Chapter 1

General introduction
**General aspects of obesity**

Obesity is one of today’s most obvious – yet most neglected – public health problems. The global epidemic of overweight and obesity – “globesity” – is escalating.

In Europe the prevalence of obesity has been increasing by approximately 30% the last decades (1). In The Netherlands about 40% of the population is overweight and 10% is obese (Table 1) (2). It is important to keep in mind that already a relative small increase in body weight is associated with increased health risk and even in the preobese range there is an increased risk of comorbidities (3). On the other hand, underweight has been linked to increased risk of osteoporosis and falls.

**Table 1.** Classification of adults according to BMI (kg/m²)

<table>
<thead>
<tr>
<th>Classification</th>
<th>BMI</th>
<th>Risk of comorbidities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Underweight</td>
<td>&lt;18.50</td>
<td>Low (but risk of other clinical problems increased)</td>
</tr>
<tr>
<td>Normal range</td>
<td>18.50-24.99</td>
<td>Average</td>
</tr>
<tr>
<td>Overweight:</td>
<td>≥25.00</td>
<td></td>
</tr>
<tr>
<td>Preobese</td>
<td>25.99-29.99</td>
<td>Increased</td>
</tr>
<tr>
<td>Obese class I</td>
<td>30.00-34.99</td>
<td>Moderate</td>
</tr>
<tr>
<td>Obese class II</td>
<td>35.99-39.99</td>
<td>Severe</td>
</tr>
<tr>
<td>Obese class III</td>
<td>≥40.00</td>
<td>Very severe</td>
</tr>
</tbody>
</table>


Furthermore, obesity as well as reduced physical activity are independent predictors of mortality. It has been estimated that being overweight and physical inactive could account for over 30%
of premature deaths and 59% of deaths from cardiovascular diseases (4). This risk can be reduced with even small increases in activity in inactive individuals (5). The well-known health consequences of overweight and obesity are insulin resistance, diabetes mellitus, coronary heart disease, sleep apnea, hypertension, gallbladder disease, infertility, psychosocial problems and some types of cancer (3). Obesity is a disease condition with increased white adipose tissue mass. The accumulation of fat in the liver, skeletal muscles and visceral fat depots is important in the development of insulin resistance and the metabolic syndrome (6). The visceral fat depots have more metabolic consequences than the subcutaneous depots. There is for instance an enhanced triglyceride turnover in visceral fat, and the venous outflow is directed to the liver via the portal system leading to an excess of free fatty acids in the liver (7, 8). Furthermore, it is known that especially visceral adipose tissue is highly active, and acts as an endocrine organ. It expresses and secretes various factors involved in appetite and energy balance such as leptin, adiponectin and resistin. Many of these adipokines, and more recently described hepatokines and myokines are potential biomarkers for insulin sensitivity (9). In obesity and insulin resistant subjects, there is a chronic state of low grade inflammation as pro-inflammatory cytokines and components of the coagulation and fibrinolytic cascades (IL-6, TNFα, PAI-1) are being excreted (10, 11). Several adipokines contribute to this state of inflammation and are therefore an interesting drug target (12).

Lately, some very interesting insights on the association of intestinal microbiota and obesity and metabolic disorders have been published, linking some of the species to metabolic risk factors in obesity while other species are associated with a healthier metabolic status (13, 14, 15). Some very obese individuals have normal metabolic profiles despite large fat mass and are thus called metabolic healthy obese (MHO). The exact definition of MHO is still a subject of debate and this
metabolically benign phenotype probably has risks intermediate between healthy normal weight and unhealthy obese individuals (16).

**Energy balance and signaling in obesity**

Obesity is the consequence of a positive energy balance resulting from excess energy intake with regard to energy expenditure (3). A positive energy balance emerges from an obesogenic environment, including extensive food availability, high energy density of food and a sedentary lifestyle (17). During evolution dealing with a restrictive environment has led to limited energy expenditure and increased food intake whenever possible. This has been engraved into the genes, built in our physiology and psychology and therefore obesity can be seen as a normal physiological response to a changed environment in prone subjects (18, 19). Studies in twins, adoption and families have shown that 40 to 70% of BMI variation is due to genetic factors. Both mono- and polygenic loci have been specified which in the future will most likely lead to more personalized approaches in treating obesity (20, 21). Energy balance is essential to maintain a stable normal body weight. A minor energy imbalance leads to a gradual but persistent weight gain. Once the obesity state has been established, physical processes tend to maintain this new weight and lead to a less favorable “steady state”. Energy balance is influenced by multiple factors deriving from the adipose tissue as well as originating from the digestive tract and the “classical” endocrine systems. All these factors communicate the state of the peripheral energy stores to the central nervous system. In the brain appetite and energy expenditure is then being modulated via a highly interconnected system.

As mentioned above, in the past decades the knowledge on the regulation of energy balance as well as the interaction between signaling from the periphery and the brain is increasing (Figure
1). Little is known about the effect of locally produced factors such as IGF-1 which is present both in brain and peripheral tissues and GLP-1 which is also known to be a neurotransmitter. Presumably these factors act as a part of a fine tuning system. Also some neuropeptides such as orexins and neuromedin (22) and metabolic factors for example free fatty acids and glucose possibly act as regional modulators.

**Figure 1**: Central and peripheral interaction. OFC: Orbitofrontal Cortex, VC: Visual Cortex

The central regulation of energy balance is based on a fine tuned system involving afferent signaling, monitoring and processing of this information in the main centers in the hypothalamus and further distributed to other brain areas as well as sending signals via efferent pathways to the periphery. The normal processing of these signals can be influenced by various factors such as the nutritional state and the state of mind. In this context the term reward driven brain has sometimes been used. The hypothalamus integrates peripheral signals to regulate long term
energy balance and body weight (23). In the hypothalamus various nuclei especially in the middle area such as the arcuate, ventromedial and the dorsomedial nucleus are involved in the initiation of eating and drinking, circadian rhythms, emotion and satiety. The key neurotransmitters involved in these behavioral pathways are dopamine and serotonin (24). Information arrives via the humoral gut-brain axis or indirectly via the vagal gut-brain axis. In the arcuate nucleus of the hypothalamus neurons co-expressing agouti-related peptide, and neuropeptide Y (NPY) are involved in appetite regulation and stimulate food intake (orexogenic) and pro-opiomelanocortin (POMC) which inhibits food intake. In this network information processing on hunger and satiety via ghrelin (produced in the stomach), the gut hormones cholecystokinin (CCK) and peptide YY (PYY) as well as information on energy stores via leptin and insulin take place (25, 26, 27). Further processing of the information occurs in the corticolimbic areas such as the amygdala where the biological relevance of the information is evaluated, the hippocampus involved in memory and recognition and the ventromedial, orbitofrontal and prefrontal cortex involved in reward, satiety and motivational salience. An emerging important participant in the control of food intake and energy balance is the endocannabinoid system. The neuromodulatory effects of the endogenous cannabinoids are mediated via cannabinoid receptors (CB1) which are widely distributed in the brain. They have numerous effects on energy, glucose homeostasis and the reward system promoting weight gain and decrease of insulin sensitivity under influence of leptin, ghrelin, glucocorticoids and dopamine (28, 29). Next to this appetite regulatory system, there are metabolic factors acting directly on some brain areas. For example, fatty acid sensitive neurons have been located in the hypothalamus and hippocampus detecting daily fatty acid levels and playing a role in the energy balance by controlling food intake and insulin secretion (30).
Introduction

The efferent signaling to the peripheral organs occurs by release of hormones via the pituitary gland and by the two components of the autonomic nervous system: the parasympathetic and sympathetic nervous system.

Factors involved in body composition, hunger and satiety

In the last decades many studies have been published on the hormones and neuropeptides involved in appetite control. As yet more than 25 adipocyte-derived hormones or adipokines have been detected and examined (31). Not only adipokines, but also hepatokines and myokines, gut hormones and microbioms, pituitary hormones and neurotransmitters, and specific brain areas are a part of this regulatory system. A few well-known factors will be mentioned here. The main known postprandial satiety regulators are Glucagon-like peptide-1 (GLP-1) and PYY (32).

GLP-1 is a gut hormone which also acts as a neuropeptide. Receptors have been found in several brain regions such as the brainstem, hypothalamus but also in the ganglions of the vagal nerve, pancreas, gastrointestinal tract, kidney, lung, heart and are richly represented in the portal vein system. The main effect is improving glucose homeostasis by enhancing meal-related insulin secretion, regulating glucose production and suppressing feeding (33, 34). The level of this hormone is decreased in obesity. Already treatment with GLP-1 analogues is widely being used in obese type 2 diabetic patients with glucose dysregulation. PYY is also a gut hormone signaling information about food ingestion from the gut to the appetite regulatory brain areas. In humans, PYY given peripherally at a postprandial dose, significantly decreases appetite and reduces food intake by 33% over 24 hours. It is involved in the long term regulation of body weight and control of energy expenditure (35, 36, 37).
Leptin signals information from the peripheral tissues to the brain. It is produced in the adipose tissue and released into the circulatory system as a function of energy stores. It then crosses the blood brain barrier via specific transporter systems (38) and depending on the energy balance it either stimulates melanocyte stimulating hormone (MSH) and corticotropin-releasing hormone (CRH) leading to decrease in food intake and weight loss, or stimulates NPY leading to increased food intake and weight gain. Besides its function in the central regulation of energy balance and food regulation it has been suggested that it is involved in the short term control of energy balance. Production of leptin in the stomach (39) influencing digestion and absorption in the intestine under influence of some intestinal peptides, insulin and meal components is a supposed mechanism. In obesity this highly complicated regulatory system is disrupted. There is less suppression of the overactive response to food stimuli in the reward related areas such as the orbitofrontal cortex (40). Obese subjects become resistant to the central regulatory satiety effect of leptin, and consequently have higher leptin levels than lean individuals (41, 42, 43).

Ghrelin is secreted by the stomach but has also been isolated from other tissues such as the pancreas, adrenal cortex and ovaries. Furthermore ghrelin producing neurons have been identified in the pituitary, hypothalamus and in some paraventricular nuclei. It is a natural growth hormone (GH) secretagogue but also acts on other central and peripheral receptors having orexigenic, metabolic and antiproliferative effects (44). Besides meal initiation with increased pre-prandial levels, the secretion of ghrelin is decreased by various factors such as BMI, age, growth hormone (GH), glucose and insulin. It is predominantly seen as a central modulator of energy homeostasis (45). It reaches the hypothalamus and mesolimbic reward system via the blood brain barrier and is involved in the long-term body weight regulation (46). Ghrelin has recently been identified as one of the main contributors to reward driven feeding that can override the state of satiation. It has been suggested that ghrelin interacts with the corticotropin-
Introduction

releasing-factor system and chronic stress (47). In contrast to what would be expected, the ghrelin level is decreased in obesity. Possibly it is a physiological adaptation to the positive energy balance associated with obesity (45, 48). One of the most abundant adipocytokines is adiponectin, which circulates as multimeric species and seems to have a regulatory role in the mechanism of insulin resistance and the development of atherosclerosis (49). It has been suggested that it improves insulin sensitivity by increasing energy expenditure and fatty acid oxidation (50). Furthermore, it has various other beneficial effects peripherally such as anti-inflammatory, anti-fibrotic and anti-atherogenic effects by slowing down macrophage and lymphocyte activation and decreasing platelet aggregation (51, 52). It seems to regulate energy metabolism in a tissue specific manner, enhancing oxidative metabolism in skeletal muscle and inhibiting lipolysis in adipocytes (31). Its levels are decreased in insulin resistance, obesity and cardiovascular diseases (53). With weight reduction an increase in adiponectin can be achieved although the results have been controversial, mostly related to the amount and speed of weight loss (54).

Gastric inhibitory polypeptide (GIP) is an insulinotropic hormone locally produced in the proximal small intestine. This hormone also has glucagenotropic and adipogenic effects and could play a crucial role in the pathophysiology of obesity and diabetes type 2, yet data regarding GIP secretion have been inconsistent (55).

Also the inflammatory factors, such as tumor necrosis factor alpha (TNFα), interleukin-6 (IL-6) and c-reactive protein (CRP) have been implicated to play a role in the development of obesity, insulin resistance and cardiovascular diseases (52, 56, 57) yet the exact role is not clear. TNFα is a key regulator of the immune cells causing increased cell infiltration and acute phase responses. In insulin resistance there is an increased TNF gene expression in the adipose tissue (58). IL-6 is not only involved in the immune process peripherally but also seems to have
an important role in the central nervous system (59). For example, peripheral administration of IL-6 induces hyperlipidemia, hyperglycemia and insulin resistance in the hepatocytes (60, 61). However, increased levels in skeletal muscle after exercise suggest a possible anti-inflammatory role (11). In cerebral spinal fluid the IL-6 levels are inversely related to the degree of obesity suggesting a relative central IL-6 deficiency in obesity (62). Another adipose derived protein is resistin. There is very little known about the function of this adipocyte-specific secretory factor. High levels are found in obese subjects and are related to fat cell size, with high levels in pre-adipocytes and rarely detectable in mature cells. A role in the chronic inflammation associated with obesity has been suggested. However, reports on changes in resistin levels after weight loss are very inconsistent (44, 63-65).

Additionally, various pituitary derived hormones play an important role in controlling metabolism and body composition. GH has primarily anabolic effects and induces lipolysis. Another essential action of GH is to stimulate the production of insulin-like growth factor -1 (IGF-1) in the liver. IGF-1 promotes growth and induces proliferation of pre-adipocytes into adipocytes. Most of the bioavailability of IGF-1 is influenced by the binding proteins (IGFBPs). IGF-1 and insulin are the two main regulators of central nervous system (CNS) function and development, with highly specific distribution of their receptors especially in the hippocampus (66). The hippocampus as well as the cortex are most sensitive for glycopenia, but the function of the hypothalamus and pituitary are also influenced by insulin and glucose. There is a strong hormonal response of the pituitary to hypoglycemia (67). In obesity and with aging GH secretion is decreased possibly due to adaptation (68) resulting, amongst other things, in blunted lipolysis. The total IGF-1 levels are reported to be normal/high with high GH-binding protein levels. This is probably due to the effect of the higher insulin levels in obesity which is known to regulate the levels of one of the binding proteins (IGFBP1) (69). The abnormal expression of IGFBP has been seen in various metabolic
disorders and could possibly be used as a sensitive marker of insulin resistance (70). Little is known about the association between IGF-1 levels and body composition measurements in older people. The corticotrophic releasing system is also involved in the energy homeostasis. The abdominal obesity phenotype is characterized by hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis. This axis plays a key role in stress response and therefore an altered energetic state such as in obesity affects the stress response (“energetic stress”). When this is prolonged it could lead to a dysfunctional stress response (71). Some of the actions of the corticotrophin releasing system are mediated independently of the HPA axis. Via two subtypes of brain corticotrophic releasing factor (CRF) receptors, it induces appetite suppression, adrenocorticotropic hormone (ACTH) secretion, locomotor activation and stress response. CRF and ghrelin have opposed functions but there seems to be an interplay between these systems (47). During menopause and aging various changes in body composition occur. Some studies have shown an increase in incidence of the metabolic syndrome with menopause. The menopause status and waist circumference have an independent effect on most of the metabolic risk factors. During menopause an increase in body fat mass, but also an increase in visceral adiposity leads to a more atherogenic situation with a notable change in the metabolic active adipokines such as significantly lower adiponectin and higher levels of ghrelin (72, 73, 74).

With aging the main changes are: decreased energy expenditure, a physiological decline in hormone secretion such as GH (somatopenia), sex steroids and thyroid hormones as well as a decrease in muscle mass (sarcopenia), and bone mineral density and an increase in fat mass. The homeostasis of appetite and energy homeostasis is complex in the elderly. Insulin resistance has often been seen in older adults with increased proinflammatory cytokines and visceral fat (75). Body weight increases up to the age of 65-75 years but after that a decrease in
body weight is commonly seen due to decline in appetite and food intake. This has been referred to as the anorexia of aging (76).

In summary a very strong homeostatic system is involved in maintaining weight. In obesity, there is a deregulation of various factors such as the presence of chronic low grade inflammation, leptin resistance, lower levels of ghrelin and adiponectin, a blunted effect of GH, dysregulation of the HPA-axis and dyslipidaemia. Due to many of these factors there is an increased morbidity and mortality. The last decades, due to the discovery of various humoral factors as well as improving imaging techniques, more insight has been gained into this very complex system involved in appetite and food intake regulation. In obesity, changes in brain function are observed and summarized in the term reward driven brain. Acknowledgment of the fact that obesity has multiple underlying mechanisms could lead to a more individual treatment plan in the near future.

Aim and outline of this thesis

The aim of this thesis is to gain more insight into the changes of various hormones, metabolic factors and neuropeptides involved in the regulation of body composition and energy balance in healthy and aging subjects. Also to observe early changes in overweight post-menopausal women, peripherally as well as in the brain, that occur during caloric restriction even before significant weight loss is achieved.

Chapter 2 is a review of the role of the two major hormones known to influence both the long and short-term energy balance and their role in food intake: leptin and ghrelin.
To evaluate the neuromodulatory effect of glucose and insulin we conducted a clamp study with glucose levels near to the normal range and measured the response to stimulated CRH and GH, the results are shown in chapter 3.

In chapter 4 we evaluated the IGF-1 levels across different age categories in elderly and investigated the association of these levels with measurements of body composition. For this study we were able to use data from a large longitudinal cohort study (Longitudinal Aging Study Amsterdam).

In order to visualize the areas involved in the perception and processing of food stimulation we conducted a fMRI study, both in the hungry and in the satiety state. First this was performed in healthy men (chapter 5), before and after consuming a standardized meal. During the fMRI they were exposed to food pictures and performed simple tasks. The same study design was used in overweight postmenopausal women before and after weight loss (chapter 6). In addition various anthropological and humoral measurements were performed before, after and during an iso-caloric diet for four weeks. These measurements were obtained frequently to be able to observe early changes. Some of the neuro-humoral and metabolic factors involved in the energy balance and the processing of food related stimuli were evaluated and correlated with the findings in the brain areas. Finally, the main findings of these studies and further perspectives on this subject are discussed in chapter 7.
REFERENCES


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Chapter 2

The role of leptin and ghrelin in the regulation of food intake and body weight in humans: a review

**List of abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>OB</td>
<td>obese</td>
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<tr>
<td>Kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>LEPR</td>
<td>leptin receptor</td>
</tr>
<tr>
<td>OBR</td>
<td>leptin receptor</td>
</tr>
<tr>
<td>BBB</td>
<td>blood-brain barrier</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>NPY</td>
<td>neuropeptide Y</td>
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<tr>
<td>MCH</td>
<td>melanin-concentrating hormone</td>
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<tr>
<td>AgRP</td>
<td>agouti-related protein</td>
</tr>
<tr>
<td>GAL</td>
<td>galanin</td>
</tr>
<tr>
<td>GALP</td>
<td>galanin-like peptide</td>
</tr>
<tr>
<td>AMPK</td>
<td>hypothalamic 5’-AMP-activated protein kinase</td>
</tr>
<tr>
<td>POMC</td>
<td>pro-opiomelanocortin</td>
</tr>
<tr>
<td>CART</td>
<td>cocaine- and amphetamine-regulated transcript</td>
</tr>
<tr>
<td>NT</td>
<td>neurotensin</td>
</tr>
<tr>
<td>CRH</td>
<td>corticotropin-releasing hormone</td>
</tr>
<tr>
<td>BDNF</td>
<td>bain-derived neurotrophic factor</td>
</tr>
<tr>
<td>ARC</td>
<td>arcuate nucleus</td>
</tr>
<tr>
<td>LH</td>
<td>lateral hypothalamus</td>
</tr>
<tr>
<td>PFH</td>
<td>perifornical hypothalamus</td>
</tr>
<tr>
<td>PVN</td>
<td>paraventricular nucleus</td>
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<tr>
<td>RMR</td>
<td>resting metabolic rate</td>
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<tr>
<td>GHRL</td>
<td>human prepro-ghrelin</td>
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<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
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The role of leptin and ghrelin

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>GHS-R</td>
<td>growth hormone secretagogue receptor</td>
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<tr>
<td>GH</td>
<td>growth hormone</td>
</tr>
<tr>
<td>NTS</td>
<td>nucleus tractus solitarius</td>
</tr>
<tr>
<td>PWS</td>
<td>Prader-Willi syndrome</td>
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<tr>
<td>DIO</td>
<td>diet-induced obese</td>
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<tr>
<td>DR</td>
<td>diet-resistant</td>
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<tr>
<td>JAK</td>
<td>janus kinase</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>ICV</td>
<td>intracerebroventricular</td>
</tr>
<tr>
<td>PTP1B</td>
<td>protein-tyrosine phosphatase 1b</td>
</tr>
<tr>
<td>SHP2</td>
<td>SH2-containing phosphatase 2</td>
</tr>
<tr>
<td>SOCS3</td>
<td>suppressor of cytokine signaling 3</td>
</tr>
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</table>
Abstract
Leptin and ghrelin are two hormones that have been recognized to have a major influence on energy balance. Leptin is a mediator of long-term regulation of energy balance, suppressing food intake and thereby inducing weight loss. Ghrelin on the other hand is a fast-acting hormone, seemingly playing a role in meal initiation. As a growing number of people suffer from obesity, understanding the mechanisms by which various hormones and neurotransmitters have influence on energy balance has been subject of intensive research. In obese subjects the circulating level of the anorexigenic hormone leptin is increased, whereas surprisingly, the level of the orexigenic hormone ghrelin is decreased. It is now established that obese patients are leptin resistant. However, the manner in which both the leptin and ghrelin systems contribute to the development or maintenance of obesity is as yet not clear.

