Obesity and food reward regulation by the brain: genetic and environmental factors

Stieneke Doornweerd

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General Introduction

In the last 40 years the world has changed from an era in which underweight was twice as common as obesity, to a time when more people are obese than underweight [1]. Currently, more than 600 million people are obese worldwide, and numbers are still growing. Whereas previously, obesity was predominantly a problem in Western high-income countries, its prevalence is now emerging in developing countries. In the Netherlands, 12 percent of men and 16 percent of women are obese (i.e. body mass index (BMI) ≥30 kg/m^2), while 54 percent and 46 percent are overweight (i.e. BMI ≥25 kg/m^2), respectively [2]. Adiposity increases the risk of type 2 diabetes, cardiovascular disease and several forms of cancer [3]. Furthermore, obese people are more likely to suffer from joint problems, sleep apnoea and depression. Due to these co-morbidities, adiposity strongly increases the risk of mortality [4, 5].

Despite the abundance of diet books and heavily promoted schemes for rapid weight loss, lifestyle interventions have been largely unsuccessful in maintaining healthy body weights [6]. More long-term success on weight loss is obtained with bariatric surgery, such as gastric banding and bypass [7]. However, surgical treatments are expensive and not without risks, given the association with peri-operative complications and long-term nutritional deficiencies [8]. Also the search of anti-obesity medication has been rather disappointing, mostly because of observed, sometimes severe, side effects [9]. Therefore, it is important to further elucidate risk factors that contribute to overeating and weight gain, since this might add in the development of new prevention and treatment strategies against obesity.

FOOD INTAKE REGULATION BY THE BRAIN

Obesity results if there is an imbalance between energy intake and energy expenditure. Energy expenditure can be divided into resting metabolic rate, which is the amount of energy burnt while being at complete rest, and physical activity, which comprises all additional activity such as standing, walking around and the performance of exercise. If energy intake exceeds energy expenditure in the long run, the surplus of energy is stored as body mass, of which 60-80 percent as body fat [10].

To maintain the body in a state of energy balance (i.e. homeostasis), intake and expenditure of energy are controlled by the central nervous system through complex interactions with key informative sites of the body [11]. For the control of food intake, the brain receives information about short-term meal-related energy intake mainly from the gustatory system and the gastro-intestinal tract. These signals are mediated either by neural connections provided by the autonomic nervous system, or by hormones and metabolites. Whereas some of the peptides produced in the gut stimulate feeding and the initiation of a meal, such as ghrelin, others mediate satiety and the termination of a meal, such as cholecystokinin and glucagon-like peptide-1 [12]. For the regulation of food intake in the long-term, the brain receives information about

the amount of fat stored in the body by the hormone leptin, which is secreted by adipocytes in proportion to the amount of body fat mass. Increased leptin signalling limits food intake and supports energy expenditure through negative feedback in the brain. Key brain sites that regulate this short-term and long-term homeostatic feeding are the brainstem and the hypothalamus (Figure 1) [13].

In addition to this homeostatic feeding, palatable food is also consumed for its hedonic properties independent of energy status [14]. Such reward-related eating is highly influenced by external cues that signal the availability of palatable foods, such as its sight, smell or taste. These reward signals can override the homeostatic signals of the body, which may result in energy intake exceeding energy requirements. Numerous brain areas are involved in the processing of food reward, including cortico-limbic and midbrain areas such as the insula, amygdala, striatum, nucleus accumbens and orbitofrontal cortex (Figure 1) [15]. Neurotransmitters that are responsible for these neural processes are mainly dopamine and opioids. Signalling by dopamine is thought to contribute to the 'wanting' of food (e.g. motivational aspects and craving), whereas opioids are involved in the 'liking' of food (e.g. hedonic value or palatability) [16]. Brain circuits involved in food reward are highly integrated with those regulating homeostatic feeding, for example energy depletion increases the rewarding value of food. Since the obesity epidemic is characterized by energy intakes that go beyond metabolic needs, reward-related hedonic feeding is likely to be an important contributor to weight gain and the development of obesity.

