Regulation of food intake:
A focus on ghrelin

Wendy A.M. Blom

Promotoren

Prof. Dr. Ir. Frans J. Kok
Hoogleraar Voeding en Gezondheid
Wageningen Universiteit

Prof. Dr. Ir. Gertjan Schaafsma
Hoogleraar Voeding en Levensmiddelen
Wageningen Universiteit

Copromotor

Dr. Henk F.J. Hendriks
Produkt Manager In Vivo Voedingsfysiologisch Onderzoek
TNO Kwaliteit van Leven, Zeist

Promotiecommissie

Dr. A.J. van der Lely
Erasmus Medisch Centrum, Rotterdam

Prof. Dr. W.H.M. Saris
Universiteit Maastricht

Prof. Dr. H.A.M. Daanen
TNO Defensie en Veiligheid, Soesterberg
Vrije Universiteit Amsterdam

Prof Dr. Ir. M.W.A. Verstegen
Wageningen Universiteit

Dit onderzoek is uitgevoerd binnen de onderzoekschool VLAG

Regulatie van voedselinname: Een focus op ghreline

Wendy A.M. Blom

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de Rector Magnificus
van Wageningen Universiteit
Prof. Dr. M.J. Kropff,
in het openbaar te verdedigen
op woensdag 7 december 2005
des namiddags te half twee in de Aula.

Title: Regulation of food intake: A focus on ghrelin

Author: Wendy A.M. Blom

Thesis Wageningen University, Wageningen, The Netherlands
With abstract - with references - with summary in Dutch

ISBN 90-8504-272-0

Abstract

The objective of this thesis was to gain insight into the mechanisms of food intake regulation in order to facilitate the design of foods that could help to regulate energy intake. The focus of this thesis was on the recently discovered gastric hormone ghrelin, since the first available data on ghrelin suggested that it is the most potent hunger signal, known so far. Several intervention studies were initiated to investigate the role of ghrelin in food intake regulation.

Firstly, the association between (changes in) ghrelin concentrations and appetite was investigated. Ghrelin concentrations were positively associated with subjective appetite scores, supporting the role of ghrelin as a hunger signal. Furthermore, there was an inverse association between the decrease in ghrelin concentrations following a meal and the spontaneous time interval between two meals (the intermeal interval, a measure of meal initiation), in normal weight subjects ($r = -0.54$, $p < 0.05$), but not in obese subjects ($r = 0.08$, $p = 0.72$). This association suggests that stronger suppression of ghrelin concentrations, postpones initiation of the next meal. However, ghrelin concentrations were not related to the amount of energy consumed during the next meal.

Secondly, the effects of energy content and meal composition on the postprandial ghrelin response were investigated. It was shown that the postprandial ghrelin response to a carbohydrate enriched meal is dependent on the amount of carbohydrate and is unaffected by intake of the same volume of water. In contrast to other investigations, it was shown that a high protein meal more effectively (± 45%) decreased ghrelin concentrations than an isocaloric high carbohydrate meal ($p < 0.01$). Since the source of protein used differed from the other investigations, it was hypothesized that the effect of protein on the ghrelin response may be dependent on its composition.

Ghrelin concentrations following a meal were strongly inversely associated with the gastric emptying rate, suggesting that ghrelin may require post gastric feedback (i.e. feedback from other factors that are released when food has entered the intestine). This feedback may be provided by insulin and GIP (glucose-dependent insulinotropic peptide), whereas concentrations of both factors were inversely associated with ghrelin concentrations. The association between ghrelin and GIP being the strongest (GIP: $r \approx -0.70$; insulin: $r \approx -0.50$). Ghrelin concentrations were also associated with cholecystokinin (CCK) concentrations, although not consistently over all treatments.

Finally, also the role of ghrelin in the restoration of energy balance following energy restriction was investigated. However, neither 2 nor 3 days of 64%- energy restriction significantly increased fasting ghrelin concentrations in normal-weight men. In obese

men, ghrelin concentrations increased approximately 8% after 3 days of severe energy restriction. Changes in fasting ghrelin concentrations during energy restriction, were not associated with subsequent *ad libitum* food intake ($r = 0.22$; $p = 0.21$), suggesting that ghrelin does not act as a hunger signal during short-term energy restriction.

In conclusion, ghrelin concentrations were associated with subjective measures of appetite and with the intermeal interval, but not with *ad libitum* food intake. Therefore, it is concluded that ghrelin is a hunger signal that is not involved in the determination of meal size (satiation), but that appears to be involved in the regulation of meal initiation (satiety) in normal weight men. Furthermore, ghrelin concentrations were associated with the gastric emptying rate, supporting the hypothesis that post gastric feedback is required. This feedback may be provided by GIP and other regulators of food intake, such as insulin, CCK and PYY. The postprandial ghrelin response is dependent on the energy content of the food consumed and on the type and composition of the macronutrients. Foods that contain for example dairy proteins may effectively suppress ghrelin concentrations for a longer period. These foods may then be used to postpone meal initiation, and may contribute to the prevention and treatment of overweight and obesity.

Contents

1

Introduction

In this thesis, the mechanism of food intake regulation is investigated, with a specific focus on the role of the gastric hormone ghrelin. In this introduction, background information is provided on the prevalence and aetiology of obesity, the regulation of food intake and the involvement of ghrelin therein. In the last paragraphs of this introduction, the rationale and research questions of this thesis are described, followed by the outline of this thesis.

Prevalence of obesity

Obesity is defined as a condition of abnormal or excessive fat accumulation in adipose tissue, to the extent that health may be impaired (365). The Body Mass Index (BMI) is commonly used to classify underweight (BMI < 18.5 kg/m^2), overweight (BMI ≥ 25.0 kg/m^2) and obesity (BMI ≥ 30.0 kg/m^2) in adults.
Worldwide the prevalence of overweight and obesity is rapidly increasing (34). In the United States of America, 25-30% of the adult population is obese (100), and in Europe, the prevalence of obesity is also rising, now ranging between 10 and 25% (365). In the Netherlands, about 45% of the males, and 35% of the females are overweight or obese (338). Data from the National Institute for Public Health and the Environment (RIVM) show that the prevalence of obesity in the Netherlands grows rapidly. Between 1976 and 1980 the prevalence of obesity was 4.9% in adult men and 6.2% in adult women. Twenty years later, the prevalence of obesity had increased to 8.5% in men and 9.3% in women (338). If this trend continues, the prevalence of obesity will be doubled in the next twenty years (29). Not only in adults, but also in children and adolescents, the prevalence of overweight and obesity is rapidly increasing (105). Children and adolescents within the largest BMI percentiles are at greater risk to develop overweight or obesity in adulthood (124).

Aetiology of obesity

In principle, overweight and obesity are caused by an imbalance between energy intake and energy expenditure. When energy intake exceeds the energy expenditure, excess energy will be stored. If this imbalance in energy intake and expenditure persists over a longer period, this will lead to the development of overweight and obesity. The aetiology of obesity is multifactorial and includes environmental, genetic (308), nutritional, endocrine, psychological, toxicological, seasonal and viral (17;85;334) factors. Obesity and particularly obesity with intra-abdominal fat accumulation, is associated with an increased risk for several diseases and with increased mortality (230). Obesity is a risk factor for non-insulin dependent diabetes

mellitus (NIDDM) (237;345;365), cardiovascular disease (151;345;365), hypertension (237), psychosocial problems, osteoarthritis, sleep apnoea and certain types of cancer (365).

Genetic predisposition to obesity

There are large differences between subjects regarding their susceptibility to weight gain (42). Twin and family studies have been performed to investigate the heritability of obesity (42). The genetic contribution to the variation in BMI is estimated to be 40-80%, whereas cultural and societal factors explain the other part of the variation (6;27;42;61;203;211;263;288). Several genes linked to human obesity have been identified (308). However, these genes cannot explain the dramatic rise in overweight and obesity during the past decades, as a period of 20 years is far too short for a genetic drift. Genetic factors do largely determine the susceptibility to obesity, but the environment determines the phenotypic expression of obesity. The effects of high genetic susceptibility for weight gain are enhanced by a high-risk environment (23;211). Thus changes in lifestyle such as reduction of physical activity and increased consumption of energy-dense foods leads to increased weight gain in susceptible subjects (140). Possible mechanisms through which genetic susceptibility could affect weight gain are resting metabolic rate, macronutrient oxidation, adipogenesis, energy intake, energy expenditure and behaviour (204).

Regulation of food intake

Food intake is regulated by both non-physiological (e.g. behaviour) and physiological factors. This thesis mainly focuses on the physiological factors involved in food intake regulation. These physiological factors interact with each other and act both peripherally and centrally. The regulation of food intake can be divided in two different phases: satiation and satiety. Satiation is the process that determines when we stop eating, and therefore influences meal size. Satiety is the process that postpones the next meal (absence of satiety leads to meal initiation), and determines the time between two voluntary meals, the intermeal interval. The satiety cascade is the concept that distinguishes four mediating processes that inhibit eating (and hunger) during the early and late phases of satiety: sensory, cognitive, post-ingestive and post-absorptive processes (37) (see figure 1.1). The satiating capacity of foods and ingredients depends on their effects on these four processes.

Figure 1.1 The satiety cascade (37). The satiety cascade is the concept that distinguishes four mediating processes that inhibit eating (and hunger) during the early and late phases of satiety: sensory, cognitive, post-ingestive and post-absorptive processes. It operates on three levels of the psychobiological system: behaviour, peripheral physiology and metabolism, and brain activity. The change in the level of satiation and satiety is indicated by the change in colour, black representing the highest level of satiety and satiation.

The different phases of food intake regulation

Sensory effects are mainly determined by the palatability, taste, texture, temperature, colour and odour of the foods. The cognitive effects influencing satiety include the beliefs held about the properties of foods and their presumed effect upon the eater. Examples of such cognitive effects are the preferences and aversions for specific foods and dieting behaviour. The extent to which different individuals respond to these sensory and cognitive factors and to other external factors (e.g. presence of other people, time of day) may vary markedly. This may explain some discrepancies among human food intake studies, and some reports of high between-subject variability.

During the post-ingestive phase, the central nervous system (CNS) receives sensory afferent input reflecting the amount of food eaten and initial estimations of its nutrient content (32;132). Distension of the gut caused by the presence of food is detected by mechanoreceptors. These mechanoreceptors signal to the brain through stimulation of the vagal nerve and help to estimate the volume of food consumed (32;132). Moreover, chemoreceptors in the gastro-intestinal tract detect the chemical presence of nutrients, and provide information on the composition of the foods consumed (32;132). The degree to which these receptors are stimulated affects the level of satiation. Following food consumption, factors such as cholecystokinin (CCK) and glucagon-like peptide 1 (GLP-1) are released from the gut, stimulating satiation and reducing meal size (201). The effects of CCK and GLP-1 on satiation are at least partly mediated by their inhibition of the gastric emptying rate, thereby increasing stomach distension, leading to sensations of fullness and satiation (54;117;227;283).

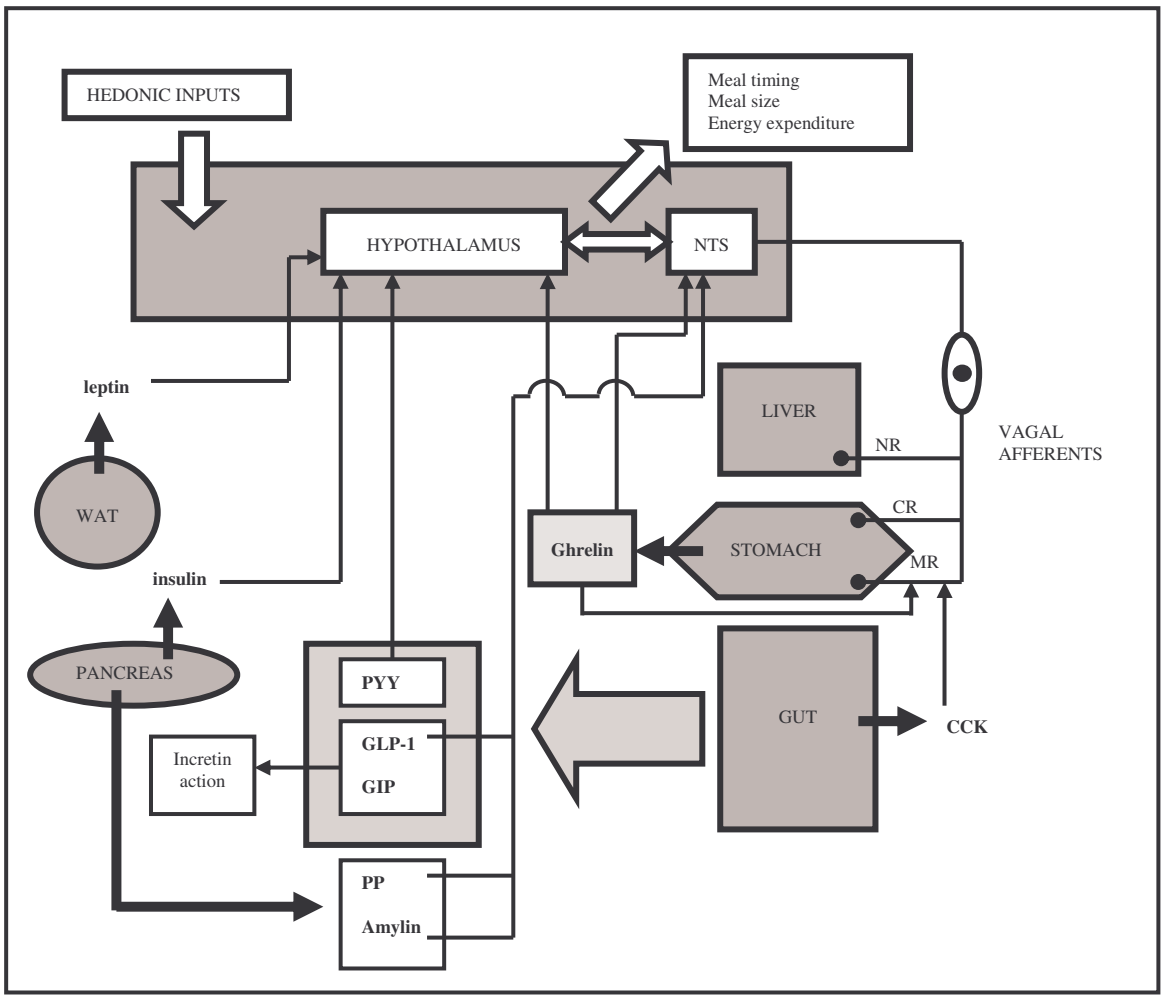


Figure 1.2 Peripheral signals related to the long- and short-term appetite regulation are mainly secreted from adipose tissue and the gastro-intestinal tract, including pancreas, and are integrated in the brain (i.e. the NTS and hypothalamus). This figure provides an overview of the main sources and signalling pathways of these peripheral signals (figure adapted from Badman and Flier (19)). In contrast with all other peripheral signals (e.g. leptin, insulin, PYY, GLP-1 and CCK) that are known to affect food intake, ghrelin stimulates appetite. The interaction between the peripheral signals together with other factors (such as hedonic inputs) determine appetite and food intake. The interactions between the peripheral signals are complex and in case of ghrelin and PYY, not very well investigated, and are therefore not shown in this figure. NTS; nucleus of tractus solitarius, NR; nutrient receptor, CR; chemoreceptor, MR; mechanoreceptor, WAT; white adipose tissue, GIP; glucose-dependent insulinotropic polypeptide, PP; pancreatic polypeptide.

The appetite-suppressing effects of CCK require the presence of food in the stomach and are enhanced when the stomach is distended (160). CCK and GLP-1 do not interact in the regulation of food consumption, but they do interact in reducing appetite (128).
The post-absorptive phase includes the processes mediated by the metabolites in the blood after absorption. The presence of glucose, free fatty acids and amino acids in the blood specifically affects the release of factors such as insulin and glucagon. These factors, subsequently, affect plasma concentrations of hormones such as ghrelin and peptide YY (PYY).

The role of the gastric peptide ghrelin in food intake regulation is the subject of this thesis and will therefore be extensively discussed in following paragraphs and chapters. PYY is secreted in the distal gastrointestinal tract in response to food intake and its concentrations remain elevated for up to 6 hours (2). Postprandial PYY concentrations reflect meal size and the nature of food, fat being the most potent stimulator of PYY secretion (2). Intravenous infusion of PYY decreases appetite and reduces food intake by 33% over 24 hours (25), but also reduces plasma ghrelin concentrations by about 16% in lean, and 26% in obese subjects (24;278).
The effects of food intake on these post-ingestive and post-absorptive processes depend largely on the type of macronutrient and amount of energy consumed.
In figure 1.2 a schematic representation of the post-ingestive and post-absorptive regulation of food intake is given. In chapter 2, the role of these and other physiological factors in the regulation of food intake and their potential as biomarkers of satiety and satiation are being reviewed.

Central regulation of food intake

Signals generated by the satiety cascade reach the brain via several routes. The main route through which the brain receives information from the periphery is the vagal nerve. The vagal nerve receives information from mechano- and chemoreceptors in the gastro-intestinal tract (75;275), and relays this information to the nucleus of tractus solitarius (NTS) in the hindbrain. The vagal nerve also mediates the effects of CCK (296) and, at least partly, the effects of GLP-1 (72;359), ghrelin (32;87;329) and PYY (1). Also metabolic changes in the liver (32) signal to the brain through the vagal nerve. Circulating levels of nutrients, metabolites and other factors are also detected by receptors in the brain stem. In addition, metabolites and hormonal signals such as ghrelin, PYY, leptin, and insulin can cross the blood brain barrier and directly alter CNS activity (22;25;248). Animal studies have shown that ghrelin, leptin, PYY and insulin, exert their effects on food intake by

modifying the activity of the anorexic (appetite-reducing) pro-opiomelanocortin (POMC) neurons, and orexigenic (appetite-stimulating) agouti related protein (AgRP) and neuropeptide Y (NPY) neurons in the arcuate nucleus of the hypothalamus in animals (153;154;243;303). Ghrelin administration significantly increases mRNA expression of the NPY and agouti-related protein (AGRP) neurons in the hypothalamus (55;92;266), stimulating energy intake (see figure 1.3). Leptin and PYY suppress the activity of NPY and AgRP neurons (55;300), and activate the POMC neurons, leading to inhibition of energy intake (243), through the melanocortin system.

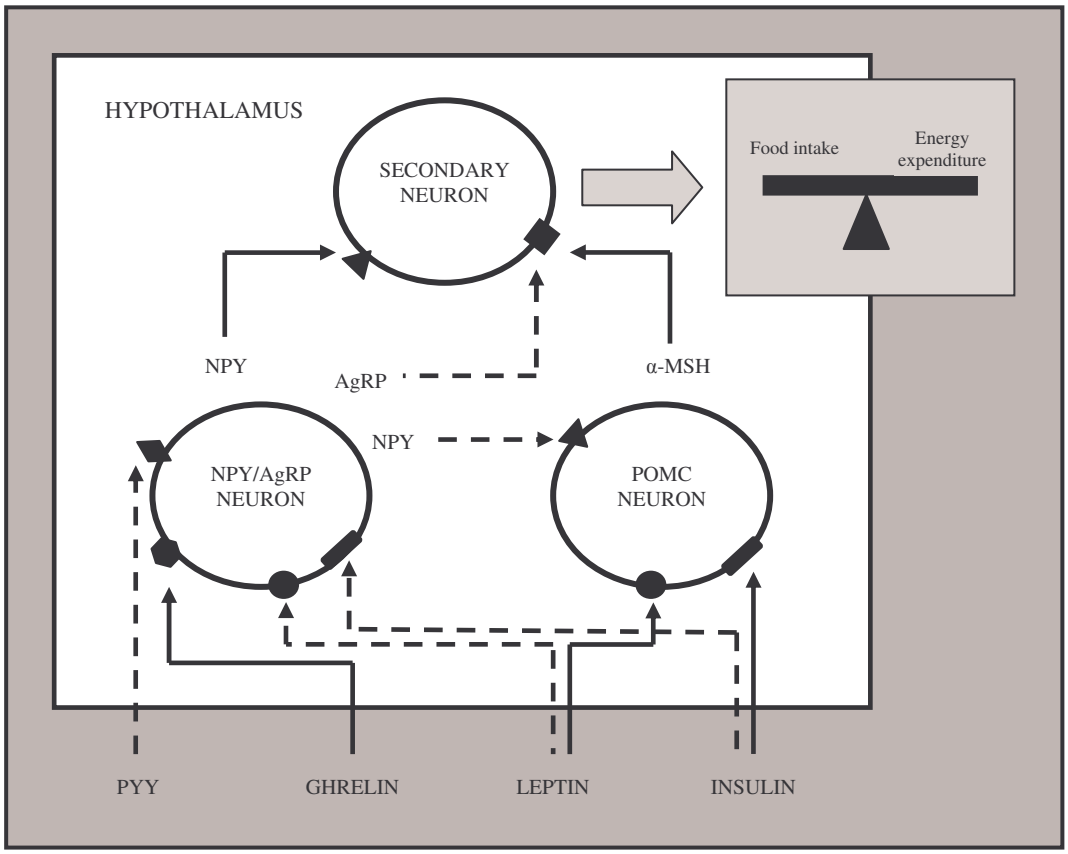


Figure 1.3 Hypothalamic regulation of food intake and energy expenditure (Figure adapted from Schwartz and Morton (299)). →; stimulation, ⇢; inhibition, ▲; NPY receptor (Y1R), ◆; NPY/PYY_{3-36} receptor (Y2R), ■; melanocortin receptor (MC4R) (blocked by AgRP) ⬢; ghrelin receptor (GHS-R), ●; leptin receptor (LEPR), ▬; insulin receptor (INS-R), α-MSH; alpha melanocyte-stimulating hormone.

The central regulation of food intake described above is mainly based on animal studies. It is much more difficult to investigate the role of the CNS in food intake regulation in humans. The development of non-invasive techniques such as functional Magnetic Resonance Imaging (fMRI) and Positron Emission Tomography (PET) gives the opportunity to investigate brain activity. Recently, Smeets et al (307) have shown an energy-dose dependent decrease in the fMRI signal in the hypothalamus following glucose ingestion, supporting the role of the hypothalamus in the regulation of food intake in humans. These techniques in combination with intravenous infusion of peptides involved in food intake (e.g. ghrelin, GLP-1, CCK) and blood sampling may provide more insight into central food intake regulation in humans.

In summary, the regulation of food intake is complex. The hierarchy and integration of the determining factors is not known. Ghrelin appears to be a hunger signal. The relevance of ghrelin in this complex system of food intake regulation is the current topic of investigation.

Project
The research described in this thesis was part of a TNO project entitled “Biomarkers for the satiating action of (components of) foods: a matter of great importance”. The objective of this project was to develop new methods and techniques for the identification and measurement of biomarkers that provide insight in and act as a standard for the effects of (components of) foods on satiety and satiation. Topics of the project were sensory specific satiety, psychomotor functioning and thermophysiology. The development of new analytical tools, characterization and production of new food grade components and products that induce satiety and/or satiation, were also objectives within the project. Within this project, two PhD-projects were initiated. One focusing on the central regulation of food intake and the other, described in this thesis, focusing on the peripheral regulation of food intake, and especially on the role of ghrelin in food intake regulation.

Ghrelin

At the time this PhD-project was initiated (in 2001), the gastric peptide ghrelin had just recently been discovered. The first studies reported that ghrelin was involved in the regulation of food intake. Unlike most other factors involved in the regulation of food intake, ghrelin appeared to be an appetite stimulating (orexigenic) factor, and

even the most potent one. The fact that ghrelin is secreted peripherally, but also acts on the food intake regulating neurons in the hypothalamus, strengthens its role in food intake regulation.
As the objective of the project was to gain more insight into the regulation of food intake, we started to focus on this new and exciting peptide.

Ghrelin is the endogenous ligand for the growth hormone secretagogue receptor (GHS-R) and was first discovered in rat stomach (13;164;235;260). GH secretagogues are synthetic peptide and non-peptide compounds that stimulate the release of growth hormone from the pituitary. Ghrelin also stimulates growth hormone release (164). Ghrelin is a 28 amino-acid peptide, in which the serine-3 is n-octanoylated (164). Plasma concentrations of desacyl ghrelin, the nonacylated form of ghrelin are far greater (10 to 15 times) than concentrations of acylated ghrelin. Until recently, only the acylated form of ghrelin was thought to be biologically active. The current perspective is that also desacyl ghrelin exerts some biological activities (14;45;46;110). For example, desacyl ghrelin does not affect growth hormone, prolactin, acetylcholine, insulin or glucose levels (45), but it does antagonize the effects of acylated ghrelin on insulin secretion and glucose levels (46;110) and even counteracts the negative effects of acylated ghrelin on insulin sensitivity (110). Furthermore, desacyl ghrelin seems to decrease food intake and gastric emptying rate, in contrast to acylated ghrelin (14).

Distribution and localization

Ghrelin is abundantly synthesized in the fundus of human stomach by X/A-like cells (12;71), but is also synthesized in a broad range of other tissues, such as the intestine (secretion decreasing from duodenum to colon), pancreas, pituitary and fat (120). Also the GHS-R receptor distribution is widespread. GHS-R mRNA is expressed in the skin, myocardium, pituitary, thyroid, pancreas, gastro-intestinal tract and the hypothalamus (120;234) but also in a wide range of other tissues (e.g. liver, spleen, lung, lymphocytes, muscle and fat) (120), suggesting that ghrelin might have widespread physiological effects.

Effects on food intake

The following observations from rodent and human studies suggest that (acylated) ghrelin is involved in food intake regulation. First, ghrelin is primarily synthesized by the stomach (12), where it stimulates secretion of gastric acid (73) and increases gastric motility (259). Second, ghrelin is the most potent stimulator of food intake, known so far. Both central and intraperitoneal administration of ghrelin in *ad libitum*

fed rats stimulates food intake (323;368). Intravenous infusion of ghrelin in humans has also shown to potently enhance appetite and to increase food intake by 28% (369). Third, in humans plasma ghrelin levels show a rise before each meal and a fall to nadir levels shortly after eating (67;68). Fourth, plasma ghrelin concentrations in normal weight subjects decrease after oral and intravenous administration of glucose, whereas intake of water does not decrease ghrelin concentrations (44;302;358).

Ghrelin knockout mice have been bred to investigate the role of ghrelin in animal physiology. Against expectations, ghrelin knockout mice show normal spontaneous food intake patterns, normal responses to starvation and diet induced obesity, normal basal levels of hypothalamic orexigenic and anorexigenic neuropeptides and normal growth (315;367). The ghrelin knockout model suggests that ghrelin antagonism may not be useful in the prevention of obesity. Nevertheless, administration of ghrelin receptor (GHS-R) antagonists does decrease energy intake and gastric emptying in lean mice, in mice with diet induced obesity and in ob/ob mice, a genetic model of obesity, fed a high fat diet. Repeated administration of the GHS-R antagonist even decreases body weight gain and improves glycaemic control in ob/ob mice, suggesting that ghrelin is closely associated to excess weight gain, adiposity and insulin resistance (15). Ghrelin induced weight gain may be caused by a reduction of fat utilization (323;367). The functions and actions of the different regulators of food intake are overlapping. Therefore, the possibility exists that one or more of the other factors involved in food intake regulation has compensated for the loss of ghrelin in the ghrelin knockout mice, resulting in mice with normal function of food intake regulation.

Regulation of body weight

Ghrelin is not only suggested to be involved in the regulation of food intake but it may also be associated with body weight regulation. This hypothesis is based on several findings. Firstly, body mass index (BMI) is negatively correlated with fasting plasma ghrelin concentrations. Obesity is associated with lowered fasting plasma ghrelin concentrations (325). Plasma ghrelin concentrations in obese Pima Indians, a population very susceptible to obesity and type II Diabetes, are even lower as compared to obese Caucasians (325). Patients with bulimia nervosa and anorexia nervosa show elevated fasting plasma ghrelin levels as compared to normal-weight subjects (255;316). Lowered plasma ghrelin concentrations in obese subjects and elevated ghrelin concentrations in patients with anorexia nervosa and bulimia nervosa suggest a physiological adaptation to the BMI, rather than a causal role of ghrelin in the development of obesity and these eating disorders, whereas ghrelin

stimulates food intake. This is supported by the finding that under conditions of negative energy balance, ghrelin mRNA expression in the rat stomach is up regulated (321). Furthermore, also in obese humans, weight loss is associated with increased mean plasma ghrelin levels throughout the day (135;255), suggesting that ghrelin may act as a starvation signal during energy restriction. In contrast, weight gain is associated with decreasing plasma ghrelin concentrations (255;256;279).

Rationale and research questions

The objective of this thesis was to gain insight into the mechanisms of food intake regulation in order to facilitate the design of foods that could help to regulate energy intake. As described, it is known that intravenous infusion of ghrelin potently stimulates food intake and appetite, that ghrelin is peripherally secreted but acts also in the central nervous system, and that its concentrations decrease fast, postprandially (after food intake) and increase preprandially (before a meal).

Several important aspects of ghrelin were not described in detail. First of all, the association between physiological ghrelin concentrations and different measures of appetite had hardly been investigated. A second aspect concerned the response of ghrelin to food intake. It was known that ghrelin decreases rapidly following intake of a meal or a glucose solution, but in order to design foods that can help to regulate energy intake, more knowledge about the effects of energy content and meal composition on the postprandial ghrelin response was required. The third point that needed to be addressed was the association between ghrelin concentrations and other hormones involved in the regulation of food intake to gain more insight into the mechanism of food intake regulation and the role of ghrelin in this complex system. Another aspect concerns the role of ghrelin in the long term regulation of food intake. Fasting ghrelin concentrations are negatively associated with BMI, and increase during weight loss, suggesting that during energy restriction ghrelin might act as a hunger signal to the brain in order to restore energy balance.
Therefore, the above aspects of ghrelin were investigated, following four research questions:

1. **Are ghrelin concentrations related to appetite?**
2. **Is the postprandial ghrelin response dependent on energy or macronutrient intake?**
3. **Is ghrelin related to other regulators of food intake?**
4. **Is ghrelin involved in the restoration of energy balance, following energy restriction?**

Outline of this thesis

In order to gain more insight into the physiological factors and processes involved in food intake regulation, we started with an extensive literature research. The literature research focused on the identification and evaluation of potential central en peripheral biomarkers of satiety and satiation. The results of this literature research are presented in chapter 2.

Following this literature research, we initiated several human clinical intervention studies to answer the research questions. These studies, described in chapters 3-7, were performed in healthy normal-weight subjects, because we aimed to investigate the role of ghrelin in food intake regulation under normal physiological circumstances. In addition to ghrelin, other physiological factors important in the regulation of food intake, identified in the literature research, were investigated, as well as their interrelationships. The physiological measures were compared with different measures of appetite.

In chapters 3 and 4, we investigated whether the postprandial ghrelin response is dependent on energy or macronutrient intake or both, by either manipulating the amount and type of carbohydrate (chapter 3), or the amount of both carbohydrate and protein (chapter 4) in the meals. An animal study reported by Williams et al (358), suggested that the postprandial ghrelin response requires post gastric feedback. We investigated this hypothesis in chapter 5, by infusing either GLP-1, which delays gastric emptying, or saline, the control treatment. The role of ghrelin in voluntary meal initiation was investigated in chapter 6. The role of ghrelin as a starvation signal and its association with leptin concentrations during energy restriction was investigated in chapter 7.

In chapter 8, the final chapter of this thesis, the results of the preceding chapters are being discussed, to answer the research questions and to come to an overall conclusion on the role of ghrelin in the regulation of food intake.

2

Biomarkers of satiation and satiety

Cees de Graaf
Wendy Blom
Paul Smeets
Annette Stafleu
Henk Hendriks

Wageningen University, Wageningen, Netherlands
TNO Quality of Life, Zeist, Netherlands
Imaging Sciences Institute, Utrecht University, Utrecht, Netherlands

American Journal of Clinical Nutrition 2004; 79:946-61

Abstract

This review's objective is to give a critical summary of studies that focused on physiological parameters relating to subjectively rated appetite, actual food intake or both. Biomarkers of satiation and satiety may be used as a tool for assessing the satiating efficiency of foods and for understanding the regulation of food intake and energy balance. Markers should be feasible, valid, sensitive, specific, reproducible, and have been shown to be a causal factor in appetite regulation. We made a distinction between biomarkers of satiation or meal termination and those of meal initiation related to satiety and between markers in the brain [central nervous system (CNS)] and those related to signals from the periphery to the CNS. Various studies showed that physicochemical measures related to stomach distension and blood concentrations of cholecystokinin and glucagon-like peptide 1 are peripheral biomarkers associated with meal termination. CNS biomarkers related to meal termination identified by functional magnetic resonance imaging and positron emission tomography are indicators of neural activity related to sensory-specific satiety. These measures cannot yet serve as a tool for assessing the satiating effect of foods, because they are not yet feasible. CNS biomarkers related to satiety are not yet specific enough to serve as biomarkers, although they can distinguish between extreme hunger and fullness. Three currently available biomarkers for satiety are decreases in blood glucose in the short term (< 5 min), which have been shown to be involved in meal initiation; leptin changes during longer term (> 2-4 d) negative energy balance; and ghrelin concentrations, which have been implicated in both short-term and long-term energy balance. The next challenge in this research area is to identify food ingredients that have an effect on biomarkers of satiation, satiety or both. These ingredients may help consumers to maintain their energy intake at a level consistent with a healthy body weight.

Introduction

Humans eat in episodes, i.e. meals and snacks (118;178). With meals, people usually eat until they are comfortably full (satiation), after which they do not eat for a certain time (satiety) (36;39). Immediately after a meal, there is a low drive to eat. This drive builds up again until the moment of the next eating episode. The moment of the next episode is not only dependent on internal factors, but to a large extent is also determined by external (conditioned) environmental factors (cues) (33;41;364). Many of environmental cues are highly dependent on the time of the day. Humans eat not only to satisfy their appetite but also for many other reasons, e.g., sensory hedonics, sensory stimulation, tension reduction, social pressure, and boredom (222;291). This review paper focuses on the internal factors that deal with appetite.

Appetite is the internal driving force for the search, choice, and ingestion of food. Appetite in humans can be measured in 2 ways.

First, it can be measured with the help of subjective ratings. Humans have a capacity for introspection and can rate the strength of their conscious drive or motivation to eat. When used appropriately, subjective ratings have been shown to be reproducible, sensitive to exposures of food components, and predictive of food intake (76;103;314). However, it should be realized that “appetite” may not always be accessible for introspection (31). In addition, people do not always eat when they are hungry, and they do not always refrain from eating when satiated (213) .

Most investigators who use rating scales to assess appetite use the terminology developed by Rogers and Blundell at the end of the 1970’s (282), i.e. hunger, desire to eat, prospective consumption, and fullness. These terms relate to slightly different aspects of the motivation to eat. Prospective consumption (or “How much can you eat?”) seems to be an easier and more concrete question than a more abstract question about hunger. “Hunger” may refer to the appetite for a meal, whereas “desire to eat” may refer to a milder, pleasant feeling of appetite for a snack. “Fullness” refers to a fullness sensation in the stomach. Because subjects may differ in their response behaviour, these scales are preferably used in within-subjects studies, where subjects participate in more than one experimental condition.

Second, appetite can be measured by actual food intake; that is, the amount of food eaten within a certain context can be considered as a measure of appetite. The degree to which actual food intake reflects appetite is debatable. There are many factors that may intervene between appetite and actual food intake: cognitive factors, such as dietary restraint, but external factors, such as availability, hedonic properties of food, and social circumstances. However, when measured under standardized

conditions, actual food intake serves as a post hoc indicator of appetite. One important consideration in this respect is that the actual food intake should be observed (i.e., directly measured), and not derived from dietary records in which subjects record their own food intake. It is difficult to obtain a precise and valid estimate of energy intake on an individual level from dietary records alone (80;121). Measurements of food intake in experimental artificial circumstances suffer from a lack of external validity in relation to eating in the normal context of eating behaviour (222). However, because the measurement of food intake in this context has the purpose of reflecting the internal drive to eat, that seems the appropriate way of measuring appetite.

In the view of Blundell et al (36;39), the expression of appetite is reflected in the relation between 3 operational levels, 1) the level of psychological events and behaviour, 2) the peripheral physiology, and 3) the central nervous system (CNS). The objective of this review is to give a critical summary of published data on the association between biological or physiological measures and either subjective ratings of appetite or actual measures of food intake.

The physiological measures that relate to subjectively rated appetite, actual food intake, or both are defined as biomarkers of satiety and satiation. Markers can be either indicators of appetite, or they can be proven to be causal factors of appetite (86). According to Diplock et al, markers should be feasible, valid, reproducible, sensitive and specific (86). The requirement of feasibility means that markers must represent relatively immediate outcomes, which can be used to assess effects of interventions within a reasonable time. This is usually not a problem with short-term markers of appetite, i.e., within meals or between meals, but this is a problem with markers considered to be involved in the long-term regulation of energy homeostasis. Markers should be measurable in easily accessible material or obtainable by using methods that are both ethical and minimally invasive. The requirement of validity in this context has to do with the notion that the markers must be clearly linked to the physiology of appetite. The sensitivity and specificity in this context reflect the strength of the association between the marker and the measures of appetite. The requirement of reproducibility reflects the consistency of effects or associations between different studies. In this review, we evaluate the usefulness of the markers according to these criteria (see table 2.1).

Knowledge of and insight into biomarkers of satiation and satiety serve 2 main purposes. First, biomarkers of satiety could be used as a tool or index with which to measure the satiating efficiency of foods. These tools may serve as a basis for type A claims with respect to functional foods, i.e., that a certain food or food ingredient enhances satiety, reduces appetite, or does both (86). Secondly it helps to

understand the physiological mechanisms behind the regulation of food intake and energy balance in humans. Of course, this process also works the other way around, i.e., an understanding of physiology of appetite may yield biomarkers of satiety.
The conceptual framework of Blundell (36;132) is used as the guiding principle for the organization of this review. Therefore, the main division in this review is between factors that influence meal termination (satiation) and factors that determine meal initiation (satiety). In many reports, the term “hunger” is used, and this can be considered the opposite of “satiety”. A lesser feeling of satiety or a higher level of hunger is related to meal initiation. A second division is that between peripheral physiology markers and CNS markers. This review uses those studies that have produced actual data on the association between physiological measures and behavioural (i.e. intake) or subjective measures or both. Physiological measures in this review include blood parameters, measures derived from imaging techniques, and measures of thermogenesis.

Search methods

Reports were identified with the help of the Medline database accessed at http://www.ncbi.nlm.nih.gov/pubmed/. The keywords or terms used were *appetite*, *food intake*, *human* in combination with the names of substances [e.g. glucose, leptin, cholecystokinin, glucagon-like peptide 1 (GLP-1), peptide YY (PYY), insulin, glucose, leptin, and ghrelin], techniques [i.e., *functional magnetic resonance imaging* (fMRI) and *positron emission tomography* (PET-scan)], concepts (*sensory-specific satiety*), and other potential biomarkers (e.g. *thermogenesis*). The closing date for searches was 15 October, 2003. Additional reports were identified from a review of references cited in the reports located by using a Medline search.

Peripheral and CNS markers involved in satiation

Studies on meal termination show that the main reason to stop eating at the end of a meal is fullness or absence of hunger, which refers to a sensation of fullness in the stomach (233;326). Another reported reason is a decline in the pleasantness or reward value of the food being eaten (233;326). The sensation of fullness is related to peripheral physiological measures. The sensory reasons to stop eating are primary CNS phenomena. The relative contribution of pleasantness and fullness to meal termination depends on the balance between these 2 factors. Very pleasant-tasting meals may result in a higher food intake and a greater fullness at meal termination.

Table 2.1 Evaluation of potential biomarkers of satiety, according to six criteria[1]

Candidate biomarkers	Causal factor in appetite, or indirect measure	Feasibility of measurement	Validity (plausible mechanism)	Sensitivity or specificity (strength of relation with appetite)	Reproducibility (consistency in findings)	Effect of food components
Satiation						
Brain image SSS	Indirect	-	+	+	+	-
Stomach fullness	Indirect	+	+	+	+	+/-
CCK	Causal	+	+	+	+	+
GLP1	Causal	+	+	+	+	+/-
Bombesin	Unknown	+	+	-	-	-
Somatostatin	Unknown	+	-	-	-	-
Satiety						
Brain imaging satiety	Indirect	-	+	-	+	-
DIT	Indirect	-	+/-	+/-	-	+
Body temperature	Indirect	+	+/-	-	-	-
Absolute glucose	Indirect	+	-	-	-	+
Glucose declines	Causal	+/-	+	+	+/-	+/-
Insulin	Causal	+	+	-	-	+
Leptin, short term	Causal	+	+	-	-	-
Leptin, neg. E-balance	Causal	+	+	+	+/-	+/-
GIP	Causal	+	+	-	-	-
Ghrelin	Causal	+	+	+	+	+/-
PYY	Causal	+	+	+/-	+/-	+/-
Enterostatin	Causal	+	+	-	-	-

[1]CCK, cholecystokinin; GLP-1, glucagon-like peptide 1; GIP, glucose-dependent insulinotropic polypeptide; PYY, peptide YY

Meal termination depends on short-term signals such as stomach distension and on gut hormones such as CCK and GLP-1. Sensitivity to these short term signals is affected by signals that work in the long term, such as leptin, insulin, and ghrelin (136;219;344) . A low leptin concentration (e.g., that observed after a few days of energy restriction) may limit the satiating effect of CCK, which leads to a higher food intake during a meal, thereby restoring energy balance. This mechanism explains how long-term signals operate to affect short-term intake. The long-term regulators are most relevant to the pathophysiology of obesity. However, knowledge about the operation of the short-term signals is essential in the understanding of the regulation of energy intake.

Biomarkers of satiation in the CNS

Before dealing with central biomarkers of satiation, we provide a short explanation of the two main techniques that are currently available to measure human brain responses that relate to appetite.

Introduction to functional neuroimaging techniques

The rapid development of brain imaging techniques during the past decade has led to non-invasive methods of measuring brain function in response to various stimuli. The two most important techniques employed in the study of appetite are PET and fMRI. For comparative reviews, see previous publications (4;30).

In PET the positron-emitting radioisotope ^{15}O incorporated in water-molecules, is administered intravenously and distributed to tissues throughout the body. Because it readily crosses the blood-brain barrier, it can be used to measure cerebral blood flow (CBF). At the site of a brain activation, blood flow increases, which leads to greater uptake of the ^{15}O water tracer into brain tissue, which in turn results in an increase in the number of gamma rays detected at that site. Thus, with PET, the local hemodynamic changes accompanying neuronal activity can be measured (18). Because the half-life of ^{15}O is about 2 minutes, it is possible in practice to acquire a PET-image every 8-10 minutes. This time interval makes PET scans more suitable as a marker of satiety than as a marker of satiation. Subtraction of an experimental image from a baseline image yields an image of the changes in regional CBF (rCBF). The spatial resolution of these images is 5 mm at best.

During a MRI procedure, the subject is placed in a strong magnetic field, which magnetizes the tissues. Then, radiofrequency pulses are applied to excite protons (hydrogen atoms, chosen because they are abundant in biological tissues). On returning to a state of equilibrium, the protons emit radio waves, which are detected

by a receiver coil. The time course of this relaxation process differs among tissues, and that difference is the source of contrast in MRI. In fMRI, the blood oxygen level-dependent (BOLD) signal is used as a measure for neuronal activity. BOLD fMRI makes use of the paramagnetic properties of endogenous deoxygenated haemoglobin as a source of contrast (21;169;252). Deoxygenated haemoglobin locally distorts the magnetic field and thus affects the relaxation process. At the site of brain activation, increased local blood flow leads to a decreased concentration of deoxygenated haemoglobin, which in turn attenuates the local distortion of the magnetic field and results in a small increase (1-5%) in the fMRI signal. Because the BOLD signal relies on the mismatch between the increase in local blood flow and local oxygen uptake, which varies among subjects and occasions, it cannot be used to quantify rCBF. The spatial resolution of BOLD fMRI can be as high as 1 mm^3, depending on the field strength and other scanner characteristics. However, the BOLD response does not co localize perfectly with the actual spot of neuronal activation. Temporal resolution in scanning terms can be as high as 64 images/s, but it is ultimately limited by the temporal characteristics of the hemodynamic response, which is the basis of the BOLD signal. The BOLD signal rises 2-3 s after neuronal activation and is back at baseline after about 10 s (251). The high temporal resolution of fMRI makes it suitable for measuring brain responses that can serve as markers for satiation.

CNS measures related to pleasantness of food and sensory specific satiety

Numerous studies have shown that the food intake during a meal is positively related to the sensory pleasantness of the food (77). Apart from that, humans eat more from meals with a variety of foods than they eat from meals containing a single food (274). This phenomenon is caused by sensory-specific satiety, which was defined by Rolls et al. (285;286) as a greater decrease in the pleasantness of an eaten food than in the pleasantness of an uneaten food. Sensory-specific satiety can be conceived as an important driver for meal termination and the variety in food choices that humans make from meal to meal and from day to day (223;274).

Studies on brain biomarkers of satiety conducted by using fMRI or PET scans showed that the (un)pleasantness of taste and olfactory stimuli is represented in the amygdala and the orbitofrontal cortex (249;371-373). Zald and Pardo (373) found an association between neural activities in the left amygdala and subjective ratings of perceived aversiveness of olfactory stimuli. The role of the left amygdala in aversiveness was confirmed by the response to the taste of a strong quinine solution (371).

In one study focusing on sensory-specific satiety, subjects rated the pleasantness of banana and vanilla odours before and after eating bananas to satiety (250). As could be expected from earlier sensory-specific satiety studies, the subjectively rated pleasantness of the banana odour decreased more than did the pleasantness of the vanilla odour. Although various other brain areas were involved in the perception of the odours, the orbitofrontal cortex was the only area in which, in all subjects, there was a decrease in neural activity parallel to the decrease in pleasantness (250). A PET study on brain activity changes in subjects who had eaten chocolate beyond satiety showed that the medial orbitofrontal cortex was activated when the chocolate was liked, whereas the lateral orbitofrontal cortex was activated when the consumption of chocolate became aversive (305).

The recent studies that used PET and fMRI techniques to study brain activity clearly show that the neural correlates of the pleasantness of foods and changes in rated pleasantness of foods during meal consumption can be reliably detected in the brain. Limitations to these techniques are that fMRI and PET scans are not easily carried out or widely available and are relatively expensive. Data from fMRI and PET scans are indirect indicators of neural activity, and therefore they cannot be considered as causal factors in the chain of events leading to satiation. The fMRI and PET scan techniques can be performed only in subjects in the supine position and with the head restricted to prevent movement, and these circumstances for carrying out the measurements are rather artificial. All of the above makes it unlikely that these techniques will be used in the near future to support a claim for the satiety-enhancing capability of functional foods. However, these techniques do represent an exciting contribution to the understanding of the biology of food choice and food intake regulation.

Biomarkers of satiation in the peripheral physiology

Physical measures related to stomach distension

The results of many short-term intake studies show that the weight or volume, rather than the energy content, of foods is one of the most important determinants of meal size (e.g. (264) . For example, when one serves human subjects *ad libitum* a familiar food (e.g., yogurt) with covertly varied fat concentrations, the weight or volume intakes are similar, but the fat and energy intakes are linearly related to the fat concentration (38;175). These findings indicate that physical measures (biomarkers) that are directly related to the effect of weight or volume of food may also be related

to satiation. Stomach distension, fullness, or both seem to be the most obvious candidates for such a measure.

The role of stomach distension in long-term energy homeostasis is less clear. In the short term, a higher energy density linearly increases energy intake, but, in the long-term, a high energy density appears more effective in decreasing food intake (313). Gastric capacity may also change over time because of dieting (116).

The role of stomach distension in satiety and food intake is clear from a series of studies of Geliebter (114-117). In one of the earliest of these studies, Geliebter showed that stomach capacity, measured by filling a balloon in the stomach, had a correlation of 0.44 (n = 8 subjects) with the *ad libitum* intake from a liquid lunch meal (114). In that study, gastric balloons with a volume of more than 400 mL reduced food intake. In a later study that included normal and bulimic subjects, the correlation coefficient between gastric capacity and *ad libitum* liquid meal intake was 0.53 (n =18 subjects) (115).

Other studies providing insight into the role of stomach distension are those with the gut hormones glucagon-like-peptide-1 (GLP-1) and cholecystokinin (CCK). GLP-1 and CCK serve as a kind of traffic-police assisting with a constant manageable influx of nutrients from the stomach into the gut. GLP-1 and CCK work through effects on pyloric pressure, stomach motility, and stomach muscle relaxation, causing a delay in gastric emptying and a subsequent increase in gastric distension (104). Gastric filling is even a required condition for the satiating effect of CCK (227).

More direct evidence for the role of gastric distension in appetite comes from studies by Melton et al (227) and Cecil et al (53). Melton et al (227) showed in 4 subjects a positive correlation between gastric pressure rise due to balloon inflation and fullness ratings. Cecil, French, and Read (53) showed in a study with 9 subjects that covert and overt intragastric infusion of tomato soup suppressed subjectively rated appetite, whereas intraduodenal infusions of soup did not lead to a reduction in subjectively rated appetite. The correlation coefficients between mean appetite ratings and mean gastric content measures were about 0.99. Regression analyses within subjects showed that gastric content measures could explain about 50-60% of the variance in the fullness ratings during overt and covert intragastric soup delivery. Rolls and Roe (284), showed that increasing the volume, but not the energy content, of gastric infused food reduced hunger ratings and food intake in 29 obese and 25 nonobese women.

In summary, there is much indirect and some direct evidence that there is a direct, inverse association between gastric distension and appetite. A number of methods have become available to measure this “biomarker”. For example, the volume

required to produce a rise of 5 cm in water pressure (116), gamma-radiation camera measures of radioactive isotopes in the stomach of radioactive isotopes mixed with ingested food (53), paracetamol absorption in the blood of paracetamol mixed with food (104), and MRI (81) are indirect measures of gastric distension. These notions suggest that markers of stomach distension are feasible, valid, reproducible, sensitive and specific. Moreover, stomach distension is likely to be a causal factor in the chain of events leading to meal termination or satiation. From this perspective, it is clear that measures of gastric distension or fullness may serve as a useful biomarker of satiation. More research may also be focused on more direct physiological measures of gastric distension, which are the direct biomarkers.

Hormonal/physiological measures

When food enters the stomach and the gut, numerous hormones with different functions are released into the blood. These hormones include CCK, GLP-1, bombesin or gastrin-releasing peptide, PYY, ghrelin, enterostatin, glucose-dependent insulinotropic polypeptide (GIP), pancreatic polypeptide, and somatostatin. From this series of hormones, CCK, GLP-1, and bombesin have a direct effect on gastric emptying (82;104), whereas the others are supposed to have longer-lasting postprandial effects on satiety and meal initiation (25;67;167).

Cholecystokinin

The most-widely investigated gut hormone in relation to appetite is CCK. CCK is released in the blood as a function of the presence of fat (i.e. long-chain free fatty acids), or protein (i.e., amino acids) in the duodenum, where CCK has an effect on receptors of the nervus vagus (82). The nervus vagus transports the signal to the nucleus tractus solitarius in the brainstem, and from there to the CNS (132).

Most studies on CCK follow a particular design in line with its presumed mode of action. In general, exogenous or endogenous CCK is infused or produced, and, during the same time, *ad libitum* food intake or subjectively rated appetite is measured. Outcome parameters are the amount of food ingested, subjectively rated appetite, or both. Endogenous CCK production is often induced by oral or intraduodenal administration of fat or protein. In some studies, specific CCK receptor blockers (e.g., loxiglumide) are administered to investigate the mechanism by which CCK exerts its action.

The first report of the appetite suppressing effect of CCK in humans, is a study by Kissileff et al (161) showing that the exogenous, peripheral (intravenous) administration of high nonphysiological doses of CCK suppressed food intake in a test meal in humans by 19%. Since that study, there have been many studies on the

effect of CCK on appetite (e.g., (20;26;47;126;181;186;189;192;198;238;239;262) (see Table 2.2). Overall, these studies give a fairly consistent picture about the effect of CCK on appetite. The weighted average of intake suppression in the first ten studies (total *n*= 214 subjects) that compared the effects of exogenous CCK and saline on actual food intake is 22.5 %. Two studies showed a dose-dependent effect of CCK on appetite (202;295). Depending on the dose, subject characteristics, and other experimental conditions, intake suppression varied between 0 % (202) and 63 % (295).

A full stomach (after preloads of about 400-500 mL) is a necessary condition for the appetite-suppressing effect of CCK. This indicates that the mechanism by which CCK suppresses appetite is the delay of stomach emptying (227). In a recent publication, Kissileff et al (160) show that CCK's suppression of food intake is enhanced when the stomach is distended.

Studies on the endogenously produced CCK also show that CCK acts as an appetite suppressant, although this effect is not clear from all studies (107;108;362). An elegant study by Matzinger et al (216) showed that the satiating effect (of intraduodenal administration) of fat could be counteracted by a specific CCK receptor blocker, loxiglumide. This finding implies that CCK mediates the effect of fat on satiation (i.e., meal termination; see table 2.2).

The effects of CCK on subjectively rated appetite are less clear than are the effects of CCK on food intake. All of the 16 studies on the effects of CCK or CCK blockers on food intake indicated that CCK suppressed food intake. The effects of CCK on subjectively rated appetite were apparent in only 8 of the 17 studies that included subjective ratings of appetite, which is probably related to the higher degree of random or systematic error in measures of subjective ratings than in measures of food intake. These bigger error components with subjective ratings imply that a larger number of subjects is needed to show systematic effects (103).

The results of the studies of CCK show that CCK can be used as a biomarker of satiation. Both endogenous and exogenous CCK suppresses appetite, and higher concentrations of CCK produce larger appetite-suppressing effects. CCK has an important role in the causal chain leading to satiation or meal termination. Observations from other studies have shown, for example, that fats with long-chain fatty acids result in higher CCK concentrations than do fats with short-chain fatty acids (106;215). Hall et al (133) recently reported on a study in which they showed that casein and whey proteins exert different effects on CCK, GLP-1 release and appetite. These studies imply that (ingredients of) foods that have a high potency for releasing CCK may be used to produce foods with a higher satiating effect. This observation creates a major and exciting challenge for future research.

One of the limitations of the role of CCK as a biomarker is the technical difficulty of its quantitative assessment in blood. Attempts to develop a radioimmunoassay for CCK had to overcome numerous challenges, such as the multiple molecular forms of CCK, low concentrations, and an amino acid sequence similar to that of gastrin (180). Plasma concentrations of gastrin are 20-100 times higher, so that even slight antibody cross-reactivity with gastrin poses a substantial problem for the accurate measurement of blood concentrations of CCK (180). Accordingly, the sensitivity and specificity of an accurate CCK assay must be extremely high (180).

Glucagon-like peptide 1

Glucagon-like peptide 1 is produced primarily in the ileum (330), in response to the presence of nutrients, i.e., carbohydrates and fat (200). GLP-1 stimulates the islet β cells in the pancreas to secrete insulin, thereby contributing to the lowering of the blood glucose concentrations in response to carbohydrate ingestion (330). GLP-1 is thought to play an important part in the "ileal brake" mechanism (i.e., adjustments of stomach and gut motility after food ingestion) that causes a moderate and stable (digestible) flow of nutrients from the stomach into the small intestines. This is probably also the mechanism by which GLP-1 exerts its effect on appetite (374). It is important to notice that the biological active form of GLP-1, GLP-$1_{(7\text{-}36\ \text{amide})}$ is rapidly degraded by the enzyme dipeptidyl peptidase IV (DPP-IV) to the inactive form GLP-$1_{(9\text{-}36)}$ (159).

The first report of the effect of GLP-1 on human appetite comes from Flint et al (102), who showed that the exogenous intravenous infusion of GLP-$1_{(7\text{-}36}$ amide) reduced the ad-libitum energy intake from a test meal in 20 nonobese men by about 12%. During GLP-1(7-36 amide) infusion, hunger and prospective food consumption were lower than during saline infusion (see also (129;195;244;245;273); see table 2.3.

A published meta-analysis on 115 subjects with respect to the effects of GLP-1(7-36 amide) infusion on *ad libitum* energy intake during test meals showed an intake reduction of 12 % during GLP-1(7-36 amide) infusions but non during saline (control) infusion. Reductions were similar for obese (9 %) and nonobese subjects (13%). An interesting finding in this meta-analysis was that differences between blood GLP-1(total) (i.e., the sum of biologically active and nonactive forms) concentration during placebo and GLP-1(7-36 amide) infusion (n = 43 subjects) were negatively correlated with differences in ratings of prospective consumption (r = -0.43), and hunger (r = -0.26), and positively correlated with differences in fullness ratings (r = 0.38)(335). GLP-1 reduces appetite in normal, obese, and diabetic subjects.

