Finnish Diabetes Prevention Study
ELISA	enzyme-linked immunosorbent assay
FSIGT	frequently sampled intravenous glucose tolerance test
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GH	growth hormone
GHRH	growth hormone releasing hormone
GHRL	ghrelin gene
GHS	growth hormone secretagogue
GHSR	growth hormone secretagogue receptor
GHSR	growth hormone secretagogue receptor gene
GPR39	G protein-coupled receptor 39
HOMA-IR	homeostasis model assessment for insulin resistance
IFG	impaired fasting glucose
IGF-1	insulin-like growth factor 1
IGFBP-1	insulin-like growth factor binding protein 1
IGT	impaired glucose tolerance
IL	interleukin
kb	kilo base pair
LD	linkage disequilibrium
LHA	lateral hypothalamic area
MAPK	mitogen-activated protein kinase
MC4R	melanocortin-4 receptor
MetS	metabolic syndrome
NCBI	National Centre for Biotechnology Information
NF-1	nuclear factor-1
NPY	neuropeptide Y
OGTT	oral glucose tolerance test
PBS	phosphate buffered saline
PBMCs	peripheral blood mononuclear cells
PCOS	polycystic ovary syndrome
PCR	polymerase chain reaction
PI3K	phosphatidylinositol 3 kinase
POMC	proopiomelanocortin
PVN	paraventricular nucleus of the hypothalamus
RFLP	restriction fragment length polymorphism
RIA	radioimmunoassay
RQ	respiratory quotient
RYGBP	Roux-en-Y gastric bypass
S_G	glucose effectiveness
S_I	insulin sensitivity
SNP	single nucleotide polymorphism
SD	standard deviation
SEM	standard error of mean
T2D	type 2 diabetes mellitus
TF	transcription factor
TG	triglycerides
TNF-α	tumour necrosis factor α
WHO	World Health Organisation
95% CI	95% confidence interval

List of original publications

This dissertation is based on the following publications, referred to in the text by their Roman numerals (I-IV):

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II. Mager U., Kolehmainen M., Lindström J., Eriksson J. G., Valle T. T., Hämäläinen H., Ilanne-Parikka P., Keinänen-Kiukaanniemi S., Tuomilehto J. O., Pulkkinen L. and Uusitupa M. I. for the Finnish Diabetes Prevention Study Group: Association between ghrelin gene variations and blood pressure in subjects with impaired glucose tolerance. Am J Hypertens 2006; 19(9): 920-926.

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In addition, the thesis presents some unpublished data.

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1 Introduction

Obesity has emerged as a pre-eminent public health problem. The prevalence and incidence of overweight and obesity are steadily rising worldwide. The so-called “obesity epidemic” gives rise to a multitude of metabolic disorders, most notably type 2 diabetes, for which obesity is a strong risk factor. Obesity, and especially abdominal obesity, is believed to be the core of the metabolic syndrome, composed of a range of metabolic abnormalities, including insulin resistance, dyslipidaemia, hypertension, impaired glucose tolerance and type 2 diabetes.

Obesity is a complex disease caused by the interplay between environmental and genetic factors. Generally speaking, overweight and obesity are caused by an imbalance in energy input and energy expenditure, with the major environmental factors involved in obesity being dietary and physical activity habits, which act in conjunction with predetermined genetic parameters. Obesity is a multifactorial disease, the onset of which is influenced by susceptibility genes interacting with each other and influencing environmental factors through determinants such as energy expenditure, fuel metabolism, muscle fibre function and appetite or food preferences. Body weight is regulated by long-term and short-term energy balance signals. Energy homeostasis is controlled by peripheral signals from adipose tissue, the pancreas and the gastrointestinal tract. These signals influence circuits in the hypothalamus and brain stem to produce positive and negative effects on the energy balance.

One of the gut-derived peptides involved in the regulation of energy balance is ghrelin. Ghrelin was originally discovered as a natural growth hormone secretagogue. Subsequently, it was noted that ghrelin acts as an appetite stimulator and a meal initiator and was therefore called the first “hunger hormone”. This peptide is produced by the stomach and circulates in the blood stream. Ghrelin acts in the hypothalamic arcuate nucleus, the centre of weight control in the brain, through its receptor to enhance expression of potent appetite stimulants.

In the present work, the role of ghrelin in obesity and insulin resistance was investigated 1) by studying genetic variations of ghrelin and its receptor in association with obesity and type 2 diabetes, 2) by examining the expression of ghrelin and ghrelin receptors in peripheral blood mononuclear cells and subcutaneous adipose tissue and 3) by investigating the possible impact of lifestyle modification on ghrelin expression and circulating levels of ghrelin.

2 Review of literature

2.1 Ghrelin

In December 1999, ghrelin was first reported as an endogenous ligand for the former orphan receptor growth hormone secretagogue receptor (GHSR) 1a [1]. Until then, only synthetic growth hormone secretagogues (GHS), compounds that are potent stimulators of growth hormone (GH) release, were known to stimulate the GHSR. Ghrelin was purified from the rat stomach, and the ghrelins in rats and humans were found to differ in only two amino acid residues [1, 2]. Ghrelin exists in several mammalian, avian and fish species [2]. The amino acid sequences of mammalian ghrelins are well conserved, especially the NH_2-terminal region with its unique fatty acid modification, indicating that this region is of central importance for the activity of the peptide [2]. Ghrelin shows high homology to motilin, in a manner similar to the high homology shown by GHSR to the motilin receptor [2], suggesting that the ghrelin and motilin systems may have evolved from a common ancestral system. A few months after ghrelin's discovery by Kojima *et al.* [1], Tomasetto *et al.* [3] reported the identification of a gastric peptide named motilin-related peptide that has an amino acid sequence identical to that of ghrelin.

2.1.1 Gene and precursor structures of ghrelin

Figure 1 describes the processing of the human ghrelin gene (*GHRL*) into the active ghrelin peptide. The human *GHRL* gene is located at chromosome 3p25-26 and consists of five exons and four introns [4]. The short first exon contains only 20 bp and is present in the noncoding region of the human *GHRL* [4]. There are two different transcriptional initiation sites, resulting in two distinct mRNA transcripts, of which one is the main form of human ghrelin mRNA *in vivo* [4]. The mRNA is translated into a precursor peptide (prepro-ghrelin), with protease cleavage and acyl-modification of the precursor resulting in a mature ghrelin peptide, a 28-amino acid peptide esterified by an octanoyl group ($C_7H_{15}CO$) at the serine-3 (Ser3) residue [1]. Acylation is necessary for ghrelin to bind to its receptor GHSR 1a [5], GH-releasing activity *in vivo,* [6] as well as other central and peripheral endocrine and non-endocrine actions [7, 8] of the peptide. The modification also determines the extent and the direction of ghrelin transport across the blood-brain barrier (BBB) [9]. The enzyme which attaches the octanoyl group to Ser3 of ghrelin has been recently identified and named GOAT (Ghrelin O-Acyltransferase) [10].

2.1.2 Regulation of *GHRL* transcription

The 5'-flanking region of the human *GHRL* contains a TATA box-like sequence, though reports on its functionality are controversial [4, 11, 12]. Putative binding sites for several

transcription factors (TF), such as activator protein-2 (AP2), basic helix-loop-helix (bHLH), PEA-3, Myb, NF-IL6, hepatocyte nuclear factor-5, NF-κB, and several E-box consensus sequences, and half-sites for oestrogen and glucocorticoid response elements have been found [4, 11, 12]. Studies of ghrelin promoter activity in different cell lines recognized several different activating sequences within 2000 bp upstream of the translation initiation site, suggesting a cell-type-specific activity for the human ghrelin promoter [4, 12, 13]. Recently, one single nucleotide polymorphism (SNP) 1062 bp upstream of the translation initiation site has been reported to disrupt the binding site for the TF MyoD, a bHLH TF, with this sequence variation resulting in lower promoter activity in the luciferase reporter gene assay [14].

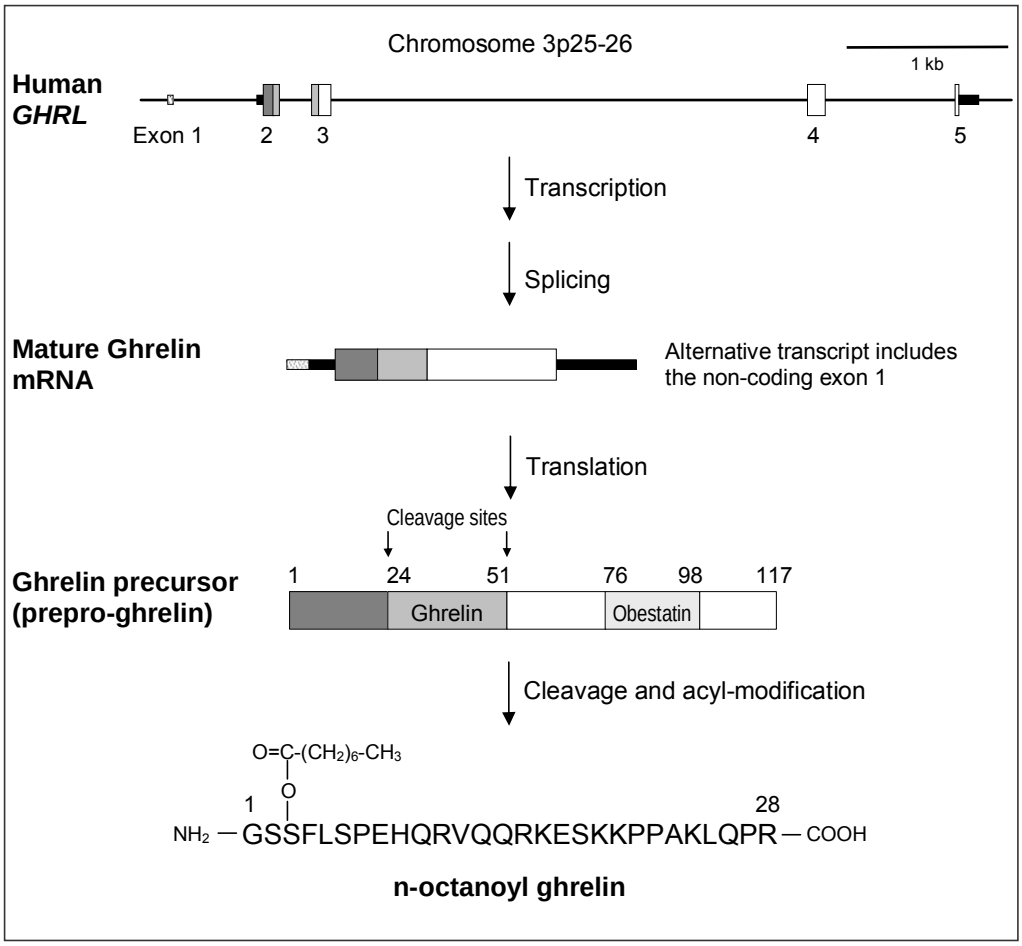

Figure 1. Conversion from the human *GHRL* gene into an active peptide (modified from Kojima and Kangawa 2005 [2]).
▫, non-coding exon 1; ▬, untranslated region; ▮, signal peptide; ▯, ghrelin; □, cleaved from mature form

2.1.3 Structural variants of ghrelin

2.1.3.1 Des-acyl ghrelin

Ghrelin has a unique fatty acid chain on its N-terminal end. However, the majority of circulating ghrelin actually lacks this acyl group and is known as des-acyl or des-octanoyl ghrelin (non-acylated or unacylated ghrelin). In the blood stream, acylated ghrelin is present at a 2.5 fold lower concentration than des-acyl ghrelin [15]. Des-acyl ghrelin was initially assumed to be an inactive peptide, since it had been shown to lack the ability to bind to the GHSR 1a [16] and showed no GH-releasing activity *in vivo* in rats or humans [1, 2, 17]. However, recent research shows that des-acyl ghrelin has physiological effects that are mediated by unknown receptors other than GHSR 1a and thus des-acyl ghrelin may be considered a metabolically active peptide affecting glucose and lipid metabolism and possibly food intake and appears to interact with acylated ghrelin in the control of metabolism, thus having non-endocrine activities [2, 18, 19].

2.1.3.2 Des-Gln14-ghrelin

Naturally occurring variants of ghrelin include an alternative splice variant des-Gln14-ghrelin, which lacks Gln at residue 14 [20]. Des-Gln14-ghrelin has been purified from rat and mouse stomach and was found in circulation with the same biological activities as ghrelin [20, 21], but whether it exists in humans as well is unclear [2]. In rodent experiments ghrelin and des-Gln14-ghrelin have been shown to stimulate gastric emptying in a dose-dependent manner while des-acyl ghrelin and motilin lack this effect. The C-terminally truncated ghrelin fragments (ghrelin-18, -10 and -5) are effective but much less potent than ghrelin itself. Ghrelin, des-Gln14-ghrelin and des-acyl ghrelin neither stimulated nor inhibited gastric acid secretion [22].

2.1.3.3 Other variants

In humans, multiple ghrelin-derived molecules differing in their acyl groups on Ser3 (decanoyl (C10:0) or decenoyl-esterified (C10:1) molecules) have also been isolated from the stomach and circulation [23]. These acyl-modified ghrelins are capable of stimulating GH release in rats to a degree similar to that of octanoylated ghrelin [23], and peptides with 27 amino acids were found that lack the C-terminal Arg28 [23].

2.1.3.4 Obestatin

It was recently discovered that another putative peptide hormone derives from the same pro-protein as ghrelin, obestatin. The 23-amino-acid polypeptide obestatin was initially reported to be the endogenous ligand for the orphan receptor G protein-coupled receptor 39 (GPR39) and to act as an appetite suppressant [24-26]. However, recent reports indicate that obestatin is unlikely to be the endogenous ligand for GPR39 [27-30].

2.2 Tissue distribution of ghrelin

Ghrelin is expressed primarily by the stomach and secondarily by the lower gastrointestinal tract (small and large intestine). Ghrelin expression levels in other organs are relatively low in comparison, and it might have physiological relevance as a paracrine or autocrine factor in these tissues. An endocrine role for extra-gastrointestinal ghrelin appears to be unlikely [18].

2.2.1 Stomach and gastrointestinal organs

Ghrelin was originally isolated from the stomach [1]. There are a number of different types of endocrine cells in the stomach, of which ghrelin-containing cells form a distinct cell type found in the mucosal layer of the stomach [31, 32]. Ghrelin has been localized in the oxyntic glands, specifically in the X/A-like cells of the gastrointestinal tract. These ghrelin-producing cells account for about 20% of the endocrine cell population in the human oxyntic mucosa [31]. Ghrelin cells develop during early foetal life [32] and are characterised by round, compact, electrondense granules that are filled with ghrelin [2, 31, 32]. Ghrelin cells are also found in the duodenum, jejunum, ileum, and colon [33], with the ghrelin concentration gradually decreasing from the duodenum to the colon [8].

Circulating ghrelin levels mostly reflect gastric secretion; in fact, they are reduced by 70% after gastrectomy [34]. After total gastrectomy, ghrelin levels gradually increase again, suggesting that the stomach is the major source of circulating ghrelin, though other tissues may compensate for the loss of ghrelin production after gastrectomy [35].

2.2.2 Endocrine pancreas

Expression of ghrelin in the human pancreatic islet remains somewhat controversial because it has variably been reported to be present in the α cells [36] or β cells [37], and it has been suggested that ghrelin cells may constitute a new islet cell type [38, 39]. Ghrelin is expressed in foetal, neonatal, and adult human pancreatic islets and is upregulated during development [32, 38]. It is anticipated that ghrelin may play a role in modifying pancreatic β cell function. The exact function of ghrelin within the islets is still unknown, but it may have a paracrine role in regulating insulin secretion or glucose metabolism.

2.2.3 Brain

Ghrelin has been detected in the hypothalamic arcuate nucleus (ARC) of the rat brain [1, 40-42]. In addition, it has been reported that ghrelin is present in hypothalamic neurons adjacent to the third ventricle between the dorsal, ventral, paraventricular and arcuate hypothalamic nuclei [43]. In humans, ghrelin expression was found in six different brain regions, namely frontal cortex, temporal cortex, visual cortex, pons, medulla, and hypothalamus [44]. In

addition, ghrelin expression in the hypothalamus was shown to be higher in obese than in lean persons [45].

2.2.4 Other tissues

Ghrelin mRNA has been detected very widely at low levels. Ghrelin mRNA and peptide are synthesized locally within the anterior pituitary gland [46-48]. In human placenta, ghrelin appears to be mainly expressed in the first half of pregnancy, whereas it could not be detected at term [49]. Human ghrelin mRNA expression was shown in T-lymphocytes, B-lymphocytes, and neutrophils from venous blood of healthy volunteers [48, 50, 51] as well as in subcutaneous and visceral adipose tissue with large intraindividual variations [52]. Ghrelin was also detected in human ovaries and testis [48, 53-55]. Furthermore, ghrelin is present in a number of different tumours including neuroendocrine tumours, pituitary adenomas, endocrine tumours of the pancreas, breast tumours and thyroid and medullary thyroid carcinomas [56].

2.3 Regulation of ghrelin plasma concentration

Numerous studies have been conducted on the possible factors influencing plasma ghrelin concentrations. The following chapter reviews the most important and most discussed factors. Most studies have investigated plasma total ghrelin concentrations (acylated and des-acyl ghrelin together). However, when acylated and/or des-acyl ghrelin levels have been measured separately, this will be pointed out. Table 1 summarises some of the factors influencing total and acylated ghrelin plasma concentrations.

Table 1. Factors affecting total and acylated ghrelin concentrations.

Factors	Total ghrelin	Acylated ghrelin
Dietary components:		
Carbohydrates	↓ [57-64] ↔ [65]	↓ [61-65]
Fat	↓ [57, 58, 64, 66] ↑ [60]	↓ [62, 64] ↔ [61, 67]
Protein	↓ [63, 64, 68] ↔ [66] ↑ [58, 60]	↓ [62-64, 67]
Glucose	↓ [69-72]	-
Insulin	↓ [73, 74]	-
Leptin	negative correlation [75-78] no correlation [79-82] ↔ [78]	-
Exercise	↔ [83-92] ↑ [86, 93]	↑ [90]

↓, decrease; ↔, no change; ↑, increase; -, no studies available

2.3.1 Fasting and food intake

Circulating plasma ghrelin levels show a diurnal pattern with preprandial increases and postprandial decreases, resulting in a characteristic secretion pattern [71, 94]. Moreover, acylated ghrelin falls after ingestion of a balanced meal [67, 95]. Interestingly, even in fasting subjects, a characteristic diurnal course with spontaneous rises and declines at customary mealtimes occurs when food is anticipated, regardless of whether it is actually later received, nor has a marked change been found in insulin or glucose levels [96, 97]. This may suggest that preprandial ghrelin secretion may represent some type of conditioned physiological reflex [96]. However, it has been observed that prolonged fasting in healthy subjects results in an overall decrease in ghrelin levels [96-98].

The volume of the ingested meals is rather unlikely to have an effect on plasma ghrelin levels, as it has been shown that gastric distension (by water or guar solution) has no effect on plasma ghrelin levels [58, 71, 99]. Instead, the extent of the ingested caloric load seems to determine the postprandial suppression of plasma ghrelin [100]. Moreover, cephalic vagal activation modified by sham feeding (chew and spit) do not contribute to the postprandial alterations of plasma ghrelin levels [58].

The effects of individual macronutrients on postprandial ghrelin concentration have been intensively studied, though these studies are not always consistent. Total and acylated ghrelin levels decrease after carbohydrate-rich test meals [57-65]. The initial decrease is followed by a subsequent increase back to the basal levels in the late postprandial and interdigestive period [58, 61, 64]. Only one report has failed to show a decrease in total ghrelin after consumption of a carbohydrate-rich liquid meal [65]. Given this rise in ghrelin plasma levels before meal intake and a fall thereafter, it has been proposed that this pattern might contribute to early postprandial satiety. The early postprandial reduction in circulating ghrelin would attenuate the hormonal gastric feeding drive, thereby supporting neurally activated satiety signals. The progressively increasing ghrelin levels during the later postprandial and interdigestive phase back to baseline could then contribute to the recurrence of appetite and hunger sensations [94]. However, no associations of postprandial total ghrelin [60, 63, 101, 102] or acylated ghrelin concentrations [63] with measures of satiety have been obsvered. Only one study showed a correlation between the increase in satiety and a decrease in ghrelin postprandially [103].

It has been shown that a high-fat meal not only induces suppression in circulating ghrelin [57, 58, 64, 66], but also increases the plasma ghrelin concentration postprandially [60]. Acylated plasma ghrelin either decreases after a high-fat meal [62, 64] or does not change [61, 67]. Protein intake has been reported to decrease [63, 64, 68], increase [58, 60] or not to affect [66] circulating ghrelin levels, while acylated plasma ghrelin has been shown to decrease after a high-protein meal [62-64, 67].

2.3.2 Glucose and insulin

Insulin has an inhibitory influence on total ghrelin levels in healthy normal-weight [74, 104] and overweight [105] persons during euglycaemic hyperinsulinaemic clamp with pharmacological insulin concentrations. Total ghrelin is also suppressed by insulin at concentrations that resemble those seen in insulin-resistant subjects [73, 106-109], in healthy normal-weight persons and in obese subjects [110], although others have reported controversial findings [104]. Acyl-ghrelin has been shown to be decreased by insulin in type 2 diabetic patients [111] and in insulin sensitive overweight patients [105] in studies using insulin in pharmacological doses, but not in insulin resistant overweight subjects [105] or in normal-weight persons using a lower dose of insulin infusion [106].

It has been suggested that postprandial hyperinsulinaemia may be responsible for the reduction in plasma ghrelin that occurs after meal intake, and it has been shown that insulin is required for prandial suppression of ghrelin [112]. However, further studies indicate that other metabolic factors are likely to be involved in postprandial ghrelin suppression [113].

Both oral and intravenous glucose loads inhibit ghrelin secretion in humans [69-72]. An increase in insulin after oral or intravenous glucose administration could contribute to the inhibitory effect of glucose on ghrelin concentrations. However, the administration of a combined pulse of glucose and insulin does not acutely suppress ghrelin levels [72]. An intravenous glucose bolus reduces ghrelin both in controls and in subjects with type 2 diabetes, which may suggest that early insulin response may have no effect on plasma ghrelin, since early insulin response to intravenous glucose infusion is abolished in patients with type 2 diabetes [114].

The exact mechanisms by which variations in insulin and glucose concentrations regulate ghrelin secretion are still unknown, and it is unclear whether insulin or glucose per se plays a direct inhibitory role in ghrelin secretion [72, 104]. Furthermore, the decrease in ghrelin levels after an oral glucose load is modulated by such factors as sex, obesity status and level of insulin resistance [66].

2.3.3 Leptin

It has been suggested that leptin may constitute a possible determinant of circulating ghrelin concentrations [115], as chronic leptin administration has been shown to upregulate ghrelin mRNA expression in the gastric fundus in rats [116]. Leptin is released from adipocytes as a function of the amount of fat, and in concert with the leptin produced locally in the hypothalamus, engages distinct hypothalamic effector pathways to restrain appetite and augment energy expenditure, acting in an opposite manner to that of ghrelin [117-119]. An inverse relationship between ghrelin and leptin concentrations has been reported by some studies [75-78], whereas others were unable to show such an association [79-82]. One study showed an inverse relation between circulating leptin and ghrelin in lean subjects and no

correlation in obese subjects [120]. Furthermore, administration of recombinant leptin has no effect on plasma ghrelin concentrations in normal-weight subjects [78].

2.3.4 Exercise

Studies investigating the effect of exercise on circulating ghrelin levels have been inconsistent [121]. Acute exercise may induce an acute negative energy balance. Short-term exercise trials have investigated the immediate effect of aerobic or resistance exercise load on ghrelin levels. Most studies investigating ghrelin response suggest that exercise alone may not alter ghrelin concentration in adults [83-89, 91, 92], whereas others have shown an increase after exercise [86, 93]. In another study, total ghrelin was unaffected by short-term exercise, whereas acylated ghrelin increased [90].

2.3.5 Sex

There are studies showing higher plasma total ghrelin levels in women than in men [66, 122-124], while acylated ghrelin has been reported to be comparable among genders [124, 125]. In contrast, acylated as well as des-acyl ghrelin levels have also been reported to be higher in women compared to men [125, 126]. Most studies have not specifically reported on a difference between males and females in their studies, thus it may be presumed that they have observed no difference, whereas others have specifically pointed out that they observed no difference between genders [79, 127].