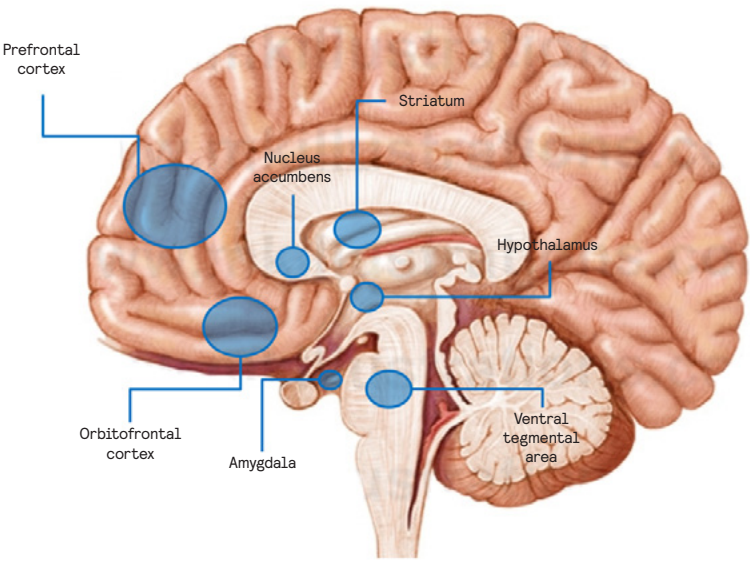

FIGURE 1
Sagittal image of the brain with schematic representation of regions involved in homeostatic feeding (hypothalamus and ventral tegmental area), hedonic feeding (striatum, amygdala, orbitofrontal cortex, nucleus accumbens) and inhibitory control (prefrontal cortex).

ALTERED BRAIN REWARD FUNCTION IN OBESITY

Studies in animals and humans have documented that chronic overeating leads to neuroadaptations of reward circuits that are comparable to changes observed in individuals with drug addiction [17]. Animal experiments demonstrated that overfeeding results in lower dopamine signalling and reduced responsivity of reward regions to food intake [18]. This is consistent with evidence from human studies using neuroimaging techniques, such as positron emission tomography and functional magnetic resonance imaging (fMRI) [19]. These studies reported lower dopamine receptor availability [20] and decreased striatal responses to palatable food intake [21] in obese compared to lean individuals. This hypofunction of the reward system is suggested to result from overeating and weight gain [22] and could, in turn, lead to more overeating by means of compensation for a lack of reward during eating.

In contrast to the *lower* reward response to actual food consumption, the reward response to cues of palatable food availability has repeatedly shown to be *higher* in obese compared to lean individuals. FMRI studies repeatedly observed higher brain activation in reward regions when comparing obese an lean participants who were presented highly palatable food pictures [23]. Such increased reward responsiveness to food cues may result in higher craving for food until the food is obtained and consumed. Interestingly, this hyper-reward responsiveness has been demonstrated to predict future weight gain [24], which suggests that it reflects an initial vulnerability factor to the development of overeating and obesity.

In addition to these alterations in the reward system, obesity has been associated with lower inhibitory control over food intake, which may result in overeating due to greater impulsivity. Brain regions that are responsible for this inhibition deficit are prefrontal regions implicated in behavioural control, such as the dorsolateral and ventral lateral prefrontal cortex [25].

Except of the evidence coming from prospective studies [22, 24], most insights for these etiological hypotheses come from cross-sectional studies, which makes it difficult to determine the nature and direction of causality within the observations. Therefore, one could argue that none of the observed alterations in reward system function cause overeating and weight gain but, rather, that they all develop secondary to overweight. This would disqualify them as meaningful targets for intervention.

ENVIRONMENTAL AND GENETIC FACTORS

Environmental factors Obesity is a complex disease, arising from a multitude of genetic and environmental factors, and their interactions. The increase in obesity prevalence has occurred very rapidly during the last 40 years, and is seen in many parts of the world. Since our genes have not substantially changed during this period, the obesity epidemic

is largely explained by environmental factors [26]. Changes in the environment include the increased availability of highly palatable foods and increased meal sizes. Between 1971 and 2004 the mean energy intake per individual in the US is suggested to have increased with 314 kcal per day [27]. Due to the industrialization of food processing, the cost of food has fallen drastically, especially of energy-dense foods high in fat and sugar. Importantly, these palatable energy-dense foods have rewarding properties which, through positive reinforcement, increases its consumption [15]. On the energy expenditure side, simultaneous advances in technology (e.g. development of computers and television) and transportation reduced the need of physical activity during work and leisure time [26].

Genetic factors However, in any given environment not all individuals become obese, which suggests that individual susceptibly factors determine how people respond to certain environments.

These individual differences in body weight regulation reflect differences in genetic make-up as well as the exposure to a host of environmental factors that influence body weight throughout the life course. The evidence supporting the importance of both genetic and environmental factors contributing to obesity can be structured based on their level of influence, i.e. 1) the population at large, 2) individual human beings, or 3) specific organ systems, in this case the brain reward system (Figure 2).

Twin and adoption studies confirmed that variation in body mass index (BMI) has a strong genetic component. The correlation of BMI between genetically identical (i.e. monozygotic) twins has consistently shown to be higher than BMI correlations between same-sex non-identical (i.e. dizygotic) twins [28,29]. Furthermore, adoption studies demonstrated that, with respect to BMI, children were more similar to their biological parents than to their adoptive parents [30]. In addition, overfeeding studies showed higher intra-pair similarity that between-pair similarity in the amount of weight gain in response to a positive energy balance [31]. During the years, the contribution of genetic effects to BMI variation has been estimated to be 40-70% [32]. Interestingly, high heritability estimates have also been documented for food-related functioning of the brain reward system, by studies showing high intra-pair correlations in multiple aspects of appetite and eating behaviour, including the rewarding value of food [33].