Table 2.2 Summary of results of studies in humans that investigated the effects of CCK, or CCK-receptor blockers on appetite, that is food intake and subjectively rated appetite; IV = intravenous, ID = intraduodenal, CCK, 8,9,33 = cholecystokinin with 8,9,33 amino-acids

First author, and year of publication	Design and stimuli used	Number and type of subjects	Results with respect to food intake	Results with respect to appetite
Exogenous				
Kissilef, 1981 (161)	Counterbalanced, cross-over -IV saline -IV CCK 8, 3.6 pmol/kg min infusion: 0 min before – 12 min after end ad libitum liquid test meal (yogurt + fruit) 12 min before test meal, appetizer was served (0.9 MJ)	12 non-obese men, on average 105% of average desirable weight age (mean ± sd): 25 ± 4 y	Mean test meal intake: saline = 644 g CCK 8 = 522 g (= -19%) difference = - 122 ± 50 g (s.e.d.)	No differences in hunger and satiety ratings
Pi-Sunyer, 1982 (262)	Double blind, randomized, cross-over -IV saline -IV CCK8, 3.6 pmol/kg min infusion: 0 min before – 12 min after end ad libitum liquid test meal (yogurt + fruit) 12 min before test meal, appetizer was served (0.9 MJ)	8 obese men, on average 137 % of average desirable weight age (mean ± sd): 25 ± 4y	Mean test meal intake: saline = 977 ± 423 g CCK-8 = 852 ± 472 g (=-13%) Difference = - 126 ± 65 (s.e.d.)	No differences in hunger and satiety ratings
Muurahainen, 1998 (238)	Counterbalanced, double blind, cross-over -IV saline -IV CCK8, total dose 2025 pmol (203 pmol/min) Infusion: 6 min before start – 4 min after start ad libitum test meal (macaroni + beef) 20 min before test meal, soup preload of 500 g (0.8 MJ)	12 non-obese men within 15 % of desirable weight age (mean ± sd): 21±3 y	Test meal intake (mean) Saline = 602 g CCK8 = 362 g (-40%) Difference = - 240 ± 81 g	Not measured

Schick, 1991 (295)	Double blind, randomized, cross-over - IV saline - IV CCK9, 1.6 pmol/kg min - IV CCK9, 8 pmol/kg min Infusion: 15 m before – 45 m after start ad libitum test meal (sandwich quarters)	18 normal weight men Age range 21-26 y	Test meal intake at end infusion (mean ± SEM): Saline = 32 ± 2 sandwich quarters CCK9-100 = 28 ± 2 (= -13%) CCK9-500 = 12 ± 3 (= -63%)	Not measured
Muurahainen, 1991 (239)	Counterbalanced, cross-over -IV saline + 100 ml preload -IV saline + 500 ml preload -IV CCK8 (203 pmol/min) + 100 ml preload -IV CCK8 (203 pmol/min) + 500 ml preload Infusion: 5 min before start- 5 min after test meal (macaroni-beef) preload (tomato soup): 20 min before start test meal	12 normal weight men Age range: 18-35 y Weight within 15 % of desirable body weight	Test meal intake (g) at (mean ± SD): saline/100 = 778 ± 274 Saline/500 = 721 ± 352 (-7%) CCK/100 = 709 ± 288 (-9%) CCK/500 = 494 ± 300 (-36%)	Hunger lower in CCK condition compared to saline condition Hunger ratings lower after 500 ml preload compared to 100 ml preload
Melton et al, 1992 (227)	Double blind for CCK/saline -IV CCK8, 98 pmol/min, 500ml gastric bal -IV saline, 500 ml gastric balloon -IV CCK8, max tolerated ball. volume -IV saline, max tolerated balloon volume infusion with 500 ml balloon: for 25 min starting 5 min before balloon inflation infusion with max. tolerated volume: started 22 min before inflation, and lasted until balloon deflation	4 normal weight women, age range: 23-28 y BMI range: 18-25 kg/m^2	Not measured	Higher mean fullness rating with CCK vs. saline at 500 ml (6.0 vs. 4.7 on 10 pt scale. With 500 ml volume, no differences in hunger, satiety ratings. CCK enhanced effect of gastric pressure on fullness
Lieverse, 1993 (Chapter IV PhD-thesis) (181)	Double blind, randomized, cross over -IV saline -IV CCK33, 0.2 pmol/kg ideal weight min infusion: for 60 min, leading to physiologically relevant CCK levels i.e. 10-15 pMol in plasma	32 subjects, 14 obese women, age (mean ± sd): 41 ± 3 y BMI: 40 ± 2 kg/m^2 18 normal weight (4m, 14 f), age: 34 ± 2 y BMI: 22 ± 0.3 kg/m^2	Not measured	CCK vs. saline reduced hunger, desire to eat, prospective consumption, and increased fullness. Difference was about 10 mm on 100 mm VAS scale. No differences between lean and mean

First author, and year of publication	Design and stimuli used	Number and type of subjects	Results with respect to food intake	Results with respect to appetite
Lieverse, 1993 (191)	Double blind, randomized, cross-over -IV saline -IV CCK33 0.2 pmol/kg ideal weight min Infusion: for 150 min leading to physiologically relevant CCK levels i.e. 10-15 pMol in plasma 60 min after start infusion ad libitum test meal (banana's)	18 subjects, 9 normal weight (5f, 4m) age range: 22-36 y, BMI range 20-25 kg/m^2 9 obese (9f), age range: 30-59, BMI range 33-49 kg/m^2	Test meal intake (mean ± s.e.m.) Saline = 553 ± 55g CCK = 486 ± 52 g (= - 12 %) (intakes were not significantly different; p = 0.09)	CCK vs. saline reduced hunger slightly more than saline (NS), no differences in desire to eat, prospective consumption. No differences between obese and normal weight.
Lieverse, 1995 (185)	Double blind, randomized, cross-over -IV saline, -IV CCK33 0.2 pmol/kg ideal weight min infusion for 165 min leading to physiologically relevant CCK levels, i.e., 10-15 pMol in plasma. 60 min after start infusion: 300 ml shake containing 100 g (132 kcal) bananas, 75 min after infusion ad libitum test meal (bananas)	18 women, 10 normal weight, age (mean ± sd): 41 ± 2 y, BMI: 22 ± 3 kg/m^2; 8 obese, age: 41 ± 3y, BMI: 39 ± 2 kg/m^2	Test meal intake (mean ± s.e.m.): saline = 346 ± 31 g CCK = 282 ± 29 g (-18%),	CCK vs. saline reduced hunger, desire to eat, prospective consumption, and increased fullness. Difference was about 10 mm on 100 VAS scale. No difference between obese and normal weight.
Ballinger, 1995 (20)	Single blind, randomized, cross-over -IV saline -IV CCK8 0.54 pmol/kg min Infusion for 40 min, leading to physiological relevant CCK levels, i.e. 6-8 pMol in plasma 20 min after start infusion: 200 ml water 25 min after start infusion: ad libitum test meal (mixed attractive buffet)	6 normal weight (4m, 2f) mean age: 31 y Range BMI: 21-25 kg/ m^2	test meal intake (mean ± SEM) saline = 6.4 ± 0.7 MJ CCK8 = 5.1 ± 0.7 MJ (= -21%)	Not measured
Gutzwiller, 2000 (126)	Double blind, randomized, cross over -IV saline, IV saline -IV saline, IV CCK: 67.5 pmol/min -IV loxiglumide 10 mg/kg h, IV saline -IV loxiglumide 10 mg/kg h, IV CCK Infusion 1 from 0-125 min, after 45 min 400 ml 0.6 MJ banana preload, from 60-70 min infusion 2, 65-125 min ad libitum mixed test meal	32 normal weight men, age range: 21-33 y	Test meal intake (mean ± SEM): Saline-sal: 7.5 ± 0.3 MJ Saline-CCK: 7.0 ± 0.3 MJ (-7%) Loxiglumide - sal: 8.3 ± 0.2 MJ (+10%) Loxiglumide - CCK: 7.3 ± 0.2 MJ (-3%)	Hunger reduced in saline-CCK condition by 3 units on 10 pts, scale Other condition produced similar hunger ratings, i.e. loxiglumide counteracted CCK effect

MacIntosh, 2001 (202)	Double blind, randomized, cross-over -IV saline milkshake (744 KJ, 375 g). -IV CCK8 0.9 pmol/kg min (Low Dose) -IV CCK8 2.7 pmol/kg min (High Dose) Infusion for 25 min, 375 g 0.7 MJ banana shake preload after min, after 10 min ad libitum mixed test-meal	24 normal weight subjects 12 (6 m, 6 f) young age 18-33 y, mean BMI = 23.8 kg/m^2 12 (6 m, 6 f) elderly age 67-83 y, mean BMI = 24.1 kg/m^2	Energy intake at test-meal Young: -saline: about 4 MJ -CCK8 LD = 4 MJ (-0%) -CCK8 HD = 2.8 MJ (-35%) Elderly: - saline: about 2.7 MJ -CCK8 LD = 2.2 MJ (-18%) -CCK8 HD = 1.6 MJ (48%) Correlation between EI-test meal and CCK 8 levels was -0.34	Lower hunger ratings in elderly vs. young No effect of treatment on hunger ratings
Kissileff, 2003 (160)	Single-blind, randomized, cross-over Treatments: - IV CCK without gastric distension - IV CCK with gastric distension - IV saline without gastric distension - IV saline with gastric distension - 150 min: breakfast (300 kcal) 0-10 min: balloon filling (300 ml) 10 min: start IV infusion 30 min: end infusion start ad libitum lunch consisting of a strawberry yogurt shake (1.04 kcal/g) 40 min: end meal 55 min: end test CCK-8 infusion: 112 ng/ml 1 ml/min Saline infusion: 1.0 ml/min 0.9% saline	16 non-obese subjects (8 m, 8 f) age men: 25.7 ± 2.4y BMI men: 22.3 ± 1.6 kg/m^2 age women: 23 ± 3 y BMI women: 21.71 ± 1.96 kg/m^2	Reduction food intake compared to IV saline without distension. CCK + distension: 200 ± 43 g CCK no distension: 96 g (SED) Saline + distension: 31 ± 43 g (ns) CCK no dis vs CCK dis: 104 g CCK distension vs saline distension: 169 ± 43 g	After balloon filling, and before CCK infusion begun, subjects were significantly more full than when the balloon was not filled. No effect of CCK was observed.
Endogenous				
Wolkowitz, 1990 (362)	Double blind, randomized cross-over -Oral 10 mg MK-329 (CCK receptor blocker) -placebo -120 min after treatment, subjects ate a 614 kcal mixed meal	8 healthy normal weight men, age range: 23-44 y weight within 15% of ideal weight.	Not measured	90 min after treatment, hunger ratings were 17 mm higher on 100 mm scale after MK329 vs. placebo. ($p < 0.05$) 155 min after treatment, hunger ratings were 8 mm higher after MK329 vs. placebo (NS)

First author, and year of publication	Design and stimuli used	Number and type of subjects	Results with respect to food intake	Results with respect to appetite
French, 1993 (108)	Subjects were given meal of 150 g beef burger, and could eat ad libitum from bacon, tomatoes, bread, butter, and orange juice. CCK levels, and hunger ratings were measured just before meal, and, 12 times after meal at 30 m intervals	9 healthy non-obese subjects (no further specifications)	Not measured	Means over subjects (n =13) correlation (r) over time r(CCK, hunger) = -64 r(CCK, fullness)= 0.68 within subject correlations: r(CCK, hunger) < 0 for 3 of 9 subjects r(CCK, fullness) > 0 for 4 of 9 subjects
French, 1994 (107)	Double blind, randomized cross-over -Oral Loxiglumide tablets 3 x 400mg/d 15 min before meals, during 3 days -placebo	11 normal weight age range: 18-44 y BMI range: 20-25 kg/m^2	Daily energy intake across 3 d according dietary records saline = 7.9 ± 0.6 MJ Loxiglumide = 8.6 ± 0.6 MJ (+ 9 %) differences in intake NS	No effects on hunger and satiety
Lieverse, 1994 (188)	Single blind, randomized cross-over -IV infusion of saline for 210 min, after 60 min ID infusion of saline -IV infusion of saline for 210 m, after 60 min ID infusion of fat (6g/h) -IV infusion of loxiglumide (10 mg/kg h), after 60 m ID infusion of fat (6 g/h) After 150 m and ad libitum test meal (sandwiches with cheese and butter)	10 normal weight subjects, (5 m, 5 f) mean age = 26 y	Test meal intake (mean ± SEM): IV saline, ID saline = 269 ± 37 g IV saline, ID fat = 206 ± 35 g (-24%) IV loxiglumide, ID loxiglumide = 245 ± 30g (-9%) differences in intake NS	Hunger was about 10 mm lower on 100 mm scale after ID fat/IV saline than after ID saline/IV saline. ID fat/IV loxiglumide produced hunger ratings in between other two conditions
Maas, 1999 (198)	Double blind, randomized, cross-over -ID saline at 0.4 ml/ kg h -ID fat at 13.6 kJ/ kg h -ID non-digestible fat (SPE) at same rate infusion for 160 min, after 90 min ad libitum test meal (cheese sandwiches)	18 normal weight (9 m, 9 f), age (mean ± SEM) 24 ± 1 y, BMI: 22 ± 0.4 kg/m^2	Test meal intake (mean) Saline = 3.7 MJ Fat = 3.1 MJ (-16%) SPE = 3.3 MJ (-7%) Correlation between CCK increase and food intake was -0.27	Fat reduced hunger in women, but not in men. SPE effects in between saline and fat effects

Matzinger, 1999 Study 1 (216)	Double blind, randomized, cross over -ID saline, 400 ml water preload -ID saline, 400 ml (0.6 MJ) banana preload -ID fat (41 g), 400 ml water preload -ID fat (41 g), 400 ml banana preload -ID infusion for 120 min; preload after 40 min, ad libitum mixed meal from 60-120 min	12 normal weight men, age range: 20-44 y	Test meal intake (mean ± SEM) ID saline, water = 7.4 ± 0.4 MJ ID saline, banana = 6.3 ± 0.4 MJ (-16%) ID fat, water = 6.6 ± 0.3 MK (-12%) ID fat, banana = 5.0 ± 0.5 MJ (-32%)	Hunger, fullness ratings in line with food intake data; ID-fat, and banana preload reduced hunger
Matzinger, 1999 Study 2 (216)	Double blind, randomized, cross over -ID saline, 400 ml ban preload, IV saline -ID fat, 400 ml ban preload, IV saline -ID fat, 400 ml (0.6 MJ) banana preload, IV loxiglumide, 10 mg/ kg h -IV infusion for 150 min; ID infusion after 30 min, preload after 70 min, ad libitum mixed meal after 90 min	12 normal weight men, age range: 20-44 y	Test meal intake (mean ± SEM) ID saline, IV saline = 7.8 ± 0.8 MJ ID fat, IV saline = 6.1 ± 0.5 MJ (-23%) ID fat, IV loxiglumide = 8.1 ± 0.5 MJ (+3%)	ID fat resulted in 1 point on 10 point scale lower hunger rating; IV loxiglumide counteracted this effect
Beglinger, 2001 (26)	Double blind, randomized, cross over -IV saline -IV loxiglumide 22 u mol/ kg h -IV infusion for 120 min; after 60 min ad libitum mixed meal	40 normal weight men age 21-34 y	Test meal intake (mean ± SEM) saline = 7.0 ± 0.2 MJ loxiglumide = 7.8 ± 0.2 MJ (+10%)	Loxiglumide reduced satiety 1 unit/10 point scale
Burton-Freeman, 2002 (47)	Randomized, cross over -Low fibre, low fat breakfast 3.6 MJ -High fibre, low fat breakfast 3.6 MJ -Low fibre, high fat breakfast 3.6 MJ 6 hours after breakfast and ad libitum meal was served	8 men and 7 women age range: 20-50 y BMI-range: 22-28 kg/m^2	No effects on food intake during test-meal	Highly significant relation between measures of hunger and satiety and plasma CCK response. For every 1% CCK increase, the amount subjects wanted to eat declined 0.45 mm on 100 mm rating scale

The study by Gutzwiller et al (127) clearly suggested a dose-response effect in the appetite-suppressing effect of GLP-1, because the most effective suppression was found at concentrations slightly above normal physiological concentrations (104;127) (see table 2.3).
The effect of GLP-1 on meal size is a typical short-term effect, and in animals the effects of GLP-1 on energy intake reduction were shown to be effective in the short term but not in the long term. However, a recent 6-week study with human diabetic patients showed that continuous subcutaneous infusion of GLP-1(7-36 amide) reduced appetite and body weight (374). The rapid degradation of GLP-1(7-36) in GLP-1(9-36) could explain why continuous infusion of GLP-1(7-36 amide) exerts long-term effects on appetite, whereas a bolus or endogenous release of GLP-1(7-36 amide) exerts short-term effects on meal size and appetite.
Studies of endogenous stimulation of GLP-1 production under influence of different nutrients (e.g., glucose and fructose) have not yet reached the same level of progress as have studies of CCK and fat. Oral glucose has a bigger effect on GLP-1(total) release than does fructose, but glucose and fructose have similar effects on appetite (166).
In summary, studies of GLP-1 show that it may be used as a biomarker of satiation. GLP-1 measures are feasible, valid, reproducible, sensitive, and specific. GLP-1 is likely to be a causal factor in the process of satiation. Food intake and subjectively rated appetite decrease as a function of GLP-1 administration. However, little is known about the possible effects of foods, which may have different satiation efficiencies through differential effects on GLP-1 production. Answering these questions may be a challenge for future studies.

Bombesin and gastrin-releasing peptide
Bombesin, isolated from the skin of the European amphibian *Bombina bombina*, and its mammalian counterpart gastrin releasing peptide (GRP) are neurotransmitters involved in several gastrointestinal functions, among them stimulation of CCK release and antrum and pyloric contraction. These effects are related to the observation that bombesin can inhibit gastric emptying in humans (343).
Lieverse et al (182-184;187) conducted most of the studies of the effects of bombesin on hunger and satiety in humans in the mid-1990s. In an early study, they showed that mean (± SEM) test meal (i.e., banana) intake in nine lean men was 482 ± 74 during bombesin infusion and 602 ± 68 g during saline infusion (183). Infusion of bombesin in combination with loxiglumide, a CCK receptor blocker, resulted in a similar suppression of food intake, which shows that the appetite suppressing effect of bombesin is independent from CCK. The results of subjective ratings of hunger

and satiety were in line with the food intake data. The independence of the appetite-suppressant effect of bombesin from CCK was confirmed in a later study (187).
The appetite-suppressing effects of intravenous infusions of bombesin and GRP in humans were also shown by Muurahainen et al (240), and Gutzwiller et al (130). Lieverse et al (182) compared the effects of bombesin and saline infusion in 9 obese women and 9 lean women and found that test meal intake was significantly reduced in the lean subjects (bombesin: 294 ± 55 g; saline: 467 ± 69 g) but not in obese subjects (bombesin: 431 ± 60 g; saline: 499 ± 99 g). Subjective ratings of hunger and satiety were in agreement with this finding. The lower sensitivity of obese subjects than of lean subjects to the appetite-suppressing effect of bombesin was confirmed in a second study (184).
The results of studies on bombesin suggest that GRP may be an interesting biomarker for satiation. However, the number of human studies is very limited, and there are no data on how various nutrient loads may affect GRP levels. More research is needed to establish whether GRP is a useful biomarker in appetite research.

Somatostatin
Data on human appetite in relation to other gastrointestinal hormones involved in meal termination are limited. In a study in 10 humans by Lieverse et al (190), intravenously infused somatostatin was shown to suppress food intake and feelings of hunger. Lavin (173) found that, under hyperinsulinemic, euglycemic conditions, intravenous infusion of the somatostatin analogue octreotide suppressed the release of GIP and GLP-1 and the corresponding rise in insulin induced by intraduodenal glucose infusion. Moreover, octreotide reversed the suppression of appetite and the reduction in energy intake induced by intraduodenal glucose infusion (173). These were the only human studies of somatostatin in relation to appetite that we found.

Table 2.3. Summary of results of studies in humans that investigated the effects of GLP-1 (Glucagon-like peptide 1), on appetite, that is food intake and subjectively rated appetite; IV = intravenous, ID = intraduodenal

First author, and year of publication	Design and stimuli used	Number and type of subjects	Results with respect to food intake	Results with respect to appetite ratings
Exogenous				
Flint, 1998 (102)	Double blind, randomized, cross-over -IV saline -IV GLP-1, 0.8 pmol/kg min Infusion: from 0 min before start fixed breakfast – 240 min before start breakfast, infusion was stopped 30 min before ad-libitum lunch, infusion continued for 30 min during ad lib mixed lunch (pasta, meat, vegetables)	20 non-obese men, age = 20-31 y BMI = 20.3-25.7 kg/m^2	Test meal intake (mean ± SEM) Saline = 4.2 ± 0.2 MJ GLP1 = 3.7 ± 0.3 MJ (-12%)	Hunger, satiety, prospective consumption ratings about 5-10 mm lower on 100 mm scale during GLP1 infusion vs. saline
Naslund, 1998 (245)	Double blind, randomized, cross-over -IV saline -IV GLP1 0.75 pmol/kg min Infusion: for 210 min, 0 min after infusion and ad libitum test meal was served (Swedish hash)	6 obese men Age = 34.7 ± 3.3 y BMI = 35.6 ± 1.8 kg/m^2 (mean ± sem)	Test meal intake (mean, range) Placebo = 493 g (216 -687 g) GLP1 = 464 g (207-685 g) (-5.9%)	After meal consumption, hunger ratings were lower during GLP1 infusion, at t = 240 min more than 30 mm difference on 100 mm scale
Gutzwiller, 1999a (127)	Double blind, randomized, cross-over -IV 5% glucose (placebo) -IV 5% gluc + GLP1, 0.375 pmol/kg min -IV 5% gluc + GLP1, 0.75 pmol/kg min -IV 5% gluc + GLP1, 1.5 pmol/kg min Infusion: for 120 min, 60 min after start Infusion, ad lib mixed test meal was served	16 non-obese men age = 23.6 ± 0.5 y (mean ± sem)	Test meal intake (mean ± SEM) Placebo = 6.8 ± 0.4 MJ 0.375 pmol GLP1 = 6.4 ± 0.4 MJ (-6.6%) 0.75 pmol GLP1 = 6.1 ± 0.4 MJ (-10.8%) 1.5 pmol GLP1 = 4.6 ± 0.3 MJ (-32.0%)	Hunger ratings dose-dependently lower after GLP1 vs. placebo, with maximal difference of 3 points on 10 point scale, between placebo and 1.5 pmol GLP1 60 min after start infusion
Long, 1999 (195)	Single blind, randomized, cross-over -IV saline -IV 1.2 pmol GLP1/kg min Infusion: for 60 min; 20 min after start Infusion 400 ml water preload; 40 min after start infusion, ad libitum mixed test meal + 200 ml water	10 non-obese men age = 20-29 y BMI = 20-27 kg/m^2	Test meal intake (mean ± SEM) Saline = 5.9 ± 0.4 MJ GLP1 = 5.5 ± 0.5 MJ (-7.1%) (NS; p = 0.27)	Hunger ratings were lower during GLP1 infusions, but differences were not statistically significant

Gutzwiller, 199b (129)	Double blind, randomized, cross-over -IV saline -IV GLP-1 1.5 pmol/kg min infusion for 120 m, 60 m after infusion an ad libitum mixed meal was served	12 diabetic men, age = 55 ± 2 y BMI = 29.4 ± 1.2 kg/m^2 (mean ± sem)	Test meal intake (mean ± sem) saline = 3.9 ± 0.4 MJ GLP1 = 2.9 ± 0.3 MJ (-26.5%)	Changes in hunger/satiety ratings compared to baseline about 1 point on 10 point scale less hungry/more, full on GLP1 vs. saline
Naslund, 1999 (244)	Double blind, randomized, cross-over -IV saline -IV GLP-1 0.75 pmol/kg min infusion for 480 m, at t = 0 fixed breakfast at t = 240 ad libitum lunch (pasta dish), at t = 480 ad libitum diner (mixed meal)	6 obese men, age = 35 ± 4 y BMI = 46 ± 3 kg/m^2 (mean ± sem)	Test meal intakes (mean – range) saline lunch = 4.3 (3.5 - 4.8) MJ GLP1 lunch = 3.8 (2.1-4.6) MJ (-12%) Saline dinner = 3.1 (1.4-5.0) MJ GLP1 dinner = 2.3 (0.8 -3.0) MJ (-26%) Reduction total intake: 21%	Hunger, desire to eat, prospective consumption ratings lower during GLP1 infusion, up to 4 points difference on 10 point scale (just before lunch)
Flint, 2001 (104)	Single blind, randomized cross-over -IV saline -IV GLP-1 0.75 pmol/ kg fat free mass min Infusion: for 300 min minus a break for 30 m before lunch. At t = 0 a fixed breakfast, at t = 270 an ad libitum lunch (mixed meal)	18 obese men, age = 43 ± 2 y BMI = 34 ± 1 kg/m^2 (mean ± sem)	Test meal intake (mean ± sem) Saline = 2.92 ± 0.23 MJ GLP1 = 2.83 ± 0.28 MJ (-3.0%)	Hunger, prospective consumption ratings about 5 mm lower on 100 mm scale during GLP1 infusions no differences in satiety, fullness ratings
Zander, 2002 (374)	Single blind, parallel -placebo -GLP1 4.8 pmol/kg min Infusion continuously subcutaneous for 6 weeks No food intake measured Hunger, satiety measured for 2 h after fixed breakfasts and lunches at week 0, 1 and 6	GLP1 group: 10 diabetics (4m/6F) age = 55 ± 4 y BMI = 35 ± 6 kg/m^2 Placebo group: 10 diabetics (4m/6F) age = 54 ± 6 y BMI = 32 ± 4 kg/m^2 (mean ± sem)	Body weight changes over 6 wks Placebo = -0.7kg GLP1 = -1.9 kg (p = 0.16)	AUC hunger, prospective consumption decreased more in GLP1 group than in placebo group; difference only significant in week 1 vs. week 0.

First author, and year of publication	Design and stimuli used	Number and type of subjects	Results with respect to food intake	Results with respect to appetite ratings
Endogenous				
Kong, 1999 (166)	Single, randomized, cross-over -Oral 75 g glucose in 300 ml -Oral 75 g fructose in 300 ml -Oral 75 g glucose in 300 ml, followed by 75 g fructose in 300 ml, 60 min later; Glucose had much stronger effect on GLP1 Blood levels than fructose. At t= 120 a ad lib mixed meal was served	8 men age = 27 ± 7 y BMI = 24 ± 3 kg/m^2 (mean ± sd)	Test meal intake (mean ± sem) glucose = 4.3 ± 0.9 MJ Fructose = 4.3 ± 1.0 MJ Gluc + fructose = 3.6 ± 1.0 MJ ($p < 0.005$ compared glucose and fructose)	No difference in hunger and satiety rating between three conditions
Rayner, 2000 (273)	Single blind, randomized, cross over -ID saline -ID glucose, 0.5 g/ min -ID fructose, 0.5 g/ min Infusion for 90 min, at t = 90 min, an ad libitum test meal was served GLP1 blood concentration were similar after glucose compared to fructose	10 subjects, 2 f, 8 m Age = 25 (19 – 37) y BMI = 25 (21-28) kg/m^2 (mean – range)	Test meal intake (mean; visually estimated from figure) saline = ≈ 4.8 MJ Glucose ≈ 4.7 MJ Fructose ≈ 4.2 MJ (intake after fructose significantly lower)	Hunger lower after glucose and fructose compared to saline, about 10 mm on 100 mm scale ($p < 0.05$ glucose vs. saline $p = 0.08$ for fructose vs. saline

Peripheral and CNS markers involved in satiety

In general, it is assumed that people start eating when they get hungry. However, meal initiation does not depend only on internal factors. Environmental cues related to the time of the day or food cues and social events are also important triggers of the next eating moment. In many of the studies discussed below, environmental factors are kept constant. In some of these studies, subjects are even isolated from time cues, so that the focus of the study is on the internal signals that drive meal initiation and satiety. In others of these studies, the time between the preload and the next spontaneous eating moment is defined as the measure of satiety.

Biomarkers of satiety and meal initiation in the central nervous system

There are 4 PET and 2 fMRI studies on brain activity related to hunger and satiety (83;111;112;194;317). In the PET studies, the state of extreme hunger (36-h fast) was compared with the state of extreme fullness (about 30 min after the beginning of ingestion of a test meal containing 50% of the estimated 24-h energy expenditure). In the fMRI studies, subjects who fasted overnight ingested 75 g glucose dissolved in 300 mL water, while they were undergoing scanning (see table 2.4).

The PET studies highlighted a large number of areas in which the regional cerebral blood flow (rCBF), a marker of neuronal activity, differed between the state of hunger and that of satiety. Among others, satiety was associated with increased rCBF in the prefrontal cortex (PFC). This is an area, known to exert an inhibitory control on brain activation in response to external and internal stimuli (162;276;277). It has efferent projections to limbic and paralimbic areas, which are involved in drive-related and emotional behaviours. It is interesting that subjects with impaired PFC function suffer from hyperphagia (122). Therefore, it has been postulated that the activation of the PFC in response to a meal contributes significantly to the onset of satiety (83;111;112;317).

The rCBF in the hypothalamus [an area known to be involved in the regulation of food intake (28;43)], the hippocampus (memory function), the thalamus (an area that integrates and relays sensory information to the cortex), and the insular and temporal cortex (both areas deal with gustatory sensory information), which are all limbic or paralimbic areas, was lower in the satiety condition than in the hunger condition (83;111;112;317). There were also consistent decreases in rCBF in the caudate

Table 2.4. Summary of results of studies in humans that investigated the relationship between hunger and satiety and the responses of the human brain.

First author, year of publication	Study Design / stimuli	Measured parameters	Number and type of subjects	Central effects of food intake / stimulus
Tataranni, 1999 (317)	Satiety versus hunger in normal-weight men. 36h fast, liquid meal 50% of DEE* delivered over 25 min. Two 1-minute PET-scans at fasted baseline and two after feeding with 10-15 minutes between the two scans.	Plasma levels of glucose, insulin, leptin and FFA from blood samples collected immediately after each scan. Subjective ratings of hunger and satiety (VAS), recorded after each scan. rCBF*.	11 normal-weight men; 35±8 yrs, 73±9 kg, 19 ± 6% body fat.	Hunger was associated with increased rCBF near the hypothalamus, insular cortex and in the anterior cingulate cortex, parahippocampal and hippocampal formation, anterior temporal and posterior orbitofrontal cortex, thalamus, caudate, precuneus, putamen and cerebellum. Satiety was associated with increased rCBF bilaterally near the ventromedial prefrontal cortex, dorsolateral prefrontal cortex and the inferior parietal lobule. Post meal insulin increase correlated negatively with post meal rCBF changes near the insular cortex (LH: r =-0.69, p = 0.02; RH: r =-0.57, p = 0.06) and the orbitofrontal cortex (LH: r =-0.72, p = 0.01; RH: r =-0.59, p = 0.05).
Gautier, 2000 (111)	Satiety versus hunger in obese and lean men. 36h fast, liquid meal 50% of DEE* delivered over 25 min. Two 1-minute PET-scans at fasted baseline and two after feeding with ~10 minutes between the two scans.	Plasma levels of glucose, insulin, leptin, FFA, gastrin, PP, and GLP-1 from blood samples collected immediately after each scan. Subjective ratings of hunger and satiety (VAS) recorded after each scan. rCBF*.	11 obese men; BMI* ≥ 35, 27±5 yrs, 115±11 kg, 38±7% body fat, and 11 lean men; BMI* ≤ 25, 35±8 yrs, 73±9 kg, 19±6 % body fat.	Obese vs. lean men in response to satiety: greater rCBF increase near the right dorsolateral and ventromedial prefrontal cortex and bilaterally in the dorsomedial prefrontal cortex, greater rCBF decrease in the right insular/anterior temporal region, right hippocampal formation, bilaterally in a large region including the orbitofrontal cortex and temporal pole and the cerebellum. rCBF decreases tended to be smaller in obese subjects in the hypothalamus, thalamus, and anterior cingulate. Negative correlation between changes in plasma insulin levels and changes in rCBF in various brain areas. Changes in hunger ratings correlated negatively with changes in rCBF in left and right prenuceus, in both lean and obese subjects.
Gautier, 2001 (112)	Satiety versus hunger in obese and lean women. 36h fast, liquid meal 50% of DEE* delivered over 25 min. Two 1-minute PET-scans at fasted baseline and two after feeding with ~10 minutes between the two scans.	Plasma levels of glucose, insulin, leptin, and FFA from blood samples collected immediately after each scan. Subjective ratings of hunger and satiety (VAS), recorded after each scan. rCBF*.	12 obese women; BMI* 41±5, 30±7 yrs, 110±14 kg, 40 ± 2% body fat and 10 lean women; BMI* 23±2, 32±10 yrs, 61±7 kg, 26 ± 6% body fat.	Obese versus lean women had a greater increase in rCBF in the ventral prefrontal cortex, greater decrease in the paralimbic areas and in areas of the frontal and temporal cortex. The correlation between post-prandial changes in rCBF and plasma levels of FFA differed between obese and lean women in the right hippocampus/hippocampal gyrus, left ventral, and dorsomedial prefrontal cortex (group effect $p < 0.02$).Lean and obese women showed opposite correlations between plasma levels of glucose and FFA and postprandial changes in rCBF in the dorsomedial prefrontal cortex (glucose: lean women r =0.77, obese r =-0.62; FFA: lean r =0.07,obese women r = -0.72, and in the right hippocampus/parahippocampal gyrus (glucose: lean women r = 0.79; obese r = -0.31; FFA, lean r = -0.58; obese r = 0.49.

Del Parigi, 2002 (84)	Satiety versus hunger in men and women. 36h fast, liquid meal 50% of DEE* delivered over 25 min. Two 1-minute PET-scans at fasted baseline and two after feeding with ~10 minutes between the two scans.	Plasma levels of glucose, insulin, leptin, FFA, gastrin, PP and GLP-1 from blood samples collected immediately after each scan. Subjective ratings of hunger and satiety (VAS), recorded after each scan. rCBF*.	44 subjects; 22 males and 22 females, 31±8 and 31±9 yrs, 28±12 and 34±9 % body fat respectively.	Many similarities between men and women. In the fasted state men versus women showed greater rCBF in the fronto-temporal and paralimbic areas. In the satiated versus the fasted state women showed greater increases in rCBF in the dorsolateral prefrontal cortex, precuneus and the occipito-temporal cortex than men. Men versus women showed greater increases in rCBF in the ventromedial prefrontal cortex (all $p < 0.005$).
Matsuda, 1999 (212)	Satiation and satiety in obese and lean subjects. 12h fast, 75g dextrose in 296ml flavoured water/distilled water as control. Continuous fMRI of a midsagittal slice for 50 minutes, drinking after 10 minutes. Temporal resolution 12s per image (250 images).	Plasma levels of glucose, insulin and leptin from blood samples taken at 15min intervals, starting 15 min before glucose ingestion. BOLD fMRI-signal*. For each ROI*: the time lag between the onset of stimulus intake and the maximum inhibition of the fMRI signal and the averaged inhibition over time.	10 obese subjects; 5 males and 5 females, BMI* 34.2±1.3, 34±2 yrs, 10 lean subjects, 5 males and 5 females, BMI* 22.0±0.9, 32±4 yrs.	Both lean and obese subjects showed an inhibition of the fMRI signal in the upper anterior (UAH) and lower posterior (LPH) hypothalamus (the paraventricular and ventromedial nuclei). Obese vs. lean subjects showed a smaller (4.8±1.3 vs. 7.0±0.6 % inhibition) and delayed (9.4±0.5 vs. 6.4±0.5 min) decrease in fMRI signal in the UAH and LPH. The time taken to reach the maximum inhibitory response correlated with the fasting plasma glucose (UAH $r = 0.68$, $p < 0.01$, LPH $r = 0.75$, $p < 0.001$) and with plasma insulin concentrations (UAH $r = 0.43$, $p < 0.05$, LPH $r = 0.47$, $p < 0.05$) in all subjects.
Liu, 2000 (194)	Satiation and satiety in subjects of both sexes. 12h fast, 75g dextrose in 296ml flavoured water/distilled water as control. Continuous fMRI of a midsagittal slice for 48 minutes, drinking after 10 minutes, Temporal resolution 12s per image (240 images).	Plasma levels of insulin from blood samples taken at 15min intervals, starting 15 min before glucose ingestion. BOLD fMRI-signal/ fMRI activity index*.	21 subjects;11 males and 10 females, 34±3 yrs.	Temporal peaks around 1.9 min (signal increase) and 10.2 min (signal decrease) after the onset of drinking, in the sensorimotor cortex and the hypothalamus respectively. Significant negative correlation ($r = -0.68$, $p < 0.01$) between the fasting plasma insulin level and the fMRI activity* index in the hypothalamus 10 min after the onset of drinking the glucose solution.

* Abbreviations in alphabetical order: BOLD-signal. Blood Oxygen Level Dependent signal, a measure for neuronal activity; BMI. Body Mass Index (kg/m2); DEE. Daily Energy Expenditure; fMRI activity index. Average normalized signal change in a region of interest (Liu et al 1999); rCBF. regional Cerebral Blood Flow, measure for neuronal activity; ROI. Region of Interest; VAS. Visual Analogue Scale.

nucleus and cerebellum, which are involved in motor activity. The relation of these changes to hunger and satiety is not yet clear. In the reports cited, there were no comments on these findings.

After the brain's responses to food in general were mapped, investigators began to investigate differences between obese and nonobese subjects. In response to satiety, both obese men and women were reported to have greater increases in the rCBF in the PFC but greater decreases in the rCBF in the orbitofrontal and temporal cortex than do their lean counterparts (111;112). Obese and lean women also differed with respect to the association between changes in plasma glucose and free fatty acids and the amount of rCBF in the PFC (112). This, again, pinpoints the PFC as an area that reflects differences in the response to satiety of obese and nonobese subjects.

Common fMRI study design and analysis are not very well suited to the use of food stimuli, because of the problems associated with head movement and the unknown timing of the brain's response to such a stimulus. From this perspective, it is interesting to note the work of Matsuda et al (212) and Liu et al (194), who showed that, by using BOLD fMRI, it is possible to measure spatial and temporal characteristics of the brain's responses to food stimuli. Both studies reported a decrease in BOLD signal in the hypothalamus about 10 minutes after the subjects began drinking a glucose solution (194;212). It is interesting that Matsuda et al (212) found that this inhibitory response was delayed as well as attenuated in obese subjects. Furthermore, Liu et al (194) reported that this hypothalamic response to a glucose load was negatively correlated with fasting plasma insulin levels.

In summary, these functional neuroimaging studies are exciting developments in the study of the mechanisms involved in the regulation of appetite. To date, the focus in the PET studies was on the comparison between extreme hunger and extreme fullness, which includes more sensations than simple common feelings of hunger. The temporal (1 scan/8 min) and spatial (5 mm) resolutions of PET are too low to be used for measuring brain responses that could serve as biomarkers of meal initiation. This would require a temporal resolution of much less than one minute, as is clear from data for glucose (see below). The spatial resolution is also too low to detect meaningful changes in the different loci of the hypothalamus, which are strongly involved in hunger and satiety. These spatial and temporal limitations make it unlikely that PET scans techniques will soon be used for measuring biomarkers of satiety or meal initiation (277). The use of fMRI to study CNS effects of food stimuli has proven to be particularly useful for taste and odour (250), but the data with respect to hunger and satiety are limited. The studies of Matsuda et al. (212) and Liu et al. (194) are promising, but they require replication by other research groups.

Biomarkers of satiety and meal initiation in the peripheral physiology

Physical measures

At first sight, body temperature and diet induced thermogenesis (DIT) seem to be attractive candidates for use as biomarkers involved in the satiety process. Heat production and the loss of heat during the oxidation of macronutrients may serve as integrative measures of energy, nutrient balance, or both. In the theory of Friedman (109), hunger depends on the amount of oxidative phosphorylation and ATP production in the liver. Thermogenesis partly reflects this level of oxidation (109). This idea is in line with observations from Westerterp-Plantenga (350), who showed that, under conditions of low oxygen availability (high altitudes), humans have a low appetite.

Diet induced thermogenesis

As far as we are aware, there are five human studies that have investigated the relationship between diet induced thermogenesis on the one hand and appetite on the other hand. Raben et al (269) found that differences in 6 hours postprandial DIT and satiety in 10 men were positively correlated after iso-energetic meals with different fibre levels. Westerterp-Plantenga et al (355) observed a positive association with a correlation coefficient of about 0.2 between DIT and satiety after lunches with different proportions of fat and energy in 32 men and women. Crovetti et al (63), studying 10 women, and Westerterp-Plantenga et al (353), studying 8 women in a respiratory chamber, found that DIT after protein-rich meals was higher than DIT after carbohydrate- or fat-rich meals. In both studies, higher DIT was correlated with higher satiety and lower hunger ratings. However, in the study by Crovetti et al, differences between protein and carbohydrate or fat DIT only emerged more than 3 h after ingestion of the preloads (63). This is a time span in which differential effects of macronutrients on appetite have disappeared (78). Another issue is that DIT and satiety after a meal are not synchronous over time (353), which makes it difficult to accept DIT as a causal factor for satiety.

In the fifth and most recent study on the relationship between DIT and appetite, Raben et al (268) found that alcohol and protein produced larger effects on thermogenesis than did carbohydrates and fats. However, there were no significant differences in rated appetite and food intake after the ingestion of amounts of these macronutrients with equal energy. These data do not support the proposed association between the macronutrient oxidation hierarchy and the satiety hierarchy (268).

DIT measurements are not easy to carry out; they require facilities for indirect

calorimetry, such as respiration chambers, ventilated hoods, or both. With the ventilated hoods, DIT measurements require subjects to sit still for several hours, whereas, in respiration chambers subjects may move as they wish, which increases random error in DIT measurements. Differences between the DIT values of different macronutrients are difficult to assess, and the association between DIT and appetite has not been determined. These observations make DIT measurements unattractive candidates for biomarkers of satiety.

Body temperature

The effects of body temperature on appetite have not been studied in great detail. The common-sense observation that fever reduces appetite may indicate that a higher body temperature is related to a low appetite. A recent study by Westerterp-Plantenga et al (352) found that a low ambient temperature was associated with a lower body temperature and a higher *ad libitum* food intake.

At present, because of a lack of data, body temperature measurement cannot be used as biomarkers of satiety. Body temperature measurements at various places of the body, e.g., in the neighbourhood of the liver, may be relatively easy to obtain with the use of infrared scanning techniques (257;356). Therefore, from a theoretical and practical perspective, this might be an interesting area for future research.

Hormonal and biochemical measures

Glucose

Glucose uptake and use have long been central features of many hypotheses about meal initiation, because of the central role of glucose in the regulation of energy metabolism, which is due to its exclusivity as an energy source for the central nervous system, its limited storage, its high turnover rate, and its tight regulation (217). In the 1950s Mayer (217) proposed the glucostatic theory for short-term appetite regulation, which postulated that glucoreceptors in the brain detect changes in the rate of glucose utilization. A decrease in glucose utilization represented the stimulus for meal initiation and an increase in glucose utilization represented the onset of satiety (217).

The clamp studies of Gielkens et al (119), comparing 5 mmol and 15 mmol glucose; of Chapman et al (58), comparing 5 mmol and 12 mmol glucose; and of Andrews et al (11), comparing 4 mmol and 8 mmol of glucose, suggest slightly but not consistent lower hunger levels at higher glucose concentrations. Glucoprivation induced by intravenous infusion of 2-deoxy-D-glucose, which competitively inhibits intracellular glucose utilization, induces hunger (319;348) and thirst in humans (348). Lavin et al (174) showed that intraduodenally administered glucose reduced subsequent energy intake about 20% more than did intravenously administered glucose. Hunger ratings

were lower and fullness and satiety were greater with intraduodenal glucose than they were with intravenous glucose (174). These appetite suppressive effects of intraduodenal glucose were abolished by the infusion of octreotide, a somatostatin analogue that inhibits gut hormone secretion. These results indicate that the effects of intestinal glucose on food intake and appetite are not regulated by increased blood glucose concentrations. Lavin et al (174) argued that these effects are more likely to be induced by small-intestine stimulation of glucoreceptors or osmoreceptors, which may induce satiety through either direct vagal stimulation or the release of insulin, incretin peptides, or both (174). In summary, there is some evidence that high blood glucose concentrations are associated with lower appetite, but this association is weak (see table 2.5).

Other research has shown that, instead of the absolute concentrations of blood glucose, the decreases in glucose utilization or intracellular glucose concentration act as the stimulus for meal initiation. This idea is in line with the original glucostatic theory of Mayer (217). Louis-Sylvestre and Le Magnen were the first to find that, in rats, meal initiations were preceded by a transient decline in blood glucose, starting 5-6 minutes before meal onset (196). In humans, declines in blood glucose also seem to precede meal requests (51) (Table 2.5). A distinction is made between transient and dynamic declines. The endogenous transient decline in blood glucose is defined as a deviation of >5% from a stable baseline blood glucose concentration that lasts at least 5 minutes. A dynamic decline is a rapid drop in blood glucose after a rise induced by the ingestion of a drink or a meal (226). There is a high correlation between dynamic and transient declines in blood glucose and meal requests (224-226). The strong association between meal request and declines in blood glucose seems to disappear when subjects are in a negative energy balance. In one report, subjects also had meal requests when their blood glucose concentrations were stable (168).

One other possible way of investigating the association between blood glucose concentrations and satiety is with the help of foods with different types of carbohydrates, because the postprandial response of blood glucose differs between carbohydrates (322). However, it should be realized that incretin hormones, vagal stimulation, and other metabolic processes mediate the blood glucose response to foods (10). The glycemic index (GI) of a carbohydrate reflects the postprandial glucose response after consumption of a standard amount of carbohydrate from a test food in comparison with the postprandial responses after consumption of a control food (either glucose or white bread) (322). It could be hypothesized that high-GI foods would lead to steep rises in glucose and related steep rises in satiety and subsequent steep decreases in satiety, and that lower-GI foods would lead to a more

Table 2.5. Summary of design and results of studies in humans that investigated the effects of glucose on appetite, that is food intake and subjectively rated appetite; IV = intravenous, ID = intraduodenal.

First author, and year of publication	Design and stimuli used	Number and type of subjects	Results with respect to food intake	Results with respect to appetite ratings
Exogenous				
Thompson, 1977 (319)	Single blind, cross-over -IV saline -IV 2-deoxy-D-glucose (50 mg/kg) 0-20 min: infusion saline or 2DG 125 min: intensity + pleasantness rating of sucrose solutions 185 -210 min: chocolate-flavoured liquid lunch 240 min: end experiment	5 normal-weight men	mean test meal intake: saline = 797 ml 2DG = 1170 ml (+47%) difference = + 373 ml	2DG increased hunger significantly from 30 -180 minutes compared to saline
Welle, 1980 (348)	Single blind, cross-over Day 1: IV 2 deoxy-D-glucose (50 mg/kg) Day 2: IV saline (50 ml, 0.9% saline) Day 3: IV 2 deoxy-D-glucose (50 mg/kg) 0-20 min: infusion saline or 2DG 150-180 min:lunch consisting of four sandwiches, pie and a beverage	5 men, within 10% of ideal weight for height Age: 19-25 y	Mean test meal intake: 2DG day 1= 1312 ± 228 kcal 2DG day 2 = 1345 ± 155 kcal saline = 981 ± 228 kcal	2DG increased hunger significantly ($p < 0.05$) at 90, 120 and 150 min, compared to saline
Chapman, 1998 (58)	Single blind, randomized, cross-over 25-170 min: IV 20% glucose to maintain glucose levels at baseline (5 mmol/L) in first three conditions 20-170 min: IV treatments -IV control, normal glucose, normal insulin -IV + 0.8 mU*kg-1*min-1 insulin, normal glucose, high insulin -IV + 1.6 mU*kg-1*min-1 insulin, normal glucose, high insulin -IV 25% glucose infusion as on 1.6 mU*kg-1*min-1 insulin condition, high glucose/high insulin (12 subjects) 140-170 min: ad libitum test meal	14 healthy young subjects (12m +2f) BMI: 23.6 ± 1.9 kg/m^2 Age range: 20-33 y Non-restrained eaters 12 subjects completed the fourth, glucose only condition.	Test meal intake (mean ± sem) Control = 4.4 ± 0.4 MJ Insulin 0.8 = 4.5 ± 0.4 MJ (+ 1%) Insulin 1.6 = 4.5 ± 0.4 MJ (+1%) Glucose + = 3.8 ± 0.4 MJ (-15 %) Intake glucose condition was significantly lower ($p < 0.05$), compared to the other three conditions.	Hunger ratings did not differ significantly between the four conditions.

Gielkens, 1998 (119)	Single blind, randomized, cross-over -IV saline (normal glucose, normal insulin) -IV 20 % glucose to maintain blood glucose at 15 mmol/L (high glucose /high insulin) -IV insulin (80-100 mU/L) + IV 20% glucose in order to maintain normal glucose levels, i.e. 4-5 mmol/L) (normal glucose/high insulin)	6 healthy subjects (1m, 5f) BMI range: 20-25 kg/m^2 Age: 22 ± 1 y	Not measured	IV glucose significantly reduced feelings of hunger and prospective food consumption compared to control and insulin infusion (about 35 mm on a 100 mm scale). The wish to eat was not significantly (P = 0.07) reduced. No effects on fullness were found.
Andrews, 1998b (11)	Single blind, randomized, cross-over 0-180 min: -IV 20% glucose, to maintain blood glucose at 5 mmol/L, normal glucose, normal insulin -IV 20% glucose, to maintain blood glucose at 8 mmol/L, high glucose, high insulin 90 - 180 min: -ID infusion of triglyceride emulsion (1,5 kcal/min, 82 ml/h)	10 healthy men BMI range: 22.5-29.6 kg/m^2 Age range: 19-40 y	Not measured	0-90 min: fullness was greater hunger was lower (about 10 mm at a 100 mm scale) at 8 mmol/l glucose infusion compared with 5 mmol/l infusion. 90-180 min: appetite decreased with blood glucose levels of 8 mmol/l compared with 5 mmol/l. Fullness increased at blood glucose levels of 5 mmol/l, but not at 8 mmol/l leading to higher scores of fullness with 5 mmol/l glucose
Lavin, 1996 (174)	- ID 20% glucose (4 ml/min) + IV 0.9% saline (2 ml/min) (A) - ID 9% saline (4 ml/min) + IV 25% glucose (blood glucose matched concentrations after ID glucose) (B) - ID 20 % glucose (4 ml/min) + IV octreotide (250µg/h in 0.9% saline) (C) Infusion (A+B): t=30 – t=120 minutes Infusion (C): octreotide: t= 0-120 minutes, ID glucose: t=30-t=120, IV glucose: t=0 till absorption ID glucose. Octreotide inhibits release of insulin and GI-hormones After ID infusion a cold buffet-style meal was presented for 30 min	7 healthy men BMI range: 20-25 kg/m^2 Age range: 19-35 y Non-restrained eaters 5 subjects for condition C	Test meal intake at end infusion (mean ± SEM ID glucose + IV saline: 907 ± 150 kcal ID saline + IV glucose: 1093 ± 152 kcal ID glucose + octreotide: + 30% compared to ID glucose	ID glucose decreased hunger and increased fullness and satiety compared with IV glucose (about 1.8, 1.5 and 1.0 res. on a 10 pt scale). Plasma glucose levels were the same although total amount of energy from ID infusions of glucose was greater (288 ~ 152 kcal)) from 60-75 min after start of infusion.

First author, and year of publication	Design and stimuli used	Number and type of subjects	Results with respect to food intake	Results with respect to appetite ratings
Endogenous				
Campfield, 1996 (51) Study 1	Time-blinded After an overnight fast subjects had to request a meal when they felt hungry. Blood glucose was monitored for 2-6 hours	18 healthy subjects (9m, 9f)	Not measured	In 83% of the 18 subjects, changes in hunger ratings and spoken meal requests were preceded by, and significantly correlated with, brief transient declines in blood glucose. Unchanged hunger ratings were associated with stable blood glucose concentrations
Campfield, 1996 (51) Study 2	Crossover, time-blinded Overnight fast, followed by either - IV saline (5 mU/kg) - IV insulin (5 mU/kg)	5 healthy subjects; examples of results of 2 subjects were reported	Not measured	Increased measures of desire to eat (about 37 mm on a 100 mm scale) and hunger (about 22 mm on a 100 mm scale) after insulin-induced transient declines in blood glucose concentration
Andrews, 1998a (10)	Single blind, randomized, crossover Visit 1 0-90 min: ID glucose (2.9 kcal/min) or ID lipid (2.9 kcal/min) 90-180 min: ID saline (0.9%, 3ml/m) 180-270 min: ID infusion of the alternate nutrient (glucose of lipid). ID-glucose lead to plasma glucose levels of 8.5-9 mmol/l After visit 1, subjects consumed 400 g glucose supplement per day for 7 days immediately before visit 2. Visit 2: same protocol as Visit 1	10 healthy men BMIrange: 21.7-26.9 kg/m^2 Age range: 19-38 y	Not measured	Day 1: Lipid reduced the desire to eat (about 10 mm on a 100 mm scale) and increased fullness (about 15 mm on a 100 mm scale). ID glucose did not change appetite ratings Day 2: ID glucose did not change appetite ratings. ID lipid was not more satiating compared to glucose
Melanson, 1999a (224)	Blind, cross-over, time-blinded Oral preloads: - 350 g, high-carbohydrate, lemon-flavoured drink (1000 kJ) - 350 g, high-fat lemon-flavoured drink (999 kJ) First meal request: one of the preloads. Second meal request: ad libitum lunch	10 weight-stable men non-restrained eaters BMI: 22.2 ± 1.8 kg/m^2 Age range: 18-30 y	Ad libitum lunch intake (mean ± sem) Fat = 4519 ± 677 kJ ; CHO = 4013 ± 789 kJ ($p > 0.05$)	71 % of dynamic and transient declines in blood glucose were associated with meal requests. Of all meal requests, 72 % were associated with a decline in blood glucose.

Melanson, 1999b (226)	Blind, randomized, crossover, time-blinded Preload: - 350 g, simple carbohydrate, lemon-flavoured drink (1000 kJ) - 350 g, high-fat lemon-flavoured drink (999 kJ) - 350 g, aspartame, lemon-flavoured drink (150 kJ) First meal request: one of the preloads. Second meal request: ad libitum lunch	10 weight-stable men BMI: 23.4 ± 1.9 kg/m^2 Age: 25.2 ± 4.0 y Non-restrained eaters	Ad libitum lunch intake (mean ± sem) Fat = 5617 ± 661 kJ; Aspartame:= 5861 ± 1652 kJ Sugar (CHO) = 6112 ± 910 kJ (p >0.05)	Duration of blood glucose response until baseline is positively correlated with the duration of the intermeal interval and post-drink satiety 81% of dynamic and transient declines in blood glucose were associated with meal requests. Of all meal requests, 73% were associated with a decline in blood glucose.
Melanson, 1999c (225)	Visit 1: max aerobic capacity and power output assessment Visit 2, 24-h time-blinded stay: Evening: Glycogen depletion exercise. After meal request; low-carbohydrate isoenergetic dinner. Next morning: 1st meal request: ad libitum high-carbohydrate and high-fat food and beverages (high-fat drink (999 kJ, 350 g),simple carbohydrate drink (1000 kJ, 350 g)	10 weight-stable men BMI: 21.9 ± 1.9 kg/m^2 Age: 23.1 ± 3.1 y Non-restrained eaters	Not measured	No effects of glycogen depletion on appetite ratings were found postabsorptively (when glycogen buffer is depleted, before first meal), 8 of 10 meals were initiated during stable blood glucose. 77% of all postprandial (after meal) declines in blood glucose were associated with meal requests. Of all postprandial meal requests, 87% were associated with a decline in blood glucose
Kovacs, 2002 (168)	Randomized, cross-over, time-blinded Treatments: - 2-week diet; 3 times/day semi-solid meal without guar gum, 947 kJ - 2-week diet; 3 times/day semi-solid meal with guar gum, 947 kJ - 2-week diet; 3 times/day, solid meal, 947 kJ After treatment and overnight fast, time-blinded subjects could request a meal. 1st, 2nd and 3rd meal request: low energy meal provided the two weeks before 4th meal request: ad libitum standardized meal (pasta + tomato sauce)	15 overweight men BMI: 28.6 ± 1.8 kg/m^2 Age: 43.7 ± 9.3 y	Not measured	Post-absorptive transient declines before meal request were not associated with meal initiation. 48% of the postprandial dynamic declines were associated with meal initiation and all 4 postprandial transient declines were associated with meal initiation.

stable pattern of glucose concentrations and satiety (147-149). However, the results of studies are yet ambiguous. Some investigators found no effect of GI on food intake and appetite (166), while others found a stronger suppression of hunger and energy intake after consumption of carbohydrates with a low GI (141;142;309). A recent study by Anderson et al (7) showed a higher short-term (within 1 hour) appetite-suppressing effect of high-GI foods than of low-GI foods (7).

The results of the studies of glucose show that glucose may be used as a biomarker of satiety (meal initiation) in certain conditions. It is clear that absolute glucose concentrations have no straightforward association with appetite. Transient and dynamic declines in blood glucose concentrations within a short time frame (5 min) are strongly related to meal initiation. These observations imply that meal initiation can be postponed by delaying transient or dynamic declines in blood glucose. It is not clear how this can be achieved in relationship with the carbohydrate structure of foods, but that could be an interesting subject of future research. The measurement of small declines in blood glucose concentrations within a short time is not easy and is rather invasive, because blood glucose has to be measured continuously (i.e. 8-10 times/min)., which is not feasible in many situations.

Insulin

Insulin, which has also been implicated in the long-term regulation of energy balance (301), is produced in the β-cells of the pancreatic islets and secreted in the blood in response to small increases in blood glucose concentrations. In healthy subjects, it stabilizes blood glucose by stimulating the uptake of glucose by peripheral tissues and by suppressing hepatic glucose production. The insulin response to a meal is also mediated, in part, by the insulinotropic incretin hormones glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), which are secreted from endocrine cells in the intestinal mucosa. Incretin hormones enhance insulin secretion in excess of that elicited by the absorbed nutrients themselves.

Studies with exogenous insulin give mixed results. An early clamp study by Rodin et al (280) in 20 subjects found that high insulin concentrations, independent of changes in blood glucose, increase hunger ratings and fluid intake (8). However, results of the studies of Woo et al (363), Gielkens et al (119) and Lavin et al (173;174) suggest that under euglycemic or hyperglycemic conditions, or both, insulin does not affect food intake or appetite (119;363) (see table 2.6).

Studies of the effect of endogenous insulin on food intake and subjective satiety and food intake suggest that insulin has an appetite-suppressing effect in lean subjects, but less so in obese subjects. Holt et al found that the insulin response (AUC) was negatively correlated (r = -0.40) with energy intake in a subsequent *ad libitum* test meal (142). Results from a study of 6 lean and 6 obese men conducted by Speechly

and Buffenstein (310), showed a negative correlation between insulin concentrations and subsequent food intake in lean men, but not in obese men. A similar finding was reported by Verdich et al (336) in 12 lean and 19 obese men.

It could be argued that the negative associations between endogenous insulin concentrations and both food intake and subjective appetite are the result of changes in substances other than insulin. It could be that glucose plays a role here because, in studies where glucose concentrations were kept constant, there was no effect of insulin on appetite and food intake (119;174;363). However, from the previous paragraph on glucose it is apparent that absolute glucose concentrations do not relate strongly to appetite. An alternative explanation for the abovementioned negative associations is that the release of incretin hormones after food intake and the subsequent release of insulin may explain why endogenous insulin concentrations do correlate with appetite and energy intake, whereas exogenous insulin concentrations do not correlate with appetite and energy intake.

Fasting insulin concentrations during energy restriction decrease (90;138;210). The relationship between these fasting insulin concentrations and increases in appetite or food intake are not clear yet. Heini et al (138) found no association between fasting insulin concentrations and appetite ratings in obese women at weeks 3 and 5 of an energy-restricted diet, whereas Mars et al (205) found a correlation ($r = -0.41$, $p < 0.01$) after 2 days of energy restriction that disappeared after 4 days of energy restriction ($r = -0.19$, NS) in a group of lean and overweight men .

Altogether, it seems improbable that insulin can act as a biomarker of satiety. There is no straightforward association between blood insulin concentrations and appetite because that association is confounded or moderated by many metabolic processes. The effects of glucose and incretin hormones on insulin concentrations and the effect of obesity on the association between insulin concentrations and appetite illustrate this. Insulin plays such a central role in the energy metabolism that it cannot be a specific biomarker of satiety.

Leptin

Leptin, the product of the *ob* gene, is synthesized mainly by adipose tissue, provides information on the availability of body fat stores to the hypothalamus. The studies in animals that described its discovery (52;131) showed that leptin reduces food intake and body weight. Plasma leptin concentrations in humans correlate positively with the total body fat stores (62;304).

Table 2.6 Summary of design and results of studies in humans that investigated the effects of insulin on appetite, that is food intake and subjectively rated appetite; IV = intravenous, ID = intraduodenal.

