The acute leptin decline after energy restriction. A biomarker for the susceptibility to weight gain?

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De acute daling van leptine na een energiearme voeding. Een biologische indicator voor de gevoeligheid voor het ontwikkelen van overgewicht?

Monica Mars

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Abstract

The last decades the prevalence of obesity has been increasing. However, large inter-individual differences in weight gain are observed. Poor energy intake regulation is one of the possible mechanisms behind a large susceptibility to weight gain. The hormone leptin is suggested to have a key-role in the regulation and restoration of energy balance. Therefore, we hypothesized that the acute leptin decline after energy restriction might be a marker for the susceptibility to weight gain. In other words, is the leptin response an individual trait that is related to the ability to restore energy balance and is it therefore related to the susceptibility to weight gain? To test this hypothesis we conducted three controlled intervention studies in which male subjects received an energy restriction of ~65% during a few days. First of all, we performed a reliability study in we which assessed the reliability of the insulin and ghrelin response on short-term (3 weeks) and long-term (1½ year). On the short-term, the leptin decline had a relatively high reliability (Intra Class Correlation [95%-Confidence interval]=0.66 [0.33; 0.85]) compared to the decline in insulin (ICC=0.45 [0.03; 0.74]) and the increase in ghrelin (ICC=0.34 [0; 0.67]). On the long-term however, the acute leptin response showed a lower reliability (ICC=0.34 [0; 0.67]). After that, we hypothesized that the leptin responsiveness to energy restriction is affected by the functionality of the leptin receptor; therefore we explored the effect of three common polymorphisms in the leptin receptor gene (Lys109Arg, Gln223Arg and Lys656Asn) on the acute leptin decline. The analyses revealed no statistically significant differences in leptin response between genotypes, *i.e.* between carriers and non-carriers of the mutant allele. Next, we investigated whether the acute decline in leptin is a biomarker for weight gain with different (intermediate) endpoints, *i.e.* appetite, energy intake compensation, and retrospective weight gain. We hypothesised that the decline in leptin is related to subjective appetite ratings, ratings that reflect hunger, fullness, desire to eat, prospective consumption, and total appetite. We observed that the magnitude of the acute decline was indeed positively associated with the increase in hunger ($r=0.42$; $p<0.05$), desire to eat ($r=0.39$; $p<0.05$), and total appetite ($r=0.35$; $p<0.05$). Next, we investigated the association between the amount of energy that is compensated in the days following energy restriction and the magnitude of the leptin decline during energy

restriction. Although we found that leptin levels declined by 24% [95%-Confidence Interval: -33%; -15.9%] and subjects showed compensatory behaviour (143 ± 27% on the first day and 124 ± 20% on the second day after energy restriction), no association was observed between the magnitude of the leptin decline and energy intake compensation (r=0.22; ns). Lastly, we investigated whether individuals with stable weight show larger leptin declines to energy restriction than individuals who gained weight. Proportionally, an 8% smaller decrease in leptin was observed in men with retrospective weight gain. However, in a selected subgroup with a larger difference in retrospective weight gain, this difference in leptin response was not found; therefore, we concluded that the data did not provide convincing evidence for our hypothesis that men who gained weight are less leptin responsive to changes in energy balance than men who were weight stable.

Overall, we conclude that the leptin decline to energy restriction is not a good biomarker for the susceptibility to weight gain. However, it may be a good indicator for energy balance or the increase in appetite during energy restriction.

Contents

1

Introduction

The last decades, the prevalence of obesity has been increasing. This is mainly a consequence of a modern lifestyle; large amounts of palatable high calorie food and a limited requirement for physical activity. However, large individual differences are observed in the susceptibility to weight gain. In this thesis the role of individual differences in food intake regulation and weight gain are investigated, giving due attention to the hormone leptin. In this introduction the biological role of leptin in the regulation of energy intake is described along with that of other interacting hormones: insulin and ghrelin. Based on this background, the rationale, research questions, and the outline of the thesis, are presented in the final paragraphs of this chapter. Prior to that, the current epidemic of obesity and its consequences are addressed.

THE EPIDEMIC OF OBESITY

Obesity can be defined as a disease in which an excess of body fat has accumulated to such an extent that health may be adversely affected (1). In The Netherlands 45% of the men and 35% of the women aged 20-59 y are overweight (BMI $\geq$ 25 kg/m^2) (2). Circa 10% of the adults is obese (BMI $\geq$ 30 kg/m^2), and it is estimated that 1-1.5% is morbidly obese (BMI $\geq$ 40 kg/m^2) (3). According to data of the National Institute of Public Health and the Environment, the prevalence of obesity has been increasing rapidly the last decades. The prevalence of obesity in men and women aged 37-43 y was 4.9% and 6.2% in the period 1976-1980, 7.4% and 7.6% in the period 1987-1991, and 8.5% and 9.3% in the period 1993-1997, respectively (4). Data of the National Food Consumption Survey and Statistics Netherlands, which is based on self-reported weight and height, confirm this trend (5, 6). In view of these developments, the Dutch Health Council foresees that in 2015 15-20% of the Dutch adults will be obese (3).

Obese individuals have a higher risk of various chronic diseases. Especially, the risk of Type II Diabetes is elevated for obese individuals compared to lean individuals; the relative risk (RR) amounts to 12.7 in men and 5.2 in women. It has been estimated that 52.7% and 26.3% of the cases of Type II Diabetes can be attributed to obesity (3). Increased risks are also found for: hypertension (RR men=4.2; RR women=2.6); cardio-vascular disease, *e.g.* myocardial infarction (RR men=3.2; RR women=1.5); gallbladder disease (RR men and women=1.8); and certain types of cancer, *e.g.* colon cancer (RR men=2.7; RR women=3.0) (3). Moreover, the high prevalence of obesity

has a major impact on the costs of health care. In 1999, the direct costs associated with obesity were estimated at 550 million euros; the indirect costs were estimated at 2 billion Euros per year (3).

Weight gain and obesity are mainly the result of a prolonged positive energy balance; more energy is consumed than expended over a longer period of time. Theoretically, one gains circa 1 kg of body weight per year if one consumes ~8 kJ more per day than expending (7). This amount of energy is comparable to 0.5 g of sugar (one tenth of a sugar lump) (8). Data of the National Institute of Public Health and the Environment show that adults increase on average 0.4-0.5 kg of body weight per year (9). However, individuals who have an inherited susceptibility are more prone to weight gain than those without this susceptibility. Worldwide scans of the DNA of obese and lean people have resulted in several polymorphisms and genetic markers for this susceptibility; the most promising candidate markers are currently verified in epidemiological studies (10). Unfortunately, the relevance of most of these polymorphisms and markers in the development of obesity is not yet clear (10). Presumably, lifestyle factors in combination with a number of genes affect the susceptibility to weight gain. Possible mechanisms through which the susceptibility to weight gain can operate are, *e.g.* low resting metabolic rate, low rate of lipid oxidation, and poor energy intake regulation (1).

ENERGY INTAKE REGULATION

Food intake is determined by many factors, including: socio-cultural factors (*e.g.* price, availability), psychological factors (*e.g.* mood, attitude towards a food), and properties of food (*e.g.* sensory characteristics, energy content) (11). One of the most important factors is the internal drive to ingest food, *i.e.* appetite. Appetite is mostly determined by peripheral signals on the state of energy balance. These signals are then sent to various nuclei in the brain and translated into a stimulation or inhibition of food intake. Important peripheral signals that are involved in the regulation of energy intake are the hormones insulin, leptin, and ghrelin (12, 13). These hormones and their role in the regulation of energy intake by the hypothalamus will be addressed in the following paragraphs.

Peripheral signals

Leptin

Leptin is a hormone that is secreted by adipose tissue in the bloodstream (14). It signals to the brain by binding to the leptin receptor that is present in the arcuate nucleus (ARC) of the hypothalamus (15). Serum leptin concentrations are positively associated with BMI and reflect the amount of fat mass of the body (16). If people lose weight, leptin concentrations are decreasing, due to loss of fat mass (17, 18). However, after acute energy restriction leptin concentrations decrease far more than can be expected from loss of fat mass (19-22). Thus, an additional function of leptin might be to signal on the state of energy balance. It has been hypothesized that leptin plays a central role in the defence of energy stores during times of food scarcity (13). Additionally, it has been thought that subjects with high leptin levels are becoming resistant to the signals of leptin (16).

Animal models provide the strongest evidence that leptin plays a central role in energy intake regulation. The two models most intensively described are the *ob*/*ob*-mouse (14), and the *db*/*db*-mouse (23, 24). The *ob*/*ob*-mouse is lacking the feed back signal of leptin to the brain, while the *db*/*db*-mouse has an inactive leptin receptor (24). Both animals are extremely obese and hyperphagic due to a genetic mutation. In humans only a few cases of obesity have been explained by mutations in the leptin gene (25) or the leptin receptor gene (26). However, these mutations are extremely rare.

Still, leptin may be an important peripheral signal for the central regulation of energy balance in humans, as it has been shown that failures in this regulation have marked consequences for energy intake regulation and weight maintenance.

Insulin

The most important function of insulin is to enhance glucose uptake by cells and consequently it regulates blood glucose concentrations after the ingestion of food. However, insulin has probably an additional function on the longer-term; in the regulation of energy balance. Not only are insulin concentrations increased after the ingestion of food, they are also increased during a prolonged positive energy balance and after increases in fat mass (27). This is demonstrated by the increased concentrations of insulin in patients with glucose intolerance or non-insulin dependent

diabetes (28). During energy restriction and weight loss, insulin concentrations decrease to normal levels (29). Thus, it is likely that insulin plays a role in long-term food intake regulation, like leptin. Moreover, like leptin receptors, insulin receptors have been localised in the Arcuate Nucleus of the hypothalamus (27). It has been speculated that the decrease in insulin concentration during energy restriction is related to the decrease of leptin (21, 27, 29). Thus, next to leptin, also insulin might be an important peripheral signal for the central regulation of energy balance, maybe via an effect on leptin secretion.

Ghrelin

Ghrelin is a hormone, that has been discovered only recently (30) and that is possibly involved in both short-term and long-term energy intake regulation (31). Ghrelin is produced by mucosa cells of the stomach (30). Ghrelin concentrations decrease shortly after the ingestion of glucose (32) or other carbohydrates (33). Also ghrelin concentrations increase during diet induced weight loss or after bypass surgery (34). For these reasons, it might have a role in energy balance regulation. However, the exact regulation of the secretion of ghrelin is still unknown (35).

Hypothalamic regulation of energy balance

As discussed in the paragraphs above, leptin, insulin and ghrelin concentrations respond to the state of energy balance, presumably in order to restore energy balance. In Figure 1.1, this regulation of the energy balance is schematically depicted (12). Under conditions of energy imbalance, the secretion of ghrelin, insulin and leptin changes, which affects several nuclei in the hypothalamus; proopiomelanocortin (POMC), Neuropeptide Y (NPY), and Agouti related protein (AgRP). POMC inhibits energy intake, while NPY and AgRP stimulate energy intake. In Figure 1.1 a positive energy balance is depicted. In this case, leptin and insulin concentrations rise. Subsequently, NPY and AgRP are inhibited, while POMC is stimulated, which results in an inhibition of energy intake. Eventually, this leads to a restoration of the energy balance. As stated, ghrelin is recently added to this mechanism. It has been suggested that ghrelin concentrations decrease during a positive energy balance (12). This might result in a decrease in the inhibition of POMC and a decrease in the stimulation of

NPY and AgRP. As a consequence, energy intake is inhibited and the energy balance will be restored.

In order to signal the nuclei in the hypothalamus, the peripheral signals operate via receptors; these are the leptin receptor (LEP-R), the insulin receptor (INS-R) and the growth hormone secretagogue receptor (GHS-R) for leptin, insulin and ghrelin, respectively. These receptors might be essential in the hypothalamic regulation of energy balance. This has been demonstrated by the large consequences of defects in the leptin receptor in mice (24) and humans (26).

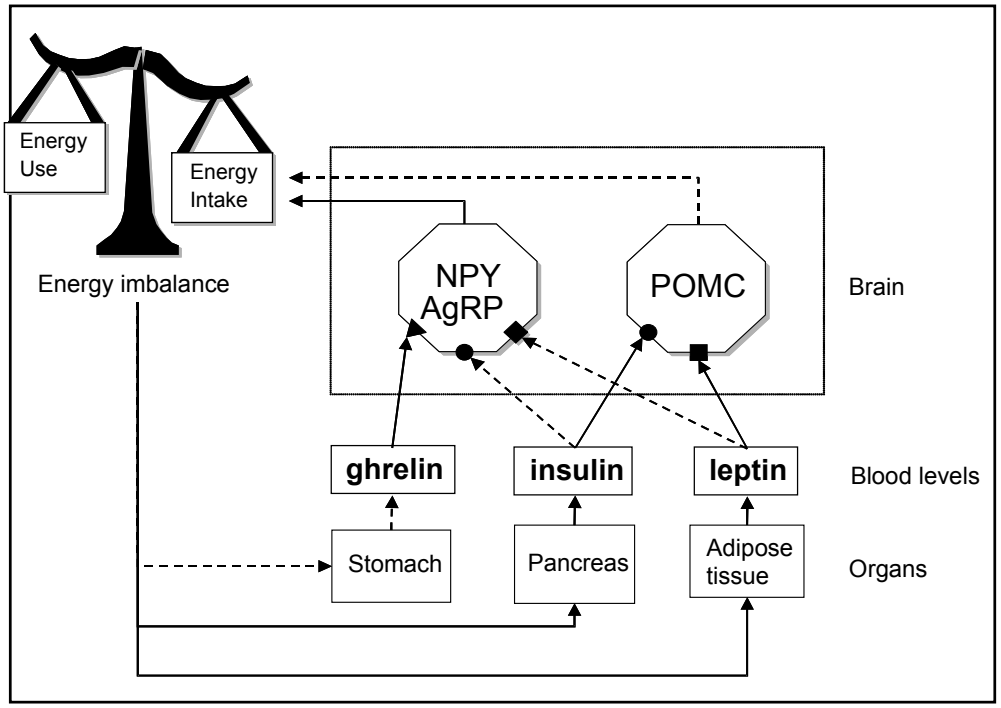

Figure 1.1 Peripheral signals on energy balance and hypothalamic regulation of energy intake (figure adapted from Korner and Leibell (12)). → =stimulation, ⇢ = inhibition, NPY=Neuro Peptide Y, AgRP=Agouti Related Protein, POMC= proopiomelanocortin, ▼ =Growth hormone secretagogue receptor (GHS-R), ● =insulin receptor (INS-R), ■ =leptin receptor (LEPR).

LEPTIN AND WEIGHT GAIN

Leptin, insulin, and ghrelin might be important peripheral signals for the state of energy balance. They all respond to short-term changes in energy balance, presumably in order to restore energy balance. As stated before, weight gain is mostly due to a small but prolonged positive energy balance, which may be caused by poor energy intake regulation. Therefore, the individual variation in acute hormonal responses to short-term changes in energy balance might be related to the individual variation in weight gain. We hypothesized that the acute hormonal responses to short-term changes in energy balance might be biomarkers for the susceptibility to weight gain.

In this thesis, we focused our research on the acute decline of leptin to energy restriction. At the time of research, the most promising biomarker for the susceptibility to weight gain was leptin. Leptin had been shown to respond within days to energy restriction (19-22) and it had been thought to have a central role in the restoration of energy balance (36). Therefore, we speculated that the acute leptin decline might be a biomarker for the ability to restore energy balance, and thus for the susceptibility to weight gain.

As depicted in Figure 1.2, we disentangled several steps from this hypothesis. The following paragraphs discuss the current knowledge on each of these steps. First, the reliability of the decline and the influence of genetic variation on it are addressed. And second, the present literature on the association of the magnitude of the acute decline in leptin with subjective appetite, energy intake compensation, and weight gain are discussed.

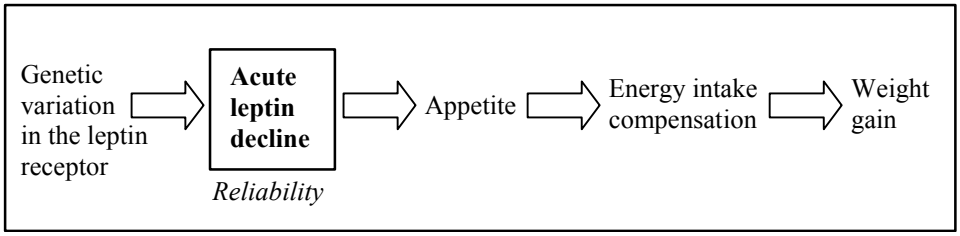

Figure 1.2 Steps disentangled from the hypothesis that the acute leptin response is a marker for the susceptibility to weight gain.

Reliability

If the acute leptin decline to energy restriction is a biomarker for the susceptibility to weight gain, then it is necessary that this decline has a high reliability. First, the decline should show a relatively low intra-individual variation. The decline should be an individual trait and therefore be reproducible within persons on both the short-term and the long-term. Second, there should be a relatively high variation in decline between persons; differences between subjects should be large enough to be biologically relevant. Currently, no information is present on the reliability of this decline. Our hypothesis is that the acute decline in leptin induced by energy restriction has a high reliability on both short-term and long-term.

Genetic variation in the leptin receptor

If the acute leptin decline to energy restriction is an individual trait, then the decline may be affected by genetic variation in the leptin receptor. It is likely that leptin exerts its action trough the leptin receptor in the hypothalamus. It has been speculated that this receptor is involved in the feed back loop for the regulation of leptin secretion (37). If so, the leptin responsiveness to energy restriction will depend on the functionality of the leptin receptor. Genome scans have revealed several polymorphisms in the leptin receptor that may affect the functionality of this receptor (38). Although no empirical data on the functionality of these polymorphisms in the leptin receptor have been reported, one population-based study found that polymorphisms in codon 109 and 223 modified the effect of leptin concentrations on weight gain (39). Therefore, we hypothesized that polymorphisms in the leptin receptor might also modify the acute decline in leptin induced by energy restriction.

Appetite

If the acute decline in leptin is a biomarker for the susceptibility to weight gain, then it should be related to weight gain, or an intermediate endpoint, *i.e.* food intake compensation or subjective appetite. In this paragraph we address current research on the intermediate endpoint ‘appetite’.

Up till now, several human studies have investigated the association between changes in leptin concentrations and their effect on subjective appetite or food intake in

humans. These studies have been conducted either during conditions of energy balance (40) or energy imbalance (41). Especially, the studies that included an energy restriction for several weeks have found an association between the decline in leptin and the increase in appetite. Although these researchers did not study the acute leptin decline that takes place within days, their findings are in line with the hypothesis that the acute decline of leptin is involved in the restoration of energy balance via appetite. First, Keim and colleagues found a strong inverse association (range of r: -0.6 to -0.7) between leptin concentrations and appetite ratings during energy restriction. Like the acute decline in leptin, this association was independent of the weight lost during the intervention (42). Second, Heini and colleagues observed 22 moderately to severely overweight women during a very low calorie diet (3.3 MJ) of five weeks. In multivariate analyses, within subjects they observed an inverse linear association between leptin concentrations and the appetite feelings (43). Third, Westerterp-Plantenga and colleagues have found in a double blind trial that appetite decreased in subjects receiving exogenous leptin during energy restriction, while the appetite increased in subjects receiving a placebo treatment (41). This difference in appetite was observed in the first weeks of the trial, and might be related to the early response in leptin.

Based on these findings and the evidence from animals that leptin is involved in energy balance restoration, we hypothesized that the magnitude of the acute decline in leptin is positively associated with the increase in subjective appetite.

Energy intake compensation

Another intermediate endpoint in weight gain is energy intake compensation, *i.e.* the amount of energy that is ingested after a period of energy restriction. If the acute decline in leptin after energy restriction is important for susceptibility to weight gain, then it may be that its magnitude relates to the amount of energy that is compensated during the following period.

As yet, only one human study has investigated the acute decline of leptin and subsequent energy intake (44). Despite several methodological drawbacks, an interesting finding was observed in this study. Six healthy obese subjects were studied during two intervention periods of three days. The first intervention contained 130% of

the estimated energy needs and the second 70% of the estimated energy needs, or vice versa. Before each intervention, subjects consumed 100% of their energy needs for three days, which was also the wash-out period between interventions. After each intervention the *ad libitum* energy intake during breakfast was measured. Although leptin levels did not return to baseline levels in the wash-out period, a surprisingly high inverse association (R^2=0.4) between the declines in leptin and the *ad libitum* energy intake was observed (44). Even though this study has several limitations, it does not keep us from hypothesizing that the magnitude of the leptin decline to energy restriction is positively associated with energy intake compensation during the following period.

Weight gain

Several population-based studies have investigated the association between weight gain and leptin concentrations (39, 45-50). A number of these studies have observed no association between leptin concentrations and subsequent weight gain (45), but also several studies have found that a high leptin level predicts weight gain (39, 46, 47). Thus, if leptin levels affect weight gain, then it is likely that high leptin levels promote weight gain. This is in line with the hypothesis that subjects with high leptin levels are leptin resistant (51) and thus more likely to gain weight. Individuals who are leptin resistant might also respond differentially to changes in energy balance. Therefore, we hypothesized that individuals who are highly leptin responsive to deviations in energy balance are better capable of restoring their energy balance, and keep their weight stable for long periods, while individuals who are less responsive, may be less capable to do so and therefore gain weight.

RATIONALE AND RESEARCH QUESTIONS

In summary, leptin decline to energy restriction might be a potential biomarker for the susceptibility to weight gain. However, this hypothesis is mainly based on animal studies and theoretical speculations; only a small number of human studies are conducted to underpin this hypothesis. The main aim of this thesis was to investigate whether the acute decline in leptin to energy restriction is a biomarker for the susceptibility to weight gain. We translated the main aim into three major research questions:

1 Is the leptin decline to energy restriction a reliable measure?
2 Is the leptin decline to energy restriction associated with genetic variation?
3 Is the leptin decline to energy restriction associated with the susceptibility to weight gain?
   a Is the decline positively related to subjective appetite?
   b Is the decline positively related to energy intake compensation in the period following energy restriction?
   c Do individuals with stable weight show larger leptin declines to energy restriction than individuals who gained weight?

We conducted three controlled intervention studies. In all three interventions a group of healthy men underwent a severe energy restricted intervention during a few days, during these days they consumed only one-third of their estimated energy needs. Before and after intervention leptin levels were measured. The studies were all limited to men, as both food intake (52) and leptin concentrations (53) are influenced by menstrual cycles.

OUTLINE

This paragraph gives an outline of the following chapters in this thesis. In Chapter 2 we examined whether the acute leptin decline is a reliable measure. Is the leptin response reproducible when we measure it repeatedly under similar circumstances? To answer this question we conducted the *LEPTOS*-study. A total of 20 subjects participated three times in a controlled intervention study in which we measured the leptin decline after 2 days. The first intervention took place in 2001, the second intervention 1½ year later, and the third intervention another 3 weeks later. This design made it possible to compare the leptin declines both on short-term and long-term. Additionally, in these analyses the reliability of insulin and ghrelin responses was assessed. As described earlier these hormones may also have an important role in the regulation of energy intake.

In Chapter 3 we investigated the influence of genetic variation in the leptin receptor on the acute leptin decline. For these analyses, the "*Eetlust*"-study was conducted. We recruited 44 men from a selection of the Doetinchem-cohort (n=358), and measured serum leptin concentrations before and after four days of energy restriction. Then, DNA from white blood cells of the participants was isolated and genotyped for three common and promising polymorphisms in the leptin receptor gene: Lys109Arg, Gln223Arg, and Lys656Asn. Next, differences in acute leptin declines between genotypes were tested. In general, many studies that investigate the effect of polymorphisms lack power. At the time of our analyses, the expected effect between genotypes was unknown, and the number of subjects in our study was relatively low. Therefore, we also performed power calculations for future studies and presented feasible suggestions to improve the power.

In Chapter 4, 5 and 6 we investigated whether the acute decline in leptin is a biomarker for weight gain with different (intermediate) endpoints. Chapter 4 focuses on hunger and appetite feelings. In these analyses, we used the data of the "*Eetlust*"-study. During this study we assessed subjective hunger and appetite by means of questionnaires on several occasions. As the leptin response is probably a starvation signal to the brain, we hypothesized that subjects with a higher response should also perceive a higher motivation to eat and thus have a higher appetite.

In Chapter 5 we studied the amount of energy that is compensated after an acute decline in leptin. A group of 35 subjects was recruited among employees and students of Wageningen University to participate in the *KALOZ*-study. First, the subjects received an energy-restricted diet for two days in order to induce an acute leptin decline. After these two days, we provided food *ad libitum, i.e.* unlimited, without restriction. In this way, we could investigate whether the magnitude of the leptin response was associated to subsequent energy intake compensation.

As mentioned before, the subjects from the "*Eetlust*"-study were recruited from the Doetinchem-cohort study. This study has monitored the body weight of its participants during the last 12 years, which gave us the opportunity to combine longitudinal data on body weight with intervention data. We selected 22 men who had gained body weight and 22 men who had remained stable in body weight over a period of 6 years. These subjects then participated in the controlled intervention study. In this manner, we could compare the acute leptin decline between the two groups in order to assess its relation with weight history (Chapter 6).

In the final chapter of this thesis we reflect on the main findings of the previous chapters, and discuss their implications for food intake regulation (Chapter 7). Taken this, we consider the role of leptin in the susceptibility to weight gain. Subsequently, this leads to the conclusion of the thesis and to directions and suggestions for future research.

At the start of this project, the field of genetic variation and obesity was promising, however during the last years it has become clear that the genetic influence on weight gain is not determined by single polymorphisms, but presumably by lifestyle factors together with several interacting genes. Therefore, we changed the initial plan of studying several polymorphisms in the leptin receptor gene and focused on leptin response as a potential biomarker for weight gain. Consequently, the studies described in this thesis are not presented in chronological order, but are organized so that the research questions are in a logical order, based on the current knowledge about the acute decline of leptin and the susceptibility to weight gain.

2

Leptin, insulin and ghrelin responses to energy restriction: biomarkers for restoring energy balance?

Monica Mars, Cees de Graaf, Lisette de Groot, Chantal Vrijhof, Caroline van Rossum, Frans Kok

The response of leptin, insulin and/or ghrelin to acute energy restriction may be a suitable indicator for the individual ability to restore energy balance. A prerequisite for a biomarker is a high reproducibility on the long- and the short-term; therefore, we performed a reliability study.

A total of 20 men (aged: 43.2 ± 4.3 y, BMI: 27.8 ± 4.1 kg/m^2) participated three times in a controlled intervention (35% of estimated energy needs for 2 days). The reliability of responses in leptin, insulin and ghrelin responses were assessed after a 3-week (short-term) and a 1½-year (long-term) interval, using the intra-class correlation coefficient (ICC) as a measure.

On average leptin declined 0.9 ng/mL (-16%), insulin declined 1.2 µU/mL (-16%), and ghrelin increased 0.25 ng/mL (+17%). On the short-term, the ICC was 0.66 [95%-Confidende Interval: 0.33;0.85] for leptin, 0.45 [95%CI: 0.03;0.74] for insulin, and 0.34 [95%CI: 0;0.67] for ghrelin. On the long-term, the ICC was 0.34 [95%CI: 0;0.67] for leptin, 0.13 [95%CI: 0;0.53] for insulin, and 0 [95%CI: 0;0.36] for ghrelin.

In contrast to ghrelin and insulin, the leptin decline after energy restriction showed a relatively high reliability on the short-term. Therefore, this response is the most promising biomarker for the individual ability to restore energy balance.