Recent advances in DNA analysis and computational techniques have allowed to study the effects of specific genetic loci on a variety of traits, including adiposity [34]. In the candidate-gene approach, alleles of a pre-specified gene that may be involved in a disease are associated with that specific disease itself. In obesity, several candidate-genes have been reported, however, due to limited study sizes many have never been replicated [35]. Another disadvantage of this approach is that the selection of candidate genes relies on a priori knowledge of

pathophysiology. Since obesity is a complex disease, it involves many biological pathways which makes the identification of candidate genes a difficult task. In the last decade, progress has been made due to the rise of large population-based cohorts and high throughput techniques that facilitate the genotyping of thousands of genetic variants [36]. Genome-wide association studies (GWAS) investigate the association between genetic variants and a disease in a hypothesis-free exploratory approach, which allows the discovery of novel variants without a priori assumptions about their function. To date, GWAS have identified 97 genetic loci associated with BMI and body fat distribution [37]. These common genetic variants have modest effect sizes and, together, explain a small proportion (less than 5%) of BMI variation. Nevertheless, the discovery of genetic variants can gain insight in the biological pathways that underlie the aetiology of obesity. For instance, variants in or nearby the fat mass and obesity associated (*FTO*) gene, which have the strongest effects on BMI, have shown to impact on food intake and resting energy expenditure [38].

Interestingly, many recently discovered variants associated with BMI are suggested to act through the central nervous system, specifically in regions implicated in homeostatic and hedonic feeding such as the hypothalamus and cortico-limbic areas, as observed by gene enrichment analyses [37]. These findings align with studies in patients with rare, monogenic forms of obesity, which demonstrated that genetic mutations, such as in the melanocortin 4 receptor (*MC4R*) and leptin receptor (*LEPR*), can cause severe obesity by disrupting pathways involved in food intake regulation by the brain [39]. In line with this, recent neuroimaging studies observed altered brain responses to food cues in regions mediating reward in humans with rare genetic mutations or common obesity-associated variants [40-42].

With heritability of BMI estimated between 40% and 70%, a large amount of the individual differences in BMI (30%-60%) must arise from exposure to environmental factors that influence body weight regulation. Although an 'obesogenic' lifestyle can be a characteristic of an entire population, compared to other populations globally or that population's past, large variation can exist within the population in the degree of exposure to palatable foods, a physical inactive lifestyle, and factors mediating these deviant behaviours. In contrast to the influence of genetic factors, the contribution of environmental factors to obesity-related alterations in brain reward responsiveness to food in humans remains largely unexplored.

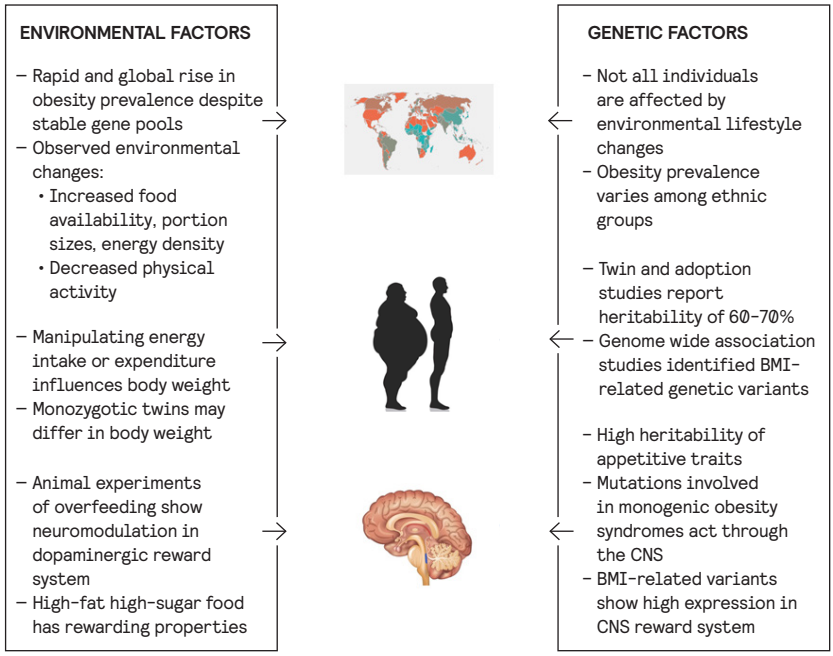

FIGURE 2
Evidence from the literature supporting the importance of genetic and environmental factors to obesity, structured by their level of influence, i.e. the population at large, the individual human being and the brain reward system. BMI, body mass index; CNS, central nervous system.