First author, and year of publication	Design and stimuli used	Number and type of subjects	Results with respect to food intake	Results with respect to appetite
Exogenous				
Woo, 1984 (363) Study 1	Randomized, cross-over T=0 minutes: breakfast T=180 minutes: start infusion; - IV saline (0.15 M) - IV insulin (0.03 U/kg/h) and IV glucose (0.25 g/kg/h) as isotonic solution T=210 minutes: ad libitum liquid meal infusion stopped 15 minutes after end meal	8 nonobese subjects (4m + 4f), age men (mean ± sd): 24.7 ± 2.2 y, average desirable weight men: 96.5 ± 11.1% Age women: 23.5 ± 1.7 y, average desirable weight women: 104.5 ± 13.7%	The insulin-glucose infusion (hyperinsulinemia + hyperglycemia) did not influence food intake.	Not measured
Woo, 1984 (363) Study 2	Randomized, cross-over T=0 minutes: breakfast T=168 minutes: appetizer T=180 minutes: lunch and start infusion; - IV saline (0.15 M) - bolus injection insulin (12 mU/kg) + IV insulin (0.03 U/kg/h) and IV glucose (0.125 g/kg/h) as isotonic solution T=210 minutes: ad libitum liquid meal infusion stopped 12 minutes after end meal	4 males, age (mean ± sd): 23.5 ± 6.8 y, average desirable weight: 96.6 ± 6.7%	Glucose concentrations were not different between the two types of infusion. No significant differences in food intake were observed between mean intake during saline infusion (1100 g) and during insulin infusion (1106 g	Not measured

Rodin, 1985 (280)	Four experimental groups: - *Basal Insulin, euglycemia (control):* 120 minutes saline infusion (1 mL/min) - *Hyperinsulinemia, hypoglycaemia:* insulin infusion (concentration maintained at 100 mU/mL) and glucose infusion (concentration decline from 90 to 59 mg/dL over 150 min study period) - *Hyperinsulinemia, hyperglycemia:* glucose infusion (concentration maintained at 125 mg/dL above fasting levels for 150 min) - *Euinsulinemia, hyperglycemia:* somatostatin infusion (9 µg/min) (inhibits endogenous insulin secretion) and insulin infusion (0.08 mU/kg/min). Glucose was maintained at 200 mg/dL for 150 minutes, starting10 minutes after start somatostatin infusion.	20 healthy young subjects (7 f, 13 m), age range: 20-31 y	Mean fluid intake after infusion stopped: Control = 718 ml Hyperinsulinemia, hypoglycaemia = 1110 ml Hyperinsulinemia, hyperglycemia = 1225 ml	Hyperinsulinemic conditions showed significant increased levels of hunger (4.8 and 5.2) compared to baseline (3.4 and 3.8) and euinsulinemic (3.7) conditions on a 7-point category rating scale. No significant differences between the hyper- and hypoglycemic conditions were observed. Hyperinsulinemia was also associated with enhanced palatability of sweet solutions
Lavin, 1996 (174)	- ID 20% glucose (4 ml/min) + IV 0.9% saline (2 ml/min) (A) -> high insulin - ID 9% saline (4 ml/min) + IV 25% glucose (blood glucose matched concentrations after ID glucose) (B) - ID glucose (4 ml/min) + IV octreotide (250 µg/h in 0.9% saline) (C) Infusion (A+B): t=30 – t=120 minutes Infusion (C): octreotide: t= 0-120 minutes, ID glucose: t=30-t=120, IV glucose: t=0 till absorption ID glucose. Octreotide inhibits release of insulin and GI-hormones) After ID infusion a cold buffet-style meal was presented for 30 min	7 healthy men, age range: 19-35 y, BMI range: 20-25 kg/m^2 Non-restrained eaters Condition C was carried out with 5 of 7 subjects.	Test meal intake at end infusion (mean ± SE) ID glucose + IV saline: 907 ± 150 kcal ID saline + IV glucose: 1093 ± 152 kcal ID glucose + IV glucose and octreotide: + 30% compared to ID glucose	ID glucose, with corresponding high insulin levels, decreased hunger and increased fullness and satiety compared with IV glucose (about 1.8, 1.5 and 1.0 res. on a 10 pt scale). Octreotide infusion (with corresponding lower insulin levels) decreased hunger and increased fullness

First author, and year of publication	Design and stimuli used	Number and type of subjects	Results with respect to food intake	Results with respect to appetite
Chapman, 1998 (58)	Single blind, randomized, cross-over T = 25 -170 min: treatment - IV control, normal glucose, normal insulin -IV + 0.8 mU*kg-1*min^{-1} insulin, normal glucose, high insulin - IV + 1.6 mU*kg^{-1} *min^{-1} insulin, normal glucose and high insulin - IV 25% glucose infusion as on insulin (1.6 mU*kg^{-1}*min^{-1}) condition, high glucose, high insulin (12 subjects) The glucose infusion rate was varied to maintain glucose levels at baseline. 140-170 min: ad lib. cold buffet meal	14 healthy young subjects (12m +2f), age range: 20-33 y, BMI (mean ± sd): 23.6 ± 1.9 kg/m^2 Non-restrained eaters 12 subjects completed fourth condition, glucose only condition	Test meal intake (mean ± sem) Control = 4.4 ± 0.4 MJ Insulin 0.8 = 4.5 ± 0.4 MJ (+ 1%) Insulin 1.6 = 4.5 ± 0.4 MJ (+1%) Glucose + = 3.8 ± 0.4 MJ (-15%) Intake glucose condition was significantly lower ($p < 0.05$), compared to the other three conditions.	Hunger ratings did not differ significantly between the four conditions
Gielkens, 1998 (119)	Single blind, randomized, cross-over Treatments t= 0-240 min - IV saline -IV 20% glucose (15 mmol/L) (hyperglycemia + hyperinsulinemia) -IV insulin (80-100 mU/L) + IV 20% glucose (4-5 mmol/L) (hyperinsulinemia + euglycemia) 10 mmol KCl was added to 500 ml 20% glucose.	6 healthy subjects (1m, 5f), age (mean ± sd): 22 ± 1 y, BMI range: 20-25 kg/m^2	Not measured	Hyperinsulinemia without hyperglycemia did not influence appetite ratings. Hyperinsulinemia in combination with hyperglycemia significantly reduced feelings of hunger and prospective feeding intentions compared to contro l and insulin infusion (about 35 mm on a 100 mm scale). The wish to eat was not significantly (P = 0.07) reduced. No effects on fullness were found.
Endogenous				
Holt, 1996 (141)	Randomized, cross-over In total 38 foods separated in 6 categories were tested. At the start of each food group, subjects were given a 1000 kJ portion of white bread (reference food). Each test food was served as a standard 1000 kJ portion with 220 mL of water. Each food item was consumed within 10 minutes. An ad libitum test meal was served at 120 minutes.	41 healthy subjects, age (mean ± sd): 22.1 ± 2.9 y, BMI: 22.7 ± 0.4 kg/m^2 (11-13 subjects per food category) non-restrained eaters	A negative correlation ($r = 0.40$, $p < 0.01$) between insulin AUC responses and ad libitum food intake at 120 minutes was found.	No significant correlations between glucose and insulin measures (e.g. AUC, index, mean peak values) and subjective satiety measures were observed

Speechly, 2000 (310)	Randomized, cross-over - High fat preload meal, containing 20% of daily energy requirement - Low fat preload meal, containing 55% of daily energy requirement. Ad libitum lunch 5 hours after preload meal	6 healthy lean men, age (mean ± sd): 26.67 ± 5.47 y, BMI: 22.50 ±1.08 kg/m ^{2} 6 healthy obese men, age: 39.83 ± 19.03 y, BMI: 39.05 ± 11.63 kg/m^2	Insulin levels immediately before lunch showed a significantly negative correlation with food intake at lunch in lean (r = - 0.63) but not in obese subjects (r = -0.31)	Not measured
Verdich, 2001 (336)	Subjects were served a 2.5 MJ solid test meal (bread with omelette), followed by an *ad libitum* lunch 190 minutes later.	12 healthy lean men, mean age: 34.2, age range: 28.7-39.6 y, mean BMI: 23.1, BMI range: 22.3-23.9 kg/m^2 19 healthy obese men mean age: 35.0, age range: 30.1-39.9 y, mean BMI: 38.7, BMI range 37.3-40.1 kg/m^2	In lean subjects energy intake was inversely related to; fasting insulin concentration prior to the fixed test-meal; insulin concentration immediately before the ad libitum test meal; AUCtotal insulin and AUCincremental, insulin. AUCtotal, insulin was found to explain 67% of the variation in ad libitum energy intake. No correlation between insulin and intake in obese subjects.	Not measured

When humans are in energy balance (i.e. weigh stable during the studies), the association between leptin concentrations and food intake and appetite is not clear. In general, leptin concentrations do not change acutely (i.e. within 3-4 hours) in response to meals, and most studies find that there is no association between leptin concentrations and subjective measures of appetite before and after meals (150;156;287). As hunger ratings change dramatically after a meal, and thus there cannot be a strong direct association between hunger ratings and leptin concentrations.

A study by Chapelot et al (57) in 6 lean men did find strong negative correlations (r = -0.95, -0.85) between leptin concentrations before lunch and dinner, and the energy intake during the first course of these meals. However, changing leptin concentrations in this study were tied to the diurnal rhythm of leptin (70;287). In the study of Chapelot (57), it was the leptin concentration in relation to the baseline in each subject that predicted food intake. This finding relates to the rhythmicity in leptin signalling to the brain that may play an important role in predicting appetite and energy intake (152).

Energy deficits of more than 24 h lead to decreases in plasma leptin concentrations (40;59;139;158;304;347;361), whereas an energy surplus of more than 24 h results in increased leptin concentrations (59;165). Plasma leptin is strongly negatively correlated with appetite and food intake when the energy balance is distorted (59;139;158;347). The association between leptin and appetite after energy restriction is independent from fat mass, which indicates that the low leptin concentrations are instrumental in restoring energy balance (158). Two intervention studies, in which 30 (354)and 12 (143) obese men following a weight-loss regimen were given pegylated human recombinant leptin also showed that leptin reduced appetite.

In the study of Chin-Chance et al (59), changes in baseline leptin values after 72 h of overcaloric, undercaloric, or eucaloric feeding were found to predict subsequent *ad libitum* intakes at breakfast (R^2 = 0.41). However, in this regression equation, each of the 6 participating subjects was represented 3 times. Because these measurements are dependent on each other, it is difficult to take this study as conclusive evidence for the role of leptin in the restoration of energy balance.

It is interesting that the results of a recent study by Weigle et al (346) suggest that a low-fat, high-carbohydrate *ad libitum* diet accompanied by weight loss leads to lower leptin without an increase in appetite. The authors attributed this effect to an increased leptin sensitivity during the low-fat, high-carbohydrate diet (346). In this study, the proportional amplitude of the 24 h leptin profile was increased after 12

weeks on the 15% fat diet. This increase in amplitude was strongly negatively correlated to the percentage change in body weight and body fat (346).
In summary, leptin is negatively correlated with appetite and food intake when subjects are not in energy balance, whereas the association between leptin and appetite during energy balance is less straightforward. Therefore, leptin seems to have a role in the regulation of food intake when energy stores change. This is also confirmed by Mars et al (205), who found a stronger negative correlation between leptin and appetite ratings after 2-4 days of 66% energy restriction than before the energy restriction protocol. Thus, leptin is suitable as a long-term biomarker of satiety when subjects are not in energy balance. However, leptin cannot serve as a simple short-term biomarker of satiety.

Glucose dependent insulinotropic polypeptide (GIP)
GIP is released not only in response to glucose ingestion, as its name suggests, but also in response to fat ingestion (94). It shares with GLP-1 its insulinotropic effect. Few studies have investigated GIP responses in relation to appetite. In a study of Verdich et al (336), GIP responses to a fixed preload (2.5 MJ) were inversely correlated with energy intake at an *ad libitum* test meal 3 h after the preload. This finding was consistent across a group of 12 lean subjects and a group of 19 obese subjects.
Although this study by Verdich et al suggested a role for GIP in human appetite regulation, a study by Vozzo et al (342) presented data that do not support this idea. Vozzo et al studied in 20 subjects the effects of 300 mL water, 75 g glucose/300 mL water, and 75 g fructose/300 mL water on GIP concentrations and on *ad libitum* test meal intake 3 h later. They found that glucose and fructose were equally effective in suppressing food intake in the test meal, but there were large differences in GIP concentrations after glucose and fructose ingestion. This finding does not support a major role for GIP in appetite.

Ghrelin
Ghrelin is abundantly synthesized in the fundus of the human stomach (12) and also in other tissues and other parts of the gastro-intestinal tract (120). Ghrelin is the endogenous ligand for the growth hormone secretagogue receptor (164), and therefore stimulates the release of growth hormone.
The results of recent studies on ghrelin suggest that it may serve as an excellent biomarker for satiety. People with the Prader-Willi Syndrome (PWS), which is characterized by severe hyperphagia, have 4.5 times higher ghrelin concentrations than do equally obese controls (64). In another study with 7 subjects with Prader-Willi

syndrome after an overnight and 5 control subjects after a 36-h fast, subjective hunger ratings were significantly correlated with ghrelin concentrations (R^2 = 0.50) (84). Intravenous infusions of ghrelin in 9 healthy humans were shown to potently enhance subjectively rated appetite and to increase energy intake during lunch by 28% (369). Diurnal rhythms in ghrelin concentrations before and after weight loss concur with diurnal rhythms in appetite in humans (67;68;79). On average, ghrelin concentrations were 24 % higher when obese subjects lost 17% of their initial weight (68). Ghrelin concentrations decline quickly after each meal, returning to premeal concentrations before the next meal is initiated (67). Plasma ghrelin concentrations in normal-weight subjects decrease after oral and intravenous administration of glucose, but the intake of an equivalent volume of water does not influence ghrelin concentrations (302), which suggests that ghrelin secretion is not affected by stomach expansion. In a recent study, Blom et al (35) found that different carbohydrate preloads, in proportion to their energy content, suppressed ghrelin concentrations in humans. Ghrelin concentrations were strongly inversely correlated ($r < -0.80$) with subjective appetite ratings. However, the infusion of lipids or the ingestion of a high-fat diet does not suppress the postprandial ghrelin concentrations as effectively as does the infusion or ingestion of glucose-containing carbohydrates (229;346). Fat restriction seems to avoid the increase in ghrelin concentrations caused by dietary energy restriction (346).
The data so far on ghrelin are very exciting, because there appears to be a close correspondence between ghrelin concentrations and appetite. Ghrelin is one of the first hormones that has a stimulating effect on appetite, and it seems to work both in the short term with meal initiation, and in the longer term after weight loss.

Peptide YY
PYY is released primarily from the distal gastrointestinal tract, i.e. the colon, and acts as an agonist (stimulator) on the Y2 receptor in the hypothalamus. This receptor inhibits the release of neuropeptide Y, the most potent CNS stimulant of appetite (25).
In two recent studies, intravenous infusion of exogenous $PYY_{(3-36)}$ (the biologically active form of PYY) was shown to suppress 24-h food intake in humans (24;25). Subjective ratings of hunger and satiety were in line with the lower food intake (24;25). In both obese and in lean subjects, food intake during a buffet lunch was decreased with approximately 30% (24). Endogenous fasting and postprandial concentrations of $PYY_{(total)}$ (the sum of biologically active and non-active forms) were significantly lower in obese subjects than in lean subjects, and fasting concentrations of $PYY_{(total)}$ were negatively associated with BMI (R^2 = 0.71) (24). MacIntosh et al

(200) studied the effects of intraduodenal infusion of lipids and glucose on the release of gastrointestinal hormones and subjectively rated appetite in young and elderly subjects. There were significant positive correlations during lipid infusion between changes in plasma $PYY_{(total)}$ concentrations and changes in fullness rating in both young (R^2 = 0.29) and elderly (R^2 = 0.29) subjects. Similar results were obtained during glucose infusion. Other studies have shown that PYY is also released in response to carbohydrate-, protein- and fat-rich meals, although not after an equal volume of water (2;258), or fat replacers (199).
The studies of Batterham et al (24;25) found that exogenous infused $PYY_{(3-36)}$ exerts a suppressive effect on food intake, which shows that PYY is one of the causal agents in the appetite cascade. However, data on the association between PYY and appetite are still very limited. Much more work seems necessary before PYY can be said to serve as a biomarker of satiety.

Enterostatin
Enterostatin is a gastrointestinal peptide that, according to data from animal studies, is hypothesized to be involved in the regulation of fat intake, the preference for food with a high fat content, or both (179). Three studies investigated the effect of enterostatin on appetite and food intake in humans, one of which used intravenous administration (290), and the other two which used oral administration (167;306). None of the 3 studies found an effect of enterostatin on *ad libitum* food intake.

Discussion

This overview of studies shows that a number of physiological measures are available that can serve as biomarker of satiation, satiety, or both. With respect to satiation (meal termination), physical and chemical measures of stomach distension and blood plasma concentrations of CCK and GLP-1 are useful. With respect to satiety and meal initiation, glucose dynamics within a short time frame (< 5 min), leptin concentrations during longer-term negative energy balance (more than 2-4 d), and ghrelin concentrations at both the short-term and long-term intervals are physiological markers. More work is needed to establish whether the other potential biomarkers of satiation, satiety, or both can be useful (Table 2.1).
Greater stomach fullness and higher concentrations of CCK and GLP-1 are associated with lower subjective hunger ratings and with lower food intake. These measures are also part of the causal chain that leads to meal termination, which implies that they can be valid biomarkers of satiation. Measures of stomach fullness, CCK, and GLP-1 are feasible because they represent immediate outcome measures

during the consumption of a meal. They are specific and sensitive because stomach fullness, CCK, and GLP-1 are different measures, but all 3 have a clear and straightforward association with subjectively rated appetite and food intake. Reproducibility follows from the observation that different research groups report similar findings.

Absolute glucose concentrations do not relate to reported appetite; however, small declines within short time frames have been shown to relate to meal requests and reported hunger. This makes blood glucose dynamics an interesting biomarker for meal initiation and satiety. The frequent sampling (10 times/minute) that is necessary and the required experimental control in these studies, such as time blinding and the long waiting times for subjects, makes this technique less feasible for most research groups.

Whereas short-term glucose signals relate to appetite, leptin relates to long-term appetite. Leptin acts as a long-term signal that is instrumental to the restoration of energy balance after energy restriction. This makes leptin less feasible as a biomarker of short-term satiety, because the time needed to achieve an effect is not obtainable within or between meals. However, for long-term studies on energy balance, leptin may serve a very useful purpose, e.g. to investigate the effect of different dietary regimens on long-term appetite responses. This might well be a very fruitful area for future research, because the essence in the problem of obesity is the long-term energy balance. The observations in various studies that most dietary carbohydrates are more potent stimulators of leptin than is fat (137;287) may offer an explanation for the observation that high-carbohydrate, low-fat diets in humans lead to a lower *ad libitum* food intake and body weight (fat) than do low-carbohydrate, high-fat diets (16) . The results of the studies of Weigle et al (346) and Chapelot et al (57) suggest a role for the relative changes (proportional amplitudes) in leptin during the day as an appetite signal to the brain (152).

Ghrelin is a hormone that acts both on the short term and the long term. From this perspective, ghrelin is one of the most exciting discoveries in appetite research in the last 5 y. The data so far suggest that ghrelin is an excellent biomarker for satiety. It acts as a peripheral hormone on receptors in the hypothalamus, thereby stimulating neuropeptide Y and agouti-related protein (154), which implies that ghrelin plays a causal role in the satiety cascade. Therefore, it can be a valid biomarker of satiety. Ghrelin's associations with hunger responses and food intake are also clear (64;68;369). It will be a challenge to investigate whether ghrelin is a functional hormone to restore energy balance after energy restriction, as well as to ascertain whether various ingredients or nutrients result in different ghrelin responses. The recent reports on PYY may indicate that PYY can serve as a biomarker of satiety

(25). PYY acts as a peripheral signal on CNS receptors in the neuropeptide Y pathway, which gives it a clear role in the satiety cascade. It has also been shown to relate to subjective appetite and actual food intake. However, the data are still scarce, and thus it is too early to declare PYY a biomarker of satiety.

DIT and body temperature are dependent on nutrient oxidation, and may therefore serve as an integrative measure of energy balance. From this perspective, they may be attractive candidates as biomarkers of satiety and satiation. However, DIT and satiety after meals are not synchronous over time (353). A recent study failed to show a clear association between DIT and appetite (268). DIT is an integrative measure of oxidation of nutrients over the entire body, from head to toe. Such a measure seems not specific enough to be related to appetite. The same is true for whole-body temperature. This issue might be different if temperature measurements could be focused on the liver, which is the primary peripheral organ for the distribution of macronutrients.

In this review, we have referred to a number of physiological parameters that were investigated in relation to appetite. This list of parameters is likely to expand in the near future. Chapelot et al (57) suggested that leptin acts on appetite through its effects on fatty acids concentrations. Fatty acid concentrations may also be an interesting candidate as a biomarker of satiety. Other biomarkers could lie in patterns in amino-acid profiles in the blood, electrophysiological recordings, and the discoveries of new hormones in relation to food intake.

CNS markers that have been investigated in relation to satiation reflect sensory-specific satiety, i.e., the decline in pleasantness of a food during its consumption. This decline in pleasantness occurs within 2 min after the first bite, and thus it is probable that fMRI, which has a much higher time resolution (> 10 scans/min) than do PET scans (1 scan/ 8-10 min), is a more suitable technique than is PET with which to assess this response. A similar notion applies for satiety responses: because hunger or satiety sensations can change very quickly, fMRI is more likely than is PET to play an important role in appetite research. An additional disadvantage of PET from an ethical point of view is its use of radioactive isotopes, which makes it a more invasive technique. Clearly, more quantitative data are needed to make these techniques suitable as biomarkers of satiation, satiety, or both.

In this review, we discussed about 80 studies in which appetite was assessed by rating subjective feelings of appetite or by assessing food intake in standardized settings. In almost all of these studies, these measures were in line with each other; that is, lower appetite ratings correlate with a lower food intake in a standardized

setting. This observation reinforces the validity of the rating scales and the food intake assessment as a measure of appetite.

The major division between satiation and satiety, as described in this review, came from the model of Blundell (36) and Halford and Blundell (132), which helped to organize this complex area of food intake research. We decided to place several hormones such as CCK, GLP-1, under the heading of satiation and another several measures, such as glucose, insulin, GIP and PYY, under the heading of satiety. The distinction between satiation and satiety and the involvement of hormones in either meal termination, meal initiation, or both may not be as strict as suggested. For example, we believe that glucose and insulin in humans are mainly involved in satiety or meal initiation. However, Langhans et al (172) showed that glucose and insulin might also be involved in meal termination. The distinction that we made was the result from theoretical considerations and from the experimental designs in which the biomarkers were studied.

The next exciting challenge in this field is to find ingredients or specific fractions in foods that have a beneficial effect on these biomarkers. For the past 10-15 y, there has been an intensive discussion on the role of macronutrients in the regulation of energy intake and body weight (16). Now, more and more work seems to focus on specific kind of fats, carbohydrates or proteins and their effect on physiological parameters that are causally related to energy and food intake. This development may help in the design of foods that are beneficial in the regulation of food intake. We end this review by mentioning a few studies that focus on this new field of research. Two of these studies showed that long chain fatty acids are more effective in releasing CCK than are short chain fatty acids (106;215). Hall et al (133) found that whey protein was more effective than was casein protein in the release of CCK and GLP-1 and the reduction of appetite. Data from Havel et al (137) suggest that high-fat diets lead to low circulating leptin concentrations, whereas carbohydrates were earlier shown to increase leptin concentrations (287). High-carbohydrate, low-fat diets may lead to a higher leptin sensitivity and therefore a lower *ad libitum* food intake and body weight (346). In this respect, Elliot et al (95) suggested that dietary fructose leads to lower insulin and leptin concentrations, which may contribute to an higher energy intake.

In summary, different amino acids, fatty acids and carbohydrates have differential effects on the release of biomarkers of satiation and satiety. This is a very fruitful and exciting area for future research.

3

Ghrelin response to carbohydrate-enriched breakfast is related to insulin

Wendy Blom
Annette Stafleu
Cees de Graaf
Frans Kok
Gertjan Schaafsma
Henk Hendriks

Wageningen University, Wageningen, Netherlands
TNO Quality of Life, Zeist, Netherlands

American Journal of Clinical Nutrition 2005; 81:367-375

Abstract

Background:
Ghrelin plays an important role in the regulation of food intake. Little is known about how ghrelin concentrations are modified by dietary factors.
Objective:
We examined the effects of both amount and type of carbohydrate on ghrelin concentrations and all correlations among the variables ghrelin, glucose, insulin, leptin, and all four subjective measures of appetite.
Design:
Twenty healthy nonobese men were studied in a double-blind, randomized, crossover design. Subjective measures of appetite and concentrations of ghrelin, glucose, insulin and leptin were frequently assessed for 4 h after liquid breakfast meals differing in energy content and carbohydrate structure, i.e., water, low-calorie (LC) meal, high-calorie simple carbohydrate (HC-SC) meal and high-calorie complex carbohydrate (HC-CC) meal.
Results:
Ghrelin concentrations decreased after the HC-SC breakfast by 41%, after the HC-CC breakfast by 33% and after the LC breakfast by 24%. No significant differences in ghrelin concentration among the three breakfasts were observed until 120 minutes. Ghrelin concentrations were correlated with subjective measures of hunger ($r = 0.51$) and fullness ($r = -0.44$). The percentage decrease in ghrelin between 0 and 30 minutes was inversely correlated with the percentage increases in insulin ($r = -0.76$) and glucose ($r = -0.79$) but not with changes in leptin ($r = 0.10$). The percentage changes in ghrelin concentrations between 30 and 180 minutes were correlated with percentage changes in insulin ($r = -0.53$) and leptin ($r = -0.47$) but not with changes in glucose ($r = 0.22$).
Conclusions:
The results support the hypothesis that ghrelin requires postgastric feedback, which may be regulated through insulin.

Introduction

The gastric peptide ghrelin (12) appears to play a pivotal role in the regulation of food intake. Ghrelin concentrations in plasma rise gradually before a meal and decrease immediately after a meal (67;68;302;324). In addition, intravenous infusion of ghrelin increases food intake and enhances appetite (69;369), and these effects suggest that ghrelin plays a role in feelings of hunger and in meal initiation. Ghrelin is suggested to be involved not only in meal initiation but also in body weight control, because body mass index (BMI; in kg/m^2) is negatively correlated with fasting plasma ghrelin concentrations (68;69;96;255;302;325).

Leptin and insulin are two other hormones involved in the regulation of energy balance and food intake (139;142;158;301;304;310;336). Ghrelin, leptin, and insulin are secreted in peripheral tissues, and they act on the central nervous system. Ghrelin stimulates the expression of neuropeptide Y (NPY) and agouti-related protein (AgRP) in the hypothalamus, and that expression stimulates food intake (153;154;243;303). Leptin and insulin both suppress food intake partly through the suppression of NPY and AgRP (92;266), and partly through the activation of the hypothalamic melanocortin system (300).

Although the central mechanisms of action have been and are still being characterized, little is known about the effects of dietary factors (e.g., structure and energy content) on plasma ghrelin concentrations, ghrelin's interactions with leptin and insulin, and the correlation between ghrelin and appetite (i.e., feelings of hunger and satiety). Plasma ghrelin concentrations are known to decrease after oral and intravenous administration of glucose (229;241;242;302), whereas lipids or high-fat diets suppress the postprandial ghrelin concentrations less effectively (229;231;346). This suggests that the postprandial ghrelin response may be modulated by glucose and insulin. Carbohydrate structure is one of the important factors determining the glucose and insulin concentrations after carbohydrate consumption. Complex carbohydrates and fibres are known to decrease feelings of hunger and to increase fullness (141;142;309). We hypothesized that the amount and type of carbohydrate may influence the ghrelin response. Therefore the associations among ghrelin, glucose, insulin and leptin concentrations and subjective measures of appetite were studied by analyzing the postprandial responses to water and to three liquid breakfasts that differed in the amount and type of carbohydrate.

Subjects and methods

Subjects

The study was conducted at TNO Nutrition and Food Research, Zeist, the Netherlands, where subjects were recruited from a pool of volunteers. All subjects completed a questionnaire on life-style, medical history and dietary habits. The medical investigator physically examined each of the subjects. Blood and urine was collected after an overnight fast for routine analysis. Each subject reported a Western lifestyle, regular Dutch dietary habits, and a stable body weight for at least 1 month before the study. Smokers, restrained eaters (a score for restrained eating > 2.5 on the Dutch Eating Behaviour Questionnaire (312)), and subjects who reported that they were following either a weight-reduction diet or a medically prescribed diet were excluded from participation. Subjects who were taking medication that may have influenced appetite and sensory functioning or who reported metabolic or endocrine disease, gastrointestinal disorders, or a history of medical or surgical events that may have affected study outcome were also excluded.
A total of 20 healthy nonobese men with a mean body mass index (BMI) of 22.6 ± 1.5 kg/m^2 (range: 19.9 - 25.4) and a mean age of 36.1 ± 13.4 y (range: 19 – 57 y) completed the study (Table 3.1).
The study was performed according to the ICH Guidelines for Good Clinical Practice (ICH topic E6, adopted 01-05-1996 and implemented 17-01-1997) and the protocol was approved by the independent Medical Ethics Committee of TNO. Each subject gave written informed consent after being informed about the study, both verbally and in writing.

Study design

The experiment had a randomized crossover design. Each subject received four treatments on separate days, with a washout period of 1 week preceding each subsequent treatment. Subjects were randomly assigned to 1 of the 4 treatment orders. With the assignment of treatment order, we ensured that the average body weight and age of the subjects in all 4 groups were more or less the same. Treatment orders were balanced according to a Latin square design. Subjects and personnel were blinded to the treatment order except the water condition. The study had a staggered start: 5 subjects started per day.

Table 3.1 Subject characteristics at the beginning of the study[1]

	Values
Age (y)	36.1 ± 13.4 (19 - 57)
Weight (kg)	76.4 ± 6.3 (65.1 - 90.7)
Height (cm)	183.7 ± 6.5 (167.4 - 191.2)
BMI (kg/m^2)	22.6 ± 1.5 (19.9 - 25.4)
Waist:hip ratio	0.86 ± 0.05 (0.77 - 0.97)
DEBQ[2]	1.6 ± 0.5 (1.0 - 2.5)

[1] All values are mean ± SD; range in parentheses. *n* = 20. DEBQ, Dutch Eating Behaviour Questionnaire.

[2] Score on the restrained-eating scale of the DEBQ. Range of possible scores on the restrained-eating scale, 1.0 - 5.0.

Liquid breakfasts

Liquid breakfasts were prepared for each person according to the estimated daily energy requirement (MJ/24 h) of that person, which was estimated by calculating the vassal metabolic rate according to Schofield's equations (298) and multiplying that value by a correction factor for physical activity level. All subjects were considered moderately active, and therefore the same correction factor of 1.79 was applied for all subjects (349).

The four liquid breakfasts (volume: 578 ± 5 mL) consisted of 1) noncarbonated mineral water (Spa Reine; Spadel Nederland B.V., Maarssen, Netherlands); 2) a low-calorie (LC; 128 KJ/100 m/L) yoghurt drink flavoured with red fruits (e.g., strawberry, cherry, raspberry, cherry and blackberry) and containing intensive (i.e., non-energy-containing) sweeteners only (Fristi; Friesland Dairy & Drinks Group, Ede, Netherlands), which is the LC breakfast; 3) an LC yogurt drink with Maltodextrin (Avebe, Veendam, Netherlands), a carbohydrate with a simple structure, which is the high-calorie simple carbohydrate (HC-SC) breakfast; and 4) an LC yogurt drink with both the exopolysaccharide Reuteran (TNO Nutrition and Food Research, Zeist, Netherlands; (113;271;272), a carbohydrate with a complex structure, and Maltodextrin, which is the HC complex carbohydrate (HC-CC) breakfast. The ratio of Reuteran to Maltodextrin was fixed at 4:21. Relatively low amounts of Reuteran were added to the HC-CC breakfast to ensure that the viscosities for the three yoghurt-

based treatments (LC, HC-SC and HC-CC) were similar. Both the HC-SC and HC-CC breakfasts provided a total of 20% of the estimated daily energy requirement. The LC breakfast provided about 6% of the estimated daily energy requirement.
Reuteran and Maltodextrin were added to 500 mL of the yogurt drink. Adding carbohydrates to beverages (or liquid breakfasts) resulted in a volume increase (71-88 mL, depending on the energy requirement of the subject). We corrected for this volume increase by adjusting the volumes of water and of the LC yogurt drink. The average volume and energy content of the four liquid breakfasts are shown in Table 3.2.
We also made sure that the taste of the three yogurt drinks was very similar by choosing a sweet yogurt drink with a distinct, red fruit flavour and by adding two carbohydrates that are almost tasteless.

Study protocol

After an overnight fast (nothing to eat or drink except for water after 22.00 h), subjects arrived at the research centre, filled out a questionnaire on their current well-being, and were weighed. A cannula was placed in the antecubital vein, and a blood sample was taken. After about 30 minutes, subjects drank one of the liquid breakfasts within 5 minutes. Thereafter, subjects were not allowed to eat or drink anything during four hours. Blood was collected at 15, 30, 45, 60, 75, 90, 105, 120, 150, 180, 210 and 240 minutes. Immediately after each blood sample was taken, subjects filled out Visual Analogue Scales (VAS) to measure subjective feelings of hunger, fullness, desire to eat and prospective food consumption.

Blood samples

For plasma, blood was collected in evacuated tubes containing K_3EDTA as coagulant and put in ice water immediately. For serum, blood was collected in evacuated tubes containing clot activator. All tubes were centrifuged for 15 minutes at 2000 X *g* at 4 ºC. Plasma and serum were removed and stored at -70 ºC and -18 ºC, respectively until they were analyzed.
Serum glucose concentrations were measured by using a commercial test kit (Boehringer, Mannheim, Germany) on a Hitachi 911 automatic analyzer (Hitachi Instrument Division, Ibaraki-ken, Japan), with intraassay CVs that ranged between 0.7% and 0.9%, depending on the concentration. Serum insulin concentrations were measured by using AIA-600 Immunoassay Analyzer (Tosoh Corporation, Toyama, Japan), with intraassay CVs that ranged between 4.3% and 5.8%, depending on the concentration. Plasma concentrations of leptin and ghrelin were analyzed by using a radioimmunoassay. Leptin was measured in duplicate with the use of a commercial

Sensitive Human Leptin kit (Linco Research Inc, St Charles, MO), with intraassay CVs that ranged between 4.9% and 6.4%, depending on the concentration. Ghrelin was measured in duplicate at 0, 30, 45, 60, 90, 120, 180 and 240 minutes in 18 subjects with the use of a commercial human radioimmunoassay kit (Phoenix Pharmaceuticals, Belmont, CA). The mean intraassay CV was 1.8% at a concentration of 0.40 µg/L and 4.5% at a concentration of 0.10 - 0.20 µg/L.

Subjective appetite

Subjective appetite was evaluated by using VASs for hunger, fullness, desire to eat and prospective food consumption (103). VASs consisted of 150-mm horizontal lines with phrases in Dutch anchored at each end that expressed the most positive or most negative sensation (i.e., I have never been more hungry/ I am not hungry at all). Subjects drew a vertical line at the point on the horizontal line that corresponded to their hunger sensation. VASs were automatically processed with the use of TELE*form* ELITE software (Version 6.1; Cardiff Software Inc., Sunnyvale, CA). Distances on the VASs were converted into scores between 0 and 100.

Table 3.2 Energy and macronutrient composition of the liquid breakfasts [1]

	Water	LC	HC-SC	HC-CC
Volume (mL)	578 ± 5[2]	578 ± 5	578 ± 5	578 ± 5
Weight (g)	578 ± 5	601 ± 5	641 ± 8	641 ± 8
Energy (kJ)	0	736 ± 7	2674 ± 137	2674 ± 137
Protein (g)	0	15[3]	13	13
Fat (g)	0	0.3	0.3	0.3
Carbohydrate (g)	0	29	146 ± 8	146 ± 8
Maltodextrin (g)	0	0	121 ± 8	102 ± 7
Reuteran (g)	0	0	0	19 ± 1
Fibre (g)	0	14	12	12

[1] LC, low-calorie breakfast; HC-SC, high-calorie, simple carbohydrate breakfast; HC-CC, high-calorie, complex carbohydrate breakfast.

[2] mean ± SD (all such values)

[3] mean (all such values)

Statistical analyses

With analysis of variance (ANOVA) for repeated measures, the response curves of ghrelin, leptin, glucose, insulin and the VAS scores after the 4 liquid breakfasts were compared, and we tested for time X treatment interactions and the effect of time separately. Incremental areas under or over the baseline were calculated. In this report, we use the term *area under the curve* (AUC) to refer to both values, delineated as negative AUC and positive AUC (the latter for the area over the curve). Evaluation of the residual plots showed that the negative or positive AUC of all variables except ghrelin and glucose could not be used for the analysis, and therefore we used the total AUC, which we defined as the sum of the areas under and over the baseline. With the use of a mixed-model ANOVA, the AUCs of the different variables were tested for an overall treatment effect. If there was a treatment effect, partial tests were performed to compare treatments pair wise, and Tukey's adjustments were used for multiple comparisons. Correlation coefficients were calculated to evaluate the association among subjective measures of appetite and blood variables. Pearson's correlation coefficient was calculated for each subject, based on 52 (13 time points, 4 treatments) or 32 (8 time points, 4 treatments for ghrelin) pairs of data. On these individual correlations a Fisher's *z* transformation was applied, to correct for deviations from the normal distribution. The mean of these 20 (18 in case of ghrelin) coefficients was calculated, the inverse of the Fisher transformation was performed, and the 95% C.I. for each correlation coefficient was calculated. Associations among changes in blood concentrations over different time intervals were investigated. The percentage change in concentration between 2 time points [(30-0)/0 x 100, (180-0)/0 x 100 and (180-30)/30 x 100] was calculated for each subject. Pearson's correlation coefficients were calculated per subject on the basis of 4 pairs of data. After correction with Fisher's *z* transformation, the average and 95% CIs were calculated. To test whether the correlation coefficients are significantly different from each other, a paired *t* test of the *z* scores was performed (Bonferroni corrected). In addition, the percentage change from baseline to the highest (i.e. glucose, insulin and fullness) or lowest (i.e. ghrelin, leptin and hunger) value was calculated.

Statistical analyses of the data were carried out with SAS/STAT statistical software (version 8.2; SAS Institute, Cary, NC). A P value <0.05 (two-sided) was considered significant in all analyses. Results are given as means ± SDs.

Results

Ghrelin

Postprandial ghrelin responses are presented in Figure 3.1. Ghrelin concentrations decreased rapidly after the LC (-24%), HC-SC (-41%) and the HC-CC (-33%) breakfasts, but less so after water (-2%). Until 90 min, ghrelin concentrations were the same after the LC and HC treatments. Ghrelin concentrations returned to their starting value between 90 and 120 min after the LC breakfast, but not until 240 min after the HC breakfasts. The repeated-measures ANOVA showed a time X treatment interaction for ghrelin ($p < 0.0001$). The negative AUC of the ghrelin response differed significantly ($p < 0.01$) between all of the treatments except between the water and LC treatment ($p = 0.09$).

Glucose

Postprandial glucose responses are shown in Figure 3.1. Blood glucose concentrations increased after the LC (12%), HC-SC (47%) and HC-CC (40%) breakfasts, reaching peak values at 30 min. By 45 min after the LC breakfast, blood glucose concentrations had returned to baseline values, and they dropped below baseline values and remained there until 120 min after the breakfast. The glucose responses showed a significant time X treatment interaction ($p < 0.0001$). The positive AUC of glucose was significantly different between all of the treatments ($p < 0.0001$), except between the water and LC treatment and between the HC-SC and HC-CC treatment.

Insulin

Serum insulin responses are presented in Figure 3.1. Insulin concentrations increased ≈ 6-fold after the LC breakfast and ≈ 12- and 10-fold after the HC-SC and HC-CC breakfasts, respectively, reaching peak values at 30 (LC) and 45 (HC) min. Serum insulin concentrations had returned to baseline values by 105 min after consumption of the LC breakfast and by 210-240 min after the consumption of the HC treatments. The insulin responses showed a significant time X treatment interaction ($p < 0.0001$). The total AUCs of insulin differed significantly between all 4 treatments ($p < 0.05$), except between the HC-SC and HC-CC treatments ($p = 0.08$) (Figure 3.1).

Leptin

The plasma leptin responses during the 4 h after the 4 liquid breakfasts are shown in Figure 3.1. Plasma leptin concentrations decreased after breakfast intake by ≈ 20%, independent of the liquid breakfast consumed. The lowest leptin concentrations were observed at 180 (water and LC), 120 (HC-SC) and 90 (HC-CC) min. The repeated-measures ANOVA showed a significant time X treatment interaction ($p < 0.05$). The AUCs of leptin did not show a significant overall treatment effect of leptin ($p = 0.125$).

Subjective Appetite

VAS scores are shown in Figure 3.2. Analysis of total AUC showed a significant overall treatment effect on hunger ($p < 0.05$), fullness ($p < 0.01$), desire to eat ($p < 0.001$) and prospective food consumption ($p < 0.01$). There was also a significant time X treatment interaction ($p < 0.01$) for all 4 postprandial appetite responses. Subjective measures of hunger decreased by ≈ 30% after the LC, HC-SC and HC-CC breakfasts, reaching the lowest values at 15 minutes. No decrease in hunger scores was observed after consumption of water. The total AUC of the hunger response was significantly smaller after water consumption than after the HC-SC ($p < 0.01$) or HC-CC ($p < 0.05$) breakfast. Subjective measures of fullness increased ≈ 14% after water, by ≈ 67% after LC, 68% after HC-SC and 91% after HC-CC breakfast, reaching peak values at 15 (LC and HC-CC) and 30 (HC-SC) min (see Figure 3.2). Fullness scores with the LC and the HC -CC treatments differed significantly ($p < 0.05$) between 90 and 180 min. The total AUC of the fullness response was significantly smaller after water consumption than after the HC-SC and HC-CC breakfasts (both: $p < 0.01$). The scores for the subjective measures desire to eat and prospective food consumption were essentially similar to those for hunger, although the decreases were generally smaller (Figure 3.2). The total AUC of desire to eat was significantly smaller after water consumption than after the HC-SC ($p < 0.001$) and HC-CC ($p < 0.05$) breakfasts and borderline significantly ($p = 0.06$) lower after the LC breakfast than after the HC-SC breakfast. The total AUC of prospective food consumption was significantly smaller after water consumption than after the HC-SC ($p < 0.001$) and HC-CC ($p < 0.01$) breakfasts and significantly smaller after the LC breakfast than after both HC treatments (both: $p < 0.05$).

Correlations

Ghrelin and appetite

Ghrelin concentrations were positively correlated with hunger ($r = 0.51$; 95% C.I. = 0.09, 0.78), desire to eat ($r = 0.51$; 95% C.I. = 0.09, 0.78) and prospective food

consumption (r = 0.52; 95% C.I. = 0.09, 0.78) and negatively correlated with fullness (r = -0.44; 95% C.I. = 0.00, -0.74), as shown in Table 3.3.

Ghrelin and other parameters

Fasting ghrelin concentrations were not significantly correlated with age or BMI. The percentage decrease in ghrelin concentrations over the first 30 min correlated with the percentage increases in insulin (r = -0.76; 95% C.I. = -0.48, -0.90) and glucose (r = -0.79; 95% C.I. = -0.53, -0.91) but not with the percentage decreases in leptin (r = 0.10; 95% C.I. = -0.36, 0.52), as shown in Table 3.4. Moreover, the percentage increase in ghrelin concentrations between 30 and 180 min was correlated with the percentage decreases in insulin concentrations (r = -0.53; 95% C.I. = -0.11, -0.79) and leptin (r = -0.47; 95% C.I. = -0.03, -0.75) but not with the percentage decreases in glucose (r = 0.22; 95% C.I. = -0.24, 0.61*)* concentrations (see Table 4). The percentage decrease in ghrelin concentrations between 0 and 180 minutes was correlated with the percentage increases in insulin concentrations (r = -0.89; 95% C.I. = -0.73, -0.95). No such correlations were found between ghrelin and glucose (r =0.06; 95% C.I. = -0.39, 0.49) or between ghrelin and leptin (r = -0.38; 95% C.I. = -0.70, 0.08), as shown in Table 3.5. The correlation coefficient of the percentage changes in ghrelin and insulin concentrations between 0 and 30 minutes did not differ significantly from the correlation coefficient of the percentage changes in ghrelin and glucose concentrations between 0 and 30 min (p = 0.69). However, the correlation coefficient of percentage changes in ghrelin and insulin between 30 and 180 min (p < 0.0001) and between 0 and 180 minutes (p < 0.0001) was different from the correlation coefficients of the percentage changes in ghrelin and glucose within these time periods.

Table 3.3 The relation between ghrelin (*n* = 18 subjects) and measures of appetite (*n* =20 subjects)[1]

	Fullness	Desire	PFC	Ghrelin
Hunger	-0.66 (-0.31, -0.85)	0.90 (0.75, 0.96)	0.86 (0.68, 0.94)	0.51(0.09, 0.78)
Fullness		-0.61 (-0.23, -0.83)	-0.59 (-0.21, -0.82)	-0.44 (0.00, -0.74)
Desire			0.92 (0.81, 0.97)	0.51 (0.09, 0.78)
PFC				0.52 (0.09, 0.78)

[1] All values are mean [correlation coefficient (r)]; 95% CIs after Fisher's *z* transformation in parenthesis. Pearson's correlation coefficients of the relation between subjective measures of appetite and ghrelin were calculated per subject. PFC: prospective food consumption, Desire: Desire to eat.

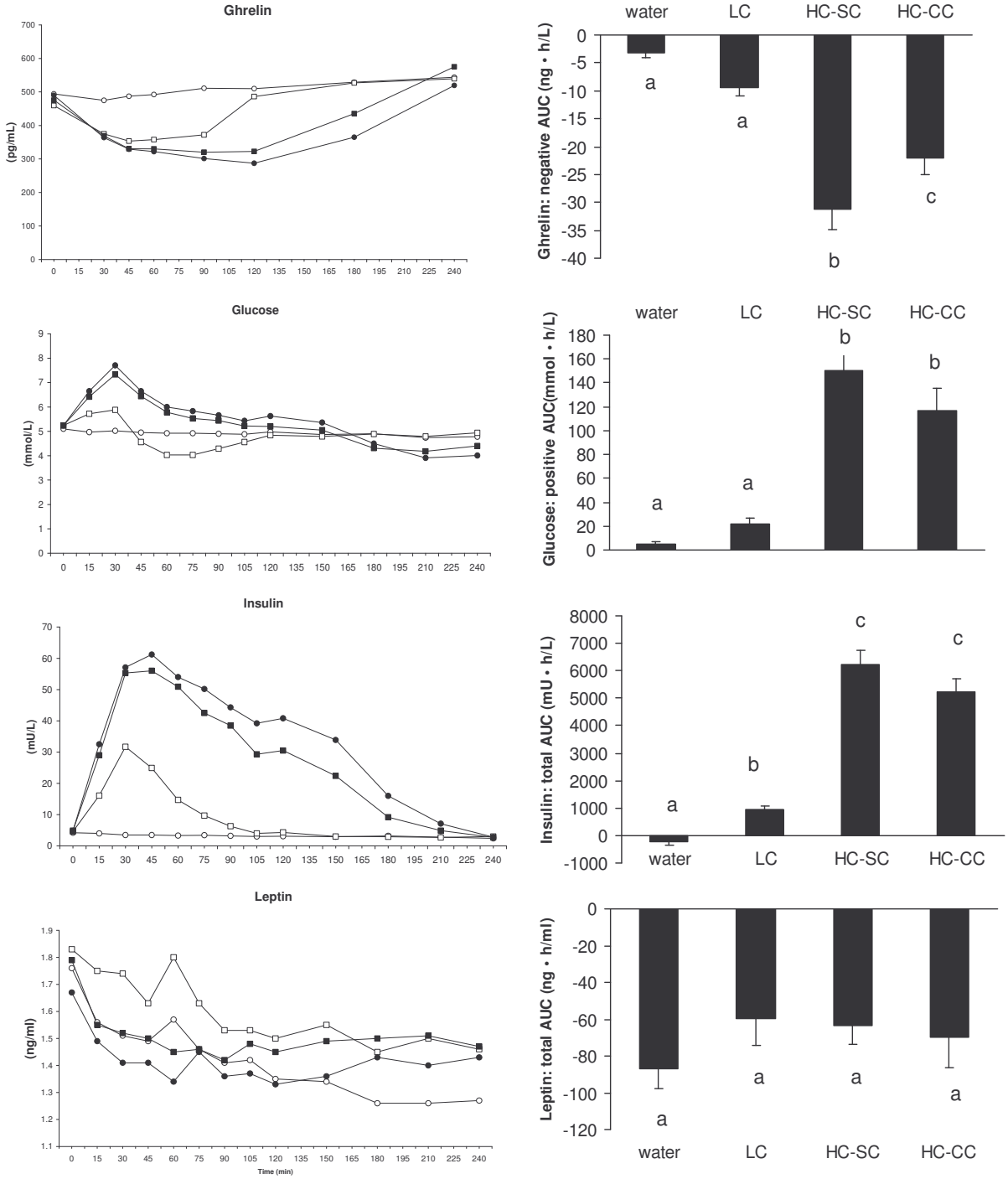


Figure 3.1 Mean (± SEM) responses of ghrelin (n = 18), glucose (n = 20), insulin (n = 20), and leptin (n = 20) 4 h after the intake of 4 liquid breakfasts. Total AUC, total area under and over the curve (or baseline); negative AUC, area under the curve; positive AUC, area over the curve. Left: ○, water; □, low-calorie (LC) meal; ●, high-calorie simple carbohydrate (HC-SC) meal; ■, HC complex carbohydrate (HC-CC) meal. There was a significant time X treatment interaction for ghrelin, glucose, insulin (all $p < 0.0001$), and leptin ($p < 0.05$) and a significant time effect (all: $p < 0.0001$). Right: bars in the same panel with different letters are significantly different, $p < 0.05$ (Tukey's adjustment).

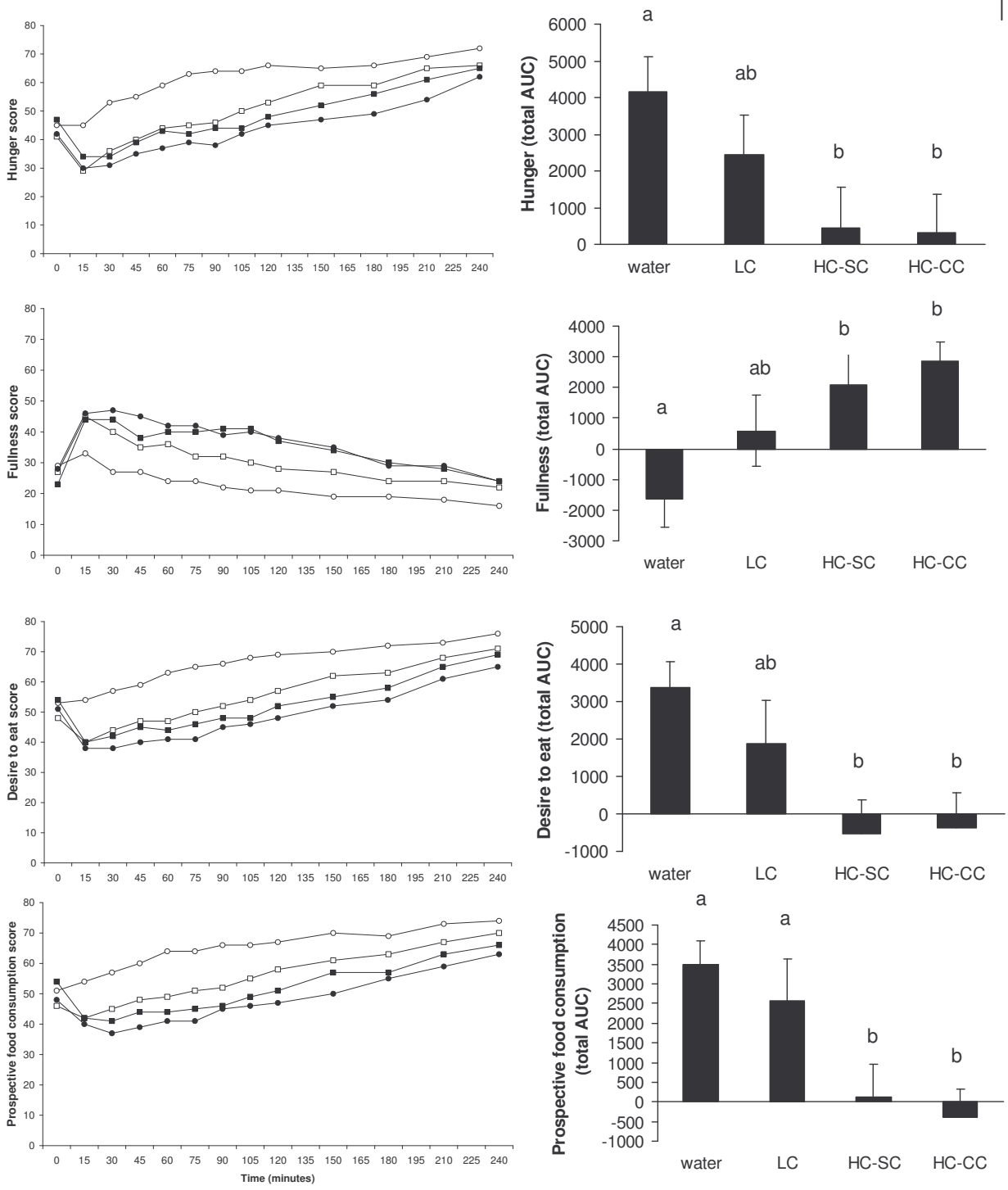


Figure 3.2 Mean (± SEM) responses of hunger, fullness, desire to eat and prospective food consumption in 20 men 4 h after the intake of 4 liquid breakfasts. Total AUC, total area under and over the curve (or baseline). Left: ○, water; □, low-calorie (LC) meal; ●, high-calorie simple carbohydrate (HC-SC) meal; ■, HC complex carbohydrate (HC-CC) meal. There was a significant time X treatment interaction for hunger, prospective food consumption (both: $p < 0.0001$), fullness, and desire to eat (both: $p < 0.01$) and a significant time effect (all: $p < 0.0001$). Right: bars in the same panel with different letters are significantly different, $p < 0.05$) (Tukey's adjustment).

Table 3.4 Correlations of percentage changes in concentrations between 0-30 and 30-180 min[1]

	0 -30	30 -180
Ghrelin - insulin	-0.76 (-0.48, -0.90)	-0.53 [a] (-0.11, -0.79)
Ghrelin - glucose	-0.79 (-0.53, -0.91)	0.22 [b] (-0.24, 0.61)
Ghrelin - leptin	0.10 (-0.36, 0.52)	-0.47 (-0.03, -0.75)
Insulin - glucose	0.82 (0.59, 0.93)	0.70 (0.37, 0.87)
Insulin - leptin	-0.12 (-0.53, 0.34)	-0.61 (-0.23, -0.83)
Glucose - leptin	-0.04 (-0.48, 0.41)	-0.68 (-0.34, -0.86)

[1] All values are mean [correlation coefficient (r)]; 95% CIs after Fisher's z transformation in parentheses. Pearson's correlation coefficients of the percentage changes in plasma and serum concentrations of ghrelin, insulin, glucose and leptin between 0 and 30 min [(30-0)/0 x 100] and between 30 and 180 min [(180-30)/30 x 100] were calculated per subject. n = 20 (except ghrelin, n = 18). Correlation coefficients were compared over the 2 time periods. The correlation coefficients of the correlation between percentage changes in ghrelin and insulin and of that between percentage changes in ghrelin and glucose were compared (paired t test of the z values, Bonferroni corrected) within a time period. Correlation coefficients with different superscript letters are significantly different from each other, $p < 0.05$.

Table 3.5 Correlations of percentage changes in concentrations between 0-180 min[1]

	Ghrelin	Insulin	Glucose
Insulin	-0.89[a] (-0.73, -0.95)	-	-
Glucose	0.06[b] (-0.39, 0.49)	- 0.24 (-0.62, 0.22)	-
Leptin	-0.38 (-0.70, 0.08)	0.49 (0.06, 0.77)	-0.36 (-0.69, 0.09)

[1] All values are mean [correlation coefficient (r)]; 95% CIs after Fisher's *z* transformation in parentheses. Pearson's correlation coefficients of the percentage changes in plasma and serum concentrations of ghrelin, insulin, glucose and leptin between 0 and 180 min [(180-0)/0 x 100] were calculated per subject. *n* = 20 (except ghrelin, *n* = 18). The correlation coefficients of the correlation between percentage changes in ghrelin and insulin and of that between percentage changes in ghrelin and glucose were compared (paired *t* test of the *z* values, Bonferroni corrected). Correlation coefficients with different superscript letters are significantly different from each other, $p < 0.05$.

Appetite and other blood parameters

Glucose concentrations were negatively correlated with hunger scores (r = -0.38; 95% C.I. = -0.70, 0.08) and positively correlated with fullness scores (r = 0.31; 95% C.I. = -0.16, 0.66) scores. Insulin concentrations also were negatively correlated with hunger scores (r = -0.51; 95% C.I. = -0.09, -0.78) and positively correlated with fullness scores (r = 0.46; 95% C.I. = 0.03, 0.75). Leptin concentrations and

sensations of hunger and fullness were less correlated with hunger (r = -0.15; 95% C.I. = -0.56, 0.31) and fullness (r = 0.13; 95% C.I. = -0.33, 0.54).

Discussion

Postprandial ghrelin responses have been investigated, but the response of ghrelin after different amounts of carbohydrates was not studied until now. In this study, we show that ghrelin responds according to the amount of carbohydrate given, although ghrelin responses differed no sooner than at 120 min, which suggests that ghrelin requires postgastric feedback. The volume of intake itself did not appear to influence ghrelin secretion, because the consumption of water hardly affected ghrelin concentrations. The postprandial ghrelin concentrations are correlated with subjective measures of appetite and with insulin concentrations but less so with glucose concentrations, which suggests that ghrelin is directly or indirectly regulated by insulin. We found no evidence for such an involvement of leptin.

In the current study, subjective measures of appetite were correlated with ghrelin concentrations. This correlation was stronger than that between appetite and glucose and comparable with that between appetite and insulin. Our observation that ghrelin concentrations after the LC and HC treatments did not differ until 120 min after consumption is in accordance with the findings of Williams et al (358). They showed that, when gastric emptying was prevented in rats, neither glucose nor water administration affected ghrelin concentrations. However, when gastric emptying was not prevented, ghrelin was suppressed by glucose only. This suggests that gastric distension and gastric chemo sensitization are insufficient to induce a ghrelin response. It is possible that these postgastric processes involve insulin secretion either directly or indirectly by stimulating the incretin hormones glucagons-like peptide 1 and gastric inhibitory peptide. Our observation that the postprandial change in ghrelin concentrations is highly and inversely correlated with the postprandial change in insulin concentrations supports this. Although postprandial changes in ghrelin concentrations during the first 30 min were correlated with both glucose and insulin, changes in ghrelin concentrations between 30 and 180 min were highly correlated with changes in insulin but not with changes in glucose.

Using clamp studies, several research groups have investigated the relation among ghrelin, insulin and glucose. Most researchers found that insulin decreases ghrelin concentrations, independent of glucose (99;197;236;293). The mechanism by which insulin has this inhibitory effect on ghrelin concentrations has not yet been ascertained. The effect of insulin may be mediated by direct effects on ghrelin-secreting cells or by indirect effects on other humoral or central mechanisms.

The data retrieved from clamp studies together with our observations suggest that the postprandial ghrelin response is dependent on insulin. Because fasting ghrelin concentrations are negatively associated with BMI and insulin resistance (68;145;218;265), postprandial ghrelin responses may also be associated with insulin sensitivity. Such a correlation was confirmed in a study of Lucidi et al (197), who found a strong positive correlation between insulin sensitivity and the percentage decrease in ghrelin after insulin infusion. This observation may be relevant in view of the obesity epidemic. People consuming meals with a high glycaemic load may have higher insulin concentrations during the postprandial phase and consequently may be temporarily less insulin sensitive. This insulin insensitivity may blunt the postprandial ghrelin response and decrease satiety.

Our results with respect to leptin are in line with the observation that leptin concentrations do not change acutely (i.e. within 3-4 h) in response to meals (150;156;287). Although leptin does not seem to play an important role in the short-term regulation of food intake when subjects are in energy balance, plasma leptin is negatively correlated with appetite and food intake when the energy balance is disturbed (158). Leptin therefore seems to have a role in the regulation of food intake when energy stores change.

In summary, ghrelin responds rapidly and dose-dependently to carbohydrate intake and is correlated with subjective measures of appetite, which suggests that ghrelin plays an important role in the regulation of food intake. The mechanism is not clear yet, although our results support the previous finding that ghrelin requires postgastric feedback, and ghrelin concentrations seem to be associated with insulin more than with glucose or leptin. However, these results are based only on a carbohydrate-rich liquid breakfast, in studies of healthy nonobese men. There is some evidence that liquid meals are less satiating than are solid meals, independent on the energy density of the meals (144). The effects of BMI, sex, insulin sensitivity and different macronutrients on the postprandial ghrelin response should also be investigated. To clarify whether ghrelin regulates meal initiation (satiety) or meal termination (satiation), the interval between meals and *ad libitum* food intake should be investigated. The current results support the hypothesis that ghrelin requires postgastric feedback, which may be regulated through insulin.

Acknowledgements

We express our gratitude to all those involved in the conduct of the study. We also thank the volunteers who participated in the study.
WAMB was involved in the design of the protocol, collection and analysis of the data, and writing of the manuscript. AS and CG were involved in the design of the protocol and provided significant advice during the writing of the manuscript. FJK and GS provided significant advice during the writing of the manuscript. HFJH was involved in the design of the protocol (Principal Investigator according to Good Clinical Practice guidelines) and writing of the manuscript and provided significant advice during the intervention study and data analysis. None of the authors had personal or financial conflicts of interest.

This study is financially supported by: Dutch Ministry of Education, Culture and Science and the Dutch Ministry of Health, Welfare and Sport.