Submitted for publication

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INTRODUCTION

Leptin, insulin and ghrelin are probably key-role players in the regulation of energy intake (54). Leptin is produced by adipocytes and informs the brain about the amount of adipose tissue (spare fuel supplies) (16) and the state of energy balance (current fuel supplies) (55). Ghrelin is produced by the stomach and duodenum, and is also involved in both the long-term and the short-term regulation of body weight (31, 34). An acute energy restriction results in a dramatic decrease in leptin levels (44), a decrease in insulin (56) and a rise in ghrelin levels (34). These hormonal responses to energy restriction may be biomarkers for the individual ability to restore energy balance.

If the individual ability to restore energy balance is associated with the leptin, insulin, and/or ghrelin response, it is necessary that these responses have a high reliability. First, the hormonal responses should show a relatively low intra-individual variation. The responses should be an individual trait and therefore adequately reproducible within persons on the short-term and on the long-term. Second, there should be a relatively high variation in response between persons; differences between subjects should be large enough to be biologically relevant. The intra-class correlation coefficient, which comprises the ratio of the intra- and inter- individual variance, is an often-used reliability measure (57). The higher this intra-class correlation, the more suitable these hormonal responses to energy restriction are as indicators for the individual ability to restore energy balance.

Currently, no information is present on the intra- and inter-individual variation in the ghrelin response to energy restriction. As ghrelin has been discovered only recently (58), it has not been studied as thoroughly as leptin and insulin. Differences in leptin responses induced by energy restriction have been shown to correlate with changes in subjective appetite (59) and, although less consistent, with weight history (56). Although these observations show that differences in leptin response between persons may be large enough to be biologically relevant, information on the intra-individual variation is not present.

In the current study, we studied the acute leptin, insulin and ghrelin response after two days of 65%-caloric restriction, and assessed the intra-class correlation in these responses, both on the short-term (3 wk) and on the long-term (1½ y).

MATERIALS AND METHODS

Subjects

Three dietary interventions were conducted as presented in Figure 2.1. The first intervention study took place in 2001, and has been described earlier (56). For the second and third intervention, which were carried out in 2002, a sub sample of 20 men was recruited from the participants of the first intervention study.

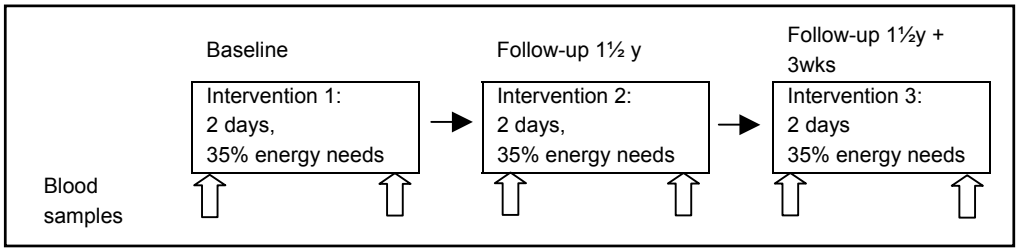

Figure 2.1 Schematic overview of the design of the three energy restriction studies performed in 20 adult men.

28 Subjects were eligible if they did not use any drugs known to affect energy metabolism, and if they were not on a prescribed or weight loss diet during the two months prior to the study. Persons with a history of diabetes mellitus, diseases affecting the thyroid gland, liver or the gastrointestinal tract were excluded, as well as those with glucosuria, anaemia or fasting plasma glucose concentrations >6.9 mmol/L. Inclusion criteria were met prior to the first and to the second intervention study. The Ethics Committee of Wageningen University approved the protocol. All subjects gave their written informed consent.

Energy restricted diet

The energy-restricted diet was calculated individually for the first intervention study as described earlier (56; chapter 6). During the second and third intervention, subjects received a diet containing a similar amount of energy as during intervention 1; 6 subjects received 4.2 MJ/d, 12 subjects received 5.0 MJ/d, 1 subject received 5.9 MJ/d and 1 subject received 6.7 MJ/d. The diet consisted of micro nutrient dense snack and meal replacements (Nutricia, Zoetermeer, The Netherlands), which were supplied at

the beginning of each intervention. Compliance was measured by pre-printed diaries, in which subjects wrote down the time of consumption. Subjects were advised to only use the provided food products, but if necessary, additional foods were written down in full detail. Non-caloric beverages were allowed.

During intervention 1, complete duplicate portions of the intervention diet were collected for one imaginary subject with an intervention diet containing 5.0 MJ/d. These portions were mixed and stored at –20°C until chemical analysis. At the end of the study, for each subject the average daily intake of energy, macronutrients and fibre was calculated based on the chemical analyses and the daily food records. For additional products, the food composition data from the Dutch Food composition Database were used (8). Subjects consumed on average 35% (range 24-40%) of their estimated energy needs, *i.e.* 4.9 ± 0.7 MJ/d (21.7% protein, 21.5% fat, 56.8% carbohydrates as percent of total energy consumed).

Measures

Blood sampling

During the intervention study, fasting samples were taken to determine serum leptin, serum ghrelin, and serum insulin concentrations. Blood sampling took place in the morning (7:45-9:00) after an overnight fast, which was defined as consuming no calorie containing products 12 h prior to blood sampling. Before bio-chemical analyses, serum and plasma samples were stored at –20°C for 17-20 months after the first intervention study and at –70°C for 1-2 months after the second and third intervention.

To test whether the storage of samples or time of analyses had affected hormone concentrations, we determined leptin in all samples from intervention 1 both directly after intervention 1 and after intervention 3. We observed a decrease of 0.4 ± 0.2 ng/mL in leptin ($p<0.01$). Because it is possible that these lower concentrations were due to variation in the bio-chemical analyses, we chose to include the results of the samples that were stored for a longer time, but were analysed in one run together with the other samples. For insulin and ghrelin only one determination was available; the samples determined directly after intervention 1.

Bio-chemical analyses

Serum leptin was assessed in duplicate by radio immunoassay (Linco Research Inc., St. Charles Missouri, USA), with a detection limit of 0.5 ng/mL. The intra-assay coefficient of variation was 8%, the inter-assay coefficient of variation 3-4%. Serum ghrelin was assessed in duplicate by radio-immunoassay (Linco Research Inc., St. Charles Missouri, USA), with a detection limit of 0.1 ng/mL. The intra-assay coefficient of variation was 4%; the inter-assay coefficient of variation was 4-11%. Serum insulin was measured by immunoassay (Immulite® 2000 analyser), with an analytical sensitivity of 2.0 U/mL. The intra-assay coefficient of variation was 3.3-5.5% the inter-assay coefficient of variation 4.1-7.3%. All samples of each subject were analysed in one run and in duplicate. To minimize variation in the analyses, means of the duplicates were used for data-analyses.

Descriptive measures

Height was measured to the nearest 0.5 cm, using a wall-mounted stadiometer. Weight was determined to the nearest 0.5 kg on an analogue scale with subjects wearing light clothes, without shoes and with empty pockets (Seca, Germany). The initial purpose of intervention 1 was to evaluate weight loss after 4 days of energy restriction, therefore weight was not measured on day 2. For the current study, we estimated weight lost at day 2 by averaging weight at day 0 with weight at day 4, hereby assuming that weight loss was linear over time. Body Mass Index (BMI) was calculated by weight (kg) divided by height (m) squared. Waist circumference was measured midway between the lower rib margin and the iliac crest, accurate to 0.5 cm. Hip circumference was measured at the point yielding the maximum circumference over the buttocks with the tape held horizontal, to 0.5 cm accurate. Waist-hip circumference was calculated as the waist circumference divided by the hip circumference (60). Body conductivity was determined by hand-to-foot bioelectrical impedance at 100 kHz (Xitron 4000). Fat mass and percentage fat mass were calculated by using the equation of Deurenberg (61). All anthropometric measurements were performed in fasting state after voiding. Additionally, an indication of the energy expended during intervention 2 and 3 was obtained by means of a diary. Physical activities where categorized per 15 minutes, subjects were instructed to fill the diary in retrospect during eating moments. Lists of energy costs per activity were used to estimate the energy expended (62).

Statistical analyses

Changes in subject characteristics during follow-up were tested by means of paired t-tests between subsequent interventions (intervention 1 *vs.* intervention 2, and intervention 2 *vs.* intervention 3). As a result of non-normal distribution, fasting levels of leptin, insulin, and ghrelin were log-transformed (ln) before testing; these variables are shown as geometric mean and 95%-Confidence Intervals (95%CI). Normally distributed variables are shown as arithmetic mean and standard error (± se). Pearson product-moment correlation coefficients (r) were calculated between baseline variables and hormonal responses.

For calculations on short-term variability, the hormonal responses to intervention 2 (int_2) and the hormonal responses to intervention 3 (int_3) were used. For calculations on long-term variability, the hormonal responses to intervention 1 (int_1) and the averaged responses during intervention 2 and 3 ($int_{2,3}$) were used. We used averaged responses in order to get a more precise estimate of the response after the 1½-year follow up. Paired differences between interventions were calculated by: int_3- int_2, and: $int_{2,3}$-int_1. The intra-individual variation (SD_{intra}) and inter-individual variation (SD_{inter}) in the hormonal responses were calculated by using the VARCOMP procedure of the SAS statistical package (v 8.0). A time-factor in the model was used to correct for the observed decrease in leptin during storage of the samples of intervention 1. This time-factor, however, did not affect the variance components; therefore, we decided not to show the results of these additional analyses. The intra-class correlation coefficient (ICC) was then calculated by the following equation:

$$ICC = \frac{SD^2_{inter}}{SD^2_{inter} + SD^2_{intra}}$$

The maximum value of the ICC is 1 and the minimum value is 0. The closer the ICC is to 1, the higher the reliability.

RESULTS

At the first intervention, subjects were 43.2 ± 4.3 y old. In Table 2.1, subject characteristics are given per intervention study. There was a decrease in fasting insulin after the 1½-year follow up ($p<0.05$). No statistically significant differences were observed in serum leptin concentrations nor in body weight. After an additional three-week follow-up, subjects had a small (<1 kg), but statistically significant, higher fat mass ($p<0.05$). During all three interventions a similar amount of energy was consumed. However, the energy expended during intervention 3 was on average 0.8 MJ/d lower than during intervention 2 ($p<0.01$). Pre-intervention 1, BMI was positively associated with leptin levels ($r=0.74$; $p<0.001$) and insulin levels ($r=0.47$; $p<0.05$), and was negatively associated with ghrelin levels ($r=-0.44$; $p<0.05$).

Table 2.1 Subject characteristics of the 20 men participating 3 times in a 2 day 65%-energy restriction intervention.

	Intervention 1 Baseline	Intervention 2 Follow-up 1½ year	Intervention 3 Follow-up 1½y + 3 weeks
Anthropometrical measures			
Weight (kg)	89.9 ± 2.4	89.5 ± 2.7	89.7 ± 2.8
BMI (kg/m^2)	27.8 ± 0.9	27.7 ± 1.0	27.8 ± 1.0
Waist circumference (m)	0.98 ± 0.02	0.99 ± 0.02	n.a.
Fat mass (%)	n.a.	28 ± 1*	29 ± 1*
Biochemical measures			
Leptin (ng/mL)	5.3 [4.2; 6.9]	5.4 [4.1; 7.2]	5.5 [4.2; 7.4]
Insulin (µU/mL)	9.2 [6.9; 12.3]*	6.5 [4.5; 9.2]*	7.4 [5.5; 9.9]
Ghrelin (ng/mL)	1.35 [1.13; 1.64]	1.50 [1.26; 1.78]	1.49 [1.25; 1.76]
Energy balance			
Energy consumed (MJ/d)	4.9 ± 0.2	5.0 ± 0.1	4.9 ± 0.2
Energy expended (MJ/d)	n.a.	14.5 ± 0.6†	13.7 ± 0.6†

n.a. = not available

Biochemical parameters are expressed as geometric mean [95%CI], other variables are expressed as arithmetic mean ± se

* Columns are statistically significant different, $p<0.05$

† Columns are statistically significant different, $p<0.01$

All three interventions showed similar and significant mean declines in body weight ($p<0.001$). No statistically significant changes in insulin were observed during the first ($p=0.14$) and second ($p=0.19$) intervention study; however, the third intervention did show a significant decline in insulin levels ($p<0.01$). All three interventions showed a statistically significant decline in leptin levels and an increase in ghrelin levels (all p-values<0.05) (Table 2.2). We observed no statistically significant differences in mean changes in weight, leptin, ghrelin, or insulin levels between interventions.

Table 2.2 Changes in anthropometrical and biochemical parameters after 2 days of 65%-energy restriction. Twenty men participated 3 times in identical intervention studies.

	Intervention 1 Baseline	Intervention 2 Follow-up 1½ year	Intervention 3 Follow-up 1½ y + 3 weeks
Δ Weight (kg)	-1.2 ± 0.1 (-1.3%) *	-1.4 ± 0.1 (-1.6%) *	-1.7 ± 0.2 (-1.9) *
Δ Leptin (ng/mL)	-0.7 ± 0.3 (-13%) ‡	-1.0 ± 0.2 (-19%) †	-0.9 ± 0.3 (-16%) †
Δ Insulin (μU/mL)	-1.0 ± 0.6 (-11%)	-1.0 ± 0.7 (-15%)	-1.7 ± 0.5 (-23%) ‡
Δ Ghrelin (ng/mL)	0.24 ± 0.06 (+18%) †	0.24 ± 0.04 (+16%) †	0.26 ± 0.06 (+18%) †

Changes are expressed as arithmetic mean ± se (proportional change).

* Statistically significant different from 0, $p<0.001$

† Statistically significant different from 0, $p<0.05$

‡ Statistically significant different from 0, $p<0.01$

In Table 2.3 the intra-individual and inter-individual variation in responses and the intra-class correlation coefficients between responses are presented. For leptin responses, we observed an intra-class correlation coefficient of 0.66 [95%CI: 0.33; 0.85] on the short-term, and an intra-class correlation of 0.34 [95%CI: 0; 67] on the long-term. Insulin showed lower intra-class correlations; ICC=0.45 [95%CI: 0.03; 0.74] on the short-term and ICC=0.13 [95%CI: 0; 0.53] on the long-term. On the short-term an ICC of 0.34 [95%CI: 0; 0.67] was observed for ghrelin and on the long-term a very small ICC was observed (~0 [95%CI: 0; 0.36]).

Table 2.3. Long-term and short-term reliability in leptin, insulin, and ghrelin responses after a 2 day 65%-energy restriction.

	Short term (3 wk) * (n=20)			Long term (1 ½ y) † (n=20)		
	Leptin (ng/mL)	Insulin (μU/mL)	Ghrelin (ng/mL)	Leptin (ng/mL)	Insulin (μU/mL)	Ghrelin (ng/mL)
Paired difference ‡	-0.12 [-0.58; 0.34]	-0.71 [-2.10; 0.69]	-0.02 [-0.16; 0.11]	0.26 [-0.89; 0.38]	-0.34 [-2.0; 1.3]	0.02 [-0.15; 0.19]
Variance components						
SD_{total}	1.28	2.83	0.58	1.16	2.98	0.23
SD_{intra}	0.75	1.91	0.52	0.95	2.66	0.24
SD_{inter}	1.05	2.10	0.24	0.68	1.35	~0
Intra-class correlation	0.66 [0.33; 0.85]	0.45 [0.03; 0.74]	0.34 [0; 0.67]	0.34 [0; 0.67]	0.13 [0; 0.53]	0 [0; 0.36]

* Data of intervention 2 and data of intervention 3. Paired differences in responses are calculated as: $\Delta int3 - \Delta int2$

† Data of intervention 1 and averaged data of intervention 2 and 3. Paired differences in responses are calculated as: $\left(\frac{\Delta int2 + \Delta int3}{2}\right) - \Delta int1$

‡ Expressed as arithmetic mean [95%CI]

DISCUSSION

Our objective was to assess the reliability of leptin, insulin and ghrelin responses to acute energy restriction on both the short-term (3 wk) and the long-term (1½ y). On the short-term we observed an intra-class correlation of 0.66 in the leptin decrease. However, the insulin decrease and ghrelin increase showed lower reliability; an intra-class correlation of 0.45 and 0.34, respectively. On the long-term, the reliability was lower than on the short-term; an intraclass correlation of 0.34, 0.13 and 0 were observed for the leptin decrease, insulin decrease, ghrelin increase, respectively.

In our study, leptin, ghrelin, and insulin all showed larger intra-individual variation on the long-term than on the short-term. Changes in variables, such as body weight, food intake, macro-nutrient intake and physical activity pattern (63), might be responsible for the variation in these responses. This is also supported in our data; the reliability increased after refraining 4 subjects with large weight changes.

Overall, leptin responses showed less intra-individual variation than insulin, and insulin less than ghrelin. For leptin, this is what we expected; leptin is a fairly stable hormone when measured in fasting state within a subject (64). Next to that, leptin is also involved in longer-term weight maintenance (65). The function of insulin, on the other hand, is not only to maintain energy balance within days (27), but it is, more importantly, necessary for the entry of glucose and amino acids into (muscle) cells. Insulin may therefore change within minutes after physical activity or other catabolic factors and thus insulin is likely to show larger intra-individual variation than leptin.

Although we expected ghrelin levels to be stable, they showed a very high intra-individual variation. Ghrelin is produced by endocrine cells in the gastric mucosa (30). During energy restriction the synthesis of ghrelin is stimulated (66) and gives a starvation signal to the brain via the vagal afferent (67). It has been observed that ghrelin, like leptin, affects the NPY-pathway (68), but ghrelin also influences gastric secretion and motility (69). The exact mechanism behind ghrelin secretion regulation is still unknown. Ghrelin, like insulin, has shown to respond within hours to stimuli, *e.g.* glucose (32) and other carbohydrate loads (33), and shows high diurnal variation in the fasting state compared to insulin and leptin (34). Under conditions of energy balance, it has shown to be strongly inversely associated to appetite (33). It might be that ghrelin

is more important in satiety and satiation, *i.e.* meal initiation or meal termination, than in the conservation of energy stores.

The intraclass correlation we found for the leptin response on the short-term was 0.66. This implies that circa 50% of the variation in response in intervention 2 could be explained by the response in intervention 3. Although, the leptin response shows a somewhat lower ICC than biomarkers that are generally accepted, like cholesterol and blood pressure (ICC: 0.7-0.9) (70, 71), it shows a higher ICC than many items measured by food frequency questionnaires (ICC: 0.3-0.8) (72). We therefore think the reliability of the leptin response on short-term is adequately to be used as a marker.

We found that leptin shows a good reliability, and thus might be a suitable indicator for the individual ability for the restoration of energy balance. Yet, the biological relevance of this response is not clear; is it a trait that affects the susceptibility to diet induced weight gain? Recently, it has been shown that patients with a high initial leptin decline during a low caloric diet respond better to leptin injections than patients with low initial declines; they show larger weight loss (73). Additionally, it as been shown that subjects with a high leptin response after energy restriction perceive higher subjective hunger feelings than subjects with a low response (59). On the other hand, it has also been shown that obese patients with an initial high leptin response were better capable of maintaining stable body weight after weight loss (74).

Although large inter-individual variation exists, its relevance is unknown. It is still uncertain whether the inter-individual variation in leptin responses is large enough to be associated with the large variation in weight gain in the population. Next, one should question which factors might cause this inter-individual variation; it may be caused by genetic determinants (*e.g.* functional polymorphisms in the leptin-pathway), but can also be caused by individually determined environmental factors (*e.g.* physical activity or food intake patterns). Although larger intervention studies might be conducted, it remains difficult to distinguish variation caused by environmental and genetic factors.

In conclusion, the leptin decline after energy restriction showed a relatively high intra-class correlation coefficient on the short-term and is therefore, the most promising biomarker of the hormones studied. Further studies should quantify the biological

relevance of the variation in the leptin response to energy restriction. Preferably, controlled intervention studies study the effect of leptin declines, induced by caloric restriction, on subsequent food intake.

3

Leptin responsiveness to energy restriction: genetic variation in the leptin receptor gene

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Serum leptin concentrations are an important afferent signal in energy balance homeostasis. It has been speculated that the leptin responsiveness to energy restriction is affected by the functionality of the leptin receptor. The purpose of this analysis is to explore the effect of polymorphisms in the leptin receptor gene on the acute decline in leptin after four days of 65%-energy restriction.

Leptin concentrations of the studied group (n=44; all men) declined by 2.3 ± 1.5 µg/L (–39.4% [95%-Confidence Interval: -43.6; -34.9]). Leptin responses did not statistically differ between noncarriers and carriers of three mutant variants of the polymorphisms; Lys109/Lys109: -41.4% *vs.* Arg109/+: -37.0% (p=0.33); Gln223/Gln223: -41.5% *vs.* Arg223/+: -37.8% (p=0.40); Lys656/Lys656:-39.5% *vs.* Asn656/+: -39.3% (p=0.96).

No effect of the assessed polymorphisms in the leptin receptor gene on the acute decline in leptin after energy restriction was observed. Power calculations are provided for future studies on the leptin responsiveness to energy restriction.

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Leptin is a hormone, produced mainly by adipose tissue and binds to receptors in the hypothalamus, resulting in an afferent signal in energy balance homeostasis; it stimulates catabolic processes and suppresses anabolic processes. The concentration of leptin is not only affected by the amount of adipose tissue, but also by the state of energy balance. Short-term overfeeding causes a rise in leptin concentrations (21), and short-term energy restriction causes a fall in leptin concentrations (44). These adjustments in leptin levels are considered important for the restoration of energy balance and, consequently, for body weight regulation (75).

If leptin has an effective role in the restoration of energy balance, it is likely to exert this action through the leptin receptor in the hypothalamus. It has been speculated that this receptor is involved in the feedback loop for the regulation of leptin excretion (37). If so, the leptin responsiveness to energy restriction would depend on the functionality of the leptin receptor.

We explored the effects of three polymorphisms in the leptin receptor gene on the acute decline in leptin after energy restriction. The three polymorphisms studied are potentially functional, they are all situated in the intracellular domain of the receptor, and could, therefore, alter leptin signaling. The polymorphism on codon 109 causes a conservative change, *i.e.* no change in charge (Lysine to Arginine). The polymorphisms on codon 223 and 656 do result in a change in charge, making those two the most likely to be functional.

Although several population-based studies have been conducted, no studies on the functionality have been reported thus far. One of these population-based studies investigated the effects of polymorphisms in the leptin receptor and leptin levels on weight gain (39). In this study, polymorphisms in codon 109 and 223 modified the effect of leptin levels on weight gain. Moreover, we observed a difference in leptin responsiveness between men who gained weight and men who had stable weight (56). These observations are in line with the hypothesis that polymorphisms in the leptin receptor could affect the leptin responsiveness to energy restriction as well.

The hypothesis of the current analysis was that genetic variation in the leptin receptor is associated with the functionality of this receptor and, therefore, influences the leptin

response to energy restriction. The leptin response after energy restriction would, thus, be differential for individuals with different genotypes.

As described in Table 3.1, the leptin concentrations of the total study group (n=44, all men) declined with an average of 2.3 ± 1.5 µg/L (-39.4% [95%CI: -43.6;-34.9]). Differences according to genotypes (carriers *vs.* non-carriers), were small and showed large variation. For the polymorphisms at codons 109, 223 and 656 a non-statistically significant difference in leptin response was observed of 4.4% (p=0.33), 3.7% (p=0.40) and 0.2% (p=0.96), respectively. For the entire group, insulin decreased by 2.3 ± 4.4 µIU/mL (-24.4 % [95%CI: -34.3; -13.0]). No statistically significant differences in insulin decreases were observed between genotypes (data not shown).

Thus, we could not observe statistically significant differences in leptin response among genotypes of the polymorphisms at codons 109, 223 and 656, *i.e.* there were no significant differences between carriers and noncarriers of the mutant allele.

Little is known about the clinical relevance of differences in leptin response. Large differences are not expected, as small differences in leptin responses might already be crucial for weight changes over long-term periods. In previous analyses, we investigated whether leptin responses were associated with body weight gain (56). Although we studied this association with retrospective data, we found that men who had gained weight (> 1 kg/year in 12 years) showed a 7% lower proportional leptin response compared with men who had stable weight in the same period. This implies that the clinically relevant difference in leptin response may be 7%, or even lower. In the current study we found maximum differences of 4.4% between carriers and non-carriers (Table 3.1), along with large standard deviations. Therefore, large numbers of subjects are needed to study the effects of leptin responses on obesity related phenotypes.

With a power of 80%, a fully controlled intervention with minimum 140 subjects (70 in each group) would be needed for testing the current hypotheses. These power calculations are based on the log-transformed proportional leptin response, an estimated difference of 4.4% between two groups (Table 3.1), with a standard deviation of 1.3% (two-sided t-test; α=0.05).

Including large numbers of subjects in a controlled intervention is unrealistic; therefore, it would be useful to have repeated measurements. In this manner, the intra-individual variation of the leptin response can be taken into account. Repeating the measure three times can reduce the number of subjects to around 30 subjects per group (power 80%).

We, therefore, realized that, due to small power, we could not find statistical evidence for the effect of polymorphisms in the leptin receptor gene. However, to be able to conduct further studies with enough statistical power, more information has to be available on the expected differences in leptin responses. Therefore we consider the current analyses as a pilot, and, with these analyses, we provide information for further study.

Table 3.1 Leptin levels and changes in leptin levels (adjusted for BMI) during the intervention according to genotype (carriers vs. non-carriers) of three polymorphisms in the LEPR-gene.

	Geometric mean (95%CI) *		Mean (95%CI) *	Geometric mean (95%CI) *
	Day_0 (µg/L)	Day_4 (µg/L)	Day_4-day_0 (µg/L)	Day_4-day_0 (%)
All subjects (n=44)	5.5 [4.7; 6.4]	3.3 [2.8; 3.9]	-2.3 [-1.9;-2.8]	-39.4 [-43.6;-34.9]
LEPR Lys109Arg †				
Lys109/Lys109 (n=24)	5.7 [4.9; 6.6]	3.3 [2.8; 3.9]	-2.4 [-3.0; -1.8]	-41.4 [-46.8; -35.3]
Arg109/+ (n=20)	5.3 [4.5; 6.2]	3.4 [2.8; 4.1]	-2.2 [-2.8; -1.6]	-37.0 [-43.4; -29.8]
Difference ‡			0.2 (p=0.64)	4.4 (p=0.33)
LEPR Gln223Arg †				
Gln223/Gln223 (n=19)	5.5 [4.6; 6.6]	3.2 [2.6; 3.9]	-2.2 [-2.9; -1.6]	-41.5 [-47.6; -34.7]
Arg223/+ (n=25)	5.5 [4.7; 6.4]	3.4 [2.9; 4.1]	-2.4 [-2.9; -1.8]	-37.8 [-43.4; -31.5]
Difference ‡			0.2 (p=0.72)	3.7 (p=0.40)
LEPR Lys656Asn †				
Lys656/Lys656 (n=26)	5.4 [4.7; 6.3]	3.3 [2.8; 3.9]	-2.3 [-2.9; -1.8]	-39.5 [-44.9; -33.5]
Asn656/+ (n=18)	5.6 [4.7; 6.8]	3.4 [2.8; 4.2]	-2.3 [-2.9; -1.6]	-39.3 [-45.8; 32.0]
Difference ‡			~0.0 (p=0.91)	0.2 (p=0.96)

* CI = confidence interval

† Leptin levels are corrected for BMI or change in BMI by lineair regression (GLM)

‡ Tested by linear regression models (GLM), tested on natural log-transformed variable

On the other hand, if the function of the leptin receptor would be decreased, other hormones and peptides regulating energy homeostasis, *e.g.* insulin, ghrelin or PYY_{3-36}, may take over, as energy homeostasis is controlled by a complex system with many different routes. However, rare cases of patients lacking leptin receptors show that the absence of this receptor results in an extremely obese phenotype (26), which illustrates the importance of this receptor for weight regulation.

In conclusion, no statistically significant associations between polymorphisms in the leptin receptor and leptin responsiveness were observed. However, these results do not exclude a genetic influence on the leptin responsiveness to energy restriction. Further studies should focus on the intra-individual variation of leptin response, and have to take into account that large numbers of subjects are needed to detect clinically relevant differences in leptin responsiveness.