THE INTRAUTERINE ENVIRONMENT AND FOOD INTAKE

In addition to environmental factors that exert their effect during life after birth, risk to disease in later life is influenced by environmental conditions during foetal development in utero. In 1990 Barker and colleagues observed that reduced foetal growth was associated with increased risk of cardiovascular disease, type 2 diabetes and associated mortality [43]. The 'foetal origins of disease' hypothesis postulates that during critical periods in foetal development, environmental factors may induce persisting changes in the body structure and function, which influences the risk of disease in later life, especially when the prenatal period is followed by an adverse environment in adulthood [44]. This foetal programming may occur through altering the expression of genes in response to environmental conditions. Since these alterations in gene regulation relate to modification without changing the DNA sequence, this phenomenon is known as 'epigenetic' mechanisms [45].

In the last decade, studies have investigated which tissues and organs mediate the deleterious effects of foetal programming. Several studies observed that individuals with poor intrauterine conditions before birth not only ate more food in general, but also had specific food preferences for palatable high-calorie foods in later life, which suggests involvement of mechanisms underlying the brain's regulation of food intake [46].

The main criticism on the foetal origins hypothesis, which was

primarily based on epidemiological studies, was its vulnerability to confounding by factors, such as social class, that influence both the intrauterine as the adult environment. Part of this confounding was reduced by pseudo-experiments of intrauterine malnutrition in which humans born during the Dutch hunger winter of 1944-1945 were compared with unexposed time-controls or siblings. In these studies, it was again observed that the intrauterine malnourished individuals had a preference for high-fat foods in adult life [47, 48]. Despite these more convincing results, however, the possibility of confounding remains, in particular by genetic factors. Hence, genes that influence the way the foetus responds to unfavourable prenatal conditions could also explain the way it develops feeding preferences in later life. Clarification of the relation between intrauterine conditions and food preferences in later life is needed, since possible interventions that could modify the intrauterine environment would only reduce disease risk in later life if the association between intrauterine growth and adult dietary preferences resulted from a true causal relationship.

AIMS AND OUTLINE OF THESIS

Although the understanding of the complex regulation of food intake and the development of obesity has expanded greatly in the last decade, there are still many unresolved issues that should be addressed. The overarching aim of this thesis was to investigate the contribution of genetic and environmental factors to food intake, physical activity, and brain reward responsiveness to food. Further, we aimed to disentangle whether the altered reward system functioning in individuals with obesity precedes overeating and weight gain, or develops secondary to overweight itself.

This thesis is subdivided into three parts which consecutively describe the contribution of 1) the intrauterine environment, 2) the environment in general and 3) genetic factors to food intake, physical activity and the regulation of food reward by the brain. Since for each of these parts a different study design was used, this thesis starts with a detailed description of why and how the data were collected in each of these study designs (Chapter 2). Then, in the first part of the thesis, the influence of intrauterine environmental factors is investigated (Chapter 3). This chapter investigates whether the previously observed association between intrauterine growth restriction and unfavourable feeding preferences in later life is indeed a result of intrauterine environmental factors, independent of genetic confounding. To this end, birth weight was associated with dietary intake of adolescent dizygotic and monozygotic twin pairs. The second part of this thesis deals with the role of unique environmental influences. In Chapter 4 and 5 rare monozygotic twin pairs with an intra-pair difference in BMI were investigated to study the contribution of unique environmental factors to food intake and physical activity (Chapter 4) and brain reward responsiveness to food (Chapter 5). This brain reward responsiveness to

food was examined using fMRI measurements during the presentation of appealing food pictures and the anticipation and receipt of a palatable food stimulus. Chapter 6 investigates the nature of the correlation between environmentally-induced overweight and altered functional connectivity of so-called resting state brain networks involved in food intake and motivation. To this end, brain activation was measured during the resting state using fMRI within BMI discordant monozygotic twins. In the third and final part of this thesis, the influence of genetic factors is studied. Chapter 7 and 8 investigate whether genetic susceptibility to obesity is related to differences in food intake and physical activity (Chapter 7) and brain reward responsiveness to food (Chapter 8). To this end, a genetic risk score based on previously identified obesity-related genetic variants was used to identify individuals with either a low or high genetic risk for obesity. Further classification of this study sample into individuals with either a low or high current BMI allowed to investigate whether altered food intake, physical activity and brain reward responsiveness to food are a cause or, rather, a consequence of obesity. Chapter 9 is the closing chapter, which summarizes the main findings of this thesis, discusses the relevance and clinical implications, reviews the possible study limitations and recommends suggestions for future research.