2.3.6 Age

Plasma total and acylated ghrelin levels have been reported to be lower with increasing age [125, 128, 129], while no age-related variation has been shown with des-acyl ghrelin [125]. Despite these findings, the vast majority of reports have remained unable to detect a difference in ghrelin levels for different age groups [122, 127, 130-133], while only one study reports a positive correlation between ghrelin concentration and age [94].

Different levels of obesity could influence the relationship between ghrelin and age. Older women showed lower ghrelin levels only within the lean group, whereas the overweight and obese groups showed no difference between younger and older persons. Furthermore, only the ghrelin levels of young women decreased with increasing levels of obesity, while no association was seen in older women [128]. In addition to BMI, other modifying factors, such as menopause, should be considered as possibly affecting plasma acylated, des-acyl or total ghrelin levels in women, as markedly higher ghrelin levels have been recently shown in the perimenopause stage compared to both premenopause and postmenopause stages [134]. Oestrogen replacement therapy has been shown to increase plasma levels of acylated ghrelin [135].

2.4 The ghrelin receptor

2.4.1 Ghrelin receptor family

Howard *et al.* [136] cloned a G protein-coupled receptor of the pituitary and hypothalamus of humans and swine and showed this receptor to be the target of GHSs, a class of peptide and non-peptide compounds leading to GH release from the anterior pituitary. Until the discovery of ghrelin in 1999 [1], GHSR was designated as an orphan receptor.

The GHSR is expressed by a single gene found at human chromosomal location 3q26.2 (Figure 2) [136, 137]. Two types of GHSR cDNAs, which are presumably produced by an alternative splicing mechanism, have been identified and designated as receptors 1a and 1b [136]. The *GHSR* gene consists of two exons: the first exon encodes transmembrane regions I to V, and the second exon encodes transmembrane regions VI and VII. The human GHSR 1a consists of 366 amino acids with 7 transmembrane regions (Figure 2). The cDNA for GHSR 1b encodes a shorter form, which consists of 289 amino acids with only 5 predicted transmembrane regions (encoded by exon 1) plus a unique 24-amino acid "tail" encoded by an alternatively spliced intronic sequence using an alternate stop codon (Figure 2) [136, 138].

Multiple transcription start sites have been proposed for *GHSR*, with one major transcription start site being located 227 nucleotides upstream from the translation initiation codon and the other one located at -453 [138-140]. The proximal promoter region of the *GHSR* gene does not have any of the typical characteristics of promoter regions, such as a TATA box, CAAT box, or the GC-rich region, though consensus sequences have been found for initiator elements [138-140]. Putative binding sites for several TF were identified on the *GHSR* promoter. These included putative binding sites for the enhancer factor AP-1, consensus sequences for the nuclear factor 1 (NF-1), as well as consensus sequences for the POU-domain TFs, Pit-1, Oct-1, and Ptx1, which have been shown to be involved in pituitary-specific expression [138, 140]. Studies of *GHSR* promoter activity in different cell lines have recognized several different repressor and enhancer elements within 3000 bp upstream of the translation initiation site [138, 139].

The existence of an alternative novel unidentified subtype of ghrelin receptor has been suggested by various studies using des-acyl ghrelin [141]. This putative subtype of ghrelin receptor is distinct from GHSR 1a, but has the same binding affinity for des-acyl and acyl-ghrelin as shown by competition binding studies using radiolabelled ghrelin in several cell lines not expressing GHSR 1a [142-146]. In addition, central administration of des-acyl ghrelin to GHSR-deficient mice was shown to stimulate feeding, while ghrelin treatment had no effect on food intake, suggesting that des-acyl ghrelin acts through a target protein that is specific for des-acyl ghrelin and independent of the known GHSR [147, 148].

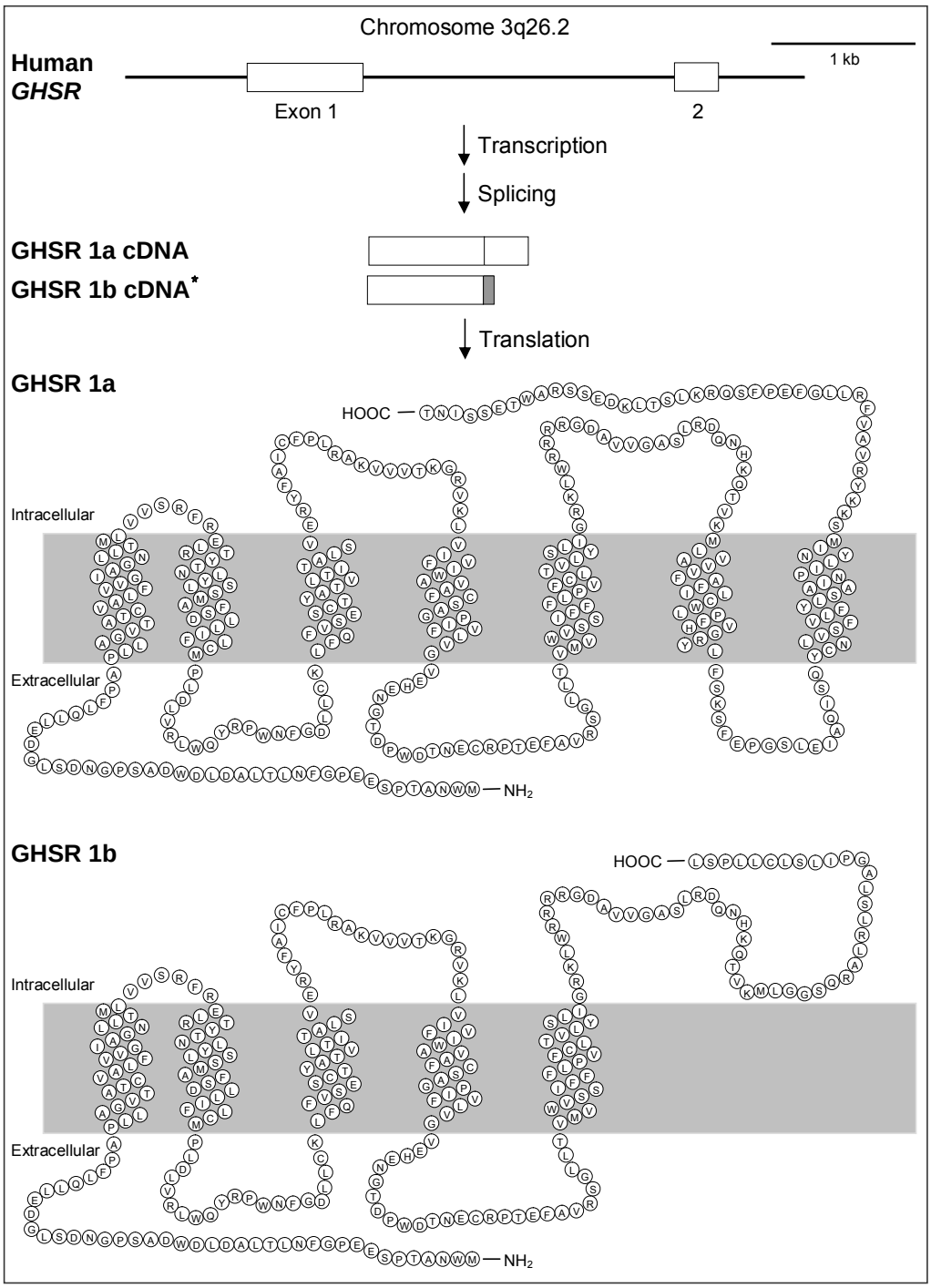

Figure 2. Schematic representation of the *GHSR* gene and amino acid sequence with proposed two-dimensional structure of the human GHSR (constructed using data from the Swiss-Prot protein knowledge database [http://ca.expasy.org/sprot/]; primary accession number Q92847).
* GHSR 1b cDNA includes 74 nucleotides of intronic sequence of GHSR 1a (▮).

2.4.2 Ghrelin receptor activation

The binding of ghrelin and synthetic GHS to the GHSR 1a activates the phospholipase C signalling pathway, leading to increased inositol (1,4,5)-trisphosphate (IP_3) turnover, followed by the release of Ca^{2+} from intracellular stores through stimulation of the G protein subunit $G\alpha_{11}$ [136]. The intracellular rise in free Ca^{2+} provokes depolarization of the somatotropes and release of GH [136, 149, 150]. Unlike the GHSR 1a, the GHSR 1b cDNA fails to bind synthetic GHS and cannot be activated by GHSR ligands [136]. Thus, the functional role of GHSR 1b remains to be defined. Nevertheless, since it is widely expressed in many normal GHSR 1a-positive or -negative tissues [48], it is possible that this receptor possesses still unknown biological functions.

In vitro studies have shown that short peptides encompassing the first four or five residues of ghrelin were found to activate the human GHSR 1a almost as efficiently as the full-length ghrelin, thus implying that the N-terminal Gly-Ser-Ser(n-octanoyl)-Phe segment constitutes the “essential core” required for efficient binding to and activation of GHSR 1a [5, 151].

GHSR 1a exhibits high constitutive activity, when overexpressed *in vitro* [152]. GHSR 1a signals with approximately 50% efficacy in the absence of an agonist [152, 153]. However, the *in vivo* physiological importance of high ligand-independent signalling activity remains to be determined. Furthermore, the constitutive activity has been shown in COS-7 (African green monkey kidney), HEK293 (human embryonic kidney) and CHO-hGHS-R (Chinese hamster ovary cells expressing human GHSR) cell lines, but not in the RC-4B/C (rat pituitary tumour) cell line, which might indicate that GHSR 1a may be turned off or on depending on the cellular context [150]. A recent report has revealed that GHSR 1b acts as a repressor of the constitutive activity of GHSR 1a when overexpressed *in vitro* [154]. Thus GHSR 1b may represent an endogenous candidate for the modulation of the activity of GHSR 1a. It has been shown that GHSR 1b can form heterodimers with GHSR 1a, which may have functional consequences [155]. Furthermore, when GHSR 1b expression exceeds that of GHSR 1a, this leads to a decrease in cell surface expression of GHSR 1a, thus leading to attenuation in GHSR 1a constitutive signalling [155].

2.4.3 Ghrelin receptor tissue distribution

GHSR 1a is highly expressed in central neuroendocrine tissues, such as the human hypothalamus and anterior pituitary gland, consistent with its role in regulating GH release [48, 136]. GHSR 1a expression has also been detected in human ovaries and testis [53, 54] and human omental fat [156], as well as in the thyroid gland, pancreas, spleen, myocardium and adrenal gland [48, 157, 158]. By contrast, GHSR 1b has been widely detected in endocrine and non-endocrine human tissues [48]. In the rat, GHSR has been found in multiple hypothalamic nuclei and in the pituitary gland [157].

2.5 Physiological functions of ghrelin

2.5.1 Effects on food intake and regulation of energy homeostasis

In general, two systems operate in the regulation of food intake: short-term and long-term regulation. Short-term regulation is concerned primarily with preventing overeating at each meal, and long-term regulation is primarily related to maintaining normal quantities of energy stores in the form of fat in the body [159]. Thus, body weight is regulated by a process of energy homeostasis, whereby total energy intake and expenditure are closely matched over long periods of time [160].

Feeding behaviour is regulated by complex mechanisms in the central nervous system (CNS). The hypothalamus and the brainstem are the main brain regions responsible for the regulation of energy homeostasis [161, 162]. They receive neural and hormonal signals from the periphery that encode information about acute nutritional state and adiposity. Mechanoreceptors and chemoreceptors in the gastrointestinal tract signal through the vagus nerve to the brainstem. A number of hormones released from the gastrointestinal tract and its associated structures stimulate ascending vagal pathways from the gut to the brainstem or act directly on neurons in the brain [162].

The central pathways that regulate body weight in response to afferent information from peripheral signals comprise a complex web of neuropeptides [161]. Catabolic neuropeptides promote weight loss by decreasing food intake and increasing energy expenditure. Anabolic neuropeptides promote weight gain by increasing food intake and decreasing energy expenditure [160]. The ARC in the hypothalamus is highly important in the regulation of feeding and appetite [161]. The anabolic neuropeptides neuropeptide Y (NPY) and agouti-related protein (AgRP) are co-localized in ARC neurons, whereas the catabolic proopiomelanocortin (POMC) is expressed in a distinct, but adjacent, subset of ARC neurons [161]. These neurons exert opposing effects on the energy balance and are reciprocally regulated by peripheral signals. POMC is cleaved into melanocortins including α melanocyte-stimulating hormone (αMSH), which exerts catabolic actions via melanocortin-4 receptors (MC4R) [160, 163]. Arcuate POMC neurons co-express the cocaine- and amphetamine-regulated transcript (CART), an anorexic neuropeptide that is regulated similarly to POMC by alterations in energy stores. Arcuate NPY/AgRP and POMC neurons project to the lateral hypothalamic area (LHA) which was denoted as a “hunger centre” [161]. Arcuate NPY/AgRP and POMC neurons also signal to the paraventricular nucleus (PVN), which mostly sends catabolic output [160, 161, 163, 164].

2.5.1.1 Short-term energy balance and meal initiation

Ghrelin may play a role in meal initiation. Peripheral or central administration of ghrelin to rodents stimulates short-term food intake and triggers eating at times of minimal spontaneous food intake [165-168]. In humans, ghrelin administration enhances appetite and increases food intake [169-171]. Moreover, a preprandial rise and a postprandial fall in plasma ghrelin levels suggests a role for ghrelin as a physiological meal initiator [94, 172]. However, it is not yet clear whether the preprandial rise in ghrelin levels does actually act to initiate eating or whether ghrelin levels rise as a consequence of the expectation of food [94]; or possibly the rise in ghrelin levels simply happens to coincide with meal times and is not directly related to hunger or meal initiation at all [173]. One study, however, showed that ghrelin levels also rise preprandially in humans in the absence of cues related to time or food [174].

2.5.1.2 Long-term energy balance

Ghrelin seems to be part of the body's mechanism for controlling the long-term energy balance. Peripheral daily administration of ghrelin causes weight gain by reducing fat utilization in mice and rats, whereas intracerebroventricular administration generated a dose-dependent increase in food intake and a subsequent increase in body weight [99]. Ghrelin also increased feeding in GH-deficient rats, indicating that ghrelin modifies energy homeostasis independently of its GH-releasing activity [165]. In humans, endogenous ghrelin levels are negatively correlated with body weight and percentage of body fat [75, 175]. This is thought to be physiologically relevant both in times of starvation and food shortage, when the elevated ghrelin levels cause the body to be more energy-efficient [99] and conversely in situations of positive energy balance, reduction in ghrelin secretion may reflect an adaptive counter-regulatory response, to eat less and store less fat [176].

Centrally or peripherally administered des-acyl ghrelin has been shown to induce a state of negative energy balance and to decrease body weight by inhibiting food intake in an inverse manner to acylated ghrelin [177]. Others have shown a decrease in food intake upon injection of des-acyl ghrelin [178, 179], whereas another study showed no alterations in feeding after administration of des-acyl ghrelin to mice [180].

2.5.1.3 Mechanisms of action

Circulating peripheral ghrelin can exert effects directly, via the blood stream, by entering the anterior pituitary gland and other areas of the brain not protected by the BBB [181], or directly, by crossing the BBB via a saturable transport system [9]. The vagus afferent nerve may be the major pathway conveying peripheral signals of ghrelin for starvation and GH secretion to the brain [182].

Ghrelin's orexigenic action is independent of its GH-releasing activity and is mediated via activation of neurons in the hypothalamic ARC that co-express the well-known orexigens, NPY and AgRP and inhibition of POMC neurons (Figure 3) [183]. Almost all arcuate NPY/AgRP neurons express the ghrelin receptor and can be stimulated by ghrelin, i.e., ghrelin induces *c-fos* expression, a marker of neuronal activation [165-167, 184]. The effect of ghrelin in the hypothalamus is opposite to that of leptin. Leptin stimulates the POMC anorexigenic pathway and inhibits the NPY/AgRP orexigenic pathway, resulting in reduced food intake (Figure 3).

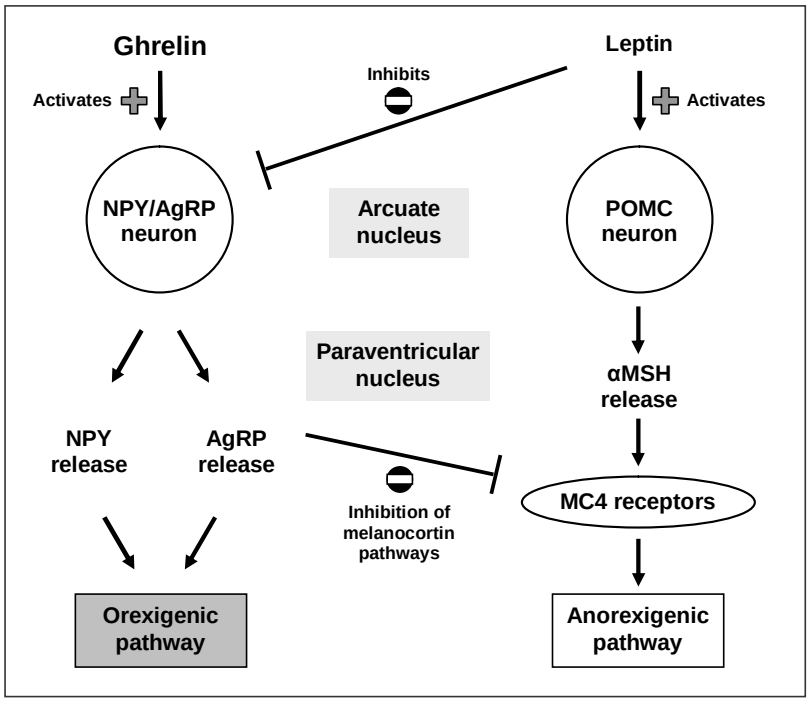

Figure 3. A simplified model of the feeding-regulatory signalling of ghrelin and leptin. (adapted from Hosoda *et al*. 2002 [185])
NPY, neuropeptide Y; AgRP, agouti-related protein; POMC, proopiomelanocortin; αMSH, α melanocyte-stimulating hormone; MC4, melanocortin-4.

Furthermore, ghrelin has been shown to stimulate the metabolic regulatory enzyme adenosine monophosphate-induced protein kinase (AMPK) in the hypothalamus [186, 187]. More recently, it has been demonstrated that ghrelin directly activates AMPK signalling in NPY neurons in the ARC leading to activation of NPY neurons and thus potentially to increased food intake [188]. AMPK acts as an intracellular energy sensor and maintains an appropriate energy level in the cell [189], and activation of AMPK in the hypothalamus increases food intake [187, 190].

2.5.2 Growth hormone-releasing activity

Ghrelin possesses a strong and dose-related GH-releasing effect [1, 191-195]. In addition, ghrelin potentiates growth hormone releasing hormone (GHRH)-dependent GH-secretion, that is, co-administration results in more GH release than does either GHRH or ghrelin alone [194, 196]. The GH-releasing effect of ghrelin is most likely mediated via the vagus nerve [167, 182] and/or by direct action on the hypothalamus/pituitary region [197, 198].

The activity of ghrelin is not fully specific for GH, because it also includes stimulatory effects on both the lactotroph and corticotroph system [191, 192, 194], and the adrenocorticotrophin (ACTH) response to ghrelin is, in turn, followed by an increase in cortisol and even aldosterone levels [194]. The effect of ghrelin on prolactin- and ACTH-secretion is age- and gender-independent [199].

2.5.3 Effects on cardiovascular system

Ghrelin has diverse cardiovascular effects which are most probably GH-independent, since expression of ghrelin receptor has been reported within the cardiovascular system [48, 200-202]. Ghrelin has a vasodilatory effect in humans [203], showing a decrease in blood pressure (BP) without an increase in heart rate [201] and additional haemodynamic effects by increasing cardiac output [204]. Ghrelin exerts a therapeutic effect in heart failure [202, 205] by improving left ventricular dysfunction and attenuation of the development of cardiac cachexia in patients with chronic heart failure [206]. In addition, the ghrelin receptor shows an upregulation and increased expression in the heart muscle of patients suffering from end-stage heart failure [207].

In animal experiments, ghrelin has been shown to inhibit sympathetic activity and to cause a decrease in arterial pressure [208, 209]. Furthermore, administration of ghrelin improves endothelial dysfunction in rats [210]. It has been suggested that ghrelin, as well as des-acyl ghrelin, act at the neurons of the nucleus of the solitary tract to suppress sympathetic nerve activity and to decrease arterial pressure [208, 209, 211]. The nucleus of the solitary tract, where baroreceptor and chemoreceptor afferents terminate, is one of the most important brain regions to regulate BP and the sympathetic nervous system [212, 213].

Potential antiapoptotic effects of ghrelin and des-acyl ghrelin at the cardiovascular level have been shown by inhibition of cell death of cultured cardiomyocytes and endothelial cells [144]. In addition to the reports *in vitro* or in animals *in vivo* [21, 144, 211], there is evidence of biological activity for des-acyl ghrelin in the human cardiovascular system, showing beneficial cardiotropic effects and vasodilation in human arteries [214].

2.5.4 Ghrelin and insulin secretion

In human studies, ghrelin has been shown to inhibit insulin secretion [215-217] and to increase plasma glucose concentration [215, 217]. Similarly, acylated ghrelin has been reported to induce a decrease in insulin and a rise in glucose levels [15]. Des-acyl ghrelin alone seems to be devoid of any endocrine effects [17], though it is able to antagonize the effects of acylated ghrelin on insulin secretion and glucose levels in normal human subjects [15]. Furthermore, the combination of acylated and des-acyl ghrelin has been shown to improve insulin sensitivity [218].

The hyperglycaemic effect of ghrelin may be mediated through activation of glycogenolysis either indirectly by stimulation of catecholamine release or directly by acting on hepatocytes, where it has already been shown to be able to modulate gluconeogenesis [19, 199, 219]. Acylated ghrelin stimulates glucose output by primary hepatocytes, whereas des-acyl ghrelin has an inhibitory effect and, moreover, counteracts the stimulatory effect of acylated ghrelin on glucose release [146]. In mice, ghrelin hampers the capacity of insulin to suppress endogenous glucose production, whereas it reinforces the action of insulin on glucose disposal; furthermore, simultaneous administration of des-acyl ghrelin abolishes the inhibitory effect of ghrelin on hepatic insulin action [220]. However, a recent human study showed that ghrelin infusion decreased insulin-stimulated glucose disposal and induced peripheral insulin resistance, but did not affect hepatic insulin production [221].

Depending on the experimental conditions, ghrelin has been reported to be able to inhibit or to stimulate insulin secretion in animals. Discrepancies may also reflect species differences. Ghrelin stimulates insulin secretion from isolated rat and mice pancreatic islets and in rats *in vivo* [36, 222-224]. Des-acyl ghrelin strongly stimulates insulin secretion by pancreatic β cells [225] and a rat insulinoma cell line [226]. However, ghrelin was also found to inhibit glucose-induced insulin release in rodents [227, 228], and ghrelin seems to directly inhibit insulin secretion in isolated islets *in vitro* [229, 230]. *In vitro*, des-acyl ghrelin promotes survival of human islets of Langerhans [225] and prevents cell death and apoptosis of pancreatic β cells [231].

2.5.5 Immunomodulation

Given the wide distribution of functional GHSR in various immune cell subsets [51], it has been hypothesized that ghrelin may exert immunoregulatory effects on immune cell subpopulations [232]. *In vitro*, ghrelin treatment was shown to inhibit production of pro-inflammatory cytokines (interleukin [IL]-1β, IL-6 and tumour necrosis factor [TNF]-α) by peripheral blood mononuclear cells s(PBMCs) via a GHSR-specific pathway [51]. It has been further reported that ghrelin inhibits IL-6 and TNF-α mRNA expression in primary human T cells [51]. These findings support a role for ghrelin in the transcriptional regulation of inflammatory cytokine expression [51].

2.5.6 Effects on adipose tissue

Several studies suggest that ghrelin may play an important role in the process of adipogenesis and storage of energy. As mentioned earlier, chronic ghrelin administration has been shown to increase body fat content in rodents [99]. Ghrelin has been shown to inhibit AMPK in adipose tissue, which could lead to increased lipid stores [186], thus potentially explaining the effect of ghrelin on fat storage. Central ghrelin infusion in rats promotes glucose and triglyceride (TG) uptake, increases lipogenesis and inhibits lipid oxidation in white adipocytes, whereas central ghrelin infusion in brown adipocytes results in decreased expression of uncoupling proteins, which usually contribute to energy dissipation. These effects seem to be mediated via the sympathetic nervous system [233].