4

The effect of a high protein breakfast on the postprandial ghrelin response

Wendy Blom
Anne Lluch
Annette Stafleu
Sophie Vinoy
Jens Juul Holst
Gertjan Schaafsma
Henk Hendriks

Wageningen University, Wageningen, Netherlands
TNO Quality of Life, Zeist, Netherlands
Danone Vitapole Nutrivaleur, Palaiseau Cedex, France
The Panum Institute, Copenhagen, Denmark

Submitted

Abstract

Background:
Dietary protein appears to be the most satiating macronutrient. Only few studies have investigated the effects of dietary protein on ghrelin secretion in humans.
Objective:
This study was designed to investigate whether a high protein breakfast is more satiating through suppression of postprandial ghrelin concentrations, or through other physiological processes.
Design:
Fifteen healthy men were studied in a single blind, crossover design. Subjective measures of satiety and blood samples were frequently assessed for 3 h after two iso-caloric breakfasts differing in protein and carbohydrate content (58.1 EN% protein, 14.1 EN% carbohydrate versus 19.3 EN% protein, 47.3 EN% carbohydrate). Gastric emptying rate was indirectly assessed with the acetaminophen absorption test.
Results:
The high protein breakfast (HP) decreased postprandial ghrelin secretion more as compared to the high carbohydrate breakfast (HC) ($p < 0.01$). Ghrelin concentrations were correlated with GIP ($r = -0.70$, 95% C.I. = -0.87, -0.38) and glucagon concentrations ($r = -0.77$, 95% C.I. = -0.90, -0.49) after the HP breakfast. The HP breakfast increased secretion of glucagon ($p < 0.0001$), GIP ($p = 0.07$), CCK ($p < 0.01$) and GLP-1 ($p = 0.10$) as compared to the HC breakfast, and decreased the gastric emptying rate ($p < 0.0001$). The HP breakfast did not affect satiety scores or *ad libitum* energy intake during lunch.
Conclusions:
The high protein breakfast decreased postprandial ghrelin concentrations. High associations between ghrelin and GIP and glucagon suggest that stimulation of these peptides may mediate the postprandial ghrelin response. The high protein breakfast also reduced gastric emptying, probably through increased secretion of CCK and GLP-1.

Introduction

Dietary protein appears to be the most satiating macronutrient. In most cases, high-protein meals increase feelings of satiety and decrease subsequent energy intake as compared to high carbohydrate or high fat meals (8;134). There are at least a few possible mechanisms by which protein induces satiety; these include thermic effects and physiological processes related to metabolic factors, gut hormones and gastrointestinal function. Proteins have a greater thermic effect compared to carbohydrates and fats (63;134;228;353). This effect may be larger because proteins need to be metabolized immediately, as proteins cannot be stored in the body. Increased amino acid concentrations may also contribute to satiety by stimulation of gluconeogenesis, thereby preventing a decrease in glycaemia (320). Another physiological process through which proteins appear to induce satiety is stimulation of secretion of the gut peptides cholecystokinin (CCK) and glucagon-like peptide 1 (GLP-1) (48;133;247). CCK and GLP-1 are known to enhance satiety and to decrease gastric emptying (126;160;202;245;246;267). In this study, we tested whether dietary protein affects satiety through other physiological effects and specifically through postprandial ghrelin secretion. Ghrelin is a peptide secreted from the stomach. There are two major molecular forms of ghrelin: acylated ghrelin, which has a n-octanoylation at serine 3; and unacylated ghrelin (164). Until recently, only the acylated form of ghrelin was thought to be biologically active. The current perspective is that also unacylated ghrelin exerts some biological activities (14;45;46;110). Ghrelin appears to be a hunger signal (243;369). Intravenous infusion of ghrelin increases food intake and enhances appetite (69;369), suggesting a role of ghrelin in meal initiation. In addition, ghrelin concentrations in plasma rise gradually before a meal and decrease immediately after eating (67;68;302;324). This postprandial decrease in ghrelin secretion is independent of the volume of the meal, as intake of water does not decrease ghrelin concentrations (35;302). The association between carbohydrate intake and ghrelin concentrations has been investigated extensively. Both oral and intravenous administration of glucose strongly, and dose-dependently, decreases ghrelin concentrations (35;242;302). Oral fat intake also decreases ghrelin concentrations (231), whereas intravenous infusion of lipids has no effect on ghrelin concentrations (229). Intake of carbohydrate-rich meals more potently decreases ghrelin concentrations than intake of high fat meals (231). Only few studies have investigated the effects of dietary protein on ghrelin secretion in humans. In these studies protein intake did not influence postprandial ghrelin concentrations (97;98;123).

The objective of this study was to investigate whether a high protein breakfast is more satiating than a high carbohydrate breakfast through suppression of postprandial ghrelin concentrations, or through other physiological processes (GLP-1, CCK, glucose-dependent insulinotropic polypeptide (GIP)).

Subjects and Methods

Subjects

The study was conducted at TNO Quality of Life, Zeist, the Netherlands, where subjects were recruited from a pool of volunteers. Each subject gave written informed consent after being informed about the study, both verbally and in writing. All subjects filled out a questionnaire on life-style, medical history and dietary habits. The medical investigator physically examined each of the subjects. Blood and urine were collected after an overnight fast for routine analysis. Each subject reported a Western lifestyle, regular Dutch dietary habits and a stable body weight for at least 1 month prior to the study. Smokers, restrained eaters, as assessed with the Dutch Eating Behaviour Questionnaire (312) (score of restriction > 2.5), subjects with haemoglobin concentrations below 8.4 mmol/L, and subjects who reported slimming or who were on a medically prescribed diet were excluded from participation. Also subjects who were on medication that may have influenced appetite and sensory functioning or who reported metabolic or endocrine disease, gastro-intestinal disorders or a history of medical or surgical events that may have affected study outcomes were not included.
Fifteen healthy, lean young men with a mean body mass index (BMI) of 21.6 ± 1.9 kg/m^2 (range: 19.0 - 25.0) and a mean age of 20.5 ± 2.5 y (range: 18 - 26) completed the study (Table 4.1).

Study design

The experiment had a crossover design. Each subject received three treatments on separate days, with a washout period of one week. For practical reasons, all subjects received the same treatment order. Subjects were blinded for treatment order and were informed that the treatment order was randomized. The study had a staggered start, with 5 subjects starting per day and was designed to investigate two separate hypotheses. In this manuscript, only one hypothesis will be presented, i.e., that protein exerts its satiating effects partly through suppression of postprandial ghrelin concentrations. The second hypothesis, i.e., that the postprandial ghrelin response requires post gastric feedback is described in a separate paper. Although we only

use two treatments to investigate the hypothesis described in this paper, we mention all three treatments because the statistical plan was based on the three treatments, as we must proceed for a clinical trial with several treatments administered.

Table 4.1 Subject characteristics at the beginning of the study (n=15)[1]

	Mean ± SD	Range
Age (y)	20.5 ± 2.5	18.0 - 26.0
Height (m)	1.85 ± 0.06	1.72 - 1.94
Body weight (kg)	73.8 ± 7.4	62.5 - 85.3
BMI (kg/m$^{2)}$	21.6 ± 1.9	19.0 - 25.0
Waist:Hip Ratio	0.86 ± 0.06	0.77 - 0.97
DEBQ[2]	1.3 ± 0.4	1.0 - 2.3

[1] DEBQ, Dutch Eating Behaviour Questionnaire.

Score on the restrained-eating scale of the DEBQ.

[2] Range of possible scores on the restrained-eating scale, 1.0 - 5.0.

Dairy breakfasts

Subjects received two iso-caloric dairy breakfasts differing in protein and carbohydrate content. These two breakfasts (weight: 400 g) consisted of: 1) plain yogurt through which 20 gram of saccharose and 1.5 grams of acetaminophen was mixed. The final product had a high carbohydrate content (47.3 En%) and a moderate protein content (19.3 En%) (HC); 2) a dairy product enriched with a whey protein isolate through which 1.5 grams of acetaminophen was thoroughly mixed. Sweeteners (aspartame and Acesulfame K) were added to obtain sweetness comparable with the other breakfast. The final product had high protein (58.1 En%) and low carbohydrate (14.1 En%) content (HP). Subjects were blinded for treatment-order, because breakfasts were kept constant in weight, volume, fat and energy content, viscosity and taste. Table 4.2 presents the energy and macronutrient contents of the breakfasts.

The two treatments described in this paper consisted of either the HC or the HP breakfast in combination with a 3h intravenous infusion of saline. The third treatment, not further mentioned in this paper, consisted of the HC breakfast in combination with a 3h intravenous infusion of GLP-1.

Study protocol

Subjects were instructed to eat and drink the same foods the evening before a test day and to record this in a diary. After an overnight fast (nothing to eat or drink except for water after 20.00 h), subjects handed in their diary, filled out a well-being

questionnaire and were weighed. Subjects were seated in a semi-supine position for the rest of the treatment to prevent effects of position on gastric emptying. An indwelling cannula was placed in the antecubital vein of each forearm, the first for the infusion of saline (0.9% NaCl) (control treatment for the other hypothesis) or GLP-1 (0.75 pmol/kg body weight/min) (main treatment for the other hypothesis) and the second for blood sampling. A pre-ingestion blood sample was collected. The infusion of saline was started when subjects started their meal. The infusion rate was kept constant (2.5 ml/min) for the whole period (180 minutes). After breakfast, consumed within 10 minutes, subjects were not allowed to eat or drink anything during three hours. Blood was collected at 15, 30, 45, 60, 90, 120, and 180 minutes. Immediately after each blood sample was taken, subjects filled out Visual Analogue Scales (VAS) to measure subjective feelings of hunger, fullness, desire to eat and prospective food consumption. Subjects received a buffet-style *ad libitum* lunch, when the infusion was stopped and the cannulas were removed. Subjects consumed the lunch, consisting of standard Dutch food items, within 30 minutes and in separate rooms. They were instructed to eat until they were satiated. In order to prevent habitual intake, foods were provided in unusual portions sizes (e.g. slices of bread were cut in 4 pieces, and peanut butter was provided in a jar of 500 grams).
The study was performed according to the ICH Guideline for Good Clinical Practice (ICH topic E6, adopted 01-05-1996 and implemented 17-01-1997) and was approved by the independent Medical Ethics Committee of the Academic Hospital in Utrecht.

Table 4.2 Energy and macronutrient composition of the breakfasts[1]

	HC	HP
Weight (g)	400	400
Energy (MJ)	1.63	1.65
Protein (g)	18.8	57.2
Carbohydrate (g)	46.0	13.9
Fat (g)	14.4	12.2
Protein (En%)	19.3	58.1
Carbohydrate (En%)	47.3	14.1
Fat (En%)	33.3	27.8

[1] HC, high carbohydrate breakfast; HP, high protein breakfast

Blood samples

Blood was collected as previously described (35). Plasma acetaminophen was analyzed using a commercially available ELISA kit (Immunalysis Corporation, Pomona, CA, USA) with an intra-assay CV of 3.7% at a concentration of 5 µg/ml, and 0.9% at a concentration of 25 µg/ml. GLP-1 (total) concentrations in plasma were measured by radioimmunoassay after extraction of plasma with 70% ethanol (vol/vol, final concentration). Carboxy-terminal GLP-1 immunoreactivity was determined using antiserum 89390 (253) which has an absolute requirement for the intact amidated carboxy-terminus of GLP-1 7-36 amide and cross reacts less than 0.01% with carboxy-terminally truncated fragments and 89% with GLP-1 (9-36) amide (253). Sensitivity was below 5 pmol/l, and intra-assay coefficient of variation below 10%. Serum glucose was determined using a commercially available test kit (Roche Diagnostics GmbH, Mannheim, Germany) on a Hitachi 911 automatic analyser (Hitachi Instrument Division, Ibaraki-ken, Japan), with intra-assay CVs ranging from 0.7% to 0.9% depending on the concentration. Serum insulin was determined as previously described (35). Plasma ghrelin (total and active) concentrations were measured using commercially available human RIA kits (Linco Research Inc., St. Charles, MO). The intra-assay CV of the total ghrelin RIA kit was 10% at a concentration of 1000 pg/ml, and 3.3% at a concentration of 1500 pg/ml. The intra-assay CV of the active ghrelin RIA kit was 6.7% at a concentration of 139 pg/ml, and 9.5% at a concentration of 237 pg/ml. Plasma glucagon concentrations were measured using a commercially available human RIA kit (Linco Research Inc., St. Charles, MO) with an intra-assay CV of 6.8% at a concentration of 60 pg/ml, and 4.0% at a concentration of 220 pg/ml. Plasma GIP concentrations were measured using a commercial human RIA kit (Phoenix Peptide, Belmont, California, USA) with an intra-assay CV of GIP was 3.3 % at a concentration of 0.40 µg/L and 2.5% at a concentration of 0.80 µg/L. Plasma CCK-8 (Cholecystokinin 26-33) concentrations were measured using an optimized and validated commercial human RIA kit (Euro-Diagnostica, Malmö, Sweden). This improved assay system has been optimized to reach a very high sensitivity of 0.05 pmol/L and no cross-reactivity towards to gastrin-17, and sulphated gastrin. The intra-assay CV was 8.9% at a concentration of 0.84 pmol/L and 4.9% at a concentration of 1.98 pmol/L.

Subjective satiety

Subjective satiety was evaluated using Visual Analogue Scales (VAS) for hunger, fullness, desire to eat and prospective food consumption (103). In addition, subjects also filled out Visual Analogue Scales, 30 minutes after breakfast, to evaluate taste,

texture and enjoyment of the meals. Visual Analogue Scales consisted of 150 mm horizontal lines, with Dutch wordings anchored at each end, expressing the most positive or negative sensation (i.e. I have never been more hungry/ I am not hungry at all). Subjects drew a vertical line on the horizontal line corresponding to their hunger sensation. Visual Analogue Scales were automatically processed, using TELE*form* Elite™ software (TELE*form* Elite™ Version 6.1, Cardiff Software Inc., California, USA). Distances on the Visual Analogue Scales were converted into scores between 0 and 100.

Statistical analyses

Analysis of variance (ANOVA) for repeated measures was used to compare the response curves of ghrelin, GLP-1, CCK, GIP, glucose, insulin, glucagon and the VAS scores after the 3 treatments, testing for time X treatment interactions. Only in case of an overall time X treatment effect, partial tests were performed comparing the high protein and high carbohydrate breakfasts. Incremental areas under or over the baseline were calculated. In this report, we use the term area under the curve (AUC) to refer to both values, delineated as negative AUC and positive AUC (the latter for the area over the curve). Evaluation of the residual plots showed that the negative AUC of ghrelin total, ghrelin active and desire to eat could not be used for the analysis, and therefore we used the total AUC, which we defined as the sum of the areas under and over the baseline. With the use of a mixed model ANOVA, the AUCs of the different variables were tested for an overall treatment effect. If there was a treatment effect, partial tests were performed comparing the HC and HP breakfasts. Mixed model ANOVA was also used to test whether taste, texture and enjoyment of the breakfasts differed. Correlation coefficients were calculated to evaluate the relation among subjective measures of satiety and blood parameters. The Pearson correlation coefficient was calculated for each subject, based on 8 (8 time points) pairs of data. On these individual correlations a Fisher's z-transformation was applied, in order to correct for deviations from the normal distribution. The mean of these 15 coefficients was calculated, the inverse of the Fisher transformation was performed and the 95% confidence interval (95% C.I.) for each correlation coefficient was calculated. In addition, correlation coefficients were calculated to evaluate the association between energy and macronutrient intake during lunch and the AUC of the different blood parameters. The correlation coefficient was calculated by treatment, based on 15 (15 subjects) observations. Also the proportional change from mean baseline concentration to the highest (glucose, insulin, glucagon, GIP, CCK, GLP-1 and fullness) or lowest (ghrelin, hunger, desire to eat and prospective food consumption) value was calculated.

Statistical analysis of the data was carried out using the SAS statistical software package (SAS/STAT Version 8.2, SAS Institute, Cary, NC). A P value <0.05 (two-sided) was considered statistically significant in all analyses. Results are given as mean ± SD.

Results

Gastric emptying

Gastric emptying was indirectly estimated by acetaminophen absorption. Figure 4.1 shows the postprandial acetaminophen concentrations and the AUCs. After the HC treatment, acetaminophen concentrations in plasma increased rapidly, reaching a maximum value of 16.2 ± 4.0 µg/ml at 120 minutes. Acetaminophen concentrations after the HP treatment rose more slowly reaching a maximum concentration of 13.0 ± 2.7 µg/ml at 120 minutes. The acetaminophen responses showed a time X treatment interaction ($p < 0.0001$). The AUC of the acetaminophen response was smaller (about 18%) after the HP breakfast ($p < 0.0001$), suggesting that the HP breakfast reduced gastric emptying as compared to the HC treatment.

Blood parameters

The 3 h postprandial responses and the AUCs of the different blood parameters are presented in figures 4.2 and 4.3.

Ghrelin

Total ghrelin (ghrelin)

Ghrelin concentrations decreased after both the HC treatment (-18%) and the HP treatment (-25%), reaching lowest values at 60 and 120 minutes, respectively. The ghrelin responses showed an overall interaction between time X treatment ($p < 0.0001$). Partial tests showed that the ghrelin responses after the HP and HC breakfast were different ($p < 0.0001$). The total AUC of the ghrelin response was larger (about 45%) after the HP breakfast than after the HC breakfast ($p < 0.01$).

Active ghrelin

Active ghrelin concentrations decreased after both the HC (-18%) and the HP (-34%) treatment, reaching lowest values at 45 and 120 minutes, respectively. ANOVA for repeated measures showed no overall interaction between time X treatment. Total AUC of the active ghrelin response did also not differ between the two breakfasts.

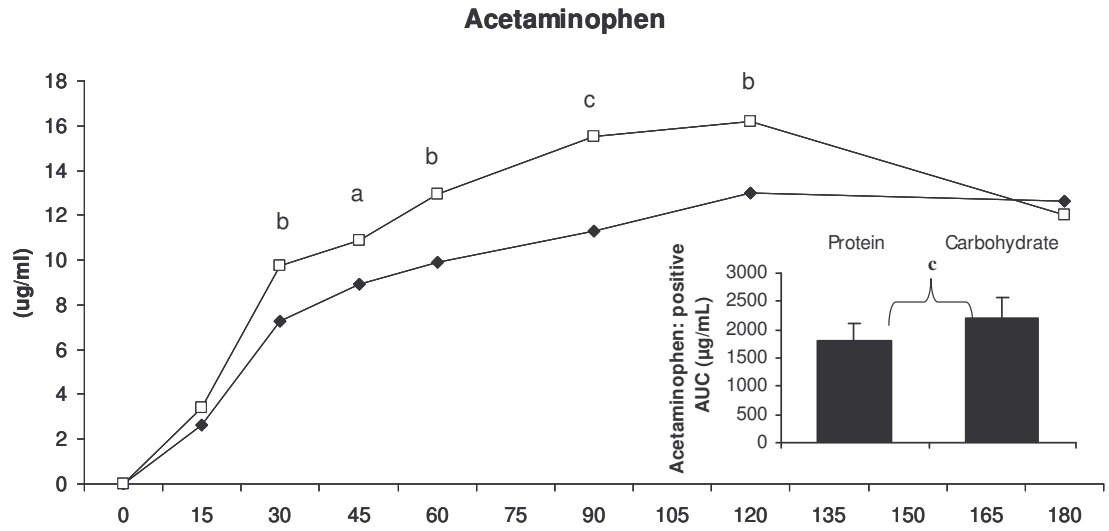


Figure 4.1: Mean responses of acetaminophen (n=15) during 3 h after the intake of the 2 breakfasts. Positive AUC, area over the curve (or baseline). □, high carbohydrate (HC) breakfast; ♦, high protein (HP) breakfast. By ANOVA, there was a significant time X treatment interaction for acetaminophen ($p < 0.0001$). Inserted graph: mean (± SD) AUC of acetaminophen (n=15). By ANOVA, there was a significant treatment effect of the AUC of the acetaminophen response ($p < 0.0001$). The different letters indicate the level of statistical significance. a: $p < 0.05$, b: $p < 0.001$, c: $p < 0.0001$

Glucose

Serum glucose concentrations increased about 24% after the HC treatment, reaching peak values at 30 minutes. In contrast, glucose concentrations did not increase after the HP treatment, but decreased about 10%, reaching lowest values at 60 minutes. The glucose responses showed an overall interaction between time X treatment ($p < 0.0001$) and partial tests showed that the glucose responses after the two breakfasts differed from each other ($p < 0.0001$). In addition, the AUC of glucose was smaller (about 76%) after the HP breakfast compared to the HC breakfast ($p = 0.0001$).

Insulin

Serum insulin concentrations increased about 8 fold after the HC treatment, and about 5.5 fold after the HP treatment, reaching peak values at 30 minutes. The insulin responses showed an overall interaction between time X treatment ($p < 0.0001$). Partial tests showed that insulin responses differed after HC and HP breakfasts ($p < 0.0001$). Insulin concentrations were lower after the HP breakfast than after the HC breakfast between 15 and 45 minutes. However, the AUCs were not significantly different.

Glucagon

Glucagon concentrations increased about 31% after the HC treatment reaching peak values at 30 minutes. Glucagon concentrations increased about 130% after the HP treatment, reaching peak values at 60 minutes. The glucagon responses showed a time X treatment effect ($p < 0.0001$) and partial tests showed that the glucagon responses after the two breakfasts differed from each other ($p < 0.0001$). The AUC of the glucagon response was larger (about 380%) after the HP breakfast compared to the HC breakfast ($p < 0.0001$).

Glucose-dependent insulinotropic polypeptide

Plasma GIP concentrations increased about 150% after both the HC treatment and the HP treatment, reaching peak values at 30 and 45 minutes, respectively. The GIP responses showed a time X treatment interaction ($p < 0.0001$). The partial test showed that the GIP responses after the HC and HP breakfast were different ($p < 0.0001$). The AUC of the GIP response was borderline significantly larger (about 21%) after the HP breakfast compared to the HC breakfast ($p = 0.07$) (Figure 4.2). GIP concentrations at 120 and 180 minutes were higher after the HP breakfast compared to the HC breakfast.

Cholecystokinin

Plasma CCK concentrations increased about 3 fold after the HC treatment reaching peak values at 15 minutes. After the HP breakfast, CCK concentrations showed a biphasic response. CCK concentrations initially increased about 6.5 fold, then dropped about 40% at 30 minutes, followed by a steadily increase after 45 minutes, reaching peak values at 60 minutes (6.5 fold increase compared to baseline values). The CCK responses showed a time X treatment interaction ($p < 0.01$) and partial testing showed that the CCK response differed between the two treatments ($p < 0.05$). The AUC of the CCK response was higher (about 54%) after the HP breakfast compared to the HC breakfast ($p < 0.01$).

Glucagon-Like Peptide 1

GLP-1 concentrations increased about 50% after the HC breakfast, and about 80% after the HP breakfast, reaching peak values at 90 and 120 minutes, respectively. The GLP-1 responses showed an overall interaction between time X treatment ($p < 0.0005$). Partial tests showed that GLP-1 responses after the HP breakfast were not different from the HC breakfast. In contrast, the AUC of GLP-1 was borderline significantly higher (about 66%) after the HP breakfast compared to the HC breakfast ($p = 0.10$).

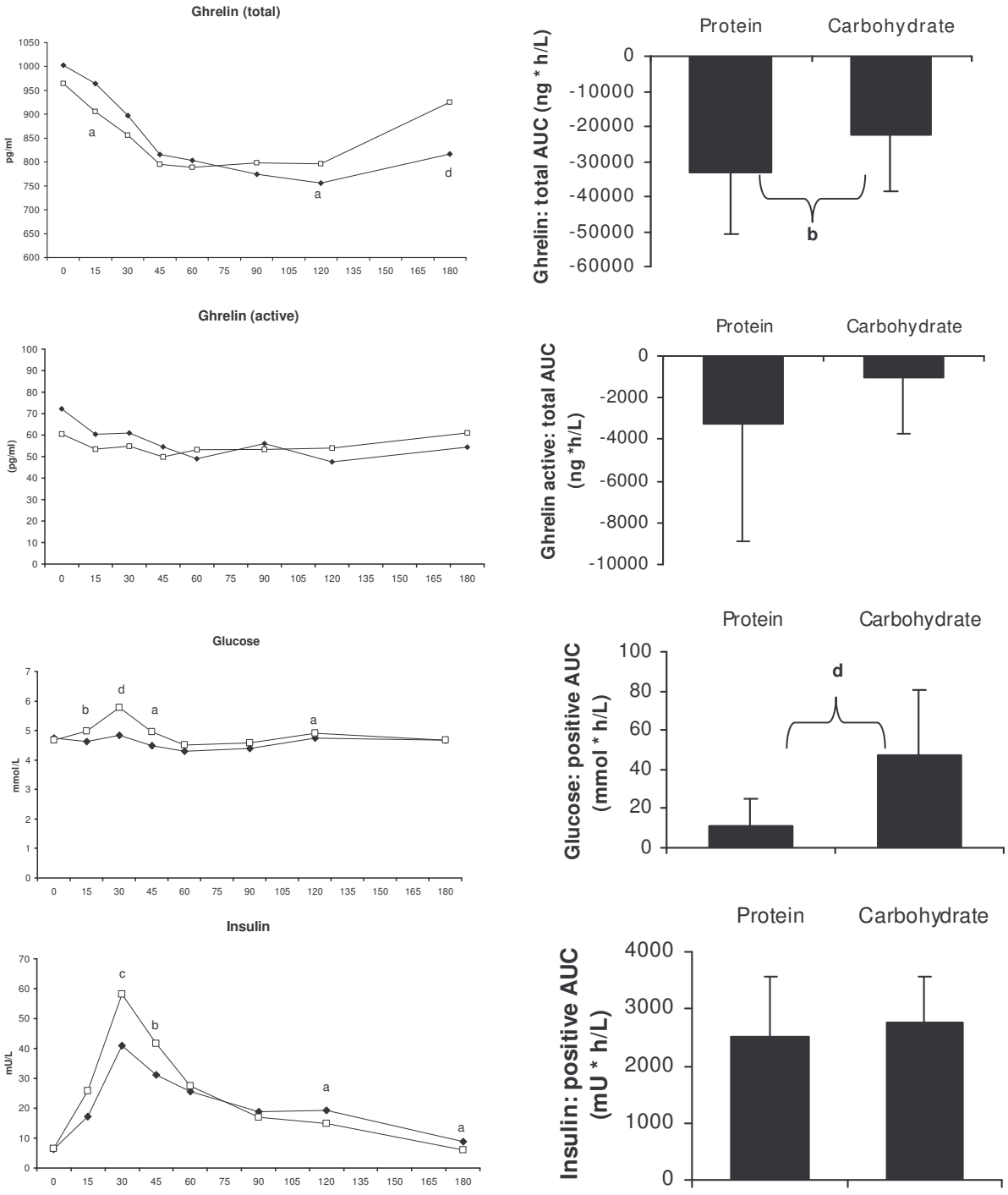


Figure 4.2 Mean responses of total ghrelin, active ghrelin, glucose, insulin (n=15) during 3 h after the intake of the 2 breakfasts. Total AUC, total area under and over the curve (or baseline); positive AUC, area over the curve. Left: □, high carbohydrate (HC) breakfast; ♦, high protein (HP) breakfast. By ANOVA, there was a significant time X treatment interaction for total ghrelin, insulin, glucose (all $p < 0.0001$) Right: mean (± SD) AUC of the different responses (n=15). By ANOVA, there was a significant treatment effect of the AUCs of the total ghrelin ($p < 0.01$) and glucose ($p \leq 0.0001$) responses. The different letters indicate the level of statistical significance. a: $p < 0.05$, b: $p < 0.01$, c: $p < 0.001$, d: $p < 0.0001$.

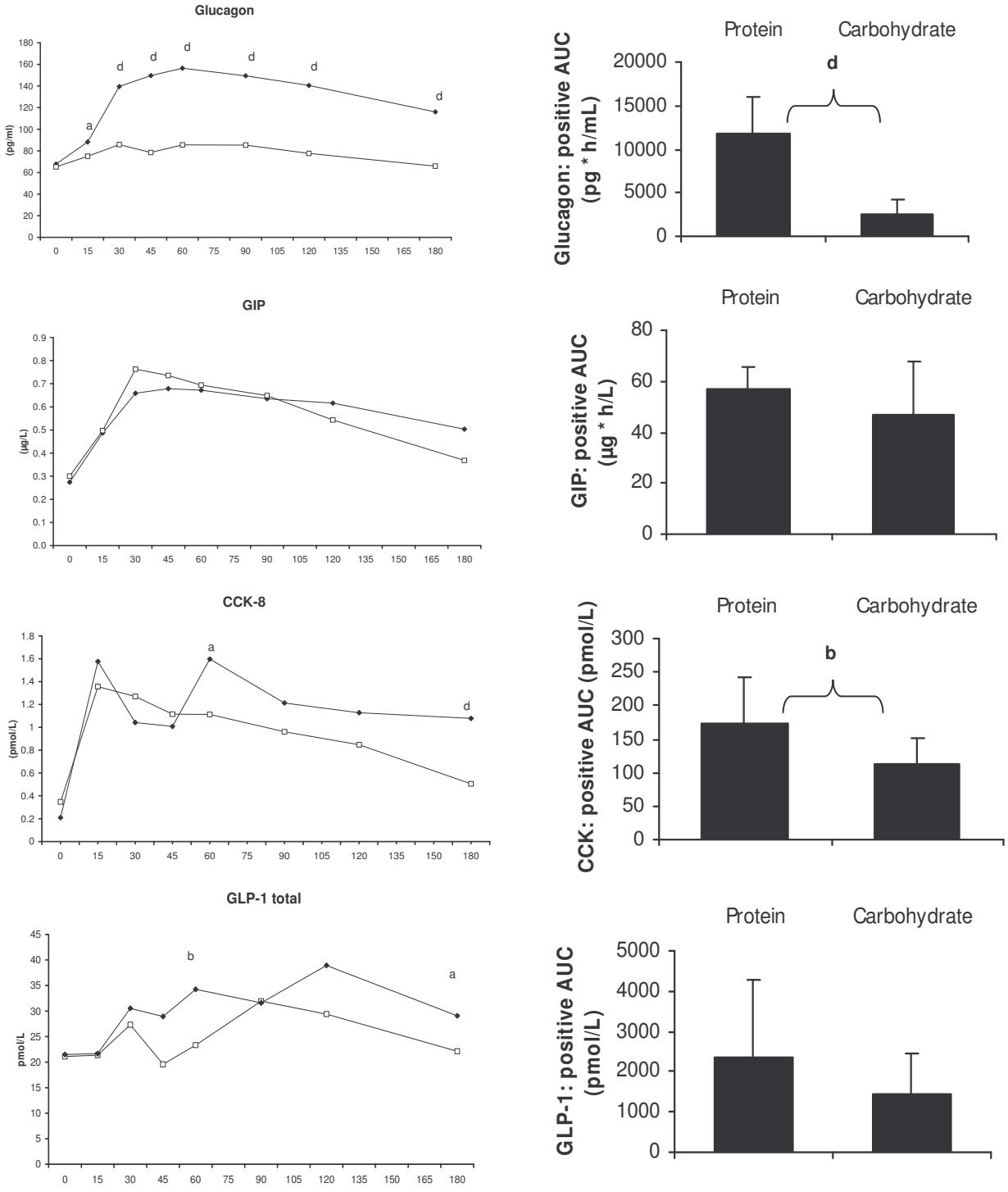


Figure 4.3 Mean responses of glucagon, GIP, CCK and GLP-1 (n=15) during 3 h after the intake of the 2 breakfasts. Total AUC, total area under and over the curve (or baseline); positive AUC, area over the curve. Left: □, high carbohydrate (HC) breakfast; ♦, high protein (HP) breakfast. By ANOVA, there was a significant time X treatment interaction for glucagon, GIP (both $p < 0.0001$), CCK ($p < 0.01$) and GLP-1 ($p < 0.001$). Right: mean (± SD) AUC of the different responses (n=15). By ANOVA, there was a borderline significant treatment effect of the AUCs of the GIP and GLP-1 responses ($p < 0.10$) and a significant treatment effect of the AUCs of the total CCK ($p < 0.01$) and glucagon ($p < 0.0001$) responses. The different letters indicate the level of statistical significance. a: $p < 0.05$, b: $p < 0.01$, c: $p < 0.001$, d: $p < 0.0001$.

Questionnaires

Subjective satiety

Subjective measures of satiety and the AUCs are presented in Figure 4.4. Fasting scores of the four satiety scales did not differ between treatments. Subjective measures of hunger decreased by about 50% after the HP treatment, and 35% after the HC treatment, reaching the lowest values at 15 minutes. Subjective measures of fullness increased following both breakfasts, reaching peak values at 15 minutes. Scores for desire to eat decreased about 50% after the HP treatment and decreased about 35% after the HC treatment. Subjective measures of prospective food consumption decreased about 45% after the HP treatment, and about 30% after the HC treatment. Analysis of the total AUC showed no treatment effect on hunger, fullness, desire to eat or prospective food consumption. There was a trend for an overall interaction between time X treatment for prospective food consumption ($p = 0.08$), but the responses of the two treatments did not differ. There was no overall interaction between time X treatment for hunger, fullness and desire to eat.

Palatability of the test meals

Subjects rated the palatability of the two test meals 30 minutes after the start of consumption on Visual Analogue Scales (figure 4.5). The ANOVA showed no treatment effects on taste, texture or enjoyment of the two meals.

Energy and macronutrient intake

Energy and macronutrient intake during the *ad libitum* lunch are presented in table 4.3. Compared to the HC treatment, the HP treatment reduced fat intake ($P = 0.05$) during the subsequent *ad libitum* lunch. No significant differences in energy, carbohydrate or protein intake were observed during lunch.

Correlations

Ghrelin and other blood parameters

Total ghrelin and active ghrelin concentrations were correlated after the HP breakfast ($r = 0.56$; 95% C.I. = 0.16, 0.80), but not after the HC breakfast ($r = 0.35$; 95% C.I. = -0.11, 0.69). Ghrelin concentrations were not associated with insulin concentrations (LP: $r = -0.36$; 95% C.I. = -0.69, 0.09; HP: $r = -0.25$; 95% C.I. = -0.63, 0.21), but were strongly and inversely associated with concentrations of the insulinotropic peptide GIP (LP: $r = -0.74$; 95% C.I. = -0.89, -0.45; HP: $r = -0.70$; 95% C.I. = -0.87, -0.38) and with acetaminophen concentrations (LP: $r = -0.76$; 95% C.I. = -0.90, -0.49; HP: $r = -0.89$; 95% C.I. = -0.95, -0.73).

Furthermore, ghrelin concentrations were inversely associated with glucagon concentrations (LP: r = -0.52; 95% C.I. = -0.78, -0.10; HP: r = -0.77; 95% C.I. = -0.90, -0.49) and with CCK concentrations during the HC (r = -0.54; 95% C.I. = -0.80, -0.13), but not during the HP (r = -0.32; 95% C.I. = -0.67, 0.18) treatment. Active ghrelin concentrations were inversely associated with acetaminophen concentrations (r = -0.50; 95% C.I. = -0.77, -0.07) and glucagon concentrations (r = -0.47; 95% C.I. = -0.75, -0.03) during the high protein treatment. No further associations between active ghrelin concentrations and other physiological parameters were observed (see table 4.4).

Subjective satiety and blood parameters

Correlations between blood parameters and measures of satiety are presented in table 4.5. Neither total nor active ghrelin concentrations were correlated with measures of satiety. GIP concentrations were correlated with scores on all appetite scales, with the exception of scores for desires to eat after the HC breakfast, and scores of hunger and prospective food consumption after the HP breakfast. Insulin concentrations were correlated with measures of hunger and fullness after the HC treatment and with all appetite measures after the HP treatment. CCK concentrations were inversely correlated with hunger and fullness during the HC treatment, but not during the HP treatment.

Table 4.3 Energy and macronutrient intake during *ad libitum* lunch[1]

	HC	HP
Energy (kJ)	5136 ± 1205	4697 ± 1784
Fat (g)*	41 ± 12	33 ± 15
Protein (g)	48 ± 14	43 ± 20
Carbohydrate (g)	166 ± 58	161 ± 61

[1] HC, high carbohydrate breakfast; HP, high protein breakfast

[2] Mean ± SD

* Significantly different (P = 0.05) between the two treatments tested by mixed model ANOVA

Food intake and blood parameters

No associations between the AUC of the ghrelin response and subsequent energy intake during the *ad libitum* lunch (HC: r = -0.15, p = 0.59; HP: r = 0.03, p = 0.93) were observed. The AUC of the ghrelin response was also not associated with fat intake during lunch, after the HC (r = 0.41; p = 0.13) or HP breakfast (r = 0.30; p = 0.28). The AUC of the glucagon response was inversely associated with energy

intake (r = -0.74; p < 0.01), protein intake (r = -0.82; p < 0.001), carbohydrate intake (r = -0.59; p < 0.05) and fat intake (r = -0.75; p < 0.01) during lunch after the HP breakfast. There was no significant association between the AUC of the glucagon response and energy (r = -0.24; p = 0.39) and macronutrient intake after the HC breakfast. The AUC of the insulin response was borderline positively correlated with protein intake during lunch after the HC breakfast (r = 0.47, p = 0.08), though not after the HP breakfast (r = 0.10, p = 0.72). There was a trend for a correlation between the positive AUC of the CCK response and fat intake during lunch (r = 0.50; p = 0.06) after the HC breakfast.

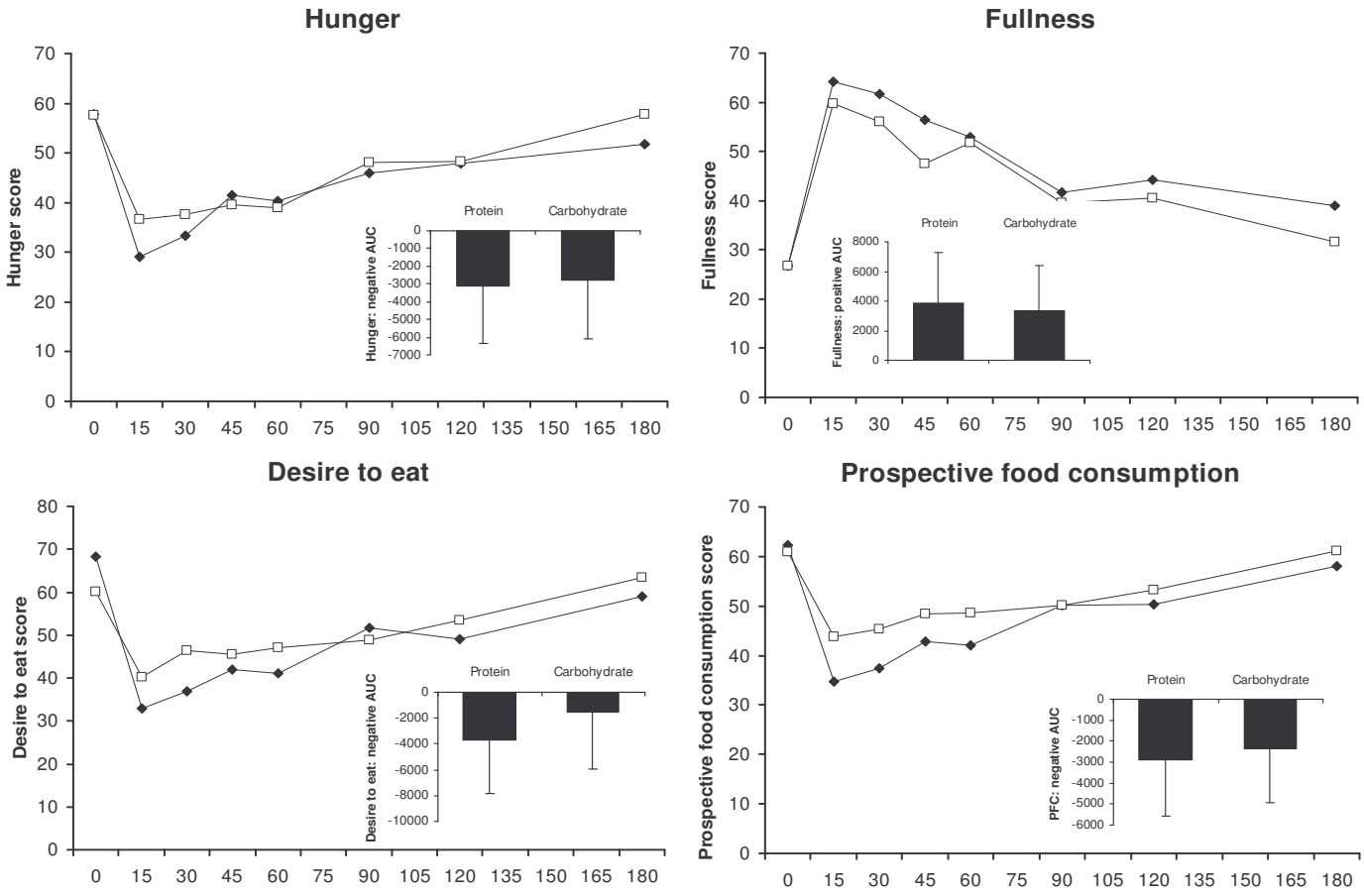


Figure 4.4 Mean responses of hunger, fullness, desire to eat and prospective food consumption (n=15) during 3 h after the intake of the 2 breakfasts. Negative AUC, area under the curve (or baseline); positive AUC, area over the curve. □, high carbohydrate (HC) breakfast; ♦, high protein (HP) breakfast. By ANOVA, there was no significant time X treatment interaction for the four satiety scales. Inserted graph: mean (± SD) AUC of the different responses (n=15).There was no significant treatment effect of the AUCs of the four appetite responses.

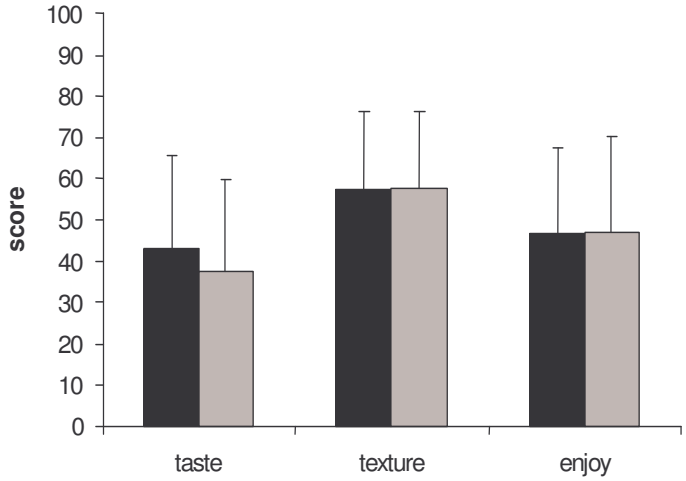


Figure 4.5 Mean (± SD) rating of the taste, texture and enjoyment of the high protein and high carbohydrate meals, 30 minutes after consumption (n=15). By ANOVA, there was no significant treatment effect for taste, texture and enjoyment. Black bars represent the high protein meal, and grey bars represent the high carbohydrate meal.

Discussion

In this study, we investigated whether a high protein meal is more satiating through suppression of the hunger peptide ghrelin, or through other mechanisms. However, both subjective sensations of appetite and *ad libitum* energy intake during lunch did not differ between the high protein and high carbohydrate breakfasts. The protein breakfast did suppress total ghrelin concentrations more than the carbohydrate breakfast. The high association between total ghrelin concentrations and GIP and glucagon concentrations suggests that the postprandial decrease in ghrelin may be mediated through stimulation of GIP and glucagon secretion. The high protein breakfast also reduced gastric emptying, and stimulated CCK and GLP-1 secretion.
For practical reasons, the design of the study was not randomized for treatment order. Consequently the period is entangled with treatment, and period effects can therefore not be eliminated. We have however, reason to believe that the lack of randomization has not influenced the results, whereas a washout period of one week is sufficient to prevent carry-over effects of the treatments, and stress hormone concentrations (data available but not shown) were not different between the treatments. In addition, baseline values of all parameters did not differ between the periods. This study was designed to compare the effects of meals differing in amount of protein and carbohydrate on subjective and physiological measures of appetite. Therefore, other factors possibly affecting appetite were kept constant. There was a

Table 4.4 The relation between different physiological parameters by treatment (n = 15)[1]

	Ghrelin total		Ghrelin active		GIP	
	Carbohydrate	Protein	Carbohydrate	Protein	Carbohydrate	Protein
Ghrelin total			0.35	0.56	-0.74	-0.70
	-	-	(-0.11, 0.69)	(0.16, 0.80)	(-0.89, -0.45)	(-0.87, -0.38)
Ghrelin active	0.35	0.56			-0.28	-0.43
	(-0.11, 0.69)	(0.16, 0.80)	-	-	(-0.64, 0.18)	(-0.74, 0.01)
acetaminophen	-0.76	-0.89	-0.10	-0.50	0.54	0.70
	(-0.90, -0.49)	(-0.95, -0.73)	(-0.52, 0.35)	(-0.77, -0.07)	(0.13, 0.79)	(0.37, 0.87)
GLP-1	-0.16	-0.41	-0.01	-0.26	0.19	0.34
	(-0.56, 0.31)	(-0.72, 0.03)	(-0.45, 0.44)	(-0.63, 0.21)	(-0.27, 0.58)	(-0.12, 0.68)
insulin	-0.36	-0.25	-0.27	-0.17	0.81	0.74
	(-0.69, 0.09)	(-0.63, 0.21)	(-0.64, 0.20)	(-0.57, 0.30)	(0.57, 0.92)	(0.44, 0.89)
glucose	0.06	0.30	-0.01	0.24	0.33	-0.19
	(-0.39, 0.49)	(-0.17, 0.65)	(-0.45, 0.44)	(-0.23, 0.62)	(-0.13, 0.68)	(-0.58, 0.28)
glucagon	-0.52	-0.77	-0.10	-0.47	0.78	0.93
	(-0.78, -0.10)	(-0.90, -0.49)	(-0.52, 0.36)	(-0.75, -0.03)	(0.52, 0.91)	(0.83, 0.97)
CCK	-0.54	-0.32	-0.21	-0.29	0.71	0.48
	(-0.80, -0.13)	(-0.67, 0.18)	(-0.60, 0.26)	(-0.65, 0.18)	(0.38, 0.87)	(0.05, 0.76)

[1] Pearson's correlation coefficients of the relation between the different physiological parameters were calculated per subject and treatment. The mean correlation coefficients (r) together with the 95% confidence intervals (in parentheses) after Fisher *z* transformation are presented

small difference in fat content of the meal (difference of 2.2 g per 400 g portion which corresponds to a difference of 5.5% energy from fat), but we do not expect that this small difference in fat content has dramatically affected the outcome of the study. Subjects were blinded for treatment order. Hedonic aspects of the two breakfasts were similar based on subjects' ratings of the two breakfasts.

Acetaminophen was added to the breakfast because its absorption is an indirect measure of the gastric emptying rate (294;357). Its bitter taste may explain the rather low scores for taste of the breakfasts.

We expected that the high protein breakfast would increase subjective satiety and possibly decrease energy intake as compared to the high carbohydrate breakfast. However, none of the appetite ratings were statistically different between the two treatments and the high protein breakfast did not affect ad libitum energy intake. We probably had not sufficient statistical power to detect the small differences in appetite and energy intake between the two breakfasts (103). It is also possible that assessing subjective satiety during infusions and blood samplings may have decreased the amplitude of the results. In this experiment, although not significant, the protein breakfast reduced appetite and energy intake at the next meal by about 439 kJ. Longer experiments would be useful to see if these beneficial effects on the regulation of appetite can be maintained and have a clinical relevance. This study was also designed to investigate the effects of a high protein meal on physiological parameters involved in the regulation of hunger and satiety, with special focus on postprandial ghrelin secretion. We observed a larger decrease in postprandial ghrelin (total) concentrations after the protein breakfast than after the carbohydrate breakfast. Also active ghrelin concentrations decreased in the postprandial period, but these concentrations were not significantly different between the two treatments. The effects observed for total ghrelin may be mediated by active ghrelin, but due to the large variations in active ghrelin concentrations we had not sufficient statistical power. The postprandial decrease in total ghrelin concentrations following protein intake was not apparent in studies performed by Erdmann et al. and by Greenman et al. (97;98;123). This discrepancy may be explained by the type of protein used. Our high protein meal consisted of a dairy product enriched with whey protein, whereas in the other three studies the high protein meals consisted of meat (97;98;123). Few studies compared the effects of the type of dietary proteins on satiety and subsequent food intake (9;133;170;171;328). Among papers showing differences between proteins, whey, for example, was found to be more satiating as compared to casein (133) and had larger effects on food intake suppression compared to egg and soy protein (9). The type of protein is reflected in the amino acid composition, and differentially affects insulin, GIP and glucagon secretion (49;247;331). In fact, plasma amino acid concentrations following intake of a high protein meal may almost completely account for the postprandial increase in insulin concentrations (297). Specifically, branched chain amino acids (BCAA), like leucine, valine and isoleucine are insulinotropic (49;247). Whey and casein both contain high concentrations of these amino acids, but intake of whey protein induces the largest insulin response (247). This may also indicate that the insulinotropic effect of amino acids is dependent on the bioavailability of amino acids, because whey is a soluble milk protein in contrast to casein which coagulates in the stomach (49;247).

Table 4.5 Mean correlation coefficient (r) with 95% confidence intervals of the relation between several blood variables and measures of appetite (n=15)

	High carbohydrate breakfast				High protein breakfast			
	Hunger	**Fullness**	**Desire to eat**	**Prospective**	**Hunger**	**Fullness**	**Desire to eat**	**Prospective**
Ghrelin total	0.22 (-0.24, 0.61)	-0.23 (-0.61, 0.24)	0.19 (-0.28, 0.58)	0.29 (-0.17, 0.65)	-0.19 (-0.58, 0.27)	0.02 (-0.43, 0.45)	-0.08 (-0.51, 0.37)	-0.07 (-0.49, 0.39)
Ghrelin active	0.11 (-0.35, 0.53)	-0.11 (-0.52, 0.35)	0.21 (-0.26, 0.59)	0.25 (-0.22, 0.62)	-0.10 (-0.52, 0.36)	-0.09 (-0.51, 0.37)	0. 07 (-0.39, 0.49)	0.08 (-0.38, 0.50)
Glucose	-0.19 (-0.59, 0.27)	0.24 (-0.23, 0.62)	-0.08 (-0.51, 0.37)	-0.19 (-0.59, 0.27)	-0.03 (-0.47, 0.41)	-0.07 (-0.49, 0.39)	0.00 (-0.44, 0.46)	0.07 (-0.39, 0.49)
Insulin	-0.51 (-0.78, -0.09)	0.55 (0.15, 0.80)	-0.34 (-0.68, 0.12)	-0.41 (-0.72, 0.04)	-0.53 (-0.79, -0.11)	0.61 (0.22, 0.83)	-0.61 (-0.83, -0.23)	-0.54 (-0.79, -0.13)
Glucagon	-0.41 (-0.72, 0.04)	0.39 (-0.07, 0.71)	-0.31 (-0.66, 0.16)	-0.37 (-0.70, 0.09)	-0.14 (-0.55, 0.32)	0.28 (-0.18, 0.65)	-0.30 (-0.66, 0.16)	-0.18 (-0.58, 0.28)
GIP	-0.51 (-0.77, -0.08)	0.54 (0.12, 0.79)	-0.36 (-0.69, 0.10)	-0.44 (-0.74, -0.00)	-0.33 (-0.67, 0.14)	0.50 (0.07, 0.77)	-0.48 (-0.76, -0.04)	-0.35 (-0.69, 0.11)
GLP-1	-0.06 (-0.49, 0.39)	0.03 (-0.42, 0.47)	-0.18 (-0.58, 0.28)	-0.28 (-0.64, 0.19)	0.05 (-0.40, 0.48)	0.03 (-0.42, 0.46)	-0.04 (-0.47, 0.41)	0.02 (-0.42, 0.46)
CCK	-0.45 (-0.74, -0.01)	0.59 (0.19, 0.82)	-0.42 (-0.73, 0.03)	-0.44 (-0.74, 0.00)	-0.21 (-0.60, 0.25)	0.37 (-0.08, 0.70)	-0.34 (-0.68, 0.12)	-0.28 (-0.64, 0.18)
Acetaminophen	-0.04 (-0.47, 0.41)	-0.01 (-0.45, 0.44)	0.03 (-0.42, 0.47)	-0.16 (-0.56, 0.31)	0.19 (-0.28, 0.58)	-0.02 (-0.46, 0.42)	0.01 (-0.43, 0.45)	0.14 (-0.32, 0.55)

Apart from the insulin stimulating effects of amino acids, the type of protein may also affect GIP concentrations (247). GIP is not only secreted from the gut in response to carbohydrate and fat ingestion (94), but also in response to milk protein (48;133;247). A high protein meal consisting of turkey does not stimulate GIP secretion (94), whereas the GIP response is pronounced after intake of whey protein (247). As GIP is an insulinotropic peptide (221;337), GIP may mediate the insulinotropic effect of milk proteins.

The postprandial increase in amino acid concentrations is also responsible for the rise in glucagon concentrations following protein intake (49). Whey proteins elicit the largest glucagon response, because of the greater availability of amino acids following whey protein consumption (49). In the present study, we observed a strong increase in GIP and glucagon concentrations following the high protein breakfast. Both GIP and glucagon concentrations were inversely associated with ghrelin concentrations. Possibly, this high protein dairy product specifically stimulates both GIP and glucagon, which may provide a strong stimulus to decrease further postprandial ghrelin concentrations.

Consumption of the high protein meal reduced acetaminophen absorption more than consumption of the high carbohydrate breakfast. This suggests that the high protein breakfast reduced the gastric emptying rate (294;357). This effect of protein on gastric emptying has been reported before (133), and may be one of the mechanisms by which protein induces satiety. The high protein meal also increased concentrations of the gut peptides CCK and GLP-1. These peptides both potently reduce appetite and food intake, which is at least partly mediated by their ability to decrease gastric emptying (245;246;267). This suggests that the effects of protein on the gastric emptying rate may be induced by the enhanced secretion of CCK and GLP-1.

We also hypothesized that protein exerts its satiating effects partly through suppression of postprandial ghrelin concentrations. Although protein intake indeed decreased ghrelin concentrations, we did not find an association between ghrelin concentrations and subjective satiety or energy intake. However, intake of a high (milk) protein breakfast affected several other physiological parameters involved in the regulation of food intake, which were associated with subjective satiety or energy intake. GIP and insulin concentrations were increased following intake of the protein meal, and were associated with increased satiety. In addition, glucagon concentrations, which were associated with decreased energy and macronutrient intake during the *ad libitum* lunch, were increased. Besides these effects, the high protein breakfast also increased concentrations of CCK and GLP-1 and decreased

gastric emptying, but these factors were not associated with subjective satiety or energy intake in this study.
In this study, we compared a high protein breakfast with a high carbohydrate breakfast that also contained a moderate amount of protein. The difference in protein quantity between the two breakfasts can also explain the observed effects. Like other studies investigating the effects of protein on satiety, our high protein treatment contained a large dose of protein. At the moment, the “active” dose of protein is still unknown (8;9). As several studies have shown that dietary protein can be helpful in weight management (91;351;375), also studies investigating the long-term effects of different amounts or types of proteins on physiological parameters and body weight regulation should be initiated.

In conclusion, the high protein breakfast decreased postprandial ghrelin concentrations more than the high carbohydrate breakfast, despite the lack of effect on satiety. Ghrelin concentrations were strongly associated with GIP and glucagon concentrations, suggesting that the postprandial decrease in ghrelin concentrations following consumption of the high protein breakfast may be mediated through stimulation of these peptides. The high protein breakfast also reduced gastric emptying rate, probably through increased secretion of CCK and GLP-1.

Acknowledgements

We want to express our gratitude to the volunteers who participated in the study; Henriëtte Fick, Inge van den Assum, José Jacobs, Soesila Sukhraj, Linda Kok, Eric Busink, Jan Catsburg, Robin van den Berg, Hans Verplanke and all other people from the Metabolic Research Unit and laboratories who assisted in the organization of the study and analyses of the blood samples; Stéphane Doat, Susanne Westenbrink and Petra van Aken for the preparation of the study substances; Cees de Graaf for his advice on the design of the study; Diane ter Doest and Linda van den Bosch for performing data management; and Cor Kistemaker and Carina Rubingh for their support with the statistical analyses.
Wendy Blom was involved in the design of the protocol, collection of the data, analysis of the data and writing of the manuscript. Anne Lluch, Sophie Vinoy and Annette Stafleu were involved in the design of the protocol and provided significant advice during the writing of the manuscript. Jens Juul Holst was involved in the GLP-1 analyses and provided significant advice on the GLP-1 infusion. Gertjan Schaafsma provided significant advice during the writing of the manuscript. Henk Hendriks was involved in the design of the protocol (Principal Investigator according

to Good Clinical Practice guidelines), writing of the manuscript and provided significant advice during the intervention study and data analysis. Anne Lluch and Sophie Vinoy are employees of Danone Vitapole. None of the other authors has any conflict of interest.

This study is financially supported by: Dutch Ministry of Economic Affairs, Dutch Ministry of Education, Culture and Science, Dutch Ministry of Health, Welfare and Sport and Danone Vitapole.

5

The effects of gastric emptying on the postprandial ghrelin response

Wendy Blom
Anne Lluch
Sophie Vinoy
Annette Stafleu
Robin van den Berg
Jens Juul Holst
Frans Kok
Henk Hendriks

Wageningen University, Wageningen, Netherlands
TNO Quality of Life, Zeist, Netherlands
Danone Vitapole Nutrivaleur, Palaiseau Cedex, France
The Panum Institute, Copenhagen, Denmark

American Journal of Physiology – Endocrinology and Metabolism
Accepted for publication (electronically published DOI, 10.1152/ajpendo.00238.2005)

Abstract

Distension and chemo sensitization of the stomach are insufficient to induce a ghrelin response, suggesting that post gastric feedback is required. This post gastric feedback may be regulated through insulin. We investigated the relation between gastric emptying rate and the postprandial ghrelin response, as well as the role of insulin and other hormones possibly mediating this response. Fifteen healthy men (BMI: 21.6 ± 1.9 kg/m^2, age: 20.5 ± 2.5 y) were studied in a single blind, crossover design. Subjects received two treatments separated by one week; 1) a dairy breakfast in combination with a 3-h intravenous infusion of GLP-1, which delays gastric emptying 2) a dairy breakfast in combination with a 3-h intravenous infusion of saline. Blood samples were drawn before breakfast and during the infusion. Postprandial ghrelin (total) responses were lower following the saline infusion as compared to the GLP-1 infusion ($p < 0.05$). Acetaminophen concentrations, an indirect measurement of gastric emptying rate, were inversely correlated with total ghrelin concentrations (saline: r = -0.76; 95% C.I. = -0.90, -0.49, GLP-1: r = -0.47; 95% C.I. = -0.76, -0.04). Ghrelin concentrations were only weakly correlated with insulin concentrations (saline: r = -0.36; 95% C.I. = -0.69, 0.09; GLP-1: r = -0.42; 95% C.I. = -0.73, 0.03), but strongly inversely correlated with GIP concentrations (saline: r = -0.74; 95% C.I. = -0.89, -0.45; GLP-1: r = -0.63; 95% C.I. = -0.84, -0.27).
In conclusion, our results support the hypothesis that ghrelin requires post gastric feedback, which may not be regulated through insulin. Conversely, our data suggest a role of GIP in ghrelin secretion.

Introduction

Ghrelin is a peptide that is predominantly secreted by the oxyntic glands of the stomach (12;71;164) and is involved in the regulation of food intake (243;369). Ghrelin concentrations decrease rapidly following nutrient intake (12;35;97;123), but not after intake of water (35;302). Williams and colleagues (358) have shown that when gastric emptying was prevented in rats, neither glucose nor water administration affected ghrelin concentrations. These observations suggest that distension and chemo sensitization of the stomach are insufficient to induce a ghrelin response, and that post gastric processes are required. These post gastric processes may involve insulin concentrations, because postprandial changes in ghrelin concentrations are associated with postprandial changes in insulin concentrations (35;98). This association is supported by clamp studies which provided some evidence that insulin decreases ghrelin concentrations, independent of glucose (99;197;236;293).
Gastric emptying is regulated by several post gastric hormones such as cholecystokinin (CCK) and glucagon-like peptide 1 (GLP-1), which both decrease the gastric emptying rate (160;202;245;246), but also by ghrelin, which appears to increase the gastric emptying rate (88;259).