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Study Design and Data Collection

II

This thesis comprises three parts which describe three different studies performed as part of the larger Netherlands Twin Register (NTR), which was established in 1987 at the VU University in Amsterdam [1]. By collecting a wealth of information on health, lifestyle and personality from twins and their families, the NTR has allowed the investigation of important questions concerning heritability, gene-environment interaction, causality and gene finding of many health-related traits [2,3]. In addition to longitudinal survey data, the NTR has collected DNA samples for the assessment of not only zygosity, but also genome wide DNA marker data. These genome-wide data from more than 10,000 individuals have been used in several large international gene-finding consortia [4], including those investigating BMI and body fat distribution [5]. Participation in the NTR is entirely voluntary and participants can get regularly updated about novel research findings.

In each of the three parts of this thesis, a different study design was used. Data of Part 1 were collected during the early years of the NTR in the 1990s, and analysed in 2013, whereas data of Part 2 and 3 were collected and analysed between 2014 and 2016.

PART 1 – INTRAUTERINE ENVIRONMENTAL FACTORS AND FOOD INTAKE

This part aimed at investigating whether the previously observed association between the intrauterine environment and adult food intake is independent of possible confounding by genetic factors.

RATIONALE OF THE STUDY DESIGN

Since 1914, twin studies have provided the opportunity to disentangle the influence of genetic and environmental factors to many (behavioural) traits [6]. In the classical twin design, the resemblance of monozygotic twin pairs on a trait is compared to the resemblance of dizygotic twins. Monozygotic twins are derived from a single fertilized egg cell and share (nearly) all of their genes, whereas dizygotic twins are derived from two distinct egg cells and share on average 50% of their genes. Therefore, a larger phenotypic resemblance of monozygotic twins than dizygotic twins must be due to genetic influences. The remaining variance is explained by environmental factors that are either shared (i.e. common environment), or unshared by the twins of a pair (i.e. unique environment). Therefore, classical twin designs are used to estimate to what extent variation in a (behavioural) trait is explained by genetic or (un)shared environmental factors.

In addition, the study of monozygotic and dizygotic twins can be used to examine whether associations between traits (e.g. a risk factor and a disease) reflect true causal relationships or result from con-

founding factors that impact on both risk factor and the disease, such as social-economic class, age or genetic background [7,8]. Epidemiological studies in unrelated individuals often cannot exclude the possibility that their observed associations emerged from confounding, since these studies can only correct for confounding variables that are 1) expected to be present and 2) possible to measure [9]. Randomized controlled trials (RCT's), in which subjects are randomly assigned to a condition, substantially reduce this influence of confounding. However, RCT's are limited to hypothetical causal conditions that can be experimentally manipulated. In addition, results from experimental studies may not always be generalizable to the population at large, due to their widely used specific inclusion and exclusion criteria. Fortunately, twin studies allow to 1) control for many of such confounding factors and 2) examine causal conditions that are otherwise impossible or unethical to investigate [7,8].These models capitalize on the relatedness of genetic and environmental factors within families. Whereas unrelated individuals share genetic and environmental factors at a random level, twins share many environmental factors, and 50% (in dizygotic twins) or 100% (in monozygotic twins) of their genes. Therefore, comparisons within twin pairs allow to control for such environmental and genetic factors. In specific, differences within dizygotic twin pairs can be explained by both genetic and non-genetic factors, whereas in monozygotic twins differences can only be explained by non-genetic factors, since here genetic factors are eliminated. Thus, in the case of a causal relation between risk factor and disease without confounding by genetic factors, one would expect that both for dizygotic and monozygotic twins, the co-twin with the risk factor would also show the highest disease prevalence, compared to the co-twin without the risk factor [10]. If, however, the relation is not causal but influenced by genetic factors, the association between risk factor and disease would be observed only in dizygotic, and not in monozygotic twins [11].

In Chapter 3 of this thesis we investigated whether the previously observed association between intrauterine growth retardation and dietary preferences in later life resulted from a true causal relationship or, rather, from an influence of genetic factors. To this end, we associated birth weight with dietary intake in a group of adolescent dizygotic and monozygotic twin pairs.

STUDY POPULATION

The study performed in this part of the thesis is part of a larger project carried out by the NTR between 1985 and 1990 in which cardiovascular risk factors were studied in 160 adolescent twin pairs and their parents [11,12]. Addresses of twins living in Amsterdam and neighbouring cities were obtained from City Council population registries. Twins still living with their biological parents were contacted by letter and, together with their parents, invited for participation in this study. Overall, between 30 and 40% of families complied. Of the 160 families that initially par-

ticipated in the study, members of 120 families additionally recorded data on dietary intake. For the final analysis of these data, opposite-sex dizygotic twins (n=17 pairs) were excluded because of possible effects of gender on both birth weight and dietary intake. Further, because of missing or unreliable dietary intake data, another 8 and 9 pairs were excluded, respectively. Thus, data of 39 dizygotic and 47 monozygotic twin pairs were available for analysis. A flow chart of the sample selection is provided in Chapter 3.