The stimulatory effects of ghrelin on adipogenesis have been shown *in vivo* in bone marrow (for acylated and des-acyl ghrelin) [234] and *in vitro* on differentiated adipocytes [235]. In different adipose tissue culture systems *in vitro*, ghrelin has been shown to stimulate insulin-induced glucose uptake, whereas des-acyl ghrelin has no effect [236]. Furthermore, ghrelin has been shown to mediate mitogenic and antiapoptotic effects through activation of the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3 kinase (PI3K)-Akt pathways [142, 237], as well as having antilipolytic properties [238]. Additionally, des-acyl ghrelin has been shown to act directly as antilipolytic factor on rat adipocytes [238]. Moreover, during adipocyte differentiation *in vitro*, ghrelin treatment was shown to stimulate fat accumulation as well as mRNA expressions of adipogenic markers, like peroxisome proliferator-activated receptor-γ, adipocyte determination and differentiation-dependent factor 1 and adipose protein 2/fatty acid binding protein [237]. In addition, in brown adipocytes *in vitro* ghrelin has been shown to inhibit the expression of adiponectin, which has been implicated in the pathogenesis of insulin resistance and obesity [239], and ghrelin exerts a receptor-mediated stimulatory effect on the leptin production of cultured rat white adipocytes [240].

2.5.7 Gastrointestinal effects

Ghrelin stimulates gastric acid secretion and gastric motility in rodents, probably through activation of the vagus nerve [241-243]. In humans, ghrelin accelerates gastric emptying and the passage of food through the small intestine, and it induces the motility of the upper gastrointestinal tract [244, 245]. The effect of ghrelin on gastric emptying may be a direct one on the stomach itself [170] or by altering the signalling to the motor centres of the brain stem through the vagus nerve [246, 247].

2.5.8 Other effects

Numerous other effects have been attributed to ghrelin, including an influence on sleep [248-250], learning and memory [251-253], stress [254], and either an antiproliferative [144, 255, 256] or positive proliferative effect on various cell lines *in vitro* [219, 234, 235, 239, 257-263]

2.6 Ghrelin concentration in different pathophysiological conditions

2.6.1 Obesity

Both circulating total and acylated ghrelin levels have been shown to be reduced in obesity [61, 71, 75, 264]. However, one study has shown higher acylated ghrelin in obese and otherwise healthy subjects compared to non-obese healthy subjects [124].

In obesity, postprandial plasma ghrelin suppression is reduced compared to normal-weight controls [265], while acylated plasma ghrelin does not change postprandially [266]. It is thought that ghrelin is linked to excessive food intake in two ways. Firstly, the lesser postprandial reduction in ghrelin levels may directly increase the length of time for which the subject feels hungry. Secondly, as a consequence of the elevated ghrelin levels, the speed of gastric emptying may not be reduced, and the resulting feeling of satiety not elicited. Without these feelings of satiety, obese individuals eat more than they need, and thus gain weight [173].

2.6.2 Effect of weight loss on ghrelin concentration

2.6.2.1 Diet-induced weight loss

So far, the majority of studies have investigated total ghrelin levels in diet-induced and combined exercise/diet weight loss studies. A detailed summary is shown in Table 2. The studies are very diverse, with different interventions, intervention periods, number and age of participants, as well as inclusion criteria. Previous studies have shown either an increase in ghrelin concentrations in obese subjects [267-273], or no change in either overweight healthy adults [274] or obese children [275, 276], in response to diet-induced weight loss. However, during weight maintenance after weight loss, ghrelin levels decreased back to the same levels observed before weight loss [277]. Even an initial decrease along with weight loss and subsequent increase in plasma ghrelin has been reported [278]. Weight loss was also shown to increase ghrelin levels in normal-weight individuals [279].

2.6.2.2 Exercise-induced weight loss

Few studies have been conducted to investigate the exclusive effect of weight loss through exercise on plasma ghrelin levels, showing either an increase [280, 281] or no change [282]. Table 3 shows all studies conducted so far on this topic.

2.6.2.3 Bariatric surgery

The effect of bariatric surgery on circulating ghrelin levels has been studied intensively but not yet clarified. Bariatric surgery involves drastic changes in gastrointestinal anatomy and physiology and different methods can have different effects.

Ghrelin levels decrease after the Roux-en-Y gastric bypass (RYGBP) operation despite massive weight loss, as shown by many studies [267, 283, 284]. However, other studies suggest that RYGBP affects ghrelin concentration not directly but through the changes in BMI with an increase in ghrelin in those subjects with substantial weight loss [285-287]. Following vertical banded gastroplasty or adjustable gastric banding, most studies agree that fasting ghrelin levels either increase [284, 288, 289] or do not change [290]. An increase in ghrelin levels was found after biliopancreatic diversion [284, 291, 292].

Table 2. Diet-induced weight loss and combined exercise/diet weight loss studies.

Type of intervention	Duration of intervention	Initial body weight (BW) (kg)/BMI (kg/m^2)	After intervention BW (kg)/BMI (kg/m^2) or changes (Δ)	Plasma ghrelin measured	Change in plasma ghrelin	Number of subjects (adults/children)	Age (years)	Refer-ence
Low-fat, high-protein, liquid-formula diet (1000 kcal/d); solid diet: 55E% C, 30E% F, 15E% P	3 mo WL-phase, 3 mo weight stabilizing phase	BW: 99.8±5.6 BMI: 35.6±1.6	BW: 82.5±5.2 BMI: 29.4±1.5 Δ: -17.4% ±1.5	total ghrelin AUC	increase by 24%	13 obese subjects	43±2	[267]
Dietary counselling, psychological support, 3-mo fitness program	6 mo WL course	BW: 95.6±5.6 BMI: 34.5±2.4	BW: 90.6±5.3 BMI: 32.7±2.3 Δ: -5% of initial weight	fasting total ghrelin	12% increase after WL	8 women	49±4	[268]
2 wks: 45E% C, 35E% F, 20E% P; 2 wks: low-fat, high-carb isocaloric diet (65E% C, 15E% F, 20E% P); 12 wks: ad libitum 15E% F diet	12 wks	BW: 74.9±10.2 BMI: 27.1±2.3	BW: 70.8±2.7	total ghrelin AUC	no difference at different study visits	18 healthy adults	45±14	[274]
Diet with daily energy deficit of 500-1000 kcal (54E% C, 30E% F, 16E% P)	6 mo	BMI: 43.7±1.5	BMI: 33.0±1.6 BWΔ: -47.0±6.6 kg	fasting total ghrelin	increase	8 male obese Caucasians [with IFG]	35±4	[284]
Hypocaloric diet	12 mo	BMI SD 4.44±1.81	after 6 mo: BMI SD 2.6±1.19 after 12 mo: BMI SD 1.71±0.77	fasting total ghrelin	increase	26 obese pre-pubertal children	8±1	[82]
Diet (55E% C, 30E% F, 15E% P); exercise	3 mo	WLE: BW: 60.4±4.9 BMI: 22.2±2.1	BWΔ: -3.2±0.8 kg BMIΔ: -1.3±0.3	fasting total ghrelin	increased in WLE, no change in other groups; negative correlation to Δweight	22 NW young women (7 controls, 5 weight-stable exercisers, 10 weight loss exercisers (WLE))	WLE: 20±2	[293]
Energy restriction (-800 kcal/d) prescribed diet: 55E% C, 30E% F, 14E% P	4 d	BW: 89.2±15.5 BMI: 28.3±4.6	BW: 87.9±15.3 BMI: 27.9±4.6	fasting total ghrelin	no change	15 healthy males	24±4	[294]

Table 2. Continued

Type of intervention	Duration of intervention	Initial body weight (BW) (kg)/BMI (kg/m^2)	After intervention BW (kg)/BMI (kg/m^2) or changes (Δ)	Plasma ghrelin measured	Change in plasma ghrelin	Number of subjects (adults/children)	Age (years)	Refer-ence
1000 kcal/d balanced diet, physical exercise, group instruction	3 mo	BW: 96.5±16.5 BMI: 36.8±5.3	BW: 87.8±15.2 BWΔ: -8.7±4.5 kg BMI: 33.6±5.2	fasting total ghrelin	increase; no difference between obese patients and controls at baseline; positive correlation Δghrelin and Δbody fat (%)	35 obese women; age-matched controls	41±11	[269]
Physical exercise, nutrition education, behaviour therapy; low-fat, high-carb diet (55E% C, 30E% F, 15E% P)	1 y	BMI: 26 (24-29) SDS-BMI: 2.4 (2.0-2.7)	*Subgroups*: I: substantial WL SDS-BMI decrease ≥0.5 Δ: -29% (25-38%) II: decrease <0.5 Δ: -12% (8-16%)	fasting total serum ghrelin	no change in either group; obese children lower ghrelin levels than NW children	37 obese children, 16 NW children	10 (8-12)	[275]
2 wks baseline diet (50E% C, 35E% F, 15E% P); 2 wks isocaloric high protein diet (50E% C, 20E% F, 30E% P); 12 wks ad libitum high-protein diet	12 wks	BW: 72.0±8.9 BMI: 26.2±2.1	BWΔ: -4.9±0.5 kg	total ghrelin AUC	increase during 12 wks ad libitum diet	19 healthy adults	41±11	[295]
7 d: usual diet, 14 d: low-carb, high-protein, high-fat diet (21g/d)	2 wks	BW: 114.75±12.9 BMI: 40.3±5.7	BW: 112.4 BWΔ: -2.0 kg	total ghrelin AUC	increased marginally	10 obese patients with T2D	51±10	[270]
2 wks weight maintaining diet (45E% C, 35E% F, 20E% P); 2 wks diet with 30% calorie restriction; 4 wks ad libitum diet	2 wks	younger: BW: 72.9±12.4 BMI: 24.7±3 older: BW: 73.6±12.7 BMI: 26.9±3	younger: BW: 71.1±2.6 older: BW: 72.1±3.0	total ghrelin 24-h levels	increase after calorie restriction in both groups, then decrease after ad libitum diet; no significant correlation with Δweight, ΔBMI	21 younger, 18 older men and women	25±5 75±4	[130]

Table 2. Continued

Type of intervention	Duration of intervention	Initial body weight (BW) (kg)/BMI (kg/m^2)	After intervention BW (kg)/BMI (kg/m^2) or changes (Δ)	Plasma ghrelin measured	Change in plasma ghrelin	Number of subjects (adults/children)	Age (years)	Refer-ence
Lifestyle intervention program (lower calorie intake, max. 30E% F, physical activity) plus 120 mg orlistat	6 mo WL, 6 mo weight maintenance	WL: BW: 97.2±22 BMI: 37.7±7.9 CG: BW: 92±14 BMI: 35.8±5.28	WL: BMI: 34.4±1.5 (6 mo), 33.9±1.4 (12 mo) Δ: -8.5% CG: 35.8±1.1 (6 mo), 36.1±1.2 (12 mo)	fasting total ghrelin	WL: increased at 6 mo, decreased to even lower than baseline at 12 mo; no correlation to ΔBMI	obese Mexican-American women; 25 in WL, 23 in control group (CG)	WL: 44±8 CG: 44±10	[277]
Diet with daily energy deficit of 500-1000 kcal	6 mo (originally 18 mo, but patients failed to continue diet so long)	BW: 105.1±21.7 BMI: 38.6±6.83	BW: 95.64±20.48 BMI: 35.11±6.32 Δ: -9.2% ±2.4	fasting total ghrelin	increase	14 obese (1 diabetic patient)	45±12	[296]
In-patient treatment program: weight reducing diet, sport, improvement of eating habits, psychological training	6 wks	BMI SDS: +2.85	BMI SDS: +2.47	total ghrelin AUC	increase by 26%; correlation with ΔBMI SDS	23 obese children (1 with IGT)	14 (10-16)	[297]
3 mo: liquid formula diet; 3 mo: solid diet: 55E% C, 30E% F, 15E% P	3 mo WL-phase, 3 mo weight stabilising phase	BMI: 35±4.8	BWΔ: -17 kg BMI: 29±5.0 Δ: -17%	total ghrelin AUC	increase; negative correlation with ΔBMI	13 obese subjects	43±2	[271]
Diet and exercise intervention (negative energy balance between -30 to -60%), diet (55E% C, 30E% F, 15E% P)	3 mo	EDG: BW: 59.6±1.8 BMI: 21.9±0.6	EDG: BWΔ: -2.5±0.9 BMIΔ: -0.91±0.3	24-h average and AUC total ghrelin	increase in EDG	12 non-obese women (4 non-exercise controls, 8 energy deficit (EDG) group)	20±1	[279]
Commercial South Beach diet (carbohydrate-restricted diet): 2 wks: 10E% C, 62E% F, 28E% P 10 wks: 27E% C, 43E% F, 30E% P	12 wks	BW: 93.5±3.6 BMI: 33.9±1.3	BW: 88.3±3.4 BMI: 32.0±1.3	fasting total ghrelin	increase; increase in ghrelin correlation with decrease in weight	24 subjects with MetS	48±7	[272]

Table 2. Continued

Type of intervention	Duration of intervention	Initial body weight (BW) (kg)/BMI (kg/m^2)	After intervention BW (kg)/BMI (kg/m^2) or changes (Δ)	Plasma ghrelin measured	Change in plasma ghrelin	Number of subjects (adults/children)	Age (years)	Refer-ence
46 d restricted energy intake of 2.1 MJ/d (500 kcal/d): protein-enriched formula diet; pegylated-recombinant leptin	6 wks	treatment: BW: 97.9±6.6 BMI: 29.1±0.4 placebo: BW: 96.6±11.8 BMI: 29.0±0.6	treatment: BW: 83.3±5.6 BWΔ: -14.6±0.8 kg BMI: 24.8±1.5 BMIΔ: -4.3±0.7 placebo: BW: 84.8±12.7 BWΔ: -11.8±0.9 kg BMI: 25.4±2.3 BMIΔ: -3.6±1.0	fasting total ghrelin	treatment: initial decrease (day 25), then increase placebo: increase (not clear if significant or not)	24 moderately overweight/obese men (12 in treatment, 12 in placebo group)	35±1	[278]
Decrease in energy intake by 20%, increase energy expenditure by 10%; individual dietary instructions: 50-60E% C, <30E% F, 20E% P	20 wks weight loss period	BW: 81.4±9.5 BMI: 31.4±2.8	BW: 69.7±9.1 BWΔ: -11.7±2.5 kg BMI: 26.9±2.9 BMIΔ: -4.5±0.9 Δ: -14.5±3.1%	fasting total ghrelin	increase by 21.2±26.7%	35 hyperlipidaemic overweight and obese females	49±7	[273]
Physical exercise, nutrition education, behaviour therapy; low-fat, sugar-reduced, high-carb diet (55E% C, 30E% F, 15E% P)	1 y	I: BMI: 26.7±3.4 SDS-BMI: 2.2±0.4	I: BMI: 23.4±2.3 SDS-BMI: 1.5±0.4	fasting total ghrelin	I: no change (mean increase 2%) II: no change, trend for decrease	44 obese Caucasian children; I: 31 children: decrease ≥0.5 SDS-BMI II: 13 children: weight-stable	11±2	[276]
Weight reduction program including physical activity (live-in summer camp); mixed diet 1000 kcal/d (50E% C, 30E% F, 20E% P) at least 4 h fitness training/d (jogging, biking, ball games)	10 d	BW: 73.6±18.5 BMI: 28.6±4.3	BW: 72.3±19.8 BMI: 27.5±4.2	fasting total ghrelin	tended to increase (p=0.067)	18 obese children and adolescents	13±2	[298]

Table 2. Continued

Type of intervention	Duration of intervention	Initial body weight (BW) (kg)/BMI (kg/m^2)	After intervention BW (kg)/BMI (kg/m^2) or changes (Δ)	Plasma ghrelin measured	Change in plasma ghrelin	Number of subjects (adults/children)	Age (years)	Refer-ence
Lifestyle modification program: hypocaloric diet (1520 kcal, 52E% C, 25E% F, 23E% P), exercise (min. 3x per wk, 60 min each)	3 mo	I: BW: 90.1±15.2 BMI: 35.1±4.8 II: BW: 88.6±17.5 BMI:34.4±5.3	I: BW: 88.6±17.5 BMI: 33.9±4.7 II: BW: 80.7±15.9 BMI: 31.5±5.1	fasting active ghrelin	I: increase II: no change (trend to decrease) note: not adjusted for basal values	66 obese, non-diabetic patients: Subgroups: I: WL <5% of BW (n=46) II: WL >5% of BW (n=20)	46±17	[299]
WL through negative energy balance: increasing training volume and decreasing caloric intake (between 200 and 1000 kcal/d) (calculated individually)	10 wks	I: BW: 82.9±9.3 BMI: 26.7±2.8 II: BW: 85.3±10.5 BMI: 25.6±2.2	I: BW: 78.8±8.4 BMI: 25.6±2.3 loss in body fat II: BW: 84.7±9.4 BMI: 25.3±2.1	fasting total ghrelin	I: increase II: no change	14 male bodybuilders: 7 in WL group (I), 7 in CG (II)	25±8	[300]

BW, body weight; Δ, change; E%, energy%; C, carbohydrates; F, fat; P, protein; WL, weight loss; AUC, area under the curve; IFG, impaired fasting glucose; BMI SD, extent of standard deviation from Spanish standards [82]; NW, normal-weight; T2D, type 2 diabetes; WLE, weight loss exercisers; SDS-BMI, standard deviation score of body mass index; CG, control group; IGT, impaired glucose tolerance; EDG, energy deficit group; MetS, metabolic syndrome

Table 3. Exercise-induced weight loss studies.

Type of intervention	Duration of intervention	Initial body weight (BW) (kg)/BMI (kg/m^2)	After intervention BW (kg)/BMI (kg/m^2) or changes (Δ)	Plasma ghrelin measured	Change in plasma ghrelin	Number of subjects (adults/children)	Age (years)	Refer-ence
Exclusively by exercise (2x daily ergometer)	93 d negative energy balance	BW: 82.1±5.3 BMI: 26.2±1.5	BWΔ: -5.0±0.6 kg BMIΔ: -1.6±0.2	total ghrelin	no change (increase not statistically significant)	7 pairs of monozygotic male twins	21±1	[282]
45 min of moderate-intensity aerobic exercise, 5 d per week; control group: 1x per wk stretching	12 mo	EG: BW: 81.4±14.1 BMI: 30.4±14.1 CG: BW: 81.7±12.1 BMI: 30.5±3.7	EG: BW: -1.3 kg BMI: -0.3 CG: BW: 0.1 kg BMI: 0.3	fasting total ghrelin	EG: progressive increase CG: no change	173 post-menopausal overweight women; 87 exercisers (EG), 86 controls (CG)	61	[280]
Combined exercise program (aerobic exercise and resistance training); subjects kept usual diet (60E% C, 25E% F, 15E% P)	12 wks	EG: BW: 51.2 (48.2-68.4) BMI: 23.6 (22.3-29.1) CG: BW:59.8 (57.0-63.2) BMI: 24.3 (24.1-25.8)	EG: BW: 49.1 (44.8-63.1) BMI: 22.2 (20.4-26.1) CG: BW: 60.5 (58.4-68.7) BMI: 24.6 (23.9-26.1)	fasting total and acylated ghrelin	EG: increase in total ghrelin to 132% of baseline at wk 12, acylated ghrelin unchanged; negative correlation Δtotal ghrelin and Δweight, ΔBMI	17 overweight Korean boys: 8 in exercise group (EG), 9 in CG	11	[281]

BW, body weight; Δ, change; EG, exercise group; CG, control group; E%, energy%; C, carbohydrates; F, fat; P, protein

2.6.3 Type 2 diabetes mellitus

Low ghrelin levels are associated with type 2 diabetes [76, 301]. Among patients with type 2 diabetes, fasting ghrelin levels are lower in obese subjects and higher in lean persons than in normal-weight patients with type 2 diabetes [71, 76], with the same finding being observed for acylated ghrelin levels [111]. Circulating ghrelin concentrations are also reduced in the healthy offspring of type 2 diabetic patients [302].

2.6.4 Insulin resistance and metabolic syndrome

Individuals with the metabolic syndrome have lower ghrelin concentration compared to normal-weight subjects [303-306]. Lower ghrelin concentrations have also been shown to associate with a higher prevalence of the metabolic syndrome, with progressively lower levels being found as the number of components of the metabolic syndrome is increased [127, 304]. This may most likely be explained by higher BMI in subjects with lower ghrelin levels, since adiposity also influences other features of the metabolic syndrome [127, 304]. In fact, it was shown that plasma total ghrelin as well as des-acyl ghrelin is lower in obese patients with metabolic syndrome compared to non-obese counterparts, though acylated ghrelin was comparable in both groups [124] or only lower in obese but not in morbidly obese compared to normal-weight persons [306].

Among obese subjects, plasma ghrelin levels have been shown to be lower in insulin-resistant than in insulin-sensitive persons [307], but neither total ghrelin, nor acylated or des-acyl ghrelin separately differ between insulin sensitive and insulin resistant persons [105]. Among overweight and obese patients, the ratio of acyl/des-acyl ghrelin is lower in insulin-sensitive than in insulin-resistant subjects [105].

A study comparing metabolically obese but normal-weight women with healthy, normal women showed no difference between plasma total ghrelin levels for these two groups [308], suggesting that obesity alone does not affect ghrelin levels, but rather metabolic disturbances have an independent effect.

2.6.5 Hypertension

Low plasma ghrelin levels are associated with hypertension [76] and inversely correlated with elevated blood pressure (BP) in different study populations [76, 309, 310]. Acylated ghrelin levels are also negatively correlated with systolic BP [125]. Pregnant women with pregnancy-induced hypertension, however, had higher ghrelin levels than normal pregnant women [309].

2.6.6 Chronic conditions with negative energy balance

Anorexia nervosa is a syndrome seen in young women characterized by a combination of weight loss, amenorrhea, and behavioural changes. Circulating ghrelin levels in patients with anorexia nervosa are significantly elevated outside of the normal range as compared to constitutionally thin controls [311] and fall back to within the normal range upon weight gain by renutrition [34, 71, 82, 312, 313]. It was found that when comparing patients with anorexia nervosa to constitutionally thin but healthy controls, the ghrelin infusions had no significant effect on appetite [314]. This suggests a certain level of ghrelin resistance in anorexia nervosa which could be the result of down-regulation of GHSR expression due to chronically high levels of circulating ghrelin [173]. In bulimia nervosa, an eating disorder characterised by habitual abnormal binge eating behaviour, higher ghrelin levels were found compared to BMI-matched normal subjects [315, 316].

Circulating ghrelin levels are also elevated in cachexia associated with chronic heart failure [317] or cancer [318], and administration of ghrelin has been suggested as a new therapeutic strategy for the treatment of cachexia [319].

2.7 Animal models of ghrelin or ghrelin receptor deficiency

Knocking out the activity of a gene provides powerful information for studying the biological function of gene products. Table 4 shows a summary of the results from studies investigating transgenic and knockout models for ghrelin and GHSR. The global deletion of the *GHRL* gene in mice results in a normal energy balance, food intake and adiposity on a standard diet [176, 320-322]. These findings were surprising and lead to the interpretation that endogenous ghrelin may not be of crucial relevance for physiological energy homeostasis. Others have attributed the lack of a metabolic phenotype to compensatory processes during early developmental phases or the presumably redundant multiplicity of pathways controlling energy balance. Furthermore, some studies have suggested that the GHSR 1a exhibits basal constitutive activity [152, 323] and thus could still provide possible residual signalling in ghrelin target cells, even in the absence of the primary ligand in mice with targeted disruption of the *GHRL* gene [153]. However, chronic exposure of ghrelin-deficient mice to a high-fat diet resulted in a reduction in weight gain and increased use of fat as fuel [176, 321]. When ghrelin deficiency was induced in adult animals, reduced food intake, reduced fat storage and weight loss were observed [324, 325]. This suggests that ghrelin is an important player in the field of energy homeostasis and appetite regulation, as part of a bigger network of regulatory molecules, which can only compensate for its loss if this occurs early in development [173].