The purpose of this review is to provide background information on the leptin and ghrelin hormones, their role in food intake and body weight in humans, and their mechanism of action. Possible abnormalities in the leptin and ghrelin systems that may contribute to the development of obesity will be mentioned. In addition, the potentials of leptin and ghrelin as drug targets will be discussed. Finally, the influence of the diet on leptin and ghrelin secretion and functioning will be described.

Introduction
In most humans, body weight is maintained in a stable condition. Humans can have the same body weight for many years. To have a constant weight, there must be an energy balance; energy intake has to be equal to energy expenditure. However, when the energy balance gets disturbed this may eventually lead to sustained weight problems like for example in obese subjects. A growing number of people, including children, suffer from obesity, particularly in the
western society. In the United States the prevalence of obesity is very high. In 1999-2002 65.1% of the adults were overweight, of which 30.4% were obese (1). In 2002 the prevalence of obesity in Europe ranged from 9% in Italy to 30% in Greece (2). Morbidity and mortality increase gradually with excess of body mass index (BMI) (3). Therefore, many investigators try to identify the underlying mechanisms behind the imbalance between energy intake and energy expenditure.

Body weight is regulated by a complex system, including both peripheral and central factors. Two of the hormones that seem to play an important role in the regulation of food intake and body weight are leptin and ghrelin. Both originate in the periphery and signal through different pathways to the brain, particularly to the hypothalamus (4-6). In the hypothalamus, activation of the leptin or ghrelin receptor initiates different signaling cascades leading to changes in food intake (6, 7). As both the leptin and ghrelin systems are disturbed in obesity, it is important to reveal their mechanism of action for the purpose of developing novel therapeutic interventions.

**Leptin is a hormone produced mainly by adipose tissue**

In 1994 the human OB (obese) gene and its product leptin were identified and characterized by Zhang et al. (8). The OB gene is located on chromosome 7 (7q31.3) and is composed of three exons and two introns spanning 18 kb (9, 10). It encodes a protein consisting of 166 amino acids with a putative signal sequence (11). Only one OB mRNA species has been found in abundance in human adipose tissues (11). In addition to adipose tissue, leptin is also produced in small amount in other human tissues such as the stomach, mammary epithelium, placenta, and heart (12-16).

Leptin acts through the leptin receptor (LEPR or OBR). The OBR gene is located on chromosome 1 (1p31), is constituted of 18 exons and 17 introns, and encodes a protein
consisting of 1162 amino acids (17, 18). One of the splice variants of the *OBR* gene, the one with the longest intracellular domain (*OB-Rb*) and full signaling capabilities, is widely expressed in the human brain (19-21). *OB-Rb* is highly expressed in the hypothalamus and cerebellum (20, 22). In addition, the leptin receptor is expressed in other tissues, such as the human vasculature, stomach, and placenta (15, 23, 24).

Importantly, leptin is released into the circulatory system by the adipose tissue as a function of the energy stores (4, 25). In 1996 Schwartz *et al.* showed that serum and plasma leptin levels are higher in subjects with a higher body mass index (BMI) and a higher percent total body fat (26). In addition, it was demonstrated that plasma leptin can cross the blood-brain barrier (BBB), and cerebral spinal fluid (CSF) leptin levels also turned out to be correlated with BMI. After release by the adipose tissue, leptin signals to the brain, giving information about the status of the body energy stores. In rodents and in humans, this results in a decrease in food intake and an increase in energy expenditure to maintain the size of the body fat stores (27-32).

Table 1 gives an overview of several factors, which have a regulatory influence on the circulating leptin levels. For example, the expression of leptin by adipose tissue is also influenced by feeding behavior (25, 33-36). Short-term (twelve hours) or long-term (two or eight weeks) overfeeding results in an increase in adipocyte leptin expression and circulating leptin in healthy human subjects (33, 36). Furthermore, circulating leptin levels show a diurnal pattern and are influenced by gender, age, exercise, and glucose uptake (37-43).
The role of leptin and ghrelin

**Table 1 Regulators of circulating leptin levels.** The release of leptin by adipose tissue is influenced by various factors.

<table>
<thead>
<tr>
<th>Effect on circulating leptin</th>
<th>Energy stores (4, 25)</th>
<th>↑ with increase in body mass index and percent total body fat</th>
</tr>
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<tbody>
<tr>
<td>Food intake (25, 33-36)</td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>Gender (38-40)</td>
<td>Higher in females compared to males</td>
<td></td>
</tr>
<tr>
<td>Age (40)</td>
<td>↓ with increasing age</td>
<td></td>
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<tr>
<td>Exercise (41, 42)</td>
<td>↓</td>
<td></td>
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<tr>
<td>Glucose uptake (43)</td>
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**Leptin's role in energy balance is mediated through the hypothalamus**

Leptin has been reported to have influence on various biological mechanisms, including reproduction (initiation of human puberty), the immune and inflammatory response, hematopoiesis, angiogenesis, bone formation, and wound healing (44-47). Most interestingly, leptin functions as a feedback mechanism that signals to key regulatory centers in the brain to inhibit food intake and to regulate body weight and energy homeostasis. This has been demonstrated by many studies in rodents (27, 28).

Based on studies in mice and rats it has been demonstrated that the hypothalamus is the primary center for regulation of food intake and body weight (48-50). After release of leptin by the adipose tissue into the bloodstream, leptin crosses the BBB and binds to the hypothalamic leptin receptors, giving information about the status of the body energy stores (6, 26, 51, 52, Figure 1). By binding to its receptors, leptin has influence on the activity of various hypothalamic neurons and on the expression of various orexigenic and anorexigenic neuropeptides.
Orexigenic peptides which levels are influenced by leptin include neuropeptide Y (NPY), melanin-concentrating hormone (MCH), agouti-related protein (AgRP), galanin (GAL), orexin, and galanin-like peptide (GALP; 48, 52-56). Furthermore, regulation of the effects of ghrelin on hypothalamic neurons (ghrelin blocks leptin’s action through the activation of the hypothalamic NPY/Y1 receptor pathway) has been suggested to be one of the important mechanisms by which leptin may control food intake and body weight (6, 57, 58). However, studies on the effects of leptin on circulating ghrelin levels in humans have given conflicting results (59-63). It is therefore still possible that leptin is not an upstream regulator of ghrelin.

Anorexigenic peptides, which expressions seem to be modulated by leptin, include POMC, cocaine- and amphetamine-regulated transcript (CART), neurotensin (NT), corticotropin-releasing hormone (CRH), and brain-derived neurotrophic factor (BDNF) (51-53, 64, 65). The orexigenic and anorexigenic neurons, which are located in the various hypothalamic regions (ARC, LH, PFH, and PVN), interact with each other (66-68). Compromise in interactions between orexigenic peptides or in their effects on anorexigenic peptides has been suggested to be one of the possible mechanisms of leptin action in the hypothalamus (6).

**Leptin induces weight loss by suppression of food intake and by stimulation of metabolic rate**

Montague *et al.* (1997) provided the first genetic evidence that leptin is an important regulator of energy balance in humans (69). The investigators studied two severely obese children. Congenital leptin deficiency, due to a homozygous frameshift mutation in the *OB* gene, was found to be associated with normal birth weight, followed by a rapid development of severe obesity associated with hyperphagia (overeating) and impaired satiety. Farooqi *et al.* (2001) examined subjects who were heterozygous for the same frameshift mutation (30). Serum leptin
concentrations were lower compared to controls and were accompanied by an increased prevalence of obesity. Leptin treatment results in decreased appetite, weight loss, increased physical activity, changes in endocrine function and metabolism, and beneficial effects on ingestive and noningestive behavior in leptin deficient patients (30, 32). Furthermore, Weigle et al. (2003) showed that leptin seems to contribute to ongoing weight loss after twelve weeks of dietary fat restriction in healthy humans (70). The effect of leptin on energy expenditure in humans is less clear. Several investigators showed that circulating leptin is not correlated with metabolism in lean or obese subjects (36, 39, 71, 72). On the other hand, Jorgensen et al. (1998) showed that the serum leptin level is a strong positive determinant of resting metabolic rate (RMR) in healthy men (29). In addition, Kennedy et al. (1997) and Jeon et al. (2003) also found a correlation between serum or plasma leptin levels and RMR (31, 39).

Until several years ago, leptin was thought only to play a significant role in long-term regulation of energy balance. More recent data indicate that leptin also seems to play a role in short-term regulation of food intake and body weight. Leptin is not only produced by adipose tissue, but also in small amount by the stomach (15). Therefore, it has been suggested that leptin might play a role in the control of meal size in cooperation with other satiety peptides (73, 74, 75). It has been shown that several intestinal peptides induce gastric leptin release (15, 75). In addition, gastric leptin secretion is stimulated by the administration of insulin, which is a hormone released into the bloodstream shortly after food intake (76). Furthermore, high-fat meals but also mixed meals lower 24h circulating leptin levels (77, 78). It is however possible that gastric leptin serves more as a local stimulus, for example by playing a role in food digestion and absorption in the intestines (15, 74, 75). Additional studies are necessary to confirm this hypothesis.

For a long time, many investigators focused their attention on the role of leptin in the pathogenesis of obesity. However, several years ago, many researchers started to realize that
leptin might be more importantly involved in adaptation to energy deprivation. Fasting for 36 hours (or three days) has been shown to result in a significant decrease of plasma leptin concentration (25, 34). This decline in plasma leptin was much greater than the change in adipose mass, indicating that this change in adipose mass is not solely responsible for the decrease in circulating leptin concentration. Several studies have demonstrated that leptin is involved in the neuroendocrine response to starvation, including changes in hormone concentrations, and possibly changes in sympathetic nervous system activity and reproductive function (79, 80). Disease states like exercise-induced amenorrhoea and anorexia nervosa are also associated with low leptin concentrations and show similar changes in neuroendocrine functioning (81). Importantly, many of the neuroendocrine alterations that occur during fasting are blunted in obese individuals (79, 82).

**Ghrelin is a hormone secreted by the stomach**

The gene coding for human prepro-ghrelin, *GHRL*, is located on chromosome 3 (3p25-26), and is composed of 4 exons and 3 introns spanning 5kb (83, 84). Human prepro-ghrelin consists of 117 amino acids, and the mature ghrelin peptide is constituted of 28 amino acids with a fatty acid chain modification (octanoyl group) on the third amino acid (85). Ghrelin peptide was originally isolated from the stomach, but ghrelin protein has also been identified in other peripheral tissues, such as the gastrointestinal tract, pancreas, ovary, and adrenal cortex (85-89). In the brain, ghrelin producing neurons have been identified in the pituitary, in the hypothalamic ARC, and in a group of neurons adjacent to the third ventricle between the dorsal, ventral, paraventricular, and arcuate hypothalamic nuclei (68, 85, 90).
Ghrelin binds to the growth hormone secretagogue receptor (GHS-R). By nucleotide sequence analysis Howard et al. (1996) identified two types of cDNA encoding for the GHS-R, which were derived from the same gene and were referred to as GHS-R1a and GHS-R1b (91, 92). The gene encoding for the human GHS-R1 receptor is located on chromosome 3 (3q26.2) and is constituted of 2 exons and 1 intron spanning 4kb (84, 92, 93). The GHS-R1a receptor is constituted of 366 amino acids. Of the GHS-R1b variant it is not clear if it is transcribed into protein in vivo, but theoretically it would code for 289 amino acids (92). The GHS-R1 receptor was originally cloned from the human pituitary and arcuate ventro-medial and infundibular hypothalamus (91). In addition, GHS-R1 receptors have been identified in other human tissues, such as the gastrointestinal tract, ovary, and testis (94-96).

The secretion of ghrelin by the stomach depends largely on the nutritional state. Ghrelin levels show preprandial increases and postprandial decreases (59, 97, 98). In addition, ghrelin levels show a diurnal variation and seem to be influenced by age, gender, BMI, growth hormone (GH), glucose, and insulin (Table 2; 59, 63, 97, 99-105). However, several of these correlations could not be confirmed (100, 106). Notably, also leptin has been suggested to have influence on circulating ghrelin levels. It has been hypothesized that the satiety-inducing effects of leptin includes the suppression of ghrelin secretion (107). Indeed the effects of leptin on energy homeostasis are opposite (though not complementary) to those of ghrelin; leptin induces weight loss by suppression of food intake, whereas ghrelin functions as an appetite-stimulatory signal. Moreover, leptin has been shown to be an upstream regulator of ghrelin in rodents (57, 84, 108). However, several studies in humans have given conflicting results. For example, Tschop et al. (2001) demonstrated that in obese patients fasting plasma ghrelin levels are negatively correlated with fasting plasma leptin levels (60). However, in another study fasting plasma leptin and ghrelin concentrations were not correlated in obese children and adolescents (61).
addition, intermeal ghrelin levels are displaying a diurnal rhythm that is in phase with that of leptin in healthy humans (59). Furthermore, a recent study showed that leptin administration to healthy volunteers does not regulate ghrelin levels over several hours to a few days (63). These results suggest that leptin does not regulate circulating ghrelin levels. It is therefore possible that the leptin and ghrelin systems function independently of each other in the control of energy homeostasis.

Table 2 Regulators of circulating ghrelin. The release of ghrelin by the stomach is influenced by various factors.

<table>
<thead>
<tr>
<th>Effect on circulating ghrelin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Food intake (59, 93, 94)</td>
</tr>
<tr>
<td>Age (95)</td>
</tr>
<tr>
<td>Gender (63, 96)</td>
</tr>
<tr>
<td>BMI (93, 97, 98)</td>
</tr>
<tr>
<td>GH (99)</td>
</tr>
<tr>
<td>Glucose (100)</td>
</tr>
<tr>
<td>Insulin (101)</td>
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</table>

The role of ghrelin in food intake is mediated through the hypothalamus

The effects of ghrelin on energy balance are at least in a large part mediated by the hypothalamus. Korbonits et al. (2004) proposed three different pathways for the appetite-inducing effects of ghrelin (103). First, after release into the bloodstream by the stomach, ghrelin may cross the BBB and bind to its receptors in the hypothalamus (89, 103, 109). Second, ghrelin may reach the brain through the vagal nerve and nucleus tractus solitarius (NTS) (84, 103).
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Third, ghrelin is produced locally in the hypothalamus, where it may directly affect the various hypothalamic nuclei (68, 103).

Ghrelin attenuates leptin-induced reduction in food intake and body weight by modulating the expression of various hypothalamic peptides. Ghrelin stimulates the activity of neurons expressing NPY, AgRP, and orexin (57, 110, 111). On the other hand, ghrelin has an inhibitory effect onto POMC neurons and CRH-producing neurons (68). Ghrelin does not seem to be a direct regulator of leptin, as fasting produces identical decreases in serum leptin in ghrelin null and wild-type mice (112). The results gathered so far indicate that leptin and ghrelin have different effects on the hypothalamic neurons producing the various orexigenic and anorexigenic peptides, resulting in more or less opposing effects on energy balance, Figure 1.
Figure 1 Pathways by which leptin and ghrelin may have effect on energy balance in humans. This schematic drawing shows the pathways by which leptin and ghrelin may reach the hypothalamus, in order to have an effect on food intake and body weight. Leptin is secreted by adipose tissue and ghrelin is secreted by the stomach. Both hormones may enter the brain through the bloodstream (long arrow with straight line). In addition, ghrelin and gastric leptin may reach the hypothalamus through the vagal nerve and nucleus tractus solitarus (short arrows with straight line). In addition, central ghrelin may affect the energy center in the hypothalamus (curved arrow). Leptin and ghrelin both stimulate (+) and suppress (−) hypothalamic neurons containing various neuropeptides, resulting in anorexic or orexic effects on energy balance (open arrows). Studies on the effect of leptin on circulating ghrelin levels gave conflicting results, whether ghrelin has influence on circulating leptin levels has not been demonstrated yet (curved arrows with dashed line). AgRP, agouti-related protein; BDNF, brain-derived neurotrophic factor; CART, cocaine- and amphetamine-regulated transcript; CRH, corticotropin-releasing hormone; GALP, galanin-like peptide; MCH, melanin-concentrating hormone; NPY, neuropeptide Y; NT, neotensin; POMC, pro-opiomelanocortin (6, 11, 14, 30, 32, 47, 51-58, 64, 65, 68, 75, 80, 81, 85, 99, 105-108, 112-115).
Ghrelin presumably functions as an appetite-stimulatory signal

Ghrelin has been shown to regulate the secretion of GH by the pituitary (85). In addition, ghrelin has effect on the gastrointestinal tract, immune cell activation, and inflammation (113, 114). Interestingly, in 2000 Tschop et al. reported that ghrelin seemed to be involved in the regulation of food intake and energy balance in mice and rats (115). Based on the results, it was postulated that ghrelin signals to the hypothalamus when an increase in metabolic efficiency is necessary. It has been demonstrated that the preprandial increase in ghrelin levels correlates with hunger scores in healthy humans, initiating meals voluntarily in the absence of time- and food-related cues (116). In addition, an intravenous injection or infusion of ghrelin also induces hunger and food intake among healthy and obese humans (117-119). Together, this indicates that ghrelin seems to function as a meal-initiation signal in the system for short-term regulation of energy balance. Based on results of studies with mice, Asakawa et al. (2005) postulated that this increase in food intake after ghrelin administration is mediated through its stimulatory effect on gastric emptying (120). This might also be the case in humans, as it has been demonstrated that circulating ghrelin levels are correlated with gastric emptying in human subjects (121). If ghrelin also has an influence on the regulation of energy expenditure is not clear. It has been reported that rodents show decreased energy expenditure after peripheral administration of ghrelin (115). However, this has not yet been demonstrated in humans.

Besides playing a role in short-term regulation of food-intake, ghrelin might also play a role in long-term regulation of energy balance. Peripheral daily administration of ghrelin induces adiposity in rodents by reducing fat utilization (115). In addition, circulating ghrelin concentrations are negatively correlated with BMI in humans, and these levels increase when obese humans lose weight, and decrease when anorexia nervosa patients gain weight. This suggests that ghrelin levels change in response to dieting to maintain body weight (101, 102).
Also in Prader-Willi syndrome (PWS), which is a syndrome resulting from a genetic defect and among other things is characterized by insatiable appetite and obesity, plasma ghrelin concentrations are higher compared to healthy subjects (122). Again, these ghrelin concentrations are negatively correlated with BMI. Furthermore, plasma ghrelin levels decrease after gastrectomy, which most likely contributes to the weight-reducing effect of this procedure (97). However, this might also be due to alterations in other gut peptides involved in regulation of appetite.