DATA COLLECTION

Data on cardiovascular risk factors were collected during a test visit at the department of Biological Psychology at the VU University [12]. Some weeks ahead of the visit, a survey was sent to the mothers, allowing them to obtain data on birth weight and gestational age of their offspring from birth certificates. Measurements of height and weight were done in a standardized way. Zygosity of the twin pairs was determined by typing DNA polymorphisms in blood samples. After the test visit, data on dietary intake was collected using two-day dietary records on one weekday and one weekend day. Oral and written instructions were provided by trained dieticians.

Food items recorded in the food diaries were coded in 2010 by two clinical dieticians. The dieticians were unaware of the birth weight of the participants. Coding was done with the use of a dietary analysis program based on the Dutch Food Composition Database (NEVO) [13]. Food products that were not included in this database were evaluated on caloric and macronutrient content, and matched to similar products that were available in the database. In 2013, the coded data were analysed within the context of the foetal origins hypothesis, following previous publications on the association between birth weight and adolescent dietary intake, as described in Chapter 3.

PART 2 – ENVIRONMENTAL FACTORS AND FOOD INTAKE REGULATION

This part of the thesis aimed at investigating the contribution of unique environmental factors to food intake, physical activity and brain reward responsiveness to food.

RATIONALE OF STUDY DESIGN

Heritability of BMI has been estimated to be relatively large [14], which implies that the regulation of body weight is under high genetic control. However, these heritability estimates tend to change during life. Whereas heritability increases during childhood, the impact of genetic effects decreases with increasing age during adulthood. These obser-

vations suggest that genetic influences are not deterministic, but that the influence of genetic factors partly depends on non-genetic factors.

Furthermore, despite the fact that monozygotic twins are nearly genetically identical, within-pair differences may occur in their BMI [15]. This discordance is explained by unique environmental factors that exert their effect on BMI either directly or indirectly through epigenetic mechanisms [16]. Therefore, studying these monozygotic twins discordant for BMI allows to study the influence of unique environmental factors, since all genetic effects are eliminated (Figure 1).

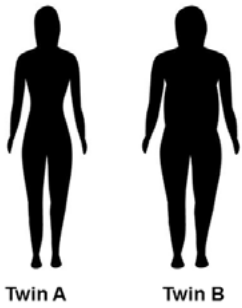

FIGURE 1
Monozygotic twin intra-pair differences model as used in of Part 2, comprising monozygotic twins with intra-pair BMI discordance.

In addition and in line with the reasoning of Part 1, discordant monozygotic twins allow to investigate the nature of causality of observed associations between traits, an ability that is not obtainable by ordinary study designs with unrelated participants. In specific, monozygotic twins discordant for a risk factor can be used to examine causal effects of this risk factor on an outcome, since all genetic and shared environmental factors, that could act as confounders between risk factor and outcome, are controlled for [17]. Thus, the observation of an association between a risk factor and an outcome variable within monozygotic twins, is suggestive for a causal relation between risk factor and outcome variable, independent of genetic confounding. For example, this so called co-twin control method successfully identified a causal link between smoking and lung cancer [18]. In contrast, a similarly designed study demonstrated that the apparent association between increased exercise behaviour and reductions in depression symptoms is actually explained by shared genetic effects [19].

In Chapter 4 and 5 we used a design of monozygotic twins discordant for BMI to investigate the influence of unique environmental factors on BMI-related alterations in physical activity, food intake, eating behaviour and brain reward responsiveness to food stimuli. In Chapter 6, we used a co-twin control method to examine whether the previously reported association between BMI and alterations in resting state network functional connectivity is explained by a true causal relationship or by genetic confounding.

STUDY POPULATION

Monozygotic twin pairs were selected for participation in this study based on BMI measurements during earlier NTR biobank projects and/or survey studies [3]. Preparation of the selected sample in the current study was done as part of a larger NTR study on longitudinal BMI discordance in monozygotic twins [20]. A flow chart of the selection procedure is presented in Chapter 4.In short, from a total of 2755 monozygotic pairs with available BMI data, pairs were selected if a) BMI discordance was >3 kg/m^2 during an NTR biobank project and/or b) if BMI discordance was >3 kg/m^2 during at least 1 survey, if BMI discordance was >2 kg/m^2 during at least 3 surveys, or if BMI discordance was >2 kg/m^2 at the most recent survey. After removal of males, incomplete pairs and individuals that had been deregistered by request, we invited 54 female twin pairs for participation by sending them a detailed information letter.