Table 4. Animal models for ghrelin and GHSR (modified from Higgins *et al*, 2007 [173])

Ghrelin	Embryonic/Adult	Finding/phenotype compared to WT littermates	Reference
	Embryonic ghrelin KO	No detected difference as compared to WT	[320]
	Embryonic ghrelin KO	KO animals on a high-fat diet showed preferential use of fat as a metabolic substrate	[176]
	Embryonic ghrelin KO	Males on high-fat diet showed less weight gain and higher locomotor activity	[321]
	Embryonic ghrelin KO	Young animals showed lower RQ and higher heat production	[326]
	Embryonic ghrelin KO	Normal body weight and feeding pattern	[327]
	Embryonic ghrelin KO	Normal feeding behaviour; increase in locomotor activity	[328]
	Transgenic mice overexpressing des-acyl ghrelin	Lower body weight and fat mass with decreased food intake and gastric emptying rate	[177]
	Transgenic mice overexpressing des-acyl ghrelin	Small phenotype, lower IGF-1 levels	[329]
	Transgenic mice overexpressing ghrelin	Normal size animal (no difference in food intake, body growth, body weights, and fat depots compared to WT). No desensitisation of the orexigenic effect of exogenous ghrelin. Desensitization of epididymal fat pads.	[330]
	Embryonic ghrelin/leptin double KO	Obesity and hyperphagia shown, but with improved insulin sensitivity	[331]
	Congenic adult ghrelin KO	Lower glucose and insulin levels	[332]
	Adult (given synthetic oligonucleotides to neutralise effects of ghrelin)	Weight loss occurred in diet-induced obese mice	[324]
	Adult (immunisation against ghrelin)	Weight loss and reduced food intake in pigs	[325]
GHSR	**Embryonic/Adult**	**Finding/phenotype compared to WT littermates**	**Reference**
	Embryonic GHSR KO	Lower body weight and IGF-1 levels	[148]
	Embryonic GHSR KO	Reduced food intake and weight gain on high-fat diet, increased fat burning	[322]
	Embryonic GHSR KO	Normal feeding behaviour	[328]
	Embryonic (given antisense GHSR mRNA, which selectively attenuates GHSR protein expression in the ARC)	Lower body weight and less adipose tissue, reduced food intake, abolition of the stimulatory effect of GHS on feeding	[333]
	Adult (given GHSR antagonist (D-Lys-3)-GHRP-6)	Reduced food intake and weight gain on high-fat diet, increased fat burning	[334]
	Congenic adult GHSR KO	Lower body, increased insulin sensitivity	[332]
Ghrelin and GHSR	**Embryonic/Adult**	**Finding/phenotype compared to WT littermates**	**Reference**
	Embryonic ghrelin/GHSR double KO	Lower body weight, decreased fat mass; normal feeding behaviour; increased energy expenditure	[328]

WT, wild type; KO, knockout; RQ, respiratory quotient; IGF-1, insulin-like growth factor 1; ARC, arcuate nucleus of the hypothalamus; GHS, growth hormone secretagogue

Similar to ghrelin-deficient mice, the appearance of *GHSR*-deficient mice is not dramatically different from that of their wild-type littermates [148]. However, the body weights of mature *GHSR*-null mice are modestly reduced compared to wild-type littermates, which is consistent with ghrelin's property as an amplifier of GH pulsatility [148]. In a model

selectively attenuating GHSR protein expression in the ARC, transgenic rats had lower body weight and less adipose tissue than did control rats [333], which is in agreement with the role of ghrelin and its receptor in the regulation of GH secretion, food intake, and adiposity. On a high-fat diet, *GHSR*-deficient mice showed reduced food intake and weight gain and utilized fat in preference to other metabolic fuels [322, 334]. When a high-fat diet was induced in adult ghrelin- or *GHSR*-deficient mice, they were not protected from weight gain, suggesting that as animals reach adulthood, they develop compensatory pathways to adjust for the loss of a ghrelin/GHSR signal [332].

Simultaneous knockout of the *GHRL* and the *GHSR* genes leads to changes in energy balance, which are not observed in mice deficient for either the ligand or the receptor alone on normal standard chow [328]. It was therefore speculated that either ghrelin may also act through an additional, as yet unknown, receptor, or that there exists another ghrelin-like ligand [328].

2.8 Genetic variations in the *GHRL* and *GHSR* genes in humans

Polymorphisms in the human *GHRL* gene and the 5'-flanking region have been intensively studied. The most studied SNP is the Leu72Met located in exon 3, which changes the amino acid sequence from Leucine to Methionine at position 72 in the prepro-ghrelin protein. The Arg51Gln SNP of *GHRL* is located in exon 3 within the last codon of the mature ghrelin protein and disrupts the recognition site of the endoprotease, leading to proteolytic cleavage of the carboxy-terminal 66 amino acids to produce mature ghrelin [335]. Table 5 shows NCBI RefSNP accession ID's (rs numbers) [336] corresponding to the name of SNPs based on their position and their location in *GHRL*. Table 6 summarizes most genetic association studies dealing with SNPs in the *GHRL* gene to date. Nonetheless, reports on SNPs in the *GHSR* gene are sparse. All studies carried out so far are described in Table 7.

Table 5. *GHRL* SNPs and their corresponding NCBI RefSNP accession IDs (rs-numbers), position and SNP location.

Rs number	Position	SNP location	Rs number	Position	SNP location
rs1629816	-4427G>A[1]	5'-region	rs34911341	Arg51Gln[2]	Exon 3
rs10490815	-3281A>G[1]	5'-region	rs696217	Leu72Met[2]	Exon 3
rs27498	-2157G>A[1]	5'-region	rs2075356	+3056T>C[1]	Intron 3
rs3755777	-1500C>G[1]	5'-region	rs4684677	Gln90Leu[2]	Exon 4
rs26311	-1062G>C[1]	5'-region	rs35684	+5179A>G[1]	3'-region
rs26312	-994C>T[1]	5'-region	rs171407	+5696A>G[1]	3'-region
rs27647	-604G/A[1]	5'-region	rs171336	+6115G>T[1]	3'-region
rs26802	-501A/C[1]	5'-region	rs2072578	+9344G>A[1]	3'-region
novel	-473G/A[1]	5'-region			

[1] number denotes the nucleotide position either upstream (-) or downstream (+) of the translation initiation site;
[2] number denotes amino acid position

2.8.1 Association with obesity

Several studies have found associations between *GHRL* SNPs and obesity or related traits, although the results are contradictory. The Met72 allele of *GHRL* was shown to be associated with earlier age at onset of obesity and higher BMI [264, 337-340], whereas the opposite has also been reported [335, 341]. The -501A>C SNP in *GHRL* promoter and the intronic +3056T>C SNP were also associated with obesity [342, 343], although some studies have failed to find any association between *GHRL* SNPs and obesity [76, 264, 339, 344-350]. Furthermore, *GHRL* SNPs were associated with metabolic syndrome in one study [346], but not in another [345]. Only one study has shown an association with the haplotypes of five *GHSR* SNPs and obesity [351], whereas another study could not show such a relationship [352].

Table 6. Studies on the associations of SNPs in the *GHRL* gene.

SNP	Risk allele	Association	Subjects	Reference
Leu72Met Arg51Gln	Met72 Gln51	Lower age of onset of self-reported obesity Not found among normal-weight subjects	96 obese and 96 normal-weight Swedish women	[337]
Leu72Met Arg51Gln	Leu72 Gln51	Higher BMI, fat mass, visceral fat, total TG and RQ; lower IGF-1 levels in Blacks Lower frequency in Whites than in Blacks Not observed among Blacks	784 French-Canadian subjects (Quebec Family Study) 778 subjects (276 Blacks and 502 Whites; HERITAGE Family Study) 1442 subjects (741 from obese registry, 701 from normal reference population; SOS)	[335]
Leu72Met	Met72	Higher BMI, earlier age of onset of obesity, reduced first phase insulin secretion	70 tall and obese children	[338]
Gln90Leu	Gln90	Higher frequency in obese children, but also in underweight students	215 extremely obese German children and adolescents 93 normal-weight students 134 underweight students 44 normal-weight adults	[353]
Leu72Met	Met72	Lower serum creatinine and lipoprotein a levels	258 Finnish Caucasians with T2D and 522 controls	[354]
Arg51Gln	Gln51	Risk factor for hypertension and T2D, predictor of 2-h plasma glucose in OGTT; lower IGF-1 and higher IGFBP-1 concentrations in normotensives: lower AUC insulin	519 hypertensive and 526 normotensive Finnish Caucasians	[355]
Arg51Gln Leu72Met		Both: no association	519 hypertensive and 526 normotensive Finnish Caucasians	[76]
Arg51Gln Met72Leu	 Met72	No association Earlier onset of obesity	300 obese and 200 lean Italian children and adolescents	[339]
Leu72Met Gln90Leu Arg51Gln	Met72	In obese/overweight: greater neonatal weight-for-age, earlier age at onset of obesity, greater IGF-1 concentration. No association with obesity phenotypes No association with obesity phenotypes	81 obese or overweight and 96 normal-weight Italian children and adolescents and 72 normal-weight young adults	[264]
-4427G>A Leu72Met +5179A>G +9344G>A	G	Diffuse large cell lymphoma All three: no associations with BMI	684 healthy controls and 308 North American subjects with non-Hodgkin Lymphoma	[344]

Table 6. Continued

SNP	Risk allele	Association	Subjects	Reference
Leu72Met		No difference between genotype and allele frequency between healthy and subjects with MetS, no association with related quantitative traits	2413 Danish Caucasian subjects (of which 279 with MetS)	[345]
Arg51Gln Leu72Met Gln90Leu	Gln51 Met72	Less MetS Higher fasting glucose, lower HDL cholesterol, higher TG; more MetS No significant associations All three: no association with BMI, waist, T2D	856 Old Order Amish from US	[346]
Arg51Gln Leu72Met Gln90Leu		All three: no association with obesity- or T2D-related traits	234 juvenile-onset obese and 323 lean men, 557 T2D and 233 glucose tolerant subjects	[347]
Leu72Met	Met72	More depressed and anxious in patients with MetDep; no association with MetDep	118 Koreans with methamphetamine dependence (MetDep), 144 controls	[356]
Leu72Met		No association with obesity	222 obese Korean children	[348]
-1500C>G -1062G>C -994C>T Leu72Met	 C	 Lower HDL cholesterol. All four: no association with T2D	760 T2D and 641 non-diabetic Koreans	[14]
Leu72Met	Met72	Lower allele frequency and lower total cholesterol levels in patients with diabetic nephropathy with renal dysfunction	138 subjects with diabetic nephropathy, 69 diabetics without nephropathy	[357]
Leu72Met	Met72	Lower creatinine levels in diabetic group, no association with T2D	206 T2D, 80 controls	[358]
-501A>C	A	Higher BMI	1045 Finnish subjects from the Oulu Project Elucidating Risk for Atherosclerosis (OPERA) study	[343]
Leu72Met		No association with weight loss	771 obese Caucasian Europeans	[349]
Leu72Met	Met72	Higher allele frequency in higher BMI group than in normal-weight group Higher BMI, waist circumference and change in body weight from age 18 No difference in allele frequency between diabetics and non-diabetics	2238 middle-aged and older Japanese people	[340]
Leu72Met	Leu72	Higher BMI in CAD patients; no association with CAD no association with hypertension, T2D or dyslipidaemia	317 Chinese CAD patients, 323 controls	[341]
Leu72Met +3056T>C	Met72 C	Higher scores on Drive for Thinness-Body Dissatisfaction subscale Higher weight, BMI, fat mass, waist circumference, sum of skinfold thicknesses, self-reported past min and max BMIs and lower HDL cholesterol	264 Japanese women	[342]
-604C>T Leu72Met Gln90Leu Haplotypes: *-604G/Leu72* versus *-604A/Leu72* *-604G/Met72*	C Leu72	Higher fasting insulin and HOMA-IR, but only in Leu72 subjects Higher TG, fasting insulin and HOMA-IR No associations *-604G/Leu72* higher insulin and HOMA-IR than other haplotypes	1420 Caucasian subjects (500 normal-weight, 920 overweight/obese)	[359]

Table 6. Continued

SNP	Risk allele	Association	Subjects	Reference
Arg51Gln	Gln51	Gln51 allele carriers developed higher cholesterol levels over time	210 haemodialysed patients, prospectively followed up for 15 months	[360]
Leu72Met		No association		
Genotype combination		Subjects with Gln51 and/or Met72 allele lost body weight faster than patients with Arg51Arg/Leu72Leu (no weight loss)		
Arg51Gln Leu72Met Gln90Leu		All three: no associations	198 children with short stature (117 with GH-deficiency and 81 with idiopathic short stature); 125 age and gender-matched healthy children as controls	[361]
rs10490815		No associations	1359 breast cancer cases and 2389 matched controls participating in the EPIC study	[350]
rs27498	A	Lower height		
rs3755777	G	Associated with 5% lower IGF-1 levels; lower height		
-604T>C		No associations		
-501T>G		No associations		
Leu72Met		No associations		
+3056A>G		No associations		
Gln90Leu		No associations		
rs35684		No associations		
rs171407	A	Higher BMI; lower height;		
	G	Higher breast cancer risk		
rs171336	G	Lower height		

TG, triglycerides; RQ, respiratory quotient; IGF-1, insulin-like growth factor 1; T2D, type 2 diabetes mellitus; IGFBP-1, insulin-like growth factor binding protein 1; AUC, area under the curve; MetS, metabolic syndrome; HOMA-IR, homeostasis model assessment for insulin resistance; MetDep, methamphetamine dependence; CAD, coronary artery disease; GH, growth hormone; EPIC, European Prospective Investigation into Cancer and Nutrition

2.8.2 Association with type 2 diabetes

The Gln51 allele of Arg51Gln in *GHRL* was reported to be a risk factor for type 2 diabetes [355] and the Leu72Met SNP was associated with type 2 diabetes-related phenotypes in some studies [346, 359], while others have reported negative results [14, 340, 341, 346, 358]. Only one report has shown an association between *GHSR* SNPs and insulin metabolism [362].

2.8.3 Association with hypertension

The Gln51 allele of the Arg51Gln SNP in *GHRL* has been found to be a risk factor for hypertension [355], though the data concerning the Leu72Met SNP are inconsistent [76, 335, 341, 354]. *GHSR* SNPs have not been associated with hypertension.

Table 7. Studies on the associations of SNPs in the *GHSR* gene.

SNP	Risk allele	Association	Subjects	Reference
rs572169		No association	746 obese German children and adolescents, 232 underweight, 96 normal-weight students, 43 children with short normal stature	[352]
rs495225	T	Initially higher allele and genotype frequency in obese compared to underweight; could not be replicated		
rs2232165		No associations	Finnish hypertensive subjects, 96 with high and 96 with low total IGF-1 plasma concentrations	[362]
rs495225	CC	Highest AUCIN values, lowest IGFBP-1 plasma levels		
rs2232169		No associations		
rs572169	GG	Highest AUCIN values		
rs4988509		No associations		
rs582780		No association with obesity	1095 Caucasians (from families), 1418 subjects from general population	[351]
rs544672		No association with obesity		
rs578015		No association with obesity		
rs509035	MA	Haplotype with 5 minor alleles (SNPs italic) associated with obesity; those 5 SNPs are also individually associated with obesity		
rs572169	MA			
rs519384	MA			
rs512692	MA			
rs490683	MA			
rs1523324		No association with obesity		
rs1992869		No association with obesity		
rs495225	C	Higher genotype frequency in bulimia nervosa	228 Japanese patients with eating disorders, 284 controls	[363]
rs582780		No association with LVH	1230 Germans	[364]
rs544672		No association with LVH		
rs578015		No association with LVH		
rs509035	MA	Association with LVH		
rs572169	MA	Association with LVH		
rs519384	MA	Association with LVH		
rs512692	MA	Association with LVH		
rs490683	MA	Association with LVH		
rs1523324		No association with LVH		
rs1992869		No association with LVH		
rs579015		No association with MI or CAD	1390 Germans with myocardial infarction (MI), 940 with MI or coronary artery disease (CAD), 1418 German controls, 638 controls (non-affected siblings)	[365]
rs509035	MA	Higher risk of MI or CAD		
rs572169	MA	Higher risk of MI or CAD		
rs519384	MA	Higher risk of MI or CAD		
rs512692	MA	Higher risk of MI or CAD		
rs490683	MA	Higher risk of MI or CAD		
rs1523324		No association with MI or CAD		
rs2948694		No associations	1359 breast cancer cases and 2389 matched controls participating in the EPIC study	[350]
rs495225		No associations		
rs572169	C	Lower height		
rs2922126	A	Higher BMI		

IGF-1, insulin-like growth factor 1; AUCIN, area under the insulin curve; IGFBP-1, insulin-like growth factor binding protein 1; MA, minor allele; LVH, left ventricular hypertrophy; MI, myocardial infarction; CAD, coronary artery disease; EPIC, European Prospective Investigation into Cancer and Nutrition. SNPs belonging to a haplotype are in italic font.

2.8.4 Association with ghrelin plasma concentration

Only a few studies have investigated the association between ghrelin polymorphisms and plasma ghrelin levels and have created inconsistent results (Table 8).

Table 8. Studies on the associations between polymorphisms in the *GHRL* gene with plasma ghrelin concentrations.

SNP	Association with plasma ghrelin concentration	Total, acyl- or des-acyl ghrelin, ratio	Subjects	Reference
Arg51Gln Leu72Met	Arg51Gln (n=6) lower than Arg51Arg subjects (n=14) No sign. difference between 3 genotypes (Met72Met (n=4) tended to have highest levels)	Total ghrelin	784 subjects (Quebec Family Study)	[335]
Arg51Gln Leu72Met	Gln51 allele carriers lower concentrations Only in hypertensive group: Leu72Leu higher than Leu72Met persons, but Met72Met highest levels	Total ghrelin	519 hypertensive and 526 normotensive Finnish Caucasians	[76]
Leu72Met Gln90Leu Arg51Gln	All three: no associations	Total and acylated ghrelin, acyl/total ratio	81 obese or overweight and 96 normal-weight Italian children and adolescents and 72 normal-weight young adults	[264]
-501A>C	Genotype distribution different between the groups (AA genotype in low ghrelin group and C allele carriers in high ghrelin group more common)	Total ghrelin	50 Finnish patients with low and 50 with high plasma ghrelin concentrations	[343]
-501A>C	No difference between 3 genotypes	Total ghrelin	1045 Finnish subjects from the Oulu Project Elucidating Risk for Atherosclerosis (OPERA) study	[343]
Leu72Met +3056T>C	Met72 allele carriers higher acylated ghrelin concentration C allele carriers higher acylated ghrelin concentration	Acylated and des-acyl ghrelin, acyl/des-acyl ratio	264 Japanese women	[342]
-604C>T Gln90Leu Leu72Met Haplotypes: *-604G/Leu72* versus *-604A/Leu72* *-604G/Met72*	No association No association Leu72Leu < Leu72Met < Met72Met *-604G/Leu72* lower ghrelin levels than other haplotypes	Total ghrelin	1420 Caucasian subjects (500 normal-weight, 920 overweight or obese), subpopulation 300 overweight or obese	[359]
Arg51Gln Leu72Met Gln90Leu	All three: no association	Total ghrelin	80 children with GH-deficiency, 57 with idiopathic short stature and 35 healthy controls	[361]

GH, growth hormone

3 Aims of the study

The aim of the work presented in this thesis was to study the role of ghrelin and the ghrelin receptor in the development of obesity and impaired glucose metabolism. The studies conducted within the scope of this thesis are mostly based on data collected in large intervention projects.

Consequently, the specific aims of this work were to:

- Study the associations between polymorphisms in the *GHRL* gene and the incidence of type 2 diabetes (*Study I*), obesity (*additional results*) or hypertension (*Study II*) in persons with impaired glucose tolerance participating in the Finnish Diabetes Prevention Study (DPS).
- Study the associations between SNPs in the *GHSR* gene and obesity and type 2 diabetes-related phenotypes in the Finnish DPS (*Study III*).
- Conduct a promoter analysis of the *GHSR* gene to investigate SNPs disrupting putative transcription factor binding sites and subsequently test their potential functionality *in vitro* (*Study III*).
- Examine the expression of ghrelin and ghrelin receptors in peripheral blood mononuclear cells (PBMCs) and subcutaneous adipose tissue in individuals with features of metabolic syndrome in the Genobin study (*Study IV*).
- Evaluate the effects of weight reduction or exercise intervention on plasma ghrelin concentrations, as well as on ghrelin expression in PBMCs and adipose tissue from participants in the Genobin study (*Study IV*).

4 Subjects and Methods

4.1 Study populations and study designs

4.1.1 The Finnish Diabetes Prevention Study *(Studies I-III)*

The Finnish Diabetes Prevention Study (DPS) is a multicentre study with five participating centres in Finland, located in Helsinki, Kuopio, Oulu, Tampere and Turku [366]. The main aim of the DPS was to assess the efficacy of an intensive and individually designed diet and exercise program to prevent or delay the onset of type 2 diabetes in Finnish subjects with IGT.

Overweight subjects (BMI over 25 kg/m^2) aged 40 to 64 years at randomization and who had IGT were eligible for the study. Impaired glucose tolerance (IGT) was defined according to the WHO 1985 criteria [367], i.e., as a plasma glucose concentration of 7.8-11.0 mmol/L two hours after the oral administration of 75 g of glucose with a non-diabetic fasting plasma glucose concentration (<7.8 mmol/L). After the first screening OGTT, a second OGTT was carried out in subjects with IGT, and the mean of the two 2-hour glucose concentrations was used as the criterion for inclusion in the study. A total of 522 subjects were randomly assigned to one of the two treatment groups: the intensive diet and exercise counselling group (n=265) or the control group (n=257). The subjects in the control group were given general oral and written information about diet and exercise at baseline and at subsequent annual visits, but were offered no specific individualized programs. The subjects in the intervention group were given detailed advice about how to achieve the goals of the intervention [366]. At baseline and at each annual visit, all study subjects completed a medical-history questionnaire and underwent a physical examination that included anthropometric and BP measurements, as well as an OGTT. The most intensive intervention was carried out during the first year of the study, and the study was terminated after a mean follow-up of 3.2 years when the risk of diabetes had been reduced by 58% in the intervention group compared with the control group [368]. However, participants were thereafter invited to take part in the extended follow-up period, and the follow-up of the diabetes incidence is still ongoing.

4.1.2 The Genetics of obesity and insulin resistance study *(Study IV)*

Originally, the *Gen*etics of *Ob*esity and *In*sulin resistance (Genobin) Study included 75 overweight or obese (BMI range 28 to 40 kg/m^2) men and women aged 40 to 70 years with impaired fasting glucose (IFG) or IGT, and at least two other features of metabolic syndrome according to the Adult Treatment Panel III criteria as modified by the American Heart Association [369, 370]. Subjects were randomized into one of the following groups: a weight reduction group (n=28), aerobic exercise training group (n=15), resistance exercise training

group (n=14) or control group (n=18). Subjects were matched for age, gender and the status of glucose metabolism. In addition, 11 normal-weight subjects (mean age 48±9 years, mean BMI 23.7±1.9 kg/m^2) were recruited. The weight-reduction group underwent a 12-week intensive weight-reduction period followed by a weight-maintenance period during study weeks 12-33 [369]. Subjects were asked to maintain their habitual level of physical exercise unchanged. For subjects in the exercise training groups, aerobic and resistance training group, respectively, the individualized and progressive training programs, which lasted for 33 weeks, were prescribed based on measured cardiorespiratory fitness and muscular strength levels. Muscle strength was measured using 5-repetition maximum tests, which were conducted before every modification in the program as well as at the end of the program. Participants also had follow-up visits once a month. The subjects in the control group were advised to continue their normal lifestyle during the study and to keep their diet and exercise habits unchanged.

Biochemical, anthropometric and BP measurements were performed at baseline, week 12 and at the end of the study at week 33. Frequently sampled intravenous glucose tolerance test (FSIGT) and adipose tissue biopsy were performed at baseline and at the end of the study.

4.1.3 Approval of the Ethics Committee

The study protocol was approved by the ethics committee of the National Public Health Institute in Helsinki, Finland (*Studies I-III*). The Ethics Committee of the District Hospital Region of Northern Savo and Kuopio University Hospital approved the study plan, and the intervention was performed in accordance with the standards of the Helsinki Declaration (*Study IV*). Written informed consent was obtained from all study subjects.

4.2 Methods

4.2.1 Clinical and biochemical examinations

4.2.1.1 Anthropometric measurements

Weight and height were measured in light clothing and BMI was calculated in all studies by dividing weight (kg) by height (m) squared. Waist circumference was measured mid-way between the lowest rib and iliac crest. BP was measured by trained study nurses using a standard sphygmomanometer twice on the right arm after 10 min of rest with the subject in a sitting position. The mean of systolic and diastolic BP was calculated from the two measurements obtained. Hypertension was defined as the mean systolic BP of ≥140 mmHg or diastolic BP of ≥90 mmHg, or taking antihypertensive medication [371, 372].