Finally, ghrelin does not seem to be crucial for the maintenance of energy homeostasis. Ghrelin knockout mice (ghrelin \(-/-\)) have a normal body size, body composition, bone density, growth rate, gastric emptying, food intake, reproduction, gross behavior, and tissue pathology (112, 123). Fasting results in normal decreases in serum insulin and leptin, and ghrelin administration stimulates appetite in ghrelin \(-/-\) mice. Moreover, also Ghsr-null mice have a normal appetite, show a normal body size, body composition, body weight, and bone density, and show normal serum leptin and insulin responses to fasting (124). Except, body weights of mature Ghsr-null mice were modestly reduced, which might be related to ghrelin’s role in GH release, resulting in subtle changes in body composition. Together this indicates that ghrelin is not critically required for growth, appetite, and fat deposition, and is not likely to be a direct regulator of leptin and insulin. It was suggested that other redundant appetite-inducing agents might compensate for loss of ghrelin functioning. Instead, De Smet et al. (2006) showed that in old mice ghrelin is a mediator of meal initiation triggered by the light/dark cycle, and in young animals ghrelin was suggested to be possibly involved in the selection of energy stores and in the partitioning of metabolizable energy into storage or dissipation as heat (123).
The role of leptin and ghrelin

Do abnormalities in leptin and ghrelin or their actions contribute to the development or maintenance of obesity?

Although it would be expected that in obese humans leptin levels are decreased and ghrelin levels are increased, circulating leptin levels turned out to be increased and circulating ghrelin levels showed to be decreased (60, 125-127). In addition, obese humans show a disturbed diurnal variation in leptin and ghrelin levels (107). It is still not clear if these abnormalities in the leptin and ghrelin systems are the cause or a consequence of obesity. Although several investigators were able to attribute obesity to polymorphisms in the genes encoding for leptin, ghrelin, and their receptors, it seems that defects in these genes are generally not involved in obesity in humans (22, 83, 126, 128-135).

As obese humans show elevated levels of leptin in serum and adipocytes, and show limited effects with leptin treatment, many researchers suggest obese humans to be leptin resistant (22, 26, 127, 136-138). The development of leptin resistance most likely involves a period of overeating, resulting in the leptin system getting so disturbed that it leads to sustained defects. Overeating results in an increase in circulating leptin levels (33, 36). This exposure of the hypothalamus to high leptin levels may have damaging effects on the hypothalamus. As a result, the hypothalamus becomes less sensitive to leptin, leading to a sustained increase in leptin levels. It has already been shown that chronic leptin infusion leads to leptin resistance in a rat model (139). In addition, Kolaczynski et al. (1996) showed that humans develop leptin resistance due to overfeeding (33).

It has been postulated that leptin resistance might be due to defective leptin transport across the BBB. Several studies support this hypothesis (26, 127, 140). It has been shown that DIO (diet-induced obese) mice develop resistance to peripherally administered leptin, while retaining sensitivity to centrally administered leptin (140). This suggests that these mice have disturbed
leptin transport through the BBB. In humans, the ratio between leptin levels in CSF and plasma has been shown to be lower in obese subjects compared to lean individuals (26, 127). This suggests that leptin enters the brain by a saturable transport system and that the capacity of leptin transport is lower in obese individuals, hereby providing a mechanism for leptin resistance. However, Levin et al. (2003) demonstrated that BBB leptin transport was not different between preobese DIO and DR (diet-resistant) rats, and impaired leptin transport developed only after DIO rats became obese and/or aged (141). Thus, defects in leptin transport appear to be an acquired defect associated with the development of obesity. In addition, preobese DIO rats had reduced leptin receptor mRNA expression in the Arc, in association with reduced leptin-induced anorexia after peripheral leptin administration. The investigators suggested that a preexisting reduction in hypothalamic leptin signaling might contribute to the development of diet-induced obesity when dietary fat and calorie intake are increased.

One other possibility is that a defect in leptin receptor expression in the hypothalamus is the cause of altered leptin sensitivity. Hypothalamic leptin receptor mRNA levels are decreased in DIO rats (141). In addition, in obese db/db and ob/ob mice Obrb mRNA levels in the Arc are increased (142). Furthermore, leptin administration reduces Obrb mRNA levels in the Arc of ob/ob mice, and fasting increases Obrb mRNA levels in the Arc of normal mice. The investigators proposed that hypothalamic Obrb expression might be sensitive to genetic and physiological interventions that alter circulating leptin levels, and that overexpression of the leptin receptor in the hypothalamus might contribute to increased leptin sensitivity (142).

However, it is important to note that in 1996 Considine et al. did not find a difference in the amount of leptin receptor mRNA between lean and obese humans (22). Therefore, this concept needs further investigation.
The role of leptin and ghrelin

It is also possible that leptin resistance is caused by defects in the downstream mediators of leptin. Based on studies with mice, AgRP and its receptor (Mc4r) have been proposed to be good candidates for human disorders of body weight regulation (143). In addition, changes in gene expression in Npy/Agrp neurons and also Pomp neurons have been demonstrated in various animal studies (6). Also defects in the signaling pathways downstream of the leptin receptor might play a role in reduced leptin response in the hypothalamus. The JAK-STAT pathway is one of the major pathways of leptin signal transduction (21, 144). El-Haschimi et al. (2000) demonstrated in studies with DIO mice that peripheral administered leptin was unable to activate hypothalamic Stat3 signaling, and the magnitude of Stat3 activation was substantially reduced after intracerebroventricular (icv) leptin (145). Several investigators have reported the negative regulators of leptin signaling (protein tyrosine phosphatase 1B, PTP1B; SH2-containing phosphatase 2, SHP2; suppressor of cytokine signaling 3, SOCS3) to be potential factors in leptin resistance (146-148). SOCS3 mRNA expression in the hypothalamus is induced by leptin (146). It mediates negative feedback on JAK-STAT activation. Excessive SOCS3 activity might therefore be involved in leptin resistance. Indeed, in 2004 Howard et al. demonstrated that mice with heterozygous Socs3 (Socs3<sup>+/−</sup>) deficiency display greater leptin sensitivity than wild-type mice; they showed enhanced weight loss and increased hypothalamic leptin receptor signaling after leptin administration (149). In addition, Socs3<sup>+/−</sup> mice seemed to be protected against the development of diet-induced obesity. Thus, the level of Socs3 expression seems to be a determinant of leptin sensitivity and susceptibility for obesity.

Whether an elevated level of circulating leptin causes a reduction in ghrelin levels is still not clear. However, it seems that leptin does not have a direct influence on ghrelin levels. It is possible that decreased plasma ghrelin concentrations represent a physiological adaptation to the positive energy balance associated with obesity (60). This is in line with the observation that
circulating ghrelin levels in obese patients increase during weight loss (102). Obese humans do not lose their responsiveness to ghrelin, or have a defect in ghrelin transport at the BBB, as peripheral administration still results in an enhanced appetite in obese subjects (118). It may be that obese patients are oversensitive to ghrelin, for example because of an overexpression of the GHS-receptor. It has been shown that a low-dose infusion of ghrelin has no effect in lean people, but does increase ad libitum energy intake in obese subjects (150). In addition, a high-dose infusion with ghrelin led to a higher increase in food intake in obese patients compared to lean subjects. However, in mice it has been shown that constitutive overexpression of Ghs-r does not affect food intake and adipose tissue response to Ghs ligands (151).

Finally, in recent studies conducted by Asakawa et al. (2005) and Zhang et al. (2005) it was demonstrated that also desacyl ghrelin and obestatin (which are peptides derived from the same ghrelin gene, that undergo differential posttranslational modifications) play a role in energy balance (120, 152). The investigators showed that treatment of rodents with desacyl ghrelin or obestatin induced a negative energy balance by decreasing food intake and delaying gastric emptying, and by decreasing body weight gain. Thus, ghrelin on one hand and desacyl ghrelin and obestatin on the other hand seem to have opposing effects on weight regulation. It might be that dysfunctioning of desacyl ghrelin or obestatin is involved in the pathophysiology of obesity. For example, disturbed posttranslational processing of the GHRL gene and therefore decreased expression of desacyl ghrelin and obestatin may result in increased food intake and body weight.

The potential of leptin and ghrelin as a drug target for weight regulation

Many studies have been performed to investigate the potential of both leptin and ghrelin as therapeutic target. Unfortunately, although leptin treatment has been shown to have beneficial
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effects in patients with leptin deficiency, it shows very limited effects in obese people (136-138). Therefore, several investigators try to find alternatives for the normal leptin hormone and to develop strategies that bypass normal central leptin functioning. In a recent study Lo et al. (2005) introduced a superior form of leptin, having enhanced pharmacological properties in comparison to recombinant leptin that has been used in former clinical trials (153). The Fc-leptin immunofusins (consisting of the Fc fragment of an immunoglobulin gamma chain followed by leptin) led to a significant weight loss in non leptin-deficient mice. In addition, Fc-leptin had an extended circulating half-life. This makes Fc-leptin an interesting compound for the treatment of non leptin-deficient obese humans. In 2003 Weigle et al. showed that leptin contributes to ongoing weight loss after twelve weeks of dietary fat restriction in healthy humans (70). Moreover, in a recent study Rosenbaum et al. (2005) showed that daily administration of leptin, in addition to a diet, could prevent adaptations normally occurring during weight loss (154).

Also the potential of the ghrelin system as a therapeutic target for obesity treatment is still under discussion. As it has been demonstrated that circulating ghrelin levels increase when obese humans lose weight, and because obese mice show an increase in sensitivity to ghrelin upon weight loss, blockage of ghrelin could prevent weight regain after weight loss (155). In a recent study with rats it was demonstrated that anti-ghrelin blocks ghrelin-induced increase in food intake after ghrelin injection (156). In addition, the ghrelin receptor constitutes a potential drug target. The GHS-receptor has been shown to be constitutively active (157). Blocking this constitutive receptor activity was suggested to possibly lower the set point for hunger between meals. It has already been demonstrated that GHS-R antagonists result in a decrease of energy intake in lean and obese mice, and repeated administration gave a decrease of body weight gain in ob/ob mice (158). However, as it is possible that the ghrelin system functions differently in humans, similar studies in human subjects are still necessary. Notably, in another study a
novel GHS-R1a antagonist was discovered, which blocks ghrelin-induced GH release in the medial arcuate nucleus, but like ghrelin induces increased body weight gain through the dorsal medial hypothalamus (159). The investigators suggested that the role of ghrelin in weight gain might be mediated by a novel receptor other than GHS-R1a. Therefore, GHS-R1a might not be a potential target to block ghrelin-induced food intake.

One other strategy is to target genes that are involved in leptin or ghrelin functioning, for example negative regulators of leptin or ghrelin signaling. Howard et al. (2004) proposed SOCS3, which has been identified as a leptin-induced negative regulator of leptin receptor signaling and potential mediator of leptin resistance, to be a potential target for therapeutic intervention (149). In addition, PTP1B has been suggested to be a valuable target for the treatment of leptin resistance in human obesity (160). Likewise, the use of agents that stimulate inhibitors of ghrelin signaling may be a potential way to suppress ghrelin’s stimulatory effect on food intake and body weight.

**Can the diet be modulated to stimulate the secretion or enhance the action of leptin and ghrelin?**

Food intake can have significant effects on circulating leptin and ghrelin levels. Overfeeding results in an increase in adipocyte leptin expression and circulating leptin in healthy human subjects (33, 36). Fasting (for 20 or 36 hours or three days) results in a decrease of adipocyte leptin mRNA and serum leptin levels, with a greater decline in leptin levels in lean subjects than in obese subjects (25, 34, 35). Refeeding is again associated with a rise in serum leptin levels, and leptin levels return to baseline after 24 hours (25, 34). On the other hand, fasting results in an increase in plasma ghrelin levels, with a nearly twofold increase immediately before each meal (59, 97). This preprandial increase in ghrelin levels correlates with hunger scores in
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humans (116). Feeding results in a decrease in plasma ghrelin levels within one to two hours (59, 98).

Not only the size and frequency of meals has an effect on circulating leptin and ghrelin levels, also the composition of a meal is a determinant of leptin and ghrelin levels in humans, Table 3. For example, low-fat/high carbohydrate meals result in an increase of circulating leptin concentrations, which is larger, compared to high-fat/low-carbohydrate meals (161). In addition, high-fat meals lower 24h circulating leptin levels relative to high-carbohydrate meals (78).

Hydrolyzed guar fiber or protein intake does not seem to have influence on circulating leptin concentrations (162, 163).

A low-fat diet seems to have an inhibitory effect on ghrelin levels, as one study reported that a low-fat/high-carbohydrate diet resulted in weight loss, without an increase in plasma ghrelin levels (70). Another study demonstrated that a high-carbohydrate diet caused a larger drop in ghrelin levels than a high-fat diet in healthy women (164). The effect of protein ingestion on ghrelin levels gives conflicting results (163, 165, 166). Finally, the use of non-caloric Psyllian fibers results in a decrease of plasma ghrelin levels in healthy women (167). Together, these data indicate that for obese subjects it is important to follow a specific diet in order to regulate food intake and body weight.
Table 3 Effects of diet composition on circulating leptin and ghrelin levels. The composition of a diet can have increasing or decreasing effect on circulating leptin and ghrelin levels.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Effect on circulating leptin</th>
<th>Effect on circulating ghrelin</th>
</tr>
</thead>
<tbody>
<tr>
<td>High-fat</td>
<td>24h circulating leptin levels ↓ relative to high-carbohydrate meal (78)</td>
<td>↓ (149)</td>
</tr>
<tr>
<td>High-carbohydrate</td>
<td></td>
<td>↓ (Larger drop compared to high-fat diet, 149)</td>
</tr>
<tr>
<td>Low-fat/high-carbohydrate</td>
<td>↑ (larger compared to high-fat/low-carbohydrate meal, 146)</td>
<td>No increase (70)</td>
</tr>
<tr>
<td>High-fat/low-carbohydrate</td>
<td>↑ (146)</td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>No effect (148)</td>
<td>Conflicting results (148, 150, 151)</td>
</tr>
<tr>
<td>Hydrolyzed guar fiber</td>
<td>No effect (147)</td>
<td></td>
</tr>
<tr>
<td>Non-caloric Psyllian fibers</td>
<td></td>
<td>↓ (152)</td>
</tr>
</tbody>
</table>
Conclusion
What becomes clear from this review is that both leptin and ghrelin play major roles in the control system for energy balance in humans. However, leptin is primarily involved in long-term regulation of energy balance; it is released into the circulatory system as a function of energy stores, whereas ghrelin is a fast-acting hormone, of which the circulatory levels show clear meal-related changes. One other difference is that, in contrast to leptin, ghrelin does not seem to be critical for normal appetite and growth. Interestingly, leptin and ghrelin functioning in the system for energy homeostasis involves several overlapping pathways. At present, it is still not clear whether abnormalities in the leptin or ghrelin systems contribute to the development of obesity. Nevertheless, disturbances in both systems seem to play a role in the maintenance of obesity. Most important, obese patients are leptin resistant and it is therefore necessary to develop a treatment which overcomes leptin insensitivity or that bypasses normal central leptin functioning. For example, by developing novel forms of leptin with stronger physiological properties. The Fc-leptin immunofusins used by Lo et al. (2005) were shown to have positive effects on body weight in mice (153). Additional studies are warranted to assess the effects of these compounds in humans. Also ghrelin is still recognized as a potential drug target for weight regulation. When obese patients lose weight ghrelin levels show an increase, as if to compensate for this weight loss (155). Therefore is seems interesting to try ghrelin antagonists while following a strict diet. Furthermore, the peptides downstream of leptin and ghrelin constitute possible targets for therapeutic interventions. For example, Makimura et al. (2002) demonstrated that a reduction of hypothalamic Agrp results in an increase of metabolic rate and a decrease of body weight without affecting food intake in mice. This suggests that agents antagonizing the effect of AgRP may be a useful strategy to treat obesity, without producing unacceptable loss of appetite (168). Interestingly, Belsham et al. (2004) created a number of hypothalamic neuronal cell lines, which can be used as models to study the regulation of neuropeptides associated with the control of
feeding behavior. Eventually, such studies may provide information that is necessary for the
design of anti-obesity agents (169).

As diet and exercise have significant effects on energy homeostasis, the use of solely therapeutic
drugs to treat obesity does not seem to be sufficient. Orzano et al. already showed that the most
effective treatment is provided by a combination of diet and exercise (3). Taken together, the best
strategy to accomplish long-term changes in body weight seems to be the use of potential anti-
obesity agents in combination with a low-fat diet and sufficient exercise.
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The role of leptin and ghrelin


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The role of leptin and ghrelin


Chapter 3

The Impact of Blood Glucose Levels on Stimulated ACTH and Growth Hormone Release in Healthy Subjects

Jakobsdóttir S, Twisk JW, Drent ML.
Chapter 3

SUMMARY

Objective In studies investigating the influence of glucose levels on the pituitary function the methods used have been variable and mainly focused on the change in function as a reaction to unphysiological low or high blood glucose levels. In the present study the impact of physiological and elevated blood glucose levels on ACTH and growth hormone release are investigated.

Design The euglycaemic and hyperglycaemic clamp techniques were used to reach stable levels of 4, 8 and 12 mmol/l blood glucose levels. After a stabilisation phase of 2 hours a CRH or a GHRH stimulation test was performed.

Subjects Seven, and eight healthy male volunteers participated in this study.

Measurements The Area Under the Curve (AUC), peak values and time to peak of ACTH, cortisol and growth hormone were calculated to evaluate the response to the CRH and GHRH stimulation test.

Results The peak values of ACTH, cortisol and growth hormone seemed to be highest during the 4 mmol/l clamp sessions, compared to the 8 and 12 mmol/l clamps, although the differences were not statistically significant when analysed for every subject individually. The AUC and time to peak measurements were comparable during the three clamp procedures.

Conclusion The pituitary reaction on CRH and GHRH was not significantly changed by various blood glucose levels.
INTRODUCTION

Blood glucose levels and insulin seem to play an important role in the neuroendocrine derangements seen in diabetes mellitus, insulin resistance and obesity. The neuromodulatory effect of glucose and insulin has been established to some extent, yet the exact site of action remains speculative. The hippocampus and cortex are most sensitive for glycopenia, but glucose and insulin seem to have influence on the hypothalamic and pituitary level also (1-6). The reaction of the pituitary to low blood glucose levels is very strong and known as the counter regulatory response of Growth Hormone (GH)-, Adrenocorticotropic Hormone (ACTH)- and cortisol secretion. As an example, hypoglycaemia leads to a five to six fold rise in ACTH peak within 45 minutes (7). The set point for activating this counter regulatory response is not fixed and depends on factors such as antecedent blood glucose, insulin and cortisol levels (8-12).

On the other hand, high blood glucose levels suppress growth hormone secretion in normal subjects (13-16). Although the exact mechanism remains unknown, GH secretion may be inhibited by blood glucose at the hypothalamic level by increased somatostatin secretion or reduced Growth Hormone Releasing Hormone (GHRH). It is also possible that elevated glucose levels inhibit GH secretion at the level of the pituitary somatotroph cells directly (4;5). It is unclear however, whether this inhibition of GH secretion is caused by the blood glucose level itself or by the secondary rise in insulin levels. In patients with diabetes GH levels are abnormally high and the response to GH stimuli is exaggerated, suggesting a central derangement caused by glucose levels, FFA, IGF-1 or insulin (17-19). In patients with diabetes an elevated set point for the reactivity to blood glucose levels of the pituitary-adrenal axis has been described either as a consequence or a cause of deranged carbohydrate metabolism or insulin insensitivity (20).

Whether the pituitary hormone release in normal subjects is related to blood glucose levels in a situation of normoglycemia is not clear. In studies which have been performed to investigate the
influence of glucose on the pituitary function in normal subjects and patients with diabetes the methods have differed widely. In most cases insulin induced hypoglycaemia and sometimes a glucose clamp procedure have been used to investigate the pituitary response to altered blood glucose levels (5;6;12;19;21-25).

The present study was designed to investigate the influence of physiological and elevated blood glucose levels on stimulated pituitary function, during a stable situation using a euglycemic hyperinsulinemic or hyperglycaemic clamp technique. We hypothesized that ACTH- and GH secretion following a standardised stimulatory test are influenced by blood glucose levels within the physiological range, resulting in a faster and/or higher peak after stimulation when the level is 4 mmol and a slower and/or lower peak response at 8 and/or 12 mmol.