The objective of this study was to investigate whether the postprandial ghrelin response requires post gastric feedback, and if so, whether insulin or other post gastric processes provide this feedback. If the postprandial ghrelin response indeed requires post gastric feedback, ghrelin concentrations should be dependent on the gastric emptying rate.
Therefore, subjects received either an intravenous infusion of GLP-1, which delays gastric emptying, or saline. Gastric emptying was indirectly measured by acetaminophen absorption (294;357). We measured the postprandial ghrelin (total and active) and insulin responses, as well as other factors involved in the regulation of food intake (e.g., glucose, glucagon and glucose-dependent insulinotropic polypeptide (GIP)).

Materials and Methods

Subjects

The study was conducted at TNO Quality of Life, Zeist, the Netherlands, where subjects were recruited from a pool of volunteers. Each subject gave his written

informed consent after being informed about the study, both verbally and in writing. All subjects filled out a questionnaire on life-style, medical history and dietary habits. The medical investigator physically examined each of the subjects. Blood and urine were collected after an overnight fast for routine analysis. Each subject reported a Western lifestyle, regular Dutch dietary habits and a stable body weight for at least 1 month prior to the study. Smokers, restrained eaters, as assessed with the Dutch Eating Behaviour Questionnaire (312) (score of restriction > 2.5), subjects with haemoglobin concentrations below 8.4 mmol/L, and subjects who reported slimming or who were on a medically prescribed diet were excluded from participation. Also subjects who were on medication that may have influenced appetite and sensory functioning or who reported metabolic or endocrine disease, gastro-intestinal disorders or a history of medical or surgical events that may have affected study outcome were not included.

Fifteen healthy, lean young men with a mean body mass index (BMI) of 21.6 ± 1.9 kg/m^2 (range: 19.0 - 25.0) and a mean age of 20.5 ± 2.5 y (range: 18 - 26) completed the study (Table 5.1).

Table 5.1 Subject characteristics (n=15)[1]

	Mean ± SD	Range
Age (y)	20.5 ± 2.5	18.0 - 26.0
Height (m)	1.85 ± 0.06	1.72 - 1.94
Body weight (kg)	73.8 ± 7.4	62.5 - 85.3
BMI (kg/m$^{2)}$	21.6 ± 1.9	19.0 - 25.0
Waist:Hip Ratio	0.86 ± 0.06	0.77 - 0.97
DEBQ[2]	1.3 ± 0.4	1.0 - 2.3

[1] Values represent measurements taken at the beginning of the study

[2] Score on the restrained eating scale of the Dutch Eating Behaviour Questionnaire. Range possible scores on the restrained eating scale: 1.0 - 5.0

Study design

The study had a crossover design. Each subject received three treatments on separate days, with a washout period of one week. For practical reasons, all subjects received the same treatment order. Subjects were blinded for treatment order and were informed that the treatment order was randomized. The study had a staggered start, with 5 subjects starting per day. Subjects were successfully randomized for body weight and age. The study was designed to investigate two separate hypotheses. In this manuscript, only one hypothesis will be presented, i.e., that the

postprandial ghrelin response requires post gastric feedback. The second hypothesis, i.e., that protein exerts its satiating effects partly through suppression of postprandial ghrelin concentrations, is described in a separate paper. We only described the outcome of the two treatments used to investigate the hypothesis presented in this paper. All three treatments are mentioned, because the statistical plan was based on these three treatments.

Study treatments

At breakfast, subjects consumed 400 grams of plain yogurt to which 20 grams of saccharose and 1.5 grams of acetaminophen was thoroughly mixed for both treatments. Table 5.2 presents the energy and macronutrient content of the breakfast. At the same time, an intravenous infusion of either saline (0.9% NaCl) (2.5 ml/min) or 0.75 pmol/kg body weight/min GLP-1 (7-36) amide (Clinalfa, Merck Biosciences Ag, Läufelfingen, Switzerland) dissolved in saline was infused during 180 minutes. Thereafter, subjects received an ad libitum, buffet-style lunch, which consisted of standard Dutch food items. Subjects ate their lunch in separate rooms within 30 minutes. They were instructed to eat until they were satiated. In order to prevent habitual intake, foods were provided in unusual portion sizes (e.g. slices of bread were cut in 4 pieces, and peanut butter was provided in a jar of 500 grams). The third treatment, not further presented in this paper, consisted of an isocaloric high protein breakfast in combination with an intravenous infusion of saline.

Table 5.2 Energy and macronutrient composition of the breakfast

Weight (g)	400
Energy (kJ)	1628
Protein (g)	18.8
Carbohydrate (g)	46.0
Fat (g)	14.4
Protein (En%)	19.3
Carbohydrate (En%)	47.3
Fat (En%)	33.3

Study protocol

Subjects were instructed to eat and drink the same food items the evening before each of the two test days by recording this in a diary. After an overnight fast (nothing to eat or drink except for water after 20.00 h), subjects handed in their diary, filled out

a well-being questionnaire and were weighed. The subjects were seated in a semi-supine position for the rest of the treatment to prevent effects of position on gastric emptying. An indwelling cannula was placed in the antecubital vein of each forearm, the first for the infusion of saline or GLP-1 (7-36) and the second for blood sampling. A pre-ingestion blood sample was collected. After breakfast, consumed within 10 minutes, subjects were not allowed to eat or drink anything during three hours. Blood was collected at 15, 30, 45, 60, 90, 120, and 180 minutes. Subjects received an *ad libitum* lunch, after 180 minutes, when the infusion was stopped and the cannulas were removed.
The study was performed according to the ICH Guideline for Good Clinical Practice (ICH topic E6, adopted 01-05-1996 and implemented 17-01-1997) and was approved by the independent Medical Ethics Committee of the Academic Hospital in Utrecht.

Blood samples

Blood was collected as previously described (35). Plasma acetaminophen was analyzed using a commercially available ELISA kit (Immunalysis Corporation, Pomona, CA, USA) with an intra-assay CV of 3.7% at a concentration of 5 µg/ml, and 0.9% at a concentration of 25 µg/ml. GLP-1 concentrations in plasma were measured by radioimmunoassay after extraction of plasma with 70% ethanol (vol/vol, final concentration). Carboxy-terminal GLP-1 immunoreactivity was determined using antiserum 89390 (253) which has an absolute requirement for the intact amidated carboxy-terminus of GLP-1 7-36 amide and cross reacts less than 0.01% with carboxy-terminally truncated fragments and 89% with GLP-1 (9-36) amide (253). Sensitivity was below 5 pmol/l, and intra-assay coefficient of variation below 10%. Serum glucose was determined using a commercially available test kit (Roche Diagnostics GmbH, Mannheim, Germany) on a Hitachi 911 automatic analyser (Hitachi Instrument Division, Ibaraki-ken, Japan), with intra-assay CVs ranging from 0.7% to 0.9% depending on the concentration. Serum insulin was determined as previously described (35). Plasma ghrelin (total and active) concentrations were measured using commercially available human RIA kits (Linco Research Inc., St. Charles, MO). The intra-assay CV of the total ghrelin RIA kit was 10% at a concentration of 1000 pg/ml, and 3.3% at a concentration of 1500 pg/ml. The intra-assay CV of the active ghrelin RIA kit was 6.7% at a concentration of 139 pg/ml, and 9.5% at a concentration of 237 pg/ml. Plasma glucagon concentrations were measured using a commercially available human RIA kit (Linco Research Inc., St. Charles, MO) with an intra-assay CV of 6.8% at a concentration of 60 pg/ml, and 4.0% at a concentration of 220 pg/ml. Plasma GIP concentrations were measured using a commercially available human RIA kit (Phoenix Peptide, Belmont, California,

USA) with an intra-assay CV of GIP was 3.3 % at a concentration of 0.40 µg/L and 2.5% at a concentration of 0.80 µg/L. Plasma CCK-8 (Cholecystokinin 26-33) concentrations were measured using an optimized and validated commercial human RIA kit (Euro-Diagnostica, Malmö, Sweden). This improved assay system has been optimized to reach a very high sensitivity of 0.05 pmol/L and no cross-reactivity towards to gastrin-17, and sulphated gastrin. The intra-assay CV was 8.9% at a concentration of 0.84 pmol/L and 4.9% at a concentration of 1.98 pmol/L.

Statistical analyses

With analysis of variance (ANOVA) for repeated measures the response curves of ghrelin, GLP-1, CCK, GIP, glucose, insulin and glucagon after the 3 treatments were compared, testing for time X treatment interactions. Tests comparing the GLP-1 and saline treatments were only performed in case an overall treatment effect was observed. With mixed model analysis of variance, differences in concentrations were investigated per time-point. Incremental areas under or over the baseline were calculated. The term area under the curve (AUC) refers to both values, delineated as negative AUC and positive AUC (the latter for the area over the curve). Evaluation of the residual plots showed that the negative AUC of “ghrelin total” and “ghrelin active” could not be used for the analysis. We defined the total AUC as the sum of the areas under and over the baseline, in case of “ghrelin total” and “ghrelin active”. With the use of a mixed model ANOVA, the AUCs of the different variables were tested for an overall treatment effect. Correlation coefficients were calculated to evaluate the relation among blood parameters. Per treatment, the Pearson correlation coefficient was calculated for each subject, based on 8 (8 time points) pairs of data. On these individual correlations, a Fisher’s z-transformation was applied, in order to correct for deviations from the normal distribution. The mean of these 15 coefficients was calculated, the inverse of the Fisher transformation was performed and the 95% confidence interval (95% C.I.) for each correlation coefficient was calculated. Also the proportional change from baseline to the highest (glucose, insulin, glucagon, GIP, CCK and GLP-1) or lowest (ghrelin) value was calculated.

Statistical analysis of the data was carried out using the SAS statistical software package (SAS/STAT Version 8.2, SAS Institute, Cary, NC). A P value <0.05 (two-sided) was considered statistically significant in all analyses. Results are given as mean ± SD.

Results

Gastric emptying

Gastric emptying was indirectly estimated using acetaminophen absorption. Figure 5.1 shows the postprandial acetaminophen concentrations and the AUCs of the acetaminophen response. After the saline infusion, acetaminophen concentrations in plasma increased rapidly, reaching maximum values of 16.2 ± 4.0 µg/ml at 120 minutes. Acetaminophen concentrations after the GLP-1 infusion reached maximum concentrations of only 12.9 ± 3.2 µg/ml at 180 minutes. The acetaminophen responses showed an overall time X treatment interaction ($p < 0.0001$). Partial tests showed that the acetaminophen responses after GLP-1 and saline were different ($p < 0.0001$), namely the AUC was smaller (about 32%) after GLP-1 infusion as compared to saline infusion ($p < 0.0001$).

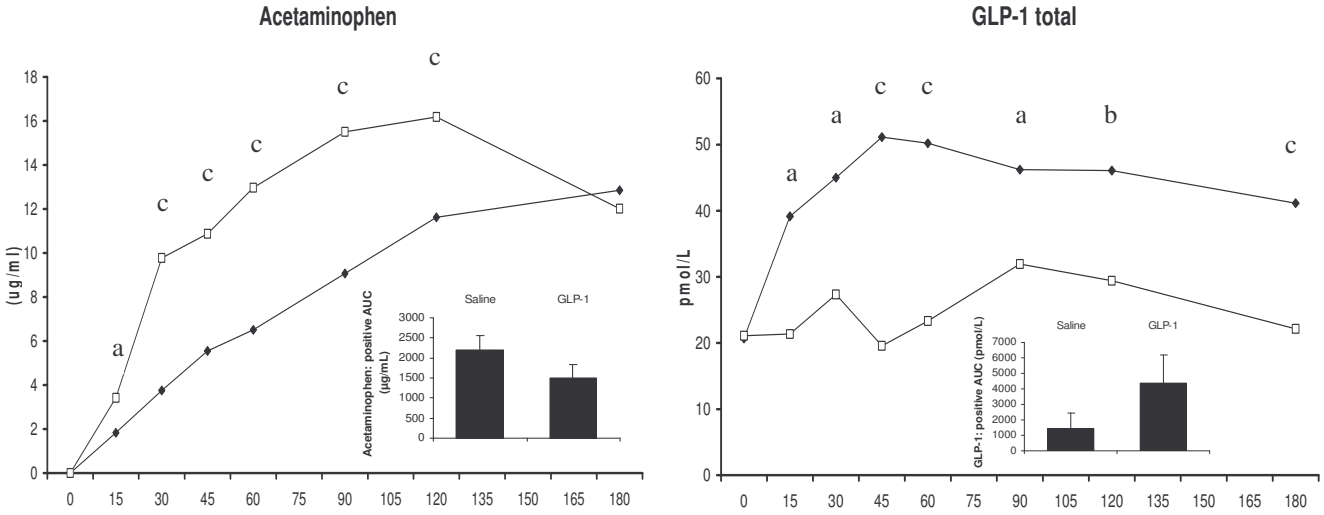


Figure 5.1: Three hour postprandial responses of acetaminophen and GLP-1 (total) (n=15) after infusion of saline or GLP-1 (active). -□-: saline, -♦-: GLP-1. By ANOVA, there was a significant time X treatment effect for acetaminophen ($p < 0.0001$) and GLP-1 ($p < 0.001$). The different letters indicate the level of statistical significance. a: $p < 0.01$, b: $p < 0.001$, c: $p < 0.0001$. Inserted graphs: mean ± SD AUC of the acetaminophen and GLP-1 responses. By ANOVA, there was a significant treatment effect of the AUCs for GLP-1 and acetaminophen (both $p < 0.0001$).

Blood parameters

Glucagon-Like Peptide 1(GLP-1)

Total GLP-1 concentrations following infusion of GLP-1 (7-36) amide were within the physiological range (220;254). GLP-1 concentrations increased about 50% after the saline infusion, and about 150% after the GLP-1 infusion, reaching peak values at 90 and 45 minutes, respectively (see figure 5.1). The GLP-1 responses showed an

overall interaction between time X treatment ($p < 0.0005$). Partial tests showed that GLP-1 responses after GLP-1 and saline infusion were different ($p < 0.0001$), as well. In addition, the AUCs of GLP-1 were larger (about 207%) after GLP-1 infusion compared to infusion of saline ($p < 0.0001$).

Ghrelin

Total ghrelin (ghrelin)

Ghrelin responses and AUCs are presented in figure 5.2. Ghrelin concentrations decreased after both the saline infusion (-18%) and the GLP-1 infusion (-15%), reaching lowest values at 60 and 120 minutes, respectively. The ghrelin responses showed an overall interaction between time X treatment ($p < 0.0001$). Partial tests showed that the ghrelin responses after GLP-1 and saline were different ($p < 0.05$). The AUCs of the ghrelin responses were not different between the GLP-1 and saline infusions. Ghrelin concentrations tended to be lower ($p < 0.10$) at 90 and 120 minutes after the saline infusion as compared to the GLP-1 infusion.

Active ghrelin

Active ghrelin concentrations decreased after the saline (-18%) and the GLP-1 (-33%) infusions, reaching lowest values at 45 and 60 minutes, respectively (figure 5.2). There was no overall treatment effect.

Glucose

Serum glucose responses and AUCs are presented in figure 5.2. Glucose concentrations increased about 24% after the saline infusion, reaching peak values at 30 minutes. In contrast, during GLP-1 infusion, glucose concentrations decreased by about 11%. The glucose responses showed an overall interaction between time X treatment ($p < 0.0001$) and partial tests showed that the GLP-1 and saline responses differed from each other ($p < 0.0001$). The AUC of glucose was smaller (about 67%) after the GLP-1 infusion than after the saline infusion ($p < 0.001$).

Insulin

Figure 5.2 presents the serum insulin responses and AUCs. GLP-1 infusion reduced postprandial insulin concentrations as compared to saline infusion (2.5 fold increase compared to 8 fold increase after saline). ANOVA for repeated measures showed an overall interaction between time X treatment ($p < 0.0001$). Partial tests showed that insulin responses after GLP-1 and saline were different ($p < 0.0001$), namely the AUC of the insulin response was smaller (about 45%) after the GLP-1 infusion as compared to the saline infusion ($p < 0.0001$).

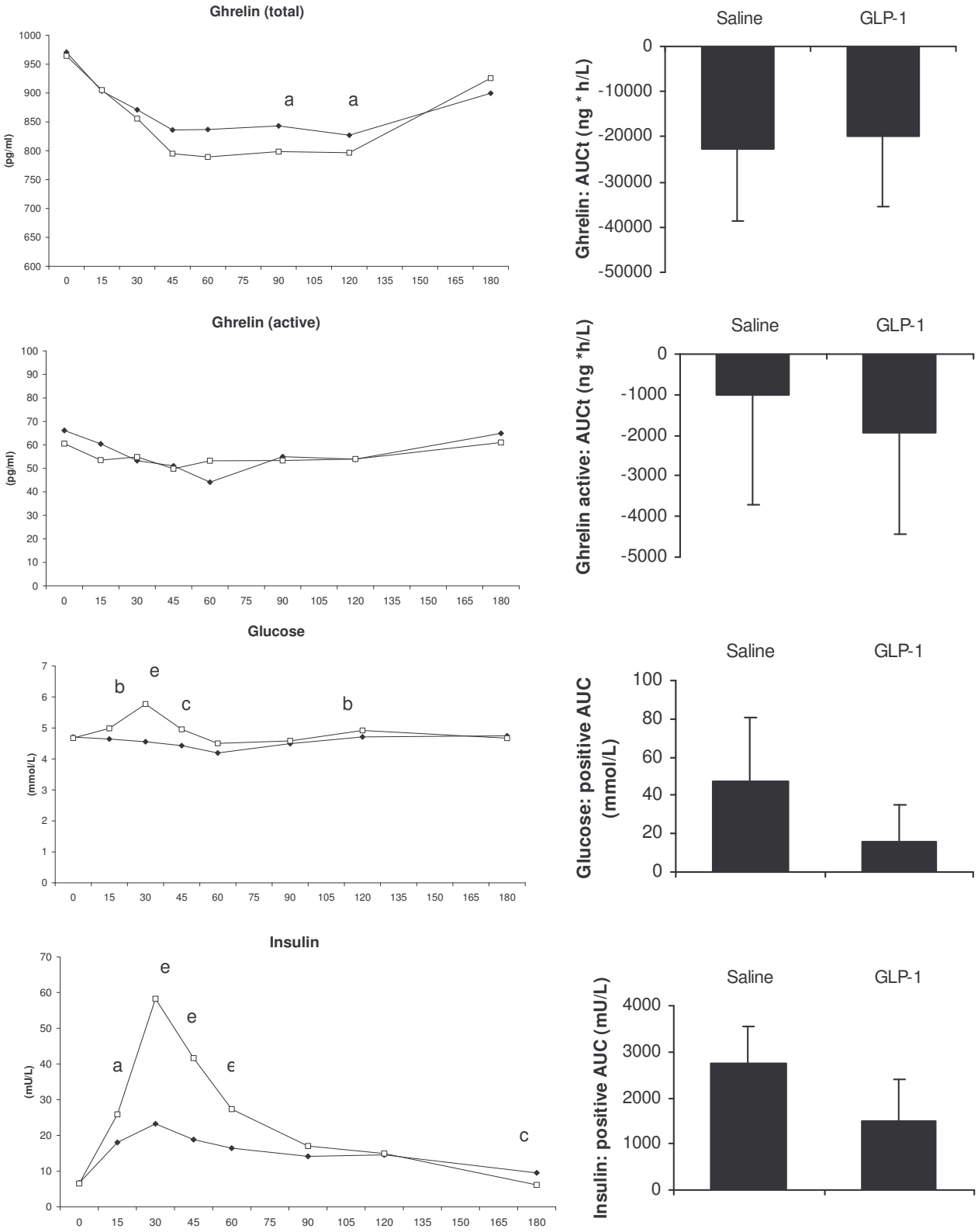


Figure 5.2 Three hour postprandial responses of ghrelin (total), ghrelin (active), glucose and insulin in 15 men after infusion of saline or GLP-1 (active). Left: -□-: saline, -♦-: GLP-1. By ANOVA, there was a significant time X treatment effect for ghrelin total, insulin and glucose (all $p < 0.0001$). The different letters indicate the level of statistical significance. a: $p < 0.10$, b: $p < 0.05$, c: $p < 0.01$, d: $p < 0.001$, e: $p < 0.0001$. Right: mean ± SD AUC of the responses. By ANOVA, there was a significant treatment effect of the AUCs for insulin ($p < 0.0001$) and glucose ($p < 0.001$).

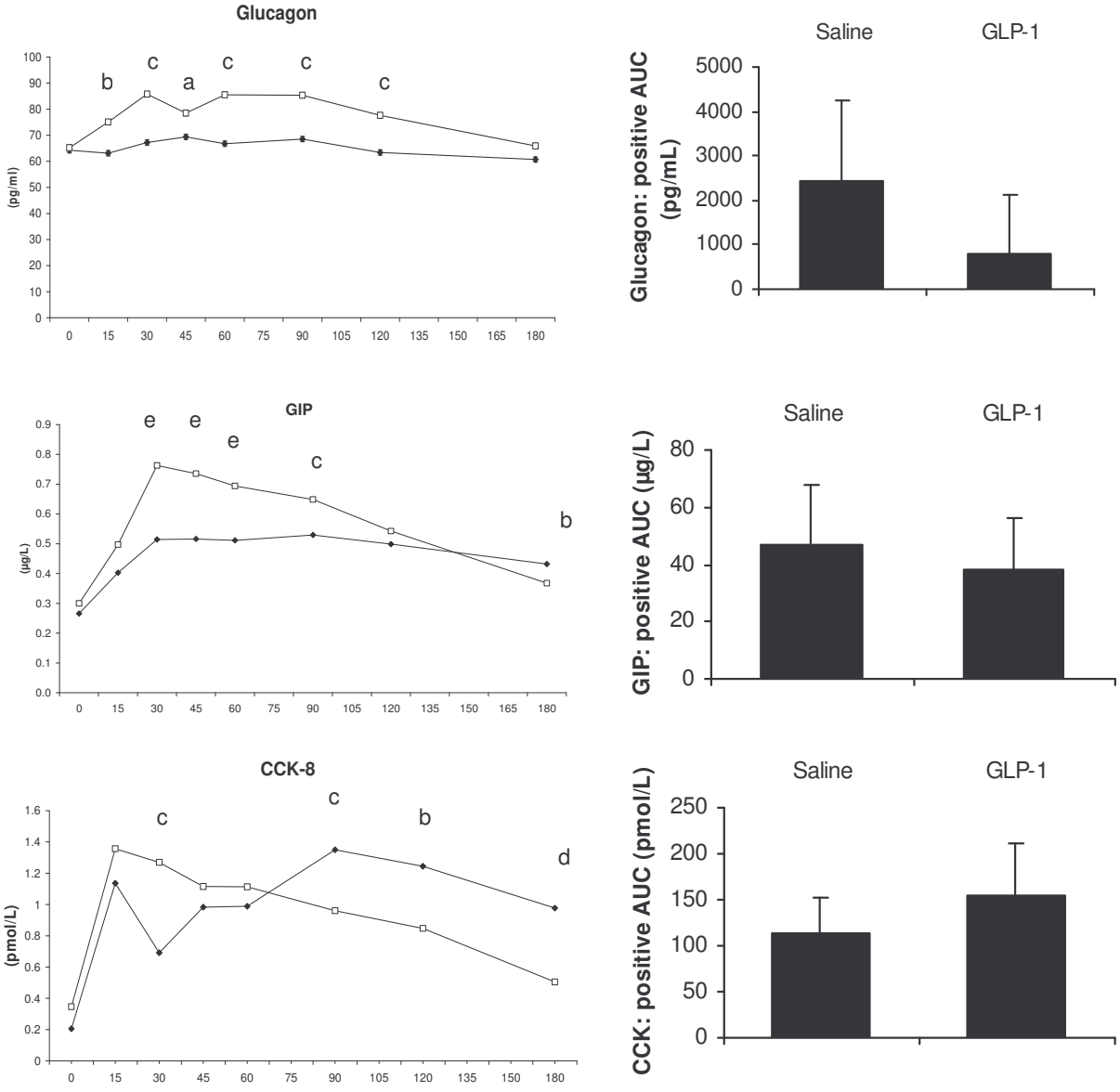


Figure 5.3 Three hour postprandial responses of glucagon, GIP and CCK-8 in 15 men after infusion of saline or GLP-1 (active). Left: -□-: saline, -♦-: GLP-1. By ANOVA, there was a significant time X treatment effect for glucagon, GIP (all $p < 0.0001$) and CCK ($p < 0.01$). The different letters indicate the level of statistical significance. a: $p < 0.10$, b: $p < 0.05$, c: $p < 0.01$, d: $p < 0.001$, e: $p < 0.0001$. Right: mean ± SD AUC of the responses. By ANOVA, there was a significant treatment effect of the AUCs for CCK and glucagon (both $p < 0.05$).

Glucagon

Glucagon concentrations increased about 31% after the saline infusion, and reached peak values at 30 minutes, but were hardly affected by the GLP-1 infusion (+ 8%) (figure 5.3). The glucagon responses showed an overall time X treatment effect ($p < 0.001$). Partial tests showed that the glucagon responses were different between the two treatments ($p < 0.05$). The AUC of glucagon was smaller (about 67%) after the GLP-1 infusion than after the saline infusion ($p < 0.05$).

Glucose-dependent insulinotropic polypeptide

Plasma GIP responses and AUCs are presented in figure 5.3. GIP concentrations increased about 150% after the saline infusion and about 100% after the GLP-1 infusion, reaching peak values at 30 and 90 minutes, respectively. The GIP responses showed an overall time X treatment interaction ($p < 0.0001$). Partial tests showed that also the GIP responses following GLP-1 and saline infusion were different ($p < 0.0001$). Although there was an overall treatment effect ($p < 0.01$) of the AUCs of the GIP responses, the AUCs of the GIP responses after saline and GLP-1 infusion were not different ($p = 0.11$). GIP concentrations were significantly higher after saline than after GLP-1 infusion between 30 and 90 minutes ($p < 0.05$).

Cholecystokinin

CCK concentrations increased about 3 fold after the saline infusion reaching peak values at 15 minutes (figure 5.3). CCK concentrations showed a biphasic response following GLP-1 infusion. Initially CCK concentrations increased about 4.5 fold, but then dropped at 30 minutes, followed by a steady increase, reaching peak values at 90 minutes (6.5 fold increases compared to baseline values). The CCK responses showed an overall time X treatment interaction ($p < 0.01$). Partial tests showed that the CCK responses following GLP-1 and saline infusion were different ($p < 0.001$). The AUC of the CCK response after GLP-1 infusion was larger (about 37%) than after the saline infusion ($p < 0.05$).

Correlations

Acetaminophen concentrations, used as an indirect measurement of gastric emptying rate, were correlated with concentrations of the other blood parameters to assess the relation of these parameters with gastric emptying.

Acetaminophen concentrations were inversely correlated with total ghrelin concentrations both during saline infusion ($r = -0.76$; 95% C.I. = -0.90, -0.49) and during GLP-1 infusion ($r = -0.47$; 95% C.I. = -0.76, -0.04) (see table 5.3). Also GIP

concentrations were positively associated with acetaminophen concentrations (saline: r = 0.54; 95% C.I. = 0.13, 0.79; GLP-1 r = 0.59; 95% C.I. = 0.19, 0.82). CCK concentrations were only positively associated with acetaminophen concentrations during GLP-1 infusion (r = 0.55; 95% C.I. = 0.14, 0.80), but not during infusion of saline (r = 0.36; 95% C.I. = -0.10, 0.69). In contrast, insulin concentrations were not correlated at all with acetaminophen concentrations (saline: r = 0.15; 95% C.I. = -0.32, 0.55, GLP-1: r = 0.12; 95% C.I. = -0.34, 0.54). There was no association between acetaminophen and active ghrelin concentrations, as well (saline: r = -0.10; 95% C.I. = -0.52, 0.35, GLP-1: r = -0.08; 95% C.I. = -0.50, 0.38).

Correlation coefficients between ghrelin concentrations and concentrations of other blood parameters were calculated to assess the relation of these blood parameters with ghrelin.
Correlations between ghrelin concentrations and other parameters are presented in table 5.3. The postprandial responses of total ghrelin and active ghrelin, were positively correlated during GLP-1 infusion (r = 0.56; 95% C.I. = 0.15, 0.80), but not during saline infusion (r = 0.35; 95% C.I. = -0.11, 0.69). Total ghrelin concentrations were strongly inversely correlated with concentrations of the insulinotropic peptide GIP (saline: r = -0.74; 95% C.I. = -0.89, -0.45; GLP-1: r = -0.63; 95% C.I. = -0.84, -0.27) and were also inversely correlated with concentrations of CCK (saline: r = -0.54; 95% C.I. = -0.80, -0.13; GLP-1: r = -0.50; 95% C.I. = -0.77, -0.08). Ghrelin concentrations were also inversely associated with concentrations of the other insulinotropic peptide, GLP-1, during GLP-1 infusion (r = -0.58; 95% C.I. = -0.81, -0.18), but this association was not present during infusion of saline (r = -0.16; 95% C.I. = -0.56, 0.31). Conversely, glucagon concentrations were inversely associated with ghrelin concentrations during saline infusion r = -0.52; 95% C.I. = -0.78, -0.10), but not after infusion of GLP-1 (r = -0.16; 95% C.I. = -0.56, 0.30). In contrast with our hypothesis, total ghrelin concentrations were not associated with insulin concentrations (saline: r = -0.36; 95% C.I. = -0.69, 0.09; GLP-1: r = -0.42; 95% C.I. = -0.73, 0.03). There were no associations between active ghrelin concentrations and other physiological parameters than total ghrelin (see table 5.3).

Table 5.3 Mean correlation coefficient (r) with 95% confidence intervals of the relation between physiological parameters by treatment (n=15)

	Saline		GLP-1	
	ghrelin total	GIP	ghrelin total	GIP
acetaminophen	-0.76 (-0.90, -0.49)	0.54 (0.13, 0.79)	-0.47 (-0.76, -0.04)	0.59 (0.19, 0.82)
GLP-1	-0.16 (-0.56, 0.31)	0.19 (-0.27, 0.58)	-0.58 (-0.81, -0.18)	0.49 (0.06, 0.77)
insulin	-0.36 (-0.69, 0.09)	0.81 (0.57, 0.92)	-0.42 (-0.73, 0.03)	0.64 (0.28, 0.84)
GIP	-0.74 (-0.89, -0.45)		-0.63 (-0.84, -0.27)	
CCK	-0.54 (-0.80, -0.13)	0.71 (0.38, 0.87)	-0.50 (-0.77, -0.08)	0.61 (0.24, 0.83)
Ghrelin active	0.35 (-0.11, 0.69)	-0.28 (-0.64, 0.18)	0.56 (0.15, 0.80)	-0.18 (-0.58, 0.28)

The Pearson correlation coefficients of the relation between ghrelin and other physiological parameters were calculated per subject. The mean correlation coefficients together with the 95% confidence intervals after Fisher Z-transformation are presented

Discussion

Animal studies show that distension and chemo sensitization of the stomach are insufficient to induce a ghrelin response (358), suggesting that post gastric feedback is required. In this study, we investigated the association between gastric emptying rate and the postprandial ghrelin response, and whether insulin or other post gastric processes are involved in the postprandial ghrelin response. The results of this study show that ghrelin responses are associated with the gastric emptying rate, supporting our hypothesis that ghrelin requires post gastric feedback. Our data did not support our hypothesis that insulin is involved in the postprandial regulation of ghrelin secretion. On the other hand, ghrelin concentrations were strongly associated with GIP and CCK concentrations.

In this study, the postprandial responses of different regulators of food intake were investigated. The association between the different measures were investigated by correlational analysis. Although correlations do provide more insight into the associations between different measures, they do not provide a causal relationship. Therefore, results should be confirmed by future experiments.

The design of the study involved infusion of GLP-1, which might have affected concentrations of the other variables. However, the relatively low dose of GLP-1 amide infused, resulted in total GLP-1 concentrations that remained within the physiological range (220;254). GLP-1 infusion reduced the insulin response following a meal, despite the fact that GLP-1 is an insulinotropic hormone. This observation has also been reported by Nauck et al (246) and suggests that the effect of GLP-1 infusion on gastric emptying outweighs the insulinotropic effects of GLP-1. Nevertheless, there are indications that GLP-1 infusion directly affected ghrelin concentrations. Our first hypothesis was that postprandial ghrelin response requires post gastric feedback. However, ghrelin concentrations were only significantly higher between 90 and 120 minutes, despite that GLP-1 infusion did reduce the gastric emptying rate and ghrelin concentrations were correlated with acetaminophen absorption. Possibly, infusion of GLP-1 suppressed ghrelin secretion directly, since a study in the isolated rat stomach showed that GLP-1 decreases ghrelin secretion (193). This direct suppressive effect may have confounded the association between ghrelin and gastric emptying. Nevertheless, the association between ghrelin and GLP-1 has not been directly tested in humans yet. GLP-1 infusion in the absence of food intake may provide more insight into the direct effects of GLP-1 on ghrelin concentrations. The inverse association between ghrelin and acetaminophen concentrations during saline and GLP-1 infusion suggests that the postprandial ghrelin response is strongly related to the gastric emptying rate, however other studies are needed to confirm this. Investigation of the effects of multiple different treatments that increase or decrease emptying rate through differing mechanisms, on ghrelin secretion, may provide more information about the importance of post-gastric feedback for postprandial ghrelin secretion.

In the second hypothesis, we tested whether insulin is the post gastric factor that is involved in postprandial ghrelin secretion. To show that insulin provides feedback to ghrelin we investigated the correlation between ghrelin and insulin concentrations. In contrast with our expectations, insulin concentrations were not significantly correlated with ghrelin concentrations (or with acetaminophen absorption). We do not believe that GLP-1 infusion may have confounded this relation, because a similar weak

correlation was found after saline infusion. Ghrelin concentrations were inversely correlated with GIP and CCK concentrations. So far, little is known about the relation between GIP and ghrelin secretion. Only few studies investigated the relation between GIP and ghrelin secretion and showed contradictive results (3;292). The strong inverse association between GIP and ghrelin concentrations, which were observed in this study, suggests that GIP, instead of insulin, might act as the post gastric feedback signal for the postprandial ghrelin response. There were also indications for a role of CCK herein. These results are however correlative and do therefore not prove a causal role of GIP and CCK in ghrelin secretion. Future studies should directly investigate this causality. For example the effect of GIP and CCK antagonists on postprandial ghrelin secretion, may provide more information, as well as infusion of GIP and CCK.

There are two major molecular forms of ghrelin: acylated ghrelin, which has a n-octanoylation at serine 3; and unacylated ghrelin (164). Until recently, only the acylated form of ghrelin was thought to be biologically active. The current perspective is that also unacylated (desacyl) ghrelin exerts some biological activities (14;45;46;110). To gain more insight into the postprandial responses of acylated (active) and unacylated ghrelin concentrations, we measured both active ghrelin as well as total ghrelin concentrations, which is the sum of acylated and unacylated ghrelin. Both active and total ghrelin concentrations decreased in the postprandial period. However, only total ghrelin concentrations were different between the two treatments. The effects observed for total ghrelin may be mediated by active ghrelin. However, due to the large variations in active ghrelin concentrations we may not have had sufficient statistical power to detect differences.

Conclusions

The results of this study show that postprandial ghrelin responses are inversely associated with the gastric emptying rate, and support the hypothesis that ghrelin requires post gastric feedback. In these experimental conditions, our data did not support the hypothesis that insulin regulates the postprandial regulation of ghrelin secretion. Conversely, total ghrelin concentrations were associated with GIP and CCK concentrations, suggesting a role of GIP and CCK in postprandial ghrelin secretion.

Acknowledgements

We want to express our gratitude to the volunteers who participated in the study; Henriëtte Fick, Inge van den Assum, José Jacobs, Soesila Sukhraj, Linda Kok, Eric Busink, Jan Catsburg, Hans Verplanke and all other people from the Metabolic Research Unit and laboratories who assisted in the organization of the study and analyses of the blood samples; Stéphane Doat, Susanne Westenbrink and Petra van Aken for the preparation of the study substances; Cees de Graaf for his advice on the design of the study; Diane ter Doest and Linda van den Bosch for performing data management; and Cor Kistemaker and Carina Rubingh for their support with the statistical analyses.

This study is financially supported by: Dutch Ministry of Economic Affairs, Dutch Ministry of Education, Culture and Science, Dutch Ministry of Health, Welfare and Sport and Danone Vitapole
None of the authors had a conflict of interest.

6

Postprandial ghrelin kinetics are associated with the intermeal interval in time-blinded normal weight men, but not in obese men

Wendy Blom
Marieke Advocaat
Kees de Graaf
Anne Lluch
Annette Stafleu
Gertjan Schaafsma
Henk Hendriks

Wageningen University, Wageningen, Netherlands
TNO Quality of Life, Zeist, Netherlands
Danone Vitapole Nutrivaleur, Palaiseau Cedex, France

Submitted

Abstract

Background:
Plasma ghrelin concentrations rise gradually before a meal and decrease immediately after eating, suggesting a role for ghrelin in meal initiation.
Objective:
To investigate the role of ghrelin in meal initiation.
Subjects and methods:
Nine normal-weight (age: 33.2 ± 4.8 y, BMI: 23.2 ± 0.5 kg/m^2) and eleven obese (age: 40.8 ± 4.7 y, BMI: 33.2 ± 0.8 kg/m^2) men were put on a three-day energy restrictive and a three-day energy balanced diet separated by one month. Each diet was followed by a time-blinded (overnight) stay at the research facility. Subjects received a breakfast (preload) and were instructed to ask for lunch when they felt hungry. To test whether ghrelin serves a critical role in spontaneous meal initiation, the relation between ghrelin kinetics and the intermeal interval (IMI), appetite scores and energy intake during lunch was assessed.
Results:
Lunch request was preceded by an increase in ghrelin, reaching at least 93% of fasting values. These preprandial increases in ghrelin concentrations were not associated with the IMI. However, postprandial decreases in ghrelin concentrations ($r = -0.54$; $p < 0.05$) and the AUC of the ghrelin response ($r = -0.57$, $p = 0.01$) were associated with the intermeal interval, but not with *ad libitum* energy intake during lunch. The association was observed after an energy balanced and energy restricted diet, in normal weight subjects only, not in obese subjects.
Conclusion:
The results support a role for ghrelin in meal initiation in normal weight but not in obese men.

Introduction

Ghrelin, a peptide predominantly produced by the stomach (164), appears to play an important role in the regulation of food intake. Intravenous infusion of ghrelin stimulates food intake and enhances appetite in humans (369). In addition, plasma ghrelin concentrations rise gradually before a meal and decrease immediately after eating (67;324), suggesting a role for ghrelin in meal initiation. Postprandial ghrelin concentrations appear to be correlated with subjective appetite (35;66;84). Further evidence for a role of ghrelin in meal initiation is provided by the observation that the postprandial decrease in ghrelin concentrations is dependent on energy intake, and is not affected by intake of water (35). However, in almost all studies investigating pre- and postprandial ghrelin responses, meals were not voluntarily consumed, but on a scheduled basis disregarding the subject's appetite. Such design allows for the possibility that preprandial increases in ghrelin concentrations were part of a cognitive, anticipatory response to upcoming meals. Thus far, only two studies (50;66) have investigated the role of ghrelin as a meal initiator in subjects blinded for external cues related to time or food. Both studies were performed in normal weight men, but not in obese men. Ghrelin secretion may be disturbed in obese men: ghrelin secretion is not only down regulated in obese individuals as compared to lean subjects (96;302;318;370), the decline in plasma ghrelin after a meal is also blunted (96;318). Energy restriction is known to increase ghrelin concentrations in obese subjects (68) and might therefore also influence the effects of ghrelin in those subjects.

The objective of this study was to investigate whether ghrelin is involved in meal initiation and specifically whether the ghrelin response and effects are dependent on BMI and energy requirements.

If plasma ghrelin concentrations serve a critical physiological role in spontaneous meal initiation in humans, plasma ghrelin concentrations or its fluctuations should be related with the time between two meals (intermeal interval) and with subjective appetite measures. Therefore, associations between (changes in) ghrelin concentrations and intermeal interval (IMI), subjective measures of appetite and energy intake during lunch were studied. Comparisons were made according to BMI (normal weight and obese subjects) and treatments (after energy restricted diet and energy balanced diet).

Subjects and methods

Subjects

The study was conducted at TNO Quality of Life, Zeist, The Netherlands, where obese and normal weight male subjects were recruited from a pool of volunteers and by advertisements in local newspapers. After being informed about the study, both verbally and in writing, each subject gave voluntary written informed consent. Health was assessed by a health and lifestyle questionnaire, physical examination, and blood and urine analysis. Each subject reported a Western lifestyle, regular Dutch dietary habits and a stable body weight for at least one month prior to the study. Smokers, restrained eaters, as assessed with the Dutch Eating Behaviour Questionnaire (333) (obese: score of restriction > 3.25; non-obese: score of restriction > 2.5), and subjects who reported slimming or who where on a medical prescribed, vegan or macrobiotic diet were excluded. Also subjects who were on medication that may have influenced appetite and sensory functioning or who reported metabolic or endocrine disease, gastro-intestinal disorders or a history of medical or surgical events that may have affected study outcome were excluded.
Eleven obese (BMI: 33.2 ± 0.8 kg/m^2; age: 40.8 ± 4.7 y) and nine normal weight (BMI: 23.2 ± 0.5 kg/m^2; age: 33.2 ± 4.8 y) healthy subjects completed the study. Baseline characteristics of the twenty subjects are shown in table 6.1.

Study design

The experiment had a randomised and cross-over design. Each subject followed a three-day energy-restrictive (64% restriction) diet and a three-day energy-balanced diet, both followed by a 23-h time-blinded stay at the research facility. The two treatments were separated by a wash-out period of approximately 1 month. Subjects were randomised for BMI and age. The study had a staggered start, with five subjects starting per day.

Study protocol

Energy requirements
The energy-restricted and energy-balanced diets were composed based on the estimated daily energy requirement of that person. The subjects' daily energy requirement (MJ/d) was estimated by calculating the basal metabolic rate (BMR) according to Schofield's equation (298): BMR (MJ) = 0.063 * weight (kg) + 2.896 (men 18-30 y) or 0.048 * weight (kg) + 3.653 (men 30-60 y), and multiplying this estimate with a correction factor for physical activity level (PAL). The average

physical activity level was estimated by a short retrospective physical activity questionnaire containing activity concentrations based on a WHO report (366) and a compendium of physical activities (5).

Table 6.1 Subject characteristics at baseline

	All (n=20)		Normal weight (n=9)		Obese (n=11)	
	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range
Age (y)	37.0 ± 15.0	18-57	33.2 ± 4.8	19-51	40.8 ± 4.7	18-57
Weight (kg)	95.9 ± 18.2	68.9-129.6	78.7 ± 1.8*	68.9-85.4	109.9 ± 3.3	93.8-129.6
Height (m)	1.83 ± 0.05	1.73-1.94	1.84 ± 0.02	1.73-1.94	1.82 ± 0.01	1.77-1.90
BMI (kg/m^2)	28.7 ± 5.5	21.4-36.0	23.2 ± 0.5*	21.4-26.3	33.2 ± 0.8	29.1-36.0
Waist/hip ratio	0.96 ± 0.05	0.82-1.03	0.92 ± 0.02*	0.82-0.99	1.00 ± 0.01	0.96-1.03
DEBQ[1]	2.2 ± 0.6	1.0-3.1	1.8 ± 0.6*	1.0-2.5	2.6 ± 0.4	1.5-3.1
HOMA[2]	3.03 ± 3.15	0.45-14.93	1.37 ± 0.65*	0.45-2.25	4.38 ± 3.76	1.12-14.93
QUICKI[3]	0.34 ± 0.04	0.26-0.44	0.37 ± 0.03*	0.34-0.44	0.32 ± 0.03	0.26-0.38
$ISI_{0,120}$[4]	130.9 ± 86.9	44.3-456.5	182.0 ± 108.7*	102.6-456.5	89.1 ± 24.7	44.3-133.0

[1] Score on the restrained-eating scale of the Dutch Eating Behaviour Questionnaire. Range of possible scores on the restrained-eating scale, 1.0-5.0. (332;333)

[2] Homeostasis Model Assessment (insulin sensitivity index) (214)

[3] Quantitative Insulin Sensitivity Check Index (insulin sensitivity index) (157)

[4] Insulin Sensitivity Index (insulin sensitivity index) (125)

* $p < 0.05$: significant difference as compared with the obese subjects

Diets

Each subject received a three-day energy-restricted diet, containing approximately 36% of the estimated energy requirement of the subject, and a three-day energy-balanced diet, containing approximately 110% of the estimated energy requirement. Subjects were given 110% of their daily energy requirement, because our experience is that 100% of the calculated daily energy requirement gives an underestimation. The energy contents of the diets were calculated by taking 110% or 33.3% of the individual energy needs. Subjects did not receive foods with an energy content which exactly matched their calculated energy requirement, but subjects were divided in 'energy-categories' (spread: 1.0 MJ), which matched the best with their individual energy requirement. Both diets consisted of standard Dutch food products.

Each diet day, subjects were supplied with boxes containing all the food items they were allowed to eat during that day, except for water, coffee and tea, which they were allowed to drink freely (without sugar or milk). Subjects were instructed to eat only the

food items provided in the boxes and to return the packaging, containing all foods that were left over, if any, to the research facility at their next visit (the same day or the next day). In the washout period, subjects were instructed to eat the amount which they were used to eat before this study.

23h time-blinded period

On day 3, subjects arrived at the research centre at 18:00h. They were individually housed in rooms isolated from external cues such as daylight, sound and time till 17:00 h the next day. Subjects received dinner in their room. The composition of the dinner (macaroni Bolognese with cheese) was the same for each subject and during both treatments, though the amount of energy consumed differed between subjects, depending on energy-requirement and type of diet. After dinner, subjects were not allowed to eat or drink (except water) anymore. In the morning of day 4, the subjects were awakened at 07:00h (time unknown to the subjects) and an indwelling cannula was placed in the forearm of the subjects. They received a standard Dutch breakfast, containing approximately 13% of the subjects' daily energy requirement. Blood samples were collected just before (t=0 min) and after breakfast (t=30, 50, 65, 90, 120, 170, 240, 305, 355, 425, 480 min, but only until lunch request). Directly after each blood sample subjects filled out visual analogue scales (VAS) to measure subjective measures of hunger, fullness, desire to eat and prospective food consumption. Subjects were instructed to ask for lunch if they felt hungry. On request, subjects received an *ad libitum* buffet-style lunch, which consisted of standard Dutch food items. Subjects were instructed to eat until they were satiated. In order to prevent habitual intake, foods were provided in unusual portions sizes.

Blood samples were collected after lunch request, but before eating (t=0 min). Directly after each blood sample subjects filled out VAS to measure subjective appetite.

The study was performed according to the ICH Guideline for Good Clinical Practice (ICH topic E6, adopted 01-05-1996 and implemented 17-01-1997) and was approved by the independent Medical Ethics Committee of TNO.

Body weight

Subjects were weighed every morning (fasted) with indoor clothing, without shoes and with empty pockets on a digital balance accurate to 0.1 kg.

Energy intake during lunch

All food items were weighed before and after the *ad libitum* lunch. Energy intake and macronutrient composition of the consumed foods were calculated by use of the computer programme SAS (SAS/STAT Version 6.12, SAS Institute, Cary, NC).

Blood sampling and biochemical analyses

For plasma, blood was collected in Vacutainer® tubes containing K_3EDTA as coagulant and put in ice water immediately. For serum, blood was collected in Vacutainer® tubes containing clot activator. All tubes were centrifuged for 15 minutes at 2000 G at 4°C. Plasma and serum were removed and stored at -70°C and -18°C respectively, until analysis.
Serum glucose was determined using a commercial test kit (Roche Diagnostics GmbH, Mannheim, Germany) on a Hitachi 911 automatic analyser (Hitachi Instrument Division, Ibaraki-ken, Japan), with intra-assay CVs ranging from 0.7% to 0.9% depending on the concentration. Serum insulin was measured using AIA-600 Immunoassay Analysator, with intra-assay CVs which ranged between 4.3% and 5.8% dependent on the concentration. Total plasma ghrelin was measured using a commercially available radioimmunoassay (RIA) kit (Linco Research Inc., St. Charles, USA). The mean intra-assay CV was 10.0% at a concentration of 1.0 µg/L, and 3.3% at a concentration of 1.5 µg/L. Serum FFA was determined using a commercially available test kit (Randox Laboratories LTD, Ardmore, UK) on a Hitachi 911 automatic analyser (Hitachi Instrument Division, Ibaraki-ken, Japan), with intra-assay CVs ranging from 6.0% to 8.3% (n= 5) depending on the concentration.

Subjective appetite

Subjective appetite was evaluated using visual analogue scales (VAS) for hunger, fullness, desire to eat and prospective food consumption (103;314). Visual analogue scales consisted of 150 mm horizontal lines, with Dutch wordings anchored at each end expressing the most positive or negative sensation (i.e. I have never been more hungry/ I am not hungry at all). Subjects drew a vertical line on the horizontal line corresponding to their appetite sensation. Visual analogue scales were scanned using TELE*form* ELITE software (TELE*form* ELITE, Version 6.1, Cardiff Software Inc., California, USA). Distances on the visual analogue scales were converted into scores between 0 and 100.

Statistical analyses

All variables are shown as arithmetic mean ± SD. The range represents the minimum and maximum value of the variable. Baseline group comparisons were made by using an unpaired two-sided Student t-test. Absolute and percentage changes in fasting ghrelin concentrations between the day 1 and day 4 of the diets were calculated and compared to baseline values by an unpaired two-sided Students t-test. Incremental areas under or over fasting concentrations were calculated. The term area under the curve (AUC) refers to both values, delineated as negative AUC and positive AUC (the latter for the area over the curve). The percentage of ghrelin concentrations at nadir concentrations and at meal request as compared to the fasting concentration before breakfast was calculated. Using Mixed Model ANOVA, Tukey adjusted, with BMI as a fixed factor, the intermeal interval, energy intake during lunch, percentage ghrelin at nadir and meal request, and AUC characteristics and fasting concentrations of the different variables were tested for a treatment or BMI effect or an interaction between treatment and BMI. If there was an interaction effect, partial tests were performed to compare treatment and BMI groups, pair wise. Associations between AUCs or changes in blood parameters and subjective measures of appetite, *ad libitum* energy intake and intermeal interval were calculated by means of Pearson correlation coefficients (r). The association between postprandial blood and appetite responses was calculated differently. Pearson correlation coefficient was calculated for each subject, based on 16 (8 time points, 2 treatments). On these individual correlations a Fisher's z-transformation was applied, in order to correct for deviations from the normal distribution. The mean of these 20 coefficients was calculated and the inverse of the Fisher transformation was performed and the 95% confidence interval (95% C.I.) for each correlation coefficient was calculated. Postprandial (absolute) changes from fasting concentrations to nadir (in case of ghrelin and FFA) concentrations or from fasting concentrations to maximum concentrations (in case of glucose and insulin) were calculated. Also preprandial changes from nadir concentrations to concentrations at meal request (in case of ghrelin and FFA) or from maximum concentrations to concentrations at meal request (in case of glucose and insulin), were calculated. The percentage change in concentration between 0 and 30 minutes and between 30 and 170 minutes was calculated to investigate relationships among changes in blood parameter concentrations and appetite scores over different time intervals. P-values < 0.05 were considered statistically significant. Statistical analysis of the data was carried out using the SAS statistical software package (SAS/STAT Version 8.2, SAS Institute, Cary, NC).

Results

Body weight

Normal weight subjects lost 1.1 ± 1.2 kg (range: -2.8; 1.1) of body weight during the energy balanced diet ($p = 0.03$) and 2.2 ± 0.6 kg (range: -2.9; -1.3) during the energy restricted diet ($p < 0.0001$). Obese subjects gained on average 0.6 ± 0.9 kg (range: -1.1; 2.7) of body weight during the energy balanced diet ($p = 0.07$) and lost on average 2.3 ± 0.9 kg (range: -3.6; -0.7) of body weight during the energy restricted period ($p < 0.0001$).

Ghrelin

Absolute and percentage changes in fasting ghrelin concentrations during the two diets are presented in table 6.2. During the energy restricted diet, fasting ghrelin concentrations significantly ($p < 0.01$) increased (8.2 ± 8.1 %) in obese subjects, but not in normal weight subjects or after the energy balanced diet.
Fasting ghrelin concentrations on day 4 were lower in obese subjects ($p = 0.05$) compared to normal weight subjects. There was a trend for higher ghrelin concentrations ($p = 0.07$) after the energy restrictive diet as compared to the energy balanced diet, in the obese only. Postprandial ghrelin responses are presented in figure 6.1. Mean ghrelin concentrations decreased following breakfast, reaching nadir concentrations of on average 88.0 ± 5.7% of fasting values. These nadir concentrations ranged between 76.1% and 96.6% and were borderline significantly lower in lean subjects as compared to obese subjects ($p = 0.07$) (see table 6.3). The diet did not affect nadir ghrelin concentrations ($p = 0.26$). At lunch request mean ghrelin concentrations were on average 105.8 ± 7.1% compared to fasting values, ranging between 91.1 and 119.8%. In two cases, plasma ghrelin concentrations were decreasing just before meal request, but these decreases were preceded by a preprandial rise in ghrelin concentrations. In fact, only in 6 subjects, ghrelin concentrations had not reached fasting values before or at meal request, although in these 6 subjects, ghrelin concentration had reached between 93.3% and 99.4% of fasting values.

Intermeal interval

The intermeal interval (IMI) is shown in table 6.3. The IMI appeared to be shorter in the obese subjects than in the normal weight subjects, after energy restriction, but this effect was not significant ($p = 0.11$). There was no significant treatment effect on IMI ($p = 0.22$) nor an interaction between BMI and treatment ($p = 0.24$).

Table 6.2 Absolute and percentage changes in fasting ghrelin concentrations between days 01 and 04 of the diets by BMI category and treatment (mean ± SD).

	All (n=20)		Normal weight (n=9)		Obese (n=11)	
	Energy balanced	Energy restricted	Energy balanced	Energy restricted	Energy balanced	Energy restricted
Absolute change (ng/L)	-2.3 ± 91.1	50.0 ± 102.7*	4.8 ± 110.6	14.2 ± 124.9	-8.1 ± 76.9	79.3 ± 73.9†
Percentage change (%)	-0.2 ± 7.2	5.5 ± 8.9*	0.5 ± 8.2	2.3 ± 9.3	-0.7 ± 6.6	8.2 ± 8.1†

*p < 0.05: significant change from baseline

† p < 0.01: significant change from baseline

Table 6.3 Intermeal interval, energy intake during lunch and changes in ghrelin concentrations by BMI category and treatment (mean ± SD).

			Energy balanced		Energy restricted	
	Normal weight	Obese	Normal weight	Obese	Normal weight	Obese
IMI (minutes)	323 ± 63	286 ± 53	323 ± 53	303 ± 58	322 ± 75	268 ± 42
Energy intake (MJ)	6.82 ± 1.57	6.78 ± 2.36	6.27 ± 1.09*	6.82 ± 2.60	7.36 ± 1.83	6.73 ± 2.21
Nadir ghrelin (% of baseline)	85.7 ± 6.3†	89.9 ± 4.4	87.2 ± 6.9	89.7 ± 3.9	84.3 ± 5.5	90.1 ± 5.1
Ghrelin at lunch request (% of baseline)	105.3 ± 6.6	106.2 ± 7.7	105.1 ± 8.5	108.3 ± 7.3	105.5 ± 4.3	104.1 ± 7.8

*p < 0.10: energy balanced diet versus energy restricted diet in normal weight subjects

† p < 0.10: normal weight subjects versus obese subjects

Subjective appetite

Fasting subjective measures of hunger ($p < 0.05$) and prospective food consumption ($p < 0.05$) were higher after short-term energy restriction than after an energy-balanced period. There was a trend ($p = 0.08$) for higher scores of desire to eat after energy restriction as compared to energy balance, but there was no effect of energy restriction on fasting fullness concentrations. Fasting appetite scores were not

affected by BMI. Postprandial appetite scores decreased, but there was no significant BMI or treatment effect of the AUCs of the four measures of appetite.

Energy intake during lunch

The energy intake during lunch is shown in table 6.3. There was no BMI ($p = 0.96$) or treatment ($p = 0.12$) effect on energy intake during lunch. There was an interaction between BMI and treatment ($p = 0.05$). In the normal weight subjects the energy intake during lunch after an energy restricted diet was borderline significantly higher than after an energy balanced diet ($p = 0.09$). No effect of diet on energy intake during the *ad libitum* lunch was observed in the obese men ($p = 0.99$).

Other blood parameters

Fasting insulin and glucose concentrations were higher in the obese subjects than in the normal weight subjects (insulin: $p < 0.0001$; glucose: $p < 0.01$), and lower after energy restriction (insulin: $p < 0.01$; glucose $p < 0.05$). Fasting concentrations of free fatty acids (FFA) were higher after energy restriction as compared to energy balance ($p < 0.0001$) (see figure 6.1). There was no significant BMI effect ($p = 0.68$) on fasting FFA concentrations.

Postprandial insulin, glucose and FFA responses are presented in figure 6.1. The positive AUC of the insulin response was larger in the obese subjects as compared to the normal weight subjects, both after the balanced ($p < 0.001$) as well as the energy restricted diet ($p < 0.05$).

In the normal weight subjects, but not in the obese subjects the postprandial glucose response (positive AUC) was larger after three days of energy restriction than after a three day energy balanced diet ($p < 0.001$).

The negative AUC of the FFA response was larger after energy restriction than after energy balance ($p < 0.0001$) and independent of BMI.

Correlations

If ghrelin plays a critical physiological role in spontaneous meal initiation in humans, plasma ghrelin concentrations or its fluctuations should be related with subjective measures of appetite and with the intermeal interval, a measure of meal initiation. We therefore studied associations between (changes in) ghrelin concentrations and intermeal interval (IMI) and subjective measures of appetite. Comparisons were made according to BMI (normal weight and obese subjects) and treatments (after energy restricted diet and energy balanced diet).