Studies I-III. Weight change was calculated as the difference in weight between the baseline and three years. For those developing diabetes, weight change was calculated from the

baseline value to the last weight measurement available, which varied from one to three years depending on the date of diagnosis of type 2 diabetes.

Study IV. Relative weight change was calculated as:
(weight at week 33 – weight at baseline) / weight at baseline x 100.

4.2.1.2 Laboratory measurements

A 2-hour OGTT with a glucose dose of 75 g after a 12-hour overnight fast was carried out to determine glucose tolerance. Blood samples for plasma glucose and serum insulin concentrations were drawn at 0, 30, and 120 min.

Studies I-III. Plasma glucose was measured locally by standard methods, and the measurements were standardised by the central laboratory in Helsinki. Serum insulin was determined with a radioimmunoassay (RIA) (Pharmacia, Uppsala, Sweden). Serum total cholesterol, HDL cholesterol and TG were determined using an enzymatic assay method (CHOD-PAP, Boehringer Mannheim, Germany, Monotest). LDL cholesterol was calculated using the Friedewald formula: Total cholesterol – (HDL cholesterol + TG / 2.2), and applied only when TG levels were below 4.5 mmol/L [373]. For subjects with TG levels >4.5 mmol/L, no LDL cholesterol calculations were performed. The homeostasis model assessment for insulin resistance (HOMA-IR) was calculated using the following formula: fasting plasma glucose (mmol/L) x fasting serum insulin (mU/L) / 22.5 [374].

Study IV. Biochemical analyses were performed using routine methods at the Clinical Laboratory Centre of the Kuopio University Hospital and at the Clinical Unit of the University of Kuopio. Plasma glucose concentration was analysed by the hexokinase method (Thermo Clinical Labsystems, Vantaa, Finland), and insulin was determined by the chemiluminescence sandwich method (ACS, Bayer A/S, USA). Lipoproteins were separated by ultracentrifugation (Beckman Optima L-90K) preceding enzymatic methods (Roche Diagnostics, Mannheim, Germany) on Kone Pro Clinical Chemistry Analyser (Thermo Clinical Labsystems, Konelab, Espoo, Finland) to analyse cholesterol, TG and HDL-cholesterol. Serum free fatty acids were measured with a turbidometric analyser (CV% 1.5) (Kone Ltd, Espoo, Finland). Commercial RIA kits were used for the analysis of serum leptin and plasma total ghrelin (Linco Research Inc., St. Louis, MO, USA). Plasma TNF-α, IL-6 and IL1-β concentrations were measured by solid phase enzyme-linked immunosorbent assay (ELISA) (Quantikine, R&D Systems, Minneapolis, MN, USA). High-sensitivity C-reactive protein (hsCRP) was determined using the Immage Immunochemistry System (Immulite 2000 DPC, Los Angeles, LA, USA).

The FSIGT was performed according to the Minimal Model method by administrating 300 mg/kg body weight glucose through a catheter inserted into an antecubital vein as a 50% solution. Plasma glucose and serum insulin concentrations were followed for three hours, collecting altogether 25 blood samples. During the first 10 minutes, blood samples were

drawn every two minutes and just before the 20 min insulin bolus (0.03 U/kg body weight). The results were calculated with MINMOD Millennium software [375] as glucose effectiveness (S_G), insulin sensitivity (S_I) and acute phase insulin response (AIR) using the area of insulin concentration above the baseline during 0-10 minutes.

4.2.2 Selection of single nucleotide polymorphisms

4.2.2.1 *GHRL* polymorphisms

Originally, genetic variations in the *GHRL* gene were determined by direct sequencing of the coding region and the proximal promoter area (1 kb upstream of the translation start site) in 35 massively obese Finnish subjects [376]. Six SNPs were detected in this study population and subsequently genotyped in other study populations: three SNPs in the 5'-region: -604G/A (rs27647), -501A/C (rs26802) and the novel variation -473G/A, and three SNPs in the coding region: Arg51Gln (rs34911341), Leu72Met (rs696217) and Gln90Leu (rs4684677) (Figure 4).

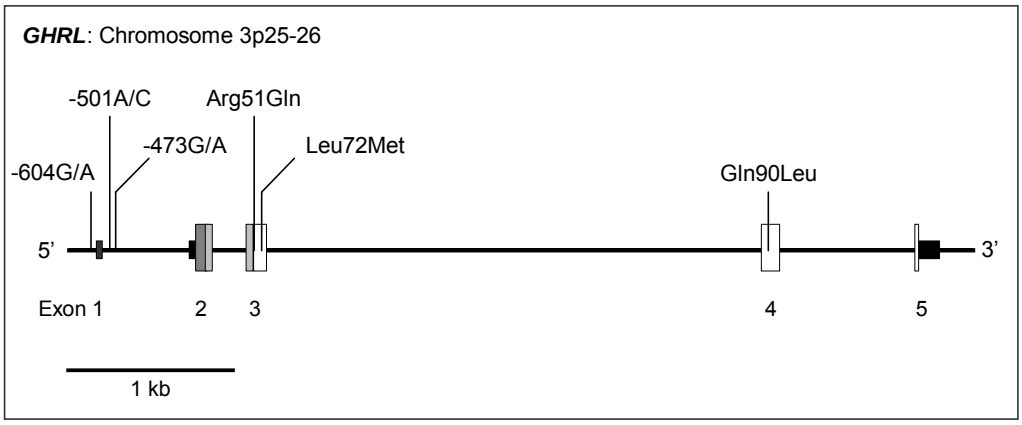

Figure 4. Polymorphic sites in the 5'-region and the coding sequence of *GHRL* (modified from *Study II* [377]). Black boxes, untranslated region; dark grey box, signal peptide; light grey boxes, mature ghrelin peptide; white boxes, cleaved from mature form.

4.2.2.2 *GHSR* polymorphisms

For selection of *GHSR* SNPs for genotyping, the HapMap [378], NCBI [336] and Ensembl [379] databases and previously published reports were used. Publicly available genotyping data for the genomic regions of interest were examined with Haploview software [380]. Linkage disequilibrium (LD) statistics were calculated and the haplotype blocks visualized. SNPs were either selected manually or using Tagger software (http://www.broad.mit.edu/mpg/tagger/server.html) [381].

Originally, six tag SNPs from two haploblocks covering 15 kb of the gene with three SNPs in the 5' end of the gene (rs474225, rs490683 (alternative name in HapMap rs863441), and rs9819506), one in the coding region in exon 1 leading to a synonymous amino acid

substitution (rs495225), one in the intron (rs509035) and one in the 3' end of the *GHSR* gene (rs565105) were selected (Figure 5). In addition, all SNPs in haploblock 1 in the 5'-regulatory region (spanning over 17 kb, chr 3: 173648897-17365897) available in the HapMap database and located in putative TF-binding sites were tested in gelshift experiments. SNPs showing differential protein binding were genotyped additionally (rs6772676).

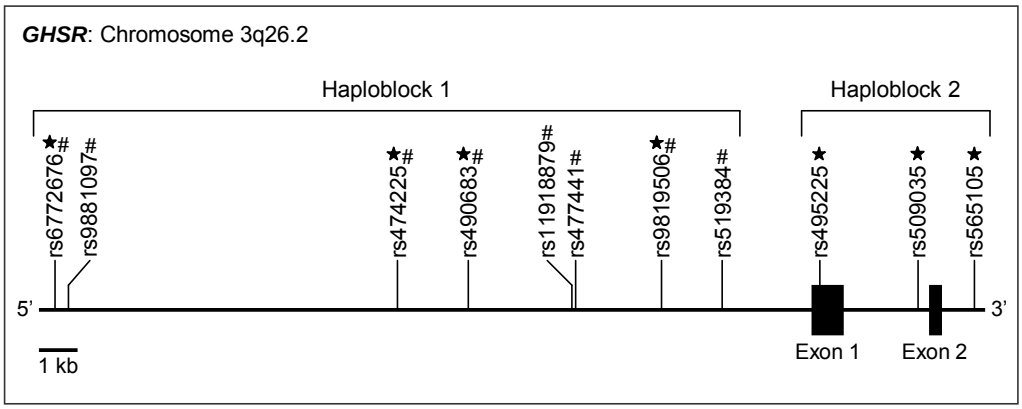

Figure 5. Schematic representation of the human *GHSR* gene indicating the locations of analysed SNPs belonging to two haploblocks (*Study III*).
★ SNPs genotyped in DPS population
SNPs from HapMap database in TF-binding sites and tested in gelshift assays

4.2.3 DNA analysis

4.2.3.1 DNA extraction

Genomic DNA was prepared from peripheral blood leukocytes by the salting out method [382].

4.2.3.2 Restriction fragment length polymorphism analysis

Polymerase chain reactions (PCR) were performed with thermo cyclers (GeneAmp PCR system 2700, Applied Biosystems, Foster City, CA, USA). The six SNPs in the *GHRL* gene were detected by PCR based-restriction fragment length polymorphism analysis (PCR-RFLP). The genomic DNA was amplified by PCR, followed by digestion with specific restriction enzymes (Fermentas, Tamro Medlab OY, Vantaa, Finland) for three hours. The primers and restriction enzymes used are presented in Table 9. The fragments were separated on an agarose gel and visualized under ultraviolet light after staining with ethidium bromide (Figure 6). Approximately 10% of the samples were repeated.

Table 9. Primers used for amplification of regions where SNPs of interest in the *GHRL* gene are located and respective restriction enzymes used for digestion of PCR fragments

SNP	Primer 5' to 3'	Restriction enzyme (Temperature)
-604G/A	F: CACAGCAACAAAGCTGCACC R: AAGTCCAGCCAGAGCATGCC	*Dra*I (37°C)
-501A/C	F: AGAACAAACGCCAGTCATCC R: GTCTTCCAGCCAGACAGTCC	*Mwo*I (37°C)
-473G/A	same as for -501A/C	*Fok*I (55°C)
Arg51Gln	F: GCTGGGCTCCTACCTGAGC R: GGACCCTGTTCACTGCCAC	*Sac*I (37°C)
Leu72Met	same as for Arg51Gln	*Bsr*I (65°C)
Gln90Leu	F: GAGGTGTCACTCAGCAGTCC R: TCTTCTTCTTCAGGGCCTGGCTGTGCTGCTAGTAC (mismatch nucleotide introduced to the primer is underlined)	*Sca*I (37°C)

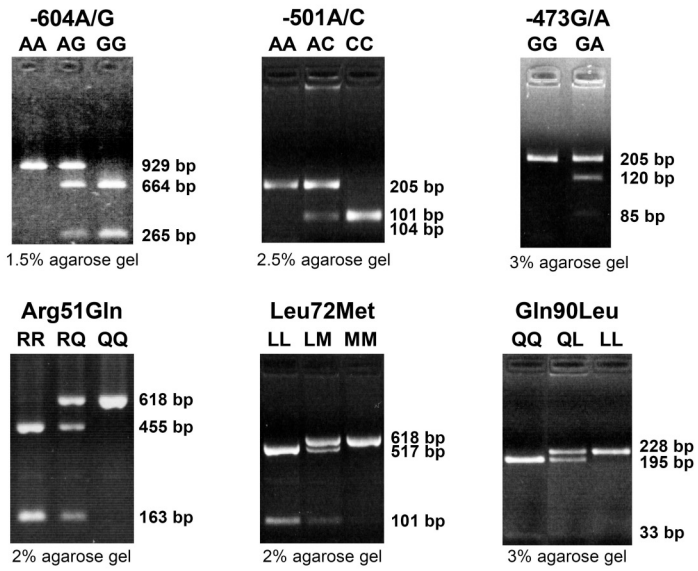

Figure 6.
PCR-RFLP analysis of the six SNPs in the *GHRL* gene and its promoter. In the gel pictures, the first lane always represents the wild-type homozygotes, the second lane the heterozygotes, and the third lane the mutated homozygotes. For the three SNPs in the prepro-ghrelin protein (Arg51Gln, Leu72Met, and Gln90Leu), the lanes are named with one-letter amino acid codes: R = Arg, Q = Gln, L = Leu, M = Met. Representative gels are shown (adapted from *Study II* [377]).

4.2.3.3 TaqMan allelic discrimination assay

Genotyping of *GHSR* SNPs was performed with TaqMan Allelic Discrimination Assays (Applied Biosystems, Foster City, CA, USA). The PCR amplification was performed in a GeneAmp PCR system 2700 (Applied Biosystems, Foster City, CA, USA) under the following conditions: 95°C for 10 min and 40 cycles of denaturation 92°C for 15 sec and annealing/extension 60°C for 1 min. The assay IDs from Applied Biosystems for the corresponding SNPs used are presented in Table 10. Fluorescence was detected on an ABI

Prism 7000 sequence detector. A subset of randomly selected samples representing 6.3% of the study cohort was repeated.

Table 10. TaqMan genotyping assay IDs for *GHSR* SNPs genotyped in *Study III*.

NCBI SNP Reference	Assay ID (Applied Biosystems)
rs6772676	C___1307047_10
rs474225	C___1079483_10
rs490683	C___2169194_10
rs9819506	C___2863288_10
rs495225	C___1079488_1
rs509035	C___2863306_10
rs565105	C___2399556_20

4.2.4 Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) from Genobin study participants (*Study IV*) were isolated from anticoagulated peripheral blood by density centrifugation using the Lymphoprep® reagent (Axis-Shield, Norway) according to the manufacturer's instructions. Altogether, 56 samples for all three time-points were available for 24 subjects in the weight-reduction group, 9 subjects in the resistance training group, 13 subjects in the aerobic training group, and 10 subjects in the control group. Only those subjects from whom PBMC samples were available at all three time-points were included in the analysis.

4.2.5 Adipose tissue biopsy

Adipose tissue samples from Genobin study participants (*Study IV*) were taken by syringe from subcutaneous abdominal adipose tissue under local anaesthesia after an overnight fast to collect 0.5-5 g of adipose tissue for adipocyte isolation and RNA extraction [369]. Adipose tissue samples for the mRNA expression studies were washed twice with PBS and treated with RNAlater (Ambion, Austin, TX) according to the instructions provided by the manufacturer, and stored at -80°C until used for RNA extraction.

4.2.6 RNA isolation

Total RNA from adipose tissue from Genobin study participants (*Study IV*) was extracted using the TRIzol method followed by further purification with RNeasy Mini Kit columns according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA, and Qiagen, Valencia, CA, USA). Total RNA from PBMCs was isolated using RNeasy Mini Kit columns according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). The RNA concentration and the A_{260}/A_{280} ratio was measured using a NanoDrop spectrophotometer (NanoDrop Technologies, DE, USA), with an acceptable ratio being 1.9-2.1. Integrity of the RNA was assessed using agarose gel electrophoresis.

4.2.7 Quantitative real time polymerase chain reaction

RNA was reverse transcribed into cDNA using High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time PCR analyses (S*tudy IV*) were performed with TaqMan® chemistry using ready made assays according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA); the assays used in this study are presented in Table 11. Samples were analysed in triplets with an Applied Biosystems 7500 Real-Time PCR System (Foster City, CA, USA). The absolute quantification was used with a standard curve, using points of 0.5, 1.5, 6, 18 and 36 ng and a calibrator of 6 ng of mRNA equivalent. The cDNA pool for the standard curve was created by combining cDNA from a representative number of subjects of all three time points for PBMC samples and from two time points for adipose tissue samples, respectively. Quantities on each plate were first corrected by the calibrator on the plate. Quantity values were finally normalised to the expression of the endogenous control, which was glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for PBMCs. Cyclophilin A1 was used as the endogenous control for human adipose tissue.

Table 11. Applied Biosystems assay IDs for studied genes.

Gene	Gene ID	AB assay ID
Ghrelin	GHRL	Hs00175082_m1
Growth hormone secretagogue receptor 1a	GHSR 1a	Hs01026313_m1
Growth hormone secretagogue receptor 1b	GHSR 1b	Hs01031463_s1
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	Hs99999905_m1
Peptidylprolyl isomerase A (cyclophilin A1)	PPIA	Hs99999904_m1
Tumour necrosis factor (TNF-alpha)	TNF	Hs00174128_m1
Interleukin 1, beta	IL1B	Hs00174097_m1
Interleukin 6	IL6	Hs00174131_m1

4.2.8 Protein extraction

Study III: Hypothalami of three male Sprague-Dawley rats (supplier Harlan, Netherlands) were pooled and the frozen tissue was homogenised in 0.5 ml of low salt buffer (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 50 mM KCl, 0.5 mM dithiothreitol, proteinase inhibitors) using a rotor-stator homogeniser (ART Labortechnik, Müllheim, Germany). Cells were then resuspended in 2-3 packed volumes of the same low salt buffer with 0.5% Nonidet P-40 and homogenised by pipetting on ice. The nuclear pellet was subsequently resuspended in one packed cell volume of high salt buffer (10 mM Hepes, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, proteinase inhibitors). Extraction was performed for 30 min on ice.

4.2.9 Gelshift assays

Gelshift assays (S*tudy III*) were performed with 10 µg of protein of the nuclear extracts. The proteins were incubated for 15 min in a total volume of 20 µl of binding buffer (150 mM KCl, 1 mM dithiothreitol, 25 ng/ml herring sperm DNA, 5% glycerol, 10 mM Hepes, pH 7.9). Constant amounts (1 ng) of ^{32}P-labeled double-stranded oligonucleotides (50,000 cpm) containing either one of the respective SNP alleles (Table 12) were then added, and incubation was continued for 20 min at room temperature. Protein-DNA complexes were resolved by electrophoresis in 8% non-denaturing polyacrylamide gels (mono- to bisacrylamide ratio 19:1) in 0.5 x TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.3) for 90 min at 200 V and quantified on a FLA-3000 reader (Fuji, Tokyo, Japan) using ScienceLab99 software (Fuji).

Table 12. Oligonucleotides used in gelshift assays (S*tudy III*).

SNP	Oligo sequence 5'-3'
rs6772676	AGCCTCAACACCTGA**C**GATTTTTCAGGG AGCCTCAACACCTGA**T**GATTTTTCAGGG
rs9881097	GGTTCTACCTTCCT**T**AGTTAAGCTTCATCC GGTTCTACCTTCCT**C**AGTTAAGCTTCATCC
rs474225	GCCTTTG**T**TTCCCTTTCATCT GCCTTTG**C**TTCCCTTTCATCT
rs490683	CCATATAAAAGAGGT**C**CCAGAAAGCT CCATATAAAAGAGGT**G**CCAGAAAGCT
rs11918879	ACTTTTTAATAAATGATG**C**TGAAACAACTG ACTTTTTAATAAATGATG**T**TGAAACAACTG
rs477441	ACTAAAGCCA**A**AAAATAATAGAACA ACTAAAGCCA**C**AAAATAATAGAACA
rs9819506	AGCTGGAAT**G**CATATAAGTGACAT AGCTGGAAT**A**CATATAAGTGACAT
rs519384	TTAAAAACTC**A**ATTTGGGGCCG TTAAAAACTC**T**ATTTGGGGCCG

Nucleotides in bold denote the two alleles for the respective SNP.

4.2.10 Promoter analysis

In silico promoter analysis (S*tudy III*) of the sequences containing all *GHSR* SNPs in haploblock 1 for putative TF binding sites was performed using Genomatix MatInspector Release professional 7.4.8.2 [383]. We determined all SNPs which lie in possible TF binding sites and consequently all TF binding sites which are disrupted by either one of each SNP allele. We used a threshold of 0.75 for core similarity and the optimized setting for matrix similarity.

4.2.11 Statistical analysis

Statistical analyses were performed using SPSS software versions 11.5.1 (*Studies I and II*) and 14.0 (*Studies III and IV*) for Windows (SPSS Inc., Chicago, IL, USA). Normal distribution was tested using the Kolmogorow-Smirnov test with Lilliefors correction. To normalise skewed distributions, logarithmic transformations or reciprocal transformations were applied when needed. Homogeneity of variances was tested using Levene's test. A p-value of 0.05 or lower was considered statistically significant. Data are given as means ± SD, unless otherwise stated. To correct for multiple hypothesis testing (*Study III*), the false discovery rate (FDR) was calculated using Q-value 1.0 software [384]. π_0 was estimated with the bootstrap method using a λ range from 0 to 0.9 by 0.05. The FDR for each p-value is reported as q. LD statistics and Hardy-Weinberg disequilibrium were calculated, and haplotype blocks were visualized using Haploview software [380].

Comparisons of continuous variables between the genotype or genotype combination groups were evaluated with the general linear model for the univariate analysis of variance (ANOVA) for normally distributed variables, and the nonparametric Mann-Whitney or Kruskal-Wallis tests for non-normally distributed variables. Categorical variables were compared with a χ^2 test or a two-sided Fisher's exact test. The paired samples t-test was applied for testing differences in continuous variables of interest between baseline and week 12 or week 33 (*Study IV*). To test the difference between the ghrelin concentrations of men and women, the independent samples t-test was used, and univariate ANOVA was applied to test the difference in ghrelin plasma levels at baseline between normal-weight and overweight/obese subjects with features of metabolic syndrome (*Study IV*). Logistic regression (*Study I*) or Cox regression analyses (*Study III*) with appropriate covariates were applied to evaluate whether the SNPs predicted the conversion from IGT to type 2 diabetes. Repeated measures ANOVA was used to analyse differences between the genotype of each SNP during the follow-up period for four consecutive measurements for the variables of interest with appropriate covariates included in the models (*Study III*). Genotype*group interaction was tested, and the study groups were analysed separately if the interaction term was significant (*Study III*). Repeated measures ANOVA was also used for testing the interaction between ghrelin concentrations and the study groups during the whole study period with the measurements from baseline, and at week 12 and week 33 (*Study IV*). P-values are for trends between all tested groups, and pairwise comparisons based on estimated marginal means were adjusted for multiple comparisons with Bonferroni correction. The linear mixed models analysis for repeated measures data was applied to analyse differences between genotype combination groups in systolic and diastolic BP levels of four measurements (*Study II*) as well as to test the relationship between plasma ghrelin levels and relevant variables longitudinally (*Study IV*). Correlations between changes in plasma ghrelin levels with other measurements were performed using Pearson's correlation, and linear

regression analysis was used to test correlations between the gene expression of ghrelin and that of GHSR 1b in PBMCs, as well as for expression of several cytokines in PBMCs with appropriate covariates (*Study IV*). Spearman correlations were used when variables were not normally distributed after logarithmic transformation (*Study IV*). Relative changes in weight and measures of glucose and insulin metabolism from baseline to year 3 were analysed with the Kruskal-Wallis test, since data were not normally distributed even after applying several transformations (*Study III*).

5 Results

5.1 General characteristics of study populations

Both the Finnish DPS and the Genobin study included middle-aged Finnish persons with overweight or obesity and insulin resistance. In the DPS, this high-risk group for developing type 2 diabetes was randomly divided into an intervention and control group, with the incidence of type 2 diabetes providing the main outcome measure [385]. During a mean intervention period of about 3 years, a 58% relative risk reduction in the progression from IGT to type 2 diabetes was achieved [366, 386]. Persons in the intervention group lost significantly more weight than those in the control group during the 3-year follow-up period (intervention group: -3.4±5.2 kg, control group: -0.6±5.2 kg, p<0.0001).

In the Genobin study, the individuals were randomised into four study groups. In this study, only participants in the weight reduction group lost weight significantly during the 33-week intervention period (-4.6±3.9 kg, p<0.001) [369]. Subjects in both exercise groups showed no significant change in their body weight (resistance training group: -1.0±2.6 kg, p=0.190; aerobic training group: -1.6±3.5 kg, p=0.103), nor did the subjects in the overweight control group (-0.2±1.5 kg, p=0.576).

Tables 13 and 14 show the baseline characteristics of the participants in the Finnish DPS and the Genobin study, respectively.

Table 13. Baseline characteristics of the DPS participants altogether and separately in the intervention and control group with available DNA.