SUBJECTS AND METHODS

Subjects
Seven, respectively eight healthy male volunteers participated in a GHRH and a Corticotropin Releasing Hormone (CRH) stimulation test. One subject in the GHRH group decided to participate in two instead of the three clamp sessions. The anthropometric characteristics are shown in table 1.

Table 1: Subject characteristics

<table>
<thead>
<tr>
<th></th>
<th>CRH stimulation test (n = 8)</th>
<th>GHRH stimulation test (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>24.6 +/- 2.9</td>
<td>25.3 +/- 2.4</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>80.6 +/- 9.1</td>
<td>85.1 +/- 12.4</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>23.1 +/- 1.4</td>
<td>23.9 +/- 2.2</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>87.1 +/- 7.3</td>
<td>88.0 +/- 9.6</td>
</tr>
</tbody>
</table>

mean levels with standard deviation
Exclusion criteria were previous history of endocrine, kidney, heart, hepatic or central system disease and taking any medication or drugs. All subjects gave written informed consent. The study was approved by the Medical Ethical Committee of the VU University Medical Center and was conducted according to the principles of the Helsinki Declaration.

**Methods**

The subjects were randomised regarding the order in which the various glucose levels had to be reached (4, 8 or 12 mmol/l) and came on three separate occasions for the clamp sessions with at least 1 week interval. At 8 a.m., after an overnight fast, the subjects were studied in the supine position. Indwelling catheters (Venflon: Viggo, Helsingborg, Sweden) with a 3-way stopcock were inserted in an antecubital vein of each arm, one for glucose (and insulin during the 4 mmol glucose clamp technique) infusion and the other one for blood sampling.

To reach the blood glucose level of 4 mmol/l a euglycemic hyperinsulinemic clamp was performed. Insulin 30 IU (Velosulin: NovoNordisk, Denmark) was diluted to 50 ml with 44.7 ml of 0.9% sodium chloride and 5 ml of 20% human albumin was infused at a rate of 40 mU / min / m² during a 4 hour clamp (26). 20% Glucose solution was infused to maintain a steady state, using a manual method as described earlier (27). Glucose was measured every 5 minutes with an automated glucose oxidase method with a glucose analyser YSI 2300 (Yellow Springs Instruments, Yellow Springs, OH, U.S.A.). After a period of two hours in steady state, the subjects received 100 µg CRH corticorelin (Ferring B.V) or, respectively, 100 µg GHRH somatorelin (Ferring B.V.) as a bolus injection i.v. Blood samples for determination of ACTH, Cortisol or GH were drawn at T = -15, 0, 15, 30, 45, 60, 90, 120 minutes. The samples for plasma ACTH were obtained in heparinized tubes (Sarsted, Nurnbrecht Germany) and directly placed on ice. All samples were immediately processed, centrifuged at 4° C for 10 minutes, at 3500 x g. They were then frozen at –20° C and stored until assays were performed.
In order to achieve a steady state of blood glucose levels at 12 mmol/l we used the hyperglycaemic clamp technique as described by de Fronzo et al. using a 15 minute glucose priming dose of 9,622 mg/m² and thereafter adjusting the maintenance dose of glucose depending on the glucose measurements every 5 minutes. To reach the level of 8 mmol/l the same method was used, except the priming dose was adjusted downwards proportionally (28). Thereafter the same clamp procedure was performed as described above during a period of 4 hours. Because not only glucose but also insulin may influence the pituitary response, insulin levels were measured throughout the clamp procedure and were used in the statistical analysis. To account for possible effect of dilution the haematocrit levels were obtained in the beginning and at the end of every clamp session (29).

**Hormone analysis**

For determination of cortisol (nmol/l) the competitive immunoassay ACS:180 System, Bayer Diagnostics, Nederland was used. The intra-assay variable with the coefficient of variation (CV) was 3% and inter assay CV 6%. Plasma ACTH was measured using a radioimmunoassay and the sensitivity of the assay was 10 pg/ml. The intra- and interassay coefficients of variation were 5% and 8%, respectively (30). Serum growth hormone was determined using chemoluminiscense Nichols Institute Diagnostics, San Juan Capistrano USA. The detection limit was 0.5 mU/l and the interassay coefficient of variation was 5%.

Insulin was measured using the Immunoradiometric assay Biosource/Medgenix Diagnostics Fleurus Belgium. The intra-assay variable was 2% at 318 pmol/l and 5% at 40 pmol/l and interassay variable CV 6%. The detection limit of the assay was 10 pmol/l.
Statistical analysis

Data are presented as median levels for the various time measurements. To measure the Area Under the Curve (AUC) the following formula was applied:

\[ AUC = \frac{1}{2} \sum_{t=1}^{T-1} (t_{t+1} - t_t)(Y_t + Y_{t+1}) \]

Where: AUC = area under the curve; T = number of measurements; and Y = observation of the outcome variable at time=t (34).

To evaluate the differences in Area Under the Curve (AUC), peak hormone level and time to peak in the three independent clamps (4, 8 and 12 mmol) the Kruskal-Wallis Test was performed. To evaluate the differences in development over time between the three clamps, Generalized Estimating Equations (GEE-analysis) was used. This method for longitudinal data analysis was also used to investigate the possible confounding effect of insulin, by adding insulin to the GEE model. To evaluate the changes in haematocrit levels before and after the clamp procedures Wilcoxon signed ranks test was used. A p-value <0.05 was considered statistically significant.

All analyses were performed using the statistical software package SPSS version 13 (SPSS, Chicago, IL, USA).
RESULTS

The baseline characteristics of the subjects participating in the CRH and GHRH clamp are shown in table 1. Mean glucose concentrations at the beginning of each of the clamp procedures were normal and did not significantly differ between test procedures (table 2).

The target glucose levels were successfully reached during the clamps (table 2) and remained stable thereafter.

Table 2: Mean glucose levels at beginning and during clamp procedure

<table>
<thead>
<tr>
<th>concentration mmol/l</th>
<th>glucose levels at beginning</th>
<th>glucose levels during clamp</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRH stimulation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>4.6 +/- 0.3</td>
<td>4.1 +/- 0.4</td>
</tr>
<tr>
<td>8</td>
<td>4.9 +/- 0.3</td>
<td>7.9 +/- 0.5</td>
</tr>
<tr>
<td>12</td>
<td>4.8 +/- 0.3</td>
<td>11.7 +/- 0.8</td>
</tr>
<tr>
<td>GHRH stimulation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>4.5 +/- 0.4</td>
<td>4.3 +/- 0.5</td>
</tr>
<tr>
<td>8</td>
<td>4.4 +/- 1.2</td>
<td>7.8 +/- 0.8</td>
</tr>
<tr>
<td>12</td>
<td>4.2 +/- 0.4</td>
<td>11.4 +/- 1.2</td>
</tr>
</tbody>
</table>

Mean levels with standard deviation

The clamp procedures were generally well tolerated, most of the subjects experienced hot flushes and slight palpitations during a period of 1-2 minutes after administration of the stimulus bolus.

One subject experienced a vasovagal syncope of short duration during his second clamp session and therefore, as a precaution, his last clamp session was cancelled.
The impact of blood glucose on pituitary hormone release

Insulin levels were stable during the 4 and 8 mmol/l clamps. During the 4 mmol/l clamps of the CRH stimulation the median level was 520 pmol/l and during the 8 mmol/l clamps the median level was 254 pmol/l. After the GHRH stimulation tests the median insulin levels were 478 pmol/l during the 4 mmol/l clamps and 397 pmol/l during the 8 mmol/l clamps. In the 12 mmol/l clamps the levels were higher, namely 591 pmol/l (CRH stimulation) and 760 pmol/l (GHRH stimulation) and kept increasing during the whole period as shown in figure 1.

![Median insulin levels during clamp procedures](image)

**Figure 1:** Median insulin levels during clamp procedures at various time measurements. CRH: corticotropin releasing hormone GHRH: growth hormone releasing hormone

The median levels of ACTH, cortisol and GH at the various time measurements are shown in figures 2, 3 and 4 respectively and in table 3. The median ACTH peak was reached 30 minutes after the CRH stimulus during the 4 mmol/l and 8 mmol/l clamps and 45 minutes after the CRH stimulus during the 12 mmol/l clamps (figure 2). The median ACTH level was highest during the 4 mmol/l clamps, but this was not statistically significant. The ACTH time to peak was delayed...
during the 8 mmol/l clamps compared to the 4 mmol/l clamps, but this difference was not statistically significant. The areas under the curves (AUC) were comparable. The subsequent cortisol peak was reached 30 minutes after the CRH stimulus during the 4 mmol/l clamps and 45 minutes after the CRH stimulus during the 8 and 12 mmol/l. The cortisol measurements showed a similar trend, with the highest peak value reached during the 4 mmol/l clamps in comparison with the 8 and 12 mmol/l clamps, but again this difference was not statistically significant. The cortisol time to peak and AUC values were also not significantly different between the 4, 8 and 12 mmol/l clamps.

**ACTH**

![Box plots of ACTH levels with CRH stimulation at 4 mmol/l and 8 mmol/l](image)
The impact of blood glucose on pituitary hormone release

Figure 2: Median ACTH levels at various time measurements during clamp procedures after stimulation with CRH.

**ACTH**: adrenocorticotropic hormone
**CRH**: corticotropin releasing hormone

Cortisol

![Cortisol graph](image)
Figure 3: Median cortisol levels at various time measurements during clamp procedures after stimulation with CRH: corticotropin releasing hormone

During the GHRH clamp sessions the peak GH level was seen 30 minutes after the GHRH stimulus during the 4 mmol/l and 12 mmol/l clamps, but was seen later (45 minutes) during the 8 mmol/l clamps.

The growth hormone response to GHRH stimulation showed the highest peak at the 4 mmol/l in comparison with the 8 and the 12 mmol/l clamps, but this difference was not statistically significant. The time to peak and AUC were not significantly different as well.
The impact of blood glucose on pituitary hormone release

Growth Hormone

Figure 4: Median growth hormone levels at various time measurements after stimulation with GHRH. GHRH: growth hormone releasing hormone.

º = outliers (IQR 1.5-3)
To evaluate the differences in peak, time to peak and AUC in every subject individually, the Kruskal-Wallis test was performed. During the clamps with CRH stimulation, there were no significant changes in the peak (p=0.58 for ACTH, p=0.99 for cortisol), time to peak (p=0.36 for ACTH and p=0.22 for cortisol) and AUC (p=0.93 for ACTH, p=0.75 for cortisol), between the subjects individually. The same was found in the GHRH stimulation clamps, namely no significant values in peak (p=0.27), time to peak (p=0.52) and AUC (p=0.91).

To account for all measurements that were made the general estimation equation model (GEE model) was used. The differences in development over time when evaluated between the three clamps were not significant.

We found no significant changes in the regression coefficients after adding insulin to this same model (GEE model) to correct for its possible confounding effect.

In some of the clamp procedures the haematocrit levels changed. In the CRH stimulation clamps, the haematocrit levels of the 4 mmol/l and the 8 mmol/l differed statistically (p=0.03 and p=0.02) but not the 12 mmol/l (p=0.12). The haematocrit levels of the GHRH stimulation clamps became significantly different in the 4 mmol/l (p=0.03) and the 12 mmol/l (p=0.04) clamps, but not in the 8 mmol/l clamps (p=0.07).
DISCUSSION

The results of this study do not prove that the stimulated ACTH and GH release is modulated by blood glucose levels in the normal and slightly elevated range. However, there seemed to be a trend in the median peak values and time to peak, but these changes were not significant. We did expect the peak of ACTH, cortisol and growth hormone to be higher at the 4 mmol/l clamps as this value lies in the vicinity of the trigger for the counter regulatory response to low blood glucose levels. During the 4 mmol/l clamp the measured ACTH peak was at 30 minutes, the cortisol peak at 30-45 minutes and the growth hormone peak at 30 minutes, in accordance with previous literature (15;31;32). With regards to the peak level and the time to peak reactions in the 8 and the 12 mmol clamps of the CRH stimulation tests there seemed to be a clamp concentration related trend, lower peak and delayed time to peak in the 8 and the 12 mmol/l clamps respectively when compared to the 4 mmol/l clamps. To our knowledge this has not previous been studied for these specific blood glucose levels using the clamp technique and stimulation tests. Surprisingly during the 12 mmol/l clamps were GHRH stimulation was applied the growth hormone peak was very quickly reached. The reason for this is not clear. Possibly the very high levels of insulin are involved, as it has been speculated that insulin can paradoxically stimulate growth hormone at least in patients with diabetes (4). On the other hand, the opposite, that insulin has an inhibitory effect on GH reaction to GHRH simulation has been suggested in normal subjects and in obese individuals (41). The high levels of insulin however seemed not to have influenced on the ACTH secretion during the 12 mmol/l clamp. When using a statistical method to correct for the confounding effect of insulin, we could not rule out nor verify an effect. In the literature it has been shown that insulin has a modulatory effect on the neuroendocrine regulation either directly or by causing local neuroglycopenia which then starts a cascade of hormone reactions (2;3;8). Since insulin receptors have been found in the hypothalamus, hippocampus and pituitary it is assumed that they play an important role in the production and secretion of the pituitary hormones (1;22;33).
When the individual differences in peak, time to peak and AUC were evaluated, there were absolutely no significant results, emphasizing the fact that there are very large inter-individual differences. The glucose levels were similar at the beginning and during the 4, 8 and 12 mmol/l clamps. There are various other factors that can interfere with the test results such as the physical condition of the subjects, the amount of sleep prior to the tests and psychosocial stress. All those factors have a direct effect on the function of the hypothalamus and explain presumably a high intra individual variation in the stimulated hormone secretion.

Another factor that has to be taken in account is the effect of haemodilution because during long clamp procedures a substantial amount of intravenous fluids are administrated. We measured the haematocrit levels before and after every clamp procedure, and found a significant change in most of these levels indicating a possible dilutional effect on the plasma/serum levels of the measured hormones.

Our results suggest an effect of physiological and marginally elevated blood glucose levels on the stimulated ACTH and growth hormone secretion, using the highly reproducible eu- and hyperglycemic clamp technique. In various studies these GH and ACTH levels have been measured, often following an insulin induced hypoglycemia and most of these studies have been performed in healthy subjects and in patients with type 1 diabetes mellitus. Further investigation evaluating the pituitary response to physiological blood glucose levels in patients with type 2 diabetes mellitus, obesity and insulin resistance using the clamp technique might increase our understanding of the modifying role of glucose, insulin and possibly other factors known to have neuromodulatory effect such as leptin and glucagon like peptide (GLP-1) in these patients (35;36;37). Recently the growth hormone releasing peptide-6 (GHRP-6) which increases GH both via the hypothalamus and the pituitary has been shown to be a reliable test for adult GH deficiency. It would be interesting to use GHRP-6 as a stimulus of the GH axis during clamp procedures, as it has been suggested not to be influenced by clinical factors that are known to
alter the GH secretion (38;39). Micic et al used GHRP-6 in combination with GHRH stimulus in patients with type 2 diabetes mellitus during eu- and hyperglycemic clamp procedures, and found a lower GH response during the hyperglycemic condition (40), the results expected by us also.

In summary, the blood glucose levels of 4, 8 and 12 mmol/l did not significantly affect the pituitary response to CRH and GHRH stimulus in healthy subjects. A large intraindividual variation in the might be responsible for our inability to proof the trend seen in the graphs.

ACKNOWLEDGEMENTS
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REFERENCES


Chapter 4

Serum Insulin-like Growth Factor-I and Body Composition in Community Dwelling Older People

SUMMARY

OBJECTIVES: The decline in the GH/IGF-I (growth hormone/insulin-like growth factor-I) axis during normal aging might be involved in the changes in body composition associated with increasing age. We conducted a study to investigate serum IGF-I levels across different age categories and a possible association between serum IGF-I and measurements of body composition in older people.

DESIGN: A cross-sectional analysis of community dwelling older people, which participated in a large longitudinal cohort study (Longitudinal Aging Study Amsterdam).

SUBJECTS: 1319 subjects, 644 men, mean age 75.6 ± 6.6 years and 675 women, mean age 75.4 ± 6.6 years.

MAIN OUTCOME MEASUREMENTS: IGF-I, body mass index (BMI), waist, waist-hip ratio (WHR), fat mass, lean body mass and total bone mineral density.

RESULTS: IGF-I levels were significantly lower in the highest age categories. BMI and biceps skinfold measurements were lower in the lowest IGF-I quartile in men aged ≥ 75.5 years. In men with a low total physical activity score (<131 min/day), BMI, WHR and skinfolds were significantly lower in the lowest IGF-I quartile. In women with a high total physical activity score (>174 min/day), WHR was lower in the lowest IGF-I quartiles.

CONCLUSION: In this large cohort of community dwelling older people we observed lower serum IGF-I levels in the higher age categories. A low serum IGF-I was associated with significantly lower measurements of body composition, such as BMI, skinfolds and WHR. These results do not support previous findings that high IGF-I levels are favourable for a healthy body composition in community dwelling older people.
INTRODUCTION

Normal aging is accompanied by various changes in body composition, such as a decrease in muscle mass, bone mineral density (BMD) and an increase in fat mass (FM) (1-4). The causes of these changes are not clear. Several factors may be involved for example changes in sex steroids, lifestyle and physical activity. It has also been suggested that the growth hormone/insulin-like growth factor-I (GH/IGF-I) axis plays a role. The activity of the GH/IGF-I axis declines with increasing age, known as the somatopause (5-8). The reduction in GH secretion is estimated to be approximately 14 % per decade between the third and eight decade (9;10). There is a strong positive association between GH and IGF-I levels, but this association is weaker in older people than in young adults (4;7;10). Interestingly, the maximum growth hormone secretory capacity of the pituitary gland seems to be unaffected by age (6). Previous studies have shown sex differences in IGF-I levels and therefore different reference ranges are used in clinical practice for men and women. Little is known about reference values in older persons, but one study reported no sex difference in plasma IGF-I levels in older subjects (11).

Reports on the association between IGF-I and body composition in older people have been variable. Some studies have shown a negative association between IGF-I and weight, body mass index (BMI), waist-hip ratio (WHR) or fat mass (12-14), while others observed no association (3;15-17). In women with a mean age of 55 years, a non linear relationship between IGF-I and various indices of adiposity was observed (18). A positive association between IGF-I levels and bone mineral density has been shown in older women and men, although this effect disappeared after correction for age in one of these studies (3;19-21). Altogether there is evidence for an association between IGF-I levels and at least some components of the body composition in older people. This is supported by studies in healthy older people showing measurable effects on body composition after a low dosage of recombinant human growth hormone with or without sex steroids for a short period of time (22-24).
IGF-I levels decline with increasing age, however, until now only a few studies on IGF-I levels in the age category above 65 years have been performed. Furthermore, the studies which have been performed in older people on IGF-I levels and the association between IGF-I and body composition or bone metabolism have used relatively small study samples. In the present study we aimed to determine the ranges of IGF-I levels per age category as well as the association between IGF-I and body composition in a large cohort of community dwelling older (65-88 years of age) men and women. We expected to find lower IGF-I levels in older people and a positive association between IGF-I and lean body mass (LBM) or total bone mineral density (BMD) and a negative association between IGF-I and fat mass.
IGF-I and body composition

METHODS

Study population

For the current study data were collected within the Longitudinal Aging Study Amsterdam (LASA). LASA is an interdisciplinary, longitudinal cohort study on the predictors and consequences of changes in physical, cognitive, emotional and social functioning in older men and women (25). The LASA population consists of a random sample of over 3000 community dwelling older men and women, 55-85 years of age, stratified by age and sex. The participants are living in 11 municipalities in three regions in the Netherlands, being a representative sample of the Dutch population. In total, 3107 subjects were enrolled in the baseline examination (1992/93). In the second data-collection (1995/96), 71% of the original respondents completed a main interview. All interviews were conducted by specially trained and intensively supervised interviewers (main interview) and nurses (medical interview), and were tape-recorded in order to monitor the quality of the data. The present study was performed in participants of the second data-collection (n=1720), in which IGF-I levels were measured and age was ≥ 65 years as of January 1, 1996 (n=1319). In participants from Amsterdam, total body Dual-energy X-ray absorptiometry (DXA) was performed (n=515). The study was approved by the Medical Ethics Review Committee of the VU University Medical Center and conducted according to the principles of the Helsinki declaration. Informed consent was obtained from all participants.