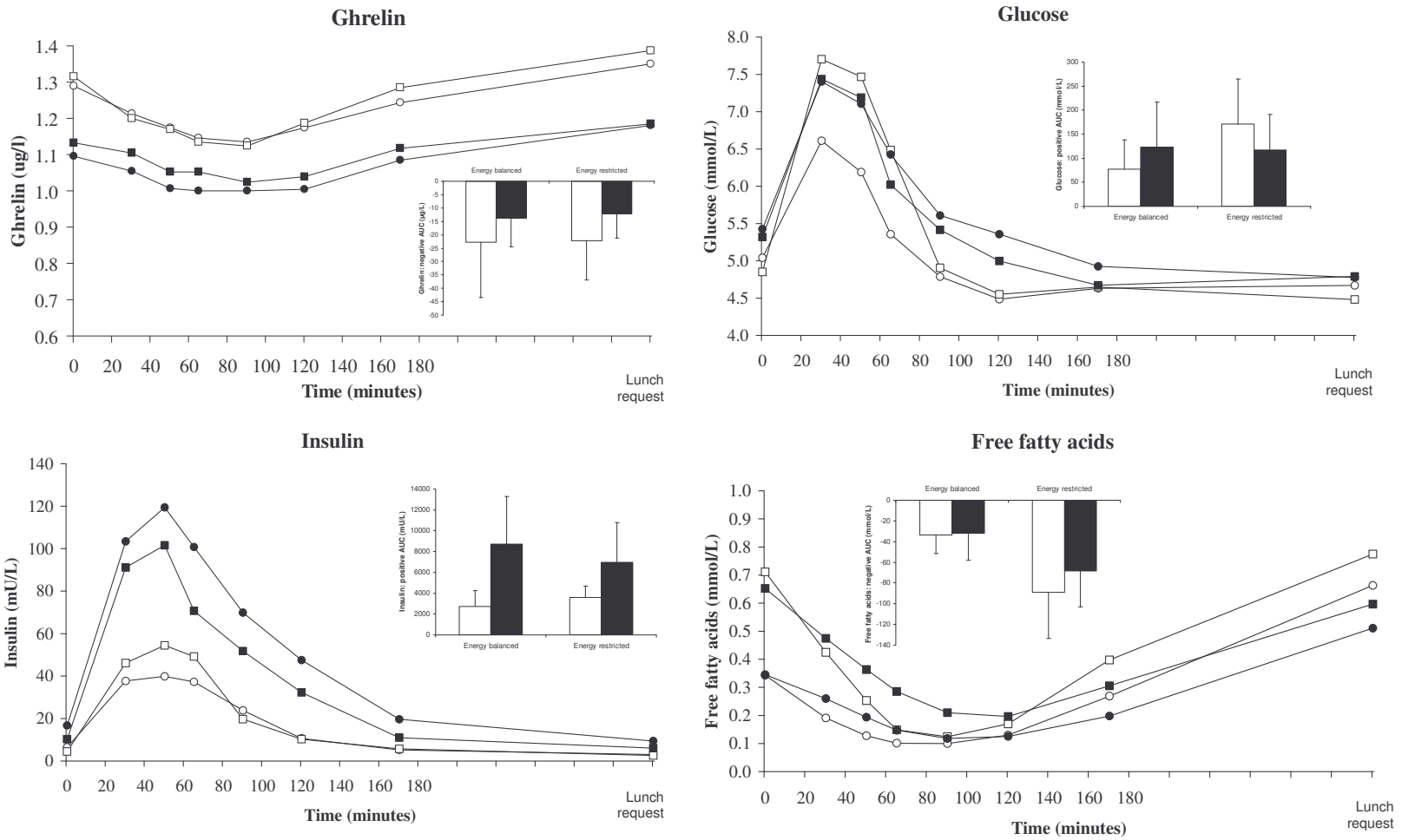


Figure 6.1 Mean ghrelin, insulin and glucose concentrations at day 04 after breakfast per BMI group and per treatment (○ = normal weight balanced, □ = normal weight restricted, ● = obese balanced, ■ = obese restricted). 170 minutes after breakfast is the last measurement point where data of all subjects was collected. After this time point some subjects requested lunch. The measurement indicated with 'lunch request' was taken after lunch request, but before eating. The time of this measurement differed between and within subjects. Inserted graphs: mean ± SD AUC of the ghrelin, glucose, insulin and free fatty acids responses. White bars: normal weight subjects, black bars: obese subjects.

Table 6.4 Pearson correlation coefficient (r) of the correlation between intermeal interval and ghrelin responses.

Intermeal interval	Preprandial ↑ ghrelin	Postprandial ↓ ghrelin	Negative AUC ghrelin
All (n=40)	0.29 (p = 0.07)	-0.37 (p < 0.05)	-0.42 (p < 0.01)
Normal weight (n=18)	0.16 (p = 0.52)	-0.54 (p < 0.05)	-0.57 (p = 0.01)
Obese (n=22)	0.27 (p = 0.23)	0.08 (p = 0.72)	0.00 (p = 1.00)
EB (n=20)	-0.02 (p = 0.94)	-0.23 (p = 0.32)	-0.34 (p = 0.14)
ER (n=20)	0.51 (p < 0.05)	-0.52 (p < 0.05)	-0.53 (p < 0.05)
Normal weight, EB (n=9)	-0.03 (p = 0.94)	-0.62 (p = 0.08)	-0.63 (p = 0.07)
Normal weight, ER (n=9)	0.40 (p = 0.28)	-0.53 (p = 0.14)	-0.58 (p = 0.10)
Obese, EB (n=11)	-0.06 (p = 0.85)	0.32 (p = 0.34)	0.11 (p = 0.76)
Obese, ER (n=11)	0.52 (p = 0.10)	-0.18 (p = 0.60)	-0.09 (p = 0.80)

EB: energy balanced diet, ER: energy restricted diet

Ghrelin and intermeal interval

Pearson correlation coefficients of the relation between IMI and the ghrelin response were calculated (see table 6.4). The IMI was associated with the AUC of the ghrelin response ($r = -0.42$; $p < 0.01$) and with the postprandial decrease in ghrelin concentrations, following breakfast ($r = -0.37$; $p < 0.05$). These associations only existed in the normal weight subjects (AUC: $r = -0.57$, $p = 0.01$; postprandial decrease: $r = -0.54$; $p < 0.05$), not in the obese subjects (AUC: $r = 0.00$; $p = 1.00$; postprandial decrease: $r = 0.08$; $p = 0.72$). Preprandial increases in ghrelin concentrations were only weakly, borderline significant, correlated with the IMI ($r = 0.29$; $p = 0.07$). This association was strong after energy restriction ($r = 0.51$; $p < 0.05$) but did not exist after energy balance ($r = -0.02$; $p = 0.94$) and was not influenced by BMI.

Ghrelin and subjective appetite

Ghrelin concentrations were associated with subjective measures of hunger (r = 0.54, 0.13-0.79), desire to eat (r = 0.57; 95% C.I. = 0.18-0.81) and prospective food consumption (r = 0.53; 95% C.I. = 0.12-0.79) but not with fullness (r = -0.22; 95%

C.I. = -0.60-0.25). The AUC of the ghrelin response was associated with the AUC of desire to eat ($r = 0.32$, $p < 0.05$) and prospective food consumption ($r = 0.38$, $p < 0.05$), but not with hunger ($r = 0.15$, $p = 0.35$) or fullness ($r = -0.06$, $p = 0.72$). Postprandial decreases or preprandial increases in ghrelin concentrations were not correlated with postprandial decreases or preprandial increases in appetite (data not shown).

Ghrelin and energy intake during lunch

Ad libitum energy intake is an indirect measure of satiation. To assess whether ghrelin is related to satiation, we also investigated the association between ghrelin and voluntary energy intake. There was no association between ghrelin concentrations and *ad libitum* energy intake during lunch.

Ghrelin and other blood parameters

Ghrelin concentrations have been shown to decrease energy dose dependently. To gain more insight into the mechanism of postprandial ghrelin secretion, we investigated the association between changes in ghrelin concentrations and changes in concentrations of glucose, insulin and free fatty acids, three important metabolic factors. Percentage changes in ghrelin concentrations over 2 time periods (0-30 and 30-170 minutes), were compared with percentage changes in the other blood parameters over the same period. An inverse correlation was observed between changes in ghrelin and insulin concentrations between 0 and 30 minutes after the energy balanced (EB) diet ($r = -0.50$; $p < 0.05$), but not after the energy restricted (ER) diet ($r = -0.35$; $p = 0.13$). This association also existed between 30 and 170 minutes (EB: $r = -0.64$; $p < 0.01$, ER: $r = -0.21$; $p = 0.38$). Changes in ghrelin concentrations were also inversely correlated with changes in glucose concentrations between 0 and 30 minutes (EB: $r = -0.33$; $p = 0.15$, ER: $r = -0.45$; $p < 0.05$), but not between 30 and 170 minutes (EB: $r = -0.10$; $p = 0.67$, ER: $r = -0.28$; $p = 0.24$). Changes in FFA concentrations were only correlated with changes in ghrelin concentrations between 30 and 170 minutes (EB: $r = 0.50$; $p < 0.05$, ER: $r = 0.42$; $p = 0.07$), not between 0 and 30 minutes (EB: $r = -0.05$; $p = 0.85$, ER: $r = 0.26$; $p = 0.27$).

Discussion

In the present study we tested the hypothesis that ghrelin may act as a meal initiator. The results indicate that in normal weight subjects, the postprandial ghrelin response is related to the intermeal interval, a measure of meal initiation. This suggests that ghrelin is indeed involved in meal initiation.

The limitations of our study deserve some attention. As expected, all subjects significantly lost weight during the energy restricted diet. However, during the energy balanced diet normal weight subjects also significantly lost weight, although less as compared to the energy restricted diet. In contrast, obese subjects gained weight during the energy balanced diet. This may have confounded the results. However, fasting FFA concentrations, which responded most sensitively to energy restriction, independent of BMI, showed no difference between obese and normal weight subjects. This suggests that the effectiveness of the two diets did not differ between the obese and normal weight men.
The postprandial ghrelin response was rather small (nadir values ranging between 76.1 – 96.6% of fasting values). This may be explained by the relatively small breakfast subjects received, as the postprandial ghrelin response is dependent on the amount of energy consumed (35;50). The relatively small ghrelin response may have negatively affected the statistical power. However, the small breakfast did represent a standard Dutch breakfast and induced variation in both ghrelin responses and in the length of the intermeal interval.
Only total ghrelin concentrations were measured, the dynamics of acylated over unacylated ghrelin could have been changed.
The study was performed in an environment devoid of time and eating cues, what makes it less likely that an association between the ghrelin responses and meal request was confounded by external stimuli. At lunch request, subjects were asked to estimate the time. The deviation between real and guessed lunchtime ranged between -134 and 140 minutes, suggesting that the time-blinding protocol was successful.

In studies, where meals were provided at fixed points in time, plasma ghrelin concentrations peaked just before meals and fell to nadir values immediately after eating (67;324), suggesting that ghrelin is involved in meal initiation. In this study, ghrelin concentrations had reached at least 93.3% of fasting concentrations at meal request. Nevertheless, the preprandial increase in ghrelin concentrations was only associated with the IMI after energy restriction, not after energy balance, suggesting that recovery of ghrelin concentrations to a certain threshold may be more relevant in meal initiation than the preprandial increase itself.
The fact that in all subjects, ghrelin concentrations had either reached or almost reached fasting values at meal request, and that ghrelin kinetics are correlated with the intermeal interval supports the concept. These results are however correlative and do therefore not show a causal role of ghrelin in meal initiation. Interventions

manipulating not only ghrelin concentrations, but also ghrelin kinetics or intermeal interval are needed to further substantiate this relation.
Our findings are, however, not in line with the studies of Callahan *et al.* (50) and Cummings *et al.* (66). In these studies no association between ghrelin kinetics and spontaneous meal request was observed. However, Callahan et al. (50) investigated not the postprandial ghrelin responses in relation to meal initiation, but rather ghrelin concentrations at meal request, and in the study of Cummings *et al.* (66), only 6 subjects were included, what may explain the lack of association.

Ghrelin kinetics were also associated with appetite. Subjective measures of desire to eat and prospective food consumption both reflect sensations of appetite related to the next meal. However, ghrelin concentrations were not correlated with energy intake during that next meal. A lack of a correlation may be observed, because energy intake is a measure of intrameal satiety (satiation), whereas ghrelin is thought to affect intermeal satiety (meal initiation). Nevertheless, energy intake during the *ad libitum* lunches was relatively high (on average more than 2 times the standard energy intake during lunch of Dutch adult men (339)), suggesting that food consumption may not have been determined by physiological signals only, but possibly also by cognitive or external factors, despite all efforts to control for these factors.

Proportional changes in ghrelin and insulin concentrations were highly correlated. Also, FFA concentrations were correlated with ghrelin concentrations. Cummings et al. hypothesized that these processes are highly dependent on each other, whereas the failure of a subjects' insulin levels to return to fasting concentrations during the IMI may have caused a lack of a preprandial increase in ghrelin concentrations. Ghrelin increases would also depend on increases in NEFA (66). In the present study, all subjects showed a preprandial increase in ghrelin concentrations and decrease in insulin concentrations. Both the strong correlations between insulin and ghrelin and between FFA and ghrelin are in accordance with the hypothesis of Cummings et al.

As reported before (96;318), the postprandial ghrelin response was blunted in the obese subjects. A blunted ghrelin response may reflect reduced ghrelin sensitivity and may therefore explain the absence of a significant relation between postprandial ghrelin concentrations and the intermeal interval in obese subjects. A decreased ghrelin sensitivity is also observed in dietary-induced obese mice (261). In these mice, the sensitivity to the orexigenic effects of exogenous ghrelin was also reduced.

Ghrelin sensitivity improved upon weight loss (261) and may in turn restore the association between ghrelin and meal initiation. In this study, we observed a significant increase in fasting ghrelin concentrations in the obese subjects, after three days of energy restriction. These results are in line with the results of Cummings et al (68) who showed an increase in fasting ghrelin concentrations following diet induced weight loss. Unfortunately, this increase was not accompanied by a clear increase in ghrelin sensitivity in these obese subjects. The period of energy restriction may have been too short, and the increase in fasting ghrelin concentrations may have been too small.

In conclusion, meal request was preceded by a preprandial increase in ghrelin concentrations in all subjects. The AUC of the ghrelin response and the postprandial decrease in ghrelin concentrations were both associated with the intermeal interval in normal weight subjects, but not in obese subjects. These results support the hypothesis that ghrelin is involved in meal initiation in normal weight subjects.

Acknowledgements

We want to express our gratitude to the volunteers who participated in the study; Henriëtte Fick, Inge van den Assum, José Jacobs, Soesila Sukhraj, Angelique Speulman, Eric Busink, Gerda Pot, Jan Catsburg, Robin van den Berg, Hans Verplanke and all other people from the Metabolic Research Unit and laboratories who assisted in the organization of the study and analyses of the blood samples; Susanne Westenbrink and Petra van Aken for the preparation of the study substances; Diane ter Doest and Linda van den Bosch for performing data management; and Cor Kistemaker and Carina Rubingh for their support with the statistical analyses.

Wendy Blom was involved in the design of the protocol, collection of the data, analysis of the data and writing of the manuscript. Marieke Advocaat was involved in the analysis of the data and writing of the manuscript. Anne Lluch, Cees de Graaf and Annette Stafleu were involved in the design of the protocol and provided significant advice. Gertjan Schaafsma provided significant advice. Henk Hendriks was involved in the design of the protocol (Principal Investigator according to Good Clinical Practice guidelines), writing of the manuscript and provided significant advice. None of the authors had a conflict of interest.

This study is financially supported by: Dutch Ministry of Education, Culture and Science, Dutch Ministry of Health, Welfare and Sport and Danone Vitapole.

7

Fasting ghrelin does not predict food intake after short-term energy restriction

Wendy Blom
Monica Mars
Henk Hendriks
Lisette de Groot
Annette Stafleu
Frans Kok
Kees de Graaf

Wageningen University, Wageningen, Netherlands
TNO Quality of Life, Zeist, Netherlands

Submitted

Abstract

Objective:
To investigate the role of ghrelin as a hunger signal during energy restriction and to test the hypothesis that changes in fasting leptin concentrations during energy restriction are associated with changes in fasting ghrelin concentrations.

Research Methods and Procedures:
Thirty-five healthy, lean men (aged: 23 ± 3 y, BMI: 22.3 ± 1.6 kg/m^2) participated in a controlled intervention study. Fasting ghrelin and leptin concentrations were measured before and after two days of 62% energy restriction and after a two-day period of *ad libitum* food intake. Energy intake during the latter period was assessed.

Results:
On average ghrelin concentrations did not change (0.05 [95% CI: -0.03; 0.12] µg/L) during energy restriction. Changes in ghrelin concentration during energy restriction were not associated with energy intake during the *ad libitum* period (r = 0.07; NS). *Ad libitum* energy intake was, however, associated with the change in ghrelin concentrations during the same period (r = -0.34; p = 0.05). Ghrelin and leptin concentrations were not associated. In addition, the ratio of percentage changes in ghrelin and leptin during energy restriction, an indirect measure of leptin's suppression of ghrelin, was not correlated with *ad libitum* food intake following energy restriction (r = -0.26; p = 0.14).

Discussion:
Fasting ghrelin concentrations did not rise following a two day energy restriction regimen. Moreover, changes in ghrelin concentrations during energy restriction were not associated with subsequent *ad libitum* food intake, suggesting that fasting ghrelin does not act as a hunger signal to the brain. The data did not support our hypothesis that leptin suppresses the effects of ghrelin.

Introduction

Fasting plasma concentrations of ghrelin, a stomach derived orexigenic peptide, are inversely associated with BMI (68;255;325), suggesting a role of ghrelin in body weight control. If ghrelin is indeed involved in the regulation of body weight, then diet induced weight loss would lead to increased ghrelin concentrations, which may serve as a hunger signal to the brain in order to restore energy balance. As far as we are aware of, the relation between changes in ghrelin concentration during energy restriction and subsequent *ad libitum* food intake have not been investigated up till now.

The role of leptin, a fat-derived anorexigenic hormone, however, has been investigated more extensively. Leptin concentrations are positively associated with body fat stores (304) and strongly inversely correlated with appetite and food intake under conditions of energy imbalance (59;158;347). Energy deficiency (> 24 hrs) leads to decreased leptin concentrations. Recently, Mars and colleagues provided evidence for an association between this decrease in leptin concentration and increased appetite (207). Possibly, the decline in leptin serves as a starvation signal to the brain (101).

Animal studies showed that both leptin and ghrelin act on the same central mechanisms in the hypothalamus. Ghrelin stimulates the orexigenic neuropeptide Y (NPY) and agouti-related protein (AgrP) neurons (155;303) and leptin acts partly through the activation of the anorexigenic melanocortin system (300), and partly through suppression of NPY and AgrP in the paraventricular nucleus (PVN) (92;266). In addition, there are some indications, from animal studies only, that leptin suppresses ghrelin secretion from the stomach (327). These data suggest an interaction between ghrelin and leptin. Ghrelin and leptin may together determine the appetite drive during energy restriction. In this study we tested four hypotheses. Firstly, we hypothesized that fasting ghrelin concentrations increase acutely during energy restriction. Secondly, we hypothesized that fasting ghrelin concentrations after energy restriction are positively related to subsequent *ad libitum* food intake. Thirdly, we tested the hypothesis that changes in fasting leptin concentrations during energy restriction are associated with reciprocal changes in fasting ghrelin concentrations. Our fourth hypothesis was that the change in the ratio of plasma ghrelin to leptin is a better predictor of subsequent change in *ad libitum* food intake than the change in either ghrelin or leptin alone.

Research methods and procedures

Subjects

The study was conducted at the Wageningen University, Wageningen, the Netherlands, where 35 male subjects (aged 18-50 y, BMI 20-30 kg/m^2) were recruited among employees and students. Each subject gave written informed consent before screening. Exclusion criteria for participation were: restraint eating as assessed with the Dutch Eating Behaviour Questionnaire (332) (score restrained eating scale > 2.38); diabetes or disturbed glucose metabolism (fasting plasma glucose > 6.1 mmol/L or glucosuria); using medication affecting energy metabolism, body weight or food intake; stomach or bowel diseases (blood in stool/constipation/diarrhoea); anaemia (Hb < 8.5 mmol/L, Ht < 41 %) and blood donation during the intervention study. The Ethics Committee of Wageningen University approved the study protocol.

Study design

During the first two study days, subjects received a controlled energy restricted diet containing one-third of their estimated energy needs, followed by a two-day period of *ad libitum* food intake.

Day 1 and 2: Energy restriction
The subjects daily energy requirement (MJ/d) was estimated by calculating the basal metabolic rate according to Schofield's equation (146): BMR (MJ) = 0.0485x weight (kg) + 3.67, and multiplying this estimate with a physical activity level. The physical activity level was estimated by a short retrospective physical activity questionnaire containing six activities (366). For each subject one third of the energy requirement was calculated. Based on this value, subjects were allocated to three different energy groups, resulting in fifteen subjects receiving 4.2 MJ/d, fifteen subjects receiving 5.0 MJ/d and five subjects receiving 5.8 MJ/d. The energy restricted diet consisted of meal and snack replacements each containing 0.8 MJ (Profiel, Nutricia, Zoetermeer, The Netherlands). In addition to this, subjects were allowed to consume non-caloric beverages *ad libitum* (e.g. diet coke, black coffee and black tea). On day 1 subjects arrived at the research centre between 7:30 and 9:30 in the morning, after an overnight fast (nothing to eat or drink except for water after 22:00 h). They were weighed after voiding and a fasting blood sample was taken. Subjects received a breakfast and were given boxes containing the meal replacements for day 1 and day 2. Compliance was measured with the help of pre-printed daily food records, in which subjects registered the time of consumption of all products.

Day 3 and 4: Ad libitum food intake

During the third and fourth day of the intervention study, subjects were allowed to eat *ad libitum.* Subjects consumed a breakfast and warm buffet-style lunch at the research centre. In order to prevent habitual intake, foods were provided in unusual portions sizes. On day 3 subjects were weighed after an overnight fast and a blood sample was taken. The breakfast on day 3 consisted of a milk shake, which was offered *ad libitum* in a blinded beaker, containing 400 g of milkshake. The macronutrient composition of this milkshake was according to the Dutch national dietary guidelines; 58, 29, 13 per cent of calories were derived from carbohydrates, fat and protein, respectively (340). One beaker contained 2.6 MJ, which reflects the average energy intake in young adult men during breakfast (341). Subjects were instructed to drink until satiation. A second and third beaker was available upon request. Subjects were not aware of the energy content of this breakfast. They took the remaining meals (except lunch) and snacks for day 3 home and returned at the research centre for a warm buffet-style lunch. At least 200% of the estimated energy needs were available for each subject. In addition to the foods provided, subjects were free to use other products. Subjects recorded the foods consumed at home in detail in a diary. During the *ad libitum* period, only standard Dutch food items were provided (see appendix I). On day 4 subjects were weighed after an overnight fast and received a buffet-style breakfast. They took the remaining meals (except lunch) and snacks for day 4 home and returned at the research facility for a warm buffet-style lunch. On day 5 subjects came to the research facility after an overnight fast, were weighed and a blood sample was drawn.

Outcome measures

Energy intake

The food diary, leftovers and empty packages were crosschecked, and portion sizes were verified with dummies of household measures by a trained dietician. Energy intake and macronutrient composition of the consumed foods were calculated by use of food composition tables and product information of manufacturers. To correct for individual differences in energy needs, energy intake proportional to estimated energy needs was calculated per day.

Anthropometric measures

A wall-mounted stadiometer was used to measure height. Height was measured without shoes with the Frankfurt plane horizontal; accurate to 0.5 cm. Subjects were weighed with indoor clothing, without shoes and with empty pockets on a digital balance accurate to 0.1 kg.

Blood sampling and bio-chemical analyses

During the study, for each subject, fasting blood samples were taken at the same time point in the morning. Blood samples were placed directly on ice after sampling, and after coagulation of the serum samples, centrifuged at 2600 rpm for 10 minutes. Serum and plasma samples were then divided among aliquots and stored at –70°C until analyses.

Serum leptin concentrations were assessed in duplicate by radio immunoassay (Linco Research Inc., St. Charles Missouri, USA), with the lowest detection limit at 0.5 ng/mL. The intra-assay coefficient of variation was 3-8%, the inter-assay coefficient of variation 4-8%. Plasma ghrelin concentrations were assessed in duplicate by radio immunoassay (Linco Research Inc., St. Charles Missouri, USA), with an intra-assay coefficient of variation of 5% at a concentration of 1866 ng/L. Serum insulin was measured in duplicate by immunoassay (Immulite 2000 Analyzer), with the lowest detection at 2.0 µU/mL. Plasma glucose was measured quantitatively by a bichromatic endpoint assay (Glu FlexTM reagent). All samples of each subject were analyzed in one run. Means of the duplicates were used for data analyses.

Statistical Analyses

Because of non-normality, fasting concentrations of insulin and leptin were transformed with the natural logarithm (ln) before statistical analyses. Of these variables, geometric means and 95%-Confidence Intervals [95%-CI] are shown. Other variables are shown as arithmetic mean ± SD or [95%-CI]. The range represents the minimum and maximum value of the variable. Percentage changes in concentrations between day 1 and day 3, and between day 3 and day 5 were calculated as follows: (day 3 – day 1)/ day 1 * 100 and (day 5 – day 3)/ day 3 * 100. To investigate the relation between changes in leptin and ghrelin, the ratios of percentage changes of ghrelin versus the percentage changes in leptin were calculated. These ratios were calculated as follows: (ghrelin day 3/ ghrelin day 1 * 100)/ (leptin day 3/ leptin day 1 * 100) and (ghrelin day 5/ ghrelin day 1 * 100)/ (leptin day 5/ leptin day 1 * 100). Because of non-normality the ratios were transformed with the natural logarithm (ln) before statistical analyses. The geometric means and 95%-Confidence Intervals [95%-CI] are shown. Associations were calculated by means of Pearson correlation coefficients (r). One subject increased 61.5% in leptin during energy restriction. Although he lost weight (3.0 kg) during energy restriction, we cannot exclude a lack of compliance. Therefore, we excluded his data from the analyses. P-values <0.05 were considered statistically significant. For all data-analyses we used the statistical package SAS (Release 8.2, SAS Institute Inc., Cary, NC, USA).

Results

Subject characteristics are presented in table 7.1. Fasting blood concentrations of ghrelin, leptin, insulin and glucose are presented in table 7.2. During the whole study period, no association was found between fasting ghrelin concentrations and fasting leptin, glucose, body weight or BMI. At baseline, day 1, fasting ghrelin concentrations were associated with insulin concentrations ($r = -0.37$; $p < 0.05$). This correlation was not present at day 3, after two days of energy restriction ($r = -0.17$; $p = 0.33$), but was restored after two days of *ad libitum* food intake at day 5 ($r = -0.56$; $p < 0.001$). Fasting leptin concentrations were associated with body weight and BMI on day 1 ($r = 0.38$; $p < 0.05$, $r = 0.58$; $p < 0.001$, respectively) and on day 5 ($r = 0.36$; $p < 0.05$, $r = 0.62$; $p < 0.0001$, respectively). This association also existed on day 3 with BMI ($r = 0.52$; $p < 0.01$) but not with body weight. No relation between fasting leptin and insulin or leptin and glucose was observed during the study.

Table 7.1 Subject characteristics (n=34).

	Arithmetic mean ± SD	Range
Age (y)	23 ± 3	19-29
Weight (kg)	73.3 ± 6.4	62.9-84.6
Body Mass Index (kg/m^2)	22.3 ± 1.6	19.8-24.9
Estimated BMR (MJ/d)[*]	7.2 ± 0.3	6.4-7.9
PAL[†]	1.8 ± 0.2	1.5-2.2
Estimated Energy needs (MJ/d) [‡]	13.0 ± 1.7	10.4-16.3
Restraint eating score[§]	1.5 ± 0.4	1.0-2.3

[*] Estimated Basal Metabolic Rate (146)

[†] PAL = Physical Activity Level (366)

[‡] Estimated by BMR x PAL

[§] Assessed by the Dutch Eating Behaviour Questionnaire (332)

Energy restriction

During the energy restrictive period subjects consumed on average 38.0 ± 2.7% (range: 32.1; 43.1%) (4.9 ± 0.5 MJ) of their estimated energy needs (table 7.3 and figure 7.1) and lost on average 1.1 ± 0.6 kg (range: -2.6; 0.3 kg) of body weight. Fasting leptin, insulin and glucose concentrations changed during the energy restrictive period by -1.0 [95% CI: -1.4; -0.5] µg/mL (-27.2%), -2.4 [95% CI: -3.3; -1.5] µU/mL (-30.7%) and -0.3 [95% CI: -0.4; -0.2] mg/mL (-5.2%), respectively (table 7.2).

In contrast with our first hypothesis, the average fasting ghrelin concentrations did not change during energy restriction (0.05 [95% CI: -0.03; 0.12] µg/L (+3.1%)) (Table 7.2). The percentage changes in fasting ghrelin, leptin and insulin per subject are presented in figure 7.2.

Table 7.2 Biochemical parameters before intervention, after 2 days of 62%-energy restriction, and after 2 days of *ad libitum* energy intake (n= 34).

	Fasting concentrations			Δ restricted energy intake *	Δ *ad libitum* energy intake *
	day 1	day 3	day 5	$\frac{\text{day 3} - \text{day 1}}{\text{day 1}} \times 100\%$	$\frac{\text{day 5} - \text{day 3}}{\text{day 3}} \times 100\%$
Glucose (mg/mL)	5.1 [4.9; 5.2] *	4.8 [4.7; 4.9] *	5.0 [4.8; 5.1] *	-5.2 [-8.0; -2.5]	3.9 [1.3; 6.6]
Insulin (µU/mL)	4.4 [2.4; 7.5] †	2.0 [2.0; 2.3] †	4.2 [3.3; 6.2] †	-30.7 [-41.0; -20.4]	84.1 [55.9; 112.3]
Leptin (µg/mL)	2.3 [1.8; 3.2] †	1.7 [1.3; 2.0] †	2.2 [1.7; 2.7] †	-27.2 [-34.4; -19.9]	37.6 [26.7; 48.5]
Ghrelin (µg/L)	1.9 [1.7; 2.0] *	1.9 [1.8; 2.1] *	1.9 [1.7; 2.0] *	3.2 [-0.8; 7.1]	-2.8 [-6.5; 0.9]
Ghrelin/leptin‡	1.0 §	1.3 [1.2; 1.6] †	1.1 [1.0; 1.2] †		

* Arithmetic mean (95% CI)

† Geometric mean (95% CI)

‡ Ratio ghrelin/leptin day 3 is calculated as follows: $\left(\frac{\text{ghrelin\_day03}}{\text{ghrelin\_day01}} * 100\right) \Big/ \left(\frac{\text{leptin\_day03}}{\text{leptin\_day01}} * 100\right)$

Ratio ghrelin/leptin day 5 is calculated as follows: $\left(\frac{\text{ghrelin\_day05}}{\text{ghrelin\_day01}} * 100\right) \Big/ \left(\frac{\text{leptin\_day05}}{\text{leptin\_day01}} * 100\right)$

§ Reference value

Although the average ghrelin concentrations did not change during energy restriction, there was a large variation in individual ghrelin responses. Therefore changes in ghrelin concentrations during energy restriction, in relation to energy intake could still be investigated. There was no correlation between changes in ghrelin and leptin concentrations (r = -0.06; p = 0.76). Changes in ghrelin concentrations were also not associated with decreases in insulin (r = -0.05; p = 0.78), body weight (r = 0.05; p = 0.76) nor with the percentage energy intake during the energy restriction period (r = -0.12; p = 0.51). To test our second hypothesis, namely that fasting ghrelin concentrations are positively correlated with energy intake during the subsequent *ad libitum* food intake period, we calculated the correlation between fasting ghrelin concentrations at day 3 and energy intake during the *ad libitum* food intake period. There was no correlation (r = 0.22; p = 0.21). There was also no association between

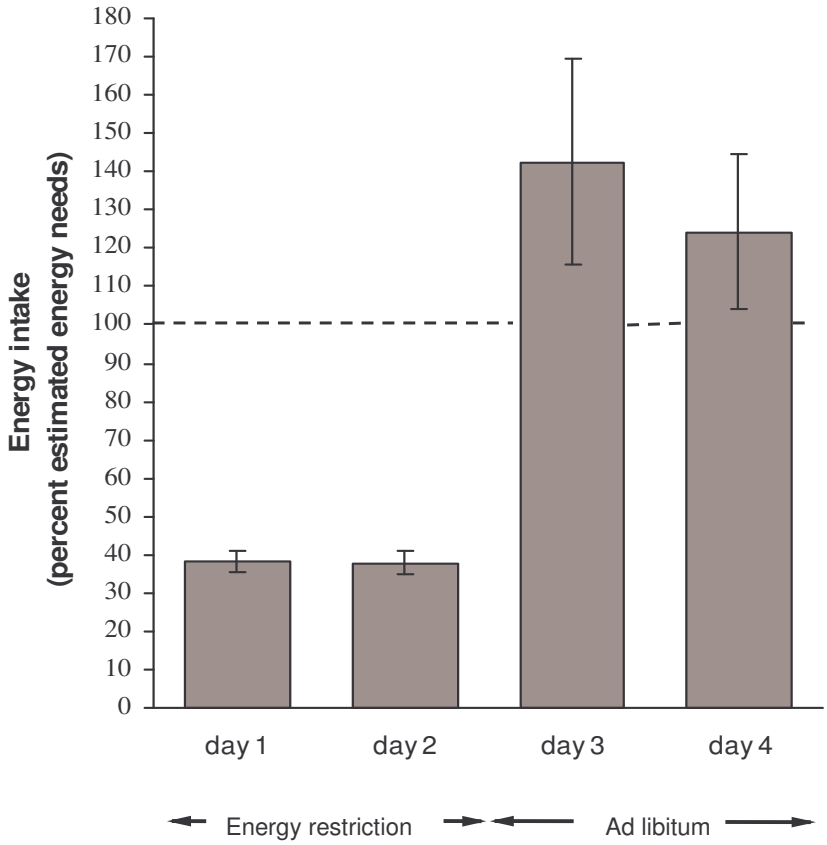


Figure 7.1 Energy intake balance during the 62%-energy restriction and subsequent *ad libitum* energy intake (n=34).

Table 7.3. Energy, macronutrient, and fibre intake of the 34 men participating in the intervention study ($\bar{x}$ ± SD).

	Restricted energy intake		*Ad libitum* energy intake	
	Day 1	Day 2	Day 3	Day 4
Energy intake (MJ)	4.9 ± 0.5	4.9 ± 0.6	18.3 ± 2.9	16.0 ± 2.6
Carbohydrates (en%)	54.5 ± 0.4	54.2 ± 0.5	52.7 ± 4.1	47.3 ± 4.8
Fat (en%)	19.9 ± 0.5	20.1 ± 0.6	33.8 ± 3.4	36.0 ± 4.7
Protein (en%)	25.3 ± 0.3	25.5 ± 0.3	11.9 ± 1.6	15.7 ± 2.4
Alcohol (en%)	-	-	1.7 ± 3.4	1.1 ± 1.9
Mono- and disaccharides (g)	113 ± 8	116 ± 10	256 ± 64	229 ± 63
Fibre (g/MJ)	6.0 ± 0.1	6.2 ± 0.2	1.8 ± 0.4	2.2 ± 0.4

changes in ghrelin concentrations during energy restriction and energy intake during the *ad libitum* food intake period (r = 0.07; p = 0.70) (table 7.4).
The decreases in fasting leptin and insulin concentrations during the energy restrictive period were also not associated with energy intake during the *ad libitum* food intake period (r = 0.19; p = 0.28 and r = 0.21; p = 0.24, respectively) (table 7.4).
The leptin results of the present study are described in more detail in a publication of Mars et al. (206) describing the role of leptin as a starvation signal.

Ad libitum food intake

During the period of *ad libitum* food intake subjects did show compensatory behaviour, i.e. they consumed on average 133.4 ± 21.4% (range: 99.1; 188.7%) (17.2 ± 2.4 MJ) of their estimated energy needs (figure 7.1). After two days of *ad libitum* food intake, fasting leptin, insulin and glucose concentrations changed on average by + 0.7 [95% CI: 0.4; 0.9] µg/mL (37.6%), + 2.5 [95% CI: 1.2; 3.7] µU/mL (84.1%) and + 0.2 [95% CI: 0.1; 0.3] mg/mL (3.9%), respectively, returning to their starting value (figure 7.2). The average ghrelin concentrations did not change during the *ad libitum* food intake period (0.06 [95% CI: -0.14; 0.01] µg/L (-2.8%)) (table 7.2).
During the *ad libitum* food intake period, changes in ghrelin concentrations were inversely associated with the percentage energy intake (r = -0.34; p = 0.05). This means that a higher food intake was associated with a larger decrease instead of a larger increase in fasting ghrelin concentrations.

Ratio of percentage changes in ghrelin and leptin

To test our fourth hypothesis, namely that the change in the ratio of plasma ghrelin to leptin is a better predictor of the subsequent change in *ad libitum* food intake, we calculated the ratio of percentage changes in ghrelin versus the percentage changes in leptin. On day 3, after the energy restrictive period the geometric mean of the ratio was on average 1.3 (95% CI: 1.2; 1.6) and during the *ad libitum* food intake period 1.1 (95% CI: 1.0; 1.2) (see table 7.2 and figure 7.3). However, in contrast with our expectations, the ratio ghrelin/leptin on day 3 was not positively associated with the percentage energy intake during the *ad libitum* food intake period (r = -0.26; p = 0.14).

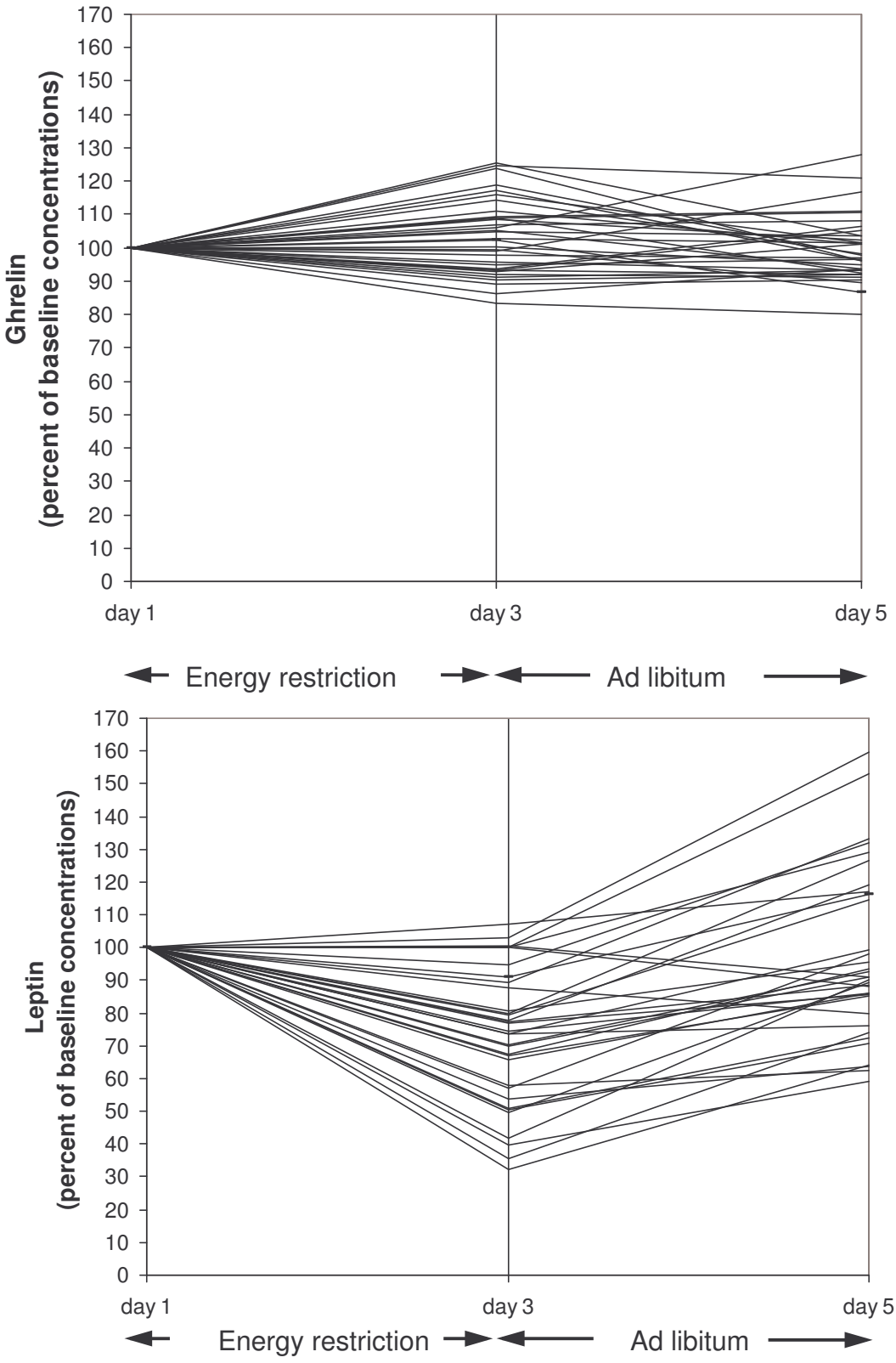


Figure 7.2 Changes in serum leptin and plasma ghrelin concentrations during the 62%-energy restriction and subsequent *ad libitum* energy intake (n=34).

Table 7.4. Associations between *ad libitum* energy intake and absolute changes in ghrelin and leptin and the ratio of percentage changes in ghrelin and leptin induced by a 2-day 62%-energy restriction (n=34).

	Pearson's r (p-value)		
	ΔGhrelin	ΔLeptin	Ratio day 3[†]
First ad libitum day (day 3)			
Energy intake (kJ)	0.11 (0.53)	0.22 (0.22)	-0.25 (0.16)
Energy intake (%)[*]	0.08 (0.66)	0.29 (0.10)	-0.29 (0.09)
Second ad libitum day (day 4)			
Energy intake (kJ)	0.06 (0.72)	0.03 (0.88)	-0.05 (0.78)
Energy intake (%)[*]	0.04 (0.81)	0.15 (0.39)	-0.15 (0.39)
Average of the ad libitum days			
Energy intake (kJ)	0.10 (0.57)	0.15 (0.41)	-0.18 (0.32)
Energy intake (%)[*]	0.07 (0.70)	0.25 (0.15)	-0.26 (0.14)

[*] Per cent of baseline estimated energy needs

[†] Ratio ghrelin/leptin day 3 is calculated as follows: $\left(\frac{ghrelin\_day03}{ghrelin\_day01}*100\right)\Big/\left(\frac{leptin\_day03}{leptin\_day01}*100\right)$

Discussion

In this study we tested four hypotheses. The first hypothesis was that fasting ghrelin concentrations increase during energy restriction. However, we did not find an acute increase in fasting ghrelin concentrations after two days of severe energy restriction, or a decrease in ghrelin concentrations after two days of *ad libitum* food intake. In contrast, body weight, leptin, insulin and glucose did respond as expected: decreasing during energy restriction and increasing during *ad libitum* food intake. Our second hypothesis was that fasting ghrelin concentrations after energy restriction are positively related with subsequent *ad libitum* food intake. Although subjects did show compensatory behaviour during the *ad libitum* period, i.e. they consumed considerably more energy than their estimated energy needs, this cannot be attributed to ghrelin. Neither fasting ghrelin concentrations at day 3 nor changes in ghrelin concentration during energy restriction were (positively) associated with energy intake during the *ad libitum* food intake period. Our third hypothesis was that changes in leptin during energy restriction are associated with reciprocal changes in ghrelin levels. However, there was no correlation between ghrelin and leptin

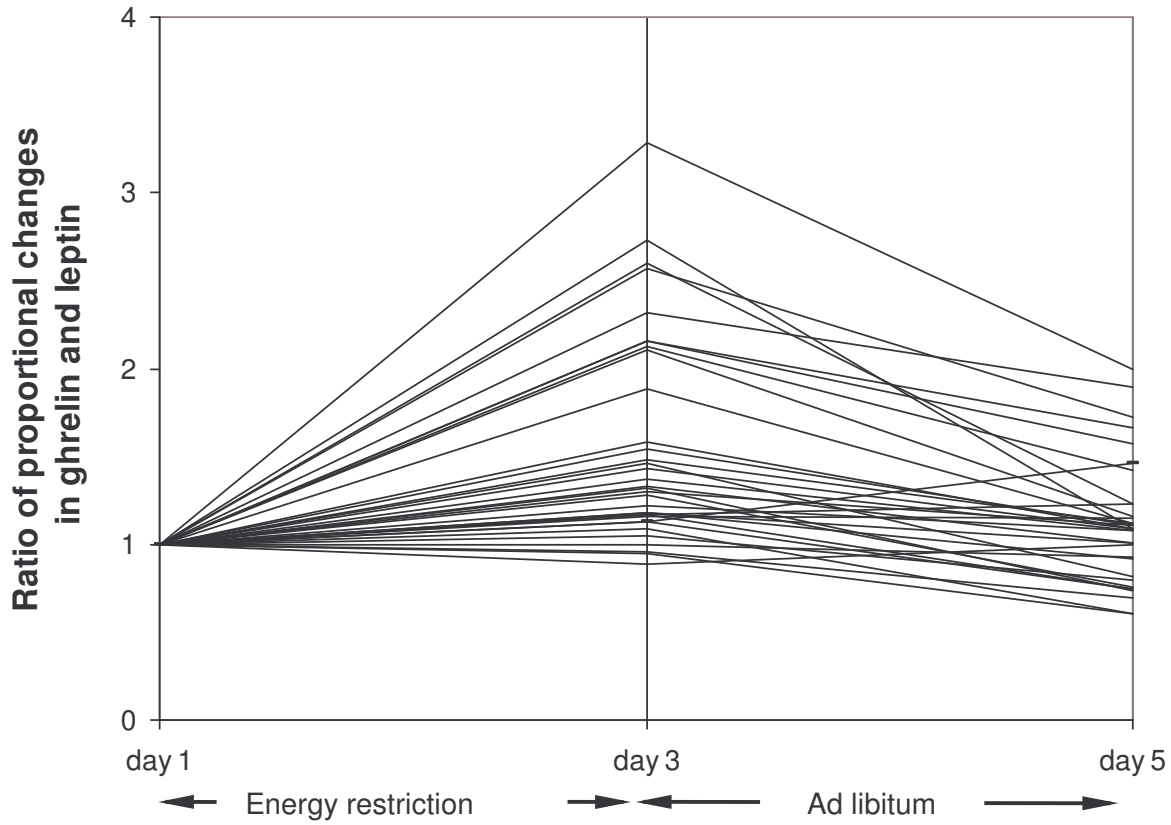


Figure 7.3 The ratio of percentage changes of ghrelin and leptin (n=34).

concentrations at day 3 or between changes in leptin and ghrelin concentrations during energy restriction. Our fourth hypothesis was that the change in the ratio of plasma ghrelin to leptin is a better predictor of the subsequent change in *ad libitum* food intake than the change in either ghrelin or leptin alone. The ratio ghrelin/leptin after energy restriction was not associated with energy intake during the *ad libitum* food intake period and therefore does not predict energy intake. The relation between ghrelin and leptin concentrations, as reflected in this ratio does therefore not act acutely as a hunger signal to the brain.

Although changes in ghrelin concentrations during energy restriction did not predict subsequent food intake, we did observe an association between energy intake and changes in ghrelin concentrations during the *ad libitum* food intake period. This association suggests that higher energy intake is related to larger decreases in fasting ghrelin concentrations. Ghrelin may therefore be regulated as a consequence rather than a cause of food intake changes.

There are some factors that might explain why we did not observe an increase in ghrelin concentrations during energy restriction. First of all it is possible that body fat stores may have to be reduced more strongly to increase fasting ghrelin concentrations. Furthermore, one may argue that food intake influences the reproducibility of the fasting ghrelin concentrations and the response to energy restriction as ghrelin is highly responsive to food intake. This may have caused a lack of power to detect changes in ghrelin concentrations. It is also possible that energy restriction increased active ghrelin concentrations only, and that this is not directly reflected into the total ghrelin pool. In future studies, both active and total ghrelin concentrations should be investigated.
Fasting ghrelin concentrations, are a good surrogate for the 24-h ghrelin concentration (67). However, a disadvantage of measuring fasting ghrelin levels is that the effect of energy restriction on ghrelin concentrations could be reflected in the diurnal pattern of ghrelin secretion or the postprandial ghrelin responses rather than in the fasting concentrations. Energy restriction can increase the amplitude of the diurnal pattern of ghrelin secretion (152) and decreases the amplitude of the diurnal pattern of leptin secretion (93). We only measured fasting concentrations of ghrelin and leptin, because measurement of spontaneous energy intake was critical in this study. Continuous or multiple blood sampling would require a medical setting, which may have affected spontaneous energy intake. We therefore chose to measure *ad libitum* energy intake as accurate as possible in a free-living situation, but we decided to only measure fasting ghrelin and leptin concentrations.

It is clear that fasting ghrelin concentrations increase after a longer period of energy restriction, i.e. a period of 3 months or more (68;177). However the relation between short-term energy restriction and fasting ghrelin concentrations is less clear. Mars and colleagues have shown in their reproducibility studies (three studies performed with same group of subjects) that fasting ghrelin concentrations increased on average with 16% or 18% during two days of 65%-energy restriction (209). However, in this study we could not reproduce this effect. Other recent studies also showed that three days of total fasting (56) or four days of energy restriction (89) did not affect fasting ghrelin concentrations. In those two studies fat mass did not change during the intervention, which might explain why ghrelin concentrations did not increase. Nevertheless, in our study and in the studies of Mars et. al. only changes in body weight and not in fat mass were measured. Possibly, ghrelin concentrations may not be directly related to fat stores, but are indirectly regulated through other parameters which do respond more directly to acute energy restriction (e.g. insulin).

Ghrelin might be more important as a hunger signal during energy balance, while other variables (e.g. PYY) may be more important during energy restriction.

Although subjects compensated their energy intake during the *ad libitum* food intake period, this compensation was not related to changes in ghrelin, leptin or insulin, suggesting that these hormones are not involved in energy intake compensation following energy restriction. However, changes in other hormones (e.g. PYY, GLP-1 and CCK) and for example glycogen may also contribute to the compensatory response to food restriction and may have modulated the effects of ghrelin and leptin. Besides their association with food intake, ghrelin and leptin have both been associated with energy expenditure (289;311), and ghrelin appears to be involved in the metabolic fuel preference (367), favouring weight gain. Via these ways, ghrelin and leptin may have affected body weight homeostasis. Energy expenditure and metabolic fuel preferences were however not measured.

In summary, this was the first study in which *ad libitum* food intake following energy restriction was investigated to test the hypothesis that ghrelin acts as a hunger signal during energy restriction. However, we did not find evidence for this hypothesis. Fasting ghrelin concentrations did not rise acutely following a two day energy restriction regimen and therefore ghrelin appears not to act as a hunger signal to the brain. In contrast, changes in ghrelin concentrations were inversely correlated with food intake and may be regulated as a consequence rather than a cause of food intake changes. We also did not find evidence for an interaction between leptin and ghrelin, since changes in fasting leptin concentrations were not associated with changes in fasting ghrelin concentrations. Moreover, the ratio of the percentage changes in ghrelin and leptin was not associated with energy intake during the *ad libitum* lunch.

Acknowledgements

We want to express our gratitude to the volunteers who participated in the study; Els Siebelink, Karin Borgonjen and Lieneke Kolmus for their help during the preparation and implementation of the intervention study; and Lucy Ockma and co-workers for their assistance in the sample collection. WAMB was involved in the analysis of the data and writing of the manuscript. MM was involved in the design of the study, data collection, and writing of the manuscript. HFJH was involved in writing of the manuscript and provided significant advice. AS provided significant advice and

consultation. LCGPMG, FJK and CG were involved in the design of the study and provided significant advice. None of the authors had a conflict of interest.

This research is supported by the Netherlands Association for Scientific Research (NWO-MW project no. 980-10-007), the Dutch Ministry of Education, Culture, and Science and the Dutch Ministry of Health, Welfare and Sport.

8

General Discussion

This thesis focuses on the role of the gastric hormone ghrelin in the regulation of food intake. Four research questions were formulated in chapter 1. In the subsequent chapters, the results of studies designed to answer these research questions were described. In this final chapter, the findings of this thesis in relation to the research questions are discussed, as well as the internal and external validity of the data. After the overall conclusions of this thesis, some directions for future research will be provided.

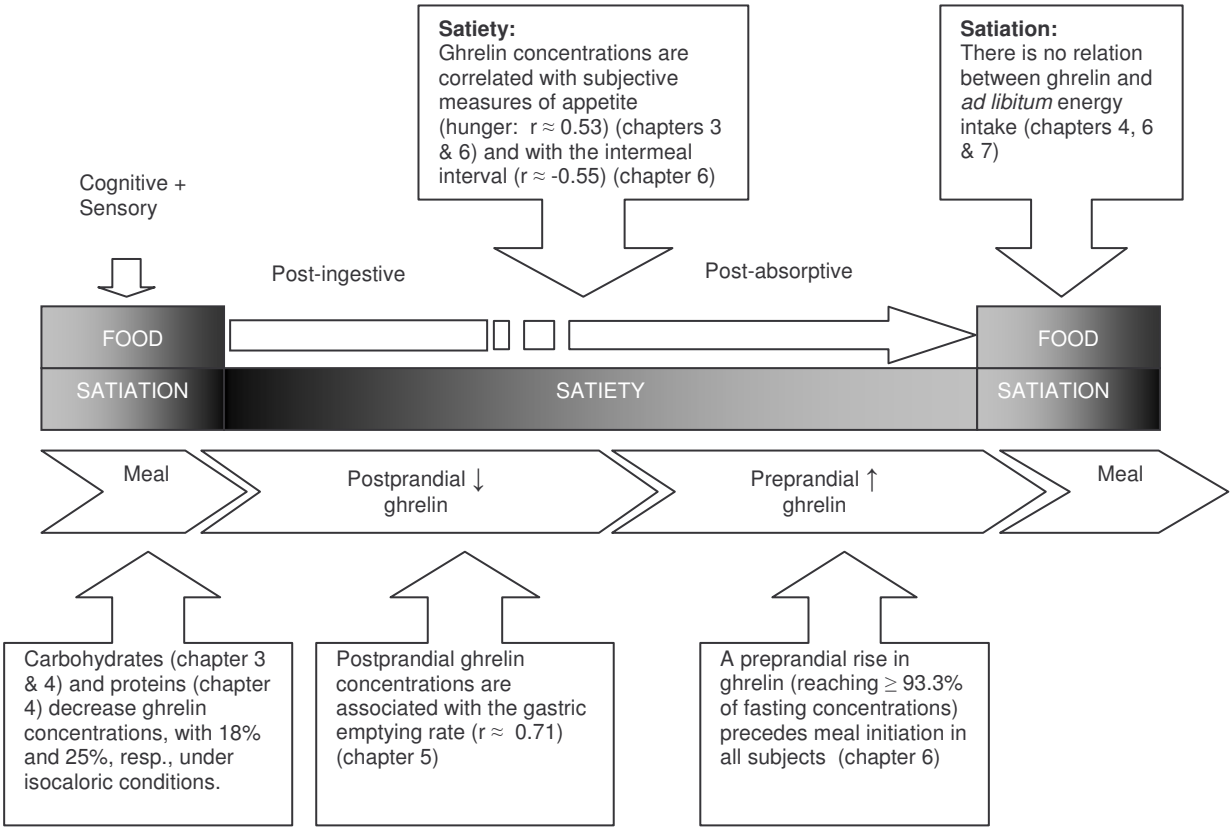
Main findings

The main results with respect to the role of ghrelin in the regulation of food intake are summarized in figure 8.1. Ghrelin concentrations were shown to decrease rapidly and dose-dependently after carbohydrate intake (chapter 3) and intake of a high protein dairy breakfast decreased ghrelin concentrations more as compared to a high carbohydrate dairy breakfast (chapter 4). The postprandial ghrelin response seemed to depend on gastric emptying (chapters 4 + 5), supporting the concept that ghrelin may require postgastric feedback. Although ghrelin concentrations were inversely associated with insulin concentrations in two studies (chapters 3 + 6), evidence from another study (chapters 4 and 5) suggests that the insulinotropic hormone GIP, is also inversely related to ghrelin and possibly even stronger than to insulin, suggesting that GIP may provide postgastric feedback to ghrelin. Postprandial ghrelin responses were positively associated with subjective measures of appetite (chapters 3 and 6). In normal weight men, the postprandial ghrelin response was associated with the intermeal interval, a measure of meal initiation (chapter 6). However, neither fasting (chapter 7) nor postprandial (chapters 4 and 6) ghrelin concentrations were associated with *ad libitum* food intake during energy balance or after energy restriction. Severe energy restriction (2 or 3 days) did not increase fasting ghrelin concentrations in normal weight men (chapters 6 and 7), although 3 days of severe energy restriction did increase fasting ghrelin concentrations in obese men (chapter 6).

Internal validity

In this section, the most critical issues of the methodology used to obtain the results described in this thesis are discussed. The focus is on the study designs, treatments, subjects and the two most important measures, i.e. ghrelin and visual analogue scales.

Figure 8.1 Main findings of this thesis



Study design

The studies described in this thesis had a randomized, cross-over design, allowing for within subject comparison of the treatments. Only one study had a non-randomized, crossover design, because of practical reasons. Consequently, period was entangled with treatment, and period effects can therefore not be eliminated. Nevertheless, carry-over effects of the treatments are unlikely whereas treatments were separated by 1 week. Moreover, baseline measurements of all parameters were similar between treatments, and so were stress hormone concentrations (data available, but not shown). It is therefore unlikely that a lack of randomization has affected the study outcome, although it may have reduced the sensitivity and reproducibility of the subjective appetite measures.

To standardize the measurements, treatments were preceded by an overnight fast, in all studies and in some studies also by a standard evening meal. Additionally, subjects were instructed to maintain their usual food intake pattern, in between treatments to prevent large fluctuations in body weight.

Treatment

The studies were further standardized by blinding the subjects for treatment order (with exception of the water treatment). Test meals were kept as similar as possible (e.g. in taste, texture, volume) to allow for objective investigation of the effects of energy or macronutrient content on ghrelin concentrations and subjective measures of appetite. In case the treatment consisted of energy restriction, energy restriction was proportional to the estimated energy requirements of the subjects, to induce a similar energy restriction in each subject. However, the method used to estimate the energy requirements and also the categorization of the subjects in energy groups may have induced variation in this energy restriction. Nevertheless, our goal was to investigate whether an acute energy restriction has an effect on fasting and postprandial ghrelin concentrations. The variation in the level of energy restriction has probably not affected these results. Moreover, one of these two studies in which the effects of energy restriction on ghrelin concentrations was investigated, had a randomized cross-over design allowing for comparison within subjects independently of the variation in energy restriction between subjects.

Subjects

Highly restrained eaters, as assessed with the Dutch Eating Behaviour Questionnaire (333), were excluded from participation in our studies, because restrained eaters are less sensitive to internal appetite cues (281). This reduced sensitivity to internal appetite cues would have confounded the association between ghrelin and appetite, the most important measure of this thesis.

The number of subjects included in an intervention study is crucial for the statistical power of the study needed to detect relevant effects (to prevent type II errors). The power analyses were based on data obtained in previous studies, performed by the same research unit. Power calculations showed that a difference of 10% in postprandial ghrelin concentrations requires about 20 subjects, and that a difference of 20% requires about 7 subjects, depending on the standard deviation of the differences. In addition, 18 subjects would be sufficient to detect a difference of 10 points on the visual analogue scales, which were considered relevant. Therefore, the lack of association between ghrelin and appetite in one study may be partly explained by inclusion of 15 subjects only. In conclusion, the statistical power was sufficient to detect relevant changes in ghrelin concentrations and in subjective appetite scores in all our intervention studies, with the exception of one study (chapter 4).

Measures

The two most important measures reported in this thesis are ghrelin concentrations and subjective appetite scores (i.e. hunger, fullness, desire to eat and prospective food consumption).

Usually total ghrelin concentrations, the sum of unacylated ghrelin and acylated ghrelin, were analyzed, because only recently, a sensitive commercial radioimmunoassay method has been made available for acylated ghrelin. The measurements were standardized by placing blood samples immediately on ice water after collection and by adding protease inhibitor to the blood samples to prevent degradation of ghrelin. In addition, ghrelin concentrations were measured in duplo, and all samples of one subject were analyzed in one run. The intraassay coefficients of variation of the ghrelin analyses were always equal or less than 10%. The postprandial ghrelin response was investigated by calculating the areas above the ghrelin curve and the postprandial decreases and preprandial increases in ghrelin concentrations. These measures of the ghrelin response were related to other physiological and non-physiological measures. Although a correlation does not provide information about causality, it will help to gain more insight into the role of ghrelin in the regulation of food intake.

Subjective appetite was assessed by visual analogue scales (VAS) which have been reported to be sensitive to experimental manipulations (e.g. alterations in diet composition and energy intake) and to changes in physiological variables involved in appetite (270;314). The appetite scores are less suitable as predictors of subsequent energy intake, since appetite scores predict subsequent energy intake only to a certain degree (± 25%) (103). Mean VAS scores are reported to be reproducible (test-retest reliability), under controlled conditions and in within-subjects, repeated-measures designs (314). The reproducibility of VAS was not investigated in the studies described in this thesis, but the studies had controlled, within-subjects, repeated-measures designs. Subjective measures of appetite may not only be a direct outcome of underlying physiological processes, but may also be influenced by cognitive and external factors. Therefore, as many external factors as possible were excluded. One of the studies was even performed in a setting devoid of external cues such as time, sunlight and sound, allowing for the investigation of the role of ghrelin in spontaneous meal initiation independent of these external cues.

External validity

The main objective of this thesis was to investigate whether ghrelin is a hunger signal. This was investigated in several ways, as described in chapters 3 to 7. In this section the external validity of the results is discussed and the four research questions, which were formulated in the introduction of this thesis, are answered.