Characteristic	All individuals (n=507)	Intervention group (n=259)	Control group (n=248)
Sex (men/women)	166/341	88/171	78/170
Age (years)	55±7 (507)	55±7 (259)	55±7 (248)
Weight (kg)	86.2±14.2 (507)	86.6±14.0 (259)	85.8±14.4 (248)
BMI (kg/m^2)	31.2±4.5 (507)	31.3±4.5 (259)	31.2±4.5 (248)
Waist circumference (cm)	101.2±11.0 (505)	101.8±11.0 (258)	100.6±11.0 (247)
Systolic blood pressure (mmHg)*	138.2±17.6 (502)	139.9±17.5 (258)#	136.3±17.6 (244)#
Diastolic blood pressure (mmHg)*	85.7±9.6 (502)	85.8±9.2 (258)	85.6±9.9 (244)
Fasting plasma glucose (mmol/L)	6.14±0.75 (507)	6.11±0.77 (259)	6.16±0.74 (248)
2-h plasma glucose (mmol/L)	8.88±1.49 (507)	8.87±1.52 (259)	8.89±1.47 (248)
Fasting serum insulin (mU/L)	14.77±7.40 (461)	14.77±7.31 (234)	14.77±7.50 (227)
2-h serum insulin (mU/L)	95.17±65.10 (458)	97.94±74.26 (234)	92.27±53.91 (224)
HOMA-IR (mmol x mU x l^{-2})	4.11±2.29 (461)	4.09±2.25 (234)	4.11±2.33 (227)

Values are means ± SD (n); to convert values for insulin to pmol/L, multiply by 6. *Antihypertensive drugs were being taken by 35.1% of subjects in the intervention group and 36.6% in the control group. #p=0.016 for the comparison between intervention and control group by two-tailed t-test.

Table 14. Baseline characteristics of the Genobin study participants altogether and separately in the intervention and control groups.

Characteristic	All individuals (n=75)	Weight reduction group (n=28)	Resistance exercise training group (n=14)	Aerobic exercise training group (n=15)	Control group (n=18)
Sex (men/women)	37/38	12/16	7/7	10/5	8/10
Age (years)	60±7 (75)	59±7 (28)	62±6 (14)	59±5 (15)	61±7 (18)
Fasting plasma ghrelin (pg/ml)	824.6±236.4 (75)	827.6±272.8 (28)	790.1±184.3 (14)	808.3±185.3 (15)	860.6±261.0 (18)
Weight (kg)	92.6±12.8 (75)	92.8±15.1 (28)	92.7±11.2 (14)	97.7±12.9 (15)	87.9±8.3 (18)
BMI (kg/m^2)	32.9±2.8 (75)	32.9±3.2 (28)	33.5±3.1 (14)	32.8±2.2 (15)	32.4±2.5 (18)
Waist circumference (cm)	108.6±8.8 (75)	108.2±8.6 (28)	110.5±9.5 (14)	111.7±9.8 (15)	105.4±7.2 (18)
Systolic blood pressure (mmHg)	136.8±13.1 (75)	137.5±16.7 (28)	139.4±10.4 (14)	133.5±9.0 (15)	136.6±11.7 (18)
Diastolic blood pressure (mmHg)	88.7±9.5 (75)	89.8±9.8 (28)	89.1±12.3 (14)	88.6±5.4 (15)	86.6±9.9 (18)
Fasting plasma glucose (mmol/L)	6.44±0.46 (75)	6.44±0.49 (28)	6.31±0.40 (14)	6.52±0.51 (15)	6.48±0.43 (18)
2-h plasma glucose (mmol/L)	7.50±2.10 (75)	6.88±1.99 (28)	7.83±1.60 (14)	7.71±2.27 (15)	8.03±2.37 (18)
Fasting serum insulin (mU/L)	12.65±7.75 (75)	12.70±6.10 (28)	14.44±10.91 (14)	11.43±4.85 (15)	12.18±9.38 (18)
2-h serum insulin (mU/L)	87.92±71.09 (75)	89.13±83.27 (28)	102.60±49.74 (14)	77.81±63.55 (15)	83.06±73.95 (18)
S_I ($(mU/l)^{-1} \times min^{-1}$)	2.17±1.10 (72)	2.32±1.38 (26)	1.66±0.62 (14)	2.09±1.13 (15)	2.43±0.79 (17)
AIR ($(mU/l)^{-1} \times min^{-1}$)	4.58±3.92 (71)	5.04±4.04 (26)	4.99±3.29 (14)	3.40±3.02 (15)	4.60±4.99 (16)
Ghrelin mRNA expression (AU)					
PBMCs	107.9±43.2 (56)	124.5±51.2 (24)	94.0±15.8 (9)	81.5±16.6 (13)	115.0±46.1 (10)*
Adipose tissue	89.3±45.3 (70)	91.0±45.0 (26)	60.5±30.1 (13)	109.8±53.9 (13)	93.0±41.2 (18)#

Values are means ± SD (n); to convert values for insulin to pmol/L, multiply by 6. *p=0.021 for the comparison between the four study groups by Kruskal-Wallis test. #p=0.025 for the comparison between the four study groups by Kruskal-Wallis test (adapted from *Study IV* [370]).

5.2 Genetic variations in the *GHRL* and *GHSR* genes

5.2.1 Genotype frequencies of *GHRL* and *GHSR* polymorphisms

The observed genotype frequencies in all *GHRL* and *GHSR* SNPs were in Hardy-Weinberg equilibrium. The genotype frequencies for all studied polymorphisms are shown in Table 15.

Table 15. Genotype frequencies of *GHRL* and *GHSR* SNPs in the DPS and Genobin study populations.

***GHRL* SNP**	**Genotype**	**Genotype frequency in DPS (*n*)**	**Genotype frequency in Genobin study (*n*)**
-604G/A	GG	0.134 (68)	0.213 (16)
	GA	0.489 (248)	0.467 (35)
	AA	0.377 (191)	0.320 (24)
-501A/C	AA	0.540 (274)	0.613 (46)
	AC	0.373 (189)	0.293 (22)
	CC	0.087 (44)	0.093 (7)
-473G/A	GG	0.976 (495)	0.987 (74)
	GA	0.024 (12)	0.013 (1)
	AA	-	-
Arg51Gln	Arg51Arg	0.943 (478)	0.933 (70)
	Arg51Gln	0.055 (28)	0.067 (5)
	Gln51Gln	0.002 (1)	-
Leu72Met	Leu72Leu	0.769 (390)	0.773 (58)
	Leu72Met	0.203 (103)	0.200 (15)
	Met72Met	0.028 (14)	0.027 (2)
Gln90Leu	Gln90Gln	0.730 (370)	0.773 (58)
	Gln90Leu	0.250 (127)	0.200 (15)
	Leu90Leu	0.020 (10)	0.027 (2)
***GHSR* SNP**	**Genotype**	**Genotype frequency in DPS (*n*)**	**Genotype frequency in Genobin study (*n*)**
rs6772676	CC	0.229 (116)	0.280 (21)
	CT	0.485 (246)	0.413 (31)
	TT	0.286 (145)	0.307 (23)
rs474225	TT	0.037 (19)	0.027 (2)
	TC	0.260 (132)	0.320 (24)
	CC	0.702 (356)	0.653 (49)
rs490683	CC	0.091 (46)	0.133 (10)
	CG	0.424 (215)	0.333 (25)
	GG	0.485 (246)	0.533 (40)
rs9819506	GG	0.389 (197)	0.413 (31)
	GA	0.465 (236)	0.440 (33)
	AA	0.146 (74)	0.147 (11)
rs495225	TT	0.481 (244)	0.547 (41)
	TC	0.412 (209)	0.360 (27)
	CC	0.107 (54)	0.093 (7)
rs509035	CC	0.426 (216)	0.467 (35)
	CT	0.464 (235)	0.373 (28)
	TT	0.110 (56)	0.160 (12)
rs565105	CC	0.099 (50)	0.093 (7)
	CA	0.406 (206)	0.360 (27)
	AA	0.495 (251)	0.547 (75)

5.2.2 Association between SNPs in the *GHRL* gene and conversion to type 2 diabetes (*Study I*)

During the first three years of the DPS, 21 subjects (8.7%) in the intervention group and 51 subjects (21.4%) in the control group developed type 2 diabetes. Only the Leu72Met SNP of *GHRL* was associated with conversion from IGT to type 2 diabetes. In the entire study population, the conversion rate to type 2 diabetes was 13.2% among subjects with the Leu72Leu genotype, 22.1% among subjects with the Leu72Met genotype and 15.4% among subjects with the Met72Met genotype (p=0.096). In the intervention group, 6.3% of the subjects with the Leu72Leu, 20.9% with the Leu72Met and none of subjects with the Met72Met genotype converted to diabetes (p=0.006; Figure 7). In the control group, 20.6% of the subjects with the Leu72Leu, 23.1% with the Leu72Met and 33.3% with the Met72Met genotype converted to diabetes (p=0.715; Figure 7).

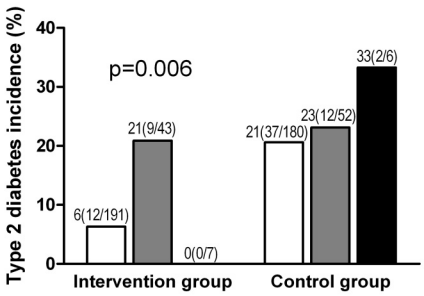

Figure 7. Three-year incidence of type 2 diabetes according to the Leu72Met polymorphism in the intervention and control group of DPS [% (number of subjects who converted to diabetes / total number of subjects)]. Intervention group p=0.006 and control group p=0.715 for comparison among all three genotypes (χ^2 test); white bars, Leu72Leu; dark grey bars, Leu72Met; black bar, Met72Met (modified from *Study I* [387]).

Individuals homozygous for the Leu72 allele were less likely to convert to type 2 diabetes than were those subjects with the other genotypes in the entire DPS population (OR 0.47, 95%CI: 0.26-0.87, p=0.016) and in the intervention group separately under a dominant model of inheritance (OR 0.28, 95%CI: 0.10-0.79, p=0.016). When a codominant model was evaluated, persons with Leu72Met genotype had a higher risk of converting to type 2 diabetes than did the Leu72Leu subjects in the entire DPS population (OR 2.16, 95%CI: 1.15-4.08, p=0.017) or in the intervention group separately (OR 4.38, 95%CI: 1.50-12.75, p=0.007). In this model, homozygosity for the Met72 allele was not associated with an increase in risk of conversion to type 2 diabetes.

None of the *GHSR* polymorphisms were statistically significantly associated with the risk of type 2 diabetes (*Study III*).

5.2.3 Association between SNPs in the *GHSR* gene and glucose metabolism (*Study III*)

Differences between genotypes were observed with the rs6772676 SNP for fasting (p/q=0.038/0.035) and 2-h plasma glucose (p/q=0.006/0.035) in the whole study population when data were analyzed longitudinally, with heterozygotes showing the highest levels.

In the entire DPS population, 2-h plasma glucose (Figure 8) and 2-h serum insulin levels differed according to the *GHSR* rs490683 genotypes when the data were analysed longitudinally (p/q=0.015/0.035, p/q=0.050/0.041, respectively). Subjects with rs490683-CC genotype tended to have the lowest values.

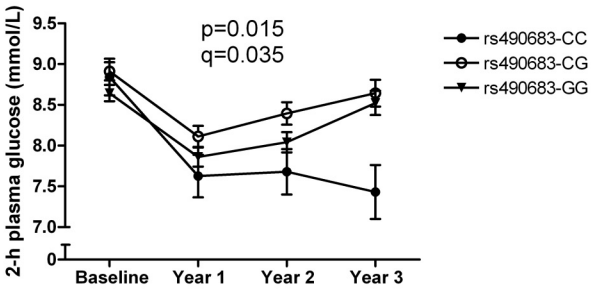

Figure 8. 2-h plasma glucose according to rs490683 genotypes in all DPS participants (*Study III*). Mean ± SEM are estimated marginal means calculated from repeated measures ANOVA with group, age and BMI at baseline as significant covariates. Pairwise comparisons: CC vs. CG p=0.020, CC vs. GG p=0.295, CG vs. GG p=0.190.

When relative changes between baseline and year 3 were analysed in the whole study population, differences between rs490683 genotypes were seen in fasting plasma glucose, 2-h plasma glucose, 2-h serum insulin, and a trend was observed for relative change in fasting serum insulin (Table 16). When the study groups were analysed separately, significant differences between the rs490683 genotypes according to fasting and 2-h plasma glucose, fasting and 2-h serum insulin were observed in the control group (Table 16), but not in the intervention group. Specifically, in the control group these concentrations were decreased in subjects with rs490683-CC genotype and increased in subjects with rs490683-GG genotype.

Table 16. Relative changes in fasting and 2-h plasma glucose, fasting and 2-h serum insulin according to genotypes of the *GHSR* rs490683 for the entire DPS population and intervention and control group separately.

	rs490683-CC (*n*)	**rs490683-CG (*n*)**	**rs490683-GG (*n*)**	**P-value**	**Q-value**
Change in fasting glucose (%)					
All subjects	-2.6 ± 1.7 (46)	2.1 ± 0.9 (212)	2.0 ± 0.8 (242)	0.014	0.069
Intervention group	-1.9 ± 2.0 (28)	0.9 ± 1.2 (104)	-0.7 ± 1.1 (122)	0.310	0.785
Control group	-3.7 ± 3.2 (18)	3.2 ± 1.3 (108)	4.7 ± 1.2 (120)	0.004	0.078
Change in 2-h glucose (%)					
All subjects	-12.9 ± 3.3 (46)	4.0 ± 2.1 (211)	1.5 ± 1.8 (242)	0.001	0.020
Intervention group	-11.1 ± 4.4 (28)	-0.4 ± 2.8 (104)	-2.0 ± 2.6 (122)	0.164	0.785
Control group	-15.6 ± 5.1 (18)	8.2 ± 3.0 (107)	5.1 ± 2.5 (120)	0.007	0.100
Change in fasting insulin (%)					
All subjects	-11.5 ± 6.0 (39)	-0.1 ± 3.0 (189)	9.0 ± 5.8 (226)	0.067	0.101
Intervention group	-7.0 ± 9.7 (22)	-5.1 ± 3.6 (91)	-8.4 ± 3.2 (116)	0.755	0.871
Control group	-17.4 ± 5.7 (17)	4.4 ± 4.6 (98)	27.3 ± 11.2 (110)	0.004	0.078
Change in 2-h insulin (%)					
All subjects	-21.5 ± 7.6 (38)	0.1 ± 5.2 (185)	1.1 ± 5.8 (219)	0.045	0.086
Intervention group	-17.8 ± 12.1 (22)	-8.0 ± 6.9 (91)	-11.7 ± 7.9 (112)	0.339	0.785
Control group	-26.5 ± 7.3 (16)	7.9 ± 7.7 (94)	14.6 ± 8.5 (107)	0.029	0.245

Data are means ± SEM.

Similar results to those with rs490683 were also observed with rs509035. In the entire study population, 2-h plasma glucose and fasting serum insulin levels differed according to the rs509035 genotypes when the data were analysed longitudinally (p/q=0.014/0.035 and p/q=0.037/0.035, respectively), with rs509035-TT genotype showing the lowest values.

When relative changes between baseline and year 3 were analysed for the whole study population, differences between rs509035 genotypes were seen in fasting plasma glucose (p/q=0.046/0.086), 2-h plasma glucose (p/q=0.011/0.069) and 2-h serum insulin (p/q=0.037/0.086). When the study groups were analysed separately, significant differences between the rs509035 genotypes according to 2-h plasma glucose, fasting and 2-h serum insulin were observed in the control group (p/q=0.038/0.267, p/q=0.050/0.300, and p/q=0.014/0.152, respectively), but not in the intervention group. These levels were decreased in subjects with rs509035-TT genotype in the control group during the study period.

5.2.4 Association between SNPs in the *GHRL* gene and blood pressure and hypertension (*Study II*)

In the DPS population at baseline, the -604G/A SNP of *GHRL* was significantly associated with systolic BP (p=0.019, -604GG: 132.7 ± 14.0, -604GA: 139.0 ± 19.0, -604AA: 138.9 ± 16.6 mmHg, respectively). When data were analysed longitudinally, *GHRL* SNPs -604G/A, -501A/C and Leu72Met were associated with systolic and diastolic BP. Persons with -604GG genotype had the lowest systolic and diastolic BP levels during the four consecutive measurements (Figure 9 A, B).

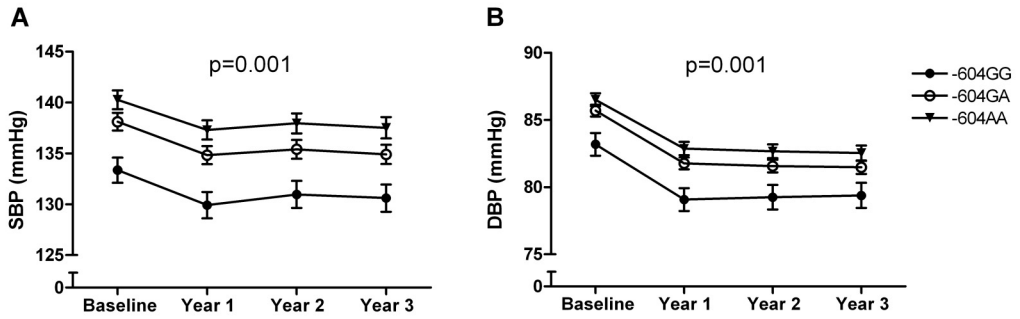

Figure 9. Mean values ± SEM calculated from linear mixed model analysis. **A**. Systolic BP levels in the entire DPS population. Pairwise comparisons: GG vs. GA: p=0.034, GG vs. AA p<0.001. **B**. Diastolic BP levels in the entire DPS population. Pairwise comparisons: GG vs. AA p=0.001, GG vs. GA p=0.071.

Individuals with -501CC genotype had the highest systolic and diastolic BP levels during the four consecutive measurements (Figure 10 A, B).

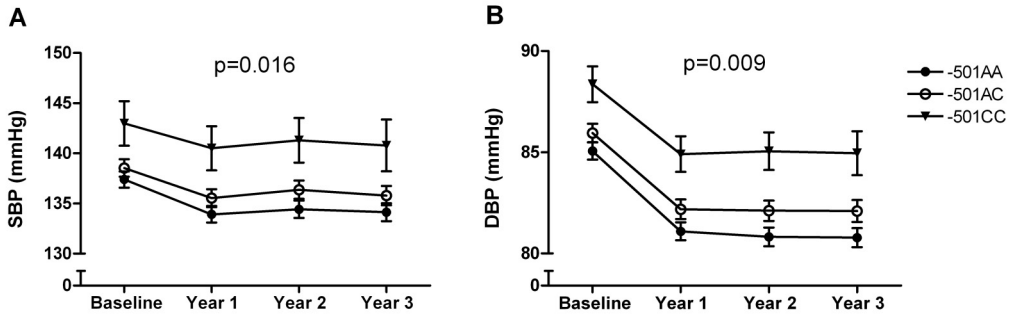

Figure 10. Mean values ± SEM calculated from linear mixed model analysis. **A**. Systolic BP levels in the entire DPS population. Pairwise comparisons: AA vs. CC: p=0.013, AC vs. CC p=0.086. **B**. Diastolic BP levels in the entire DPS population. Pairwise comparisons: AA vs. CC p=0.007, AC vs. CC p=0.059.

In addition, the Leu72Met SNP was associated longitudinally with systolic (p=0.013 for comparison among all three genotypes) and diastolic BP (p=0.017 for comparison among all three genotypes), with Leu72Leu subjects exhibiting the lowest levels.

The four most common *GHRL* SNPs (-604G/A, -501A/C, Leu72Met and Gln90Leu) represented a haplotype block and formed seven major haplotypes. The most common haplotype with a frequency of 0.346 in the DPS population included the -604G, -501A, Leu72 and Gln90 alleles of the four SNPs. Subsequently, we compared subjects who had simultaneously the -604GG, -501AA, Leu72Leu and Gln90Gln genotypes (0000), and therefore harboured the most common haplotype, with all other subjects. Similar to the single SNP analyses, subjects with the 0000 genotype combination had significantly lower systolic (p=0.003) and diastolic BP (p=0.004) than subjects with other genotype combinations at four consecutive measurements (*Study II*, Fig. 3 [377]). Although the prevalence of hypertension was not significantly different between the subjects with the 0000 genotype combination and the other subjects at baseline (57.7% vs. 64.7%, p=0.200), those subjects with the 0000 genotype combination had a significantly lower prevalence of hypertension at year 1 (40.0% vs. 58.9%, p=0.006) and year 2 (41.2% vs. 58.7%, p=0.013), and a similar trend was also seen at year 3 (46.8% vs. 59.2%, p=0.071) (*Study II*). When analysed with logistic regression, these results were reinforced, and the 0000 genotype combination was found to be protective against hypertension in year 1 (OR=0.408, 95% CI: 0.225-0.739; p=0.003), year 2 (OR=0.438, 95% CI: 0.238-0.804; p=0.008) and year 3 (OR=0.533, 95% CI: 0.285-0.999; p=0.050) (*Study II* [377]).

In the Genobin study population at baseline, the *GHRL* Leu72Met SNP was associated with systolic BP (p=0.035; Leu72Leu: 138.0 ± 13.3, Leu72Met: 130.3 ± 10.3, Met72Met: 151.0 ± 2.8 mmHg). When analysed longitudinally, the association between Leu72Met SNP and systolic BP was no longer significant (p=0.121).

5.2.5 Associations between SNPs in the *GHRL* or *GHSR* genes and obesity (*Study III*)

In the DPS population at baseline, the -604G/A SNP of *GHRL* was significantly associated with weight (p=0.033, -604GG: 87.6 ± 14.7, -604GA: 87.4 ± 14.8, -604AA: 84.1 ± 13.1 kg, respectively), and the Gln90Leu SNP was associated with waist circumference (p=0.050, Gln90Gln: 101.5 ± 10.7, Gln90Leu: 101.2 ± 11.7, Leu90Leu: 92.6 ± 9.1 cm, respectively). When the data were analysed longitudinally, subjects homozygous for the Leu90 allele (n=10) had significantly lower body weight over four consecutive measurements than did the subjects with Gln90Gln (p=0.014) or Gln90Leu genotype (p=0.029; Figure 11). Subjects with the Leu90Leu genotype also had lower BMI over the 3-year follow-up (p=0.015 for trend among all three genotype groups) compared to subjects homozygous for Gln90 (p=0.013) or the heterozygous subjects (p=0.029). Waist circumference was no longer significantly associated when the data were analysed longitudinally.

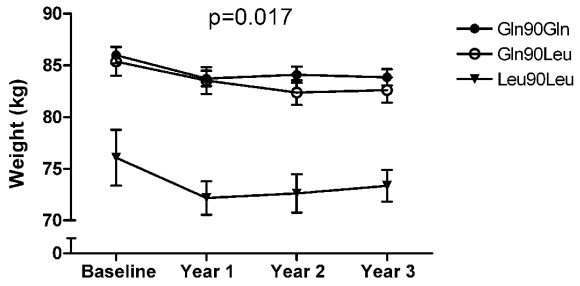

Figure 11. Weight (kg) during the study period of DPS according to the three genotypes of the Gln90Leu polymorphism. Adjusted mean values (± SEM) were calculated from linear mixed model analysis with sex, age, height and fat mass (%) as covariates included. Pairwise comparisons: Gln90Gln vs. Leu90Leu: p=0.014, Gln90Leu vs. Leu90Leu: p=0.029.

In the DPS population at baseline (*Study III*), the *GHSR* SNPs were not associated with obesity; however, in the follow-up data analysis, when the four distinct time-points were analysed simultaneously, a difference in weight was observed between the genotypes of rs9819506 (p/q=0.036/0.035, Figure 12).

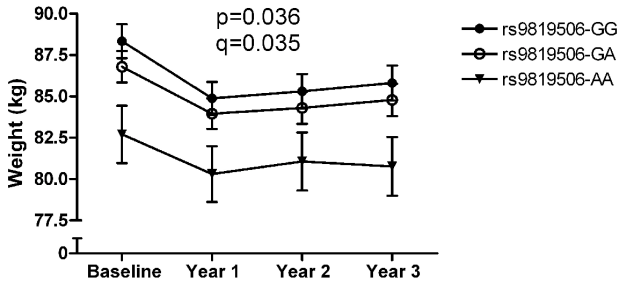

Figure 12. Weight according to rs9819506 genotypes in the entire DPS study population (*Study III*). Mean ± SEM are estimated marginal means calculated from repeated measures ANOVA with sex and age as significant covariates. Pairwise comparisons: GG vs. GA p=1.000, GG vs. AA p=0.030, GA vs. AA p=0.116.

We also studied the genotype effect on changes in body size measures during the 3-year intervention (*Study III*). When all subjects were analysed together, subjects with rs490683-CC genotype lost significantly more weight than did the others (p/q=0.032/0.086, Figure 13). When the two treatment groups were analysed separately, weight loss was most profound in the intervention group for subjects with the rs490683-CC genotype compared to the other genotypes (p/q=0.020/0.447), but no significant difference was observed in the weight change between the rs490683 genotypes in the control group (p/q=0.538/0.751). No significant differences in weight loss during the intervention period were seen with other *GHSR* SNPs.