Anthropometric measurements

Height (cm) to the nearest 0.5 cm and weight (kg) to the nearest 0.1 kg were measured. Body mass index (BMI, kg/m²) was calculated using body height from the baseline study in 1992/1993 and weight obtained in 1995/1996 to minimize possible overestimation of BMI that may occur as height tends to decline with aging. Waist and hip circumference (cm) were measured twice and mean values were used to evaluate waist-hip ratio (WHR) (26). Skinfolds were measured at the biceps and triceps according to standard procedures and the mean value of three measurements
Chapter 4

was used. DXA was performed using the Hologic QDR-2000 scanner (Hologic Inc., Waltham, MA, USA) and for each region of interest the bone area (cm²), bone mineral content (grams), bone mineral density (grams/cm²), total mass (grams), fat mass (grams) and lean mass (fat free soft tissue mass and bone mineral content in grams) were evaluated. The coefficient of variation of DXA body composition measurements is 2-3% for total body fat and 1-2% for total LBM.

Laboratory assays

Blood samples were drawn in the morning after a 12 hour fast, processed and centrifuged within 60 minutes. Samples were stored at −20°C until analysis in 1999. IGF-I levels were determined using an immunoradiometric assay after extraction (DSL, Webster, Texas, USA) with a detection limit of 1 nmol/l. Inter-assay coefficient of variation (CV) was < 14 %. The reference range (P5-P95) for IGF-I values with the used method is 11-19 nmol/l for both men and women aged > 60 years. For measurements of testosterone a radioimmunoassay (Coat-A-Count, DPC Los Angeles, USA) was used, with an inter-assay CV of 11 %, 7 % and 6 % at mean testosterone concentrations of 1.5 nmol/l, 5 nmol/l and 30 nmol/l respectively. The detection limit was 1 nmol/l. Estradiol levels were measured using radioimmunoassay (Double antibody Diasorin Biomedica, Saluggia, Italy) with an inter-assay CV of 14 % and 7 % at mean estradiol concentrations of 30 pmol/l and 100 pmol/l respectively. The detection limit was 18 pmol/l. All measurements were done at the Endocrine Laboratory of the VU University Medical Center. For albumin and creatinine measurements standard in-house procedures were used.

Potential effect modifiers

Since anthropometric measurements and IGF-I levels change with increasing age, age was expected to be a potential effect modifier. Physical activity was also expected to be an effect modifier since both body composition as well as IGF-I levels are known to be influenced by physical activity. In our study physical activity in the previous two weeks was estimated with the
IGF-I and body composition

LASA physical activity questionnaire (LAPAQ). LAPAQ is a validated questionnaire by which daily physical activity (household, sport and leisure activities) is ascertained in older people. A total physical activity score was calculated as time spent on physical activity in minutes per day (min/day) (27;28).

Potential confounders

During the medical interview, lifestyle variables were assessed, including smoking habits (yes/former/never) and alcohol use (no/light/moderate/excessive) (27). With a detailed questionnaire, self-reported chronic diseases were assessed including asthma/COPD (chronic obstructive pulmonary disease), CVD (cardiovascular disease), diabetes mellitus, stroke, osteoarthritis, rheumatoid arthritis and cancer. Three categories were used in this analysis (none/one/two or more chronic diseases). Serum creatinine (as an indicator for renal function) and serum albumin (as an indicator for nutritional status) were considered to be confounders.

Statistical analysis

Since IGF-I may have different effects in both genders, we analysed all data separately for men and women. All parameters and confounders were checked for normal distribution and when needed a logarithmic transformation was performed to approximate normal distribution. Spearman and Pearson correlation coefficients were calculated to examine multicollinearity. Multiple linear regression analysis was performed to study the association between IGF-I and body composition. For all analyses a p-value of ≤ 0.05 was considered statistically significant. IGF-I levels are divided in quartiles (Q), Q1 is the lowest IGF-I quartile and Q4 the highest (reference group). Although there were some subjects with IGF-I levels below or above the reference values, they were not excluded since IGF-I distribution was normal and the results did not change (data not shown).

Two models were used in the multiple linear regression analysis. In the first model only the four quartiles of IGF-I were included (unadjusted model). In the second model we adjusted for all
mentioned confounders. We found a significant interaction between IGF-I and age and therefore we dichotomized the study sample in two groups using the mean age of 75.5 years both for men and women. There was also a significant interaction between IGF-I and physical activity score, for men the mean score was 131 min/day and for women the mean physical activity score was 174 min/day. The groups were accordingly dichotomized and analysed separately. All analyses were performed using the statistical software package SPSS version 13 (SPSS, Chicago, IL, USA) and all values are presented as mean with standard deviation (SD) unless stated otherwise.

RESULTS

In this study we evaluated 644 men, mean age 75.6 ± 6.6 years, mean IGF-I level of 14.4 ± 5.1 nmol/l and mean body mass index (BMI) 25.9 ± 3.4 kg/m². The total number of women was 675, mean age 75.4 ± 6.6 years, mean IGF-I 13.2 ± 5.3 nmol/l, mean BMI 27.4 ± 4.7 kg/m². Overall, the mean IGF-I level was significantly higher in men than in women. Other baseline characteristics are shown in Table 1.
Table 1. Baseline characteristics of the study population

<table>
<thead>
<tr>
<th></th>
<th>Men</th>
<th>Women</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total (N=644)</td>
<td>Total (N=675)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>75.6 (6.6)</td>
<td>75.4 (6.6)</td>
</tr>
<tr>
<td>IGF-I (nmol/l) *</td>
<td>14.4 (5.1)</td>
<td>13.2 (5.3)</td>
</tr>
<tr>
<td>Physical activity (min/day) *</td>
<td>130.8 (95.4)</td>
<td>174.4 (98.4)</td>
</tr>
<tr>
<td>Alcohol (%)*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No alcohol intake</td>
<td>14</td>
<td>34.6</td>
</tr>
<tr>
<td>Light alcohol intake</td>
<td>49.2</td>
<td>51</td>
</tr>
<tr>
<td>Moderate alcohol intake</td>
<td>26.7</td>
<td>12.2</td>
</tr>
<tr>
<td>Excessive alcohol intake</td>
<td>10.1</td>
<td>2.2</td>
</tr>
<tr>
<td>Smoking (%)*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>25</td>
<td>11.9</td>
</tr>
<tr>
<td>No</td>
<td>10.1</td>
<td>59.9</td>
</tr>
<tr>
<td>Former</td>
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<td>28.3</td>
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<tr>
<td>Chronic diseases (%)</td>
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<td></td>
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<tr>
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<td>25.1</td>
</tr>
<tr>
<td>One chronic disease</td>
<td>36.3</td>
<td>38.3</td>
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<tr>
<td>Two or more chronic diseases</td>
<td>34.3</td>
<td>36.6</td>
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<td>Creatinine (µmol/l) *</td>
<td>105.5 (35.7)</td>
<td>85.6 (32.4)</td>
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<td>Albumin (g/l)</td>
<td>42.0 (4.0)</td>
<td>41.7 (4.0)</td>
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<td>Total Testosterone (nmol/l) *</td>
<td>15.4 (5.0)</td>
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<tr>
<td>Total Estradiol (pmol/l) *</td>
<td>77.3 (25.2)</td>
<td>32.1 (16.3)</td>
</tr>
<tr>
<td>BMI (kg/m²)*</td>
<td>25.9 (3.4)</td>
<td>27.4 (4.7)</td>
</tr>
<tr>
<td>Waist (cm) *</td>
<td>99.1 (10.2)</td>
<td>92.3 (11.7)</td>
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<tr>
<td>WHR*</td>
<td>0.98 (0.07)</td>
<td>0.88 (0.08)</td>
</tr>
<tr>
<td>Total fat mass (g) *‡</td>
<td>22389.3 (7427.2)</td>
<td>31378.3 (10138.5)</td>
</tr>
<tr>
<td>Total lean mass (g) *‡</td>
<td>54570.8 (6029.6)</td>
<td>39702.5 (4857.7)</td>
</tr>
<tr>
<td>Total body BMD (g/cm²)*‡</td>
<td>1.07 (0.11)</td>
<td>0.93 (0.10)</td>
</tr>
</tbody>
</table>

All values are presented as mean with standard deviation (SD) unless stated otherwise.

DXA: dual-energy X-ray absorptiometry; IGF-I: insulin-like growth factor-I; BMI: body mass index

WHR: waist-hip ratio; BMD: bone mineral density

* Significant difference between both sexes, p ≤ 0.05.
‡ DXA subsample (men n=255, women n=260).
The values of mean IGF-I per age category for men and women are shown in Figure 1. In men, the mean IGF-I levels in all age categories above 70 years were significantly lower when compared to the age category of 65-70 years. In women, on the other hand, the mean IGF-I level was only significantly different in the age categories above 80 years when compared to the youngest age category.

Figure 1. Mean IGF-I levels per age category for men and women. ** Significant difference when compared to age category 65-70 years, p <0.01

Figure 2 shows mean BMI, FM and LBM according to IGF-I deciles in men and women respectively. There was no difference in mean FM across the deciles for both men and women. Women had a higher BMI and FM and a lower LBM compared to men.
IGF-I and body composition

Men

Women

Figure 2. Mean BMI, FM and LBM per IGF-I deciles. BMI: body mass index; FM: fat mass; LBM: lean body mass; IGF-I: insulin-like growth factor-I.
In Table 2 the results of the regression analysis of IGF-I levels with body composition measurements in men aged ≥75.5 are presented. Men with low IGF-I levels had a significantly lower BMI compared to men with higher levels of IGF-I (p<0.05). This association remained significant after adjustment for potential confounders (p<0.05). Biceps skinfold measurements were significantly lower across all quartiles of IGF-I compared to the reference group (adjusted, Q1 p<0.01, Q2 p<0.05 and Q3 p<0.05). In men aged <75.5 years the BMI was lower (only without adjustment) in the lowest IGF-I quartile when compared to the highest quartile. In women there were no significant associations between IGF-I and measurements of body composition (results not shown).

Table 3 shows the results of regression analysis of IGF-I levels with body composition measurements, stratified by physical activity score, for men. Unadjusted, BMI was significantly lower in Q1, Q2 and Q3 in men with a low physical activity score (<131 min/day) as compared to the reference group. After adjustment the association remained significant in Q1 and Q2 (p<0.05). Furthermore, low IGF-I levels were associated with lower waist circumference (p<0.05), WHR (p<0.01), biceps skinfold measurements (p<0.05), triceps skinfold measurements (p<0.05) and total body mass (p<0.05) when compared to men with levels of IGF-I in the highest quartile. Total body fat and total LBM were lower in Q1 when compared to Q4, however, after adjustment these associations were no longer significant.
IGF-I and body composition

| Table 2. Results of regression analysis of IGF-I levels with body composition measurements in men with age ≥ 75.5 |
|---|---|---|---|---|
| &nbsp; | Q1 | Q2 | Q3 | Q4 |
| mean IGF-I | 7.9 | 11.9 | 15.0 | 20.7 |
| min-max IGF-I | 2.0-10.2 | 10.3-13.3 | 13.4-16.7 | 16.8-37.0 |

**Unadjusted**

<table>
<thead>
<tr>
<th> </th>
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<th>β</th>
<th>β</th>
<th>β</th>
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<tbody>
<tr>
<td>BMI</td>
<td>288</td>
<td>-0.182*</td>
<td>-0.111</td>
<td>-0.123</td>
<td>Ref</td>
</tr>
<tr>
<td>Waist circumference</td>
<td>299</td>
<td>-0.129</td>
<td>-0.023</td>
<td>-0.031</td>
<td>Ref</td>
</tr>
<tr>
<td>WHR</td>
<td>299</td>
<td>-0.100</td>
<td>0.016</td>
<td>-0.032</td>
<td>Ref</td>
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<tr>
<td>Biceps skinfold</td>
<td>301</td>
<td>-0.228**</td>
<td>-0.186*</td>
<td>-0.164*</td>
<td>Ref</td>
</tr>
<tr>
<td>Triceps skinfold</td>
<td>300</td>
<td>-0.144</td>
<td>-0.161*</td>
<td>-0.169*</td>
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</tr>
<tr>
<td>Total body mass</td>
<td>125</td>
<td>-0.139</td>
<td>-0.003</td>
<td>0.013</td>
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<td>Total body fat mass</td>
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<td>-0.011</td>
<td>-0.003</td>
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</tr>
<tr>
<td>Total lean body mass</td>
<td>125</td>
<td>-0.124</td>
<td>0.010</td>
<td>0.030</td>
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</tr>
<tr>
<td>Total body BMD</td>
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<td>-0.095</td>
<td>-0.044</td>
<td>-0.073</td>
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**Adjusted**

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<tr>
<td>BMI</td>
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<td>-0.187*</td>
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<td>Ref</td>
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<tr>
<td>Waist circumference</td>
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<td>-0.124</td>
<td>-0.04</td>
<td>-0.031</td>
<td>Ref</td>
</tr>
<tr>
<td>WHR</td>
<td>299</td>
<td>-0.054</td>
<td>0.093</td>
<td>-0.019</td>
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<tr>
<td>Biceps skinfold</td>
<td>301</td>
<td>-0.239**</td>
<td>-0.184*</td>
<td>-0.173*</td>
<td>Ref</td>
</tr>
<tr>
<td>Triceps skinfold</td>
<td>300</td>
<td>-0.143</td>
<td>-0.155</td>
<td>-0.177*</td>
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<td>Total body mass</td>
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<tr>
<td>Total body fat mass</td>
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<td>-0.112</td>
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<td>0.003</td>
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<td>0.024</td>
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<td>-0.008</td>
<td>-0.004</td>
<td>-0.091</td>
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</tbody>
</table>

*IGF-I: insulin-like growth factor-I; Q: quartile IGF-I; BMI: body mass index; WHR: waist-hip ratio; BMD: bone mineral density

* p < 0.05; **p < 0.01
Table 3 shows the results of regression analysis of IGF-I levels with body composition measurements, stratified by physical activity score, for men. Unadjusted, BMI was significantly lower in Q1, Q2 and Q3 in men with a low physical activity score (<131 min/day) as compared to the reference group. After adjustment the association remained significant in Q1 and Q2 (p<0.05). Furthermore, low IGF-I levels were associated with lower waist circumference (p<0.05), WHR (p<0.01), biceps skinfold measurements (p<0.05), triceps skinfold measurements (p<0.05) and total body mass (p<0.05) when compared to men with levels of IGF-I in the highest quartile. Total body fat and total LBM were lower in Q1 when compared to Q4, however, after adjustment these associations were no longer significant.
Table 3. Results of regression analysis of IGF-I levels with body composition measurements, stratified by total physical activity score, in men (age 75.6 ± 6.6 years)

<table>
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<tr>
<th></th>
<th>Q1</th>
<th>Q2</th>
<th>Q3</th>
<th>Q4</th>
<th>Q1</th>
<th>Q2</th>
<th>Q3</th>
<th>Q4</th>
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<tr>
<td>Mean IGF-I</td>
<td>7.9</td>
<td>12.1</td>
<td>14.9</td>
<td>20.7</td>
<td>7.8</td>
<td>11.7</td>
<td>15.0</td>
<td>20.2</td>
</tr>
<tr>
<td>Min-max</td>
<td>2.0-10.2</td>
<td>10.4-</td>
<td>13.4-</td>
<td>16.8-</td>
<td>4.3-</td>
<td>10.3-</td>
<td>13.4-</td>
<td>16.8-</td>
</tr>
<tr>
<td>IGF-I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total activity ≥ 131</strong></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean IGF-I</td>
<td>7.8</td>
<td>11.7</td>
<td>15.0</td>
<td>20.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Min-max</td>
<td>4.3-</td>
<td>10.3-</td>
<td>13.4-</td>
<td>16.8-</td>
<td>13.4-</td>
<td>16.8-</td>
<td>20.2</td>
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**Unadjusted**

<table>
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<tr>
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<th>β</th>
<th>β</th>
<th>β</th>
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<th>β</th>
<th>β</th>
<th>β</th>
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<tbody>
<tr>
<td>BMI</td>
<td>355</td>
<td>-0.172**</td>
<td>-0.140*</td>
<td>-0.124*</td>
<td>Ref</td>
<td>229</td>
<td>-0.137</td>
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<td>-0.041</td>
<td>Ref</td>
</tr>
<tr>
<td>Waist</td>
<td>367</td>
<td>-0.145*</td>
<td>-0.065</td>
<td>-0.061</td>
<td>Ref</td>
<td>235</td>
<td>-0.096</td>
<td>0.078</td>
<td>-0.016</td>
<td>Ref</td>
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<tr>
<td>WHR</td>
<td>367</td>
<td>-0.153*</td>
<td>-0.043</td>
<td>-0.062</td>
<td>Ref</td>
<td>234</td>
<td>-0.092</td>
<td>0.000</td>
<td>-0.057</td>
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</tr>
<tr>
<td>Biceps skinfold</td>
<td>369</td>
<td>-0.188**</td>
<td>-0.057</td>
<td>-0.042</td>
<td>Ref</td>
<td>233</td>
<td>-0.013</td>
<td>0.031</td>
<td>-0.016</td>
<td>Ref</td>
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<tr>
<td>Triceps skinfold</td>
<td>367</td>
<td>-0.135*</td>
<td>-0.066</td>
<td>-0.053</td>
<td>Ref</td>
<td>233</td>
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<tr>
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<td>-0.247**</td>
<td>-0.100</td>
<td>-0.074</td>
<td>Ref</td>
<td>98</td>
<td>0.012</td>
<td>0.073</td>
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</tr>
<tr>
<td>Total body fat mass</td>
<td>147</td>
<td>-0.204*</td>
<td>-0.042</td>
<td>-0.057</td>
<td>Ref</td>
<td>98</td>
<td>0.038</td>
<td>0.105</td>
<td>0.000</td>
<td>Ref</td>
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<tr>
<td>Total lean body mass</td>
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<td>-0.217*</td>
<td>-0.137</td>
<td>-0.071</td>
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<td>98</td>
<td>-0.026</td>
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<td>Ref</td>
<td>98</td>
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**Adjusted for other factors**

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<th>β</th>
<th>β</th>
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</tr>
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<tbody>
<tr>
<td>BMI</td>
<td>355</td>
<td>-0.151*</td>
<td>-0.134*</td>
<td>-0.109</td>
<td>Ref</td>
<td>229</td>
<td>-0.085</td>
<td>0.045</td>
<td>-0.026</td>
<td>Ref</td>
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</tr>
<tr>
<td>Waist</td>
<td>367</td>
<td>-0.173*</td>
<td>-0.080</td>
<td>-0.080</td>
<td>Ref</td>
<td>235</td>
<td>-0.062</td>
<td>0.107</td>
<td>-0.006</td>
<td>Ref</td>
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</tr>
<tr>
<td>WHR</td>
<td>367</td>
<td>-0.181**</td>
<td>-0.037</td>
<td>-0.100</td>
<td>Ref</td>
<td>234</td>
<td>-0.052</td>
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<td>-0.046</td>
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<tr>
<td>Biceps skinfold</td>
<td>369</td>
<td>-0.156*</td>
<td>-0.037</td>
<td>-0.037</td>
<td>Ref</td>
<td>233</td>
<td>0.016</td>
<td>0.068</td>
<td>0.009</td>
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<tr>
<td>Triceps skinfold</td>
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<td>-0.063</td>
<td>-0.056</td>
<td>Ref</td>
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<td>0.101</td>
<td>0.119</td>
<td>0.110</td>
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<td>0.103</td>
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<tr>
<td>Total body fat mass</td>
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<td>-0.023</td>
<td>-0.048</td>
<td>Ref</td>
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<td>0.040</td>
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<td>-0.021</td>
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<tr>
<td>Total lean body mass</td>
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<td>-0.143</td>
<td>-0.093</td>
<td>-0.022</td>
<td>Ref</td>
<td>98</td>
<td>0.012</td>
<td>0.069</td>
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<tr>
<td>Total body BMD</td>
<td>147</td>
<td>-0.045</td>
<td>0.052</td>
<td>0.031</td>
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<td>0.064</td>
<td>0.003</td>
<td>0.045</td>
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</table>

* p < 0.05; ** p < 0.01

Adjusted for: age, regions, alcohol use, smoking, chronic diseases, albumin, creatinine, estradiol and testosterone. IGF-I: insulin-like growth factor-I; Q: quartile IGF-I; BMI: body mass index; WHR: waist-hip ratio; BMD: bone mineral density
Table 4 shows the results of regression analysis of IGF-I levels with body composition measurements, stratified by physical activity score, for women. In contrast to the results found in men, we only found an association between IGF-I and WHR in the group with a high physical activity score. WHR was significantly lower after adjustment in Q1 (p<0.01) and Q2 (p<0.05) when compared to the highest IGF-I levels. We did not find any significant associations between IGF-I and total body fat, total LBM and total body BMD in women. In men total body fat and LBM were lower in Q1 when compared to Q4 in the unadjusted analyses, however, after adjustment these associations were no longer significant.
Table 4. Results of regression analysis of IGF-I levels with body composition measurements, stratified by total physical activity score, in women (age 75.4 ± 6.6 years)

<table>
<thead>
<tr>
<th></th>
<th>Q1</th>
<th>Q2</th>
<th>Q3</th>
<th>Q4</th>
<th>Q1</th>
<th>Q2</th>
<th>Q3</th>
<th>Q4</th>
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<tr>
<td><strong>Mean IGF-I</strong></td>
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**Adjusted for other factors**

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* p < 0.05;  ** p < 0.01

Adjusted for: age, regions, alcohol use, smoking, chronic diseases, albumin, creatinine and estradiol.