In the following weeks, participants were contacted by telephone to gauge their willingness to participate, provide more information when needed, and check in- and exclusion criteria. Inclusion criteria were age range between 18 and 75 years and stable body weight (reported weight change of <5% in previous 3 months). Exclusion criteria were current diabetes mellitus, serious heart, liver or renal disease, malignancies, uncontrolled thyroid disease, neurological or psychiatric disease (including eating disorders and depression), pregnancy or breast feeding, MRI contra-indications (e.g. metal implants, claustrophobia), alcohol or drug abuse, and the use of glucose-lowering drugs or psychoactive medication. From the 54 initially selected female twin pairs, 21 pairs (39%) were excluded due to the following reasons: pregnancy (n=2), eating disorder (n=4), diabetes mellitus (n=1), serious heart disease (n=1), neurological disease (n=3), MRI contra-indications (n=3) or reported BMI difference < 2 kg/m^2 due to recent weight change in one or both co-twins (n=7). Fourteen pairs (26%) had no interest in participation, mostly because of lack of time. Another 2 pairs (5%) could not be contacted by telephone due to loss of follow-up. Thus, 16 pairs (30%) were included in this study.

DATA COLLECTION

Data was collected during a 5 hours visit at the clinical research unit at the department of Internal Medicine at the VU University Medical Centre, and in the 2-3 weeks following this visit. Both co-twins of a pair were examined during a single test visit, which was scheduled to start between 8:00 and 10:00 AM. An overview of all measurements performed in this study is presented in Figure 2.

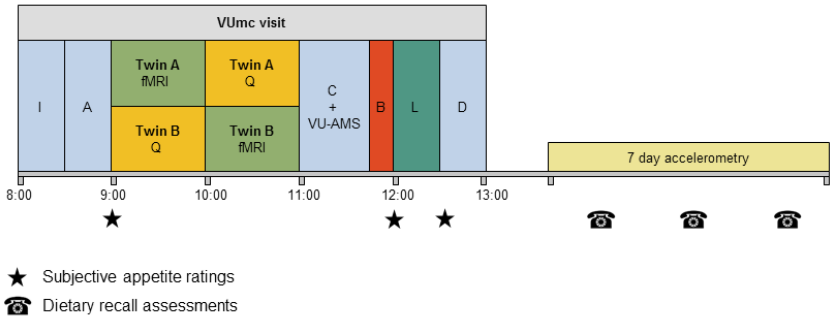

FIGURE 2
Overview of study procedures followed in Part 2 and 3. I, informed consents and standardized interview; A, anthropometry; fMRI, functional magnetic resonance imaging; Q, questionnaires; C, indirect calorimetry; VU-AMS, VU University ambulatory monitoring system; B, blood draw; L, lunch meal; D, detailed instructions and portion size estimations for data collection on dietary intake and physical activity.

Informed consent and interview Participants arrived at the research clinic after an overnight fast and after 24 hours of refraining from heavy exercise. All data collection was performed by a research physician and a clinical dietician. Measurements started after participants had read and signed informed consent forms. A standardized, oral interview was used to collect data on socio-demographics and health.

Anthropometrics After removal of shoes and heavy clothing, height was measured using a stadiometer, and weight using a digital scale. Body composition was assessed using bio-electrical impedance analysis (Maltron BF-906 body fat analyser, Maltron Ltd, Essex, UK). Body fat distribution was assessed by measuring waist circumference (at the midpoint between the lowest rib and top of the iliac crest) and hip circumference (around the widest portion of the buttocks), using a stretch- resistant tape.

Subjective appetite ratings On three time points (i.e. before the scanning session, before and after the lunch meal), participants were asked to rate their feelings of appetite and hunger on a Likert scale ranging from 0 ('not at all') to 10 ('extremely') [21]. Participants were asked the questions 1) How hungry are you now? 2) How full are you now? 3) How much could you eat right now? 4) How much is your desire right now to eat something sweet / savoury / fat?

Functional MRI Structural and functional brain scanning was performed during a 1 hour scanning session. Task paradigms for the examination of brain activation in response to 1) appealing food pictures and 2) palatable food receipt were designed as part of earlier fMRI studies on food reward at the Internal Medicine department [22, 23] and based on previous studies [24, 25].