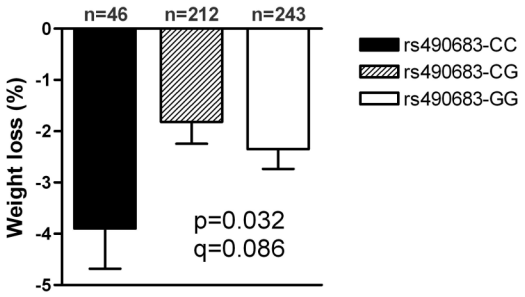

Figure 13. Relative weight loss according to the different genotypes of rs490683 in all DPS participants and separately in intervention and control group. (*Study III*).

5.3 *In silico* and *in vitro* analysis of the 5'-regulatory region of the *GHSR* gene (*Study III*)

The Genomatix MatInspector software [383] was used to identify putative TF binding sites in the 5'-region (haploblock 1) of the *GHSR* gene that may be implicated in the regulation of the gene expression. Screening of 20 kb upstream of the translation initiation site resulted in the identification of 8 SNPs which potentially disrupted one or more TF binding sites in either one of the alleles or both. Subsequently, oligonucleotides containing one or the other allele of each SNP were tested to identify possible differences in protein binding between the alleles of each SNP in gelshift assays using rat hypothalami as a TF protein source. The SNPs rs6772676 and rs490683 showed different protein binding when comparing both alleles. Specifically, when the protein binding of the so-called "wild-type" allele was normalised to 100%, the rs6772676-T allele showed only 28% protein binding compared to the rs6772676-C allele, whereas the rs490683-G allele showed 745% protein binding compared to the rs490683-C allele (Figure 14). The sequence with the rs490683-G allele contains a putative binding site for the TF NF-1, which is disrupted when G is replaced by C, changing the sequence from GCCA to CCCA.

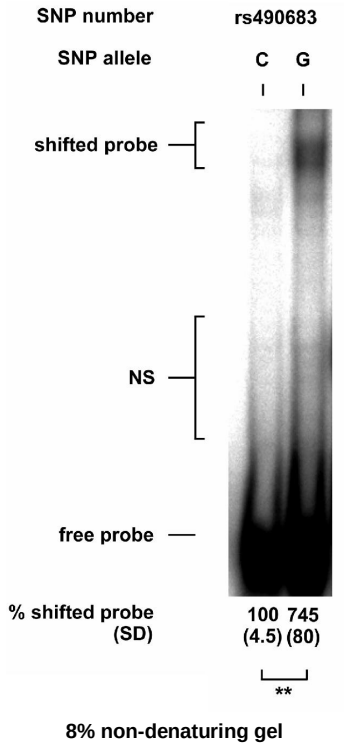

Figure 14. Differential protein binding of rat hypothalamus nuclear protein extract (*Study III*). Gelshift experiments were performed with ^{32}P-labeled oligonucleotides containing one or the other allele of respective single nucleotide polymorphism (SNP). Numbers below the gels indicate the means of three independent experiments with standard deviation (SD) in parenthesis. Protein binding of the "wild-type" allele was normalised to 100%. Representative gels are shown. NS denotes non-specific DNA-protein interactions, which are not due to specific binding between the labelled DNA and the proteins contained in the hypothalamus extract.
** $p<0.001$.

5.4 Gene expression studies

5.4.1 Subcutaneous adipose tissue

Ghrelin mRNA expression was detected in the subcutaneous adipose tissue samples of all study persons, with mean C_T-values of 33.9 (range 32.2-36.1) at baseline. The mean ghrelin expression at baseline was 89.3 in arbitrary units (AU) (range 20.8-276.4). The mean values for men and women were 96.1 and 82.5 AU, respectively (p=0.228). The intervention had no significant effect on ghrelin expression (data not shown). During the intervention, the study groups showed no difference regarding ghrelin expression in adipose tissue (ghrelin expression*group interaction p=0.409; group effect p=0.409). Ghrelin mRNA expression in subcutaneous adipose tissue was not significantly associated with ghrelin plasma levels or other metabolic parameters (data not shown).

Neither GHSR 1a nor type 1b mRNA expression could be detected in the subcutaneous adipose tissue of our study participants (data not shown).

5.4.2 Peripheral blood mononuclear cells (*Study IV*)

Both ghrelin and GHSR 1b mRNA were expressed in the PBMCs of all study participants, whereas no GHSR 1a mRNA expression could be detected. The mean ghrelin expression in

PBMCs at baseline was 107.9 in arbitrary units (AU; range 47.5-240.3), with mean C_T-values of 31.9 (range 31.1-33.3). The mean values for men and women were 99.6 and 116.9 AU at baseline, respectively (p=0.157). The intervention had no significant effect on ghrelin expression (data not shown). During the intervention, no differences were observed between the study groups regarding ghrelin expression in the PBMCs (ghrelin expression*group interaction p=0.778; group effect p=0.459); therefore, the groups were analysed together. The mean GHSR 1b expression in PBMCs at baseline was 94.8 AU (range 5.6-619.3), with mean C_T-values of 31.0 (range 27.2-33.7) and no significant difference being seen between men and women (mean 78.2±62.6 AU and 112.7±138.1 AU, respectively).

Ghrelin and GHSR 1b expressions were positively correlated at all three time points of the study (Figure 15 A). In a multivariate model, ghrelin mRNA expression in PBMCs was a significant predictor of GHSR 1b expression in PBMCs at baseline and week 33, and a trend was also seen at week 12 (Figure 15 A).

5.4.2.1 Association with inflammatory markers (*Study IV*)

The expression of several cytokines related to inflammation from PBMCs was also measured in order to determine whether a connection might exist between these cytokines and ghrelin expression in PBMCs. Ghrelin mRNA expression in PBMCs was found to have a significant positive correlation with TNF-α expression at baseline and week 12, with the same trend being seen in week 33 with borderline significance (Figure 14 B).

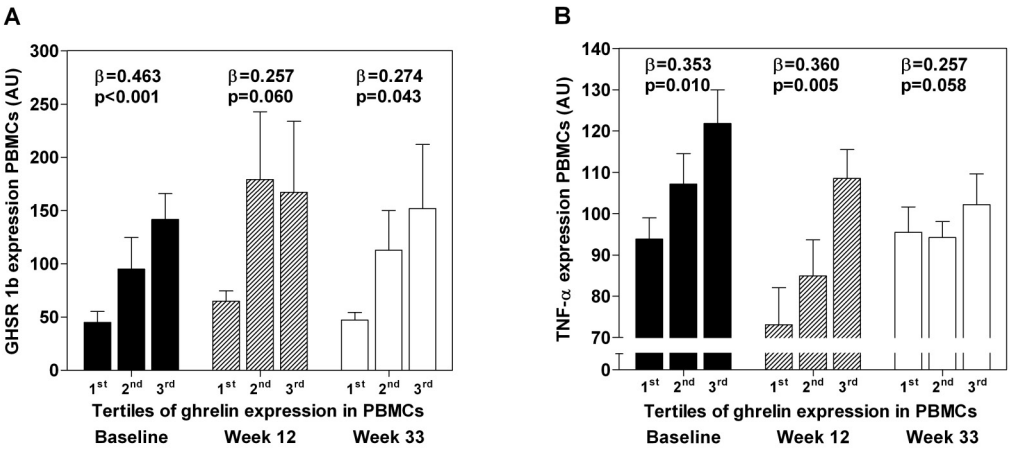

Figure 15. Variation of GHSR 1b (**A**) and TNF-α (**B**) mRNA expression in PBMCs according to tertiles of ghrelin mRNA expression in PBMCs at baseline, week 12 and week 33 of the Genobin study separately, respectively. Data are means and SEM. P-values obtained from linear regression analysis with GHSR 1b or TNF-α as dependent variable, respectively, and sex, age, BMI at the given time point as covariates; β = standardised regression coefficient.

IL-1β expression in PBMCs showed no correlation with ghrelin expression in the PBMCs at baseline (standardised regression coefficient β=0.078, p=0.581). Nevertheless, at week 12

(β=0.278, p=0.040) and week 33 (β=0.312, p=0.022) ghrelin expression was a significant predictor of IL-1β expression in PBMCs. In addition, the relative change in ghrelin expression in PBMCs from baseline to week 33 and relative change in IL-1β expression in PBMCs correlated strongly with each other (r=0.503, p<0.001).

5.4.2.2 Association between SNPs in the *GHRL* gene and ghrelin expression in PBMCs (*Study IV*)

In the Genobin study population at baseline, the *GHRL* -604G/A SNP was associated with ghrelin mRNA expression in PBMCs, with -604AA individuals having the lowest levels (p=0.001; -604GG: 137.2 ± 55.6, -604GA: 113.9 ± 40.3, -604AA: 83.3 ± 20.5 AU). Similarly, the -501A/C SNP was associated with ghrelin mRNA expression in PBMCs, with -501CC persons having the lowest levels (p=0.025; -501AA: 116.4 ± 43.1, -501AC: 101.1 ± 44.6, -501CC: 75.6 ± 20.5 AU).

Significant differences in the three measurements conducted during the study period were observed between the ghrelin mRNA expression in PBMCs and both the -604G/A and -501C/A polymorphisms. Study participants with the -604AA genotype of the -604G/A SNP had lower ghrelin expression in PBMCs than those with -604GG (p=0.003) and -604GA genotypes (p=0.060) (Figure 16 A). Similarly, individuals with the -501CC genotype of the -501A/C polymorphism had lower ghrelin expression in PBMCs than those with the -501AA genotype (p=0.009) (Figure 16 B). BMI at baseline, sex, age or study group did not significantly contribute to the different expression levels between the genotypes of either of the polymorphisms.

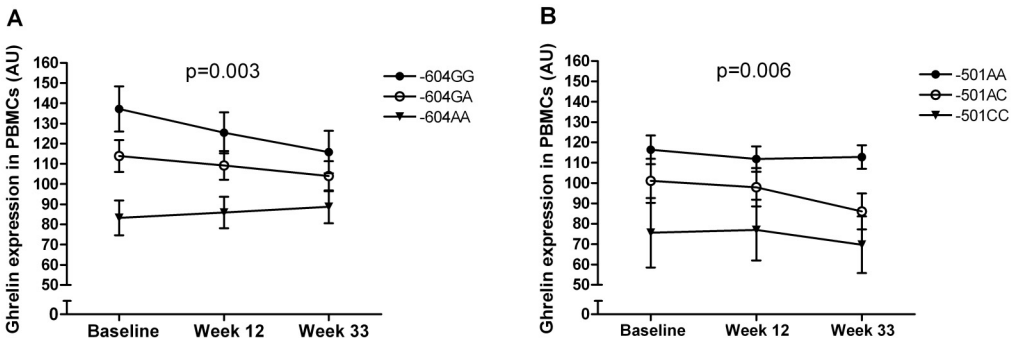

Figure 16. Ghrelin mRNA expression in PBMCs at three time points of the Genobin study according to the -604G/A (**A**) and the -501A/C (**B**) polymorphism of the *GHRL* gene (*Study IV* [370]). Values are estimated marginal means calculated from repeated measures ANOVA. **A**. Pairwise comparisons: GG vs. GA p=0.397, GG vs. AA p=0.003, GA vs. AA p=0.060. **B**. Pairwise comparisons: AA vs. AC p=0.188, AA vs. CC p=0.009, AC vs. CC p=0.332.

5.5 Plasma ghrelin concentrations (*Study IV*)

5.5.1 Plasma ghrelin concentrations at baseline

At baseline, the mean fasting plasma ghrelin concentration in the whole Genobin study population (n=75) was 825 pg/ml (range 451-1468 pg/ml). The mean values for men and women were 795 and 854 pg/ml, respectively, and did not significantly differ from each other (p=0.283). Multiple linear regression analysis demonstrated that fasting serum insulin concentrations (β=-0.483, p<0.001), insulin concentrations during an OGTT (30 min serum insulin: β=-0.368, p=0.003; 120 min serum insulin: β=-0.457, p<0.001), insulin sensitivity index (S_I) (β=0.343, p=0.006) and HDL cholesterol (β=-0.300, p=0.016) were significant independent predictors of ghrelin concentration at baseline. A trend towards a negative association between ghrelin plasma levels and acute insulin response (AIR) was seen (β=-0.236, p=0.068). Weight, BMI or waist circumference was not correlated with plasma ghrelin levels in Genobin study participants at baseline.

Plasma ghrelin levels in the overweight/obese study participants were significantly lower than those in normal-weight persons (p=0.005, adjusted for age and sex; mean fasting plasma ghrelin concentration of normal-weight persons 1056 pg/ml, range 622-1510 pg/ml).

5.5.2 Plasma ghrelin concentrations during interventions

The association between fasting plasma ghrelin levels and fasting serum insulin concentrations over the time period of the Genobin study was examined using the linear mixed models analysis. The results show that fasting serum insulin was a significant predictor of fasting plasma ghrelin levels (p=0.024). When weight, BMI or waist circumference was included separately as a covariate into the model, it was found that these measures of fat mass/obesity significantly contributed to the plasma ghrelin concentration (p=0.050, p=0.025, p=0.023, respectively), with fasting insulin concentration no longer being significant (p=0.075 - p=0.128). S_I did not significantly contribute to the ghrelin concentrations when tested with this multilinear model. However, AIR significantly influenced the fasting plasma ghrelin concentrations over the study period (p=0.001), even after adjusting for the effect of weight, BMI, waist circumference and S_I. In fact, weight, BMI and waist circumference were strongly associated with plasma ghrelin longitudinally (p=0.013 for weight, p=0.004 for BMI, p=0.005 for waist circumference).

When participants from the weight reduction group (n=28) were compared to those from the control group (n=18), the interaction term for plasma ghrelin*group indicated a trend for difference (p=0.067): the plasma ghrelin concentrations differed significantly between the groups at week 12 (p=0.042) and at week 33 (p=0.024), with higher levels being observed in the weight reduction group (Figure 17).

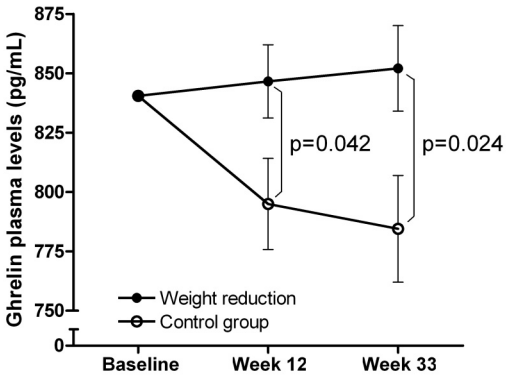

Figure 17. Plasma ghrelin concentrations in the weight reduction and control groups of the Genobin study. Repeated measures ANOVA with baseline plasma ghrelin levels as covariate. Values are estimated marginal means ± SEM. Interaction term plasma ghrelin*group p=0.067 (*Study IV* [370]).

Correlation analysis on all study participants showed an inverse relationship between the relative weight change and relative change in plasma ghrelin from baseline to week 33 (β=-0.294, p=0.010).

6 Discussion

6.1 Methodological considerations

6.1.1 Study populations

Participants of DPS and the Genobin study were carefully selected and clinically well characterised. All persons in both studies were Finnish. The current Finnish population is believed to originate from a small group of individuals who settled in the southwest part of the country about 2000 years ago. Since the initial immigration, the population has continued to be relatively isolated, with little external migration into the area [388]. Therefore, the Finnish population has been designated as a model population for human genetic studies and even smaller sample sizes can lead to reliable results.

The individuals investigated in *Studies I, II* and *III* were participants of the Finnish DPS [366]. They are a representative sample of middle-aged, overweight Finnish persons with IGT who were at high risk of developing type 2 diabetes. Nowadays, the DPS population might be considered small for genetic association studies. However, due to the fact that DPS is a longitudinal study and has consisted of a relatively homogenous population of Finnish individuals with IGT, the number of participants (n=507) seems appropriate.

The participants in the Genobin study who were investigated in *Study IV* had either IGT or IFG and two additional features of the metabolic syndrome and were thus also at high risk of developing type 2 diabetes. Because the Genobin study was designed as a controlled lifestyle intervention, the number of individuals in this study (n=75) is low for a genetic association study; nevertheless, some statistically significant associations were found. However, the small sample size diminishes the power to detect further associations.

6.1.2 Measurements of obesity

Overweight or obesity was determined by measuring body weight and height, and calculating BMI. BMI is an adequate and simple measure of obesity for large-scale studies, since it has been shown to correlate with body fat, morbidity and mortality [389]. Waist circumference is an important measure of obesity risk and a practical surrogate marker of visceral abdominal fat [389].

6.1.3 Measurements of insulin resistance

Fasting insulin concentrations and HOMA-IR were used as surrogate measures of insulin resistance in *Studies I, II* and *III*. Both fasting insulin and HOMA-IR have proved to be a reasonably good marker of insulin resistance [390]. The golden standard for insulin sensitivity is the euglycaemic hyperinsulinaemic clamp technique [391]. However, in large studies, such

as DPS, this method is not feasible. In the smaller Genobin study, S_I and AIR were calculated from FSIGT (*Study IV*). Those indices inferred from FSIGT have been shown to correlate well with those derived from clamp studies and thus provide valid measures of insulin sensitivity [392-394].

6.1.4 Determination of SNPs

Polymorphisms in the *GHRL* gene in DPS and Genobin study populations were genotyped with the PCR-RFLP method (*Studies I, II* and *IV*). RFLP analysis is a sensitive and reliable method for the detection of SNPs in candidate genes. The specificity of restriction enzymes is very high. Recently, newer and less time-consuming methods have been introduced; therefore, the SNPs in the *GHSR* gene were genotyped with TaqMan Allelic Discrimination assays (*Study III*). This method is robust and accurate and allows fast, direct detection with high sample-to-sample reproducibility. In both methods, a representative sample was repeated to confirm the genotype in addition to reanalysis of samples for which the result was unclear.

6.1.5 Gene expression

The primers and probes in the gene expression assay for GHSR 1b (Applied Biosystems assay ID Hs01031463_s1) were designed within one exon, because GHSR 1b is encoded by a single exon. Such an assay detects genomic DNA and should use as a control RNA which has not been reverse transcribed. Unfortunately, by this time, I had already reverse transcribed all of the limited amount of RNA samples available. Therefore, it was no longer possible to provide a control RNA sample to rule out DNA contamination. This may have obscured the results.

Ghrelin mRNA expression was detected in subcutaneous adipose tissue with mean C_T-values of 33.9, and a range of 32.2 to 36.1. IL-6 mRNA expression was detected in PBMCs with mean C_T-values of 34.9, and a range of 31.8 to 37.0. C_T values greater than 35 approach the sensitivity limits of the real-time PCR detection system, and thus those results have to be interpreted with caution.

6.1.6 Promoter analysis

In silico screening

As the initial step in gene expression, transcription is central to regulatory mechanisms. Components of transcriptional regulation include TFs that bind to specific TF-binding sites (either proximal or distal to a transcription start site), interactions between bound TFs and cofactors, as well as the influence of chromatin-structure [395]. Several computational methods are available for identifying the regulatory sequences that control the rate of transcription initiation of specific genes of interest [395].

In the present study, Genomatix MatInspector software was used to determine putative TF binding sites which are disrupted by SNPs in the *GHSR* promoter (*Study III*). MatInspector is a software tool that utilises a large library of position weight matrix descriptions for TF binding sites to locate matches in DNA sequences [383]. The matrices in the MatInspector library are derived from single publications with either a nucleotide distribution matrix or a list of binding sites, or from several papers that have published individual binding sites. It must be noted that not all the sites found are necessarily functional in the particular biological context [383].

Gelshift assays

For gelshift assays, rat hypothalami nuclear extract was used as a source of TF protein. Since no further supershift assays with specific antibodies against NF-1 were conducted, it can only be presumed that the results are specific and thus the oligo-sequence may as well be bound by a different unknown protein. Furthermore, protein binding to a TF-binding site *in vitro* does not always imply functionality *in vivo*.

6.1.7 Measurement of plasma ghrelin concentration

The plasma ghrelin levels reported in *Study IV* are fasting plasma total ghrelin concentrations. Overnight fasting plasma levels of ghrelin have been shown to correlate well with the 24-h integrated area under the curve values of ghrelin [79, 94]. However, by measuring only total ghrelin, individual effects of acylated and des-acyl ghrelin or a change in acyl/des-acyl ghrelin ratio may be overlooked, since acylated and des-acyl ghrelin forms may induce different physiological and metabolic effects [146, 218].

For participants in DPS, plasma ghrelin concentrations are not available.

6.1.8 Statistical analyses

Investigating many genetic markers and their interactions for an association with multiple phenotypes and quantitative traits raises the possibility of finding false positive results; that is, it increases the risk of type I errors. Bonferroni corrections for multiple testing are currently considered a rather conservative approach and may “overcorrect” the threshold level, thereby increasing the chance of making type II errors, i.e., introducing false negative results. Thus, to correct for multiple testing, we calculated the FDR, expressed as a q-value, for given p-values. Associations with a significant p-value, but a high q-value must be cautiously interpreted and are more likely to be false-positive findings.

Some associations may be overlooked because of a lack of adequate statistical power due to the low allele or genotype frequencies of some variants investigated in the studies presented in this thesis or due to the limited sample sizes of the studied populations. In

general, findings must be cautiously interpreted and can be regarded tentative until they are corroborated, i.e., replicated in other studies and populations.

6.2 General discussion

6.2.1 Ghrelin and ghrelin receptor and type 2 diabetes and glucose metabolism (*Study I*, *Study III)*

In the series of studies conducted, one SNP in the *GHRL* gene, but none of the SNPs in the *GHSR* gene, was associated with the conversion from IGT to type 2 diabetes in the DPS. Three SNPs in the *GHSR* gene were associated with measures of glucose and insulin metabolism.

In *Study I*, the Leu72Met variant of the *GHRL* gene was associated with type 2 diabetes. Individuals with the Leu72Leu genotype developed less frequently type 2 diabetes than did those with the Met72 allele or the Leu72Met genotype. This was true for the whole DPS population as well as for the intervention group separately, suggesting that subjects with the Leu72Leu genotype benefited most from lifestyle intervention to reduce the risk of conversion from IGT to type 2 diabetes. Individuals with the Met72 allele in the intervention group, on the other hand, developed almost as often diabetes as did control subjects. Whether more drastic lifestyle changes may be needed in persons with the Met72 allele to prevent the conversion to type 2 diabetes remains unknown. Moreover, in the intervention group, no converters to type 2 diabetes with Met72Met genotype were found, while individuals in the control group who were homozygous for the Met72 allele showed the highest percentage of converters. Nevertheless, the Met72Met genotype was not significantly associated with the incidence of type 2 diabetes, perhaps due to the small number of subjects with this genotype.

In a recent study, metabolic syndrome was more prevalent among persons with the Met72 allele, while the Leu72Met SNP was not associated with type 2 diabetes, HOMA-IR or insulin secretion [346]. A reduced first-phase insulin secretion in OGTT in obese children carrying the Met72 allele has been reported [338], which might suggest a defect in the insulin secretion of those subjects. However, in a more recent study, the Leu72 allele was associated with higher fasting insulin and HOMA-IR values, and individuals with the Met72Met genotype displayed the highest insulin sensitivity [359]. Furthermore, in many of the previously conducted studies, Leu72Met was not associated with type 2 diabetes or related phenotypes [14, 340, 341, 346, 358]; thus, it is difficult to speculate about a possible mechanism behind the associations seen in the present study. Moreover, the functional significance of the Leu72Met SNP remains uncertain. It lies outside the region where the mature ghrelin product is encoded, but it may play a significant role in posttranslational processing.

Regarding polymorphisms in the *GHSR* gene, Vartiainen *et al.* [362] showed that subjects with rs495225-CC genotype had highest area under the insulin curve values and IGFBP-1 concentrations. In the DPS population, rs490683 and rs509035 were associated with several measures of glucose and insulin metabolism over four consecutive measurements, as well as when analysed as relative changes of these measures. Beneficial changes in insulin and glucose metabolism could only be observed in the control group, and specifically in individuals with rs490683-CC and rs509035-TT genotypes. The control group in the DPS trial can be considered as a prospective cohort of people at high risk of diabetes. Since changes in the measures of glucose and insulin metabolism during the study period were only observed in the control group, this indicates that the rs490683-CC genotype and the rs509035-TT genotype are beneficial or protective genotypes.

6.2.2 Ghrelin and hypertension (*Study II*)

DPS participants who had simultaneously the -604GG, -501AA, Leu72Leu and Gln90Gln genotypes had the lowest systolic and diastolic BP levels at baseline and consistently throughout the 3-year follow-up compared to all other genotypes. These persons also had a lower prevalence of hypertension and a lower risk of hypertension compared to all other subjects.