IGF-I: insulin-like growth factor-I; Q: quartile IGF-I; BMI: body mass index; WHR: waist-hip ratio; BMD: bone mineral density
DISCUSSION

In our large cohort of aging subjects mean IGF-I levels were significantly higher in male than in female subjects, indicating that the sex difference is still present in older people. As expected and reported previously, mean serum estradiol levels were higher in men than in women (29). In men testosterone is aromatized to estradiol, resulting in higher estradiol levels in men than in women after the menopause. Since estrogen levels are thought to influence IGF-I levels and are different in men and women, we corrected for estradiol levels in our analysis. Furthermore, we found lower IGF-I levels in the older age categories. This is in agreement with previous studies (7;11;14). In some of the subjects we observed IGF-I levels below the reference range. There were no indications of incorrect sample treatment or storage problems. As the reference range for the age category above 70 years was not defined, it can be assumed that these low levels were at the lower part of the normal range.

Men aged >75.5 years with low IGF-I levels had significantly lower BMI and biceps skinfold measurements when compared to men with high IGF-I levels. It is known that nutritional status influences both IGF-I levels and BMI. However, it is not likely that differences in nutritional status explain the association between IGF-I levels and body composition in older men with low IGF-I levels as we found no differences in BMI, FM and LBM between the lower and higher age categories. Furthermore, we corrected for albumin levels, used as a marker for nutritional status. In women, these associations were not seen.

Men with a low physical activity score and lower IGF-I levels had significantly lower BMI, waist circumference, WHR, total body mass, biceps and triceps skinfold measurements compared to men with higher IGF-I levels. These associations were not found in men with a high physical activity score. On the contrary, in women we found a lower WHR in the lower IGF-I quartiles when compared to the highest quartile in the group with the highest physical activity score. The difference between men and women could be explained by a different mean total physical activity
score and different type of activities. For example, household tasks were included in the calculation of the physical activity score. We used the mean physical activity score to dichotomize the groups with a low and high physical activity score, but this is a statistical approach and possibly there is a threshold value for the influence of physical activity on IGF-I levels. Future studies should investigate this more thoroughly.

In other studies a negative association between IGF-I and BMI was found. However, these study populations were younger (12;13). Possibly, this negative association disappears with older age. In obese subjects, a strong association between IGF-I and BMI has been reported (20;30), but the relationship between IGF-I and fat distribution remains unclear (18;31). Our population was not obese, but overweight with a mean BMI of 25.9 kg/m² (men) and 27.4 kg/m² (women). Therefore the association between IGF-I and measurements of body composition may not be as strong as found in obese subjects. LBM, FM and BMD were analysed by DXA technique, which has been shown to be a good method for measuring body composition (32;33). Yet, we found no significant associations between IGF-I and LBM, FM and BMD. This might be due to a lack of power as DXA measurements were only available in a small subgroup. We observed lower waist circumference in men and WHR in both men and women with low IGF-I levels. In a previous study a negative association between IGF-I and WHR was reported (13). Our data do not support these findings and suggest that higher IGF-I levels are not per se favourable for healthy measurements of body composition.

There were some limitations to our study. This was a cross-sectional study and IGF-I was only assessed at one time point in LASA. It would be interesting to perform longitudinal analyses to gain more insight into this association in the future. Furthermore, we used total IGF-I levels in this study, since there were no data available on binding proteins for IGF-I, especially IGFBP-3. Therefore the effect of free IGF-I could not be evaluated. Some studies suggest that local tissue
IGF-I might play an important role in the maintenance of body composition (20;34) but this is difficult to measure with the available techniques.

There may be other factors that influence the associations between IGF-I and the different components of body composition such as leptin and adiponectin, however, these measurements were not available.

In conclusion, in our study cohort of 1319 older people aged 65 years and older, we observed lower IGF-I levels in the higher age categories. In the lowest IGF-I quartile in men with low physical activity, BMI, WHR and skinfolds were significantly lower when compared to the highest IGF-I quartile. These results do not support previous findings that high IGF-I levels are favourable for healthy body composition measurements in community dwelling older people. Further investigations in older people are recommended to elucidate the exact association between IGF-I levels and measurements of body composition.

Acknowledgments

The study is based on data from the Longitudinal Aging Study Amsterdam which is largely funded by a grant from the Netherlands Ministry of Health, Welfare and Sports, Directorate of Nursing Care and Older persons. The authors thank Jan Poppelaars for his assistance in providing the data.
REFERENCES


Chapter 5

Brain Activation by Visual Food-Related Stimuli and Correlations with Metabolic and Hormonal Parameters: a fMRI study

Jakobsdottir S, de Ruiter M, Deijen JB, Veltman DJ, Drent ML.
ABSTRACT

Background Functional Magnetic Resonance Imaging (fMRI) has increasingly been used to locate specific brain areas related to the perception and processing of food stimuli. Regional brain activity in these areas is likely to vary with metabolic state (hunger or satiety). Several important neuroendocrine adipokines may also be involved.

Aim To investigate regional brain activity and cognitive performance during processing of visual food stimuli in a satiated and a hungry state, and to correlate these with neuroendocrine factors known to be involved in hunger and satiated states.

Subjects and methods Fifteen healthy, normal weight male subjects were included; two fMRI sessions were performed with a one week interval, after overnight fasting or 1 hour after consuming a standardized meal. Blood samples and an appetite assessment score were obtained after each fMRI session.

Results Main effects of processing food versus non-food stimuli were observed in the ventral visual stream, including the fusiform gyrus and parahippocampal areas bilaterally, significantly more in the fasting state. When fasting, there was a significant positive correlation between ghrelin and food related activation in the insula areas bilaterally and the right hippocampus. A highly significant negative correlation was found between leptin and activity in the left hippocampal area and right insula during the satiation condition.

Conclusion The increased activation of food vs. non-food pictures in the ventral visual stream reflects increased salience of food pictures when subjects are hungry. Ghrelin and leptin were differentially associated with activations in areas involved in memory and emotion processing.
INTRODUCTION

The neurophysiological processing of food-related stimuli is increasingly considered relevant for the understanding of appetite regulation and the pathogenesis of obesity. Single-cell recordings in primates have shown neural activity related to food stimuli in the amygdala and the orbitofrontal cortex (OFC) [(1),(2)]. In the amygdala, information regarding biologically relevant stimuli such as food is generally perceived, and forwarded to other brain regions such as the ventromedial cortex for further processing of its rewarding value or motivational salience [(3-5)]. Functional neuroimaging modalities such as functional Magnetic Resonance (fMRI) have been used to locate specific brain areas related to the perception and processing of food related stimuli in humans. Regional brain activity associated with perception of food stimuli is likely to depend on the state of hunger and satiety, presumably reflecting processing of motivational significance of food stimuli in addition to sensory effects [(4;5) for an overview].

An interaction between perceptional, motivational and cognitive factors and changes in regional cerebral blood flow in the amygdala and orbitofrontal cortex using positron emission tomography (PET) has been demonstrated, supporting the theory that these areas constitute an integrated neural system critically involved in making adaptive responses and guiding decision making [(6) (7),(5)]. In a $^{15}$O-PET study, it was shown that other limbic structures such as the nucleus accumbens and the insula seem to participate similarly in mediating physiological and motivational states [(8)]. LaBar et al. used fMRI to assess the influence of hunger on the response of the amygdala and its related cortical structures. Their results showed that food related visual stimuli were associated with a greater response in the amygdala, parahippocampal gyrus, and anterior fusiform gyrus when participants were hungry. These findings further support the hypothesis that these regions are involved not only in visual processing but also in the integration of subjective interoceptive states [(9)].
However, in both studies, subjects were scanned using a fixed-order design, which may have biased their results [(8,9)].

Several hormones, for example ghrelin, leptin and insulin, have been shown to be involved in the central regulation of appetite, hunger and satiation [(10-12)]. Receptors for these hormones have been found in the hypothalamus, and the involvement of these hormones in the energy homeostasis and food intake has widely been hypothesized [(13-15)]. These receptors are also expressed in other areas of the brain such as the hippocampus and pituitary. One of the most important adipokines, leptin, has been implicated in a variety of functions of the central nervous system such as learning and memory processes, neuroendocrine regulation, and possibly neuroprotection [(16-19)]. Likewise, metabolic factors such as glucose and free fatty acids are likely to serve as regional modulators of postprandial neuronal events [(20)]. It is therefore relevant to include measurements of these factors when studying brain responses to food related stimuli under various conditions, but to date studies on this issue have been scarce.

Another relevant aspect of the reaction to food stimuli is the role of craving, which can be defined as an intense desire to eat specific food types and is likely to influence the development of obesity, although the process of craving is still insufficiently understood. The neural correlates of food craving have been investigated with fMRI, showing activation in areas such as the hippocampus, insula and caudate nucleus, which have also been implicated in drug craving [(21)]. In another study, viewing chocolate pictures was associated with increased activation of the ventral striatum in cravers compared to non-cravers, highlighting the importance of these regions in making salience judgments regarding food stimuli [(22)].
The present study was designed to further investigate processing of visual food stimuli both in a satiated (one hour after food ingestion with a standardized meal) and hungry (after 12 hour fasting) state, in randomised order on two separate occasions to avoid order effects [(8, 9)]. A second aim was to evaluate correlations between hormonal measurements and regional brain activity. To this end, we employed fMRI during presentation of food and non-food pictures in a crossover design, during which memory encoding and retrieval performance was registered, since it has been shown that memory for food items is associated with amygdala activity [(8)].

Also, ghrelin, leptin, insulin, glucose and free fatty acids were measured both during fasting and satiated conditions. We hypothesized that responses to food vs. non-food pictures would be greater during the fasting state relative to the satiated state, in areas known to be involved in processing visual food stimuli. Furthermore, a positive correlation between activity in these areas and ghrelin was expected, as ghrelin is an orexigenic hormone and its receptors have been located in the hippocampus and ventromedial hypothalamus. In contrast, during the satiated state we expected to find correlations between the satiation signals leptin and insulin and activity within the hypothalamus.
METHODS

Subjects

Fifteen healthy, normal weight, right-handed male subjects were included. Data from one subject had to be discarded due to scanner failure, leaving 14 subjects for subsequent analyses. The subjects’ health was determined by medical history, physical examination and laboratory screening tests. Subjects with a history of neurological or psychiatric disorder were excluded. They were all medication free. Mean age was 23.4 ± 3.5 years, range 19-27 years, mean BMI was 22.4 ± 2.0 kg/m². Written informed consent was obtained, the study was approved by the Medical Ethical Committee of the VU University Medical Center and was conducted according to the principles of the Helsinki Declaration.

Design

With a one-week interval two fMRI sessions were performed, either after 12-hour overnight fasting or 1 hour after consuming a 1600 kcal standard meal (consisting of 44.4 energy % carbohydrates, 15.8 energy % protein and 39.8 energy % fat), in randomised order. After each fMRI session, a 10-point Likert scale Appetite assessment score was obtained, consisting of items on hunger, fullness, desire to eat, prospective consumption, and total appetite [(23)]. Also, blood samples were collected for measurement of plasma glucose, free fatty acids, triglycerides, insulin, leptin, and ghrelin.

Scanning procedure

A 1.5 Tesla Sonata MR scanner (Siemens, Erlangen, Germany) was used to obtain echo-planar images (EPI) with blood oxygenation level dependent (BOLD) contrast. The subject’s head was fixed with foam pads to minimize head movements. During the scanning procedure,
Brain activation and correlations with peripheral parameters

pictures were presented by projection on a screen at the back end of the scanner, which could be seen through a mirror mounted above the subject’s head. For functional MRI, an echo planar imaging sequence (TR = 3.306 s, TE = 45 ms, flip angle = 90º) was used creating transversal whole brain acquisitions (38 slices, 3 x 3 mm in-plane resolution, slice thickness 2.5 mm with a 0.5 mm inter-slice gap). Two series of pictures were presented in a block design, depicting food or non-food items such as landscapes, people, and houses. For the encoding phase 48 pictures and for the retrieval phase 96 pictures (half new, half already seen during the encoding phase) were presented randomly selected for food (half) and nonfood (half). A button box was used to register the subject’s response and reaction times. The subjects were not asked to memorize the pictures, but were requested to make indoor/outdoor judgements to control for attention differences. During retrieval, subjects performed a two-choice recognition task (seen before/new). The encoding phase lasted ca. 5 min during which 96 fMRI volumes were collected, whereas retrieval duration was ca. 9 min (176 volumes). Between the encoding and retrieval subsessions, a T1-weighted structural MRI scan (MP-RAGE, magnetization prepared rapid acquisition gradient echo, resolution 1 mm in-plane, 160 slices) was obtained.

Image processing and analysis

Statistical Parametric Mapping (SPM2) software (Wellcome Department of Imaging Neuroscience, Institute of Neurology, London UK) was used for imaging analysis. The images were realigned to compensate for subject movement and were corrected for differences in slice acquisition timing. The T1-weighted anatomical images obtained from each subject were thereafter coregistered to the mean echo planar images (EPI). Spatial normalization was performed to match a standard template, and images were spatially smoothed using a 6 mm FWHM filter. Next, imaging data were analysed within the context of the General Linear
Chapter 5

Model, using boxcar regressors convolved with a synthetic haemodynamic response function. For each subject, we performed food vs. non-food comparisons and entered the resulting contrast images into second-level (random effects analyses). For main effects (food vs. non-food) results were thresholded at p<0.05 corrected for multiple comparisons using the FDR method [(24)], whereas stimulus x condition interaction effects are reported at p<0.001 uncorrected masked with the relevant main effect, unless indicated otherwise. Additionally, analyses of covariance were performed using hormone measurements as regressors. Regions determined by Montreal Neurological Institute (MNI) coordinates for peak effects were verified using a standard brain atlas.

Laboratory analysis

Serum leptin and ghrelin were measured with a radio-immunoassay (Linco Research Inc, St.Charles, Missouri, USA). The intra-assay coefficient of variation (CV) for leptin was 3% at 25 µg/l and 8% at 5 µg/l and the inter-assay CV was 4% at 25 µg/l and 8% at 5 µg/l. For ghrelin the intra-assay CV was 5% at 3000 ng/l, 8% at 2000 ng/l and 10% at 1000 ng/l and the inter-assay CV was 5%. Insulin was measured using the Immunoradiometric assay Biosource/Medgenix Diagnostics, Fleurus, Belgium. The intra-assay CV is 2% at 318 pmol/l and 5% at 40 pmol/l and inter-assay CV 6%. Serum concentrations of glucose, triglycerides and free fatty acids were measured in accordance with standardized techniques.

Statistical analysis

Laboratory measurements were analyzed using a standard statistical package (SPSS version 13; SPSS, Chicago, IL, USA). Paired T-tests were used to evaluate changes between the fasting and the satiated state. In addition, Pearson’s correlations (one-tailed or two-tailed, depending on specific hypotheses) were calculated between the subscales of the Hunger
questionnaire and blood sample parameters as well as the correlations between imaging and laboratory data. Correlations were computed separately for the hungry and satiated conditions.

RESULTS

Psychometric measurements
To determine the effects of conditions satiated-food, satiated-nonfood and fasting-food, fasting-nonfood on reaction time (RT) and number of correct responses two separate repeated-measures analyses of variance (ANOVA) were used with RT and number of correct responses under each condition as repeated measurements factor. Only RTs for correct trials were evaluated. With respect to RT, within-subjects contrasts indicated that the RT was significantly longer for fasting-food than for fasting-nonfood items \((F(1,14) = 25.22, p < 0.0005, \eta^2 = 0.64)\). In addition, RTs were significantly longer for satiated-food than for satiated-nonfood items \((F(1,14) = 46.90, p < 0.0005, \eta^2 = 0.77)\). There were neither significant differences between the RTs under the fasting and satiated conditions separately analysed for the food and non-food conditions, nor for those averaged across food and non-food conditions.

Furthermore, within-subjects contrasts indicated that the number of correct responses was significantly lower for fasting-food than for fasting-nonfood items \((F(1,15) = 4.56, p = 0.05, \eta^2 = 0.23)\). In addition, there was neither any significant difference between the number of correct responses under the fasting and satiated conditions separately analyzed for the food and non-food condition nor for those averaged across food and non-food conditions.


**Laboratory measurements**

Mean glucose levels did not differ between the fasting and satiated state in this study population. As expected, during fasting mean levels of ghrelin and FFA were significantly higher compared to the satiated state. During the satiated state, insulin and triglyceride levels were higher than during the fasting state. Leptin levels were also slightly higher during satiation (Table 1).

**Table 1:** Laboratory measurements in healthy men during fasting and satiated conditions presented as mean levels with standard deviation

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<td>4.79 ± 0.30</td>
<td>4.57 ± 0.33</td>
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<td>Triglycerides (mmol/l)</td>
<td>0.77 ± 0.33</td>
<td>1.35 ± 0.57**</td>
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<tr>
<td>Free fatty acids (mmol/l)</td>
<td>0.31 ± 0.11</td>
<td>0.15 ± 0.11**</td>
</tr>
<tr>
<td>Insulin (pmol/l)</td>
<td>46.8 ± 21.5</td>
<td>245.7 ± 127.3**</td>
</tr>
<tr>
<td>Leptin (µg/l)</td>
<td>3.0 ± 1.5</td>
<td>3.5 ± 2.1*</td>
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<tr>
<td>Ghrelin (pg/ml)</td>
<td>1826.7 ± 402</td>
<td>1386.9 ± 223.8**</td>
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* p < 0.05  ** p < 0.01
Brain activation and correlations with peripheral parameters

Imaging data

Results of imaging data are summarized in Table 2. Main effects for food related stimuli vs. non-food related stimuli (averaged across encoding and retrieval) were observed in ventrolateral prefrontal cortex (VLPFC) and lateral temporal areas bilaterally, the ventral visual stream with fusiform and parahippocampal gyri as well as the hippocampus proper bilaterally, whereas amygdala activity only approached significance. Interaction effects (increased activation of food vs. non-food pictures during the fasting compared to the satiated condition) were observed in right VLPFC and bilateral ventral visual stream, including the fusiform gyrus and parahippocampal gyrus. During encoding of correctly recognized pictures significant interaction effects were observed in the occipital cortex bilaterally (x=12, y=-81, z=-6, peak Z score: 3.50 and x=-12, y=-93, z=-3, peak Z score: 3.35 respectively), the left fusiform gyrus (x=-21, y=-69, z=-15, peak Z score: 3.59), insula (x=-33, y=21, z=21, peak Z score: 3.50) and the left middle temporal gyrus (x=-42, y=-57, z=-6, peak Z score: 3.13). During retrieval of correctly recognized pictures, we found left posterior hippocampus (x=-21, y=-39, z=0, peak Z score: 3.18), with ventral striatum approaching significance (x=-6, y=6, z=-6, peak Z score: 3.00).
**Table 2**: Regions showing an increase in brain activity (Blood Oxygen Level Dependent contrasts) in response to visual food versus nonfood stimuli (pooled for encoding and retrieval tasks):

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<td>X</td>
<td>Y</td>
</tr>
<tr>
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<td>30</td>
</tr>
<tr>
<td>Ventrolateral, prefrontal cortex (right)</td>
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<tr>
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<td>Lateral temporal area (left)</td>
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<td>Visual stream, fusiform gyrus and parahippocampus gyrus (left)</td>
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<tr>
<td>Posterior hippocampus (right)</td>
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</tr>
</tbody>
</table>

Main effects across fasting and sated significant corrected for multiple comparisons (false discovery rate method) unless indicated otherwise. X, Y, Z coordinates represent standardized coordinates from the MNI (Montreal Neurological Institute) brain. Peak Z shows standardized significance value

a) $P$ uncorrected = 0.005; b) $P$ uncorrected = 0.002

**Correlations between imaging and laboratory data**

**Fasting condition**: There was a significant positive correlation between ghrelin levels and activation of the right and left insula, and activation of the right hippocampus (Table 3).