METHODS

We studied the leptin response in 44 apparently healthy men (age: 43 ± 5 y, BMI: 27.3 ± 3.2 kg/m^2, BMI range 22.7-39.8 kg/m^2). Fasting leptin and insulin levels were measured before and after a four-day energy-restricted diet, which consisted of ±35% of the estimated energy needs. On average, subjects consumed 4.9 MJ/day during the intervention (21.7%, 21.5, and 56.8% of energy derived from protein, fat , and carbohydrates, respectively). The selection of the subjects and study design has been described in detail previously (53).

Leptin and insulin levels were measured in serum samples that were taken after a minimum fast of 12 h, and which had been stored at -20°C. Serum leptin was assessed by a radioimmunoassay (LINCO Research, Inc., St. Charles, MO), with an analytical sensitivity of 0.5 µg/L. Intra-assay coefficient of variation was 3 to 8%, and the inter-assay coefficient of variation was 4 to 8%. Serum insulin was measured by immunoassay (Immulite® 2000 analyzer; Pharmacia & Upjohn Diagnostics AB, Upsala, Sweden), with an analytical sensitivity of 2 µIU/mL. Plasma glucose was measured quantitatively by a bichromatic endpoint assay (Glu FlexTM Reagent; Dade Behring BV, Leusden, The Netherlands). All samples of one subject were analysed in

one run. All measurements were performed in duplicate. The means of the two measurements were used for data-analyses.

Three polymorphisms in the leptin receptor gene were genotyped from stored white blood cells: Lys109Arg, Gln223Arg, and Lys656Asn. Genotyping was performed by using polymerase chain reaction restriction fragment length polymorphism analyses (15).

Subjects were categorized according to genotype, *i.e.* either having one or two mutant alleles (carrier) or not having the mutant allele (non-carrier), for each polymorphism separately. General Linear Models (GLM), containing leptin as the dependent variable, genotype as categorical variable, and BMI as the covariable, were used to test differences in leptin response among genotypes. Because of nonnormality leptin, levels and proportional changes in leptin and insulin were log-transformed (natural logarithm) before testing. Consequently geometric means and 95%-Confidence Intervals (95%CI's) are presented for these variables. Arrhythmic means, standard deviations or 95%CI's are presented for all other variables. A level of $p<0.05$ was considered statistically significant.

4

Fasting leptin and appetite responses induced by a four-day 65%-energy restricted diet

Monica Mars, Cees de Graaf, Lisette de Groot, Caroline van Rossum, Frans Kok

Objective: Animal studies show that the leptin decline after acute severe caloric restriction is a peripheral signal to increase food intake. However, most human studies have failed to observe such a relationship. We studied the acute effects of severe caloric restriction, on the association between serum leptin concentrations and subjective appetite.

Research methods and procedures: Before, after a 2-day and after a 4-day diet, consisting of 35% of the estimated energy needs, fasting serum leptin concentrations and self perceived appetite levels were measured in 44 healthy adult men (aged: 43 ± 5 y; BMI: 27.3 ± 3.2 kg/m^2). Appetite levels were assessed with a 10 point-Likert scale, reflecting: hunger, fullness, desire to eat, prospective consumption, and total appetite.

Results: After the four-day energy deficit, fasting leptin concentrations decreased with 39.4% (95%CI: -43.6; -34.9%). This decline was associated with an increase in fasting hunger ($r=-0.42$; $p<0.01$), desire to eat ($r=-0.39$; $p<0.05$), and total appetite ($r=-0.38$; $p<0.05$). Furthermore, the association between fasting leptin concentrations and fasting appetite levels became stronger during the energy restriction period (for total appetite: day 0 $r=-0.15$; $p=0.13$ | day 2 $r=-0.31$; $p=<0.05$ | day 4 $r=-0.41$; $p<0.01$).

Conclusions: The acute proportional reduction in fasting leptin after 4-days energy restriction is associated with an increase in self-perceived appetite. Additionally, the inverse association between proportional fasting leptin concentrations and self perceived appetite response becomes stronger as energy restriction is prolonged. These findings suggest that leptin has an instrumental role in restoring energy balance in humans through the expression of appetite.

Submitted for publication

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INTRODUCTION

Leptin is a hormone that is produced by fat cells and secreted into the blood. From animal studies it is known that leptin has an inhibiting effect on food intake. Ob/ob mice, transgenic mice that lack the gene to produce leptin, are leptin deficient and extremely obese (14). Also in humans some cases of extreme obesity are known, which are caused by leptin deficiency, as a result of rare mutations in the leptin gene (25). Injecting these humans or ob/ob mice with leptin, reduces their food intake and brings them back to lower body weight (76-78).

Serum leptin concentrations are positively correlated with the amount of fat mass in the body (16). However, during short-term severe energy restriction serum leptin concentrations are rapidly falling, to a larger extent than would be expected from loss of fat mass alone (19, 21, 79). Therefore, leptin is thought to be instrumental for the restoration of negative energy balances (36, 80). This acute reduction in leptin might be the result of reductions in insulin concentrations that occur during energy restriction (81).

Leptin may regulate energy balance by affecting energy intake, *i.e.* appetite and food intake (36). Up till now, several studies have investigated the association between leptin levels and appetite or food intake in humans, either during conditions of energy balance (40, 82-87) or energy imbalance (41, 42, 88). Especially, the latter studies found evidence for the hypothesis that leptin is involved in the restoration of negative energy balances via appetite. Westerterp-Plantenga and colleagues found a decrease in appetite ratings by administration of exogenous leptin during energy restriction (41). Keim and colleagues found a strong inverse association between leptin concentrations and appetite ratings during energy restriction, independent of weight loss (42). However, both studies were based on several weeks of energy restriction, and did not study the effects of severe energy restriction that take place within days.

Thus, it is generally assumed that leptin is instrumental in restoring energy balance via appetite or food intake. However, if leptin has an important role in energy balance via appetite, leptin may affect appetite after short-term changes in energy balance. Therefore, the objective of our study was to assess the association between leptin and appetite responses induced by an acute, *i.e.* four-day, energy restriction.

RESEARCH METHODS AND PROCEDURES

Subjects

Forty-four adult men were recruited from the Doetinchem Cohort Study (56, 89). Subjects were eligible if they did not use any drugs known to affect energy metabolism, and if they were not on a prescribed or weight loss diet during the previous two months. Persons with a history of diabetes mellitus, diseases affecting the thyroid gland, liver or the gastrointestinal tract were excluded as well as those with glucosuria, anemia or fasting plasma glucose concentrations >6.9 mmol/L (28).

All measurements took place at the Municipal Health Service Center in Doetinchem (The Netherlands). All study participants gave their written informed consent. The study protocol was approved by the Ethics Committee of Wageningen University.

Experimental procedure

Before intervention, subjects came in fasting state (minimum fast of 12 hr) to the research center between 8 and 9 a.m. Fasting was defined as not consuming any food or calorie containing drinks 12 hours prior to the visit. An oral glucose tolerance test (OGTT) was performed as described earlier (56). During all 7 blood samples subjects rated their subjective appetite.

After two days of intervention, subjects came, again between 8 and 9 p.m. in fasting state (minimum fast of 12 h) to the research center. One blood sample was taken and subjective appetite was rated. After four days of intervention, the procedure of the first study day was repeated.

Caloric restriction

The energy content of the diet was calculated by taking 33.3% of the individual energy needs and rounding this up in units of 0.8 MJ. Details on the calculated individual energy needs are described previously (56). Sixteen subjects received a 4.2 MJ, 23 received a 5.0 MJ, four received a 5.9 MJ and one subject received a 6.7 MJ diet. Macronutrients were set at protein 26 energy percent, fat 19 energy percent, and carbohydrates 55 energy percent for each diet. Calculations beforehand showed that

the micronutrient and mineral content of the supplied diet met the Dutch recommended dietary allowance (90).

The diet consisted of micronutrient-dense meal and snack replacements, *e.g.* shake mixes, muesli bars, and soups (Nutricia, Zoetermeer, The Netherlands). The products were supplied at the beginning of the study, subjects were instructed to consume these products according to a fixed schedule; subjects were allowed to shift products within a day, but not between days. Compliance was measured by pre-printed daily food records; in which subjects wrote down the time of consumption of each product. Subjects were advised to only use the provided food products, but if necessary, additional foods were written down in full detail. Subjects were allowed to use non-nutritive beverages *ad libitum*. At the end of the study, food intake during the four days of intervention was calculated by means of the daily food records and chemical analyses of the meal and snack replacements. Subjects consumed on average 35% (range 24-40%) of their estimated energy needs, *i.e.* 4.9 ± 0.7 MJ/d (21.7 % protein, 21.5 % fat, 56.8 % carbohydrates as percent of total energy consumed).

Measurements

Before the experiment, restraint eating behavior was assessed by means of the Dutch Eating Behavior Questionnaire (DEBQ) (91). The score derived from this questionnaire is an indicator for cognitive awareness of food intake.

Height was measured to the nearest 0.5 cm, using a wall-mounted stadiometer. Weight was determined to the nearest 0.5 kg on an analogue scale with subjects wearing light clothes, without shoes and with empty pockets. Body Mass Index (BMI) was calculated by weight (kg) divided by height (m) squared.

Appetite was measured by means of 10 point-Likert scales, including hunger, fullness, desire to eat and prospective consumption, as described by Hill *et al.* (92). Second, a score for total appetite was calculated by adding up the scores of hunger, desire to eat, and prospective consumption, and subtracting the score of fullness. Additionally, a score for appetite after the glucose load was calculated. The area under the curve as percentage of the total area was calculated, as described previously (93). A high score on hunger, desire to eat, prospective consumption and total appetite represents high appetite. For fullness, low scores represent high appetite.

Blood samples were centrifuged at 2500 r/pm for 10 minutes, before divided into aliquots. Aliquots were stored at -20 °C before laboratory analyses. Serum leptin was assessed by a radioimmunoassay (LINCO Research, Inc.), with an analytical sensitivity of 0.5 ng/mL. Intra-assay coefficient of variation was 3-8%, the inter-assay coefficient of variation was 4-8%. Serum insulin was measured by immunoassay (Immulite® 2000 analyzer), with an analytical sensitivity of 2 mIU/mL . Intra-assay coefficient of variation was 3.3-5.5%, the inter-assay coefficient of variation was 4.1-7.3%. Plasma glucose was measured quantitatively by a bichromatic endpoint assay (Glu FlexTM reagent). All samples of each subject were analysed in one run and in duplicate. Means of the duplicates were used for data-analyses. Incremental areas under the curve (AUC's) were calculated for glucose and insulin during the OGTT, in order to assess the glucose tolerance and insulin sensitivity.

Statistical Analyses

Due to non-normality, geometric means and 95%-Confidence Intervals (95%CI) are presented for the appetite parameters, leptin and insulin levels. Also the proportional changes in leptin and insulin after intervention are presented as geometric means.

Absolute changes after intervention were normally distributed and therefore presented as arithmetic means (95%CI). Because only the acute leptin changes, which are independent of changes in body weight loss, were of interest, adjustments for changes in BMI were made with general linear models (GLM).

Pearson correlation coefficients (r_p) were calculated between changes in appetite and normally distributed variables, *i.e.* absolute changes in leptin, insulin and glucose. The non-parametric Spearman correlation coefficient (r_s) was calculated for variables that were not normally distributed, *i.e.* proportional changes in leptin, insulin and glucose. Since only the acute leptin changes, which are independent of body weight loss, were of interest, adjustments for changes in weight were calculated by means of partial correlation (R). Additionally, Pearson correlation coefficients (r_p) were calculated between appetite parameters and leptin concentrations for each intervention day.

The power, *i.e.* 1 minus the chance of type II error, of the present analyses was sufficient (80%) to detect a correlation coefficient of 0.41 (tested two-sided, with a p-value of 0.05 considered as statistically significant).

In the analyses, p-values smaller than 0.05 were considered statistically significant. All statistical tests were performed with the SAS package (v8.0).

RESULTS

General characteristics of the 44 men who completed the study are shown in Table 4.1. Subjects were on average 44 ± 5 years old, and had an average BMI of 27.3 ± 3.2 kg/m^2. At baseline, BMI was highly correlated to fasting leptin levels (r_p=0.72; p<0.0001) and fasting insulin levels (r_p=0.49; p<0.001). There was no association between BMI and any of the appetite parameters (data not shown). During the intervention subjects had lost on average 2.4 kg (range: 0.5-3.5 kg). Body weight loss was positively associated with baseline body weight (r_s=0.34; p=0.02).

Table 4.1. Characteristics of the subjects participating in the intervention study, consisting of a 64% energy restriction of 4-days (n=44).

	Mean	SD	Range
General			
Age (y)	43	5	31-52
Height (cm)	181	7	167-196
Baseline weight (kg)	89.5	10.3	71.5-118.4
Baseline BMI (kg/m^2)	27.3	3.2	22.7-39.8
Energy balance			
BMR (MJ/day) *	8.0	0.5	7.2-9.4
PAL †	1.7	0.2	1.4-2.2
Estimated energy needs (MJ/d) ‡	13.6	1.6	10.8-19.9
Energy intake during intervention (MJ/d) §	4.9	0.7	3.0-6.9
Energy deficit during intervention(%) **	63.9	2.6	60.0-75.7
Weight loss after intervention (kg)	2.4	0.7	0.8-4.4
Restrained eating score ††	2.5	0.6	1.0-4.0

* BMR = Basal Metabolic Rate at baseline, estimated by equation of Schofield (95)

† PAL = Physical Activity Level, estimated by questionnaire (100)

‡ Calculated by PAL x BMR

§ Calculated by dietary records

** Calculated by energy intake as percentage of estimated energy needs

†† Assessed with Dutch Eating Behaviour Questionnaire (92): 1=not restraint at all, 5=extremely restraint

Serum leptin concentrations were steadily decreasing during the intervention (Table 4.2). Insulin and glucose concentrations declined during the first two days, and did not decline further during the next two days. There were small increases in glucose tolerance and insulin sensitivity during the four-day intervention.

Fasting appetite levels increased between t_0 and t_2, and did not change during the subsequent two days (Table 4.3). Furthermore, levels of appetite during the glucose tolerance test, in all dimensions except fullness, increased significantly after four days of intervention.

The proportional changes in leptin concentrations after four days of intervention were associated with changes in hunger (r_s=-0.42; $p<0.01$), desire to eat (r_s=-0.33; $p<0.05$) and total appetite (r_s=-0.33; $p<0.05$) (Table 4.4). Adjusting for changes in BMI during the intervention slightly increased the correlations of desire to eat (R=-0.39; $p<0.05$) and total appetite (R=-0.38; $p<0.05$). No associations were observed between the absolute changes in leptin concentrations and the appetite parameters after two days of intervention (data not shown). For absolute changes in insulin and glucose, no statistical significant correlations were observed with the changes in appetite parameters, respectively for total appetite: r_s=0.01 (ns) and r_s=-0.14 (ns) (Table 4.4).

Table 4.2 Fasting levels on day 0, 2, and 4, and responses in physiological parameters during a 64% energy restriction of 4-days in adult men (n=44).

	Before intervention (t_0) *	After 2 days of intervention (t_2) *	After 4 days of intervention (t_4) *	Response		
				$t_2 - t_0$ †	$t_4 - t_0$ †	$t_4 - t_0$ (%)*
Fasting						
Leptin (ng/mL)	5.5 (4.7; 6.4)	4.3 (3.6; 5.0)	3.3 (2.8; 3.9)	-1.3 (-1.7; -0.8)	-2.3 (-1.7; -0.8)	-39.4 (-43.6; -34.9)
Insulin (μIU/mL)	9.2 (7.8; 10.9)	7.0 (5.8; 8.9)	7.1 (5.8; 8.6)	-2.4 (-3.5; -1.3)	-2.4 (-3.5; -1.3)	-24.4 (-32.6; -14.9)
Glucose (mmol/L)	5.8 (5.6; 6.0)	5.4 (5.2; 6.7)	5.4 (5.2; 5.5)	-0.4 (-0.5; -0.3)	-0.5 (-0.6; -0.3)	-7.8 (-10.1; -5.5)
AUC ‡						
Insulin §	3.9 (3.1; 5.0)	-	3.7 (3.0; 4.5)	-	0.3 (0.1; 0.7)	-3.5 (-13.9; 8.2)
Glucose **	1.5 (1.2; 1.8)	-	1.7 (1.5; 2.1)	-	-0.6 (-1.3; 0.1)	21.6 (-0.02; 48.1)

* Fasting levels (t_0, t_2, t_4) and proportional changes [t_4-t_0(%)] are expressed as geometric mean (95%CI)

† Absolute changes (t_2-t_0, t_4-t_0) are expressed as arithmetic mean (95%CI)

‡ Area Under the Curve, after 75g glucose load

§ μIU/mL*120min*10^3

** mmol/L*120min*10^2

Table 4.3 Fasting levels on day 0, 2, and 4, and responses in appetite parameters during a 64% energy restriction of 4-days in adult men (n=44).

	Before intervention (t_0) *	After 2 days of intervention (t_2) *	After 4 days of intervention (t_4) *	Response $t_2 - t_0$ †	Response $t_4 - t_0$ †
Fasting ‡					
Hunger	2.8 (2.3; 3.4)	4.1 (3.3; 5.0)	4.1 (3.4; 4.9)	1.5 (0.9; 2.1)	1.3 (0.8; 1.9)
Fullness	3.7 (3.2; 4.2)	3.2 (2.8; 3.8)	3.4 (2.9; 3.9)	-0.5 (-0.9; 0.0)	-0.3 (-0.8; 0.2)
Desire to eat	3.7 (3.2; 4.4)	4.8 (4.0; 5.7)	5.0 (4.3; 5.8)	1.3 (0.6; 1.9)	1.2 (0.5; 1.9)
Prospective consumption	4.0 (3.4; 4.7)	4.9 (4.2; 5.6)	4.7 (4.0; 5.4)	0.8 (0.3; 1.4)	0.7 (0.2; 1.2)
Total appetite §	7.8 (6.5; 9.3)	11.0 (9.1; 13.3)	9.6 (7.8; 11.9)	4.0 (2.2; 5.7)	3.6 (1.9; 5.3)
After 75g glucose load (%) **					
Hunger	43 (37; 49)	-	51 (46; 57)	-	8 (3; 13)
Fullness	48 (43; 53)	-	46 (41; 51)	-	-2 (-6; 11)
Desire to eat	48 (43; 54)	-	57 (52; 63)	-	9 (4; 13)
Prospective consumption	50 (44; 56)	-	55 (49; 61)	-	5 (1; 9)
Total appetite	43 (39; 47)	-	49 (44; 53)	-	6 (2; 10)

* Fasting levels (t_0, t_2, t_4) expressed as geometric mean (95%CI)

† Absolute changes (t2-t0, t4-t0) expressed as arithmetic mean (95%CI)

‡ Fasting appetite was measured by a 10-point scale; *e.g.* 1 is low hunger, 10 is high hunger

§ Individual score calculated by; Total appetite = hunger - fullness + desire to eat + prospective consumption

** Calculated by the area under the curve as percentage of the total area over a period of 2 hours after a 75g glucose load, dissolved in 200 ml tea. This method has been in full detail by Hulshof and others (94)

Table 4.4 Association between absolute and proportional responses in leptin, and changes in appetite parameters, during a 64% energy restriction of 4-days in adult men (n=44).

	Pearson correlation coefficient (p-value) Absolute response *		Spearman correlation coefficient (p-value) Proportional response †	
Leptin response				
Δ Hunger	0.18	(0.25)	-0.42	(0.005)
Δ Fullness	0.05	(0.76)	0.19	(0.21)
Δ Desire to eat	0.09	(0.57)	-0.33	(0.03)
Δ Prospective consumption	0.13	(0.42)	-0.17	(0.29)
Δ Total appetite	0.13	(0.41)	-0.33	(0.03)
Leptin response adjusted for change in BMI				
Δ Hunger	0.09	(0.55)	-0.42	(0.006)
Δ Fullness	0.09	(0.56)	0.20	(0.19)
Δ Desire to eat	0.00	(0.98)	-0.39	(0.01)
Δ Prospective consumption	0.06	(0.69)	-0.22	(0.17)
Δ Total appetite	0.05	(0.75)	-0.38	(0.01)
Insulin response				
Δ Total appetite	0.07	(0.69)	0.01	(0.94)
Glucose response				
Δ Total appetite	-0.14	(0.39)	-0.14	(0.37)

* Calculated by fasting leptin on day 4 minus fasting leptin level on day 0

† Calculated by the difference in leptin on day 4 and day 0 as a percentage of leptin level on day 0

Correlations between fasting appetite and fasting leptin became stronger during continuation of the energy restriction (Table 4.5). While no significant relation with leptin is observed at baseline of the study, *i.e.* for total appetite r_p=–0.15 (ns), after two days this association becomes significant, *i.e.* r_p=-0.31 ($p<0.05$), and after four days it increases to r_p=-0.41 ($p<0.01$).

The correlation between insulin and leptin concentrations increased with prolonged caloric restriction; r_s=0.35 ($p<0.05$) at t_0, r_s=0.63 ($p<0.0001$) at t_2, and r_s=0.71 ($P<0.0001$) at t_4. At baseline the correlation between insulin concentrations and total appetite was r_p=-0.29 ($p=0.06$), after two days this increased to r_p=-0.41 ($p<0.01$), while after four days this decreased to r_p=-0.19 (ns). For glucose, increasing associations were found; at baseline the correlation between glucose and total appetite was r_p=-0.29 ($p=0.06$), after two days this increased to r_p=-0.33 ($p<0.05$), and after four days to r_p=-0.39 ($p<0.01$).

Table 4.5 Association between leptin and appetite parameters during a 64% energy restriction in adult men, i.e. at baseline, after 2 days and after 4 days of energy restriction (n=44).

	Spearman correlation coefficients (p-values)					
	Day 0		Day 2		Day 4	
Leptin						
Hunger	-0.13	(0.42)	-0.25	(0.10)	-0.44	(<0.01)
Fullness	0.04	(0.79)	0.11	(0.49)	0.19	(0.22)
Desire to eat	0.05	(0.76)	-0.25	(0.10)	-0.27	(0.08)
Prospective consumption	-0.21	(0.16)	-0.31	(<0.05)	-0.26	(0.09)
Total appetite	-0.15	(0.32)	-0.31	(<0.05)	-0.41	(<0.01)
Leptin adjusted for BMI *						
Hunger	-0.10	(0.54)	-		-0.22	(0.16)
Fullness	0.15	(0.35)	-		0.15	(0.36)
Desire to eat	0.02	(0.91)	-		-0.23	(0.15)
Prospective consumption	-0.10	(0.51)	-		-0.10	(0.52)
Total appetite	-0.13	(0.41)	-		-0.24	(0.13)
Insulin						
Total appetite	-0.29	(0.06)	-0.41	(<0.01)	-0.19	(0.24)
Glucose						
Total appetite	-0.29	(0.06)	-0.33	(<0.05)	-0.39	(<0.01)

* Adjusted for BMI by partial correlation

DISCUSSION

The present study is the first study, which investigated the association between leptin and appetite responses induced by an acute energy restriction. We observed that the proportional decrease in leptin during the energy restriction was associated with an increase in appetite. Additionally, we observed that the association between fasting leptin and appetite became more pronounced during the energy restriction.

The aim of our intervention was to obtain a decrease in leptin, which was not caused by a reduction in body weight. From the studies of Dubuc *et al.* and Kolaczynski *et al.*, we knew that the acute decline had to take place within 36 hours when fasting and within 7 days when eating 35% of the estimated energy needs (19;21). From our data, we can conclude that a four-day diet intervention containing 35% of the estimated energy needs is sufficient to induce a decrease in leptin of about 40%, which is independent of change in body weight.

After two days of energy restriction, our subjects came in fasting state to the research center. One may speculate that they were glycogen depleted and they had lowered

insulin levels (Table 4.2), which induced the oxidation of fatty acids. This metabolic stress situation most likely caused the decrease in leptin levels. However, Kolazcynski *et al.*, found an association between changes in leptin levels and beta-OH-butyrate levels after a 36-h fast, but could not confirm a causal relation (21). Also no association was found with the decrease of leptin and insulin in that study, although they proved that glucose-infusions prevented leptin levels to drop (21). From *in vitro* studies, it has been observed that refraining fat cells from insulin treatment decreased their leptin secretion (81). In our study, we observed an increasing correlation of insulin with leptin concentrations, which is in line with the hypothesis that leptin might be excreted from the adipose tissue under control of insulin.

Before energy restriction, we observed a small correlation of insulin levels with appetite (r_s=-0.29, P=0.06), which increased after two days (r_s=-0.41; p<0.01), and disappeared after 4 days of energy restriction (r_s=-0.19; ns). Simultaneously, the association between leptin levels and appetite became stronger during continuation of the energy restriction, t_0: r_s=-0.15 (ns), t_2: r_s=-0.31 (p<0.05), t_4 r_s=0.41 (p<0.01). Based on these observations one may speculate that during the first two days of energy restriction, appetite is mainly driven by changes in insulin (and possibly fatty acid oxidation) together with leptin, and that after 4 days of intervention leptin takes over. However, with our study design it is not possible to make any causal conclusions.

Our findings are in line with other studies that were performed with longer-term energy restrictions. Keim *et al.* observed a Pearson correlation coefficient of 0.7 between changes in leptin and appetite after a moderate energy restriction of 12 weeks in women (aged: 20-40 y, BMI: 22-37 kg/m^2) (42). This association is considerably higher than the correlation found in the present study (total appetite: r=0.38). However, one may question whether our study is comparable to their study. First, they studied appetite parameters measured several times during the day, which may have increased the statistical power (94). However, appetite measured in the fasting state does not have to be representative for appetite during the day (41). Additionally, they measured baseline appetite after one week of energy restriction, while our study only lasted four days. Consequently, the appetite feelings as measured by Keim *et al.* might reflect other motivations reasons to eat than in our study, which makes it difficult to compare. Furthermore, their study took place in a laboratory setting; participants lived in a

metabolic unit at the time of the study, while the subjects in the present study were free-living.

Westerterp-Plantenga and others found lower hunger and appetite levels in obese male subjects receiving pegylated leptin intravenously than in BMI- and age-matched subjects receiving a placebo (41, 88). Like in the present study, they only observed an effect on appetite while measured in fasting state. Moreover, they observed the highest effect of the exogenous leptin after the first week of intervention. This confirms our finding that the acute decrease in leptin, which takes place within days, is important for the increase in fasting appetite during energy restriction. Although a parallel change in leptin levels and appetite is observed in the study of Westerterp-Plantenga, data on an association between leptin levels and appetite are not given. This makes it difficult to compare their study to the present study.

It has been suggested that obesity is associated with a blunted response to severe energy restriction (19). In our analyses it appeared that subjects with a high leptin level (the obese subjects) had a relatively high leptin response. We therefore additionally presented proportional changes in leptin, which are indirectly corrected for baseline BMI. The obese subjects in our study did not show blunted responses to weight gain. Even more, the large range in BMI in our study might have enlarged the variation in leptin response and therefore enlarged the power of our study.

Only men were eligible to participate in our study. It is difficult to study changes in leptin levels and appetite in women as menstrual cycles are suggested to influence both leptin levels (53) and subjective appetite responses (52). It is well known that women have higher leptin levels in energy balance (16), this might imply that they also have lower proportional changes in response to energy restriction. This suggests that lower associations would be found in women than in men. However, it has been suggested by Dubuc and colleagues that the changes in leptin may be the predominating peripheral signal for changes in energy balance in women. On the other hand, in men these predominating signals would be the changes in insulin and cortisol (19). Our results were not in line with this hypothesis.