Recently, ghrelin has been shown to participate in central cardiovascular and sympathetic regulation [201, 203, 208, 213, 396]. Ghrelin may have important direct cardiovascular effects through GH-independent mechanisms [213, 397, 398]. It has been shown to decrease BP [201, 208, 211], probably by acting at the nucleus of the solitary tract, one of the most important brain regions regulating BP and the sympathetic nervous system [209, 212, 213].

Although the Leu72Met SNP has not previously been associated with hypertension [354] or BP [76], Ukkola *et al.* [335] reported that female Met72 carriers had the lowest prevalence of hypertension compared to Leu72Leu subjects, in contrast to the findings in *Study II*. Previous studies have not found an association between the promoter polymorphisms -604G/A and -501A/C and ghrelin plasma levels [343, 359]. The Gln51 allele of the Arg51Gln SNP has been reported to be a risk allele for hypertension [355]. In our study this SNP was not associated with either BP or hypertension.

Low plasma ghrelin levels are inversely correlated with systolic and diastolic BP in different study populations [76, 309, 310] and associated with hypertension [76], but data on associations between ghrelin polymorphisms and ghrelin plasma levels are sparse. As plasma ghrelin measurements were not available in this study, it is difficult to interpret the observed relationship and its biological significance. No data are available to date concerning the functional relevance of any of the described polymorphisms. Furthermore, none of the described promoter variants is located at binding sites of known TFs. Therefore, the

mechanisms mediating an association between *GHRL* polymorphisms and BP regulation remain yet to be elucidated, and other studies are needed to confirm the present findings.

In general, the conflicting findings between genetic association studies could be due to several factors, including the heterogeneity of the study population or the phenotype. Different results could also be attributed to true differences in allelic association with disease phenotype in different populations.

6.2.3 Ghrelin and ghrelin receptor and obesity (*Study III*)

In this series of studies, one rare SNP in the *GHRL* gene was associated with weight and two SNPs in the *GHSR* gene were associated with weight and weight loss during the intervention in the Finnish DPS.

Ghrelin has been implicated in the development of obesity. Numerous studies on several polymorphisms in the *GHRL* gene have been undertaken and have provided a large amount of results. The Gln90Leu SNP of the *GHRL* gene was associated with weight in the DPS population. The Leu90Leu genotype with a low frequency, between 2 and 2.7% in the tested study populations, showed the lowest values for weight. Previously, this SNP has not been associated with obesity [264, 347, 359]. Only one study has reported a higher frequency of Leu90 allele among extremely obese children but considered this finding to be a false positive association, since a second control group of underweight students had the same Leu90 allele frequency [353]. The Leu90Gln SNP lies in the region coding for obestatin, a recently discovered peptide derived from the same *GHRL* gene and prepro-peptide but possibly having an opposite, anorexic action [24-26], although this is widely debated [29, 30, 399]. Nevertheless, this could provide an explanation for the negative results reported previously.

The Leu72Met variant is the most studied polymorphism in the *GHRL* gene. The Met72 allele has been variably associated with earlier age at onset of obesity and higher BMI [264, 337-340], or with lower BMI [335, 341]. Some studies have been unable to find an association with the Leu72Met SNP and obesity [344, 345, 347, 348], or with weight loss [349]. A recent prospective study showed that persons with the Gln51 and/or Met72 allele lost body weight faster than did those patients with a genotype combination of Arg51Arg and Leu72Leu [360]. However, these results need to be interpreted with caution, since these analyses were not adjusted for baseline weight. In the DPS population, no association could be found between Leu72Met and obesity (*Study I*).

In *Study III*, two SNPs (rs490683 and rs9819506) in the promoter region of the human *GHSR* gene were associated with body weight and BMI. The *GHSR* SNP rs9819506, which has not been examined in previous studies, showed an association, and individuals with

rs9819506-AA genotype showed the lowest values for body weight and a trend for BMI during the 3-year follow-up of the DPS. Interestingly, subjects with the rs490683-CC genotype displayed the highest weight loss in the whole study group, as well as in the intervention group, when assessed separately. Previously, Baessler *et al.* [351] showed an association between *GHSR* haplotypes and obesity. The so-called "susceptible" haplotype tested in that study was found more often in obese individuals and also included the rs490683-C and rs509035-T alleles. These results might be considered contradictory to those found in DPS, where the rs490683-CC genotype appears to be a "beneficial" genotype. However, these different results could also be attributed to differences in study designs and study populations, thus making it difficult to compare data from a longitudinal study with individuals at high risk of type 2 diabetes and a cross-sectional study carried out in a different population. A more recent study, however, could not point out a clear relationship between *GHSR* SNPs and obesity [350]. Wang *et al.* [352] showed that obese children and adolescents had a higher rs495225-T allele frequency than underweight subjects, but this trend could not be confirmed in their further studies, leading them to conclude that there is no evidence for the involvement of *GHSR* SNPs in body weight regulation.

To test the potential functional relevance of SNPs in the 5'-region of the *GHSR* gene, an *in silico* promoter analysis was carried out to identify SNPs disrupting putative TF binding sites. Gelshift assays using nuclear protein extracts from rat hypothalamus were used to test oligonucleotides with each allele of each SNP. Nuclear proteins bound to the sequence containing rs490683G with much higher affinity than to rs490683C. At this position, a putative NF-1 binding site exists and is disrupted by rs490683, changing the sequence from GCCA to CCCA. The NF-1 family of site-specific DNA-binding proteins functions both as cellular TFs in the regulation of gene expression and as replication factors for adenovirus DNA replication [400]. The binding specificity of NF-1 is equivalent to both the TGGCA-binding protein [401] and the CAAT-box TF [402, 403]. The NF-1 protein binds as a dimer to the dyad symmetric consensus sequence TTGGC(N_5)GCCAA on duplex DNA, although NF-1 can also bind specifically to individual half sites (TTGGC or GCCAA) with somewhat reduced affinity [400]. NF-1 has been shown to activate transcription [400], and it can be speculated that in individuals with rs490683-GG genotype, where the NF-1 half site is intact, GHSR expression is enhanced, thus potentially leading to an increase in receptor signalling and ultimately to an increase in appetite [404]. It has recently been shown that GHSR signals with ~50% activity even in the absence of agonist [152]. This implies that control of the expression level of the receptor is directly correlated to its signalling activity [323]. It was shown that during prolonged fasting, GHSR expression in the hypothalamus is increased, which could contribute to the amplification of ghrelin action [404] and could be expected to result in a ghrelin-independent increase in receptor signalling and thereby an increase in appetite [323]. The *GHSR* polymorphism rs490683 might lead to increased ghrelin receptor

expression, and in the present study, persons with rs490683-GG genotype lost less weight during a lifestyle intervention than did those individuals with the rs490683-CC genotype.

One of the investigated *GHSR* SNPs is located in the exon, but the substitution does not lead to an amino acid change. Therefore, it is not anticipated that the binding of ghrelin to its receptor is altered. However, naturally occurring mutations leading to amino acid changes in the ghrelin receptor have been previously characterised in patients with short stature and obesity developing during puberty [352, 405]. Those mutations have been shown to lead to a loss in constitutive activity of GHSR [405, 406]. Furthermore, *in vitro* experiments and proteometrics analysis of the constitutive and ghrelin-induced activities of wild-type and mutant ghrelin receptors have been carried out previously [407, 408]. *In vitro*, *GHSR* missense-mutations were shown to affect basal activity (ligand-independent signalling) and GHSR expression, as well as to alter the response to ghrelin (agonist activity) and inverse agonist function [409]. In addition, animals in which GHSR activity has been knocked out display lower body weight, reduced food intake, and increased fat burning on a high-fat diet [148, 322, 332-334]. Taken together, genetic factors, e.g., the rs490683 SNP in the *GHSR* gene, which lead to increased ghrelin receptor expression, may contribute to increased GHSR signalling and ultimately higher body weight.

6.2.4 Determinants of plasma ghrelin concentrations (*Study IV*)

In *Study IV*, plasma ghrelin levels correlated negatively with fasting serum insulin and insulin levels during the OGTT but positively with S_I and serum HDL cholesterol concentration cross-sectionally, a finding supported by previous literature [75, 76, 79, 307, 310]. However, in the longitudinal data, measures of central obesity seem to be more important contributing factors explaining low ghrelin concentrations in persons with the metabolic syndrome than insulin or insulin sensitivity. Interestingly, low AIR was also associated with high ghrelin concentrations longitudinally, suggesting that first-phase insulin secretion *per se* could influence ghrelin secretion. However, it was previously suggested that early insulin response has no effect on plasma ghrelin [114].

Numerous studies have been carried out to unravel the ambiguous relationship between insulin and ghrelin. In humans, insulin inhibits ghrelin secretion [73, 74]. An increase in insulin after glucose administration could contribute to the inhibitory effect of glucose on ghrelin secretion [69-72]. However, simultaneous administration of insulin and glucose does not change ghrelin levels [72] nor during euglycaemic clamp, insulin suppresses ghrelin [107, 108]. Administering ghrelin to humans has been shown to inhibit insulin secretion, though no effect was observed on insulin response during OGTT [215-217]. Co-administration of acylated and des-acyl ghrelin prevented the acute hyperglycaemic and hyperinsulinaemic effects of acylated and des-acyl ghrelin when administered alone [218].

The degree of insulin resistance or diabetes status could have an influence on the relationship between ghrelin and insulin. During hyperinsulinaemic-euglycaemic clamp tests, insulin decreased plasma ghrelin dose-dependently in healthy humans, but not in type 2 diabetic patients, perhaps due to the presence of insulin resistance [410]. Another clamp-study showed that hyperinsulinaemia (with concomitant hyperglycaemia) at concentrations typically seen in insulin-resistant persons did not affect plasma ghrelin but was decreased only at pharmacological insulin concentrations [104].

However, it is not clear whether insulin resistance plays a causal role in lower ghrelin concentrations, or whether ghrelin concentrations may be downregulated in insulin resistance as a physiological response to a hyperinsulinaemic state. Independently from the mechanisms mediating the endocrine or paracrine/autocrine impact of ghrelin on insulin secretion, it is clear that ghrelin influences insulin secretion and glucose metabolism; insulin and glucose levels, in turn, negatively influence ghrelin secretion, thus indicating the existence of a feedback mechanism linking ghrelin with the endocrine pancreas and glucose metabolism. The possible mechanisms behind the relationships between insulin, insulin resistance, insulin secretion and ghrelin concentrations cannot be explained based on the present study due to its descriptive nature.

Overweight or obese participants with metabolic syndrome in the Genobin study display lower ghrelin levels than do normal-weight persons, which is in line with the literature. However, in the present study, normal-weight persons were significantly younger than overweight/obese individuals, and it has been suggested by some authors that ghrelin levels decrease with increasing age [128, 129]. Although age and ghrelin levels did not correlate in the Genobin study, age may be another factor explaining lower ghrelin levels in persons with obesity and insulin resistance.

SNPs in the *GHRL* gene may also contribute to the variation in plasma ghrelin concentrations. The Leu72Met SNP is associated in some studies with Met72 allele carriers showing the highest total [359] or acylated ghrelin concentrations [342] and others showing a trend for high total ghrelin levels with Met72 allele carrier status [76, 335] or no association [264, 361]. The Gln51 allele of the Arg51Gln SNP was associated with low ghrelin levels [76, 335], but not in all studies [264, 361]. In *Study IV*, genetic variations in the *GHRL* gene were not associated with plasma ghrelin concentrations. However, due to the limited sample size and thus low numbers of individuals in each genotype group, it cannot be excluded that this study was underpowered to detect a difference according to different SNP genotypes. On the other hand, it must be noted that ghrelin is mostly produced by X/A-like cells of the oxyntic gland, an endocrine cell type in the submucosal layer of the stomach. The X/A-like cells contain round, compact, electrondense granules that are filled with ghrelin [2] and ghrelin is secreted into the blood stream and thereafter circulates throughout the whole body [411, 412]. Thus, other factors affecting the release of ghrelin from the stomach may be more important factors for ghrelin concentrations than genetic variation in the *GHRL* gene.

6.2.5 Effect of lifestyle intervention on plasma ghrelin concentrations (*Study IV*)

The majority of studies undertaken have shown an increase in total ghrelin levels upon diet-induced weight loss in obesity [267-273]. In the present study, however, diet-induced weight loss did not result in an increase in plasma ghrelin levels; instead, ghrelin concentrations in overweight/obese control subjects significantly decreased over the study period. Since participants in the Genobin study were all insulin resistant, it could be speculated that overweight/obese subjects with IGT or IFG experience a gradual decrease in plasma ghrelin levels, when they are not undergoing lifestyle changes. It may be that the absence of weight loss in obesity may further decrease plasma ghrelin levels over time. Supporting this hypothesis, the increased plasma ghrelin levels achieved by weight loss through dietary restrictions returned to baseline after a weight maintenance period of six months in a recent study [277].

In the previous literature, only one weight loss study reported an increase in plasma total ghrelin concomitant with a decrease in weight specifically in subjects with metabolic syndrome [272]. Their dietary approach was not comparable to the one used in the Genobin study, as the subjects were 12 weeks on a commercial South Beach diet, a carbohydrate-restricted diet with fat intake up to 62E%, whereas participants in the present study received individualised counselling on a recommended diet based on dietary records. Another recent study [273] with hyperlipidaemic overweight and obese females has reported an increase in fasting total ghrelin. The mean weight loss of 14.5±3.1% was higher than in the present study (4.8±3.8%), which may explain the difference concerning the increase in ghrelin concentrations.

The new finding in the present study is the decrease in plasma ghrelin in the control group with an absence of lifestyle intervention. Previous studies reporting no change in total plasma ghrelin during weight loss have been either conducted with children [275, 276]; in healthy young adults with moderate overweight [274, 294]; or in one study [277], the increase in total plasma ghrelin after active weight loss was not sustained during weight maintenance.

The Genobin study was not designed to test the effect of a specific macronutrient composition or a change in macronutrient composition on plasma ghrelin levels; therefore, no strict dietary regime was imposed on the persons in the weight reduction group. Rather, the objective was to give the participants advice to change their habits long-term, thus mimicking the real-life situation in clinical practice. In the present study, fat intake decreased and protein intake increased significantly from baseline to the end of the study in the weight reduction group, whereas the carbohydrate intake remained stable; however, no association was observed between the relative change in plasma ghrelin and changes in fat intake and protein intake from baseline to the end of the study. Therefore, conclusions about the contribution of specific macronutrients on ghrelin concentrations cannot be made based on the present study.

In many weight loss studies, an exercise component is also included in the lifestyle program, thus making it impossible to dissect the exclusive effect of prolonged exercise on ghrelin levels. With the design of the Genobin study, the effects of dietary intervention and exercise intervention can be distinguished. In this study, no changes in plasma ghrelin levels were observed in the resistance or aerobic exercise groups. This may be partly explained by the absence of weight loss or low intensity of exercise. On the other hand, it may suggest that exercise has little effect on ghrelin concentrations. In fact, there is evidence that at least acute exercise does not alter ghrelin levels [83-92]. Studies investigating the effect of weight loss through exercise without diet changes have shown either an increase [280, 281] or no change [282] in ghrelin concentrations.

6.2.6 Gene expression of ghrelin and ghrelin receptor in PBMCs and adipose tissue (*Study IV*)

Ghrelin and ghrelin receptor expression in PBMCs have not been studied in patients with metabolic syndrome undergoing lifestyle modification. Human ghrelin mRNA expression has been shown in T-lymphocytes, B-lymphocytes, and neutrophils from venous blood of healthy volunteers [48, 50, 51]. *Study IV* shows that ghrelin mRNA is also expressed in the PBMCs of subjects with metabolic syndrome, though with great individual variations in expression levels. The main aim of this study was to study PBMCs and their applicability as a surrogate marker for ghrelin metabolism before versus after moderate weight loss. PBMCs from venous blood samples are the most accessible tissue for analysis of gene expression and the least demanding for the patients compared to biopsies of other tissues. Metabolic derangements associated with metabolic syndrome potentially affect all cells in the body, and the resulting changes in gene expression may also be sampled in PBMCs. However, ghrelin expression in PBMCs was not influenced by the different interventions prescribed to the study participants, nor was there any evident difference between overweight/obese compared to normal-weight persons. Furthermore, ghrelin expression was not influenced by measures of adiposity, nor did it correlate with plasma ghrelin levels. Interestingly, polymorphisms of the ghrelin promoter region, the -604G/A and -501A/C variants, markedly modified ghrelin gene expression in PBMCs, which may partly explain the individual variation of ghrelin expression in PBMCs. Differences in ghrelin gene expression in PBMCs according to SNPs in the *GHRL* gene are one factor contributing to the variation, in addition to the normal extent of inter-individual variation which has been shown in healthy humans [413].

In the present study, GHSR 1b mRNA was also expressed in PBMCs, whereas GHSR 1a mRNA could not be detected. Ghrelin was positively correlated to TNF-α and IL-1β expression in PBMCs over the whole study period, and a positive correlation was seen between changes in the expression of ghrelin and IL-1β from baseline to the end of the study. A previous study has shown that ghrelin treatment inhibited production of pro-inflammatory

cytokines by PBMCs via a GHSR-specific pathway [51]. It was further reported that ghrelin inhibited IL-6 and TNF-α mRNA expression in primary human T cells, which supports a role for ghrelin in the transcriptional regulation of inflammatory cytokine expression [51]. Anti-inflammatory effects have also been reported for ghrelin mediated through activation of GHSR 1a in human endothelial cells [414].

On the contrary, in the Genobin study, ghrelin expression directly correlated with TNF-α, IL-1β and IL-6 expression in PBMCs. However, only GHSR type 1b was expressed in PBMC samples, whereas the expression of GHSR 1a was too low to be detected. Recently, GHSR 1b has been described as a dominant-negative mutant of GHSR 1a, resulting in attenuation of its constitutive signalling [155]. The inhibition of pro-inflammatory cytokines in PBMCs has been shown to be GHSR 1a-specific [51, 414]. It can be hypothesized that in the present study, higher expression of ghrelin led to a higher expression of pro-inflammatory cytokines due to the higher expression of GHSR 1b compared to GHSR 1a, and the concomitant attenuation of GHSR 1a action.

Ghrelin mRNA was expressed in the subcutaneous adipose tissue of participants in the Genobin study. Only two earlier studies reported gene expression in adipose tissue [48, 52]. Knerr *et al*. [52] reported ghrelin expression in subcutaneous and visceral adipose tissue with huge intraindividual variations. They could not find a significant correlation between ghrelin expression in different adipose tissues and serum concentration, nor did they find correlations with other metabolic parameters. In accordance with those findings, our results show that ghrelin mRNA expression in subcutaneous adipose tissue was not significantly associated with ghrelin plasma levels or other metabolic parameters in our study subjects.

In the present study, neither GHSR 1a nor GHSR 1b could be detected in subcutaneous adipose tissue. In previous reports, GHSR 1a was shown to be expressed in human omental fat [156] and GHSR 1b in adipose tissue [48]. Although the presence of ghrelin and its receptors has been shown, it is unlikely that ghrelin has an autocrine effect in adipose tissue, due to the low expression levels reported.

7 Conclusions and future implications

These studies were conducted to investigate associations between variations in the *GHRL* and *GHSR* genes and various phenotypes related to obesity and type 2 diabetes. Furthermore, the studies investigated the role of ghrelin plasma concentrations and gene expression in PBMCs and adipose tissue within the context of the metabolic syndrome and lifestyle interventions.

From the studies conducted, the following conclusions can be drawn:

Study I:
The Leu72Met SNP of the *GHRL* gene was associated with type 2 diabetes in the DPS population, but in light of the previous literature and the relative weakness of the association, this SNP cannot be regarded as a risk factor for developing type 2 diabetes.

Study II:
A common genotype combination of *GHRL* SNPs was associated with low blood pressure levels and lower risk of hypertension. The associations of SNPs in the *GHRL* gene found in the present study are not supported by previous association studies and have not been replicated by other research groups thereafter. Thus, it can be concluded that *GHRL* SNPs do not markedly modify the risk of investigated metabolic diseases and may not be used as markers for risk assessment in a clinical setting.

Study III:
One SNP in the promoter of the *GHSR* gene was associated with weight loss, and *in vitro* experiments suggested that this SNP, which disrupts a putative TF binding site, may affect protein binding to this regulatory site, and thus may affect GHSR expression and ultimately appetite and food intake. Finding genetic markers that may predispose to higher weight loss under lifestyle intervention circumstances will have great potential as a helpful tool in a clinical setting.

Study IV:
Ghrelin and the ghrelin receptor were expressed in PBMCs, but their expression was not altered upon metabolic changes, thus making ghrelin expression unsuitable as a surrogate marker for ghrelin metabolism in metabolic syndrome. Ghrelin was also expressed in adipose tissue, but with very low intensity. Therefore, it is not anticipated that ghrelin would exert autocrine effects in adipose tissue.

Ghrelin plasma levels were not restored by weight loss in persons with metabolic syndrome; instead, ghrelin concentrations further decreased in those not receiving counselling regarding eating behaviour and physical activity habits.

Although numerous studies have been conducted, no clear picture yet exists on the significance of ghrelin plasma levels. Ghrelin concentrations are paradoxically lower in obesity and type 2 diabetes and metabolic syndrome; nevertheless, persons with those diseases do not lose weight following a reduction in appetite-stimulating hormone levels, as could be anticipated. However, the mechanisms controlling appetite and eating behaviour are complicated and redundant, and supposedly do not function properly in persons with metabolic diseases. Furthermore, environmental factors have a great influence in addition to underlying genetic determinants. Nevertheless, the present study added important knowledge concerning the role of ghrelin in obesity and insulin resistance, thus contributing one piece to a puzzle which will hopefully be solved in the future.

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Appendix: Original Publications

Kuopio University Publications D. Medical Sciences

D 411. Skommer, Joanna. Novel approaches to induce apoptosis in human follicular lymphoma cell lines - precinical assessment.
2007. 80 p. Acad. Diss.

D 412. Kemppinen, Kaarina. Early maternal sensitivity: continuity and related risk factors.
2007. 80 p. Acad. Diss.

D 413. Sahlman, Janne. Chondrodysplasias Caused by Defects in the Col2a1 Gene.
2007. 86 p. Acad. Diss.

D 414. Pitkänen, Leena. Retinal pigment epithelium as a barrier in drug permeation and as a target of non-viral gene delivery.
2007. 75 p. Acad. Diss.

D 415. Suhonen, Kirsi. Prognostic Role of Cell Adhesion Factors and Angiogenesis in Epithelial Ovarian Cancer.
2007. 123 p. Acad. Diss.

D 416. Sillanpää, Sari. Prognostic significance of cell-matrix interactions in epithelial ovarian cancer.
2007. 96 p. Acad. Diss.

D 417. Hartikainen, Jaana. Genetic predisposition to breast and ovarian cancer in Eastern Finnish population.
2007. 188 p. Acad. Diss.

D 418. Udd, Marianne. The treatment and risk factors of peptic ulcer bleeding.
2007. 88 p. Acad. Diss.

D 419. Qu, Chengjuan. Articular cartilage proteoglycan biosynthesis and sulfation.
2007. 78 p. Acad. Diss.

D 420. Stark, Harri. Inflammatory airway responses caused by Aspergillus fumigatus and PVC challenges.
2007. 102 p. Acad. Diss.

D 421. Hintikka, Ulla. Changes in adolescents' cognitive and psychosocial funtioning and self-image during psychiatric inpatient treatment.
2007. 103 p. Acad. Diss.

D 422. Putkonen, Anu. Mental disorders and violent crime: epidemiological study on factors associated with severe violent offending.
2007. 88 p. Acad. Diss.

D 423. Karinen, Hannele. Genetics and family aspects of coeliac disease.
2008. 110 p. Acad. Diss.

D 424. Sutinen, Päivi. Pathophysiological effects of vibration with inner ear as a model organ.
2008. 94 p. Acad. Diss.

D 425. Koskela, Tuomas-Heikki. Terveyspalveluiden pitkäaikaisen suurkäyttäjän ennustekijät.
2008. 253 p. Acad. Diss.

D 426. Sutela, Anna. Add-on stereotactic core needle breast biopsy: diagnosis of non-palpable breast lesions detected on mammography or galactography.
2008. 127 p. Acad. Diss.

D 427. Saarelainen, Soili. Immune Response to Lipocalin Allergens: IgE and T-cell Cross-Reactivity.
2008. 127 p. Acad. Diss.