A negative correlation was found between insulin levels and activation of the nucleus accumbens. There were no correlations found between the imaging data and FFA measurements. Glucose levels were positively correlated with right insula activity. Triglycerides levels were negatively correlated with left hippocampal activity.
Sated condition: A strong negative correlation was found between leptin levels and activation of the left hippocampus and activation of the right insula (Figure 1/Table 4). A negative correlation was found between activation of the right dorsolateral prefrontal cortex and insulin levels. FFA levels were positively correlated with activation of the nucleus caudate area (Table 3).

Figure 1: Activation in the left hippocampus when correlated to leptin
Table 3: Correlations between reactions to visual food versus non-food stimuli (Blood Oxygen Level Dependent contrasts) and metabolic measurements.

<table>
<thead>
<tr>
<th></th>
<th>Positive correlation</th>
<th>Negative correlation</th>
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<tr>
<td></td>
<td>X</td>
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<tr>
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<td>Hippocampus right</td>
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<td>Thalamus left</td>
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<tr>
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<tr>
<td>Globus Pallidus</td>
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<td>9</td>
</tr>
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<tr>
<td>Insulin</td>
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<tr>
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<td>Insulin</td>
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<td>-42</td>
</tr>
<tr>
<td>Insulin</td>
<td>Temporal lobe left</td>
<td>-42</td>
</tr>
</tbody>
</table>

OF: orbitofrontal cortex, PFC: prefrontal cortex

*P uncorrected = 0.002

X, Y, Z coordinates represent standardized coordinates from the MNI (Montreal Neurological Institute) brain. Peak Z shows standardized significance value
Correlations between appetite assessment score and laboratory data

Under the fasting condition, a significant correlation was found between ghrelin levels and ‘thought of food’ scale scores (\(r = 0.48, p = 0.04\), one-tailed). In addition, higher ghrelin concentrations were related to lower insulin concentrations (\(r = -0.55, p = 0.035\), two-tailed). Finally, glucose correlated positively with insulin (\(r = 0.54, p = 0.047\), two-tailed) and with leptin (\(r = 0.60, p = 0.02\), two-tailed).

With respect to the satiated condition, ghrelin concentrations correlated negatively with scores on the ‘eagerness to eat’ scale (\(r = -0.61, p = 0.008\)). Finally, ghrelin correlated negatively with insulin (\(r = -0.58, p = 0.02\), two-tailed) and glucose correlated positively with triglycerides (\(r = 0.66, p < 0.007\), two-tailed).
DISCUSSION

In the present study we investigated the effects of processing visual food vs. non-food stimuli on brain activation during standardized fasting and satiated conditions. In order to mimic a physiological fasting state in the brain, we adopted a 12-hour fasting period, rather than an extremely long fasting period as used by others [(25)]. Also, we performed a second measurement one hour after ingestion of a standardized meal, expecting the acute metabolic and hormonal changes following a meal to be effective in producing a satiated state. Functional MRI sessions were performed in balanced order, with a one-week interval to separate the two conditions (fasting and satiated) to control for order effects. Main effects of food vs. non-food stimuli were mainly observed in the ventral visual stream, including fusiform gyrus and parahippocampal areas bilaterally, although anterior hippocampus and amygdala were only found at a lower threshold. In addition, we found right VLPFC, and bilateral fusiform and parahippocampal gyrus to be significantly more active during the fasting than the satiated state. Given that food and non-food pictures were matched for visual complexity, these effects are likely to be due to increased salience of food pictures, in particular when subjects were hungry. These results are in accordance with previous reports investigating visual processing of food stimuli using fMRI [(9;26)]. In addition to the medial temporal regions observed in the present study, the orbitofrontal cortex has been demonstrated to be involved in feeding related behaviour both in animals and humans [(20;27;28)]. Moreover, in our study, amygdala activity was observed for food vs. non-food pictures, but not in stimulus x condition interaction analyses, although salience of food pictures was likely to be higher during hungry states. Due to susceptibility artefacts (signal loss due to the presence of bone-air transitions, in particular nasal sinuses) we were not able to adequately measure BOLD activation in the medial OFC; also, ventral striatum activity was only observed during retrieval, even though the ventral striatum is likely to be involved in signalling rewarding properties of food [(22)]. For this
Brain activation and correlations with peripheral parameters

reason, some researchers have used $^{15}$O-PET rather than fMRI when investigating OFC function [(28-30)]. In addition, detecting amygdala activity may be problematic due to rapid habituation [(9)].

During the two conditions of fasting and satiation, mean reaction time for food pictures was longer than for non-food pictures. Also, memory performance for food-pictures was lower than for non-food pictures. The latter finding was somewhat unexpected given the association between salience and memory performance for food stimuli as reported by others [(8)], although we suggest that this could be due to greater semantic cohesion of the food stimuli [(31)] relative to nonfood stimuli.

Previous research has demonstrated that the gut-brain axis and various hypothalamic factors are of importance in regulating the energy balance and the perception of hunger and satiety [(14;32-34)]. In the present study, we found a positive correlation between ghrelin and food-related activation in the insula areas bilaterally, as well as in the right hippocampus, during the fasting state. As ghrelin is the only orexigenic hormone [(35;36)] this association was expected. Also, it is known to stimulate meal initiation, and receptors for ghrelin have been located in the hippocampus, arcuate nucleus and ventromedial hypothalamus. When correlating the hunger scores with ghrelin we found a positive correlation between ghrelin and “thought of food” hunger scores, consistent with the findings of others [(12)]. In contrast, we found a negative correlation between insulin and activation in the accumbens area when the subjects were fasting. This was also expected as postprandial insulin is one of the factors which lower ghrelin levels after a meal. Therefore, insulin can also be considered an anorexigenic hormone.

In the satiated condition, there was a strong negative correlation between leptin and left hippocampus and right insula activity. Thus, high leptin levels correlated with a lowered
response to food related pictures. The insula has been implicated in multiple processes, including interoceptive awareness of body states, food craving, and basic emotions. Stimulation of the hypothalamus by leptin results in the suppression of food intake, stimulation of satiated behaviour, and energy expenditure [(37-40)]. Also, in obese subjects, correlations have recently been reported between leptin and regional grey matter volumes [(41)]. In the present study, correlations between insulin and activation of limbic areas were mainly observed during the fasting state. Our findings of a decrease in activation during food related stimuli with increasing insulin levels in the medial temporal lobe and the prefrontal cortex might reflect the anorexigenic properties of insulin. Nevertheless, interpretation of the correlations between insulin and regional brain activation areas is not straightforward as insulin signalling in the brain is highly complex, sharing common pathways with leptin and serotonin [(42;43)].

In contrast to the findings of Gautier et al, we did not find correlations between postprandial FFA and activation in hippocampal and parahippocampal regions [(25)]. Also, there were no significant correlations between glucose and the activation in the limbic system during the fasting and satiated condition when viewing food vs. nonfood food pictures.

Our study has several potential limitations. The sample size was only moderate, although sample sizes of 12-15 are customary in fMRI studies. Also, recently published data have implicated adiponectin in regulating food intake and energy expenditure [(44;45)]. Moreover, glucagon-like peptide-1 (GLP-1), which is synthesized in the brain as well [(46;47)], may also be involved. Therefore, future research should attempt to investigate the correlations between these hormones/adipokines and regional brain activation using a similar fMRI paradigm.

Finally, various local regulatory circuits and locally produced/derived adipokines are presumably involved in the processing of food intake regulation and appetite, but such factors
Brain activation and correlations with peripheral parameters

are as yet difficult to assess. This regulation is likely to be highly complex, involving the
dopaminergic system, and psychosocial factors including stress [(48-51)].

In summary, in the present study, main effects of food versus non-food visual stimuli were
observed in the ventral visual stream, including fusiform gyrus and parahippocampal areas
bilaterally, and at lower threshold the hippocampus and amygdala areas. There was
significantly more activation in the fusiform and hippocampus gyrus bilaterally during the
fasting condition when compared to the satiation state, most likely due to increased salience
of food pictures. Furthermore, there was a significant positive correlation between the
orexigenic hormone ghrelin and the insula areas bilaterally and the right hippocampus area
when the subjects were hungry. When the subjects were satiated there was a strong negative
correlation between leptin and left hippocampus and right insula activity.

Using a repeated measures design with standardized method will enable further studies on
processing food related stimuli and appetite regulation in study populations such as in obesity,
binge eating or in anorexia nervosa. Further insights in the neural correlates of processing
food stimuli in these disorders will be of importance in the search for individualized treatment
such as behavioural counselling and in the search for effective drug treatments.
REFERENCES


Brain activation and correlations with peripheral parameters


Brain activation and correlations with peripheral parameters


Chapter 6

Acute and short term effects of caloric restriction on metabolic profile and brain activation in obese, postmenopausal women

ABSTRACT

Objective: Early anthropometric and metabolic changes during a caloric restricted diet in obese postmenopausal women, and correlations between these factors with activity in brain areas involved in processing of visual food related stimuli, were investigated.

Subjects and methods: An eight week prospective intervention study of 18 healthy postmenopausal women, with a BMI of 30-35 kg/m². The first two weeks subjects were on an isocaloric diet, four weeks on a 1000 kcal restricted diet followed by two weeks on an isocaloric diet. Anthropometric and laboratory analyses were performed weekly during the isocaloric diet and three times a week during the caloric restricted diet. fMRI scans were obtained before and after the caloric restriction in four separate sessions (fasting or sated). Generalized Estimating Equations analysis was used for data analysis.

Results: A mean weight loss of $4.2 \pm 0.5$ kg (4.8%) and a $4.2 \pm 0.4$ cm decline in waist circumference were achieved. In the first week of caloric restriction triglyceride, leptin, resistin and adiponectin levels as well as systolic blood pressure decreased and IGFBP-1 levels increased. During and after weight loss a significant increase in ghrelin levels was observed. Before weight loss increased activation of the right amygdala was seen in response to food stimuli, and FFA and glucose correlated with activity in various areas involved in food reward processing. After weight loss fasting ghrelin and sated leptin levels correlated with activity in these areas.

Conclusion: Already in the first week of caloric restriction in obese postmenopausal women various favourable metabolic changes occur before clinically relevant weight loss is achieved. Activity in the amygdala region, and correlations of metabolic factors with activity in brain areas involved in food reward processing, differ substantially before and after weight loss.
INTRODUCTION

The metabolic profile in obese subjects is frequently unfavourable and may precede the development of insulin resistance, diabetes mellitus and cardiovascular disease. Several adipocyte-derived factors, such as leptin, resistin and adiponectin have been suggested to play a role in both peripheral and central insulin resistance. Some of these factors are elevated in obesity. Leptin plays a role in maintaining energy balance and suppresses appetite (1). Although leptin levels are fivefold higher in obese than in normal weight subjects, its effect in the hypothalamus seems to be blunted in obesity (2). High levels of resistin have been associated with insulin resistance but the underlying mechanisms are not yet clear. In contrast, in obesity down-regulation of adiponectin, which is an important modulator of various metabolic derangements, is observed which can improve after weight loss (3). Also, there are elevated levels of other factors which are associated with the development of cardiovascular disease such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and C-reactive protein (CRP) (4). Especially in postmenopausal women an increased risk of cardiovascular disease is observed. This may be explained by the finding that in the menopause a redistribution of fat mass towards visceral fat occurs leading to an atherogenic lipid profile and notable changes in metabolic parameters.

Functional neuroimaging modalities have been used in humans to locate brain areas involved in the perception of food related stimuli. For instance, in obese compared to lean subjects different activity in areas involved in food reward processing has been shown (5). Therefore, the relationship of adipokines involved in the regulation of appetite, hunger and satiation with the activity in specific brain areas have been of interest in obesity research.

Loss of body fat obtained by caloric restriction improves the metabolic profile in obese patients. Previous studies have shown that this improvement occurs early during the first weeks of weight loss, although the exact moment of onset of metabolic improvement is yet
unclear. A minimum of 5% weight loss seems required to improve the metabolic profile in severely obese women (6). However, improvement appears to occur before a significant amount of adipose tissue is lost. In contrast, modest weight loss for nine weeks in postmenopausal women was found to improve insulin resistance and body fat while adipokine levels did not change (7). We therefore aimed to study the early changes in postmenopausal moderately obese women and link these peripheral and central findings.

In the present study we determined the early anthropometric and metabolic changes before, during and after a four week period of 1000 kcaloric restricted diet in moderately obese postmenopausal women. Anthropometric parameters, metabolic factors including adipokines were frequently measured to establish the acute and short term effects of mild caloric restriction. Furthermore, relations between metabolic changes and changes in brain activation induced by food related stimuli after caloric restriction relative to baseline were evaluated.
MATERIALS AND METHODS

Subjects

Eighteen healthy postmenopausal women with a mean age of 56.8 ± 3.3 years participated in this study. The mean body mass index (BMI) was 32.2 ± 2.0 kg/m². The inclusion criteria were: age 55-65 years, female gender, postmenopausal, a BMI of 30-35 kg/m², Caucasian and healthy as determined by medical history, physical examination and normal values for routine laboratory assays. Subjects using any type of medication were excluded. All subjects gave written informed consent and the study was approved by the Medical Ethical Committee of the VU University Medical Center. The study was conducted in agreement with the principles of the Helsinki Declaration.

Study Protocol

At the start of the 8-week study period a diet history was obtained from each subject and energy needs were estimated using the FAO/WHO equations for estimating basal metabolic rate (8). During the first two weeks the subjects maintained an isocaloric diet followed by a 1000 kcal restriction diet based on the individual diet history and estimated physical activity for four weeks. In the last two weeks the subjects maintained a weight adapted and calculated isocaloric diet. Blood samples and anthropometric measurements were obtained once a week during the isocaloric diet periods and three times a week on Monday, Wednesday and Friday during the four caloric restriction weeks. All subjects fasted overnight prior to a measurement day. Body weight was determined to the nearest 0.1 kg by means of a beam scale with the subject wearing only underwear. Waist circumference was measured at the narrowest part of the torso between the costal margin and iliac crest as described in the anthropometric standardization reference manual (9). Sagittal abdominal diameter (SAD) was measured in the supine position as the distance between the examination table and the
highest point of the abdomen. Measurements of body composition were performed using the bioimpedance analysis, using a Holtain body composition analyzer (Holtain Ltd, Crosswell, UK) (10). To estimate the mood state the profile of mood states (POMS) questionnaire was administered weekly during the whole study period (11). During the first 2-week isocaloric period two fMRI sessions were completed separated by a week interval, in randomized order after 12 hours overnight fasting or one hour after consuming a standardized meal which consisted of 1425 kcal: 46.6 energy % carbohydrates, 13.2 energy % protein and 39.8 energy % fat. After the 4-week caloric restriction the fMRI sessions were repeated, again in a randomized order. After each fMRI session an appetite assessment score on a 10-point Likert scale was acquired including items on hunger, fullness and desire to eat (12). For the scanning procedure we used a 1.5 Tesla Sonata MR scanner (Siemens, Erlangen, Germany) to obtain echo-planar images (EPI) with blood oxygenation level dependent (BOLD) contrast. An echo planar imaging sequence (TR=3.306 s, TE=45ms, flip angle=90°) was used creating transversal whole brain acquisitions (38 slices, 3x3 mm in-plane resolution, slice thickness 2.5 mm with a 0.5 mm inter-slice gap). Two series of pictures were presented (software: E-Prime) in a block design, depicting food or non-food items such as landscapes, people, and houses. These pictures were visually matched for visual complexity, both for the objects shown and their background. They were not systematically matched for color. Each picture was displayed for 4 seconds, followed by a pause of 2 seconds before the next picture was shown. For the encoding phase 48 pictures and for the retrieval phase 96 pictures (half new, half presented in the encoding phase) were presented randomly selected for food (half) and non-food (half). A button box was used to register the subject’s response and reaction times. Subjects were requested to press the button (yes or no) to indicate whether the pictures were taken indoor or outdoor to control for attention differences. Subjects were not asked to memorize the pictures.
Short term effects of caloric restriction

During retrieval, subjects performed a two-choice recognition task (picture seen in encoding phase or new), to explore memory performance for food vs. non-food stimuli, which may be modulated by motivational state (i.e., hunger vs. satiety) (55). In the encoding phase 96 fMRI volumes were collected and during retrieval 176 volumes. A T1-weighted structural MRI scan was also performed; see for further details our previous paper (13). Due to scan failure, data from one subject was excluded from the fMRI analyses.

Laboratory analysis

Serum concentrations of CRP, glucose, triglycerides and free fatty acids (FFA) were measured in accordance with standardized techniques. Serum leptin and ghrelin were determined with a radio-immunoassay (Linco Research Inc., St. Charles, Missouri, USA). The intra-assay coefficient of variation (CV) for leptin was 3% at 25 µg/l and 8% at 5 µg/l and the inter-assay CV was 4% at 25 µg/l and 8% at 5 µg/l. For ghrelin the intra-assay CV was 5% at 3000 ng/l, 8% at 2000 ng/l and 10% at 1000 ng/l and the inter-assay CV was 5%.

Insulin was measured using the Immunoradiometric assay Biosource/Medgenix Diagnostics, Fleurus, Belgium. The intra-assay CV is 2% at 318 pmol/l and 5% at 40 pmol/l and inter-assay CV 6%. For the determination of adiponectin a commercial kit was used from ALPCO Diagnostics (14). The intra-assay CV was 11% at the levels of 3.8 mg/l, 5.4% at 5.7 mg/l and 3.5% at 9 mg/l. The inter-assay CV was 29% at 2.28 mg/l, 15% at 3.07 mg/l and 19% at 6.02 mg/l. Resistin was determined with immunometric assay (colorimetric) Biovendor Laboratory Medicine Inc., Mordice, Czech Republic with lower limit of quantification 0.8 ng/ml, intra-assay CV 5% and inter-assay CV 10%. IGF-I, IGFBP-1 and IGFBP-3 levels were determined using chemoluminiscent method (Nichols, Institute Diagnostics, San Juan Capistrano, USA) with a lower limit of quantitation of 6 nmol/l (IGF-I) and 0.2 mg/l (IGFBP-3). The intra-assay CV was 5% for level 1 (level 2: 3%, level 3: 6%) and the inter-assay CV was 8% for level 1 (level 2: 6%, level 3: 5%) for both measurements. IL-6 and TNF-α were measured using the
Chapter 6

immunometric assay (colorimetric) technique, with referential values between 1–2 pg/ml. The lower limit of quantitation for IL-6 was 0.35 pg/ml. Gastric Inhibitory Polypeptide (GIP) was measured with the immunometric assay (colorimetric) with lower limit of quantitation at 9 pg/mL, intra-assay CV 7% and inter-assay CV 8%. Cortisol levels in 24 hr urine were measured with RIA, normal values 50-270 nmol/24 hours.