With this study we wanted to assess the role of the acute leptin decline after energy restriction in the regulation of food intake. For this objective, appetite would be,

preferably, measured by *ad libitum* intake. Because subjects were on an energy-restricted diet, this method was not feasible in our study setting. Therefore we used appetite ratings (92), which have been shown to be good indicators of the motivation to eat and predictors of actual food intake (94-96). We observed that the proportional decline in leptin was associated with an increase in appetite ratings. This suggests that subjects with a relatively high decrease in leptin, would also sense a relatively high motivation to eat. In an setting with *ad libitum* food these subjects may compensate the energy deficit faster than subjects with a smaller leptin response. However, further studies should be conducted before we can confirm these speculations.

Given the high variation in weight gain in the Western World, and weight management after weight loss, one may speculate that the leptin signal to energy imbalance is not adequate to control homeostasis for each individual. This implies that this leptin signal may be individually determined by genetic predisposition. However, no data are available on the inter-individual variation in the leptin response to energy imbalances or on its dose-response relationship, which are needed to confirm such a speculation. Given the high reproducibility of fasting leptin levels during energy balance, leptin levels after caloric restriction are probably also highly reproducible (64). However, reproducibility studies are needed to confirm these speculations.

On the whole, it may be concluded that the acute decline in leptin after 4-days caloric restriction is associated with an increase in appetite. Moreover, the inverse association between leptin levels and appetite response becomes stronger with continuation of energy deprivation. These findings indicate that leptin has an instrumental role in negative energy balances in humans, presumably by affecting the expression of appetite. These results have an important contribution in the understanding of energy homeostasis and its regulation by leptin and appetite.

5

Leptin decline after acute energy restriction and compensation in food intake

Monica Mars, Cees de Graaf, Lisette de Groot, Frans Kok

Background: It is generally accepted that the acute leptin decline after energy restriction is a starvation signal to the brain. Several studies have found an association between declines in leptin and subjective appetite; however, no solid data are available on the acute leptin decline and subsequent caloric compensation.

Objective: Our objective was to assess the effect of the acute leptin response, induced by energy restriction, on subsequent energy intake compensation. We hypothesized that men with a large decline would show larger *ad libitum* energy intake than men with a small decline in leptin.

Design: A total of 35 lean, unrestraint eating, men (aged: 23 ± 3 y, BMI: 22.3 ± 1.6 kg/m^2) participated in a controlled intervention study. Serum leptin concentrations were measured before and after 2 days of 62% energy restriction. Energy intake was measured during the 2 following days on which food was provided *ad libitum.*

Results: During energy restriction, subjects lost 1.1 ± 0.7 kg of body weight, and serum leptin concentrations declined by 24% [95%-Confidence Interval: -33.0; -15.9]. On the first day of *ad libitum* intake, subjects consumed 143 ± 27% of their estimated energy intake (18.3 ± 2.8 MJ). On the second day of *ad libitum* intake, this was 124 ± 20% (16.0 ± 2.5 MJ). No statistically significant correlations were observed between the leptin decline and energy intake during the *ad libitum* intake.

Conclusion: Although, leptin concentrations declined significantly during energy restriction and subjects showed compensatory behaviour during *ad libitum* food intake, we did not observe an association between the magnitude of this leptin decline and subsequent caloric compensation.

Submitted for publication

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BACKGROUND

Leptin is a hormone that is produced by adipose tissue and is secreted in the blood (14). As leptin is produced by fat cells, serum leptin concentrations are positively correlated with the amount of fat mass in the body (16). However, short-term severe energy restriction causes leptin levels to drop to a larger extent than would be expected from loss of fat mass alone (19, 21, 97). In a recent review, Flier advocates that this leptin decline serves as an important signal from fat to brain that the body is starving. Moreover, he suggests that this function is likely to be as important or more important than its role to inform the brain on the amount of fat mass (13).

Although several human intervention studies have shown that declines in leptin are associated with increased appetite feelings (41-43), only one study has shown that acute manipulations in energy balance are inversely associated with energy intake compensation (44). However, this study included only six obese subjects and the analyses included both energy restriction and overfeeding. Therefore, the results of that study have to be interpreted carefully. Thus, no solid data are available on the effect of the acute decline in leptin on energy intake compensation in humans.

For studies measuring energy intake compensation, it is important to select subjects that are lean and unrestraint-eating, because restraint eating, *i.e.* high cognitively awareness of food intake (91), and being overweight may suppress voluntary energy intake after weight loss. Therefore, we performed a study in 35 lean unrestraint-eating men. We induced an acute leptin decline by 2-days of 62% energy restriction and measured *ad libitum* compensatory energy intake during the two following days. We hypothesized that men with a large decline in leptin would show larger *ad libitum* energy intake than men with a small decline in leptin.

SUBJECTS AND METHODS

Subjects

A total of 35 male subjects (aged 18-50 y, BMI 20-30 kg/m^2) were recruited among employees and students of Wageningen University. Only men were recruited because menstrual cycles have shown to affect both leptin levels (53) and appetite (52). Exclusion criteria for participation were: restraint eating (Dutch Eeating Behaviour Questionaire-restraint scale >2.38 (91)); anemia (Hb <8.5 mmol/L, Ht < 0.41 L/L); diabetes or disturbed glucose metabolism (fasting plasma glucose >6.1 mg/L or glucosuria (28)); using medication affecting energy metabolism, weight or food intake; blood donation during the intervention study, stomach or bowel diseases (blood in stool/constipation/diarrhoea). Subject characteristics are described in Table 5.1. Before screening, all participants gave their written informed consent. The Ethics Committee of Wageningen University approved the study protocol.

Table 5.1 Subject characteristics of the 35 male subjects participating in the intervention study.

	Arithmetic mean ± SD	Range
Age (y)	23 ± 3	19-29
Weight (kg)	73.4 ± 6.3	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 ± 1.6	10.4-16.3
Restraint eating score §	1.5 ± 0.4	1.0-2.3

* Estimated Basal Metabolic Rate (95)

† PAL=Physical Activity level (100)

‡ Estimated by BMR x PAL

§ Assessed by the Dutch Eating Behaviour Questionnaire (92)

Energy restriction period

Day 1 and 2 of the dietary intervention were strictly controlled and comprised an energy restriction of two-third of the estimated energy needs. In order to calculate the individual energy needs for each subject, Basal Metabolic Rate (BMR) was estimated by using the equation of Schofield: *BMR (MJ)*= 0.0485 x *weight (kg)* + 3.67 (98). Then, Physical Activity Level (PAL) was estimated by a short retrospective physical activity

questionnaire containing six activities (99). BMR and PAL were used to estimate individual energy needs (E): E= *BMR* x *PAL*. Taking one third of the individual energy needs and rounding this up in energy groups of 0.8 MJ, resulted 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 nutrient dense meal and snack replacements (Profiel, Nutricia, Zoetermeer The Netherlands), each containing 0.8 MJ, which were supplied at the beginning of the study. Compliance was measured by pre-printed daily food records, in which subjects wrote down the time of consumption. Non-caloric beverages (*e.g.* diet coke, black coffee and black tea) were allowed during energy restriction, a list of these products was provided before the intervention. These products were also written down in the daily food record.

Ad libitum food intake

Day 3 and 4 of the intervention were not controlled, *i.e.* subjects were eating *ad libitum*. During these days, subjects ate their breakfast and warm lunch at the research center in a buffet setting. At least 200% of the estimated energy needs were available for each subject, and empty packages and leftovers were used to record food intake. The remaining meals and snacks that were provided and taken home also contained at least 200% of estimated energy needs. Subjects recorded the foods consumed at home in a diary and were asked to take empty packages and leftovers back to the research centre. The diary, leftovers, and empty packages were crosschecked, and portion sizes were verified with dummies of household measures by a trained dietician. We only provided foods that are generally consumed in the Netherlands during the *ad libitum* period (100) (see appendix I). Next to the provided products, subjects were free to use other products, and they were instructed to write these products down in detail in their diary.

In order to prevent habitual intake, foods were provided in unusual portions sizes. For example, bread rolls were smaller than the regular size (20 g instead of 30 g), and we used plates larger than regular dinner plates. Additionally, the breakfast on day 3 consisted of a milk shake, which was offered *ad libitum* in a blinded cup, containing 400 g of milkshake. The macronutrient composition of this milkshake was according to the Dutch national guidelines; 58, 29, 13 per cent of calories was derived from carbohydrates, fat and protein, respectively (90). One cup contained 2.6 MJ, which

reflects the average energy intake in young adult men during breakfast (100). Subjects were instructed to drink until satiation. If necessary, a second and third cup were available. Subjects were not aware of the energy content of this breakfast.

Measures

Energy intake

Food intake during all four days was calculated by crosschecking leftovers and food records. Energy intake and macronutrient composition of the food intake were calculated by use of food composition Tables (8) and product information of manufacturers. To correct for individual differences in energy needs, energy intake proportional to estimated energy needs was calculated per day.

Blood sampling and bio-chemical analyses

Fasting blood samples were taken between 7:30 and 9:30 in the morning. Subjects were not allowed to drink or eat calorie containing products at least 12h prior to blood sampling. 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%. 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 analysed in one run. Means of the duplicates were used for data analyses.

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, in fasting state after voiding.

Statistical Analyses

Because of non-normality, hormonal concentrations were transformed with the natural logarithm (ln) before 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 parameter. Changes in weight and bio-chemical parameters during the intervention were tested by the paired Students' T-tests. Associations were calculated by means of Pearson correlation coefficients (r). Differences between the lower and upper quartile in proportional leptin response were tested by Students' T-tests. P-values <0.05 were considered statistically significant. For all data-analyses we used the statistical package SAS (Release 8.0, SAS Institute Inc., Cary, NC, USA).

RESULTS

At baseline, serum leptin concentrations were associated with body weight (r=0.35; p<0.05), body mass index (r=0.57; p<0.001), and borderline associated with insulin concentrations (r=0.32; p<0.06). Subjects lost on average 1.1 ± 0.7 kg (range:-3.0: 0.3 kg) of body weight after energy restriction, and serum leptin, serum insulin and plasma glucose concentrations declined, by 0.9 [95%CI: -1.4; -0.5] μg/mL, 2.4 [95%CI:-3.2; -1.5] μU/mL and 0.3 [95%CI:-0.4; -0.1] mg/mL, respectively (Table 5.2).

In Figure 5.1, the proportional declines and increases in leptin are depicted per subject; on average leptin declined by 24.6% [95%CI:-33.3;-15.9%]. One subject increased notably in leptin (61.5%), this observation could not be explained by deviations from the protocol, as he lost weight during energy restriction (3.0 kg). Declines in serum leptin concentrations were not associated with changes in body weight (r=0.09; ns) and changes in insulin concentrations (r=0.24; ns). They were highly associated with baseline leptin concentrations (r=0.89; p<0.0001), the proportion of energy restriction (r=0.50; p<0.01), and baseline Body Mass Index (r=0.45; p<0.01). Declines in leptin were borderline associated with baseline body weight (r=0.32; p=0.06). During the energy restriction period, subjects consumed on average 4.9 ± 0.5 MJ (range: 4.4-6.1 MJ), this reflected 38.1 ± 2.7% (range: 32.1-43.1%) of their estimated energy needs (Table 5.3 and Figure 5.2).

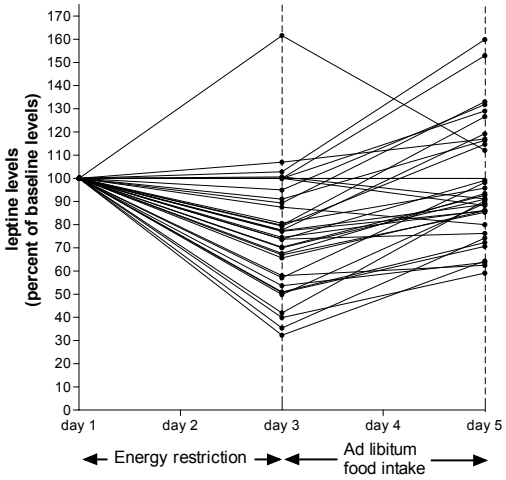

Figure 5.1 Changes in serum leptin concentrations during the 62%-energy restriction and subsequent ad libitum energy intake (n=35).

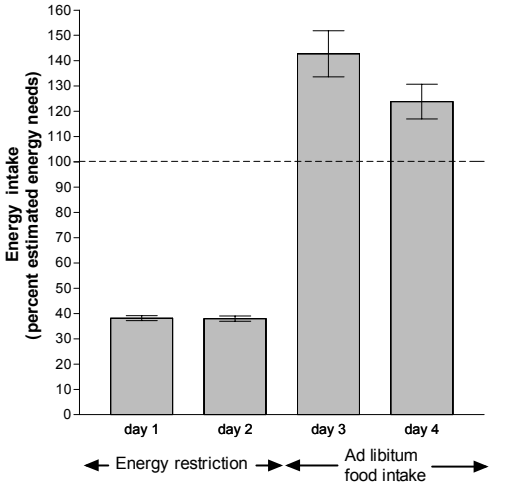

Figure 5.2 Energy intake balance during the 62%-energy restriction and subsequent ad libitum energy intake (n=35).

Table 5.2 Biochemical parameters before intervention, after 2 days of 62%-energy restriction, and after 2 days of *ad libitum* energy intake (n= 35).

	Fasting concentrations*			Δ restricted energy intake†	Δ *ad libitum* energy intake†
	day 1	day 3	day 5		
Glucose (mg/mL)	5.0 [4.9; 5.2]	4.8 [4.7; 4.9]	4.9 [4.8; 5.1]	-5.2 [-7.9; -2.5]	4.1 [1.5; 6.7]
Insulin (µU/mL)	4.5 [3.5; 5.8]	2.8 [2.3; 3.5]	4.7 [3.8; 5.9]	-29.8 [-40.0; -19.7]	84.8 [57.4; 112.1]
Leptin (µg/mL)	2.3 [1.9; 2.8]	1.6 [1.4; 1.9]	2.2 [1.8; 2.6]	-24.6 [-33.3; -15.9]	35.6 [24.4; 46.9]

* Geometric Mean [95%CI]
† Arithmic mean [95%CI]

Table 5.3 Energy, macronutrient, and fibre intake of the 35 men participating in the intervention study (mean ± 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 ± 2.8	16.0 ± 2.5
Carbohydrates (en%)	54.5 ± 0.4	54.2 ± 0.5	52.6 ± 4.0	47.1 ± 4.8
Fat (en%)	19.9 ± 0.5	20.1 ± 0.6	33.7 ± 3.3	36.0 ± 4.7
Protein (en%)	25.3 ± 0.3	25.5 ± 0.3	11.9 ± 1.6	15.7 ± 2.4
Alcohol (en%)	-	-	11.0 ± 22.4	6.2 ± 11.1
Mono- and disaccharides (g)	113 ± 8	116 ± 10	256 ± 64	227 ± 63
Fibre (g/MJ)	6.0 ± 0.1	6.2 ± 0.2	1.8 ± 0.4	2.2 ± 0.4

During breakfast of day 3, 3.7 ± 1.2 MJ (range: 1.7-5.1 MJ) of the milkshake was consumed. In total, 18.3 ± 2.8 MJ (range: 12.6-26.2 MJ) was consumed on day 3, which corresponded with 143 ± 27% (range: 100-217%) of the estimated energy needs. On day 4, 16.0 ± 2.5 MJ (range: 11.0-20.7 MJ) was consumed, which was 124 ± 20% (range: 92-181%) of the estimated energy needs. The energy consumed during day 3 was positively associated with the degree of energy restriction (r=0.36; p<0.05). During the *ad libitum* days, subjects regained 1.2 ± 0.6 kg of body weight. Plasma glucose, serum insulin and plasma leptin concentrations increased significantly with 0.2 [95%CI: 0.1; 0.3] mg/mL, 2.5 [95%CI:1.3; 3.6] µU/mL and 0.6 [95%CI: 0.4; 0.9] µg/mL, respectively (Table 5.2). On average, leptin increased by 35.6% [95%CI: 24.4; 46.9%] (Figure 5.1). These increases in leptin and insulin concentrations were associated with the declines during energy restriction; with r=0.88 (p<0.0001) and r=0.47 (p<0.01), respectively.

Table 5.4 Associations between leptin responses (per cent of baseline concentrations), induced by a 2-day 62%-energy restriction, and following *ad libitum* energy intake (n=35).

	Pearson's r (p-value)	
First ad libitum day		
Energy intake (kJ)	0.17	(0.32)
Energy intake (%)*	0.22	(0.19)
Second ad libitum day		
Energy intake (kJ)	-0.10	(0.58)
Energy intake (%)*	0.005	(0.98)

* Proportional to estimated energy needs

Correlation analyses revealed that the proportional decline in leptin during energy restriction was not associated with the energy intake compensation on day 3 or day 4 (Table 5.4). As we hypothesized that men with a large decline in leptin would show larger *ad libitum* energy intake than men with a small decline in leptin, we also compared the quartile of smallest decrease (n=9; range: -9%; 61.5%) with the quartile of largest increase (n=8; -67.8%; -46.4%). In these sub-sample analyses, we observed that the subjects with the largest decrease had, at baseline, higher leptin levels (4.7 [95%CI: 2.6; 6.8] µg/mL *vs.* 1.7 [95%CI: 0.8; 2.6] µg/mL; p<0.05), and higher insulin levels (7.0 [95%CI: 2.7; 11.3] µU/mL *vs.* 3.2 [95%CI: 1.5; 5.0] µU/mL; p<0.05). Additionally, they had somewhat higher BMI (23.3 ± 1.6 kg/m^2 *vs.* 21.7 ± 1.5 kg/m^2; p=0.05), and restraint eating score (1.8 ± 0.5 *vs.* 1.4 ± 0.3; p=0.06). Proportional to

their energy needs, the subject with the largest decrease consumed less energy during the energy restriction period than subjects with the smallest decrease (day 1: 36.4 ± 2.5% *vs.* 39.1 ± 2.5%; $p<0.05$ day 2: 35.4 ± 3.6% *vs.* 39.0 ± 3.0%; $p<0.05$). Subjects with the largest decrease had a lower food intake during the first day of the *ad libitum* food intake compared to those with the smallest decrease (122.6 ± 13.7% *vs.* 143.8 ± 14.3%; $p<0.01$). The consumption of the milkshake was not different between the two groups (3.4 ± 1.3 MJ *vs.* 3.9 ± 1.3 MJ; ns).

DISCUSSION

Although it is generally accepted that acute leptin declines, induced by severe energy restriction, are signals to the brain to stimulate energy intake, the present study is the first to investigate this in 35 young adult men. In the present study, we observed that leptin concentrations declined significantly and that subjects showed compensatory energy intake. However, we did not observe an association between the magnitude of the leptin decline and energy intake compensation.

As expected, serum leptin concentrations declined after energy restriction, and increased after refeeding. Additionally, other parameters such as body weight, insulin and glucose, also showed the expected pattern: a decrease during energy restriction and an increase during *ad libitum* intake. Moreover, subjects showed compensatory behaviour during the *ad libitum* period, *i.e.* they consumed considerably more energy than their estimated energy needs. Especially the compensatory energy intake during day 3 was apparent; the subjects consumed on average 43% on top of their estimated energy needs. Nevertheless, we did not observe that men with a large decline in leptin showed larger *ad libitum* energy intake than men with a small decline in leptin.

It might be that we did not find an association between the magnitude leptin decline and energy intake compensation because our group of subjects was too homogeneous. As stated in the introduction, restraint eating, *i.e.* high cognitively awareness of food intake (91), and being overweight may suppress voluntary energy intake after weight loss, and therefore affect energy intake compensation. For this reason, we selected a group of lean unrestraint-eating men for our intervention. However, by selecting this homogenous group, we presumably narrowed the variation in the leptin response. This

may have resulted in a small contrast in energy intake compensation between the so-called hyporesponders and hyperresponders, hereby underestimating existing associations.

During the *ad libitum* period, we used two methods to make the subjects aware of their internal cues for appetite and to prevent them from consuming their habitual meals. First, we provided the foods in unusual portion sizes, *i.e.* greater or smaller portions (see appendix I). Second, the breakfast on day 3 consisted of a milkshake of which the energy content was not known to the subjects. It may be discussed, whether these two methods might have introduced extra variation in energy intake. However, we believe that these methods were necessary to make the subjects alert to their internal cues for appetite.

In our study, the energy intake compensation was defined as the proportional energy intake compared to the estimated energy needs. It might have been that the estimation of the energy needs affected this outcome measure. We used the formula of Schofield in combination with a physical activity questionnaire to estimate individual energy needs. The Schofield equation is a simple equation based on sex, age, and weight of the subject (98), and it has been shown that over or underestimation with these type of equations is more likely to occur in obese subjects than in lean subjects, like in our study (101). The physical activity questionnaire that we used included questions on six daily activities (99), of which the number of hours of sleep and hours of working or studying were the most important questions. These two activities are rather constant within a subject. Moreover, the physical activity levels we observed were similar to those found in other studies (mean: 1.8, range; 1.5-2.2) (102). Therefore, we do not think that this estimation affected our results.

In contrast to our hypothesis, the statistical analyses in a sub-sample showed that subjects with the highest response in leptin showed lower food intake than subjects with the smallest response in leptin. The only indirect evidence that is in line with these findings is that of Torgerson and colleagues (74). They found that large declines during the first 16 weeks of a very low calorie diet were associated with large 1-year weight loss. As our subgroup analysis is based on a small group (n=17) of extremes in a homogenous group, these results have to be interpreted with caution.

There is evidence that the low availability of glucose during energy restriction might induce the decline in leptin (103, 104). During energy restriction, glycogen stores are being depleted, insulin levels are lowered, and the body switches over to fatty acid oxidation. Human and in vitro studies have shown that leptin secretion is suppressed by low insulin levels (21, 105). It has therefore been suggested that foods with high glycaemic index during energy restriction may blunt the leptin declines (103, 104), and thus lower the starvation signal to the brain, and eventually prevent weight regain after weight loss. In our study, subjects received on average 113 g and 116 g of mono- and disaccharides on day 1 and day 2, respectively. One may speculate that the leptin response would have been larger if we had lowered the amount of mono- and disaccharides in the energy restricted diet.

It has been shown that leptin has a diurnal pattern with its peak during night time (106-108). Several studies have shown that during energy restriction, not only fasting leptin levels, but also the diurnal variation is decreased (107, 108). Additionally, Weigle and colleagues have observed that a high carbohydrate diet increased the amplitude of leptin (109). This may imply that not only the fasting leptin level might be a signal on glucose availability, but also the change in diurnal pattern. Further studies should also consider this change as an important starvation signal.

Several human studies have observed that a greater decline in leptin is associated with greater appetite feelings (41-43), however, no data are available on actual energy intake. Although it has been shown that appetite feelings are good predictors of energy intake (94-96), it is possible that the relation between the motivation to eat and actual food intake is disturbed by cognitive awareness in a human situation. This may affect food intake even after excluding restraint eaters from participation. The awareness of the subjects being on an energy-restricted diet might have disturbed the association between internal cues, *i.e.* the leptin decline, and subsequent energy intake compensation.

Animal models still provide most of the direct evidence for leptin declines and their role in energy intake compensation after energy restriction (13). It might be that the role of leptin in starvation and energy intake regulation is different in rodents, and cannot be extrapolated to humans. First, leptin deficient rodents and starving rodents show several neuro-endocrine changes (14), which are not observed in leptin deficient

humans (25, 36, 110) or fasting humans (111). These observations stress that there might be important differences in the physiology of starvation between species. Second, the cognitive awareness of humans during energy restriction, which is always present, also makes it very difficult to extrapolate findings from rodents to humans.

Overall, we conclude that the leptin decline induced by a 62%-energy restricted diet of 2 days was not associated with the energy intake compensation during the following 2 days. Although, it is generally accepted that the acute response in leptin after energy restriction is a starvation response, more studies have to be conducted to confirm this assumption. Especially, controlled interventions in obese and non-obese subjects are needed to unravel the function of this starvation response in humans.

Appendix 1. Foods provided during the *ad libitum* period.

Product (portion size)	kJ/100 g
Breakfast and cold dinner	
Test meal (400g)*	641
Bread rolls (natural, with raisins or with muesli) (20g)	1013 or 1073
Margarine (75g)	2972
Cheese (±60g, 3 slices)	1571
Ham (±60g, 3 slices)	540
Boloney (±48g, 6 slices)	1292
Strawberry/cherry jam (50g)	1023
Chocolate spread (50g)	2248
Apple syrup (75g)	961
Sprinkles, chocolate flavored (75g)	1909
Sprinkles, fruit flavored (75g)	1671
Orange juice (300g)	167
Milk (300g)	202
Buttermilk (300g)	137
Warm lunch	
Day 1: Rice with spices, “Nasi” (on request)	405
Ketjap sauce (on request)	718
Scrambled egg (on request)	707
Desert (100g, 150g or 300g)	734
Day 2: Pasta (on request)	281
Chili Sauce (on request)	613
Minced meat (on request)	972
Or Quorn (vegetarians) (on request)	469
Cheese (30g)	1571
Desert (100g, 150g or 300g)	337
Snacks	
Cookie “stroopwafel” (8g)	1787
Cookie “Café noir” (10g)	1882
Gingerbread (17g)	1128
Kiwi-fruit (per piece)	168
Apple (per piece)	207
Orange (per piece)	198
Banana (per piece)	375
Chocolate bar “Twix” (60g)	2040
Chocolate bar “Snickers” (60g)	2128
Crisps, cheese flavored (45g)	2102
Crisps, paprika flavored (45g)	2288
Coffee creamer (2.5g)	2306
Sugar (5g)	1700

* Only provided during breakfast at day 3

6

Leptin and insulin responses to a four day energy deficient diet in men with different weight history

Monica Mars, Cees de Graaf, Caroline van Rossum, Lisette de Groot, Jaap Seidell, Frans Kok

Objective: To assess the leptin responses to a four-day energy restricted diet in men with different weight history; high retrospective weight gain was expected to be associated with a small decline in leptin.

Design: Changes in fasting leptin and insulin were measured during a four-day controlled intervention, in which men with high retrospective weight gain and men who had stable weight consumed 35% of their estimated energy needs.

Subjects: A total of 44 healthy men (age: 31-52 y, BMI: 22.7-39.8 kg/m^2), recruited from a cohort study: 22 men who had gained weight (weight change >1 kg/y) and 22 men whose weight had remained stable (weight change ± 0.3 kg/y) between the first (1987-1991) and the second measurement (1993-1997) of the cohort study. The intervention study was carried out in 2001.

Results: After intervention, changes in fasting leptin levels were similar for both groups of retrospective weight gain; -2.2 µIU/ml (95%ci: -2.8; -1.7) and -2.4 µIU/ml (95%ci: -3.2; -1.7) respectively (p=0.69). Proportional changes in fasting leptin levels were different: -43.3% (95%ci: -47.8; -38.4) in the men whose weight had remained stable (n=22) and -35.2% (95%ci: -42.4; -27.1) in the men who had gained weight (n=22)(p<0.05). Analyses in a subgroup of men (n=18), in which the contrast in weight history was more pronounced than in the total group, did not show this difference. A higher proportional decrease in insulin levels was seen in the men whose weight remained stable than in the men who had gained weight: -35.4% (95%CI: -46.9; -21.3) and –12.8% (95%CI: -28.1; 5.7) respectively. The proportional decrease in leptin was positively associated with the proportional decrease in insulin (r=0.52; p<0.05). The proportional decrease in leptin was positively associated with pre-intervention body weight (r=0.36 p<0.05), BMI (r=0.44; p<0.05) and waist-circumference (r=0.46; p<0.05).

Conclusion: Although we found that the 4-day energy restriction had smaller effect on the decrease in leptin in men with retrospective weight gain, our study does not show convincing evidence that men who gained weight are less leptin responsive to changes in energy balance than men who were weight stable.