Are ghrelin concentrations related to appetite?

If ghrelin acts as a hunger signal and plays an important role in meal initiation, its concentrations should be related to appetite. In this thesis the association between (changes in) ghrelin concentrations and several measures of appetite (i.e. subjective appetite, intermeal interval and *ad libitum* energy intake) was investigated.
Several studies have shown a positive association between fasting ghrelin concentrations and subjective appetite scores (3;84). Moreover, intravenous infusion of ghrelin has been shown to increase subjective appetite and energy intake (369). In addition, 2 out of 3 studies described in this thesis, showed that also postprandial ghrelin concentrations are positively associated with subjective appetite scores as is in accordance with the results of Cummings et al (66). The only study showing no association had a lack of power. Also effects of age (younger subjects), type of meal and possible distraction of the subjects may have affected the results.
The intermeal interval is the spontaneous time interval between two meals, and is a measure of meal initiation. The postprandial ghrelin response and the intermeal interval were associated, but only in time-blinded normal weight subjects. The stronger and longer ghrelin concentrations were suppressed, the longer subjects waited with their meal request. The association between ghrelin and spontaneous meal initiation has been investigated in two other studies, but these studies observed no significant association between ghrelin and meal initiation. However, in one study not the postprandial ghrelin responses, but rather ghrelin concentration at meal request were investigated (50), and in the other study only 6 subjects were included (66), what may explain the lack of association. In the study described in this thesis, no association between ghrelin and meal initiation was observed in obese subjects. This could have several causes. First of all, ghrelin sensitivity may be reduced in obese subjects. The blunted postprandial ghrelin response in obese subjects (96;176), supports this. Furthermore, meal initiation in obese subjects may be mainly regulated by cognitive and external factors, rather than internal factors, despite all efforts to control for this.
Ad libitum food intake is an indirect measure of satiation (meal termination). Despite the fact that infusion of ghrelin increases *ad libitum* food intake (369), no evidence for

an association between post- and preprandial ghrelin concentrations and *ad libitum* energy intake was observed. Energy intake during the *ad libitum* lunches was relatively high (on average more than 2 times the standard energy intake during lunch of Dutch adult men (339)), suggesting that food consumption was not only determined by physiological signals, but possibly also by cognitive or external factors, despite all efforts to control for these factors. Nevertheless, the results are in line with two other time-blinded studies (50;66), in which ghrelin and *ad libitum* food intake were also not associated.

The overall conclusion is that ghrelin is a hunger signal that is not involved in the determination of meal size (satiation), but that appears to be involved in the regulation of meal initiation (satiety) in normal weight men. The results suggest that suppression of the postprandial ghrelin concentrations may be used to postpone initiation of the next meal in normal weight men. Nevertheless, the association between ghrelin and meal initiation, and the role of ghrelin in obese subjects requires further investigation.

Is the postprandial ghrelin response dependent on energy or macronutrient intake?

Several studies have shown that ghrelin is affected in an energy dose dependent way following oral and intravenous administration of glucose (176;229;241;242;302) and consumption of a meal (50). In addition, this thesis shows that the postprandial ghrelin response to a carbohydrate enriched meal is dependent on the dose of carbohydrate and is unaffected by intake of the same volume of water.

Lipids and high-fat diets appear to suppress the postprandial ghrelin concentrations less effectively (98;229;231;346) as compared to carbohydrates. Data on the effects of protein intake on ghrelin concentrations are scarce. The only few studies available, showed a failure of protein to decrease ghrelin concentrations (97;98;123). In contrast, a study described in this thesis showed that a high protein meal is even a more potent suppressor of postprandial ghrelin concentrations than a high carbohydrate meal. These results are supported by the preliminary results of Clifton et al. (60) who also observed a stronger decrease in ghrelin concentrations following a high protein meal as compared to a high glycaemic carbohydrate meal. The protein source may be critical for the effects on ghrelin, whereas the studies in which protein decreased ghrelin concentrations, used milk protein, and the studies that reported no effect of protein on ghrelin concentrations used meat protein.

In conclusion, the postprandial ghrelin response is dependent on the energy content of the food consumed and on the type and composition of the macronutrient. In future

research, the effects of different types and sources of macronutrients on postprandial ghrelin concentrations should be more extensively investigated.

Is ghrelin related to other regulators of food intake?

Little is known about the role of ghrelin in the physiological regulation of food intake and its interaction with other regulators of food intake.

Williams et al (358) showed in rats that the postprandial ghrelin response requires postgastric feedback. When gastric emptying was prevented, neither glucose nor water administration affected ghrelin concentrations (358), suggesting that distension and chemo sensitization of the stomach are insufficient to induce a ghrelin response, and that post gastric processes are required. Therefore, the association between ghrelin concentrations and the gastric emptying rate, as indirectly assessed by acetaminophen absorption, was investigated. A strong inverse association was observed; the faster the gastric emptying rate, the stronger the postprandial decrease in ghrelin concentrations. This observation supports the hypothesis that ghrelin requires post gastric feedback.

There are several physiological parameters that may provide this post gastric feedback (e.g. insulin, leptin, GIP, GLP-1, CCK and PYY). Ghrelin concentrations were inversely associated with insulin concentrations. This association has been observed before (35;98) and is also supported by clamp studies that provided some evidence that insulin decreases ghrelin concentrations, independent of glucose (99;197;236;293). However, ghrelin concentrations were stronger inversely associated with GIP (glucose-dependent insulinotropic peptide) than with insulin. Only two studies investigated the association between GIP and ghrelin secretion. One study also observed an inverse association between ghrelin and GIP concentrations (3), but in another study no effect of intravenous GIP infusion on ghrelin secretion was found (292). However, the latter infusion was performed during a hyperglycaemic clamp and did not mimic normal postprandial responses. Other studies, with for example GIP receptor antagonists should be initiated to further elucidate the association between ghrelin and GIP. Whether GIP, an insulinotropic (insulin stimulating) hormone, also mediates the interaction between ghrelin and insulin, is not known and should be further investigated.

Furthermore an inverse association between ghrelin and CCK concentrations was observed, but only after a high carbohydrate breakfast. In humans, there is no further evidence for a association between CCK and ghrelin (3), but two animal studies have shown an interaction between peripheral ghrelin and CCK concentrations (74;163). CCK appears to inhibit the orexigenic effects of ghrelin (163). Ghrelin concentrations were not associated with GLP-1 concentrations, but may be associated with PYY,

since there are indications that postprandial PYY concentrations are inversely related to ghrelin concentrations (232) and that PYY decreases ghrelin concentrations (24). Despite some indications for an interaction between ghrelin and leptin (65;360), no association between (changes in) fasting ghrelin and leptin concentrations during energy restriction and subsequent *ad libitum* food intake was observed.

The above described association between ghrelin and other parameters were based on total ghrelin concentrations. In contrast, no associations between acylated ghrelin and other parameters related to food intake were observed. This may be explained by the high variation in acylated ghrelin concentrations. The association between acylated ghrelin and other factors involved in food intake regulation needs therefore further investigation.

In conclusion, the results support the hypothesis that post gastric feedback is required. This feedback may be provided by GIP and other factors, such as insulin, CCK and PYY. However, the data on the interactions between ghrelin and these parameters is scarce and needs further investigation.

Is ghrelin involved in the restoration of energy balance following energy restriction?

If ghrelin plays a role in the development of obesity, a positive association between ghrelin and BMI is expected. In contrast, fasting ghrelin concentrations are inversely associated with body mass index (BMI) (255;316;325), suggesting a physiological adaptation to the BMI, rather than a causal role of ghrelin in the development of obesity. However, ghrelin may play a role in restoration of energy homeostasis, whereas weight loss is associated with increased (135;255) and weight gain is associated with decreased plasma ghrelin concentrations (255;256;279). During periods of restricted energy intake, ghrelin may favour weight gain by stimulating food intake and reducing fat utilization (15;323;367). However, in the studies described in this thesis, neither 2 nor 3 days of 64%- energy restriction significantly increased fasting ghrelin concentrations in normal-weight men. In obese men, ghrelin concentrations did increase approximately 8% after 3 days of severe energy restriction. Two recent studies found no effect of 3 days of total fasting (56) (lean men), or 4 days of energy restriction (89) (overweight men), on fasting ghrelin concentrations. In contrast, Mars and colleagues have shown in their reproducibility studies (three studies performed with same group of lean and overweight subjects) that fasting ghrelin concentrations increased on average with 16% or 18% during two days of 65%-energy restriction (208). Changes in fasting ghrelin concentrations during energy restriction are probably dependent on changes in other physiological measures such as insulin sensitivity and fat cell activity. Although fasting ghrelin

concentrations did not change during the short-term energy restriction, this does not necessarily mean that the functionality of ghrelin is unchanged. Ghrelin sensitivity may have been improved.

There was a large inter-individual variation in the change in fasting ghrelin concentrations during 2 days of energy restriction, but this change was not positively associated with subsequent *ad libitum* food intake ($r = 0.22$; $p = 0.21$), suggesting that ghrelin does not act as a hunger signal during energy restriction. This lack of association may be caused by a poor reproducibility of fasting ghrelin concentrations, since the results of another study described in this thesis suggests that food intake may affect fasting ghrelin concentrations. In addition, energy restriction may increase active ghrelin concentrations only. Therefore, in future studies, both active and total ghrelin concentrations should be investigated.

In conclusion, no evidence for a role of ghrelin as a starvation signal was observed, but the results may have been confounded by a lack of reproducibility.

Conclusions

Ghrelin concentrations were associated with subjective measures of appetite and with the intermeal interval, but not with *ad libitum* food intake. Therefore, it is concluded that ghrelin is a hunger signal that is not involved in the determination of meal size (satiation), but that appears to be involved in the regulation of meal initiation (satiety) in normal weight men. Furthermore, ghrelin concentrations were associated with the gastric emptying rate, supporting the hypothesis that post gastric feedback is required. This feedback may be provided by GIP and other regulators of food intake, such as insulin, CCK and PYY. The postprandial ghrelin response is dependent on the energy content of the food consumed and on the type and composition of the macronutrients. Foods that contain for example dairy proteins may effectively suppress ghrelin concentrations for a longer period. These foods may then be used to postpone meal initiation, and may contribute to the prevention and treatment of overweight and obesity.

Recommendations for future research

- In future research, the causal role of postprandial ghrelin in meal initiation should be verified by, for example, studies in which the effects of intravenous infusion of physiological doses of ghrelin on voluntary meal initiation are investigated.
- To identify the most important regulators of food intake (candidates are, among others, ghrelin, CCK, GLP-1, leptin and PYY), the hierarchy of the different regulators should be assessed. Administration of receptor antagonists and intravenous infusion of the different factors can be useful herein.
- It is suggested that different types and compositions of (macro) nutrients and bioactive compounds are tested on their efficacy in modulating the most important regulators of food intake. The combination of food components that optimally induces satiation and satiety may prove to be helpful in weight maintenance and the regulation of long term energy intake. Addition of food components that increase energy expenditure, e.g. by increasing diet induced thermogenesis, may be of additional value.
- More knowledge about the signalling pathways of ghrelin and other regulators of food intake in humans will facilitate the development of pharmaceutical agents for the treatment and prevention of obesity. Identification of these signalling pathways will help to identify the most important targets (i.e. tissues, receptors and mechanisms) for the pharmaceutical agents, in order to optimally affect food intake regulation and energy homeostasis. Techniques that can be used to identify these signalling pathways are labelling techniques, functional magnetic resonance imaging (fMRI) and nutrigenomics. For example, infusion of labelled ghrelin will provide information about the main targets of peripheral ghrelin, and its signalling pathways. In addition, infusion of ghrelin in combination with fMRI will help to assess whether the main target of ghrelin in the central nervous system is indeed the hypothalamus. Furthermore, application of nutrigenomics techniques (i.e. transcriptomics, proteomics and metabolomics), will help to identify new biomarkers of satiety and satiation and may provide more insight into the mechanism of food intake regulation.

References

1. Abbott CR, Monteiro M, Small CJ et al. The inhibitory effects of peripheral administration of peptide YY(3-36) and glucagon-like peptide-1 on food intake are attenuated by ablation of the vagal-brainstem-hypothalamic pathway. Brain Res 2005;1044:127-31.
2. Adrian TE, Ferri GL, Bacarese-Hamilton AJ, Fuessl HS, Polak JM, Bloom SR. Human distribution and release of a putative new gut hormone, peptide YY. Gastroenterology 1985;89:1070-7.
3. Aguilera A, Cirugeda A, Amair R et al. Ghrelin plasma levels and appetite in peritoneal dialysis patients. Adv Perit Dial 2004;20:194-9.
4. Aine CJ. A conceptual overview and critique of functional neuroimaging techniques in humans: I. MRI/FMRI and PET. Crit Rev Neurobiol 1995;9:229-309.
5. Ainsworth BE, Haskell WL, Leon AS et al. Compendium of physical activities: classification of energy costs of human physical activities. Med Sci Sports Exerc 1993;25:71-80.
6. Allison DB, Kaprio J, Korkeila M, Koskenvuo M, Neale MC, Hayakawa K. The heritability of body mass index among an international sample of monozygotic twins reared apart. Int J Obes Relat Metab Disord 1996;20:501-6.
7. Anderson GH, Catherine NL, Woodend DM, Wolever TM. Inverse association between the effect of carbohydrates on blood glucose and subsequent short-term food intake in young men. Am J Clin Nutr 2002;76:1023-30.
8. Anderson GH, Moore SE. Dietary proteins in the regulation of food intake and body weight in humans. J Nutr 2004;134:974S-9S.
9. Anderson GH, Tecimer SN, Shah D, Zafar TA. Protein source, quantity, and time of consumption determine the effect of proteins on short-term food intake in young men. J Nutr 2004;134:3011-5.
10. Andrews JM, Doran S, Hebbard GS, Rassias G, Sun WM, Horowitz M. Effect of glucose supplementation on appetite and the pyloric motor response to intraduodenal glucose and lipid. Am J Physiol 1998;274:G645-G652.
11. Andrews JM, Rayner CK, Doran S, Hebbard GS, Horowitz M. Physiological changes in blood glucose affect appetite and pyloric motility during intraduodenal lipid infusion. Am J Physiol 1998;275:G797-G804.
12. Ariyasu H, Takaya K, Tagami T et al. Stomach is a major source of circulating ghrelin, and feeding state determines plasma ghrelin-like immunoreactivity levels in humans. J Clin Endocrinol Metab 2001;86:4753-8.
13. Arvat E, Di Vito L, Broglio F et al. Preliminary evidence that Ghrelin, the natural GH secretagogue (GHS)- receptor ligand, strongly stimulates GH secretion in humans. J Endocrinol Invest 2000;23:493-5.
14. Asakawa A, Inui A, Fujimiya M et al. Stomach regulates energy balance via acylated ghrelin and desacyl ghrelin. Gut 2005;54:18-24.
15. Asakawa A, Inui A, Kaga T et al. Antagonism of ghrelin receptor reduces food intake and body weight gain in mice. Gut 2003;52:947-52.
16. Astrup A, Astrup A, Buemann B, Flint A, Raben A. Low-fat diets and energy balance: how does the evidence stand in 2002? Proc Nutr Soc 2002;61:299-309.
17. Atkinson RL, Dhurandhar NV, Allison DB et al. Human adenovirus-36 is associated with increased body weight and paradoxical reduction of serum lipids. Int J Obes Relat Metab Disord 2005;29:281-6.
18. Attwell D, Iadecola C. The neural basis of functional brain imaging signals. Trends Neurosci 2002;25:621-5.
19. Badman MK, Flier JS. The gut and energy balance: visceral allies in the obesity wars. Science 2005;307:1909-14.
20. Ballinger A, McLoughlin L, Medbak S, Clark M. Cholecystokinin is a satiety hormone in humans at physiological post- prandial plasma concentrations. Clin Sci (Lond) 1995;89:375-81.
21. Bandettini PA, Wong EC, Hinks RS, Tikofsky RS, Hyde JS. Time course EPI of human brain function during task activation. Magn Reson Med 1992;25:390-7.
22. Banks WA, Tschop M, Robinson SM, Heiman ML. Extent and direction of ghrelin transport across the blood-brain barrier is determined by its unique primary structure. J Pharmacol Exp Ther 2002;302:822-7.

23. Barsh GS, Farooqi IS, O'Rahilly S. Genetics of body-weight regulation. Nature 2000;404:644-51.
24. Batterham RL, Cohen MA, Ellis SM et al. Inhibition of food intake in obese subjects by peptide YY3-36. N Engl J Med 2003;349:941-8.
25. Batterham RL, Cowley MA, Small CJ et al. Gut hormone PYY(3-36) physiologically inhibits food intake. Nature 2002;418:650-4.
26. Beglinger C, Degen L, Matzinger D, D'Amato M, Drewe J. Loxiglumide, a CCK-A receptor antagonist, stimulates calorie intake and hunger feelings in humans. Am-J-Physiol-Regul-Integr-Comp-Physiol 2001;280:R1149-R1154.
27. Bell CG, Walley AJ, Froguel P. The genetics of human obesity. Nat Rev Genet 2005;6:221-34.
28. Bellinger LL, Bernardis LL. The dorsomedial hypothalamic nucleus and its role in ingestive behavior and body weight regulation: lessons learned from lesioning studies. Physiol Behav 2002;76:431-42.
29. Bemelmans, W. J. E., Hoogenveen, R. T., Visscher, T. L., Verschuren, W. M. M., and Schuit, A. J. Toekomstige ontwikkelingen in overgewicht. Inschatting effecten op de volksgezondheid. RIVM rapport 260301003/2004. 2004. RIVM, Bilthoven.
Ref Type: Report
30. Berns GS. Functional neuroimaging. Life Sci 1999;65:2531-40.
31. Berridge KC. Food reward: brain substrates of wanting and liking. Neurosci Biobehav Rev 1996;20:1-25.
32. Berthoud HR. Multiple neural systems controlling food intake and body weight. Neurosci Biobehav Rev 2002;26:393-428.
33. Birch LL, McPhee L, Sullivan S, Johnson S. Conditioned meal initiation in young children. Appetite 1989;13:105-13.
34. Bjorntorp P. Obesity. Lancet 1997;350:423-6.
35. Blom WA, Stafleu A, de Graaf C, Kok FJ, Schaafsma G, Hendriks HF. Ghrelin response to carbohydrate-enriched breakfast is related to insulin. Am J Clin Nutr 2005;81:367-75.
36. Blundell J. Pharmacological approaches to appetite suppression. Trends Pharmacol Sci 1991;12:147-57.
37. Blundell J, hill AJ, Rogers P. Hunger and the satiety cascade - Their importance for food acceptance in the late 20th century. 2004:233-49.
38. Blundell JE, Burley VJ, Cotton JR, Lawton CL. Dietary fat and the control of energy intake: evaluating the effects of fat on meal size and postmeal satiety. Am J Clin Nutr 1993;57:772S-7S.
39. Blundell JE, Lawton CL, Cotton JR, Macdiarmid JI. Control of human appetite: implications for the intake of dietary fat. Annu Rev Nutr 1996;16:285-319.
40. Boden G, Chen X, Mozzoli M, Ryan I. Effect of fasting on serum leptin in normal human subjects. J Clin Endocrinol Metab 1996;81:3419-23.
41. Booth DA, Mather P, Fuller J. Starch content of ordinary foods associatively conditions human appetite and satiation, indexed by intake and eating pleasantness of starch-paired flavours. Appetite 1982;3:163-84.
42. Bouchard C, Tremblay A. Genetic influences on the response of body fat and fat distribution to positive and negative energy balances in human identical twins. J-Nutr 1997;127:943-7.
43. Bray GA, Gallagher TF, Jr. Manifestations of hypothalamic obesity in man: a comprehensive investigation of eight patients and a reveiw of the literature. Medicine (Baltimore) 1975;54:301-30.
44. Briatore L, Andraghetti G, Cordera R. Acute plasma glucose increase, but not early insulin response, regulates plasma ghrelin. Eur J Endocrinol 2003;149:403-6.
45. Broglio F, Benso A, Gottero C et al. Non-acylated ghrelin does not possess the pituitaric and pancreatic endocrine activity of acylated ghrelin in humans. J Endocrinol Invest 2003;26:192-6.
46. Broglio F, Gottero C, Prodam F et al. Non-acylated ghrelin counteracts the metabolic but not the neuroendocrine response to acylated ghrelin in humans. J Clin Endocrinol Metab 2004;89:3062-5.
47. Burton-Freeman B, Davis PA, Schneeman BO. Plasma cholecystokinin is associated with subjective measures of satiety in women. Am J Clin Nutr 2002;76:659-67.

48. Calbet JA, Holst JJ. Gastric emptying, gastric secretion and enterogastrone response after administration of milk proteins or their peptide hydrolysates in humans. Eur J Nutr 2004;43:127-39.
49. Calbet JA, MacLean DA. Plasma glucagon and insulin responses depend on the rate of appearance of amino acids after ingestion of different protein solutions in humans. J Nutr 2002;132:2174-82.
50. Callahan HS, Cummings DE, Pepe MS, Breen PA, Matthys CC, Weigle DS. Postprandial suppression of plasma ghrelin level is proportional to ingested caloric load but does not predict intermeal interval in humans. J Clin Endocrinol Metab 2004;89:1319-24.
51. Campfield L, Smith F, Rosenbaum M, Hirsch J. Human eating: evidence for a physiological basis using a modified paradigm. Neurosci-Biobehav-Rev 1996;20:133-7.
52. Campfield LA, Smith FJ, Guisez Y, Devos R, Burn P. Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central neural networks. Science 1995;269:546-9.
53. Cecil J, Francis J, Read N. Comparison of the effects of a high-fat and high-carbohydrate soup delivered orally and intragastrically on gastric emptying, appetite, and eating behaviour. Physiol-Behav 1999;67:299-306.
54. Cecil J, Francis J, Read N. Relative contributions of intestinal, gastric, oro-sensory influences and information to changes in appetite induced by the same liquid meal. Appetite 1998;31:377-90.
55. Challis BG, Pinnock SB, Coll AP, Carter RN, Dickson SL, O'Rahilly S. Acute effects of PYY3-36 on food intake and hypothalamic neuropeptide expression in the mouse. Biochem Biophys Res Commun 2003;311:915-9.
56. Chan JL, Bullen J, Lee JH, Yiannakouris N, Mantzoros CS. Ghrelin levels are not regulated by recombinant leptin administration and/or three days of fasting in healthy subjects. J Clin Endocrinol Metab 2004;89:335-43.
57. Chapelot D, Aubert R, Marmonier C, Chabert M, Louis-Sylvestre J. An endocrine and metabolic definition of the intermeal interval in humans: evidence for a role of leptin on the prandial pattern through fatty acid disposal. Am-J-Clin-Nutr 2000;72:421-31.
58. Chapman IM. Effect of intravenous glucose and euglycemic insulin infusions on short-term appetite and food intake. Am J Physiol 1998;274:R596-R603.
59. Chin-Chance C, Polonsky KS, Schoeller DA. Twenty-four-hour leptin levels respond to cumulative short-term energy imbalance and predict subsequent intake. J Clin Endocrinol Metab 2000;85:2685-91.
60. Clifton P, Bowen J, Noakes M. Effect of dietary protein and carbohydrate type on appetite and energy intake and plasma ghrelin and cholecystokinin. Obesity Reviews 2005;6:42 (abstr).
61. Comuzzie AG, Allison DB. The search for human obesity genes. Science 1998;280:1374-7.
62. Considine RV, Sinha MK, Heiman ML et al. Serum immunoreactive-leptin concentrations in normal-weight and obese humans. N Engl J Med 1996;334:292-5.
63. Crovetti R, Porrini M, Santangelo A, Testolin G. The influence of thermic effect of food on satiety. Eur-J-Clin-Nutr 1998;52:482-8.
64. Cummings DE, Clement K, Purnell JQ et al. Elevated plasma ghrelin levels in Prader Willi syndrome. Nat Med 2002;8:643-4.
65. Cummings DE, Foster KE. Ghrelin-leptin tango in body-weight regulation. Gastroenterology 2003;124:1532-5.
66. Cummings DE, Frayo RS, Marmonier C, Aubert R, Chapelot D. Plasma ghrelin levels and hunger scores in humans initiating meals voluntarily without time- and food-related cues. Am J Physiol Endocrinol Metab 2004;287:E297-E304.
67. Cummings DE, Purnell JQ, Frayo RS, Schmidova K, Wisse BE, Weigle DS. A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. Diabetes 2001;50:1714-9.
68. Cummings DE, Weigle DS, Frayo RS et al. Plasma ghrelin levels after diet-induced weight loss or gastric bypass surgery. N Engl J Med 2002;346:1623-30.
69. Cuntz U, Fruhauf E, Wawarta R et al. A role for the novel weight-regulating hormone ghrelin in anorexia nervosa. Am Clin Lab 2002;21:22-3.

70. Dallongeville J, Hecquet B, Lebel P et al. Short term response of circulating leptin to feeding and fasting in man: influence of circadian cycle. Int J Obes Relat Metab Disord 1998;22:728-33.
71. Date Y, Kojima M, Hosoda H et al. Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. Endocrinology 2000;141:4255-61.
72. Date Y, Murakami N, Toshinai K et al. The role of the gastric afferent vagal nerve in ghrelin-induced feeding and growth hormone secretion in rats. Gastroenterology 2002;123:1120-8.
73. Date Y, Nakazato M, Murakami N, Kojima M, Kangawa K, Matsukura S. Ghrelin acts in the central nervous system to stimulate gastric acid secretion. Biochem Biophys Res Commun 2001;280:904-7.
74. Date Y, Toshinai K, Koda S et al. Peripheral Interaction of Ghrelin with Cholecystokinin on Feeding Regulation. Endocrinology 2005.
75. Davison JS, Clarke GD. Mechanical properties and sensitivity to CCK of vagal gastric slowly adapting mechanoreceptors. Am J Physiol 1988;255:G55-G61.
76. de Graaf C. The validity of appetite ratings. Appetite 1993;21:156-60.
77. de Graaf C, De Jong LS, Lambers AC. Palatability affects satiation but not satiety. Physiol Behav 1999;66:681-8.
78. de Graaf C, Hulshof T, Weststrate JA, Jas P. Short-term effects of different amounts of protein, fats, and carbohydrates on satiety. Am J Clin Nutr 1992;55:33-8.
79. de Graaf C, Jas P, van der KK, Leenen R. Circadian rhythms of appetite at different stages of a weight loss programme. Int J Obes Relat Metab Disord 1993;17:521-6.
80. de Vries JH, Zock PL, Mensink RP, Katan MB. Underestimation of energy intake by 3-d records compared with energy intake to maintain body weight in 269 nonobese adults. Am J Clin Nutr 1994;60:855-60.
81. de Zwart IM, Mearadji B, Lamb HJ et al. Gastric motility: comparison of assessment with real-time MR imaging or barostat measurement initial experience. Radiology 2002;224:592-7.
82. Degen L, Matzinger D, Drewe J, Beglinger C. The effect of cholecystokinin in controlling appetite and food intake in humans. Peptides 2001;22:1265-9.
83. Del Parigi A, Chen K, Gautier JF et al. Sex differences in the human brain's response to hunger and satiation. Am J Clin Nutr 2002;75:1017-22.
84. DelParigi A, Tschop M, Heiman ML et al. High circulating ghrelin: a potential cause for hyperphagia and obesity in prader-willi syndrome. J Clin Endocrinol Metab 2002;87:5461-4.
85. Dhurandhar NV, Whigham LD, Abbott DH et al. Human adenovirus Ad-36 promotes weight gain in male rhesus and marmoset monkeys. J Nutr 2002;132:3155-60.
86. Diplock AT, Aggett PJ, Ashwell M, Bornet F, Fern EB, Robertfroid MB. Scientific concepts of functional foods in Europe: Consensus Document. Br-J-Nutr 1999;81:S1-S27.
87. Dockray GJ. Luminal sensing in the gut: an overview. J Physiol Pharmacol 2003;54 Suppl 4:9-17.
88. Dornonville dlC, Lindstrom E, Norlen P, Hakanson R. Ghrelin stimulates gastric emptying but is without effect on acid secretion and gastric endocrine cells. Regul Pept 2004;120:23-32.
89. Doucet E, Pomerleau M, Harper ME. Fasting and postprandial total ghrelin remain unchanged after short-term energy restriction. J Clin Endocrinol Metab 2004;89:1727-32.
90. Dubuc G, Phinney S, Stern J, Havel P. Changes of serum leptin and endocrine and metabolic parameters after 7 days of energy restriction in men and women. Metabolism 1998;47:429-34.
91. Due A, Toubro S, Skov AR, Astrup A. Effect of normal-fat diets, either medium or high in protein, on body weight in overweight subjects: a randomised 1-year trial. Int J Obes Relat Metab Disord 2004;28:1283-90.
92. Ebihara K, Ogawa Y, Katsuura G et al. Involvement of agouti-related protein, an endogenous antagonist of hypothalamic melanocortin receptor, in leptin action. Diabetes 1999;48:2028-33.
93. Elimam A, Marcus C. Meal timing, fasting and glucocorticoids interplay in serum leptin concentrations and diurnal profile. Eur J Endocrinol 2002;147:181-8.
94. Elliott R, Morgan L, Tredger J, Deacon S, Wright J, Marks V. Glucagon-like peptide-1 (7-36)amide and glucose-dependent insulinotropic polypeptide secretion in response to

nutrient ingestion in man: acute post-prandial and 24-h secretion patterns. J-Endocrinol 1993;138:159-66.
95. Elliott SS, Keim NL, Stern JS, Teff K, Havel PJ. Fructose, weight gain, and the insulin resistance syndrome. Am J Clin Nutr 2002;76:911-22.
96. English PJ, Ghatei MA, Malik IA, Bloom SR, Wilding JP. Food fails to suppress ghrelin levels in obese humans. J Clin Endocrinol Metab 2002;87:2984.
97. Erdmann J, Lippl F, Schusdziarra V. Differential effect of protein and fat on plasma ghrelin levels in man. Regul Pept 2003;116:101-7.
98. Erdmann J, Topsch R, Lippl F, Gussmann P, Schusdziarra V. Postprandial response of plasma ghrelin levels to various test meals in relation to food intake, plasma insulin, and glucose. J Clin Endocrinol Metab 2004;89:3048-54.
99. Flanagan DE, Evans ML, Monsod TP et al. The influence of insulin on circulating ghrelin. Am J Physiol Endocrinol Metab 2003;284:E313-E316.
100. Flegal KM, Carroll MD, Ogden CL, Johnson CL. Prevalence and trends in obesity among US adults, 1999-2000. JAMA 2002;288:1723-7.
101. Flier JS. Obesity wars: molecular progress confronts an expanding epidemic. Cell 2004;116:337-50.
102. Flint A, Raben A, Astrup A, Holst J. Glucagon-like peptide 1 promotes satiety and suppresses energy intake in humans. J-Clin-Invest 1998;101:515-20.
103. Flint A, Raben A, Blundell J, Astrup A. Reproducibility, power and validity of visual analogue scales in assessment of appetite sensations in single test meal studies. Int-J-Obes-Relat-Metab-Disord 2000;24:38-48.
104. Flint A, Raben A, Ersboll AK, Holst JJ, Astrup A. The effect of physiological levels of glucagon-like peptide-1 on appetite, gastric emptying, energy and substrate metabolism in obesity. Int J Obes Relat Metab Disord 2001;25:781-92.
105. Fredriks AM, van Buuren S, Wit JM, Verloove-Vanhorick SP. Body index measurements in 1996-7 compared with 1980. Arch Dis Child 2000;82:107-12.
106. French S, Conlon C, Mutuma S et al. The effects of intestinal infusion of long-chain fatty acids on food intake in humans. Gastroenterology 2000;119:943-8.
107. French SJ, Bergin A, Sepple CP, Read NW, Rovati L. The effects of loxiglumide on food intake in normal weight volunteers. Int J Obes Relat Metab Disord 1994;18:738-41.
108. French SJ, Murray B, Rumsey RD, Sepple CP, Read NW. Is cholecystokinin a satiety hormone? Correlations of plasma cholecystokinin with hunger, satiety and gastric emptying in normal volunteers. Appetite 1993;21:95-104.
109. Friedman MI. Fuel partitioning and food intake. The-American-journal-of-clinical-nutrition (USA) 1998;67:513-8.
110. Gauna C, Meyler FM, Janssen JA et al. Administration of acylated ghrelin reduces insulin sensitivity, whereas the combination of acylated plus unacylated ghrelin strongly improves insulin sensitivity. J Clin Endocrinol Metab 2004;89:5035-42.
111. Gautier J, Chen K, Salbe A et al. Differential brain responses to satiation in obese and lean men. Diabetes 2000;49:838-46.
112. Gautier JF, Del Parigi A, Chen K et al. Effect of satiation on brain activity in obese and lean women. Obes Res 2001;9:676-84.
113. Geel-Schutten GH, Faber EJ, Smit E et al. Biochemical and structural characterization of the glucan and fructan exopolysaccharides synthesized by the lactobacillus reuteri wild-type strain and by mutant strains. Appl Environ Microbiol 1999;65:3008-14.
114. Geliebter A. Gastric distension and gastric capacity in relation to food intake in humans. Physiol Behav 1988;44:665-8.
115. Geliebter A, Melton PM, McCray RS, Gallagher DR, Gage D, Hashim SA. Gastric capacity, gastric emptying, and test-meal intake in normal and bulimic women. Am J Clin Nutr 1992;56:656-61.
116. Geliebter A, Schachter S, Lohmann-Walter C, Feldman H, Hashim SA. Reduced stomach capacity in obese subjects after dieting. Am J Clin Nutr 1996;63:170-3.
117. Geliebter A, Westreich S, Gage D. Gastric distention by balloon and test-meal intake in obese and lean subjects. Am J Clin Nutr 1988;48:592-4.

118. Gibney, MJ, Wolever, TMS, and Frayn, KN. Periodicity of eating and human health. Br-J-Nutr 77 (Suppl. 1), S1-S129. 1997.
Ref Type: Journal (Full)
119. Gielkens HA, Verkijk M, Lam WF, Lamers CB, Masclee AA. Effects of hyperglycemia and hyperinsulinemia on satiety in humans. Metabolism 1998;47:321-4.
120. Gnanapavan S, Kola B, Bustin SA et al. The Tissue Distribution of the mRNA of Ghrelin and Subtypes of Its Receptor, GHS-R, in Humans. J Clin Endocrinol Metab 2002;87:2988.
121. Goris AH, Westerterp-Plantenga MS, Westerterp KR. Undereating and underrecording of habitual food intake in obese men: selective underreporting of fat intake. Am J Clin Nutr 2000;71:130-4.
122. Graff-Radford NR, Russell JW, Rezai K. Frontal degenerative dementia and neuroimaging. Adv Neurol 1995;66:37-47.
123. Greenman Y, Golani N, Gilad S, Yaron M, Limor R, Stern N. Ghrelin secretion is modulated in a nutrient- and gender-specific manner. Clin Endocrinol (Oxf) 2004;60:382-8.
124. Guo SS, Wu W, Chumlea WC, Roche AF. Predicting overweight and obesity in adulthood from body mass index values in childhood and adolescence. Am J Clin Nutr 2002;76:653-8.
125. Gutt M, Davis CL, Spitzer SB et al. Validation of the insulin sensitivity index (ISI(0,120)): comparison with other measures. Diabetes Res Clin Pract 2000;47:177-84.
126. Gutzwiller J, Drewe J, Ketterer S, Hildebrand P, Krautheim A, Beglinger C. Interaction between CCK and a preload on reduction of food intake is mediated by CCK-A receptors in humans. Am-J-Physiol-Regul-Integr-Comp-Physiol 2000;279:R189-R195.
127. Gutzwiller J, Goke B, Drewe J et al. Glucagon-like peptide-1: a potent regulator of food intake in humans. Gut 1999;44:81-6.
128. Gutzwiller JP, Degen L, Matzinger D, Prestin S, Beglinger C. Interaction between GLP-1 and CCK-33 in inhibiting food intake and appetite in men. Am J Physiol Regul Integr Comp Physiol 2004;287:R562-R567.
129. Gutzwiller JP, Drewe J, Goke B et al. Glucagon-like peptide-1 promotes satiety and reduces food intake in patients with diabetes mellitus type 2. Am J Physiol 1999;276:R1541-R1544.
130. Gutzwiller JP, Drewe J, Hildebrand P, Rossi L, Lauper JZ, Beglinger C. Effect of intravenous human gastrin-releasing peptide on food intake in humans. Gastroenterology 1994;106:1168-73.
131. Halaas JL, Gajiwala KS, Maffei M et al. Weight-reducing effects of the plasma protein encoded by the obese gene. Science 1995;269:543-6.
132. Halford J, Blundell J. Pharmacology of appetite suppression. Prog-Drug-Res 2000;5425-58.
133. Hall WL, Millward DJ, Long SJ, Morgan LM. Casein and whey exert different effects on plasma amino acid profiles, gastrointestinal hormone secretion and appetite. Br J Nutr 2003;89:239-48.
134. Halton TL, Hu FB. The effects of high protein diets on thermogenesis, satiety and weight loss: a critical review. J Am Coll Nutr 2004;23:373-85.
135. Hansen TK, Dall R, Hosoda H et al. Weight loss increases circulating levels of ghrelin in human obesity. Clin Endocrinol (Oxf) 2002;56:203-6.
136. Havel PJ. Peripheral signals conveying metabolic information to the brain: short- term and long-term regulation of food intake and energy homeostasis. Exp Biol Med (Maywood) 2001;226:963-77.
137. Havel PJ, Townsend R, Chaump L, Teff K. High-fat meals reduce 24-h circulating leptin concentrations in women. Diabetes 1999;48:334-41.
138. Heini AF, Kirk KA, Lara-Castro C, Weinsier RL. Relationship between hunger-satiety feelings and various metabolic parameters in women with obesity during controlled weight loss. Obes Res 1998;6:225-30.
139. Heini AF, Lara-Castro C, Kirk KA, Considine RV, Caro JF, Weinsier RL. Association of leptin and hunger-satiety ratings in obese women. Int J Obes Relat Metab Disord 1998;22:1084-7.
140. Hill JO, Peters JC. Environmental contributions to the obesity epidemic. Science 1998;280:1371-4.
141. Holt SH, Brand Miller JC, Petocz P. Interrelationships among postprandial satiety, glucose and insulin responses and changes in subsequent food intake. Eur J Clin Nutr 1996;50:788-97.

142. Holt SH, Miller JC, Petocz P, Farmakalidis E. A satiety index of common foods. Eur J Clin Nutr 1995;49:675-90.
143. Hukshorn CJ, Westerterp-Plantenga MS, Saris WH. Pegylated human recombinant leptin (PEG-OB) causes additional weight loss in severely energy-restricted, overweight men. Am J Clin Nutr 2003;77:771-6.
144. Hulshof T, de Graaf C, Weststrate JA. The effects of preloads varying in physical state and fat content on satiety and energy intake. Appetite 1993;21:273-86.
145. Ikezaki A, Hosoda H, Ito K et al. Fasting plasma ghrelin levels are negatively correlated with insulin resistance and PAI-1, but not with leptin, in obese children and adolescents. Diabetes 2002;51:3408-11.
146. James WPT, Schofield EC. Different levels of analysis in estimating requirements. In: James WPT, Schofield EC, eds. Human Energy Requirements. Oxford; New York; Tokyo: Oxford Medical Publications 1990:42-57.
147. Jenkins DJ, Kendall CW, Augustin LS et al. Glycemic index: overview of implications in health and disease. Am J Clin Nutr 2002;76:266S-73S.
148. Jenkins DJ, Wolever TM, Collier GR et al. Metabolic effects of a low-glycemic-index diet. Am J Clin Nutr 1987;46:968-75.
149. Jenkins DJ, Wolever TM, Taylor RH et al. Glycemic index of foods: a physiological basis for carbohydrate exchange. Am J Clin Nutr 1981;34:362-6.
150. Joannic JL, Oppert JM, Lahlou N et al. Plasma leptin and hunger ratings in healthy humans. Appetite 1998;30:129-38.
151. Jousilahti P, Tuomilehto J, Vartiainen E, Pekkanen J, Puska P. Body weight, cardiovascular risk factors, and coronary mortality. 15-year follow-up of middle-aged men and women in eastern Finland. Circulation 1996;93:1372-9.
152. Kalra SP, Bagnasco M, Otukonyong EE, Dube MG, Kalra PS. Rhythmic, reciprocal ghrelin and leptin signaling: new insight in the development of obesity. Regul Pept 2003;111:1-11.
153. Kamegai J, Tamura H, Shimizu T, Ishii S, Sugihara H, Oikawa S. Regulation of the ghrelin gene: growth hormone-releasing hormone upregulates ghrelin mRNA in the pituitary. Endocrinology 2001;142:4154-7.
154. Kamegai J, Tamura H, Shimizu T, Ishii S, Sugihara H, Wakabayashi I. Central effect of ghrelin, an endogenous growth hormone secretagogue, on hypothalamic peptide gene expression. Endocrinology 2000;141:4797-800.
155. Kamegai J, Tamura H, Shimizu T, Ishii S, Sugihara H, Wakabayashi I. Chronic central infusion of ghrelin increases hypothalamic neuropeptide Y and Agouti-related protein mRNA levels and body weight in rats. Diabetes 2001;50:2438-43.
156. Karhunen L, Haffner S, Lappalainen R, Turpeinen A, Miettinen H, Uusitupa M. Serum leptin and short-term regulation of eating in obese women. Clin Sci (Lond) 1997;92:573-8.
157. Katz A, Nambi SS, Mather K et al. Quantitative insulin sensitivity check index: a simple, accurate method for assessing insulin sensitivity in humans. J Clin Endocrinol Metab 2000;85:2402-10.
158. Keim N, Stern J, Havel P. Relation between circulating leptin concentrations and appetite during a prolonged, moderate energy deficit in women. Am-J-Clin-Nutr 1998;68:794-801.
159. Kieffer TJ, McIntosh CH, Pederson RA. Degradation of glucose-dependent insulinotropic polypeptide and truncated glucagon-like peptide 1 in vitro and in vivo by dipeptidyl peptidase IV. Endocrinology 1995;136:3585-96.
160. Kissileff HR, Carretta JC, Geliebter A, Pi-Sunyer FX. Cholecystokinin and stomach distension combine to reduce food intake in humans. Am J Physiol Regul Integr Comp Physiol 2003;285:R992-R998.
161. Kissileff HR, Pi-Sunyer FX, Thornton J, Smith GP. C-terminal octapeptide of cholecystokinin decreases food intake in man. Am J Clin Nutr 1981;34:154-60.
162. Knight RT, Grabowecky MF, Scabini D. Role of human prefrontal cortex in attention control. Adv Neurol 1995;66:21-34.
163. Kobelt P, Tebbe JJ, Tjandra I et al. CCK inhibits the orexigenic effect of peripheral ghrelin. Am J Physiol Regul Integr Comp Physiol 2005;288:R751-R758.
164. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 1999;402:656-60.

165. Kolaczynski JW, Ohannesian JP, Considine RV, Marco CC, Caro JF. Response of leptin to short-term and prolonged overfeeding in humans. J Clin Endocrinol Metab 1996;81:4162-5.
166. Kong MF, Chapman I, Goble E et al. Effects of oral fructose and glucose on plasma GLP-1 and appetite in normal subjects. Peptides 1999;20:545-51.
167. Kovacs, E. M. Satiety and body weight regulation. 2002. Maastricht University. Ref Type: Thesis/Dissertation
168. Kovacs EM, Westerterp-Plantenga MS, Saris WH et al. Associations between spontaneous meal initiations and blood glucose dynamics in overweight men in negative energy balance. Br J Nutr 2002;87:39-45.
169. Kwong KK, Belliveau JW, Chesler DA et al. Dynamic magnetic resonance imaging of human brain activity during primary sensory stimulation. Proc Natl Acad Sci U S A 1992;89:5675-9.
170. Lang V, Bellisle F, Alamowitch C et al. Varying the protein source in mixed meal modifies glucose, insulin and glucagon kinetics in healthy men, has weak effects on subjective satiety and fails to affect food intake. Eur-J-Clin-Nutr 1999;53:959-65.
171. Lang V, Bellisle F, Oppert JM et al. Satiating effect of proteins in healthy subjects: a comparison of egg albumin, casein, gelatin, soy protein, pea protein, and wheat gluten. Am J Clin Nutr 1998;67:1197-204.
172. Langhans W, Grossmann F, Geary N. Intrameal hepatic-portal infusion of glucose reduces spontaneous meal size in rats. Physiol Behav 2001;73:499-507.
173. Lavin JH. Interaction of insulin, glucagon-like peptide 1, gastric inhibitory polypeptide, and appetite in response to intraduodenal carbohydrate. Am J Clin Nutr 1998;68:591-8.
174. Lavin JH, Wittert G, Sun WM, Horowitz M, Morley JE, Read NW. Appetite regulation by carbohydrate: role of blood glucose and gastrointestinal hormones. Am J Physiol 1996;271:E209-E214.
175. Lawton CL, Burley VJ, Wales JK, Blundell JE. Dietary fat and appetite control in obese subjects: weak effects on satiation and satiety. Int J Obes Relat Metab Disord 1993;17:409-16.
176. Le Roux CW, Patterson M, Vincent RP, Hunt C, Ghatei MA, Bloom SR. Postprandial plasma ghrelin is suppressed proportional to meal calorie content in normal-weight but not obese subjects. J Clin Endocrinol Metab 2005;90:1068-71.
177. Leidy HJ, Gardner JK, Frye BR et al. Circulating ghrelin is sensitive to changes in body weight during a diet and exercise program in normal-weight young women. J Clin Endocrinol Metab 2004;89:2659-64.
178. LeMagnen J. Neurobiology of feeding and nutrition. London: Academic Press, 1992.
179. Levine AS, Kotz CM, Gosnell BA. Sugars and fats: the neurobiology of preference. J Nutr 2003;133:831S-4S.
180. Liddle RA. On the measurement of cholecystokinin. Clin Chem 1998;44:903-4.
181. Lieverse, R. CCK and bombesin and obesity. 61-73. 1993. Ref Type: Thesis/Dissertation
182. Lieverse R, Jansen J, Masclee A, Lamers C. Significant satiety effect of bombesin in lean but not in obese subjects. Int-J-Obes-Relat-Metab-Disord 1994;18:579-83.
183. Lieverse R, Jansen J, Zwan Avd et al. Bombesin reduces food intake in lean man by a cholecystokinin-independent mechanism. J-Clin-Endocrinol-Metab 1993;76:1495-8.
184. Lieverse R, Masclee A, Jansen J, Lam W, Lamers C. Obese women are less sensitive for the satiety effects of bombesin than lean women. Eur-J-Clin-Nutr 1998;52:207-12.
185. Lieverse RJ, Jansen JB, Masclee AA, Lamers CB. Satiety effects of a physiological dose of cholecystokinin in humans. Gut 1995;36:176-9.
186. Lieverse RJ, Jansen JB, Masclee AA, Lamers CB. Satiety effects of a physiological dose of cholecystokinin in humans. Gut 1995;36:176-9.
187. Lieverse RJ, Jansen JB, Masclee AA, Lamers CB. Bombesin reduces food intake after a preload in man by a cholecystokinin-independent mechanism. Clin Sci (Lond) 1993;85:277-80.
188. Lieverse RJ, Jansen JB, Masclee AA, Rovati LC, Lamers CB. Effect of a low dose of intraduodenal fat on satiety in humans: studies using the type A cholecystokinin receptor antagonist loxiglumide. Gut 1994;35:501-5.

189. Lieverse RJ, Jansen JB, Masclee AA, Rovati LC, Lamers CB. Effect of a low dose of intraduodenal fat on satiety in humans: studies using the type A cholecystokinin receptor antagonist loxiglumide. Gut 1994;35:501-5.
190. Lieverse RJ, Jansen JB, Masclee AM, Lamers CB. Effects of somatostatin on human satiety. Neuroendocrinology 1995;61:112-6.
191. Lieverse RJ, Jansen JB, van de ZA, Samson L, Masclee AA, Lamers CB. Effects of a physiological dose of cholecystokinin on food intake and postprandial satiation in man. Regul Pept 1993;43:83-9.
192. Lieverse RJ, Jansen JB, van de ZA, Samson L, Masclee AA, Lamers CB. Effects of a physiological dose of cholecystokinin on food intake and postprandial satiation in man. Regul Pept 1993;43:83-9.
193. Lippl F, Kircher F, Erdmann J, Allescher HD, Schusdziarra V. Effect of GIP, GLP-1, insulin and gastrin on ghrelin release in the isolated rat stomach. Regul Pept 2004;119:93-8.
194. Liu Y, Gao J, Liu H, Fox P. The temporal response of the brain after eating revealed by functional MRI. Nature 2000;405:1058-62.
195. Long S, Sutton J, Amaee W et al. No effect of glucagon-like peptide-1 on short-term satiety and energy intake in man. Br-J-Nutr 1999;81:273-9.
196. Louis-Sylvestre J, Le Magnen J. A fall in blood glucose level precedes meal onset in free-feeding rats. 1980. Obes Res 1996;4:497-500.
197. Lucidi P, Murdolo G, Di Loreto C et al. Ghrelin is not necessary for adequate hormonal counterregulation of insulin-induced hypoglycemia. Diabetes 2002;51:2911-4.
198. Maas MI, Hopman WP, Gelder BV et al. Does intraduodenal administration of sucrose polyester (Olestra) cause satiation in humans? Appetite 1999;33:195-208.
199. Maas MI, Hopman WP, Katan MB, Jansen JB. Release of peptide YY and inhibition of gastric acid secretion by long- chain and medium-chain triglycerides but not by sucrose polyester in men. Eur J Clin Invest 1998;28:123-30.
200. MacIntosh CG, Andrews JM, Jones KL et al. Effects of age on concentrations of plasma cholecystokinin, glucagon- like peptide 1, and peptide YY and their relation to appetite and pyloric motility. Am J Clin Nutr 1999;69:999-1006.
201. MacIntosh CG, Horowitz M, Verhagen MA et al. Effect of small intestinal nutrient infusion on appetite, gastrointestinal hormone release, and gastric myoelectrical activity in young and older men. Am J Gastroenterol 2001;96:997-1007.
202. MacIntosh CG, Morley JE, Wishart J et al. Effect of exogenous cholecystokinin (CCK)-8 on food intake and plasma CCK, leptin, and insulin concentrations in older and young adults: evidence for increased CCK activity as a cause of the anorexia of aging. J Clin Endocrinol Metab 2001;86:5830-7.
203. Maes HH, Neale MC, Eaves LJ. Genetic and environmental factors in relative body weight and human adiposity. Behav Genet 1997;27:325-51.
204. Maffeis C. Aetiology of overweight and obesity in children and adolescents. Eur J Pediatr 2000;159 Suppl 1:S35-S44.
205. Mars M, de Graaf C, van Rossum CT, de Groot CPGM, Seidell JC, Kok FJ. Leptin and appetite responses induced by a four-day energy restriction; prliminary results. Appetite 2002;39:247 (abstr).
206. Mars M, de Graaf C, de Groot LCPGM, Kok FJ. Leptin decline after acute energy restriction and compensation in food intake. AMERICAN-JOURNAL-OF-CLINICAL-NUTRITION 2005.
207. Mars M, de Graaf C, de Groot LCPGM, van Rossum C, Kok FJ. Leptin and appetite responses induced by a four-day energy restriction; preliminary results. Appetite 2002;39:247 (abstr).
208. Mars M, de Graaf C, de Groot LCPGM, Vrijhof C, van Rossum C, Kok FJ. Leptin, insulin and ghrelin responses to energy restriction: biomarkers for restoring energy balance? In: Mars M, ed. The acute leptin decline after energy restriction. A biomarker for the subsceptibility to weight gain? Thesis Wageningen University, Wageningen: 2004:25-37.
209. Mars M, de Graaf C, de Groot LCPGM, Vrijhof C, van Rossum C, Kok FJ. Leptin, insulin and ghrelin responses to energy restriction: biomarkers for restoring energy balance? In: Mars M, ed. The acute leptin decline after energy restriction. A biomarker for the subsceptibility to weight gain? Thesis Wageningen University, Wageningen: 2005:25-37.

210. Mars M, de GC, van Rossum CT, de Groot CP, Seidell JC, Kok FJ. Leptin and insulin responses to a four-day energy-deficient diet in men with different weight history. Int J Obes Relat Metab Disord 2003;27:574-81.
211. Marti A, Moreno-Aliaga MJ, Hebebrand J, Martinez JA. Genes, lifestyles and obesity. Int J Obes Relat Metab Disord 2004;28 Suppl 3:S29-S36.
212. Matsuda M, Liu Y, Mahankali S et al. Altered hypothalamic function in response to glucose ingestion in obese humans. Diabetes 1999;48:1801-6.
213. Mattes R. Hunger ratings are not a valid proxy measure of reported food intake in humans. Appetite 1990;15:103-13.
214. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 1985;28:412-9.
215. Matzinger D, Degen L, Drewe J et al. The role of long chain fatty acids in regulating food intake and cholecystokinin release in humans. Gut 2000;46:688-93.
216. Matzinger D, Gutzwiller J, Drewe J et al. Inhibition of food intake in response to intestinal lipid is mediated by cholecystokinin in humans. Am-J-Physiol 1999;277:R1718-R1724.
217. Mayer J. Regulation of the energy intake and the body weight. The glucostatic theory and the liposatic hypothesis. Ann N Y Acad Sci 1955;63:15-42.
218. McLaughlin T, Abbasi F, Lamendola C, Frayo RS, Cummings DE. Plasma ghrelin concentrations are decreased in insulin-resistant obese adults relative to equally obese insulin-sensitive controls. J Clin Endocrinol Metab 2004;89:1630-5.
219. McMinn JE, Sindelar DK, Havel PJ, Schwartz MW. Leptin deficiency induced by fasting impairs the satiety response to cholecystokinin. Endocrinology 2000;141:4442-8.
220. Meier JJ, Nauck MA, Kranz D et al. Secretion, degradation, and elimination of glucagon-like peptide 1 and gastric inhibitory polypeptide in patients with chronic renal insufficiency and healthy control subjects. Diabetes 2004;53:654-62.
221. Meier JJ, Nauck MA, Schmidt WE, Gallwitz B. Gastric inhibitory polypeptide: the neglected incretin revisited. Regul Pept 2002;107:1-13.
222. Meiselman HL. The contextual basis for food acceptance. In: Meiselman HL, MacFie HJ, eds. Food choice, acceptance and consumption. London: Blackie Academic & Professional 1996:239-63.
223. Meiselman HL, deGraaf C, Lesher LL. The effects of variety and monotony on food acceptance and intake at a midday meal. Physiol Behav 2000;70:119-25.
224. Melanson K, Westerterp PM, Saris W, Smith F, Campfield L. Blood glucose patterns and appetite in time-blinded humans: carbohydrate versus fat. Am-J-Physiol 1999;277:R337-R345.
225. Melanson KJ, Westerterp-Plantenga MS, Campfield LA, Saris WH. Appetite and blood glucose profiles in humans after glycogen-depleting exercise. J Appl Physiol 1999;87:947-54.
226. Melanson KJ, Westerterp-Plantenga MS, Campfield LA, Saris WH. Blood glucose and meal patterns in time-blinded males, after aspartame, carbohydrate, and fat consumption, in relation to sweetness perception. Br J Nutr 1999;82:437-46.
227. Melton PM, Kissileff HR, Pi-Sunyer FX. Cholecystokinin (CCK-8) affects gastric pressure and ratings of hunger and fullness in women. Am J Physiol 1992;263:R452-R456.
228. Mikkelsen PB, Toubro S, Astrup A. Effect of fat-reduced diets on 24-h energy expenditure: comparisons between animal protein, vegetable protein, and carbohydrate. Am-J-Clin-Nutr 2000;72:1135-41.
229. Mohlig M, Spranger J, Otto B, Ristow M, Tschop M, Pfeiffer AF. Euglycemic hyperinsulinemia, but not lipid infusion, decreases circulating ghrelin levels in humans. J Endocrinol Invest 2002;25:RC36-RC38.
230. Mokdad AH, Marks JS, Stroup DF, Gerberding JL. Actual causes of death in the United States, 2000. JAMA 2004;291:1238-45.
231. Monteleone P, Bencivenga R, Longobardi N, Serritella C, Maj M. Differential responses of circulating ghrelin to high-fat or high-carbohydrate meal in healthy women. J Clin Endocrinol Metab 2003;88:5510-4.

232. Monteleone P, Martiadis V, Rigamonti AE et al. Investigation of peptide YY and ghrelin responses to a test meal in bulimia nervosa. Biol Psychiatry 2005;57:926-31.
233. Mook DG, Votaw MC. How important is hedonism? Reasons given by college students for ending a meal. Appetite 1992;18:69-75.
234. Muccioli G, Papotti M, Locatelli V, Ghigo E, Deghenghi R. Binding of 125I-labeled ghrelin to membranes from human hypothalamus and pituitary gland. J Endocrinol Invest 2001;24:RC7-RC9.
235. Muller AF, Lamberts SW, Janssen JA et al. Ghrelin drives GH secretion during fasting in man. Eur J Endocrinol 2002;146:203-7.
236. Murdolo G, Lucidi P, Di Loreto C et al. Insulin is required for prandial ghrelin suppression in humans. Diabetes 2003;52:2923-7.
237. Must A, Spadano J, Coakley EH, Field AE, Colditz G, Dietz WH. The disease burden associated with overweight and obesity. JAMA 1999;282:1523-9.
238. Muurahainen N, Kissileff HR, Derogatis AJ, Pi-Sunyer FX. Effects of cholecystokinin-octapeptide (CCK-8) on food intake and gastric emptying in man. Physiol Behav 1988;44:645-9.
239. Muurahainen NE, Kissileff HR, Lachaussee J, Pi-Sunyer FX. Effect of a soup preload on reduction of food intake by cholecystokinin in humans. Am J Physiol 1991;260:R672-R680.
240. Muurahainen NE, Kissileff HR, Pi-Sunyer FX. Intravenous infusion of bombesin reduces food intake in humans. Am J Physiol 1993;264:R350-R354.
241. Nakagawa E, Nagaya N, Okumura H et al. Hyperglycaemia suppresses the secretion of ghrelin, a novel growth-hormone-releasing peptide: responses to the intravenous and oral administration of glucose. Clin Sci (Lond) 2002;103:325-8.
242. Nakai Y, Hosoda H, Nin K et al. Plasma levels of active form of ghrelin during oral glucose tolerance test in patients with anorexia nervosa. Eur J Endocrinol 2003;149:R1-R3.
243. Nakazato M, Murakami N, Date Y et al. A role for ghrelin in the central regulation of feeding. Nature 2001;409:194-8.
244. Naslund E, Barkeling B, King N et al. Energy intake and appetite are suppressed by glucagon-like peptide-1 (GLP-1) in obese men. Int J Obes Relat Metab Disord 1999;23:304-11.
245. Naslund E, Gutniak M, Skogar S, Rossner S, Hellstrom PM. Glucagon-like peptide 1 increases the period of postprandial satiety and slows gastric emptying in obese men. Am J Clin Nutr 1998;68:525-30.
246. Nauck MA, Niedereichholz U, Ettler R et al. Glucagon-like peptide 1 inhibition of gastric emptying outweighs its insulinotropic effects in healthy humans. Am J Physiol 1997;273:E981-E988.
247. Nilsson M, Stenberg M, Frid AH, Holst JJ, Bjorck IM. Glycemia and insulinemia in healthy subjects after lactose-equivalent meals of milk and other food proteins: the role of plasma amino acids and incretins. Am J Clin Nutr 2004;80:1246-53.
248. Nonaka N, Shioda S, Niehoff ML, Banks WA. Characterization of blood-brain barrier permeability to PYY3-36 in the mouse. J Pharmacol Exp Ther 2003;306:948-53.
249. O'Doherty J, Rolls ET, Francis S, Bowtell R, McGlone F. Representation of pleasant and aversive taste in the human brain. J Neurophysiol 2001;85:1315-21.
250. O'Doherty J, Rolls ET, Francis S et al. Sensory-specific satiety-related olfactory activation of the human orbitofrontal cortex. Neuroreport 2000;11:893-7.
251. Ogawa S, Menon RS, Kim SG, Ugurbil K. On the characteristics of functional magnetic resonance imaging of the brain. Annu Rev Biophys Biomol Struct 1998;27:447-74.
252. Ogawa S, Tank DW, Menon R et al. Intrinsic signal changes accompanying sensory stimulation: functional brain mapping with magnetic resonance imaging. Proc Natl Acad Sci U S A 1992;89:5951-5.
253. Orskov C, Rabenhoj L, Wettergren A, Kofod H, Holst JJ. Tissue and plasma concentrations of amidated and glycine-extended glucagon-like peptide I in humans. Diabetes 1994;43:535-9.
254. Orskov C, Wettergren A, Holst JJ. Secretion of the incretin hormones glucagon-like peptide-1 and gastric inhibitory polypeptide correlates with insulin secretion in normal man throughout the day. Scand J Gastroenterol 1996;31:665-70.