**Statistical analysis**

To evaluate the differences over time during the visits, Generalized Estimating Equations (GEE analysis) were used in which time was modelled categorical represented by dummy variables. Changes of the measurements during the isocaloric period were averaged. To evaluate changes in appetite scores before and after weight loss the paired sampled t-test was used. To evaluate the effect of hunger and satiation on reaction time and number of correct responses during fMRI analyses of variance (ANOVA) were used with diet (isocaloric/1000kcal) and type of picture (food/non-food) as independent factors. All analyses were performed using the statistical software package SPSS version 13 (SPSS, Chicago, IL, USA). For the imaging analysis the Statistical Parametric Mapping (SPM5) software (Welcome Department of Imaging Neuroscience, Institute of Neurology, London, UK) was used. fMRI images were realigned, slice timed, coregistered to the structural MRI scan, warped to a standard brain template and spatially smoothed using a 6 mm Full Width at Half Maximum (FWHM) filter. Next, imaging data were analyzed within the context of the General Linear Model, using boxcar regressors convolved with a synthetic hemodynamic response function. For each subject, we performed food versus non-food comparisons and entered the resulting contrast images into second-level (random effects) analyses of variance (ANOVA) as implemented in SPM, according to a 2 x 2 design. For the present report, we focused on effects of fasted vs sated state before and after weight loss, therefore contrast images were summed over encoding and retrieval phases to increase power. Additionally, analyses of
covariance were performed using metabolic parameters as regressors. All statistical effects were evaluated at p<0.001 uncorrected. For regions of interest, a small volume correction was applied by centering a 5 ml (10.7 mm radius) sphere around the peak voxel and considered significant at p<0.05. Anatomical regions as identified by Montreal Neurological Institute (MNI) coordinates for peak effects were verified using a standard brain atlas. Correlations between hormonal and metabolic measurements and BOLD signaling were evaluated by means of separate univariate models.

**RESULTS**

**Baseline characteristics**

Eighteen healthy postmenopausal women participated. Baseline characteristics are shown in Table 1.

**Anthropometric parameters**

During the 4-week caloric restriction there was a significant mean weight loss starting from the first week of 4.2 ± 0.5 kg, from 88.6 kg (95% CI 84.9–92.3) to 84.4 kg (95% CI 80.2–89.2). Accordingly, BMI declined significantly by 1.5 ± 0.1 kg/m². Waist circumference, fat mass and fat free mass declined significantly during and after the first week of the caloric restriction (Figure 1A). In the first week of caloric restriction a significant and sustained decrease in systolic blood pressure was observed. The diastolic blood pressure was initially declined but these changes were not consistently significant (Figure 1A).
Table 1. Baseline characteristics

<table>
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<tr>
<td>Weight, kg</td>
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</tr>
<tr>
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<td>Fat free mass, kg</td>
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<td>Systolic blood pressure, mmHg</td>
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<td>Urine cortisol, nmol/24hr</td>
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Figure 1A. Mean changes in anthropometric parameters during caloric restriction

X-axis represents measurements: isocaloric diet (week 1-2 and 7-8): measurements 1 and 14, caloric restriction (week 3-6): measurements 2-13. * P-value <0.05 compared to baseline
Metabolic parameters

Triglyceride levels increased during the beginning of the first isocaloric week and then decreased significantly during the caloric restriction which remained during the whole study period. The FFA levels increased significantly during the first week of the caloric restricted diet. Leptin decreased early in the first week of caloric restriction and this decrease remained significant. There was a significant correlation (p <0.049) between changes in leptin and changes in fat mass in the fasting state, but not in the sated state. Total adiponectin and resistin levels decreased significantly in the first week of caloric restriction but returned to baseline values in the second isocaloric period. Ghrelin levels increased significantly during the first week of caloric restriction. IGF-1 levels decreased significantly in the first and subsequent weeks of caloric restriction. IGFBP-3 levels also decreased significantly in the second week of caloric restriction. On the other hand, IGFBP-1 levels increased significantly in the first week of caloric restriction and remained increased during the whole caloric restriction period (Figure 1B). There were no significant changes in insulin, glucose, CRP, IL-6, TNF-α, GIP or urinary cortisol excretion during the study period (data not shown).
Short term effects of caloric restriction

Figure 1B. Mean changes in metabolic parameters during caloric restriction

X-axis represents measurements: isocaloric diet (week 1-2 and 7-8): measurements 1 and 14, caloric restriction (week 3-6): measurements 2-13. * P-value <0.05 compared to baseline
Neuropsychological parameters

The appetite scores changed with food ingestion compared to fasted state but the scores did not differ between the pre- and post isocaloric phase. (data not shown). With respect to the POMS scales the five subscales depression, anger, fatigue, vigour and tension were analysed. During the four weeks of caloric restriction there was a sustained significant decrease in depression, anger, tension and fatigue scores. Vigour increased but only significantly in the first and the last week of the diet (Figure 2).

Figure 2. Mean changes in POMS scores during caloric restriction
Short term effects of caloric restriction

Table 2. Regions showing an increase in brain activity (Blood Oxygen Level Dependent contrasts) in response to visual food versus non-food stimuli (pooled for encoding and retrieval tasks) before and after caloric restriction

<table>
<thead>
<tr>
<th>Regions</th>
<th>X</th>
<th>Y</th>
<th>Z</th>
<th>peak Z</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anterior insula (left)</td>
<td>-27</td>
<td>6</td>
<td>15</td>
<td>4.07</td>
</tr>
<tr>
<td>Hippocampus (left)</td>
<td>-27</td>
<td>-27</td>
<td>-9</td>
<td>3.89</td>
</tr>
<tr>
<td>Amygdala (left)</td>
<td>-24</td>
<td>-3</td>
<td>-15</td>
<td>3.61</td>
</tr>
<tr>
<td>Amygdala (right)</td>
<td>18</td>
<td>-3</td>
<td>-24</td>
<td>3.26</td>
</tr>
<tr>
<td>Limbic lobe, cingulated gyrus (right)</td>
<td>12</td>
<td>30</td>
<td>30</td>
<td>3.34</td>
</tr>
<tr>
<td>Occipital lobe, lingual gyrus (left)</td>
<td>-24</td>
<td>-72</td>
<td>0</td>
<td>3.78</td>
</tr>
<tr>
<td>Occipital lobe, cuneus (left)</td>
<td>-21</td>
<td>-93</td>
<td>0</td>
<td>3.48</td>
</tr>
<tr>
<td>Occipital lobe, fusiform gyrus (right)</td>
<td>24</td>
<td>-60</td>
<td>-15</td>
<td>3.51</td>
</tr>
</tbody>
</table>

X, Y, Z coordinates represent standardized coordinates from the MNI (Montreal Neurological Institute) brain. Peak Z shows standardized significance value.

Brain activation

The main effects across fasting and satiation for food related stimuli versus non-food related stimuli are shown in Table 2. A significant activation was found in the left anterior insula, left hippocampus, amygdala bilaterally, the right cingulate gyrus, as well as regions in the occipital lobe bilaterally. Before caloric restriction there was a significant activation in the right amygdala only during the fasting state, this effect disappeared after caloric restriction, as shown by interaction analysis (Figure 3).
Figure 3. Brain area activities before and after weight loss.

Upper panel shows BOLD activation in right hemisphere amygdala at MNI coordinates 24, -6, -15, statistical Z value = 3.69, p<0.05 corrected. Lower panel shows contrast estimates with 90% confidence intervals for the four experimental conditions at the same location.
Short term effects of caloric restriction

The correlations of metabolic parameters (ghrelin and leptin) with fMRI differences between food and non-food pictures before and after caloric restriction are shown in Table 3. During the fasting state, before caloric restriction there were no correlations between ghrelin and imaging data, after caloric restriction there were positive correlations with several areas, such as cuneus bilaterally, left putamen and the right parahippocampus. Before caloric restriction there were several significant negative correlations between glucose measurements (data not shown) and areas of interest, such as left inferior parietal lobe (X=-57, Y=-33, Z=33 peak Z: 4.53), bilateral parahippocampus (left: X=-15, Y=-15, Z=-15 peak Z: 3.42, right (X=24, Y=-15, Z=-27 peak Z: 3.56), and right insula (X=51, Y=-27, Z=18 peak Z: 3.40). After caloric restriction there were no correlations between glucose and these areas. Before caloric restriction FFA correlated with various areas frontal and parietal when the subjects were fasting, after caloric restriction these correlations disappeared (data not shown). In the sated state, before caloric restriction, there were no significant correlations between leptin and brain regions activated in response to food pictures. On the other hand, after caloric restriction there were many areas seen which correlated with leptin levels, such as amygdala, putamen (positive correlation) and thalamus, insula, parahippocampus (negative correlation).
Table 3. Correlations between reactions to visual food versus non-food stimuli (Blood Oxygen Level Dependent contrasts) and metabolic measurements: before and after caloric restriction (L=left, R=right), * p<0.05 corrected, (*) p< 0.10 corrected

<table>
<thead>
<tr>
<th></th>
<th>Positive correlations</th>
<th></th>
<th>Negative correlations</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>X</td>
<td>Y</td>
<td>Z</td>
<td>peak</td>
</tr>
<tr>
<td>Fasting</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ghrelin</td>
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<td>none</td>
</tr>
<tr>
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<td>-3</td>
<td>-21</td>
</tr>
<tr>
<td></td>
<td>Putamen R</td>
<td>15</td>
<td>9</td>
<td>-3</td>
</tr>
<tr>
<td></td>
<td>Cingulate gyrus L</td>
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<td>-57</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Hippocampus L</td>
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<tr>
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<td>Hypothalamus R</td>
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<td>-6</td>
</tr>
<tr>
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<td>Ghrelin</td>
<td>Amygdala L</td>
<td>-15</td>
<td>-21</td>
</tr>
<tr>
<td></td>
<td>Leptin</td>
<td>none</td>
<td>none</td>
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</tr>
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<table>
<thead>
<tr>
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<th>Positive correlations</th>
<th></th>
<th>Negative correlations</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>X</td>
<td>Y</td>
<td>Z</td>
<td>peak</td>
</tr>
<tr>
<td>Fasting</td>
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<td></td>
<td>Parietal lobe R</td>
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<td>Cuneus L</td>
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<td>Putamen L</td>
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<td></td>
<td>Frontal gyrus L</td>
<td>-27</td>
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<td>51</td>
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<tr>
<td>Sated</td>
<td>Ghrelin</td>
<td>Amygdala R</td>
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<tr>
<td></td>
<td>Leptin</td>
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<td>none</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Caudate nucleus L</td>
<td>-15</td>
<td>12</td>
<td>12</td>
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<tr>
<td></td>
<td>Caudate nucleus R</td>
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<td>6</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Frontal gyrus L</td>
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<td>18</td>
<td>15</td>
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<tr>
<td></td>
<td>Frontal gyrus R</td>
<td>45</td>
<td>15</td>
<td>-9</td>
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<td></td>
<td>Amygdala L</td>
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<td></td>
<td>Insula L</td>
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<td></td>
<td>none</td>
<td>none</td>
<td>none</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lingual gyrus L</td>
<td>-6</td>
<td>-72</td>
<td>0</td>
</tr>
</tbody>
</table>
Short term effects of caloric restriction

After weight loss there were no significant changes in memory performance when non-food pictures were projected compared to food pictures. Mean reaction time was significantly slower during the sated state (p<0.05) and when food pictures were observed (p<0.001).
DISCUSSION

This study shows that in the very first stage of caloric restriction several favourable metabolic changes occur, such as a decrease in systolic blood pressure, leptin, resistin, triglycerides and IGF-I levels as well as an increase in ghrelin and IGFBP-1. Brain activity in the amygdala area, and correlations of metabolic factors with activity in brain areas involved in the food reward system, differ substantially before and after weight loss.

In the present study weight loss started in the first week of caloric restriction and a modest total mean weight loss of 4.8 % was seen after four weeks of caloric restriction. Others have shown a similar weight loss after four to five weeks of caloric restriction although this was achieved with variable caloric restrictions. During the first week of caloric restriction a reduction in fat free mass was observed as well as a decrease in fat mass. A similar early loss in fat mass has been described by others (15-17). SAD measurements are useful in clinical studies as an indirect index of visceral fat. In our study these measurements showed a declining trend, but only reached significance at the end of the caloric restriction period. Possibly this is due to the moderate weight loss achieved at that time. On the other hand, there was a gradual significant decrease in the waist circumference measurements during the entire study period.

The very early decrease in systolic blood pressure at the start of weight loss was unexpected. The relationship between blood pressure decline and weight loss has been reported before, but the decrease in blood pressure was only seen after moderate (5-10% of baseline) weight loss (18). Underlying mechanisms could be a decrease in cardiac output due to loss of the increased extracellular volume (19), suppression of sympathetic nervous activity (20), or improvement of insulin sensitivity (21). From our findings it may be concluded that even before weight loss is observed these changes do take place. We did not find any
associations between systolic blood pressure decline and changes in metabolic parameters (data not shown).

Also, a very early decrease was observed in triglyceride level. This was not seen in previous studies after weight loss in obese healthy women aged 58-83 yr (22) and in postmenopausal women following various diets during nine weeks (7). Studies on low carbohydrate diet have shown decreases in triglyceride level. For example, two studies on overweight hyperlipidemic subjects showed a decline in triglyceride levels after two weeks (23) and after six weeks (24). Favourable changes in triglycerides were observed in a meta-analysis of the effect of low carbohydrate versus low fat diets on cardiovascular risk factors with a follow up of at least six months (25). In the present study, FFA levels were increased early during the caloric restriction diet but seemed to decrease thereafter. As high FFA levels are associated with hydrolysis of triglycerides this increase could be an acute metabolic effect.

Simultaneous with acute weight loss an early decrease in leptin levels was also seen. Leptin signals excess fat mass and due to leptin resistance, markedly elevated leptin levels are seen in obese subjects (26;27). An early decrease in leptin levels has been found previously after four days energy deficient diet in a group of healthy overweight men as reflected by a 39.4% decrease in leptin levels (28). In another study in postmenopausal women leptin levels were twofold increased and normalized after twelve weeks of low energy diet (29). The reason for the early decrease of leptin levels is still unclear. A possible correlation with insulin has been suggested (28), which is however not supported by our study. Speculations on whether the decrease in leptin levels is related to the amount of weight loss are still under debate (6;29;30).

We observed a very early significant decline in resistin levels. It is well known that resistin, an adipocyte specific secretory factor, is increased in obesity and in diabetes mellitus. Although the precise function of resistin is still unclear, an increase may cause insulin resistance (1).
Previously, a decrease in resistin has been reported after short term weight management (31). Our results also point to an interconnection between early weight loss and decline of resistin levels. In contrast, postmenopausal women with diabetes mellitus reaching a weight loss of 4.5 kg did not show any change in plasma resistin levels after interventions with diet and/or exercise (32). Others have reported even an increase in serum resistin during weight loss (33).

Weight reduction generally leads to an increase in adiponectin level (1). This increase is advantageous as adiponectin mediates insulin sensitivity. As it is known that proinflammatory cytokines down-regulate adiponectin gene expression (34), the increase in adiponectin would be expected to occur in synchrony with the changes in these cytokines. Studies did not show any effects after nine weeks (7), but did show a rise of adiponectin levels after twelve weeks of moderate weight loss (35). Surprisingly, in our study there was initially a very early decrease in adiponectin level. This is possibly an early effect detected due to frequent sampling, with the expected rise following later on. A decrease in adiponectin levels has been reported previously in healthy normal weight women after caloric restriction (36) and after a hyperinsulinemic-euglycemic clamp in obese postmenopausal women (37). The reason for this decrease is unknown. A possible correlation with serum lipids, independently of fat mass, has been suggested (38).

Insulin and glucose levels did not change significantly. This is possibly due to the relative small amount of weight loss achieved, not reaching the effects of 5% weight loss threshold described by others (6).

GIP promotes lipid and glucose storage and acts as a mediator of energy mobilization in a complex network of other hormones and metabolic factors. Still, the exact implication of GIP, especially in obesity is unknown (39). GIP levels did not change during weight loss in our study.
Short term effects of caloric restriction

After initial weight loss was achieved ghrelin levels increased significantly. This was expected as ghrelin is involved in the long-term regulation of body weight (40). Ghrelin levels are lower in obesity, possibly due to a physiological adaptation to the positive energy balance (41).

Another very early change observed in our study was the rise in IGFBP-1 level. IGFBP-1 levels are known to be negatively correlated to BMI (42), and an increase in the levels after fasting or during caloric restriction have been reported by others (43). IGFBP-1 is acutely regulated by food intake and insulin levels and has an important role in the acute regulation of IGF-1 availability (44). The total IGF-1 and IGFBP-3 levels decreased during the first and second week of caloric restriction, respectively. Short term hypocaloric diet in obese subjects has lead to a decrease of free IGF-1 levels (45). In another study the levels of IGF-1 remained unchanged after twelve weeks of caloric restriction whereas the IGFBP-3 decreased (43).

Our study did not demonstrate any changes in CRP, IL-6 and TNF-α levels. High levels of these inflammatory parameters have been found in obese subjects. In accordance with other studies, weight loss during a short period of time does not seem to influence these parameters (7). Also, as others have pointed out, there might be a threshold for weight loss needed to induce an improvement in the inflammatory state in obese subjects (46). On the other hand, a significant reduction of TNF-α and IL-6 has been reported after twelve weeks of weight loss (5% of initial weight) (35).

During the study period the 24-hour urinary cortisol levels did not change. This could be due to the relative small amount of weight loss achieved. Rask et al. (47) have recently shown a decrease in cortisol excretion after substantial weight loss following gastric bypass surgery. Also, in a study in 127 overweight and obese women higher urinary cortisol levels were associated with high energy intake independent of BMI (48).
A very early and sustained significant improvement in depression, anger, tension and fatigue scores was demonstrated. A positive effect of weight loss on mood has also been described by others, both in mildly overweight and obese subjects (49;50).

The present study demonstrated that food pictures significantly activated brain areas known to be involved in food reward processing such as the insula, hippocampus and amygdala. This is concordant with other studies in obese subjects comparing activity in these regions to normal weight subjects (51). Interestingly, in our study the significant activation of the right amygdala during the fasting condition disappeared after caloric restriction. The amygdala receives information on food stimuli and forwards it to other brain regions for further processing of its reward value or motivational salience (52). In a literature review it was concluded that obese subjects show an increased response to food cues, thereby contributing to increased craving for food in obese subjects (53). Until now, studies investigating these processes after short term caloric restriction are scarce. We also evaluated associations between various metabolic measurements and activity in brain regions in response to visual food stimuli. Before weight loss we found that higher levels of glucose were associated with lower levels of activity in brain regions supporting food reward processing such as hippocampus and insula. This is in agreement with studies reporting reductions of activity in food reward regions after glucose ingestion, which has been associated with reductions in food craving (56). In the current sample, these effects were not seen after weight loss, indicating that signalling of satiety was altered after weight loss. The association of leptin levels with food-related brain activity is complex (57) and dependent on weight loss and fasting status. In the present study our data overall show that before caloric restriction, leptin showed both positive and negative associations with activity in regions related to processing of food reward, as has also been described by others (57,58). Only after caloric restriction, prefrontal cortex showed increased activity with higher leptin levels.
Short term effects of caloric restriction

(one region during fasting and two regions when sated), which is in agreement with the notion of leptin signalling to the prefrontal cortex as a satiety region (53,56). It thus seems that this mechanism is normalized or restored in obese individuals after caloric restriction. In a similar vein, associations of fasting ghrelin and brain activity apparently normalized after caloric restriction. Whereas no significant correlations of the orexigenic ghrelin during fasting were found with brain activity to food stimuli before caloric restriction, this association was found to be restored after caloric restriction, with higher levels of ghrelin being associated with higher levels of corticostriatal and parahippocampal activation (59). The present results are thus in line with previous reports showing that baseline neuronal responses to food cues may predict the efficacy of weight loss interventions (60,61).

In contrast to a previous study (55), memory performance for food versus non food pictures was not different, possibly due to greater semantic cohesion of the food stimuli (54).

In summary, in the first week of caloric restriction several favourable metabolic changes occur before moderate weight loss is achieved. Furthermore, after weight loss, correlations of metabolic parameters with brain areas involved in the food reward system are more in concordance with findings in lean subjects.
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Short term effects of caloric restriction


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