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INTRODUCTION

Small-prolonged deviations in energy balance may ultimately lead to significant weight gain. It has become clear that the hormone leptin follows changes in energy balance (55). Short-term energy restriction leads to decreases in leptin levels (19, 21, 44, 112, 113) and short-term overfeeding leads to increases in leptin levels (114). These adjustments in leptin levels are conceived to be instrumental for the restoration of energy balance, and consequently important for body weight regulation (36, 75).

Several studies in general population cohorts have found a positive association between leptin levels and subsequent weight change (16, 39, 45, 46, 48-50). Differential responses in leptin to short-term deviations in energy balance might be also of high importance for weight gain. Individuals who are highly leptin responsive to deviations in energy balance may be better capable of restoring their energy balance, and keep their weight stable for longer periods, whereas individuals who are less responsive, may not be capable to do so and therefore gain weight.

To test this hypothesis, one should measure leptin decreases to an energy deficit and subsequently assess long-term weight gain. Awaiting such prospective data on weight change, we used retrospective data instead, hereby assuming that leptin responsiveness to deviations in energy balance is a fixed characteristic for an individual. A history of long-term weight gain would thus be related to relatively small leptin decreases to an energy deficit.

The primary objective of this study was to assess the leptin responses to a 4-day energy deficit, which contained 35% of the estimated energy needs, in men with different weight history; men who either had gained weight or men whose weight had remained stable. In addition, changes in insulin, glucose and insulin sensitivity were measured in order to study their association with the decrease in leptin.

METHODS

Subjects

Subjects were recruited from the Doetinchem Cohort Study, which was based on a random sample of the general population (89). The first measurements took place in the period 1987-1991 and the second measurements in the period 1993-1997, with an individual follow-up period of 6 y. These measurements included, among others, measurements of weight and height, and a non-fasting blood sample.

We selected and invited two groups of men: a group of 174 men who had gained >1 kg/y between the first and the second measurement, and a control group consisting of 174 men whose weight had been stable during the same period (changed less than 0.3 kg/y). This control group was frequency matched for age and smoking status as described previously (39). Exclusion criteria prior to selection were: being on a medically prescribed diet or weight loss diet, consuming more than five glasses of alcoholic beverages per day, having a history of chronic disease, and changing smoking habits 5 y before the start of the follow-up period or during the follow-up period (starting or quitting).

In 2001, 51 men (out of the 338 selected men) were enrolled in the screening for the intervention. The screening consisted of a medical questionnaire, a short physical activity questionnaire, weight and height measurements, and a urine and blood test. Subjects were eligible if they did not use any drugs known to affect energy metabolism, and were not on a prescribed or weight loss diet during the past 2 months. Persons with a history of diabetes mellitus, diseases affecting the thyroid gland, liver or the gastrointestinal tract were excluded, as well as those with glucosuria, anaemia or fasting plasma glucose levels >7.8 mmol/l (28). Based on these criteria, three subjects were excluded from participation; one because of anaemia, and two because of the use of drugs affecting energy metabolism. One participant withdrew before the intervention and another three withdrew during the intervention due to personal circumstances. A total of 44 subjects completed the study. All measurements took place at the Municipal Health Service Centre in Doetinchem (The Netherlands). All study participants gave

their written informed consent. The study protocol was approved by the Ethical Committee of the Wageningen University.

Intervention design

The design was a controlled parallel intervention study. Weight and biochemical parameters related to the regulation of energy balance were studied during an acute energy deficit. The intervention diet contained 35% of the estimated energy needs for a period of 4 days. Physiological parameters were measured pre-intervention (day 0), after two days (day 2), and after four days (day 4) of intervention. Weight was measured at day 0 and at day 2.

Intervention diet

Individual energy needs (E) were estimated by the following equation: E = BMR x PAL, in which Basal Metabolic Rate (BMR) was estimated by using the equation: BMR (MJ) = 0.0485 x weight (kg) + 3.67 (98). The Physical Activity Level (PAL) was estimated by a short retrospective physical activity questionnaire containing six activities (98, 99). The average PAL was 1.7 ± 0.2 (range 1.4-2.2), which equalled the average PAL found by others for adult men (102). Next, individual energy needs were divided by 3 and rounded up in units of 840 kJ (200 kcal), in order to have an energy restriction of ±67% for each participant. In all subjects received a 4.2 MJ diet, 23 received a 5.0 MJ diet, four received a 5.9 MJ diet and one subject received a 6.7 MJ diet (57 en% carbohydrates; 21 en% fat; 22 en% protein). Calculations beforehand showed that the micronutrient and mineral content of the supplied diet met the Dutch-recommended dietary allowances (90).

The diet consisted of meal replacing products (Nutricia, Zoetermeer, The Netherlands), for example, shake mixes, muesli, muesli-bars, and soups, which were supplied at the start of the intervention. Compliance was measured by daily food records. Subjects were advised to only use the provided food products, if necessary, additional foods were written down in full detail. On average, subjects consumed 35% (range 24-40%) of their estimated daily energy needs, which was similar for men with different weight history.

Measurements

During the present intervention, weight measurements were performed according to an identical protocol that was used at the first and second measurement of the Doetinchem Cohort Study. During the intervention weight was measured on day 0 and day 4. Subjects were weighed with indoor clothing, without shoes and with empty pockets on a balance accurate to 0.1 kg (Seck Bascule MT, USA). Using a wall-mounted stadiometer, height was measured without shoes with the Frankfurt plane horizontal, accurate to 0.5 cm. Body mass index was calculated by weight (kg) divided by height squared (m^2). Waist circumference was measured midway between the lower rib margin and the iliac crest, accurate to 0.5 cm. Hip circumference was measured at the point yielding the maximum circumference over the buttocks with the tape held horizontal, to 0.5 cm accurate. Waist-hip circumference was calculated as the waist circumference divided by the hip circumference (60).

The blood samples at the first and second measurement of the Doetinchem cohort study were taken in non-fasting state. Plasma was stored at max -20 °C for 6-14 years before laboratory analyses. During the intervention study, fasting samples were taken to determine serum leptin, serum insulin, and plasma glucose concentrations. Additionally, on day 0 and day 4, an oral glucose tolerance test (OGTT) was performed: serum insulin and plasma glucose concentrations were measured at 15, 30, 45, 60, 90 and 120 minutes after a 75 g glucose load (dissolved in 200 ml tea). Blood sampling took place in the morning (8:00-9:00) after an overnight fast (minimum 12 h). During the OGTT, blood was sampled, using a catheter, which was placed in the anticubital vein of one of the arms. To prevent the catheter from clogging, about 10 ml physiological salt solution (NaCl 0.9%) was injected in the catheter after each sample. With each blood sample, one extra tube was drawn and rejected to remove the physiological salt solution from the catheter. Samples were stored at -20 °C for 0-5 months before laboratory analyses.

Plasma glucose was measured quantitatively by bichromatic endpoint assay (Glu FlexTM reagent). Serum insulin was measured by immuno-assay (Immulite® 2000 analyser; analytical sensitivity of 2 µU/ml). Three samples from one subject had to be diluted before measuring, because insulin levels exceeded the calibration range of 400 µU/ml. Serum leptin was assessed by radio immunoassay (LINCO Research Inc.,

Missouri, USA; analytical sensitivity of 0.5 ng/ml). The intra-assay coefficient of variation was 3-8%, the inter-assay coefficient of variation was 4-8%. All samples of each subject were analysed in duplicate and in one run. For the second measurement, 11 out of 44 blood samples were available.

Calculations

An index for insulin sensitivity was calculated by the method of Matsuda *et al.* (108), which takes into account both fasting glucose and insulin levels, as well as average glucose and insulin levels during an OGTT.

After subtracting baseline values, incremental areas under the curve (AUC) were calculated for glucose and insulin during the OGTT by the trapezoidal method. The AUC's were calculated by use of GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego California USA).

Statistical analyses

Prior to the study, power calculations showed that the minimum detectable difference in proportional leptin decline would amounted to 6.6%, between two groups of both 20 subjects. These calculations were based on the study of Dubuc *et al.* (19), who studied the leptin decrease on a seven-day 68% energy deficit in eleven lean male subjects.

Weight measurements during the intervention study showed that the initial contrast in weight change between the weight gain group and weight stable group was less pronounced than expected. Various men in the initial weight gain group had remained stable during the last five years, whereas a number of men in the weight stable group had been gaining weight. Therefore, we did not only perform data-analyses for the initial weight gain (>1 kg/y in 6y, n=22) and weight stable group (±0.3 kg/y in 6y, n=22), but also did analyses for the group who continued to gain weight (>1 kg/y in 11 y, n=12) (consistently weight gaining men) and who continued to remain stable in weight (±0.3 kg/y in 11 y, n=6) (consistently weight stable men). Although we lost power with these subgroup analyses, these subjects were considered to represent the men with the highest contrast in weight gain and therefore were of high interest in view of our objective.

Differences between groups for continuous and categorical variables were tested by Student's *t*-tests and χ^2 tests, respectively. As a result of non-normal distribution, fasting levels and proportional changes of leptin, insulin, AUC insulin and AUC glucose were log-transformed before testing. These results are expressed as geometric means with 95%-Confidence Intervals (CI). Other variables are expressed as arithmetic mean ± SD or arithmetic mean (95%CI). Adjustments for BMI and changes in BMI were made by using analyses of covariance. Pearson correlation coefficients were calculated to test associations between variables and changes in variables. *P*-values <0.05 were considered statistically significant.

RESULTS

Weight history

For the men whose weight had remained stable during the cohort study, a median change in weight of 0.1 kg/y (range -0.3; 0.3 kg/y) was found, and for the men who had gained weight a median change in weight of 1.3 kg/y (range 1.0; 1.9 kg/y) was found (Table 6.1). However, the pre-intervention measurement showed that the contrast in weight history had become smaller during the years after the second Doetinchem cohort measurement: a median weight change of 0.5 kg/y (range: -0.1; 0.4 kg/y) was observed for the men whose weight had remained stable, and a median weight change of 1.0 kg/y (range: 0.1; 1.8 kg/y) was observed for the men who had gained weight.

In Table 6.2, weight history categories are based on the first and pre-intervention measurement of weight. Six men were consistently weight stable (median weight change: 0.1 kg/y; range -0.1; 0.3 kg/y) and twelve men consistently gained weight (median weight change: 1.3 kg/y; range 1.0; 1.8 kg/y) during this period.

Table 6.1 Weight history and subject characteristics during the first cohort measurement, second cohort measurement and pre-intervention measurement (median (minimum; maximum)).

	1st measurement 1987-1991	2nd measurement 1993-1997	Pre-intervention 2001
Men whose weight had remained stable (n=22) *			
Follow-up (y)	-	6.0 (5.5; 6.2)	12.1 (9.4;13.9)
Age (y)	33 (26; 39)	39 (31; 45)	45 (39; 52)
Weight (kg)	79.8 (67.4;100.0)	80.6 (66.0; 101.5)	84.7 (77.3; 118.4)
Weight gain (kg/y) †	-	0.1 (-0.3; 0.3)	0.5 (-0.1; 0.4)
Height (m)	1.83 (1.74; 1.96)	-	-
BMI (kg/m^2)	24.7 (21.2; 33.8)	24.7 (21.2; 34.4)	26.0 (23.0; 40.0)
Leptin (μU/ml)	3.2 (1.6; 8.7)‡	3.2 (2.4; 4.6) §	4.5 (2.4; 16.6)
Men who had gained weight (n=22)			
Follow-up (y)	-	6.0 (5.6; 6.2)	11.5 (9.4; 14.0)
Age (y)	31 (21; 36)	37 (27; 42)	41 (31; 47)
Weight (kg)	80.4 (67.1; 97.8)	88.8 (74.4; 108.7)	93.2 (71.5; 114.3)
Weight gain (kg/y) †	-	1.3 (1.0; 1.9)	1.0 (0.1; 1.8)
Height (m)	1.80 (1.67; 1.89)	-	-
BMI (kg/m^2)	24.1 (20.1; 30.1)	26.4 (22.3; 33.6)	27.5 (23.6; 35.7)
Leptin (μU/ml)	3.9 (1.4; 11.0)	5.4 (4.2; 5.6) **	6.5 (2.5; 13.3)

* Weight history based on first and second measurement: men with a weight change of ± 0.3 kg/y are categorised as "men whose weight had remained stable", men with a weight change > 1 kg/y are categorised as "men who had gained weight"

† Weight gain compared with 1st cohort measurement

‡ n=21

§ n=5

** n=6

Results for men who gained weight and men whose weight had remained stable

At baseline, men who gained weight were slightly taller than those whose weight had remained stable, and 7.5 kg heavier, consequently BMI was similar for both groups (Table 6.2). During the intervention the men lost an average of 2.4 kg (range: 0.5; 3.5 kg), which was not statistically significant between the men whose weight remained stable and the those who had gained weight (2.5 ± 0.7 kg *vs.* 2.3 ± 0.7 kg; p=0.28).

At baseline, leptin levels were higher for the men who had gained weight (p=0.04), after adjustment for baseline BMI, this difference remained statistically significant (p=0.03) (Table 6.3). There were no differences in the other biochemical parameters between the two groups at baseline.

Table 6.2 Weight history and subject characteristics during the first cohort measurement, second cohort measurement and pre-intervention measurement (median (minimum; maximum)).

	1st measurement 1987-1991	2nd measurement 1993-1997	Pre-intervention 2001
Men whose weight was consistently stable (n=6) [*]			
Follow-up (y)	-	5.9 (5.5; 6.1)	10.7 (9.4; 13.9)
Age (y)	32 (25; 39)	38 (31; 45)	43 (34; 51)
Weight (kg)	82.7 (77.4; 91.0)	84.4 (83.4; 89.5)	85.2 (79.0; 89.8)
Weight gain (kg/y) [†]	-	0.2 (-0.3; 1.2)	0.1 (-0.1; 0.3)
Height (m)	1.83 (1.76; 1.89)	-	-
BMI (kg/m^2)	25.3 (23.6; 27.6)	25.7 (24.0; 28.7)	26.1 (24.1; 27.3)
Leptin (µU/ml)	4.8 (1.6; 6.1)	3.9 (3.1; 4.7) [‡]	4.3 (2.7; 5.0)
Men who consistently gained weight (n=12)			
Follow-up (y)	-	5.9 (5.7; 6.2)	11.7 (9.4; 14.0)
Age (y)	31 (21; 36)	37 (27; 42)	43 (31; 46)
Weight (kg)	81.3 (72.6; 100.0)	90.1 (80.5; 108.7)	96.1 (87.7; 118.4)
Weight gain (kg/y) [†]	-	1.4 (0.3; 1.9)	1.3 (1.0; 1.8)
Height (m)	1.83 (1.72; 1.96)	-	-
BMI (kg/m^2)	24.5 (20.1; 33.8)	27.0 (22.3; 34.3)	29.2 (24.7; 40.0)
Leptin (µU/ml)	3.9 (2.8; 8.4) [§]	5.0 (4.2; 5.6) [**]	7.8 (5.3; 16.8)

[*] Weight history based on first and pre-intervention measurement: men with a weight change of 0.3 kg/y are categorised as "men whose weight was consistently stable", men with a weight change > 1 kg/y are categorised as "men who consistently gained weight"

[†] Weight gain compared with 1st cohort measurement

[‡] n=3

[§] n=11

[**] n=4

For the total group, leptin levels decreased significantly with 1.3 ± 1.4 µIU/ml (-22.4%) after two days of energy restriction and 2.3 ± 1.5 µIU/ml (-39.4%) after 4 days of energy restriction. The proportional change in leptin after four days was approximately 8% higher in the men whose weight had remained stable compared to those who had gained weight (-43.3% *vs.* -35.2%, P=0.04). In absolute numbers however, leptin decreases were similar for both groups (-2.2 ± 1.2 *vs.* -2.4 ± 1.7 µIU/ml; P=0.69). Adjustments for change in BMI during the intervention (data not shown) or baseline BMI did not change these results (Table 6.3).

In the total group, fasting insulin and glucose levels declined during the first two days with -2.4 ± 3.7 µIU/ml (24%) and -0.4 ± 0.4 mmol/L (6.5%) respectively, and did not further decline during the last two days (Table 6.3). Proportionally, the insulin levels

decreased more in the men whose weight remained stable than in the men who had gained weight (-35.4 *vs.* -12.8%; p=0.01). In addition, the insulin sensitivity index showed a larger increase in the men whose weight remained stable compared to the men who had gained weight (2.3 ± 2.5 *vs.* 0.6 ± 1.4; p=0.01). The AUC insulin, AUC glucose and fasting glucose did not show differences between the groups with different weight history (Table 6.3).

Results for consistently weight stable and weight gaining men

After selecting only the consistently weight stable and weight gaining men for data-analyses, the differences in weight, BMI and waist circumference between the two groups were more pronounced, 86.1 ± 4.4 *vs.* 99.8 ± 10.1 kg (p<0.01), 25.8 ± 1.3 *vs.* 29.8 ± 4.1 kg/m^2 (p<0.01), and 96 ± 4 *vs.* 106 ± 11 cm (p<0.01), respectively. Weight loss during the intervention was similar for both groups (-2.3 ± 0.7 *vs.* -2.8 ± 0.6 kg; p=0.21).

Leptin was at baseline of the intervention twice as high in the consistently weight gaining men than in the consistently weight-stable men (Table 6.4). After adjustment for baseline BMI this difference no longer reached significance. The other biochemical parameters were not different at baseline.

The consistently weight gaining men had a -2.7 ± 2.0 µIU/ml change in leptin, which was significantly higher than that of the consistently weight stable men (-1.1 ± 0.73 µIU/ml; p=0.03). No differences in proportional decreases were found (-29.6 *vs.* -33.0%, p=0.74). Adjusting for BMI changes during the intervention or BMI differences at baseline did not change these findings (Table 6.4).

No statistical significant differences were observed in the insulin and glucose metabolism-related parameters when comparing the consistently stable and the consistently weight gaining group (Table 6.4).

Table 6.3 Biochemical parameters during the intervention study (mean (95%-Confidence Interval))

				Difference between day 4 and pre-intervention	
	Pre-intervention	Day 2	Day 4	µIU/ml	%
Men whose weight had remained stable (n=22) *					
Leptin (µU/mL) †	4.8 (3.8; 6.0)	3.5 (2.8; 4.5)	2.7 (2.1; 3.5)	-2.2 (-2.8; -1.7)	-43.3 (-47.8; -38.4)
Leptin (µU/mL) †,‡	4.9 (4.2; 5.7)	3.6 (3.1; 4.3)	2.8 (2.4; 3.3)	-2.1 (-2.7; -1.5)	-43.1 (-48.5; -37.2)
Insulin (µIU/mL) †	9.2 (7.1; 11.9)	6.5 (4.7; 8.9)	6.2 (4.3; 8.9)	-2.8 (-4.6; -1.1)	-35.4 (-46.9; -21.3)
AUC insulin (10^3) †,§	3.6 (2.5; 5.3)	-	3.2 (2.3; 4.4)	-0.9 (-1.9; 0.1)	-8.3 (19.2; 4.0)
Glucose (mmol/L)	5.8 (5.5; 6.1)	5.5 (5.2; 5.7)	5.3 (4.9; 5.6)	-0.5 (-0.8; -0.3)	-9.2 (-12.8; -5.6)
AUC glucose (10^2) †,**	1.5 (1.0; 2.1)	-	2.1 (1.6; 2.7)	0.5 (-0.1; 1.1)	46.5 (7.8; 98.7)
Insulin sensitivity index ††	5.4 (4.0; 6.7)	-	7.6 (5.4; 9.7)	2.3 (1.1; 3.5)	42.6 (22.2-63.0)
*Men who had gained weight (n=22)**					
Leptin (µIU/mL) †	6.4 (5.3; 7.7) *	5.2 (4.2; 6.4)	4.1 (3.4; 5.0)	-2.4 (-3.2; -1.7)	-35.2 (-42.4; -27.1) ‡‡
Leptin (µU/MI) †,‡	6.2 (5.3; 7.2) *	5.0 (4.2; 5.9)	4.0 (3.4; 4.7)	-2.5 (-3.2; -1.9)	-35.4 (-41.5; -28.7)
Insulin (µIU/mL) †	9.2 (7.4; 11.6)	7.6 (6.2; 9.3)	8.1 (6.6; 9.8)	-1.8 (-3.9; 0.4)	-12.8 (-28.1; 5.7) ‡‡
AUC insulin (10^3) †,§	4.2 (2.9; 6.1)	-	4.1 (3.1; 5.5)	-0.3 (-1.4; 0.7)	1.8 (-16.6; 24.3)
Glucose (mmol/L)	5.9 (5.5; 6.2)	5.4 (5.1; 5.8)	5.4 (5.3; 5.6)	-0.4 (-0.6; -0.2)	-6.4 (-9.5; -3.2)
AUC glucose (10^2) †,**	1.5 (1.2; 1.8)	-	1.5 (1.1; 1.9)	0.1 (-0.3; 0.5)	1.1 (-21.0; 29.4)
Insulin sensitivity index ††	4.9 (3.8; 6.0)	-	5.5 (4.3; 6.7)	0.6 (-0.1; 1.2)	22.4 (-1.4; 46.2) ‡‡

* Weight history based on first and second measurement: men with a weight change of ± 0.3 kg/y are categorised as "men whose weight had remained stable", men with a weight change > 1 kg/y are categorised as "men who had gained weight"

† Baseline levels were log-transformed before testing, geometric mean and 95%-confidence intervals are shown

‡ Adjusted for baseline BMI by analyses of covariance

§ Units: µIU/mL/120min

** Units: mmol/L/120min

†† Calculated by the equation of Matsuda (108)

‡‡ Significantly different from the men whose weight remained stable, $p<0.05$

Table 6.4. Biochemical parameters during the intervention study (mean (95%-Confidence Interval)).

				Difference between day 4 and pre-intervention	
	Pre-intervention	Day 2	Day 4	µIU/ml	%
Men whose weight was consistently stable (n=6) [*]					
Leptin (µIU/mL) [†]	3.9 (2.9; 5.1)	3.4 (2.4; 4.7)	2.7 (1.9; 4.0)	-1.1 (-1.9; -0.4)	-29.6 (-44.7; -10.4)
Leptin (µIU/mL) [†,‡]	4.4 (3.3; 5.8)	3.8 (3.0; 4.9)	3.1 (2.3; 4.2)	-1.3 (-2.7; 0.20)	-28.5 (-44.0; -8.9)
Insulin (µIU/mL) [†]	11.5 (6.3; 21.2)	10.1 (5.0; 20.5)	8.4 (3.4; 20.8)	-1.8 (-5.1; 1.5)	-26.9 (-56.0; 21.6)
AUC insulin (103) [†,§]	3.3 (1.5; 7.6)	-	3.0 (1.8; 5.0)	-0.9 (-3.0; 1.1)	-11.0 (-36.1; 24.2)
Glucose (mmol/L)	6.1 (5.5; 6.0)	5.3 (4.3; 6.2)	5.6 (5.2; 4.8)	-0.6 (-1.0; -0.1)	-9.5 (-16.8; -2.1)
AUC glucose (102) [†,**]	1.2 (0.5; 2.9)	-	1.4 (0.8; 2.3)	-0.2 (-0.8; 0.5)	10.1 (-25.0; 61.6)
Insulin sensitivity index [††]	4.2 (2.4; 6.1)	-	4.7 (2.7; 9.7)	1.9 (-0.8; 4.6)	47.9 (-14.4; 110.3)
Men who consistently gained weight (n=12) [*]					
Leptin (µIU/mL) [†]	7.8 (6.3; 9.7)	6.9 (5.7; 8.3)	5.3 (4.2; 6.5)	-2.7 (-4.0; -1.5) [‡‡,*]	-33.0 (-42.9; -21.3)
Leptin (µIU/mL) [†,‡]	7.4 (6.1; 8.9)	6.5 (5.5; 7.6)	4.9 (4.0; 6.0)	-2.6 (-3.7; -1.6)	-33.4 (-43.5; -21.6)
Insulin (µIU/mL) [†]	10.4 (7.4; 14.6)	9.1 (6.4; 13.0)	9.4 (6.7; 13.2)	-1.2 (-3.2; 0.8)	-9.4 (-24.8; 9.3)
AUC insulin (10^3) [†,§]	4.1 (2.0; 8.3)	-	4.5 (2.7; 7.5)	-0.1 (-2.2; 2.1)	20.1 (-12.7; 65.3)
Glucose (mmol/L)	5.9 (5.3; 6.4)	5.6 (5.0; 6.2)	5.2 (5.3; 5.9)	-0.2 (-0.6; 0.1)	-3.1 (-7.5; 1.3)
AUC glucose (10^2) [†,**]	1.7 (1.3; 2.1)	-	1.8 (1.4; 2.4)	0.3 (-0.3; 0.9)	18.0 (-12.6; 59.5)
Insulin sensitivity index [††]	4.5 (3.0; 6.0)	-	6.2 (3.3; 6.1)	0.0 (-0.7; 0.7)	3.2 (-9.6; 15.9)

[*] Weight history based on first and pre-intervention measurement: men with a weight change of 0.3 kg/y are categorised as "men whose weight was consistently stable", men with a weight change > 1 kg/y are categorised as "men who consistently gained weight"

[†] Baseline levels were log-transformed before testing, geometric mean and 95%-confidence intervals are shown

[‡] Adjusted for baseline BMI by analyses of covariance

[§] Units: µIU/mL/120min

[**] Units: mmol/L/120min

[††] Calculated by the equation of Matsuda (116)

[‡‡] Significantly different from the men whose weight was consistently weight stable, $p<0.05$

Relationships with the decrease in leptin

Table 6.5 shows the correlation coefficients of several variables and the four-day leptin change. First of all, a strong association (r=0.78) between pre-intervention leptin levels and the decrease in leptin was observed. Second, both the baseline insulin levels and the proportional change in insulin levels were positively related with the leptin decrease, and had correlation coefficients of 0.26 and 0.52, respectively. Furthermore, the decline in leptin was positively related with pre-intervention weight (r=0.44; P=0.003), BMI (r=0.36; p=0.02), and waist circumference (r=0.46; p=0.002) at baseline. A weak but not significant positive association was found between the change in leptin and weight loss during the intervention (r=0.27; p=0.08).

Table 6.5 Pearson correlation coefficients (p-values) four-day decline in leptin and several variables at baseline and changes in variables during the intervention (n=44).

	Leptin change	
	Absolute	Proportional *
Baseline		
Leptin †	0.77 (<0.0001)	-
Insulin †	0.31 (0.041)	0.10 (0.53)
Glucose	0.22 (0.15)	0.10 (0.52)
Insulin sensitivity	-0.24 (0.12)	0.13 (0.40)
Age	0.02 (0.91)	-0.14 (0.38)
Weight	0.36 (0.017)	0.15 (0.33)
BMI	0.44 (0.003)	0.14 (0.36)
Waist circumference	0.46 (0.002)	0.08 (0.59)
Change		
Insulin	0.26 (0.09)	0.52 (0.0004) †,‡
Glucose	0.02 (0.89)	0.26 (0.09) †,‡
Insulin sensitivity	0.22 (0.17)	0.30 (0.06)
BMI	0.28 (0.07)	0.07 (0.64)
Weight	0.27 (0.08)	0.11 (0.49)

* Proportional change with regard to baseline levels

† Correlation with proportional change in insulin/glucose

‡ Correlation is calculated after log-transformation

DISCUSSION

After four days of energy restriction, a similar decrease in leptin levels was found in men with high retrospective weight gain as in those who had stable weight. Proportionally, an 8% smaller decrease in leptin was observed in men with retrospective weight gain. This difference in proportional decrease could not be seen in a subgroup in which the difference in retrospective weight gain was more pronounced.