255. Otto B, Cuntz U, Fruehauf E et al. Weight gain decreases elevated plasma ghrelin concentrations of patients with anorexia nervosa. Eur J Endocrinol 2001;145:669-73.
256. Otto B, Tschop M, Fruhauf E et al. Postprandial ghrelin release in anorectic patients before and after weight gain. Psychoneuroendocrinology 2005;30:577-81.
257. Pavlidis I, Eberhardt NL, Levine JA. Seeing through the face of deception. Nature 2002;415:35.
258. Pedersen-Bjergaard U, Host U, Kelbaek H et al. Influence of meal composition on postprandial peripheral plasma concentrations of vasoactive peptides in man. Scand J Clin Lab Invest 1996;56:497-503.
259. Peeters TL. Central and peripheral mechanisms by which ghrelin regulates gut motility. J Physiol Pharmacol 2003;54 Suppl 4:95-103.
260. Peino R, Baldelli R, Rogers P et al. Ghrelin-induced growth hormone secretion in humans. Eur J Endocrinol 2000;143:R11-R14.
261. Perreault M, Istrate N, Wang L, Nichols AJ, Tozzo E, Stricker-Krongrad A. Resistance to the orexigenic effect of ghrelin in dietary-induced obesity in mice: reversal upon weight loss. Int J Obes Relat Metab Disord 2004;28:879-85.
262. Pi-Sunyer X, Kissileff HR, Thornton J, Smith GP. C-terminal octapeptide of cholecystokinin decreases food intake in obese men. Physiol Behav 1982;29:627-30.
263. Pietilainen KH, Kaprio J, Rissanen A et al. Distribution and heritability of BMI in Finnish adolescents aged 16y and 17y: a study of 4884 twins and 2509 singletons. Int J Obes Relat Metab Disord 1999;23:107-15.
264. Poppitt SD, Prentice AM. Energy density and its role in the control of food intake: evidence from metabolic and community studies. Appetite 1996;26:153-74.
265. Poykko SM, Kellokoski E, Horkko S, Kauma H, Kesaniemi YA, Ukkola O. Low plasma ghrelin is associated with insulin resistance, hypertension, and the prevalence of type 2 diabetes. Diabetes 2003;52:2546-53.
266. Pralong FP, Gonzales C, Voirol MJ et al. The neuropeptide Y Y1 receptor regulates leptin-mediated control of energy homeostasis and reproductive functions. FASEB J 2002;16:712-4.
267. Pupovac J, Anderson GH. Dietary peptides induce satiety via cholecystokinin-A and peripheral opioid receptors in rats. J Nutr 2002;132:2775-80.
268. Raben A, Agerholm-Larsen L, Flint A, Holst JJ, Astrup A. Meals with similar energy densities but rich in protein, fat, carbohydrate, or alcohol have different effects on energy expenditure and substrate metabolism but not on appetite and energy intake. Am J Clin Nutr 2003;77:91-100.
269. Raben A, Christensen NJ, Madsen J, Holst JJ, Astrup A. Decreased postprandial thermogenesis and fat oxidation but increased fullness after a high-fiber meal compared with a low-fiber meal. Am J Clin Nutr 1994;59:1386-94.
270. Raben A, Tagliabue A, Astrup A. The reproducibility of subjective appetite scores. Br-J-Nutr 1995;73:517-30.
271. Rahaoui, H, Leer, R. J, Dijkhuizen, L, and Van Geel-Schutten, G. H. Lactobacillus Reuteri Glucosyltransferase. Nederlandse Organisatie voor Toegepast-Natuurwetenschappelijk Onderzoek TNO, Delft. PCT/NL01/00393(WO 01/90372). 29-11-2001. The Netherlands. 23-5-2001.
Ref Type: Patent
272. Rahaoui, H, Leer, R. J, Dijkhuizen, L, and Van Geel-Schutten, G. H. Glucan incorporating 4-, 6-, and 4, 6- linked anhydroglucose units . Nederlandse Organisatie voor Toegepast-Natuurwetenschappelijk Onderzoek TNO, Delft. US20000604957 20000628 (US6486314). 26-11-2004. The Netherlands. 28-6-2000.
Ref Type: Patent
273. Rayner CK, Park HS, Wishart JM, Kong M, Doran SM, Horowitz M. Effects of intraduodenal glucose and fructose on antropyloric motility and appetite in healthy humans. Am J Physiol Regul Integr Comp Physiol 2000;278:R360-R366.
274. Raynor HA, Epstein LH. Dietary variety, energy regulation, and obesity. Psychol Bull 2001;127:325-41.

275. Reidelberger RD, Hernandez J, Fritzsch B, Hulce M. Abdominal vagal mediation of the satiety effects of CCK in rats. Am J Physiol Regul Integr Comp Physiol 2004;286:R1005-R1012.
276. Reiman EM. The application of Positron Emission Tomography to the study of normal and pathologic emotions. J Clin Psychiatry 1997;58 (suppl 16):4-12.
277. Reiman EM, Lane RD, Ahern GL et al. Neuroanatomical correlates of externally and internally generated human emotion. Am J Psychiatry 1997;154:918-25.
278. Riediger T, Bothe C, Becskei C, Lutz TA. Peptide YY directly inhibits ghrelin-activated neurons of the arcuate nucleus and reverses fasting-induced c-Fos expression. Neuroendocrinology 2004;79:317-26.
279. Robertson MD, Henderson RA, Vist GE, Rumsey RD. Plasma ghrelin response following a period of acute overfeeding in normal weight men. Int J Obes Relat Metab Disord 2004;28:727-33.
280. Rodin J, Wack J, Ferrannini E, DeFronzo RA. Effect of insulin and glucose on feeding behavior. Metabolism 1985;34:826-31.
281. Rogers PJ. Eating habits and appetite control: a psychobiological perspective. Proc Nutr Soc 1999;58:59-67.
282. Rogers PJ, Blundell JE. Effect of anorexic drugs on food intake and the micro-structure of eating in human subjects. Psychopharmacology (Berl) 1979;66:159-65.
283. Rolls B, Castellanos V, Halford J et al. Volume of food consumed affects satiety in men. Am-J-Clin-Nutr 1998;67:1170-7.
284. Rolls BJ, Roe LS. Effect of the volume of liquid food infused intragastrically on satiety in women. Physiol Behav 2002;76:623-31.
285. Rolls BJ, Van Duijvenvoorde PM, Rolls ET. Pleasantness changes and food intake in a varied four-course meal. Appetite 1984;5:337-48.
286. Rolls ET, Rolls BJ, Rowe EA. Sensory-specific and motivation-specific satiety for the sight and taste of food and water in man. Physiol Behav 1983;30:185-92.
287. Romon M, Lebel P, Velly C, Marecaux N, Fruchart JC, Dallongeville J. Leptin response to carbohydrate or fat meal and association with subsequent satiety and energy intake. Am J Physiol 1999;277:E855-E861.
288. Rosenbaum M, Leibel RL, Hirsch J. Obesity. N Engl J Med 1997;337:396-407.
289. Rosenbaum M, Murphy EM, Heymsfield SB, Matthews DE, Leibel RL. Low dose leptin administration reverses effects of sustained weight-reduction on energy expenditure and circulating concentrations of thyroid hormones. J Clin Endocrinol Metab 2002;87:2391-4.
290. Rossner S, Barkeling B, Erlanson-Albertsson C, Larsson P, Wahlin-Boll E. Intravenous enterostatin does not affect single meal food intake in man. Appetite 1995;24:37-42.
291. Rozin P. The socio-cultural context of eating and food choice. In: Meiselman HL, MacFie HJ, eds. Food choice, acceptance and consumption. London: Blackie Academic & Professional 1996:83-104.
292. Rudovich NN, Dick D, Moehlig M et al. Ghrelin is not suppressed in hyperglycemic clamps by gastric inhibitory polypeptide and arginine. Regul Pept 2005;127:95-9.
293. Saad MF, Bernaba B, Hwu CM et al. Insulin regulates plasma ghrelin concentration. J Clin Endocrinol Metab 2002;87:3997-4000.
294. Sanaka M, Kuyama Y, Yamanaka M. Guide for judicious use of the paracetamol absorption technique in a study of gastric emptying rate of liquids. J Gastroenterol 1998;33:785-91.
295. Schick RR, Schusdziarra V, Mossner J et al. Effect of CCK on food intake in man: physiological or pharmacological effect? Z Gastroenterol 1991;29:53-8.
296. Schirra J, Goke B. The physiological role of GLP-1 in human: incretin, ileal brake or more? Regul Pept 2005;128:109-15.
297. Schmid R, Schulte-Frohlinde E, Schusdziarra V et al. Contribution of postprandial amino acid levels to stimulation of insulin, glucagon, and pancreatic polypeptide in humans. Pancreas 1992;7:698-704.
298. Schofield W. Predicting basal metabolic rate, new standards and review of previous work. Hum-Nutr-Clin-Nutr 1985;39.
299. Schwartz MW, Morton GJ. Obesity: Keeping hunger at bay. Nature 2002;418:595-7.

300. Schwartz MW, Seeley RJ, Woods SC et al. Leptin increases hypothalamic pro-opiomelanocortin mRNA expression in the rostral arcuate nucleus. Diabetes 1997;46:2119-23.
301. Schwartz MW, Woods SC, Porte D, Seeley RJ, Baskin DG. Central nervous system control of food intake. Nature 2000;404:661-71.
302. Shiiya T, Nakazato M, Mizuta M et al. Plasma ghrelin levels in lean and obese humans and the effect of glucose on ghrelin secretion. J Clin Endocrinol Metab 2002;87:240-4.
303. Shintani M, Ogawa Y, Ebihara K et al. Ghrelin, an endogenous growth hormone secretagogue, is a novel orexigenic peptide that antagonizes leptin action through the activation of hypothalamic neuropeptide Y/Y1 receptor pathway. Diabetes 2001;50:227-32.
304. Sinha M, Ohannesian J, Heiman M et al. Nocturnal rise of leptin in lean, obese, and non-insulin-dependent diabetes mellitus subjects. J-Clin-Invest 1996;97:1344-7.
305. Small DM, Zatorre RJ, Dagher A, Evans AC, Jones-Gotman M. Changes in brain activity related to eating chocolate: from pleasure to aversion. Brain 2001;124:1720-33.
306. Smeets M, Geiselman P, Bray GA, York DA. The effect of oral enetrostatin on hunger and food intake in humans volunteers. FASEB-JOURNAL 1999;13:A871 (abstr).
307. Smeets PA, de Graaf C, Stafleu A, van Osch MJ, van der Grond J. Functional MRI of human hypothalamic responses following glucose ingestion. Neuroimage 2005;24:363-8.
308. Snyder EE, Walts B, Perusse L et al. The human obesity gene map: the 2003 update. Obes Res 2004;12:369-439.
309. Sparti A, Milon H, Di Vetta V et al. Effect of diets high or low in unavailable and slowly digestible carbohydrates on the pattern of 24-h substrate oxidation and feelings of hunger in humans. Am-J-Clin-Nutr 2000;72:1461-8.
310. Speechly DP, Buffenstein R. Appetite dysfunction in obese males: evidence for role of hyperinsulinaemia in passive overconsumption with a high fat diet. Eur J Clin Nutr 2000;54:225-33.
311. St-Pierre DH, Karelis AD, Cianflone K et al. Relationship between ghrelin and energy expenditure in healthy young women. J Clin Endocrinol Metab 2004;89:5993-7.
312. Strien, T. van, Frijters, J. E. R., Bergers, G. P. A., and Defares, P. B. Nederlandse Vragenlijst voor Eetgedrag. 1986. Lisse, Swets en Zeitlinger b.v.
Ref Type: Generic
313. Stubbs J, Ferres S, Horgan G. Energy density of foods: effects on energy intake. Crit Rev Food Sci Nutr 2000;40:481-515.
314. Stubbs R, Hughes D, Johnstone A et al. The use of visual analogue scales to assess motivation to eat in human subjects: a review of their reliability and validity with an evaluation of new hand-held computerized systems for temporal tracking of appetite ratings. Br-J-Nutr 2000;84:405-15.
315. Sun Y, Ahmed S, Smith RG. Deletion of ghrelin impairs neither growth nor appetite. Mol Cell Biol 2003;23:7973-81.
316. Tanaka M, Naruo T, Muranaga T et al. Increased fasting plasma ghrelin levels in patients with bulimia nervosa. Eur J Endocrinol 2002;146:R1-R3.
317. Tataranni P, Gautier J, Chen K et al. Neuroanatomical correlates of hunger and satiation in humans using positron emission tomography. Proc-Natl-Acad-Sci-U-S-A 1999;96:4569-74.
318. Tentolouris N, Kokkinos A, Tsigos C et al. Differential Effects of High-fat and High-carbohydrate Content Isoenergetic Meals on Plasma Active Ghrelin Concentrations in Lean and Obese Women. Horm Metab Res 2004;36:559-63.
319. Thompson DA, Campbell RG. Hunger in humans induced by 2-deoxy-D-glucose: glucoprivic control of taste preference and food intake. Science 1977;198:1065-8.
320. Tome D. Protein, amino acids and the control of food intake. Br J Nutr 2004;92 Suppl 1:S27-S30.
321. Toshinai K, Mondal MS, Nakazato M et al. Upregulation of Ghrelin expression in the stomach upon fasting, insulin- induced hypoglycemia, and leptin administration. Biochem Biophys Res Commun 2001;281:1220-5.
322. Truswell A. Glycaemic index of foods. Eur-J-Clin-Nutr; Suppl 2 1992;46.
323. Tschop M, Smiley D, Heiman M. Ghrelin induces adiposity in rodents. Nature 2000;407:908-13.

324. Tschop M, Wawarta R, Riepl RL et al. Post-prandial decrease of circulating human ghrelin levels. J Endocrinol Invest 2001;24:RC19-RC21.
325. Tschop M, Weyer C, Tataranni PA, Devanarayan V, Ravussin E, Heiman ML. Circulating ghrelin levels are decreased in human obesity. Diabetes 2001;50:707-9.
326. Tuomisto T, Tuomisto MT, Hetherington M, Lappalainen R. Reasons for initiation and cessation of eating in obese men and women and the affective consequences of eating in everyday situations. Appetite 1998;30:211-22.
327. Ueno N, Dube MG, Inui A, Kalra PS, Kalra SP. Leptin modulates orexigenic effects of ghrelin and attenuates adiponectin and insulin levels and selectively the dark-phase feeding as revealed by central leptin gene therapy. Endocrinology 2004;145:4176-84.
328. Uhe AM, Collier GR, O'Dea K. A comparison of the effects of beef, chicken and fish protein on satiety and amino acid profiles in lean male subjects. J Nutr 1992;122:467-72.
329. Uyama N, Geerts A, Reynaert H. Neural connections between the hypothalamus and the liver. Anat Rec A Discov Mol Cell Evol Biol 2004;280:808-20.
330. van Dijk G, Seeley RJ, Thiele TE et al. Metabolic, gastrointestinal, and CNS neuropeptide effects of brain leptin administration in the rat. Am J Physiol 1999;276:R1425-R1433.
331. van Loon LJ, Kruijshoop M, Menheere PP, Wagenmakers AJ, Saris WH, Keizer HA. Amino acid ingestion strongly enhances insulin secretion in patients with long-term type 2 diabetes. Diabetes Care 2003;26:625-30.
332. van Strien, T. Eating behaviour personality traits and body mass. 1986. Wageningen, Landbouwhogeschool.
Ref Type: Thesis/Dissertation
333. van Strien T, Frijters JER, Bergers GPA, Defares PB. The Dutch Eating Behaviour Questionnaire (DEBQ) for assessment of restrained, emotional and external eating behaviour. Int J Eat Disord 1986;5:295-315.
334. Vangipuram SD, Sheele J, Atkinson RL, Holland TC, Dhurandhar NV. A human adenovirus enhances preadipocyte differentiation. Obes Res 2004;12:770-7.
335. Verdich C, Flint A, Gutzwiller JP et al. A meta-analysis of the effect of glucagon-like peptide-1 (7-36) amide on ad libitum energy intake in humans. J Clin Endocrinol Metab 2001;86:4382-9.
336. Verdich C, Toubro S, Buemann B, Lysgard MJ, Juul HJ, Astrup A. The role of postprandial releases of insulin and incretin hormones in meal-induced satiety--effect of obesity and weight reduction. Int J Obes Relat Metab Disord 2001;25:1206-14.
337. Vilsboll T, Krarup T, Madsbad S, Holst JJ. Both GLP-1 and GIP are insulinotropic at basal and postprandial glucose levels and contribute nearly equally to the incretin effect of a meal in healthy subjects. Regul Pept 2003;114:115-21.
338. Visscher TL, Kromhout D, Seidell JC. Long-term and recent time trends in the prevalence of obesity among Dutch men and women. Int J Obes Relat Metab Disord 2002;26:1218-24.
339. Voedingscentrum. Zo eet Nederland 1998, resultaten van de Voedselconsumptiepeiling 1998 (in Dutch). Results of the Ductch food consumption survey 1998. 1998. The Hague, Voedingscentrum.
Ref Type: Report
340. Voedingsraad. Nederlandse Voedingsnormen 1989 (in Dutch). Dutch Nutritional Guidelines. 1989. The Hague, Voorlichtingsbureau voor de voeding.
Ref Type: Report
341. Voorlichtingsbureau voor de voeding. Zo eet Nederland 1998, resultaten van de voedselconsumptiepeiling 1998 (in Dutch). Results of the Dutch food consumption survey 1998. 1998. The Hague, Voorlichtingsbureau voor de Voeding.
Ref Type: Report
342. Vozzo R, Baker B, Wittert GA et al. Glycemic, hormone, and appetite responses to monosaccharide ingestion in patients with type 2 diabetes. Metabolism 2002;51:949-57.
343. Walsh JH, Maxwell V, Ferrari J, Varner AA. Bombesin stimulates human gastric function by gastrin-dependent and independent mechanisms. Peptides 1981;2 Suppl 2:193-8.
344. Wang L, Barachina MD, Martinez V, Wei JY, Tache Y. Synergistic interaction between CCK and leptin to regulate food intake. Regul Pept 2000;92:79-85.

345. Wannamethee SG, Shaper AG, Walker M. Overweight and obesity and weight change in middle aged men: impact on cardiovascular disease and diabetes. J Epidemiol Community Health 2005;59:134-9.
346. Weigle DS, Cummings DE, Newby PD et al. Roles of leptin and ghrelin in the loss of body weight caused by a low fat, high carbohydrate diet. J Clin Endocrinol Metab 2003;88:1577-86.
347. Weigle DS, Duell PB, Connor WE, Steiner RA, Soules MR, Kuijper JL. Effect of fasting, refeeding, and dietary fat restriction on plasma leptin levels. J Clin Endocrinol Metab 1997;82:561-5.
348. Welle SL, Thompson DA, Campbell RG, Lilavivathana U. Increased hunger and thirst during glucoprivation in humans. Physiol Behav 1980;25:397-403.
349. Westerterp KR, Goran MI. Relationship between physical activity related energy expenditure and body composition: a gender difference. Int J Obes Relat Metab Disord 1997;21:184-8.
350. Westerterp-Plantenga MS. Effects of extreme environments on food intake in human subjects. Proc Nutr Soc 1999;58:791-8.
351. Westerterp-Plantenga MS, Lejeune MP, Nijs I, van Ooijen M, Kovacs EM. High protein intake sustains weight maintenance after body weight loss in humans. Int J Obes Relat Metab Disord 2004;28:57-64.
352. Westerterp-Plantenga MS, Marken Lichtenbelt WD, Strobbe H, Schrauwen P. Energy metabolism in humans at a lowered ambient temperature. Eur J Clin Nutr 2002;56:288-96.
353. Westerterp-Plantenga MS, Rolland V, Wilson SA, Westerterp KR. Satiety related to 24 h diet-induced thermogenesis during high protein/carbohydrate vs high fat diets measured in a respiration chamber. Eur J Clin Nutr 1999;53:495-502.
354. Westerterp-Plantenga MS, Saris WH, Hukshorn CJ, Campfield LA. Effects of weekly administration of pegylated recombinant human OB protein on appetite profile and energy metabolism in obese men. Am J Clin Nutr 2001;74:426-34.
355. Westerterp-Plantenga MS, Wijckmans-Duijsens NE, Verboeket-van de Venne WP, De Graaf K, Weststrate JA, Het Hof KH. Diet-induced thermogenesis and satiety in humans after full-fat and reduced-fat meals. Physiol Behav 1997;61:343-9.
356. Westerterp-Plantenga MS, Wouters L, ten Hoor F. Deceleration in cumulative food intake curves, changes in body temperature and diet-induced thermogenesis. Physiol Behav 1990;48:831-6.
357. Willems M, Quartero AO, Numans ME. How useful is paracetamol absorption as a marker of gastric emptying? A systematic literature study. Dig Dis Sci 2001;46:2256-62.
358. Williams DL, Cummings DE, Grill HJ, Kaplan JM. Meal-related ghrelin suppression requires postgastric feedback. Endocrinology 2003;144:2765-7.
359. Williams DL, Grill HJ, Cummings DE, Kaplan JM. Vagotomy dissociates short- and long-term controls of circulating ghrelin. Endocrinology 2003;144:5184-7.
360. Williams J, Mobarhan S. A critical interaction: leptin and ghrelin. Nutr Rev 2003;61:391-3.
361. Wisse BE, Campfield LA, Marliss EB, Morais JA, Tenenbaum R, Gougeon R. Effect of prolonged moderate and severe energy restriction and refeeding on plasma leptin concentrations in obese women. Am J Clin Nutr 1999;70:321-30.
362. Wolkowitz OM, Gertz B, Weingartner H, Beccaria L, Thompson K, Liddle RA. Hunger in humans induced by MK-329, a specific peripheral-type cholecystokinin receptor antagonist. Biol Psychiatry 1990;28:169-73.
363. Woo R, Kissileff H, Pi SF. Elevated postprandial insulin levels do not induce satiety in normal-weight humans. Am-J-Physiol 1984;247:R745-R749.
364. Woods SC. The eating paradox: how we tolerate food. Psychol Rev 1991;98:488-505.
365. World Health Organization. Obesity: Preventing and managing the global epidemic. WHO Consultation on Obesity. 894. 1999. Geneva, Switzerland. WHO Technical Report Series.
Ref Type: Report
366. World Health Organization. Report of a Joint FAO/WHO/UNU Expert consultation. Principles for the estimation of energy requirements. Energy and protein requirements, 34-52. 1985. Geneva, World Health Organization.
Ref Type: Report

367. Wortley KE, Anderson KD, Garcia K et al. Genetic deletion of ghrelin does not decrease food intake but influences metabolic fuel preference. Proc Natl Acad Sci U S A 2004;101:8227-32.
368. Wren A, Small C, Ward H et al. The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion. Endocrinology 2000;141:4325-8.
369. Wren AM, Seal LJ, Cohen MA et al. Ghrelin enhances appetite and increases food intake in humans. J Clin Endocrinol Metab 2001;86:5992.
370. Yildiz BO, Suchard MA, Wong ML, McCann SM, Licinio J. Alterations in the dynamics of circulating ghrelin, adiponectin, and leptin in human obesity. Proc Natl Acad Sci U S A 2004;101:10434-9.
371. Zald DH, Hagen MC, Pardo JV. Neural correlates of tasting concentrated quinine and sugar solutions. J Neurophysiol 2002;87:1068-75.
372. Zald DH, Lee JT, Fluegel KW, Pardo JV. Aversive gustatory stimulation activates limbic circuits in humans. Brain 1998;121 (Pt 6):1143-54.
373. Zald DH, Pardo JV. Emotion, olfaction, and the human amygdala: amygdala activation during aversive olfactory stimulation. Proc Natl Acad Sci U S A 1997;94:4119-24.
374. Zander M, Madsbad S, Madsen JL, Holst JJ. Effect of 6-week course of glucagon-like peptide 1 on glycaemic control, insulin sensitivity, and beta-cell function in type 2 diabetes: a parallel-group study. Lancet 2002;359:824-30.
375. Zemel MB. Role of calcium and dairy products in energy partitioning and weight management. Am J Clin Nutr 2004;79:907S-12S.

Summary

The objective of this thesis was to gain insight into the mechanisms of food intake regulation in order to facilitate the design of foods that could help to regulate energy intake. The research described in this thesis focused on the recently discovered gastric hormone ghrelin, since the first available data on ghrelin suggested that it may play an important role in meal initiation. Four research questions were formulated, to further investigate the role of ghrelin in meal initiation:

1. Are ghrelin concentrations related to appetite?
2. Is the postprandial ghrelin response dependent on energy or macronutrient intake?
3. Is ghrelin related to other regulators of food intake?
4. Is ghrelin involved in the restoration of energy balance, following energy restriction?

To assess the relevance of ghrelin, an extensive literature study was initiated, focusing on the identification and evaluation of potential central en peripheral biomarkers of satiety and satiation (chapter 2). Following this literature study, several human clinical intervention studies were performed, which are described in chapters 3-7.

Are ghrelin concentrations related to appetite?

If ghrelin acts as a hunger signal and plays an important role in meal initiation, its concentrations should be related to measures of appetite. Therefore, the association between (changes in) ghrelin concentrations and several measures of appetite, i.e. subjective appetite, intermeal interval and *ad libitum* energy intake, was investigated. In chapters 3, 4 and 6 the relation between ghrelin and subjective measures of appetite was investigated, by examining the association between postprandial ghrelin concentrations and scores of hunger, fullness, desire to eat and prospective food consumption on the visual analogue scales. A positive correlation between ghrelin concentrations and subjective appetite scores was observed in 2 out of the 3 studies. The only study showing no association (chapter 4) had a lack of power. Also age, type of meal and distraction of the subjects may have confounded the results.

Furthermore, an inverse association between the intermeal interval (a measure of meal initiation) and both the postprandial decrease in ghrelin concentrations ($r = -0.54$, $p < 0.05$) and the negative area under the ghrelin curve ($r = -0.57$, $p = 0.01$) was found, in normal weight subjects, but not in obese subjects (chapter 6). No significant association between the intermeal interval and the preprandial increase in ghrelin concentrations was observed. These associations suggest that suppression of ghrelin concentrations may postpone initiation of the next meal. However, these results need to be replicated in other studies. Although ghrelin concentrations appear

to be associated with meal initiation, they were not related to *ad libitum* energy intake (chapters 4, 6 and 7).

Is the postprandial ghrelin response dependent on energy or macronutrient intake?

In order to design foods that can help to regulate energy intake, more knowledge about the effects of energy content and meal composition on the postprandial ghrelin response is required. In chapter 3, it was shown that the postprandial ghrelin response to a carbohydrate enriched meal is dependent on the dose of carbohydrate and is unaffected by intake of the same volume of water. In contrast to others, it was shown in chapter 4 that a high protein meal more effectively reduced (± 45%) postprandial ghrelin concentration as compared to an isocaloric high carbohydrate meal ($p < 0.01$). It was hypothesized that this effect of protein may be dependent on its source and composition.

Is ghrelin related to other regulators of food intake?

Little is known about the role of ghrelin in the physiological regulation of food intake and its interaction with other regulators of food intake. Therefore, the correlation between ghrelin and several other factors was investigated. In chapters 4 and 5, a strong inverse association between postprandial ghrelin concentrations and acetaminophen absorption (an indirect measure of gastric emptying rate) was observed, after a high carbohydrate ($r = -0.76$; 95% C.I. = -0.90, -0.49) and high protein ($r = -0.89$; 95% C.I. = -0.95, -0.73) meal (chapter 4) and after GLP-1 infusion ($r = -0.47$; 95% C.I. = -0.76, -0.04) (chapter 5), suggesting that the postprandial ghrelin response is dependent on the rate of gastric emptying. This supports the hypothesis that ghrelin requires post gastric feedback. Postprandial ghrelin concentrations were also inversely associated with insulin (in chapters 3 and 6 not in chapters 4 and 5) and GIP (glucose-dependent insulinotropic peptide) (chapters 4 and 5) concentrations, the association between ghrelin and GIP being the strongest (GIP: $r \approx -0.70$; insulin: $r \approx -0.50$). CCK concentrations were also inversely associated with ghrelin concentrations, but only after a high carbohydrate breakfast ($r \approx -0.52$). Although both leptin and ghrelin are associated with BMI and are suggested to be involved in the long term regulation of food intake, no association between (changes in) fasting ghrelin and leptin concentrations during energy restriction and subsequent *ad libitum* food intake was found (chapter 7).

In contrast to total ghrelin (the sum of unacylated and acylated ghrelin) concentrations, no association between acylated ghrelin and other parameters

related to food intake was found. This may be explained by the high variation in acylated ghrelin concentrations, and needs therefore further investigation.

Is ghrelin involved in the restoration of energy balance following energy restriction?

Ghrelin may play a role in restoration of energy homeostasis, because weight loss is associated with increased plasma ghrelin concentrations and weight gain is associated with decreased plasma ghrelin concentrations. During periods of restricted energy intake, ghrelin may favour weight gain by acting as a starvation signal, stimulating food intake. However, in the studies described in this thesis, neither 2 nor 3 days of 64%- energy restriction significantly increased fasting ghrelin concentrations in normal-weight men. In obese men, ghrelin concentrations did increase approximately 8% after 3 days of severe energy restriction. Changes in fasting ghrelin concentrations during energy restriction are probably dependent on changes in other physiological measures such as insulin sensitivity and fat cell activity. Two or three days of energy restriction may have not been sufficient to induce such changes in ghrelin. However, this does not necessarily mean that the functionality of ghrelin is unchanged. Ghrelin sensitivity may have been improved. There was a large inter-individual variation in the change in fasting ghrelin concentrations during energy restriction, but these changes were not positively associated with subsequent *ad libitum* food intake ($r = 0.22$; $p = 0.21$), suggesting that ghrelin does not act as a hunger signal during energy restriction. This lack of association may be caused by a poor reproducibility of fasting ghrelin concentrations, since the one of the studies showed (chapter 6) that food intake may affect fasting ghrelin concentrations. In addition, energy restriction may increase active ghrelin concentrations only.

General conclusions

In chapter 8, the results are placed in perspective by discussing the internal and external validity of the data.

Ghrelin concentrations were associated with subjective measures of appetite and with the intermeal interval, but not with *ad libitum* food intake. Therefore, it is concluded that ghrelin is a hunger signal that is not involved in the determination of meal size (satiation), but that appears to be involved in the regulation of meal initiation (satiety) in normal weight men. Furthermore, ghrelin concentrations were associated with the gastric emptying rate, supporting the hypothesis that post gastric feedback is required. This feedback may be provided by GIP and other regulators of

food intake, such as insulin, CCK and PYY. The postprandial ghrelin response is dependent on the energy content of the food consumed and on the type and composition of the macronutrients. Foods that contain for example dairy proteins may effectively suppress ghrelin concentrations for a longer period. These foods may then be used to postpone meal initiation, and may contribute to the prevention and treatment of overweight and obesity.

Samenvatting

Overgewicht

In de westerse samenleving neemt het aantal mensen met overgewicht steeds verder toe. Nederland is hierop geen uitzondering, want ook hier heeft 45% van de volwassen mannen en 35% van de volwassen vrouwen overgewicht, waarvan 10% ernstig overgewicht (obesitas). Obesitas, verhoogt de kans op het ontwikkelen van verschillende ziekten zoals ouderdomssuikerziekte, hart- en vaatziekten en hoge bloeddruk.
Overgewicht wordt veroorzaakt door een grotere energie inname dan energie verbruik, gedurende een langere periode. Het ontstaan van overgewicht wordt beïnvloed door verschillende factoren. De combinatie van erfelijke gevoeligheid en leefstijl (mate van lichamelijke activiteit en het eetpatroon) bepaalt of je overgewicht ontwikkeld. Dit verklaart ook waarom sommige mensen sneller aankomen in gewicht dan anderen.

Regulatie van voedselinname

Zowel interne (fysiologische) signalen als externe factoren (o.a. tijdstip, aanwezigheid van andere personen en het ruiken van voedsel) bepalen het eetgedrag.
Normaal gesproken eten mensen totdat ze een comfortabel vol gevoel krijgen waardoor ze stoppen met eten. Dit wordt de verzadiging binnen een maaltijd (in het Engels ‘satiation’) genoemd. Direct na de maaltijd is de behoefte om te eten klein, maar met het verstrijken van de tijd neemt de behoefte om te eten weer toe. De tussen-maaltijd-verzadiging (in het Engels ‘satiety’) neemt dan af en het hongergevoel (eetlust) neemt weer toe.
Dit proefschrift richt zich voornamelijk op de fysiologische signalen die de voedselinname reguleren, en in het bijzonder op de rol van het hormoon ghreline in de regulatie van voedselinname. Uit een uitgebreide literatuurstudie, welke is beschreven in hoofdstuk 2, bleek namelijk dat ghreline mogelijk een belangrijke rol speelt in deze regulatie.

Ghreline

Het hormoon ghreline wordt voornamelijk in de maag aangemaakt, en wordt afgescheiden in het bloed. Ghreline concentraties stijgen wanneer er niet wordt gegeten, en dalen snel na een maaltijd. Ghreline lijkt een hongersignaal te zijn, omdat toediening van ghreline in het bloed ervoor zorgt dat mensen meer honger krijgen en meer gaan eten. Dit zou kunnen betekenen dat de stijgingen in ghreline concentraties voor een maaltijd ervoor zorgen dat je gaat eten, en dat afnames in

ghreline concentraties na een maaltijd ervoor zorgen dat je niet meteen weer gaat eten.

Doelstellingen van dit proefschrift

Het doel van de onderzoeken beschreven in dit proefschrift was om meer inzicht te krijgen in het mechanisme van voedselinnameregulatie. Dit inzicht zou kunnen helpen bij het ontwikkelen van voedingsmiddelen die gebruikt kunnen worden bij gewichtsbeheersing. Het onderzoek wat in dit proefschrift is beschreven, richt zich in het bijzonder op het recent ontdekte hormoon ghreline, aangezien de eerste gegevens erop wijzen dat dit hormoon een belangrijke rol zou kunnen spelen bij de regulatie van voedselinname. Er werden vier onderzoeksvragen geformuleerd.

1. Zijn ghreline concentraties gerelateerd aan eetlust?
2. Is de ghreline respons na een maaltijd afhankelijk van de energie- of macronutriëntinname?
3. Is ghreline gerelateerd aan andere regulatoren van voedselinname?
4. Is ghreline betrokken bij het herstel van de energie balans, na energierestrictie (een energiebeperkt dieet)?

Zijn ghreline concentraties gerelateerd aan eetlust?

Als ghreline een hongersignaal is, dan zouden ghreline concentraties gerelateerd moeten zijn aan eetlust. Daarom zijn de verbanden tussen ghreline concentraties en verschillende maten voor eetlust onderzocht.

In hoofdstukken 3, 4 en 6 is de relatie tussen ghreline en subjectieve eetlust, voor en na verschillende maaltijden, onderzocht. Subjectieve eetlust werd gemeten met behulp van een vragenlijst waarop proefpersonen moesten aangeven hoeveel honger ze hadden, hoe vol ze waren, hoe graag ze op dat moment iets zouden willen eten, en hoeveel ze op dat moment zouden kunnen eten. De scores op deze vragenlijst werden vergeleken met de ghreline concentraties op dezelfde tijdstippen. Er werd een positieve relatie gevonden tussen ghreline concentraties en subjectieve eetlust. Dit betekent dat de afname in ghreline concentraties na een maaltijd gepaard ging met een afname in eetlust, en dat de stijging in ghreline concentraties voor een maaltijd gepaard ging met een toename in eetlust.

Ook is er gekeken naar de relatie tussen ghreline en verzadiging binnen een maaltijd en naar de relatie tussen ghreline en verzadiging tussen twee maaltijden. De vrijwillige energie-inname tijdens een maaltijd (hoeveel je eet) is een maat voor de verzadiging binnen een maaltijd. Er werd geen relatie gevonden tussen ghreline concentraties en de vrijwillige energie-inname tijdens een maaltijd (hoofdstukken 4, 6 en 7). Hoe groter de verzadiging tussen twee maaltijden, des te langer het duurt

voordat er gestart wordt met een nieuwe maaltijd (maaltijdinitiatie). Om te onderzoeken of ghreline bepaalt wanneer er gestart wordt met een maaltijd (maaltijdinitiatie), werden proefpersonen geïsoleerd van tijd, geluid en zonlicht, zodat ze niet wisten hoe laat het was. In deze proefpersonen werd vervolgens gemeten hoe lang ze na een standaard ontbijt wachtten met het vragen om een lunch. De tijd tussen het ontbijt en de lunch is een maat voor maaltijdinitiatie. Hoe langer de ghreline concentraties laag bleven na een maaltijd, des te langer dat het duurde voordat de proefpersonen om de volgende maaltijd vroegen (hoofdstuk 6). Dit zou kunnen betekenen dat je door ghreline concentraties te onderdrukken, de volgende maaltijd zou kunnen uitstellen.

Is de ghreline respons na een maaltijd afhankelijk van de energie- of macronutriëntinname?
Om voedingsmiddelen te ontwikkelen die kunnen bijdragen aan gewichtsbeheersing, is meer kennis nodig over de effecten van energie en (macronutriënt)samenstelling van een maaltijd, op de ghreline respons. In dit proefschrift zijn vooral de effecten van koolhydraat en (melk)eiwit op ghreline onderzocht. De overige macronutriënten vet en alcohol zijn niet onderzocht. Hoofdstuk 3 laat zien dat de ghreline respons na een koolhydraatrijke maaltijd afhankelijk is van de hoeveelheid koolhydraat dat de maaltijd bevat. Een gelijk volume water had geen effect op de ghreline concentraties. In hoofdstuk 4 werden de effecten van een koolhydraatrijke maaltijd en een eiwitrijke maaltijd op ghreline concentraties vergeleken. Hoewel beide maaltijden even groot waren en ook evenveel calorieën bevatten, waren de ghreline concentraties na de eiwitrijke maaltijd lager dan na de koolhydraatrijke maaltijd. Mogelijk is dit effect van eiwit afhankelijk van de samenstelling van het eiwit, aangezien andere onderzoekers geen effect van eiwit op ghreline concentraties vonden met een ander soort eiwit, namelijk vleeseiwit.

Is ghreline gerelateerd aan andere regulatoren van voedselinname?
Er is weinig bekend over de relatie tussen ghreline en andere stoffen die betrokken zijn bij de regulatie van voedselinname. In hoofdstukken 4 en 5 werd een sterke negatieve correlatie gevonden tussen ghreline concentraties en de snelheid waarmee de maag zich ledigt. Hoe sneller de maaglediging, des te sneller daalden de ghreline concentraties. Dit suggereert dat de ghreline respons na een maaltijd afhankelijk is van de maagledigingssnelheid en mogelijk gereguleerd wordt door factoren die afgescheiden worden nadat nutriënten de maag gepasseerd zijn. Factoren die hier mogelijk bij betrokken zijn, zijn het darmhormoon GIP (glucose-dependent insulinotropic polypeptide) en insuline, aangezien ghreline concentraties

negatief gecorreleerd waren met beide factoren. Het verband tussen ghreline en GIP was sterker, dan het verband tussen ghreline en insuline. Concentraties van het darmhormoon cholecystokinine (CCK) waren ook negatief gecorreleerd met ghreline concentraties, maar alleen na een koolhydraatrijke maaltijd. Er werd geen relatie gevonden tussen ghreline en leptine concentraties.

Is ghreline betrokken bij het herstel van de energie balans, na energie-restrictie (een energie-arm dieet)?
Ghreline speelt mogelijk een rol bij het herstel van de energie balans, aangezien gewichtsverlies geassocieerd is met een toename in ghreline concentraties, en gewichtstoename geassocieerd is met een afname in ghreline concentraties. Tijdens een periode van beperkte energie inname, zou ghreline mogelijk gewichtstoename kunnen stimuleren door als een hongersignaal te functioneren en voedselinname te stimuleren. Echter, in de studies beschreven in dit proefschrift had een energiebeperkt dieet van 2 of 3 dagen (proefpersonen kregen maar 1/3 van hun normale hoeveelheid voedsel) geen effect op nuchtere ghreline concentraties bij mannen met een normaal lichaamsgewicht. Bij mannen met obesitas, stegen de nuchtere ghreline concentraties met slechts 8% na 3 dagen energiebeperking. Veranderingen in nuchtere ghreline concentraties tijdens energiebeperking zijn waarschijnlijk afhankelijk van veranderingen in andere fysiologische factoren, zoals de insulinegevoeligheid en de activiteit van vetcellen. Twee of drie dagen energie beperking was waarschijnlijk niet voldoende om ghreline concentraties beduidend te veranderen. Dit hoeft echter niet te betekenen dat de functionaliteit van ghreline onveranderd bleef. De gevoeligheid van het lichaam voor ghreline zou verbeterd kunnen zijn.
Er waren grote verschillen tussen personen in de veranderingen in ghreline concentraties tijdens energiebeperking, maar deze veranderingen voorspelden niet hoeveel mensen na het energiebeperkte dieet gingen eten. Dit suggereert dat ghreline niet als een honger signaal werkt tijdens energiebeperking.

Conclusies

In hoofdstuk 8 werden de resultaten in perspectief geplaatst door de sterke en zwakke punten van het onderzoek te belichten en de resultaten te vergelijken met ander onderzoek.
Uit dit onderzoek bleek dat ghreline concentraties gerelateerd waren aan subjectieve eetlust en aan het tijdsinterval tussen twee maaltijden, maar dat ghreline concentraties niet gerelateerd waren aan de energie inname tijdens een maaltijd.

Daarom is de conclusie getrokken dat ghreline een korte termijn honger signaal is, die niet bepaalt wanneer je stopt met eten (en hoeveel je eet), maar die lijkt te bepalen wanneer je begint te eten. Er werd ook een verband gevonden tussen ghreline concentraties en de maagledigingssnelheid. Dit suggereert dat de ghreline respons na een maaltijd afhankelijk is van de maagledigingssnelheid en mogelijk gereguleerd wordt door factoren die afgescheiden worden nadat nutriënten de maag gepasseerd zijn. Mogelijke factoren die hierbij betrokken zijn, zijn GIP en andere regulatoren van voedselinname zoals insuline en CCK. De ghreline respons is afhankelijk van de hoeveelheid calorieën die de maaltijd bevat en van de soort en samenstelling van de macronutriënten. De resultaten suggereren dat melkeiwitten de ghreline concentraties gedurende een langere periode onderdrukken, waardoor de volgende maaltijd mogelijk uitgesteld kan worden. Nutriënten met dergelijke eigenschappen kunnen een bijdrage leveren aan de preventie en behandeling van overgewicht en obesitas.

Dankwoord

Na ruim vier jaar is mijn proefschrift nu dan toch echt bijna af. Maar niet voordat ik alle mensen heb bedankt die op de één of andere manier betrokken waren bij de totstandkoming van dit proefschrift. Dit zijn er zoveel dat ik niet iedereen bij naam kan noemen in dit dankwoord. Daarom wil ik nu alvast tegen iedereen zeggen; bedankt! Zonder de hulp van jullie allen was het me nooit gelukt!

Natuurlijk wil ik ook een aantal mensen persoonlijk bedanken, te beginnen met mijn copromotor **Henk**. Henk, toen ik begon aan mijn promotieonderzoek was het nog niet duidelijk wie mijn dagelijkse begeleider bij TNO zou zijn. Dit veranderde al snel toen bleek dat ik bij jou en de andere fysiologen de deur plat liep met vragen over het reilen en zeilen van een humane interventiestudie. Ik heb altijd bij je terecht gekund met mijn vragen en problemen. Dit heb ik altijd erg gewaardeerd. Ook wil ik je bedanken voor je kritische blik op de vele versies van mijn manuscripten. Je enthousiasme en betrokkenheid hebben zeker bijgedragen aan de leuke tijd die ik de afgelopen 4 jaren bij TNO heb gehad. De etentjes bij jou en Lies thuis waren ook erg gezellig!

Ook wil ik mijn promotoren hartelijk bedanken. **Gertjan** en **Frans**, in het begin van mijn promotieonderzoek hielden jullie, enigszins op de achtergrond, kritisch het design van mijn studies in de gaten. In het laatste jaar toen ik me voornamelijk op het schrijven ging richten werd jullie betrokkenheid steeds intensiever. Jullie hebben er altijd voor gezorgd dat de rode draad door mijn proefschrift niet in de knoop raakte. Bedankt hiervoor!

Annette, jij was projectleider van het project “Biomerkers voor verzadiging” en daarom ook nauw betrokken bij de verschillende studies die ik heb uitgevoerd. Ook jij hebt kritisch naar de vele versies van mijn manuscripten gekeken en ik waardeer het zeer dat ik je opmerkingen altijd binnen de “deadline” terugkreeg ongeacht hoe druk je het had. Ook bij jou kon ik altijd terecht met vragen. Ik vond het erg leuk om samen met jou en Kees een hoofdstuk te schrijven over verzadiging. Daar heb ik veel van geleerd. **Kees**, jou zag ik de eerste jaren voornamelijk op de donderdagen, jouw “TNO-dagen”. We hebben aardig wat gebrainstormd over studie designs en jouw kennis over verzadiging kwam altijd erg goed van pas. Het schrijven van het overzichtsartikel over biomerkers voor verzadiging nam toch veel meer tijd in beslag dan we hadden ingeschat, maar ik vond het erg leuk om daar met jou en de anderen aan te werken.

Zonder proefpersonen geen proef en dus ook geen proefschrift. Daarom wil ik hierbij graag alle **proefpersonen** bedanken. Mede dankzij jullie inzet en enthousiasme waren de studies een succes. Bedankt mannen!
Niet alleen de proefpersonen, maar ook de vele andere mensen die hebben meegewerkt aan de studies ben ik grote dank verschuldigd. **Inge** en **Henriëtte**, jullie hebben er als “study nurses” voor gezorgd dat de studies volgens plan werden uitgevoerd en dat de proefpersonen enthousiast bleven en, nog belangrijker, zich aan de (leef)regels hielden. Maar ook de andere medewerkers van de metabole unit; **Eric**, **Hanny, Christel, José**, **Soesila**, **Angelique**, **Desiree**, **Jolanda**, **Wilfred, Ineke, Linda** en de overige verpleegkundigen, bloedprikkers en artsen wil ik bedanken voor hun inzet. Dankzij jullie liep alles gesmeerd. Mijn studies zouden ook niet uitgevoerd kunnen worden zonder de ondersteuning van diëtetiek. **Susanne** en **Petra**, jullie wil ik met name bedanken voor de hulp tijdens de voorbereiding van de studies en zeker ook voor alle ochtenden waarop jullie voor dag en dauw op zijn gestaan om de testmaaltijden te bereiden. Natuurlijk ben ik al degenen (diëtisten, datamanagers, secretaresses, stagiaires, etc) die Susanne en Petra hierin hebben ondersteund ook erg dankbaar. **Robin, Hans, Wouter, Jan C, Wilma** en alle andere mensen van het lab wil ik bedanken voor de oneindige hoeveelheid analyses die ze voor me hebben uitgevoerd. De datamanagers, **Astrid, Diane** en **Linda** wil ik bedanken voor het scannen van de talloze vragenlijsten en voor hun hulp als ik weer eens ruzie had met SAS. Zonder de statistische ondersteuning van **Cor** en **Carina** was ik er vast niet uitgekomen. **Esther,** ik vind het erg fijn dat jij een aantal TNO-rapporten voor me hebt geschreven. Daar had ik naast het schrijven van de publicaties en mijn proefschrift geen tijd meer voor.

De afgelopen jaren heb ik drie studenten mogen begeleiden. **Linda, Gerda** en **Marieke**, jullie hulp bij de uitvoering van de studies en de data-analyse was onmisbaar. Bedankt, en veel succes met jullie verdere carriere!

I would like to thank **Anne Lluch** and **Sophie Vinoy** from Danone Vitapole for the close collaboration during the last two studies and their financial support. I enjoyed our discussions very much, and of course also the short trips to Paris. I am also grateful to **Jens Juul Holst** for his input on the GLP-1 infusion protocol and for the (free) GLP-1 analyses.

Ik heb de afgelopen jaren met veel mensen samengewerkt. Hoewel het resultaat van deze samenwerking niet altijd is beschreven in dit proefschrift, wil ik de mensen die hierbij betrokken waren natuurlijk wel bedanken. Met name **Hein**, **Koen** en **Martine**

van TNO Defensie en Veiligheid in Soesterberg wil ik bedanken voor de gastvrijheid en de prettige samenwerking. Hein jouw enthousiasme werkt erg inspirerend. Ik vind het leuk dat je als lid van de promotiecommissie aanwezig zal zijn bij de verdediging van mijn proefschrift.

Ik heb het altijd erg naar mijn zin gehad bij TNO, en dat komt zeker ook door de gezellige werksfeer. Mijn collega's van de afdeling FCRA (voedingsepidemiologie) wil ik bedanken voor alle gezellige koffie- en lunchpauzes en de getoonde interesse. Hoewel ik misschien een beetje een vreemde eend in de bijt was (in ieder geval qua onderzoeksonderwerp) heb ik me altijd onderdeel van de afdeling gevoeld. **Elleny**, jou wil ik in het bijzonder bedanken voor de gezellige tijd die we bij elkaar op de kamer hebben beleefd. Hoewel we qua onderwerp erg uit elkaar liggen heb ik het toch altijd leuk gevonden om onze ervaringen als AIO uit te wisselen. **Paul**, ik vind het jammer dat het er nooit van gekomen is om samen een studie uit te voeren. Succes met het afronden van jouw proefschrift!
Ook de collega's van de afdeling Physiological Sciences (voedingsfysiologie) wil ik bedanken voor hun interesse en hulp bij het opzetten van studies. Ik heb me toch ook altijd wel een beetje onderdeel van jullie afdeling gevoeld. **Wilrike** jij was degene bij fysiologie met de meeste affiniteit met mijn onderwerp en daarom ook vraagbaak en plaatsvervangend projectleider bij mijn studies. Bedankt! **Joline**, het feit dat we qua werksituatie in hetzelfde schuitje zaten heeft toch wel een band geschept. Bedankt voor de gezelligheid, roddels en soms ook serieuze gesprekken en voor de motiverende mailtjes vanuit Boston. Ik vind het erg leuk dat je straks als paranimf naast me staat.

Als niet-Wageninger met een werkplek in Zeist duurt het even voordat je in Wageningen bent ingeburgerd. Daarom waren voor mij de AIO-reis naar Duitsland, Zwitserland en Italië en die naar Australië een leuke manier om andere AIO's te leren kennen. Ik wil iedereen die mee is geweest op deze reizen bedanken voor de gezelligheid. **Monica** jij was als AIO met een vergelijkbaar onderwerp toch wel mijn eerste aanspreekpunt in Wageningen. Ik vind het leuk dat we uiteindelijk toch ook nog hebben samengewerkt.

Verder wil ik alle **vrienden en vriendinnen** bedanken voor hun interesse in mijn werk en de nodige ontspanning. Utrecht is misschien niet vlak bij de deur, maar afstand is relatief. Ik ben erg blij dat we ondanks de afstand elkaar toch regelmatig blijven zien. Alex en ik maken de ritjes naar het oosten dan ook met plezier.

Ook de **familie Blom**, **familie van den Berg** en **familie Borger** wil ik bedanken voor het tonen van hun interesse in mijn werk. Ik hoop dat dit boekje, en met name de Nederlandse samenvatting helpt begrijpen waar ik de afgelopen jaren mee bezig was.

Pap, **Mam**, **Daniëlle** en **Leon**, jullie wil ik natuurlijk ook bedanken voor jullie interesse en steun en voor het feit dat jullie altijd voor me klaar staan. Dit boekje is ook een beetje voor jullie.

Lieve **Alex**, je bent de afgelopen jaren altijd mijn steun en toeverlaat geweest en hebt me er van overtuigd dat ik het allemaal best kan. Je bent altijd erg betrokken geweest bij mijn onderzoek, en niet alleen omdat we de eerste jaren carpoolden en jij daardoor min of meer verplicht was om naar informatiebijeenkomsten te komen of extra vroeg te beginnen op je werk. Ik vind het erg fijn dat je me ook tijdens de verdediging van dit proefschrift terzijde zal staan, als paranimf. Met jou naast me durf ik alles aan.

Curriculum Vitae

About the author

Willemina Albertha Maria Blom, Wendy, was born on the 1st of July 1978, in Deventer, The Netherlands. She grew up in Boerhaar. In 1996, she passed secondary school (Atheneum) at the “Florens Radewijns College” in Raalte. In the same year, she started her study in Biology at the State University of Groningen, to become specialized in behavioural and neuronal sciences. As part of this study, she started her first research project at the department of Biological Psychiatry of the academic hospital in Groningen, where she investigated the influences of the biological clock on mood and body temperature in patients with winter depression before and after light therapy. At the department of Animal Physiology of the Biology faculty, she performed her second research project focusing on the effects of a high fat diet on the stress response in rats. She ended her study with an internship at the department of Animal Welfare at the Institute for Animal Science and Health in Lelystad, where she performed a behavioural study focusing on fear in pigs after social defeat. In 2001 she received her MSc degree. In the same year, she started working as a PhD-fellow at the Division of Human Nutrition at the Wageningen University and the Department of Nutritional Epidemiology at TNO Nutrition and Food Research (now TNO Quality of Life) in Zeist. Her PhD-project, of which the main results are presented in this thesis, was part of a multidisciplinary TNO project focused on the identification and measurement of biomarkers of satiety and satiation. She joined several courses and congresses within the framework of the educational program of the Graduate School VLAG (Food Technology, Agrobiotechnology, Nutrition and Health Sciences).

List of publications

Peer reviewed publications

W.A.M. Blom, A. Stafleu, C. de Graaf, F.J. Kok, G. Schaafsma, H.F.J. Hendriks. Ghrelin response to carbohydrate-enriched breakfast is related to insulin. Am J Clin Nutr. 2005; 81:367-375

W.A.M. Blom, A. Lluch, A. Stafleu, S. Vinoy, J.J. Holst, G. Schaafsma, H.F.J. Hendriks. The effect of a high protein breakfast on the postprandial ghrelin response. Submitted

W.A.M. Blom, A. Lluch, S. Vinoy, A. Stafleu, R. van den Berg, J.J. Holst, F.J. Kok, H.F.J. Hendriks. The effects of gastric emptying on the postprandial ghrelin response**.** Am J Physiol Endocrinol Metab. Accepted for publication (electronically published DOI, 10.1152/ajpendo.00238.2005)

W.A.M. Blom, M. Advocaat, C. de Graaf, A. Lluch, A. Stafleu, G. Schaafsma, H.F.J. Hendriks. Postprandial ghrelin kinetics are associated with the intermeal interval in time-blinded normal weight men, but not in obese men. Submitted

W.A.M. Blom, M. Mars, H.F.J. Hendriks, C.P.G.M. de Groot, A. Stafleu, F.J. Kok, C. de Graaf. Fasting ghrelin does not predict food intake after short-term energy restriction. Submitted

C. de Graaf , **W.A.M. Blom**, P.A.M. Smeets, A. Stafleu, H.F.J. Hendriks. Biomarkers of satiation and satiety. Am J Clin Nutr. 2004; 79:946-61

B. Buwalda, **W.A.M. Blom**, J.M. Koolhaas, G. van Dijk. Behavioral and physiological responses to stress are affected by high-fat feeding in male rats. Physiol & Behav. 2001; 73; 1-7.

Abstracts

W.A.M. Blom, H.F.J. Hendriks, A. Stafleu, C. de Graaf, F.J. Kok, G. Schaafsma. Ghrelin and appetite responses after liquid breakfasts varying in energy content and carbohydrate structure. Int J Obes. 2003; 27:(supplement 1) S35.

W.A.M. Blom, A. Stafleu, H.F.J. Hendriks. The effect of energy restriction on satiation and satiety in obese and lean men. Int. J. Obes. 2004; 28: (supplement 1) S214.

W.A.M. Blom, C. de Graaf, P.A.M. Smeets, A. Stafleu, H.F.J. Hendriks. Biomarkers of satiation and satiety: a review. Int. J. Obes. 2004; 28: (supplement 1) S214.

W.A.M. Blom, A. Lluch, A. Stafleu, S. Vinoy, H.F.J. Hendriks. The effect of high versus low protein breakfast and gastric emptying on subjective and physiological measures of satiety. Obes Rev. 2005; 6: (supplement 1) 50.

W.A.M. Blom, A. Lluch, S. Vinoy, R. van den Berg, H.F.J. Hendriks. Effects of gastric emptying on the postprandial ghrelin response. European Congress on Obesity, 01-04 June 2005, Athens, Greece.

W.A.M. Blom, A. Lluch, H.F.J. Hendriks. The effect of a whey protein enriched dairy product on subjective and physiological measures of satiety. International Whey Conference, 11-14 September 2005, Chicago, USA.

Other publications

W.A.M. Blom, A. Stafleu, C. de Graaf. Satiety and the control of obesity. Chapter 18 in: Functional foods, ageing and degenerative disease. Edited by C Remacle and B Reusens. Woodhead Publishing Ltd.

W.A.M. Blom, H.F.J. Hendriks, A. Stafleu, C. de Graaf, F.J. Kok and G. Schaafsma. Effect of extra-cellular polysaccharides on satiety. Dietary Fibre – bio-active carbohydrates for food and feed. Edited by J.W. van der Kamp, N.-G. Asp, J. Miller Jones and G. Schaafsma. Wageningen Academic Publishers. 2004; 231-236

W.A.M. Blom. De verzadigende werking van darmhormonen. Voeding Nu. 2005; 4:11-13

Overview of completed training activities

Discipline specific activities

- VLAG course Regulation of energy- and substrate metabolism, 2001
- VLAG course Regulation of food intake and its implications for nutrition and obesity, 2002
- VLAG course Food perception and food preference, 2003
- VLAG course Ecophysiology of the gastrointestinal tract, 2003
- University of Utrecht: Practical statistics for microarray data, 2004
- Erasmus Summer Programme, 2004
    - Principles of research in medicine
    - Introduction to data analysis
    - Regression analysis
- RIVM symposium; Genetic causes of overweight, 2001
- Meetings NWO Nutrition, 2001, 2003, 2005
- Meeting Centre for Human Nutrigenomics, 2002
- Dietary Fiber, 2003
- Annual meeting of the Society of the Study of Ingestive Behavior (SSIB), 2003
- European Congress on Obesity, 2003 - 2005
- European Society for Clinical Investigation (ESCI), 2004
- Symposium Netherlands Association of the Study of Obesity (NASO), 2004
- Food Summit 2004
- 4th International Whey Conference, 2005

General courses

- Introduction course WMO and GCP, 2002
- SAS programming and basic statistics, 2002
- Scientific writing, 2002
- VLAG PhD week, 2002

Optional courses and activities

- Preparation PhD research proposal
- Meetings of the journal club, 2001-2004
- PhD-study tour Switzerland and Germany, 2001
- PhD-study tour Australia, 2003

The studies described in this thesis were performed at TNO Quality of Life, Zeist and at the Division of Human Nutrition of The Wageningen University, Wageningen. This work was financially supported by the Dutch Ministry of Economic Affairs, Dutch Ministry of Education, Culture and Science, Dutch Ministry of Health, Welfare and Sport and Danone Vitapole.

Financial support by the Wageningen University, TNO Quality of Life, de Dr. Ir. Van de Laar Stichting and Nuclilab (Linco Research) for the publication of this thesis is gratefully acknowledged.

Layout: Wendy A.M. Blom
Printed by: Ponsen & Looijen BV, Wageningen, The Netherlands