We recruited our subjects from an existing cohort study; this approach made it possible to combine intervention data with data from the past. However, this approach also had limitations. We hypothesised that the contrast in weight gain between the two groups was caused by differential decreases in leptin levels to fluctuations in energy balance. Therefore, we made the assumption that differential decreases in leptin were still noticeable 5 y later, when our intervention study was conducted. It is likely that weight gain itself affected these decreases in leptin. Also because weight related parameters were associated with the decrease in leptin (Table 6.5). At this moment, however, this is the only approach that is available to study decreases of leptin to deviations in energy balance and its effect on weight gain, since these data on leptin decreases have not been measured in the past.

We studied the effect of a negative energy balance on the decrease in leptin; then, differential increases of leptin to a positive energy balance might be of more importance, since weight gain is mainly caused by a small but chronic positive energy balance. On the other hand, from the literature it is known that the feedback mechanisms to restore energy homeostasis are less sensitive to a positive energy balance than to a negative energy balance (75). In other words, the highest change in leptin was expected to be found when intervening with a negative energy balance. Next to that, we also had ethical questions by overfeeding obese subjects.

Our study was conducted in free-living subjects, therefore some methodological concessions had to be made. We did not include a run-in period in energy balance before the intervention started. This may have influenced energy balance and therefore also leptin levels at baseline. However, the men's weight did not change more than 1 kg between the medical screening and the baseline measurement of the intervention.

Thus, we can assume that the men were in energy balance before energy restriction. Furthermore, energy requirements were estimated, not measured; the equation of Schofield was used, together with a short questionnaire on physical activity. After that, energy groups were formed. We assumed that the variation in energy requirements within a day is higher than the error in our estimation method. However, we cannot exclude that a part of the variation in the decrease in leptin is due to over- or underestimation of the energy requirements.

After 4 days of 65% energy restriction, leptin levels dropped by 39%. Leptin is known to be highly dependent on the size and amount of fat mass in the body. With linear regression we could, however, only explain 8% of the variation in the decrease in leptin. Dubuc and colleagues also observed a similarly decline in leptin, independent of loss of fat mass (19). They studied the leptin levels in lean men (BMI 24.7 ± 0.5 kg/m^2) during a seven-day 68% energy restriction and found a fall in leptin levels of 36%. Unfortunately, leptin data after 4 days of energy restriction were not shown, so we were not able to compare their data with ours. Other researchers, who studied the effect of energy restriction on leptin levels also have shown these clear decreases in leptin after energy restriction (21, 44, 112, 113). These studies are however, not comparable to our study because of differences in design, such as the percentage of energy restriction, duration of the intervention and/or study population. But, their results confirm the idea that adjustments in leptin levels may be instrumental for the restoration of energy balance.

The decrease in leptin was similar in the men who had gained weight as in the men whose weight had remained stable. On the other hand, the men who had gained weight had a smaller proportional decrease in leptin, which is in line with our hypothesis. However, we have to be careful with our conclusions based on this finding.

First of all, we did not find similar results in the sub-sample of consistently weight stable and weight gaining men. In this group the contrast in weight gain was higher, and therefore it was expected to find also a higher contrast in leptin decrease. Then, conclusions concerning these subgroup analyses have to be made very carefully because of the relatively small number of subjects: 6 *vs.* 12, whereas the power calculations showed that 20 subject in each group were needed.

Secondly, pre-intervention difference in fat mass may have had a role in the proportional decrease in leptin. However, after adjustment for BMI before intervention only a minor effect was seen on the proportional decrease in leptin (Table 6.3).

Lastly, the difference in proportional decrease in leptin may be due to differences in leptin levels as such. In a former study, in which leptin levels and subsequent weight gain were also studied in the Doetinchem Cohort Study, nonfasting leptin levels were related to subsequent weight gain. An increase in leptin with 0.5 μIU/ml increased the risk of gaining weight (*i.e.* gaining over 1.3 kg/y) with 27% (39). Since we recruited our subject from the same cohort, it may be that the pre-intervention difference in leptin levels, which was partly responsible for the proportional difference in decrease in leptin, is identical to the risk factor we observed in our former study, *i.e.* having relatively high leptin levels. Looking at the instrumental function of leptin, we do not know whether the absolute or the proportional adjustments to energy deviations are biologically relevant. And as far as we know, there is no clear answer to this question as yet.

Similar to the proportional change in leptin, we found insulin levels and the insulin sensitivity index to show larger changes in men whose weight remained stable compared to men who had gained weight. Moreover, the proportional decrease in insulin was associated with the decrease in leptin (r=0.52). Correlation coefficients of the same magnitude are also shown in the study of Racette *et al.* and Dubuc *et al.* of respectively 0.41 (p=0.07) and 0.54 (p=0.09) (19, 112). In these studies it has been suggested that the observed fall in leptin might have been induced by the decline in insulin.

To our knowledge this study was the first to look at leptin changes induced by deviations from energy balance in relation to long-term weight gain. If leptin levels are involved in the development of obesity, the dynamics of leptin may be of high importance. Therefore, more controlled intervention studies are needed to study responses induced by changes in energy balance and their effect on subsequent weight change and the development of obesity. Moreover, the biological mechanism by which leptin would regulate energy balance, either by proportional changes or absolute changes in leptin, has to be looked into.

Although we found men with retrospective weight gain to have relatively lower decreases in leptin to a four-day energy restriction of 65%, our study does not provide convincing evidence that men who gained weight are less responsive to changes in energy balance than men who are weight stable.

7

General Discussion

The main aim of this thesis was to investigate whether the acute decline in leptin to energy restriction is a biomarker for the susceptibility to weight gain. In other words, is the leptin response an individual trait that is related to the ability to restore energy balance and is it therefore related to the susceptibility to weight gain?

In this discussion we reflect on the most important findings of this thesis, and address the advantages and shortcomings of the methods and measures we used. Next, we discuss the implications of our findings for leptin and its role in weight gain. By using established criteria for biomarkers, we evaluate the acute leptin decline after energy restriction as a marker for the susceptibility to weight gain. Finally, directions for future research are given.

MAIN FINDINGS

In Figure 7.1 the main findings of our studies are summarized. The leptin decline after energy restriction showed a relatively high reliability on the short-term (Chapter 2). We did not observe an effect of genetic variation in the leptin receptor gene on the leptin decline (Chapter 3). We did find that the acute decline in leptin was associated with the increase in appetite during energy restriction (Chapter 4). However, we did not observe an association between the magnitude of this leptin decline and subsequent caloric compensation (Chapter 5). Additionally, no conclusive evidence was found for a difference in acute leptin decline between men who had gained weight over several years and men who were weight stable over the same period (Chapter 6).

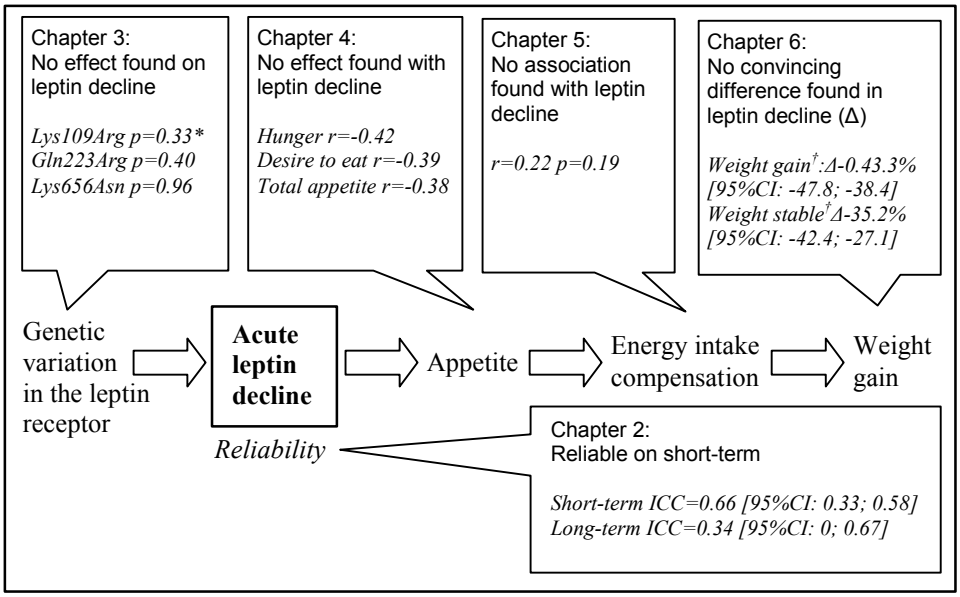


Figure 7.1 The main findings of this thesis related to the steps involved in the acute leptin response in relation to the susceptibility to weight gain. $p<0.05$ is considered statistically significant, r = Pearson correlation coefficient, 95%CI = 95%-Conficence Interval, ICC=intra-class correlation coefficient. * level of significance for the difference between carriers and non-carriers of the mutant allele. † Subjects that gained body weight. ‡ Subjects that remained stable in body weight.

METHODOLOGICAL ISSUES

In this paragraph, we address the study design, study subjects, intervention strategy, and endpoints of the interventions we conducted. We discuss the strengths and weaknesses of these methods and we reflect on their consequences for the acute leptin decline as a biomarker for the susceptibility to weight gain.

Study design

In Nutritional Sciences, critical design issues in trials usually are: a) randomisation and blinding of the treatments, b) placebo or control group, c) run-in period, d) cross-over design, e) the duration of the intervention, and f) measurement of compliance. Reflecting on our studies, we will address these issues point by point.

Randomisation, blinding and control group

We conducted three controlled intervention studies with a similar design; 2 or 4 days of energy restriction during which we measured the acute decline in leptin. Subsequently, we related the acute decline to another outcome variable, *e.g.* appetite or energy intake compensation. As all subjects received the same treatment, randomisation, and blinding was not an issue in our studies. Moreover, it is impossible to cover severe energy restriction. A control group would not add to the design, since it has been shown that fasting leptin levels (sampled at a standardized time) are fairly stable in free-living individuals (64).

Run-in period

A run-in period, *i.e.* a period in which subjects receive a standardized isocaloric diet, was very difficult to include in our design, as our subjects were free-living. However, including such a period would have better standardized the measurement of the leptin decline between subjects. Nevertheless, we standardized the blood collection by a fast of at least 12 hours prior to sampling. Additionally, we instructed the subjects not to change their usual food intake pattern. The subjects showed no large changes in weight between the screening and intervention, which confirms that the subjects did not change their energy intake (*e.g.* Chapter 6). Moreover, small changes in energy intake, for example overeating during one meal before intervention, are not very likely to induce large changes in the leptin concentration (114).

Duration of intervention

Another point of discussion might be the duration of the interventions. The aim of our intervention was to obtain a decline in leptin, not induced by a reduction in body weight. From the studies of Dubuc *et al.* and Kolaczynski *et al.*, we knew that the acute decline had to take place within 36 hours of fasting and within 7 days if subjects consumed 35% of their estimated energy needs (19, 21). In the "*Eetlust"*-study we concluded that a four-day diet intervention, containing 35% of the estimated energy needs, was sufficient to induce a decrease in leptin of 39%, which is independent of the change in body weight. Moreover, we observed that in the *LEPTOS*-study and *KALOZ*-study a two-day diet, containing 35 and 38% of the estimated energy needs respectively, was sufficient to observe a statistically significant decline in leptin of 16%

and 25%. Thus, 2 days of severe energy restriction were adequate to induce the acute leptin decline we wanted to study.

Compliance

The compliance to the fully supplied energy-restricted diet was assessed by means of pre-printed diaries in which subjects wrote down the time of consumption of the supplied products. If deviations from the protocol occurred, for example the consumption of additional products, were written down in this diary. Adding a tracer, *e.g.* lithium that is traceable in urine (115), to the meal replacements would have been a more objective measure. Using a tracer would have provided information on the consumption of the provided meal replacements; however, additional products would not have been detected by this method. This method was also not possible in our studies, as we used meal replacements that were readily available on the Dutch market. Moreover, the changes in several important indicators of compliance, such as body weight, and leptin, ghrelin, insulin and glucose concentrations supported the high compliance that was assessed by means of the food diaries.

Study subjects

The type of subjects and the number of subjects that participated in our studies is discussed in the following paragraphs.

Type of subjects

The most critical issue is the BMI of the subjects in our study. One may argue that a homogenous group with a low BMI should be studied. This has the advantage that the probability of subjects being leptin resistant is very small and that no other (interfering) variables are present, so that a biological mechanism can be studied. However, a heterogeneous group, with a large range in BMI, also has major advantages. First, in a heterogeneous group, a large variation in leptin responses is present, which makes it easier to detect associations. Second, with a view to the aim of our studies, a marker can be easily generalized to the common population if it is investigated in a heterogeneous group. Rather than studying the biological mechanism, we evaluated the leptin response as a biomarker, therefore we choose for the latter option; a heterogeneous group with a large BMI range. In the *"Eetlust"*-study the range of BMI

was 22.9-39.8 kg/m^2, and in the LEPTOS-study (a sub-sample of the *"Eetlust"*-study) this was 22.7-39.8 kg/m^2. Although we aimed for a similar range of BMI in the KALOZ-study, the individuals that participated all had a relatively low BMI, *i.e.* 19.8-24.9 kg/m^2.

Number of subjects

The number of subjects that participates in an intervention study is crucial for the inferences made from the results. The number should be adequate to provide statistical evidence for an estimated relevant effect. Before conducting our first intervention study we estimated the power using weight gain based as the endpoint (*Eetlust*-study; Chapter 6). We calculated that the minimum detectable difference in proportional leptin responses amounted to 6.6%, which we considered a relevant effect. In addition, the analyses on appetite (Chapter 4) were also based on this study. In these analyses our power was sufficient to detect an association of 0.41, which we considered relevant. For the genetic analyses (Chapter 3), our power was not sufficient to provide statistical evidence. Therefore these analyses were considered as a pilot and we provided power calculations and ideas to improve the power of future studies. Prior to the *KALOZ*-study we calculated our power based on the findings of the "*Eetlust"*-study. We calculated that 35 subjects would be sufficient to detect a minimum relevant association of 0.40 (Chapter 5). Thus, as all statistical analyses, except for the genetic analyses, had adequate statistical power; higher numbers of subjects would neither have changed our results nor our conclusions.

Intervention strategy: energy restriction

Negative vs. positive energy balance

As stated before, weight gain is mostly due to a small but prolonged positive energy balance, which may be caused by poor energy intake regulation. Therefore, the individual variation in acute hormonal responses to short-term changes in energy balance might be related to the individual variation in weight gain. In this thesis, we focused our research on the acute decline of leptin to energy restriction. Because it is very difficult to induce severe energy restriction by increasing energy expenditure we choose to restrict energy intake. Although we focused on a negative energy balance,

the increases of leptin to a positive energy balance might be of more importance for weight gain. One of the reasons for studying a negative rather than a positive energy balance was that the leptin response is probably easier to detect after energy restriction. From the literature it is known that the feedback mechanisms to restore energy homeostasis are less sensitive to a positive energy balance than to a negative energy balance (75). This is plausible from a biological perspective; a shortage of energy is life threatening, while an excess of energy may be helpful in times of scarcity. *A priori*, we assumed that the subjects with a large leptin response to a negative energy balance would also show a large response to a positive energy balance. Indeed, our own findings in Chapter 5 support this assumption. In this Chapter we describe that the decline in leptin during energy restriction was strongly associated with the increase after subsequent *ad libitum* intake ($r=0.88$; $p<0.05$). Another important reason is that, given the growing prevalence of obesity, we had ethical questions by overfeeding (obese) subjects.

Proportional energy deficit

In order to induce a similar stress in each subject, we choose an energy deficit that was proportional to the individual estimated energy needs. However, the estimation method of the energy needs and the categorisation of the subjects in energy groups may have induced variation in this stress. However, the error in our estimation method is probably lower than the variation in energy needs between days. For example, the misclassification of a subject (energy needs 15 MJ) in the 4.2 MJ group instead of in the 5.0 MJ group, would induce an error in proportional energy restriction of ~5%. However, the normal variation in energy needs between days is probably higher; the variation within subjects in resting metabolic rate is about 5% and that of total energy intake about 20% (116). Additionally, the energy restriction in our studies was only used as a tool to induce the acute leptin decline; it was not used as an endpoint in the data-analyses itself. It is therefore not likely that this may have affected our results.

Nutrient composition

The nutrient composition of the energy restriction diet might have affected the leptin decline in our studies. Jenkins and colleagues have observed that the acute decline in leptin is associated ($r=0.68$) to the change in dietary carbohydrate during energy

restriction (103). Additionally, Agus and colleagues found that an energy restricted diet containing high levels of easily absorbed carbohydrates induced a smaller decline in leptin than an energy restricted diet containing low levels (104). Our intervention diets contained more than 100 grams mono- and disaccharides per day. One may speculate that the decline in leptin would have been larger if we had lowered the amount of mono- and disaccharides in the energy restricted diet. However, we used meal replacements that were readily available on the Dutch market.

Potential marker: Acute leptin decline

Our most important measure was the marker itself; the acute leptin decline induced by energy restriction. In our studies we defined our marker as the decline in leptin in proportion to the baseline concentration. In this way, we also corrected for the baseline leptin concentrations, and indirectly for baseline BMI. It is plausible that a 2 ng/mL decline in an individual with a baseline level of 5 ng/mL, induces a smaller effect than a 2 ng/mL decline in an individual with a baseline level of 15 ng/mL. However, the proportional decline included larger measurement error than the absolute decline. In the absolute decline the error is included 2 times, *i.e.* the baseline measurement and the measurement after intervention. In a proportional response the measurement error is included 3 times in the marker, *i.e.* the baseline measurement, measurement after intervention, and, because it is divided by the baseline level of leptin, again the baseline measurement. However, we minimized this measurement error by analysing all leptin samples *in duplo* and all samples of one subject in one run.

Intermediate endpoint: subjective appetite

In order to evaluate if the acute decline in leptin is a biomarker for the susceptibility to weight gain, we investigated its relation with intermediate endpoints of weight gain, *i.e.* food intake compensation, and subjective appetite. In this paragraph we address the methodological issues concerning the intermediate endpoint "subjective appetite".

In Chapter 4 we used subjective ratings to quantify appetite (92), which have shown to be good indicators of the motivation to eat and predictors of actual food intake (94-96). As yet, these subjective ratings are the only available tools to quantify appetite. Further

research might focus on the development of objective biomarkers of appetite. Candidate biomarkers of appetite might be: ghrelin, CCK, and GLP-1 (117).

Intermediate endpoint: energy intake compensation

The next intermediate endpoint was energy intake compensation, which was assessed by the amount of energy that was compensated during two days following energy restriction (Chapter 5). If acute decline of leptin is a starvation signal it is plausible that it affects energy intake during the days after energy restriction. However, it is difficult to measure energy intake compensation. Therefore, we used two methods to ensure that subjects responded to internal cues only. First, we used unusual portion sizes, *e.g.* larger plates and smaller bread rolls. Second, we provided a blinded milk shake during the first breakfast after energy restriction. Although, we observed that subjects showed compensatory behaviour, we did not observe an association with the decline in leptin.

Endpoint: weight gain

In Chapter 6, we studied whether the acute decline in leptin was related to changes in body weight. In order to have longitudinal data on weight gain, we recruited our subjects from an existing cohort and retrospective data on weight changes were used to categorize subjects into subjects that had gained weight and subjects that remained stable during several years. However, by doing so, we assumed that this difference in weight maintenance was caused by the acute decline in leptin and that this difference in decline was still measurable after a few years. Moreover, we assumed that the acute leptin decline was an individual trait. In Chapter 2, however, we observed that the acute leptin decline was poorly reproducible after 1½ year and that it is affected by changes in weight. Nevertheless, at the moment we conducted the study described in Chapter 6 this was the only approach available to study the acute decline in relation to weight gain. Currently, the participants of the "*Eetlust*"-study are still participating in the cohort study, and future weight measurements may be used to further study the association between the acute leptin decline and prospective weight changes.

IMPLICATIONS FOR LEPTIN AND WEIGHT GAIN

In this paragraph we speculate about the implications of our findings for leptin, energy balance restoration and weight gain. But first, the mechanism behind the decline in leptin will be discussed.

Mechanism behind the acute leptin decline

After weight loss leptin levels decline, this is probably due to loss of fat mass (17, 18). However, after acute energy restriction leptin concentrations decrease far more than can be expected from loss of fat mass only (19-22). There is evidence that the low availability of glucose during energy restriction might induce this decline in leptin (21, 103-105). Normally, during energy restriction, glycogen stores are being depleted, insulin levels are lowered, and the body switches over to fatty acid oxidation. However, glucose-infusions have shown to prevent leptin levels to drop during fasting (21) and *in vitro* studies showed that refraining fat cells from insulin treatment decreased their leptin excretion (105). Also modifications in glucose content of low-energy diets have shown to affect leptin levels (104).

The acute leptin decline and energy balance restoration

In our studies we observed that the acute decline in leptin was associated to appetite (Chapter 4). However, we did not observe an association with energy intake compensation (Chapter 5) or weight gain (Chapter 6). Therefore it may be concluded that the acute decline is a marker for appetite during an energy deficit, but does not relate to the actual food intake or to eventual weight gain. It might be that this is due to a random error, which probably increases if the (intermediate) endpoint is more steps away from the leptin decline (see Figure 7.1). However, another explanation might be that the acute decline in leptin induces an increase in the motivation to eat, but that this motivation is not always translated into actual food intake. This is a phenomenon that is also seen in restraint subjects, which is due to the cognitive awareness of food intake (91).

Leptin resistance in the obese

It has been suggested that obesity is associated with leptin resistance (51), which is analogous to that of insulin resistance (28). If this is true, obese individuals might not

show responses in leptin to changes in energy balance. However, in our studies it appeared that subjects with high leptin levels, *i.e.* the obese subjects, also had a relatively high absolute and proportional leptin response (*'Eetlust'*-study and *LEPTOS*-study). Then, it is suggested that the cause of leptin resistance is an impaired ability of leptin to cross the blood brain barrier, or an impaired ability of leptin to induce responses at its receptor (118). Animal studies have shown these impaired abilities may be induced by a syndrome X-like condition, *i.e.* insulin insensitivity, elevations in glucose and insulin, and high BMI (119).

Differences between species

Animal models still provide most of the direct evidence for leptin declines and their role in energy intake compensation after energy restriction (13). It might be that the role of leptin in starvation and energy intake regulation is different in rodents, and cannot be extrapolated to humans. First, leptin deficient rodents and starving rodents show several neuro-endocrine changes (14), which are not observed in leptin deficient humans (25, 36) or fasting humans (111). These observations stress that there might be important differences in the physiology of starvation between species. Second, the cognitive awareness of humans during energy restriction, which is always present, also makes it very difficult to extrapolate findings from rodents to humans.

Other hormones and peptides affecting food intake regulation

Next to leptin, several other hormones and peptides are involved in energy balance regulation. As mentioned before, insulin might be an important signal for energy intake, as well as ghrelin (34). Another hormone is the gut-hormone PYY_{3-36}, which was first discovered in 1982 by Tatemoto (120). Recently it has been re-discovered by Batterham and colleagues; infusion of this hormone appeared to have an inhibiting effect on food intake in humans (121). It is speculated that PYY_{3-36} exerts is action through the NPY-pathway, like leptin (121). But, PYY_{3-36} is only present in very small concentrations in the blood and it is therefore very difficult to measure. However, new techniques can make this possible in the future.

Next to the energy balance signals, a complex system of meal related hormones and peptides affect food intake (117, 122). Examples of these hormones and peptides are; glucose levels and dynamics, CCK, GLP-1, bombesin, somastostatin, and ghrelin

(122). Although ghrelin might affect the NPY-pathway like leptin, it is plausible that its role in satiety and satiation is more important than that in the conservation of energy stores (Chapter 2).

Thus, although leptin probably has a central role in energy balance, many other routes exist for the regulation of energy balance and food intake. Clearly, new techniques in molecular biology will make it possible to discover also new hormones and peptides involved in energy intake regulation and will further develop the measurement of known hormones and peptides. Additionally, the interactions of several hormones and peptides involved in energy balance may be studied.

MARKER FOR THE SUSCEPTIBITILY TO WEIGHT GAIN

The main aim of this thesis was to investigate whether the acute decline in leptin to energy restriction is a biomarker for the susceptibility to weight gain. In 1999 a consensus document has been published under the co-ordination of The International Life Science Institute (ILSI) (123; page 110). Using this theoretical framework, we can evaluate the possible use of the acute leptin decline induced by energy restriction as a biomarker for weight gain.

ad 1. Hypothetically, the acute leptin decline after energy restriction represents an immediate outcome that might be used to replace a later and more remote outcome, *i.e.* weight gain.

ad 2. Currently, the acute decline is not validated rigorously; therefore no standard quality-control procedures are available.

ad 3. The marker should be clearly linked to the biological process. In our studies it appeared that the acute leptin decline was not related to retrospective weight gain or food intake compensation. However, it appeared to be related to the degree of appetite feelings during energy restriction.

ad 4. No tests are performed on the sensitivity and specificity of the acute leptin response. However, one may speculate that the specificity is high, as it has been shown that it is not likely that leptin levels decline in subjects without energy restriction (64). The sensitivity, on the other hand, might be lower. In our studies, we have observed that not all subjects showed an acute decline in leptin. Although no other centres have assessed the reproducibility of acute

decline, we found that the decline was a reliable marker within weeks, but not within years.

ad 5. The acute decline is measurable in serum, which is easily accessible. The severe energy restriction is quite invasive, but on the other hand we had only few drop-outs in the studies: 2 during the "*Eetlust*"-study, 0 during *LEPTOS*-study, and 1 during the *KALOZ*-study. However, one may have ethical questions to put lean subjects (BM<20 kg/m^2) on a severe energy-restricted diet.

ad 6. The acute decline is indeed a dynamic response and not a static measurement.

ad 7. The leptin decline is not an objective assessment of physchological or physical performance, but rather of a physiological performance.

ad 8. As a biomarker the acute leptin decline has to relate to the exposure, the food component under study, the target function or biological response, or an appropriate intermediate endpoint. The leptin response was indeed associated with the food component under study (*i.e.* energy restriction), several other biological changes in the body (*i.e.* insulin), and to one of the intermediate endpoints (*i.e.* appetite).

CONCLUSION

Thus overall, considering the theoretical framework for biomarkers provided by ILSI, we conclude that the acute decline in leptin after energy restriction is not a good biomarker for the susceptibility to weight gain. However, it may be a valid marker for the state of energy balance and for individual differences in appetite feelings during the first days of severe energy restriction.

Criteria for biomarkers:

1. Markers should represent relatively immediate outcomes, which can be used to assess interventions in a reasonable time scale; they could, therefore, wherever possible, replace later and more remote outcomes as have been used in some epidemiological studies.

2. Markers should be rigorously validated and amenable to standard quality-control procedures.

3. Markers must be clearly linked to the phenomena involved in the biological process being studied. It is important to prevent the pursuit of increasingly accurate and precise measurements, which have limited biological significance.

4. Markers should undergo single-centre studies to establish their sensitivity (*i.e.* the frequency of a negative test when the process in present) and their specificity (*i.e.* the frequency of a positive test when the process is absent). They must also show be shown to be reproducible in different centres.

5. Markers should be measurable in easily accessible material, or obtainable using methodology that must be both ethical and minimally invasive.

6. Dynamic responses might be as helpful as, or more useful than, static measurements. For example, changes in markers during clearance studies and in postprandial situations and studies of enzyme function, induction and suppression should be considered

7. Appropriate static and dynamic markers might also be based on objective assessments of psychological and physical performance and subjective assessments of quality of life or other similar outcomes.

8. Markers can either relate to the exposure, to the food component under study, relate to the target function or biological response, or relate to an appropriate intermediate endpoint.

Derived from the Scientific Concepts of Functional Foods in Europe: Consensus Document, under coordination of ILSI (123).

DIRECTIONS FOR FURTHER RESEARCH

In view of our previous reflections, this paragraph provides directions for further research.

- Further research is needed to study whether there is a dose-response relationship between the leptin decline and energy restriction within an individual. Of interest might also be the turnover point at which the leptin decline becomes a signal for the loss of fat mass instead of a signal for disturbed energy balance.
- If the magnitude of the acute decline of leptin after energy restriction is related to the increase in appetite during energy restriction, then it may be of interest to find tools to lower this response. First, it may be worthwhile to study the effect of the macronutrient composition of energy-restricted diets on the acute leptin decline and appetite feelings. Second, clinical studies might be conducted in which the effect of exogenous leptin during the first days of energy restriction is investigated.
- Although the phenomenon of 'leptin resistance' is widely used, no actual definition is given. Further studies might focus on the quantification of leptin resistance and its implications for the treatment of obese individuals. For example, the effect of changes in leptin concentrations on appetite and food intake in underweight, lean, pre-obese, and obese subjects may be studied. Additionally, the suggested causes of leptin resistance should be studied in humans, *i.e.* the impaired blood brain barrier transport, or impaired ability of leptin to induce responses at its receptor. For example, the effect of serum triglyceride levels on leptin levels in serum and leptin levels in cerebral fluid.
- In order to explain the variation in the susceptibility to weight gain other biomarkers for the regulation of energy balance may be developed. For example, new proteins and hormones may be identified by use of proteomics and metabolomics. But also gene-expression patterns may be used as a marker. The development of new biological techniques, *e.g.* micro-arrays, might be of high importance in this field. However, also other biomarkers, like diet induced thermogenesis, body temperature or fMRI- or PET-scans in which activity profiles of the brain are assessed, might be further developed.

- Next to its role in obesity, the function of leptin (and other hormones involved in food intake regulation) should be investigated in involuntary weight loss. For example in anorexia of ageing; it has been suggested that the decline in appetite and food intake in the elderly is caused by an increase in leptin levels with age (124). As in weight loss in the elderly is associated with a high risk of several morbidities and hospitalisation (125), this may be an important direction for further research.
- Lastly, effective and feasible intervention programmes aimed at the prevention of weight gain should be developed. Currently, a large project initiated by the Netherlands Hearth Foundation evaluates several intervention programmes, which consist of are tailor-made advice aimed at several age-groups that are highly susceptible for weight gain, *i.e.* adolescents, young adults and retiring middle-aged people.

References

1. World Health Organization. Obesity: preventing and managing the global epidemic - Report of a WHO Consultation on Obesity, June 1997. WHO Technical Report Series no. 894. Geneva, World Health Organization.

2. Seidell JC, Visscher TL. Hoeveel mensen hebben overgewicht? Volksgezondheid Toekomst Verkenning, Nationaal Kompas Volksgezondheid. www.nationaalkompas.nl 14-2-2-004. Bilthoven, RIVM.

3. Health Council of the Netherlands. Overgewicht en obesitas (in Dutch). Overweight and obesity. 2003. The Hague, Health Council of The Netherlands.

4. 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(9):1218-1224.

5. Health Council of the Netherlands. Commissie Trends Voedselconsumptie. Enkele belangrijke ontwikkelingen in de voedselconsumptie (in Dutch). Committee on Trends in food consumption. Significant trends in food consumption in the Netherlands. 2002. The Hague, Health Council of the Netherlands.

6. Statistics Netherlands. Statistisch jaarboek 2000 (in Dutch). Statistical year book 2000. Voorburg, Centraal bureau voor de statistiek.

7. Fogelholm M, Kukkonen-Harjula K. Does physical activity prevent weight gain--a systematic review. Obes Rev 2000;1(2):95-111.

8. Stichting NEVO. Nederlands voedingsstoffen bestand: NEVO tabel 1996. Dutch Nutrient Database. The Hague, Voorlichtingsbureau voor de voeding.

9. Van Rossum CTM, Ocke MC, Verschuren WMM, Seidell JC. Association between dietary fat and fiber with weight changes in Dutch men and women. Submitted.

10. Chagnon YC, Rankinen T, Snyder EE, Weisnagel SJ, Perusse L, Bouchard C. The human obesity gene map: the 2002 update. Obes Res 2003;11(3):313-367.

11. Shepherd R. Social determinants of food choice. Proc Nutr Soc 1999;58(4):807-812.

12. Korner J, Leibel RL. To eat or not to eat - how the gut talks to the brain. N Engl J Med 2003;349(10):926-928.

13. Flier JS. Obesity wars. Molecular progress confronts an expanding epidemic. Cell 2004;116(2):337-350.

14. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. Nature 1994;372(6505):425-432.

15. Chung WK, Power-Kehoe L, Chua M, Chu F, Aronne L, Huma Z, et al. Exonic and intronic sequence variation in the human leptin receptor gene (LEPR). Diabetes 1997;46(9):1509-1511.

16. Considine RV, Sinha MK, Heiman ML, Kriauciunas A, Stephens TW, Nyce MR, et al. Serum immunoreactive-leptin concentrations in normal-weight and obese humans. N Engl J Med 1996;334(5):292-295.

17. Wing RR, Sinha MK, Considine RV, Lang W, Caro JF. Relationship between weight loss maintenance and changes in serum leptin levels. Horm Metab Res 1996;28(12):698-703.

18. Rosenbaum M, Nicolson M, Hirsch J, Murphy E, Chu F, Leibel RL. Effects of weight change on plasma leptin concentrations and energy expenditure. J Clin Endocrinol Metab 1997;82(11):3647-3654.

19. Dubuc GR, Phinney SD, Stern JS, Havel PJ. Changes of serum leptin and endocrine and metabolic parameters after 7 days of energy restriction in men and women. Metabolism 1998;47(4):429-434.

20. Pratley RE, Nicolson M, Bogardus C, Ravussin E. Plasma leptin responses to fasting in Pima Indians. Am J Physiol 1997;273(3 Pt 1):E644-E649.

21. Kolaczynski JW, Considine RV, Ohannesian J, Marco C, Opentanova I, Nyce MR, et al. Responses of leptin to short-term fasting and refeeding in humans: a link with ketogenesis but not ketones themselves. Diabetes 1996;45(11):1511-1515.

22. 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(2):561-565.

23. Chen H, Charlat O, Tartaglia LA, Woolf EA, Weng X, Ellis SJ, et al. Evidence that the diabetes gene encodes the leptin receptor: identification of a mutation in the leptin receptor gene in db/db mice. Cell 1996;84(3):491-495.

24. Tartaglia LA, Dembski M, Weng X, Deng N, Culpepper J, Devos R, et al. Identification and expression cloning of a leptin receptor, OB-R. Cell 1995;83(7):1263-1271.

25. Montague CT, Farooqi IS, Whitehead JP, Soos MA, Rau H, Wareham NJ, et al. Congenital leptin deficiency is associated with severe early-onset obesity in humans. Nature 1997;387(6636):903-908.

26. Clement K, Vaisse C, Lahlou N, Cabrol S, Pelloux V, Cassuto D, et al. A mutation in the human leptin receptor gene causes obesity and pituitary dysfunction. Nature 1998;392(6674):398-401.

27. Baskin DG, Figlewicz LD, Seeley RJ, Woods SC, Porte D, Schwartz MW. Insulin and leptin: dual adiposity signals to the brain for the regulation of food intake and body weight. Brain Res 1999;848(1-2):114-123.

28. World Health Organization. Study group on prevention of diabetes mellitus. Prevention of diabetes mellitus. WHO Technical Report Series no. 844. 1994. Geneva, World Health Organization.

29. Porte D, Jr, Baskin DG, Schwartz MW. Leptin and insulin action in the central nervous system. Nutr Rev 2002;60(10 Pt 2):S20-S29.

30. Date Y, Kojima M, Hosoda H, Sawaguchi A, Mondal MS, Suganuma T, 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(11):4255-4261.

31. 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(8):1714-1719.

32. Shiiya T, Nakazato M, Mizuta M, Date Y, Mondal MS, Tanaka 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(1):240-244.

33. Blom W, Hendriks H, Stafleu A, De Graaf C, Kok F, Schaafsma G. Ghrelin and appetite responses after liquid breakfasts varying in energy content and corbohydrate structure. Int J Obes Relat Metab Disord 2003;27(Supplement 1):S35 (Abstract).

34. Cummings DE, Weigle DS, Frayo RS, Breen PA, Ma MK, Dellinger EP, et al. Plasma ghrelin levels after diet-induced weight loss or gastric bypass surgery. N Engl J Med 2002;346(21):1623-1630.

35. Ukkola O, Poykko S. Ghrelin, growth and obesity. Ann Med 2003;34(2):102-108.

36. Ahima RS, Prabakaran D, Mantzoros C, Qu D, Lowell B, Maratos-Flier E, et al. Role of leptin in the neuroendocrine response to fasting. Nature 1996;382(6588):250-252.

37. Hirsch J, Leibel RL. The genetics of obesity. Hosp Pract (Off Ed) 1998;33(3):55--9, 62-5, 69-70 passim. Review.

38. Chagnon YC, Chung WK, Perusse L, Chagnon M, Leibel RL, Bouchard C. Linkages and associations between the leptin receptor (LEPR) gene and human body composition in the Quebec Family Study. Int J Obes Relat Metab Disord 1999;23(3):278-286.

39. Van Rossum CTM, Hoebee B, Van Baak M, Mars M, Saris WHM, Seidell JC. Genetic variation in the LEPR gene, leptin and weight gain in Dutch adults. Obes Res 2003;11(3):377-386.

40. 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 (Colch) 1997;92(6):573-578.

41. 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(4):426-434.

42. Keim NL, Stern JS, Havel PJ. Relation between circulating leptin concentrations and appetite during a prolonged, moderate energy deficit in women. Am J Clin Nutr 1998;68(4):794-801.

43. Heini AF, Lara CC, 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(11):1084-1087.

44. 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(8):2685-2691.

45. Haffner SM, Mykkanen LA, Gonzalez CC, Stern MP. Leptin concentrations do not predict weight gain: the Mexico City Diabetes Study. Int J Obes Relat Metab Disord 1998;22(7):695-699.

46. Lindroos AK, Lissner L, Carlsson B, Carlsson LM, Torgerson J, Karlsson C, et al. Familial predisposition for obesity may modify the predictive value of serum leptin concentrations for long-term weight change in obese women. Am J Clin Nutr 1998;67(6):1119-1123.

47. Chessler SD, Fujimoto WY, Shofer JB, Boyko EJ, Weigle DS. Increased plasma leptin levels are associated with fat accumulation in Japanese Americans. Diabetes 1998;47(2):239-243.

48. Hodge AM, De Courten MP, Dowse GK, Zimmet PZ, Collier GR, Gareeboo H, et al. Do leptin levels predict weight gain?--A 5-year follow-up study in Mauritius. Mauritius Non-communicable Disease Study Group. Obes Res 1998;6(5):319-325.

49. Folsom AR, Jensen MD, Jacobs DR, Hilner JE, Tsai AW, Schreiner PJ. Serum leptin and weight gain over 8 years in African American and Caucasian young adults. Obes Res 1999;7(1):1-8.

50. Chu NF, Spiegelman D, Yu J, Rifai N, Hotamisligil GS, Rimm EB. Plasma leptin concentrations and four-year weight gain among US men. Int J Obes Relat Metab Disord 2001;25(3):346-353.

51. Arch JR, Stock MJ, Trayhurn P. Leptin resistance in obese humans: does it exist and what does it mean? Int J Obes Relat Metab Disord 1998;22(12):1159-1163.

52. Buffenstein R, Poppitt SD, McDevitt RM, Prentice AM. Food intake and the menstrual cycle: a retrospective analysis, with implications for appetite research. Physiol Behav 1995;58(6):1067-1077.

53. Riad-Gabriel MG, Jinagouda SD, Sharma A, Boyadjian R, Saad MF. Changes in plasma leptin during the menstrual cycle. Eur J Endocrinol 1998;139(5):528-531.

54. Schwartz MW, Morton GJ. Obesity: keeping hunger at bay. Nature 2002;418(6898):595-597.

55. Spiegelman BM, Flier JS. Obesity and the regulation of energy balance. Cell 2001;104(4):531-543.

56. Mars M, De Graaf C, Van Rossum CTM, De Groot CPGM, Seidell JC, F.J. K. Leptin and insulin responses to a four-day energy deficient diet in men with different weight history. Int J Obes 2003;27(5):574-581 (Chapter 6).

57. Rosner B, Willett WC. Interval estimates for correlation coefficients corrected for within-person variation: implications for study design and hypothesis testing. Am J Epidemiol 1988;127(2):377-386.

58. 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(6762):656-660.

59. Mars M, De Graaf C, Van Rossum CTM, De Groot CPGM, Seidell JC, F.J. K. Leptin and appetite responses induced by a four-day energy restriction; preliminary results. Appetite 2002;39(3):247 (Abstract).

60. World Health Organization. Report on a WHO Consultation on the Epidemiology of Obesity. Measuring Obesity - Classification and description of anthropometric data. WHO Technical Report Series no. 125. 1987. Warsaw, World Health Organization.

61. Deurenberg P, Tagliabue A, Schouten FJ. Multi-frequency impedance for the prediction of extracellular water and total body water. Br J Nutr 1995;73(3):349-358.

62. Ainsworth BE, Haskell WL, Leon AS, Jacobs DR, Jr, Montoye HJ, Sallis JF, et al. Compendium of physical activities: classification of energy costs of human physical activities. Med Sci Sports Exerc 1993;25(1):71-80.

63. Plasqui G, Kester AD, Westerterp KR. Seasonal variation in sleeping metabolic rate, thyroid activity, and leptin. Am J Physiol Endocrinol Metab 2003;285(2):E338-E343.

64. Liu J, Askari H, Dagogo-Jack S. Reproducibility of fasting plasma leptin concentration in lean and obese humans. Endocr Res 1999;25(1):1-10.

65. Friedman JM. The function of leptin in nutrition, weight, and physiology. Nutr Rev 2002;60(10 Pt 2):S1-14.

66. Toshinai K, Mondal MS, Nakazato M, Date Y, Murakami N, Kojima M, et al. Upregulation of Ghrelin expression in the stomach upon fasting, insulin-induced hypoglycemia, and leptin administration. Biochem Biophys Res Commun 2001;281(5):1220-1225.

67. Date Y, Murakami N, Toshinai K, Matsukura S, Niijima A, Matsuo H, et al. The role of the gastric afferent vagal nerve in ghrelin-induced feeding and growth hormone secretion in rats. Gastroenterology 2002;123(4):1120-1128.

68. Tamura H, Kamegai J, Shimizu T, Ishii S, Sugihara H, Oikawa S. Ghrelin stimulates GH but not food intake in arcuate nucleus ablated rats. Endocrinology 2002;143(9):3268-3275.

69. 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(3):904-907.

70. Stanforth PR, Gagnon J, Rice T, Bouchard C, Leon AS, Rao DC, et al. Reproducibility of resting blood pressure and heart rate measurements. The HERITAGE Family Study. Ann Epidemiol 2000;10(5):271-277.

71. Despres JP, Gagnon J, Bergeron J, Couillard C, Leon AS, Rao DC, et al. Plasma post-heparin lipase activities in the HERITAGE Family Study: the reproducibility, gender differences, and associations with lipoprotein levels. HEalth, RIsk factors, exercise Training and GEnetics. Clin Biochem 1999;32(3):157-165.

72. Gnardellis C, Trichopoulou A, Katsouyanni K, Polychronopoulos E, Rimm EB, Trichopoulos D. Reproducibility and validity of an extensive semiquantitative food frequency questionnaire among Greek school teachers. Epidemiology 1995;6(1):74-77.

73. Fogteloo J, Meinders E, Frolich M, McCamish M, Pijl H. The decline in plasma leptin in response to calorie restriction predicts the effects of adjunctive leptin treatment on body weight in humans. Eur J Intern Med 2003;14(7):415-418.

74. Torgerson JS, Carlsson B, Stenlof K, Carlsson LM, Bringman E, Sjostrom L. A low serum leptin level at baseline and a large early decline in leptin predict a large 1-year weight reduction in energy-restricted obese humans. J Clin Endocrinol Metab 1999;84(11):4197-4203.

75. 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 2001;226(11):963-977.

76. Halaas JL, Gajiwala KS, Maffei M, Cohen SL, Chait BT, Rabinowitz D, et al. Weight-reducing effects of the plasma protein encoded by the obese gene. Science 1995;269(5223):543-546.

77. Pelleymounter MA, Cullen MJ, Baker MB, Hecht R, Winters D, Boone T, et al. Effects of the obese gene product on body weight regulation in ob/ob mice. Science 1995;269(5223):540-543.

78. Farooqi IS, Jebb SA, Langmack G, Lawrence E, Cheetham CH, Prentice AM, et al. Effects of recombinant leptin therapy in a child with congenital leptin deficiency. N Engl J Med 1999;341(12):879-884.

79. 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(3):321-330.

80. Leibel RL. The role of leptin in the control of body weight. Nutr Rev 2002;60(10 Pt 2):S15-S19.

81. Fried SK, Ricci MR, Russell CD, Laferrere B. Regulation of leptin production in humans. J Nutr 2000;130(12):3127S-3131S.

82. 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(2):421-431.

83. 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(5 Pt 1):E855-E861.

84. Joannic JL, Oppert JM, Lahlou N, Basdevant A, Auboiron S, Raison J, et al. Plasma leptin and hunger ratings in healthy humans. Appetite 1998;30(2):129-138.

85. Larsson H, Elmstahl S, Berglund G, Ahren B. Evidence for leptin regulation of food intake in humans. J Clin Endocrinol Metab 1998;83(12):4382-4385.

86. Karhunen L, Lappalainen RI, Haffner SM, Valve RH, Tuorila H, Miettinen H, et al. Serum leptin, food intake and preferences for sugar and fat in obese women. Int J Obes Relat Metab Disord 1998;22(8):819-821.

87. Stratton RJ, Stubbs RJ, Elia M. Interrelationship between circulating leptin concentrations, hunger, and energy intake in healthy subjects receiving tube feeding. J Parenter Enteral Nutr 1998;22(6):335-339.

88. 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(4):771-776.

89. Verschuren WMM, van Leer EM, Blokstra A, Seidell JC, Smit HA, Buena de Mesquita A, et al. Cardiovascular disease risk factors in The Netherlands. Neth J Cardiol 1993;6(4):205-210.

90. Voedingsraad. Nederlandse Voedingsnormen 1989 (in Dutch). Dutch Nutritional Guidelines. 1989. The Hague, Voorlichtingsbureau voor de voeding.

91. Van Strien T. Eating behaviour personality traits and body mass. Thesis. 1986. Wageningen, Landbouwhogeschool.

92. Hill AJ, Rogers PJ, Blundell JE. Techniques for the experimental measurement of human eating behaviour and food intake: a practical guide. Int J Obes Relat Metab Disord 1995;19(6):361-375.

93. 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(3):273-286.

94. Flint A, Raben A, Blundell JE, 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(1):38-48.

95. De Graaf C. The validity of appetite ratings. Appetite 1993;21(2):156-160.

96. Stubbs RJ, Hughes DA, Johnstone AM, Rowley E, Reid C, Elia M, 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(4):405-415.

97. Haluzik M, Matoulek M, Svacina S, Hilgertova J, Haas T. The influence of short-term fasting on serum leptin levels, and selected hormonal and metabolic parameters in morbidly obese and lean females. Endocr Res 2001;27(1-2):251-260.

98. 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. p. 42-57.

99. World Health Organization. Report of a Joint FAO/WHO/UNU Expert consultation. Principles for the estimation of energy requirements. In: Energy and protein requirements. 2 ed. Geneva: World Health Organization; 1985. p. 34-52.

100. Voorlichtingsbureau voor de voeding. Zo eet Nederland 1998, resultaten van de voedselconsumptiepeiling 1998 (in Dutch). Results of the Dutch food consumption survey 1998. The Hague, Voorlichtingsbureau voor de Voeding.

101. Frankenfield DC, Rowe WA, Smith JS, Cooney RN. Validation of several established equations for resting metabolic rate in obese and nonobese people. J Am Diet Assoc 2003;103(9):1152-1159.

102. Black AE. Physical activity levels from a meta-analysis of doubly labeled water studies for validating energy intake as measured by dietary assessment. Nutr Rev 1996;54(6):170-174.

103. Jenkins AB, Markovic TP, Fleury A, Campbell LV. Carbohydrate intake and short-term regulation of leptin in humans. Diabetologia 1997;40(3):348-351.

104. Agus MS, Swain JF, Larson CL, Eckert EA, Ludwig DS. Dietary composition and physiologic adaptations to energy restriction. Am J Clin Nutr 2000;71(4):901-907.

105. Wang J, Liu R, Hawkins M, Barzilai N, Rossetti L. A nutrient-sensing pathway regulates leptin gene expression in muscle and fat. Nature JID - 0410462 1998;393(6686):684-688.

106. Sinha MK, Ohannesian JP, Heiman ML, Kriauciunas A, Stephens TW, Magosin S, et al. Nocturnal rise of leptin in lean, obese, and non-insulin-dependent diabetes mellitus subjects. J Clin Invest 1996;97(5):1344-1347.

107. Hilton LK, Loucks AB. Low energy availability, not exercise stress, suppresses the diurnal rhythm of leptin in healthy young women. Am J Physiol Endocrinol Metab 2000;278(1):E43-E49.

108. Elimam A, Marcus C. Meal timing, fasting and glucocorticoids interplay in serum leptin concentrations and diurnal profile. Eur J Endocrinol 2002;147(2):181-188.

109. Weigle DS, Cummings DE, Newby PD, Breen PA, Frayo RS, Matthys CC, 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(4):1577-1586.

110. O'Rahilly S. Leptin: defining its role in humans by the clinical study of genetic disorders. Nutr Rev 2002;60(10 Pt 2):S30-S34.

111. Chan JL, Heist K, DePaoli AM, Veldhuis JD, Mantzoros CS. The role of falling leptin levels in the neuroendocrine and metabolic adaptation to short-term starvation in healthy men. J Clin Invest 2003;111(9):1409-1421.

112. Racette SB, Kohrt WM, Landt M, Holloszy JO. Response of serum leptin concentrations to 7 d of energy restriction in centrally obese African Americans with impaired or diabetic glucose tolerance. Am J Clin Nutr 1997;66(1):33-37.

113. Cella F, Adami GF, Giordano G, Cordera R. Effects of dietary restriction on serum leptin concentration in obese women. Int J Obes Relat Metab Disord 1999;23(5):494-497.

114. 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(11):4162-4165.

115. De Roos NM, De Vries JHM, Katan MB. Serum lithium as a compliance marker for food and supplement intake. Am J Clin Nutr 2001;73(1):75-79.

116. Garrow JS. Methods for measuring energy intake and output. In: Energy balance and obesity in men. Amsterdam, North-Holland Publishing Company 1974. p. 47-91.

117. De Graaf C, Blom WAM, Smeets PAM, Stafleu A, Hendriks H. Biomarkers of satiation and satiety. Am J Clin Nutr 2004;79:946-961.

118. Levin BE, Dunn-Meynell AA, Banks WA. Obesity-prone rats have normal blood-brain barrier transport but defective central leptin signaling before obesity onset. Am J Physiol Regul Integr Comp Physiol 2004;286(1):R143-R150.

119. Banks WA, Altmann J, Sapolsky RM, Phillips-Conroy JE, Morley JE. Serum leptin levels as a marker for a syndrome X-like condition in wild baboons. J Clin Endocrinol Metab 2003;88(3):1234-1240.

120. Tatemoto K. Isolation and characterization of peptide YY (PYY), a candidate gut hormone that inhibits pancreatic exocrine secretion. Proc Natl Acad Sci 1982;79(8):2514-2518.

121. Batterham RL, Cowley MA, Small CJ, Herzog H, Cohen MA, Dakin CL, et al. Gut hormone PYY(3-36) physiologically inhibits food intake. Nature 2002;418(6898):650-654.

122. Halford JC, Blundell JE. Pharmacology of appetite suppression. Prog Drug Res 2000;54:25-58.

123. Diplock AT, Aggett PJ, Ashwell M, Bornet F, Fern EB, Roberfroid MB. Scientific concepts of funcitonal foods in Europe: concensus document. British Journal of Nutrition 1999;81(Supplement 1):S1-S27.

124. Morley JE. Decreased food intake with aging. J Gerontol A Biol Sci Med Sci 2001;56 Spec No 2:81-88.

125. Mowe M, Bohmer T. Nutrition problems among home-living elderly people may lead to disease and hospitalization. Nutr Rev 1996;54(1 Pt 2):S22-S24.

Summary

The last decades the prevalence of obesity has been increasing. This is mainly a consequence of a modern lifestyle; large amounts of palatable high calorie food and a limited requirement for physical activity. However, large individual differences are observed in the susceptibility to weight gain. Presumably, lifestyle factors in combination with a number of genes are responsible for these differences. Poor energy intake regulation is one of the possible mechanisms behind a large susceptibility to weight gain.

Leptin is a hormone that is produced by adipose tissue and is secreted in the blood. Short-term severe energy restriction causes serum leptin concentrations to drop very rapidly. Animal studies suggested that this decline has a key-role in the prevention of starvation. Next, failures in leptin metabolism are shown to have marked consequences for energy intake regulation and weight maintenance. We, therefore, hypothesized that if this leptin decline has a functional role in the restoration of energy balance and if its magnitude is an individual trait, then it might be a biomarker for the individual susceptibility to weight gain. Thus, the main aim of this thesis was to investigate whether the acute decline in leptin to energy restriction is a biomarker for the susceptibility to weight gain. In view of this aim three research questions were formulated:

1 Is the leptin decline to energy restriction a reliable measure?
2 Is the leptin decline to energy restriction associated with genetic variation?
3 Is the leptin decline to energy restriction associated with the susceptibility to weight gain or its intermediate endpoints?

To answer these questions, we conducted three controlled intervention studies: the *LEPTOS*-study (Chapter 2), the *'Eetlust'*-study (Chapter 3, 4, 6), and the *KALOZ*-study (Chapter 5). During each intervention a group of healthy men underwent dietary energy restriction during a few days. During these days they consumed one-third of their estimated energy needs. Before and after the intervention serum leptin concentrations were measured; in all three studies we observed a significant decline in leptin after a few days of energy restriction. An overview of the specific study designs is given in Table 1.

Table 1 Overview of the subjects, design, and endpoints of the three intervention studies.

Study name	Design	Endpoints (Chapter number)
LEPTOS-study	20 men (34-50 y, 22.9-39.8 kg/m^2) Reliability study; long-term (1½y) + short-term (3 wk) 65% energy restriction 2 days, 3 times per subject	Leptin decline (2)
"Eetlust"-study	44 men (31-52 y, 22.7-39.8 kg/m^2) Controlled intervention, linked to cohort data 65% energy restriction 4 days	Leptin decline and polymorphisms in the leptin receptor (3) Leptin decline and appetite scores (4) Leptin decline and weight history (6)
KALOZ-study	35 men (19-29 y 19.8-24.9 kg/m^2) Intervention; partly controlled 62% energy restriction 2 days, ad libitum intake 2 days	Leptin decline and Ad libitum energy intake (5)

Research Question 1:
Is the leptin decline to energy restriction a reliable measure?

First of all, we performed a reliability study (*LEPTOS*-study) during which we measured the leptin decline three times; at baseline, after 1½ years and after another 3 weeks. Additionally, we assessed the reliability of the insulin and ghrelin response. In Chapter 2, we describe that the leptin decline had a relatively high reliability (Intra Class Correlation [95%-Confidence interval]=0.66 [0.33; 0.85]) on the short-term compared to the decline in insulin (ICC=0.45 [0.03; 0.74]) and the increase in ghrelin (ICC=0.34 [0; 0.67]). On the long-term, however, the acute leptin showed a lower reliability (ICC=0.34 [0; 0.67]).

Research Question 2:
Is the leptin decline to energy restriction associated with genetic variation?

We hypothesized that the leptin responsiveness to energy restriction is affected by the functionality of the leptin receptor (*"Eetlust"*-study). The analyses described in Chapter 3, explored the effect of three common polymorphisms in the leptin receptor gene (Lys109Arg, Gln223Arg and Lys656Asn) on the acute leptin decline. The analyses revealed no statistically significant differences in leptin response between genotypes, *i.e.* between carriers and non-carriers of the mutant allele. Moreover, we calculated that 140 subjects (70 in each group) would be needed to statistically prove that a difference

of 4.4% (SD 1.3%) in acute leptin decline exists between groups. Theoretically, this number can be reduced to 30, if the intervention is repeated three times.

Research Question 3:
Is the leptin decline to energy restriction associated with the susceptibility to weight gain or its intermediate endpoints?

In Chapter 4, 5 and 6 we investigated whether the acute decline in leptin is a biomarker for weight gain with different (intermediate) endpoints. First, we hypothesised that the decline in leptin is related to subjective appetite ratings, *i.e.* ratings reflecting hunger, fullness, desire to eat, prospective consumption, and total appetite (Chapter 4). We observed that the magnitude of the acute decline was indeed positively associated with the increase in hunger ($r=0.42$; $P<0.05$), desire to eat ($r=0.39$; $P<0.05$), and total appetite ($r=0.35$; $P<0.05$). Additionally, we observed in this Chapter that the inverse association between leptin levels and fasting subjective appetite became stronger as energy restriction was prolonged (for total appetite: day_0 $r=-0.15$; ns | day_2 $r=-0.31$; $P<0.05$ | day_4 $r=-0.41$; $P<0.01$).

Second, we hypothesized that if the acute decline after energy restriction is a starvation signal, then its magnitude should be associated to the amount of energy that is compensated in the days following energy restriction (Chapter 5). In the *KALOZ*-study we observed that leptin levels declined by 24% [95%-Confidence Interval: -33%; -15.9%] after energy restriction. Subsequently subjects showed compensatory behaviour; they ate 143 ± 27% (18.3 ± 2.8MJ) and 124 ± 20% (16.0 ± 2.5MJ) of their estimated energy needs on the first and second day after energy restriction, respectively. However, we did not observe an association between the magnitude of the leptin decline and energy intake compensation ($r=0.22$; ns).

Third, we investigated if individuals with stable weight show larger leptin declines to energy restriction than individuals who gained weight (Chapter 6). Proportionally, an 8% smaller decrease in leptin was observed in men with retrospective weight gain. This difference in proportional decrease was not found in a subgroup in which the difference in retrospective weight gain was more pronounced. Therefore we concluded that our study did not provide convincing evidence for the hypothesis that men who gained weight are less leptin responsive to changes in energy balance than men who were weight stable.

General discussion

In Chapter 7 we reflect on several issues concerning the methodological aspects of our studies. First, we reflect on several methodological considerations of our intervention studies, such as the study design, study subjects, intervention strategy, the potential biomarker; i.e. the acute leptin decline, and the (intermediate) endpoints; i.e. subjective appetite, energy intake compensation and weight gain. Especially, the choice of a heterogeneous group of study subjects and the intervention strategy, including energy restriction rather than energy surplus, is discussed Next, we speculate on the implications of our results for leptin, energy balance restoration and weight gain. Subsequently, considering a theoretical framework, we evaluate whether the acute decline in leptin to energy restriction is a biomarker for the susceptibility to weight gain. We conclude that the acute decline in leptin after energy restriction is not a good biomarker for the susceptibility to weight gain. However, it may be a valid marker for the state of energy balance and for individual differences in appetite feelings during severe energy restriction. Lastly, several directions for future research are given. For example, other (new) biomarkers may be developed with the use of new biological techniques, and the role of leptin (and other hormones involved in food intake regulation) may be investigated in involuntary weight loss.

Samenvatting

Deze 'Nederlandse samenvatting' is geschreven voor niet-vakgenoten. Het is een vertaling van een stuk van de introductie en de Engelse samenvatting van dit proefschrift, waarbij vaktaal zoveel mogelijk is vervangen door algemeen taalgebruik.

Het probleem van overgewicht

Overgewicht en ernstig overgewicht nemen in de wereld epidemische vormen aan. Op dit moment heeft 45% van de Nederlandse mannen en 35% van de Nederlandse vrouwen overgewicht. Van alle Nederlandse volwassenen heeft zo'n 10% ernstig overgewicht. De gezondheidsraad schat dat in 2015 zo'n 15 tot 20% van de Nederlanders ernstig overgewicht heeft. Het hebben van overgewicht verhoogt de kans op een aantal ernstige chronische ziekten, zoals ouderdomssuikerziekte, hoge bloeddruk en hart- en vaatziekten.

Overgewicht wordt veroorzaakt door langere tijd meer energie (calorieën) te consumeren dan te verbruiken. Het wordt gezien als een ziekte met vele oorzaken. Eén van de oorzaken is een hoge erfelijke gevoeligheid voor het aankomen in gewicht. Op dit moment wordt hier veel onderzoek naar gedaan. Waarschijnlijk is het zo dat er een aantal stukjes op het erfelijk materiaal, gecombineerd met levensstijl, er voor zorgen dat sommige mensen sneller aankomen in gewicht dan anderen. Met levensstijl bedoelen we dan weinig lichaamsbeweging en hoge consumptie van voedingsmiddelen die veel energie bevatten.

De regulatie van de voedselinneming

De voedselinneming wordt door verschillende aspecten bepaald, zoals de prijs en de smaak van voedsel, maar ook psychologische aspecten als gemoedstoestand en de mening over bepaalde producten. Eén van de belangrijkste aspecten die de voedselinname bepaalt, is het lichaam zelf. Wanneer iemand lange tijd niet heeft gegeten, geeft het lichaam zelf signalen af naar de hersenen om te gaan eten. Belangrijke signaalstoffen zijn hierbij insuline, leptine en ghreline. In deze Nederlandse samenvatting zal ik mij met name richten op leptine.

De signaalstof "leptine"

Leptine is een signaalstof die aangemaakt wordt door het lichaamsvet. Hoe meer lichaamsvet je hebt hoe meer leptine je ook in je bloedbaan hebt. De stof wordt vanuit het lichaamsvet afgescheiden in de bloedbaan en gaat vervolgens naar de hersenen. Wanneer de hoeveelheid leptine in de hersenen snel omhoog gaat, zal het een signaal geven aan de hersenen om te stoppen met eten. Wanneer het snel omlaag gaat zal het

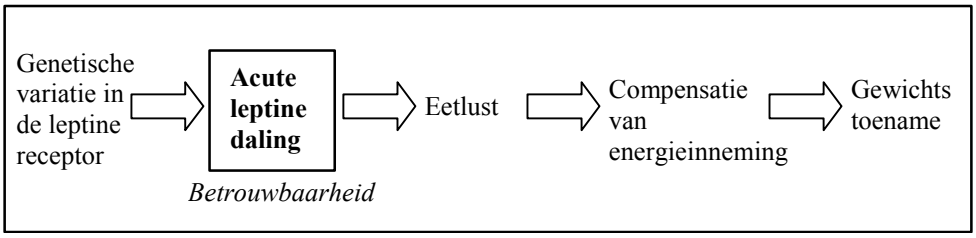
een signaal geven om juist méér te gaan eten. Het hormoon lijkt voornamelijk snel te dalen als je veel minder eet dan dat je normaal doet. De hersenen krijgen dan het signaal om weer meer te gaan eten. Onderzoekers denken dat deze daling in de hoeveelheid leptine een belangrijk signaal is voor de hersenen om allerlei processen in werking te zetten om ondervoeding te voorkomen. De stof zou ook een belangrijke rol kunnen spelen bij het op elkaar afstemmen van de hoeveelheid energie die gegeten wordt en de hoeveelheid energie die verbrand wordt door het lichaam. Het zou kunnen dat dit systeem bij mensen die snel aankomen in gewicht niet goed werkt.

Het onderzoek in dit proefschrift

In dit proefschrift staat de acute leptine-daling na een energiearme voeding centraal. De hoofdvraag van het onderzoek was:

Is de acute daling van leptine na een energiearme voeding een biologische indicator voor de gevoeligheid voor het ontwikkelen van overgewicht?

Met andere woorden: kan je door het meten van deze leptine-daling bij een persoon voorspellen of deze gevoelig is voor het ontwikkelen van overgewicht? Om deze vraag te beantwoorden hebben we deze opgedeeld in verschillende deelvragen en vervolgens hebben we drie onderzoeken uitgevoerd: het *Eetlust*-project, het *LEPTOS*-onderzoek en het *KALOZ*-onderzoek. In alle drie de onderzoeken heeft een groep mannen een energiearme voeding geconsumeerd. Voor, tijdens, en na deze voeding zijn verschillende metingen verricht die noodzakelijk waren om de onderzoeksvragen te beantwoorden waaronder de leptine-concentraties in het bloed. In het schema in figuur 1 is afgebeeld in welke stappen wij onze onderzoeksvraag hebben opgedeeld. Per blokje zullen in de volgende paragraaf de aanpak en de resultaten van het onderzoek worden uitgelegd.



Figuur 1. Schematische weergave van het onderzoek dat beschreven staat in dit proefschrift.

Aanpak en resultaten van het onderzoek

Allereerst hebben we onderzocht of de leptine-daling een betrouwbare maat is. Is de daling in leptine hetzelfde bij één persoon als je deze meerdere keren meet? Hiervoor hebben we een zogenaamde betrouwbaarheidsstudie uitgevoerd (*LEPTOS*-onderzoek). Tijdens dit onderzoek hebben 20 gezonde mannen drie keer twee dagen een energiearme voeding geconsumeerd. Voor en na deze voeding hebben we leptine-concentraties gemeten. Tussen de eerste en de tweede energiearme voeding zat 1½ jaar en tussen de tweede en de derde energie arme voeding drie weken, zodat we naar een lange en een korte termijn effect konden kijken. We zagen dat de acute daling in leptine op korte termijn een betrouwbare maat was, maar dat dit op lange termijn niet het geval was.

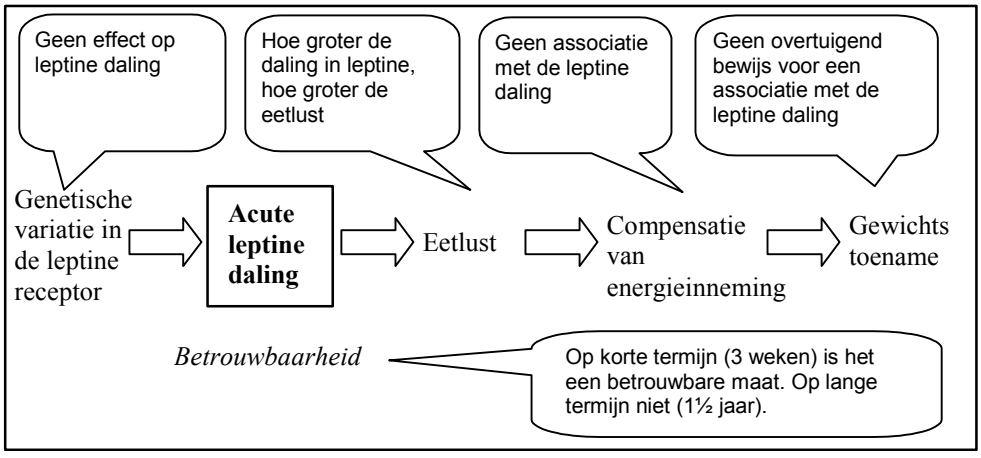
Daarna hebben we gekeken of variatie in een specifiek stukje van het erfelijk materiaal (DNA) een effect had op de leptine-daling. Voor dit onderzoek hebben we gebruik gemaakt van het erfelijk materiaal dat we verzameld hadden van de 44 mannen van het Eetlust-project, die vier dagen op een energiearme voeding waren geweest. Uit dit onderzoek bleek dat dit specifieke stukje erfelijk materiaal geen effect had op de leptine-daling. Dit betekent echter niet dat er geen erfelijke factoren zijn die de daling in leptine regelen.

Vervolgens hebben we gekeken of de leptine-daling geassocieerd was met de hoeveelheid honger die de deelnemers tijdens het onderzoek ervaarden. Met andere woorden: heeft iemand met een grote daling in leptine ook daadwerkelijk meer honger tijdens een energiearme voeding? Voor deze vraag hebben we ook gebruik gemaakt van de gegevens die we verzameld hebben tijdens het *Eetlust*-project. Aan alle 44 mannen hebben we door middel van een kort vragenlijstje, gevraagd aan te geven hoeveel honger ze hadden op een schaal van 1 tot 10. Vervolgens hebben we gekeken of deze antwoorden samen hangen met de leptine-daling. Dit bleek ook zo te zijn; hoe groter de daling in leptine hoe meer honger de deelnemers ervaarden.

Deze uitkomst bracht ons op het idee om een volgend onderzoek uit te voeren: het *KALOZ*-onderzoek. Hierin onderzochten we of mensen met een grote leptine-daling tijdens een energiearme voeding meer energie gingen eten tijdens de dagen vlak na het de energiearme voeding, dan mensen met een kleine daling. Voor dit onderzoek

hebben 35 mannelijke studenten twee dagen een energiearme voeding gevolgd en vervolgens hebben zij twee dagen alles opgeschreven wat zij aten. Het ontbijt en de warme lunch werden bij ons op de universiteit gegeten, overige voedingsmiddelen zoals avondeten en tussendoortjes mochten de deelnemers mee naar huis nemen. We zagen dat de deelnemers eerste dag na de energiearme voeding gemiddeld 43% meer energie innamen dan normaal en tweede dag was dit gemiddeld 24% meer energie dan normaal. Helaas vonden we niet dat deelnemers met een grote leptine-daling meer gingen eten.

Tenslotte hebben we onderzocht of mensen met een stijgend gewicht een kleinere daling in leptine laten zien dan mensen die hun gewicht relatief stabiel kunnen houden. Personen met een gewichtsverandering van meer dan één kilogram per jaar waren gedefinieerd als personen met een stijgend gewicht. Personen die niet meer dan één kilogram in lichaamsgewicht waren gestegen of gedaald waren gedefinieerd als personen met een stabiel gewicht. In dit onderzoek hebben we gebruik gemaakt van gegevens die tijdens het Eetlust-project zijn verzameld en van gegevens over gewichtsverleden die door het Rijksinstituut voor Volksgezondheid en Milieu zijn verzameld in het MORGEN-project. Tijdens dit onderzoek hebben we geen overtuigend bewijs gevonden dat er een verschil in leptine-daling is tussen mensen met een stijgend gewicht en mensen met een stabiel gewicht. In figuur 2 zijn alle resultaten van het proefschrift nog eens kort en overzichtelijk samengevat.



Figuur 2. Schematische weergaven van de resultaten van het onderzoek beschreven in dit proefschrift.

Conclusie van dit proefschrift

Nadat we deze resultaten op een rijtje hebben gezet en deze hebben vergeleken met internationale standaarden voor een biologische indicator, hebben wij geconcludeerd dat de acute daling in leptine na een energiearme voeding geen goede indicator is voor de gevoeligheid voor het ontwikkelen van overgewicht. Maar deze daling zou misschien wel een valide indicator kunnen zijn voor de energiebalans (als iemand te veel of te weinig eet in verhouding tot zijn/haar energiegebruik) of voor de hoeveelheid honger die iemand zou kunnen krijgen tijdens een energiearme voeding.

Tot slot

Er zijn verschillende beperkingen aan het onderzoek dat we hebben uitgevoerd. Zo zouden we bijvoorbeeld de onderzoeksopzet, de proefpersonen of de energiearme voeding anders gekozen kunnen hebben. Daarnaast hadden we ook een andere biologische indicator kunnen kiezen en ook andere uitkomstmaten dan eetlust, compensatie van energie-inneming en stijging in lichaamsgewicht. Met name de keuzes om een homogene groep te onderzoeken en om een energiearm in plaats van een energierijke voeding te onderzoeken zijn belangrijke punten van discussie. Deze punten komen uitgebreid aan de orde in de Engelstalige discussie, maar gaan te ver om er in de Nederlandse samenvatting op in te gaan. Ook wordt in deze discussie gespeculeerd en gefilosofeerd over de mogelijke consequenties van ons onderzoek voor de wetenschap en haar kennis over leptine, de regulatie van energie-inneming en energiegebruik en toename in lichaamsgewicht.

Aan het eind van de discussie hebben we enkele suggesties voor vervolgonderzoek. Op dit moment vindt er veel onderzoek plaats op het gebied van eetlusthormonen zoals leptine. Er worden steeds meer stoffen in het bloed ontdekt die betrokken zijn bij de regulatie van het lichaamsgewicht en ook de methoden om deze stoffen in het bloed te bepalen worden steeds beter. Het zou dus goed zijn om in de toekomst andere biologische indicatoren dan de leptine-daling te onderzoeken. Daarnaast zou het ook heel interessant zijn om de rol van de leptine-daling bij onvrijwillige gewichtsafname te bestuderen. Bijvoorbeeld bij oudere mensen die door gewichtsverlies een hogere kans hebben op ziekten en opname in een verzorgings- of verpleeghuis.

Dankwoord

Allereerst wil ik graag alle 85 mannen bedanken die voor dit onderzoek enkele dagen (soms meerdere keren) op een energiearme voeding hebben geleefd. De voeding bestond uit poeders en repen… leg dat maar eens uit aan je collega´s en vrienden! Het was elke keer weer geweldig om te zien hoe verschillend iedereen met zo´n voeding om gaat. Ik hoop dat jullie, naast het afzien tijdens de dieetdagen, net als ik veel plezier hebben beleefd aan het onderzoek. Mannen, hartstikke bedankt!

Naast de deelnemers hebben de afgelopen 4½ jaar hebben een heleboel andere waardevolle mensen meegewerkt aan het onderzoek, waaronder mijn (co)promotoren Kees de Graaf, Lisette de Groot en Frans Kok. Kees, bij jou kon ik altijd terecht voor de dagelijkse vragen; na een bezoekje aan jou waren de meeste vragen op mijn "boodschappenbriefje" weer beantwoord. Bedankt dat ik altijd binnen kon vallen! Lisette, vooral tijdens het schrijven heb jij veel ingebracht. Ik was altijd erg blij met je kritische blik en de verbeteringen en suggesties die je aanbracht. Ook vond ik de ritjes naar Eindhoven altijd erg gezellig. Frans, jij wilde vaak het naadje van de kous weten (of testen of ik deze wist te vinden?). Je grote interesse in mijn project heb ik altijd erg gewaardeerd.

En dan de andere mensen van het NWO-Obesitas project waar ik in Eindhoven regelmatig overleg mee had, met name Jaap Seidell en Caroline van Rossum: Jaap, jij hebt dit project destijds geïnitieerd en opgeschreven. Tijdens het project heb je meer de grote lijnen in de gaten gehouden. Caroline, ik heb het altijd heel leuk gevonden om met je samen te werken en heb ook veel van je geleerd; jij ook bedankt voor je kritische commentaar op de documenten. Ook de andere die bij dit NWO-Obesitas aanwezig waren wil ik bedanken: Barbara Hoebee, Esther van Beek, Evert van Schothorst, Jaap Keijer, Joost Oomen, en Marleen van Baak. Zo´n multidisciplinair team houdt je met de beentjes op de vloer!

Mijn eerste twee studies, het *Eetlust*-project en het *LEPTOS*-onderzoek vonden plaats binnen het MORGEN-project in Doetinchem. Ik wil hiervoor alle mensen van het MORGENproject bedanken, waaronder Monique Verschuren, voor het beschikbaar stellen van de noodzakelijke gegevens voor de interventiestudies. Het Eetlust-onderzoek werd uitgevoerd bij de GGD in Doetinchem en ik wil dan ook Caroline de Rover, Ina , Irma en de anderen medewerkers hartelijk bedanken voor hun gastvrijheid

en inzet tijdens het onderzoek (en daarna). Vooral het zetten van de infuusjes was een grote uitdaging…

Tijdens alle drie de interventies hebben veel mensen van de afdeling Humane Voeding mij geadviseerd en ondersteund. Mensen van de diëtetiek, het priklab, het chemisch lab, de lichaamsmetingen, de statistiek (vooral powerberekeningen) en de IT: Els Siebelink, Saskia Meyboom, Karin Borgonjen, Lieneke Kolmus, Joke Barendse, Lucy Okma, Betty Struijs, Paul Hulshof, Jan Burema, Pieter van 't Veer, Dirk Jochems, Ben Scholte, Anne Baars, Dione Bouchaut en alle anderen die hebben bijgedragen. Mensen allemaal hartstikke bedankt! Zonder jullie kennis zijn de (altijd ambitieuze) voedingsonderzoeken onmogelijk.

Ook afstudeerstudenten hebben heel veel werk verzet tijdens mijn onderzoek: Maartje Jansen, Carolien Bouwman, Petra Boluijt, Bas Kemps en Chantal Vrijhof. Hartstikke bedankt voor jullie inzet en ik heb het misschien nooit gezegd, maar ik heb veel geleerd van jullie!!

Astrid, Rosalie, Anouk, Linette en Marleen, spraken we nou af om Roborally te spelen of voor lotgenoten contact? In ieder geval bedankt voor alle gezellige avonden. Ook de andere (oud)collega´s van het John Snow en Agrotechnion wil ik graag bedanken voor de nodige (fantasievolle) kletspraat. Andrea, Amely, Annet, Ellen, Judith, Karen, Kim, Kristel, Liesbeth, Marielle, Maureen, Maria, Natasja, Wieke; en de rest; allemaal bedankt!

Marleen en Marleen, sinds we alledrie aangesteld waren als AIO zijn we iedere drie maanden naar de sauna gegaan om te relaxen. Misschien heeft het geholpen… het kan in ieder geval geen kwaad om het vol te houden! Gie, ons afstudeervak is af! Gie en Rik, het wordt nou wel weer eens tijd voor een activiteit met alle Witches…

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Opa en Oma van Truijen, Oma Mars, Kim, Kasper, Guurt, Rosa, Erica, Björn, Danielle, Paul, Naomi, Lisette, Ronald, Marc, Ursula, Johan, de familie Mars en familie Van Truijen. Het was soms lastig uit te leggen wat ik Wageningen aan het doen was. Was het nou studeren of toch werken? Ik ben blij dat jullie altijd veel interesse hebben getoond en gewoon hebben gevraagd wat ik deed. Ik hoop dat de Nederlandse samenvatting in dit boekje jullie een flink stuk op weg kan helpen...

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Lieve Martijn, dank je wel voor alles; op weg naar de volgende uitdaging!

Monica

Hilversum, 9 augustus 2004

Curriculum Vitae

About the author

Monica Mars was born on the 6th of April 1976 in Alkmaar, The Netherlands. She grew up in Graft-de Rijp. In 1994, she finished her secondary school (Athenaeum) at the "Da Vinci College" in Purmerend. In this year, she started her studies on Human Nutrition at the Agriculture University of Wageningen (now Wageningen University). During her studies, she specialized in epidemiology and nutritional behaviour. As part of her studies she conducted an epidemiological analysis on "Fish fatty acids and colorectal adenomas" and performed a sensory study in children "The preference for sweetness and the consumption of sugar by four and five year olds". She ended her studies with a 6-month internship at the National Public Health Institute (KTL) in Helsinki, Finland, were she performed a data analysis on mineral intake and risk of stroke in the Alpha Tocopherol Beta Carotene study. Following her studies, she started her PhD-project in 1999, which is described in this thesis. These studies were part of a multi-disciplinary project focussed on the contribution of genetic variation to diet induced weight gain financed by the Dutch Association for Scientific research (ZonMW 980-10-007). The research was conducted in cooperation with the National Institute of Public Health and the Environment (RIVM) and Maastricht University. During her PhD she joined several congresses and international courses within the educational of the VLAG (Food Technology, Agrobiotechnology, Nutrition and Health Sciences) graduate school. She was part of the organisation of the PhD-study Tour in 2001 to Italy, Switzerland, and Germany. In March 2003 she was selected for the 9th European Nutrition Leadership Programme.

Peer reviewed publications

Van Rossum CTM, Hoebee B, Van Baak M, **Mars M**, Saris WHM, Seidell JC. Genetic variation in the LEPR gene, leptin and weight gain in Dutch adults. Obesity Research 2003;11(3):377-386.

Mars M, De Graaf C, Van Rossum CTM, De Groot L, Seidell JC, Kok F.J. Leptin and insulin responses to a four-day energy deficient diet in men with different weight history. International Journal of Obesity 2003;27(5):574-581 (**Chapter 6**).

Mars M, van Rossum CTM, de Graaf C, Hoebee B, de Groot CPGM, Kok FJ. Leptin responsiveness to energy restriction: genetic variation in the leptin receptor gene. Obesity Research 2004;12(3):442-444 (**Chapter 3**).

Liem DG, **Mars M**, C de Graaf. Consistency of sensory testing in young children, Food Quality and Preference 2004;15(6):541-548.

Liem DG, **Mars M**, C de Graaf. Sweet preferences and sugar consumption of 4- and-5-year-old children: role of parents, Appetite *in press*.

Abstracts

Liem DG, **Mars M**, De Graaf C. Reliability of indirect scaling tests with respect to the intensity and pleasantness of sugar (in 4 and 5 year olds). Chemical Senses 2002;25:637

Mars M, De Graaf C, Van Rossum CTM, De Groot CPGM, Seidell JC, Kok FJ. Leptin and insulin responses to a four-day energy deficient diet in men with different weight history. Dutch Neuro Endo meeting, Doorwerth, June 4-7, 2002

Mars M, De Graaf C, Van Rossum CTM, De Groot CPGM, Seidell JC, Kok FJ. Leptin and appetite responses induced by a four-day energy restriction; preliminary results. Appetite 2002;39:(3)247

Liem DG, **Mars M**, De Graaf C. Parental influences on sugar consumption and sweet preferences of their children. Appetite 2002;39:(3)246

Blankenstein MA, Martens F, Frans M; Lomecky M, **Mars M**. Evaluation of two methods for the assessment of Ghrelin in serum. Euregio Congress of Clinical Chemistry 8-10 October 2002, Aachen, Germany

Mars M, De Graaf C, De Groot CPGM, Kok FJ. Long-term and short-term reproducibility of the leptin response after acute energy restriction. International Journal of Obesity 2003;27:(supplement 1) S138

Other publications

Bakker-Zierikzee A, Geelen A, **Mars M**, Pellis L, Rutten R, Wark P. Diversiteit binnen voedingsonderzoek in Europa. Voeding Nu 2002;(9):26-7.

Educational programme

	Organizing Institute	Year(s)
AIO-days	VLAG	1999
Journal Club	Division of Human Nutrition, WUR	1999-2002
Regulation of food intake course	VLAG	2000
Genetic variation and the consequences for health	VLAG, Nutrim	2000
Voedingsdagen, Papendal		2000, 2001, 2003
European Congress of Obesity		2000, 2001, 2003
Congress "Society for the Study of Ingestive Behavior"	SSIB	2000
Congress "Food Choice"		2000, 2002
Organizing & supervising thesis work	OWI	2000
NWO-symposium voeding en chronische ziekten	NWO	2000, 2001, 2002, 2003, 2004
Nutrition and lifestyle epidemiology course	VLAG	2001
NASO-BAASO meeting Antwerp	NASO/BAASO	2001
Neuro-endo meeting Doorwerth		2001, 2002
Symposium “Genetische oorzaken van overgewicht”	RIVM	2001
PhD-excursion to Switzerland, Italy, and Germany	Division of Human Nutrition, WUR	2001
Course "From Nutrogenomics to healthy food"	VLAG, NUTRIM	2002
Written English course	CENTA	2002
NASO-meeting	NASO	2002, 2003
European Nutrition Leadership Programme	ENLP	2003

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