1) Food picture paradigm. While lying in the MRI scanner, participants were presented 42 pictures of high-calorie food (e.g. pizza, French fries, ice cream, donuts), 42 pictures of low-calorie food (e.g. fruit salads, an apple, green vegetable salads, a cucumber) and 42 pictures of non-food items (e.g. trees, bricks, stones), all pictures being matched for colour, shape and size. Pictures were presented in a block-design, in which each block consisted of 7 pictures of the same category and each picture was presented during 2.5 seconds, separated from the following picture by a 0.5 seconds blank screen. A schematic presentation of the food picture paradigm is shown in Figure 3A.
   One hour after the scan, a recognition test was performed comprising 20 pictures of which participants needed to identify 10 pictures that were previously presented in the scanner. Also, participants viewed all 84 (low-calorie and high-calorie) food pictures again and rated each picture on how attractive the food in the picture appeared to them at that moment on a 7-point Likert scale ranging from 1 ('not at all') to 7 ('extremely').
2) Chocolate milk paradigm. In a second fMRI task, participants anticipated and received liquid sips of chocolate milk and a tasteless solution. The tasteless solution was used as a neutral stimulus, designed to mimic the natural taste of saliva. In this paradigm, two images were presented (an orange triangle or a blue star) that signalled the delivery of either 0.4 mL chocolate milk or tasteless solution, respectively. Images were presented for 2 seconds (i.e. anticipation time) in random order, followed by 3 seconds of grey screen with a fixation cross and 2 seconds of stimulus delivery. Participants were instructed to keep the solution in their mouth during 6 seconds until the sign 'swallow' appeared. The next trial was started after a time varying between 3 to 7 seconds. In 40% of the events, the cue was not followed by a stimulus delivery. Tastes were delivered using programmable syringe pumps and Vygon syringes that were inserted into the participant's mouth. In Figure 3B, a schematic presentation of the chocolate milk paradigm is provided. After the scan, the attractiveness of the receipt of the chocolate milk and tasteless solution was rated on a scale from 1 ('not at all') to 7 ('extremely').
3) Resting state fMRI. After these food-related experiments, an fMRI scanning session was performed while participants were lying at complete rest and with their eyes closed during 6 minutes. These measurements of spontaneous brain activity were used to study functional connectivity of resting state networks in the brain.

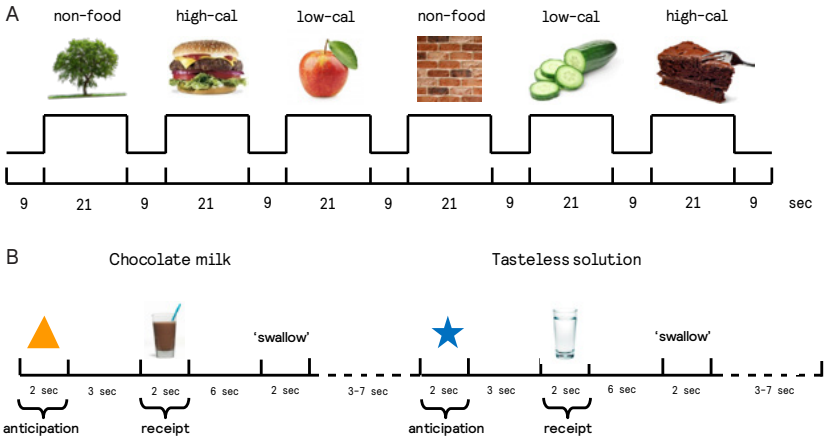

FIGURE 3
Example of timing of picture presentation during the food picture fMRI paradigm (A) and example of timing of cue presentation and stimuli delivery during the chocolate milk fMRI paradigm (B), as used in Part 2 and 3. Cal, calorie; sec, seconds.

Questionnaires Participants completed 5 validated questionnaires [26-30] for the examination of general physical and mental health (Short Form 36-item Health Survey), symptoms of depression (Centre for Epidemiologic Studies Depression Scale), eating behaviour (Dutch Eating Behaviour Questionnaire), symptoms of eating disorders (Eating Disorder Inventory version 2) and handedness (Dutch Handedness Inventory).

VU ambulatory monitoring system Subsequently, the VU ambulatory monitoring system (VU-AMS) was attached to the body of the participant, comprising 4 electrodes on the chest and 2 on the back [31]. The VU-AMS is a small non-invasive device that records both electrocardiogram and thorax impedance and can be used to examine autonomic nervous system functioning. VU-AMS recordings were performed while participants were lying at complete rest, while sitting quietly, and during a 5-min task of mental arithmetic. Meanwhile, systolic and diastolic blood pressure were measured (Dinamap Pro 100, GE Medical Systems Information Technologies, Inc., Milwaukee, Wisconsin).

Indirect calorimetry After being placed under a plastic ventilated hood (Vmax Encore n29; Viasys Healthcare, Houten, Netherlands), participants were asked to lie still with their eyes closed during 15 minutes, while breathing quietly in and out, and trying not to fall asleep. VO2 and VCO2 measurements of outgoing air were used to assess resting energy expenditure.

Blood draw Venepuncture was performed to collect 8 blood tubes (Table 1). After collection, one small heparin- and two small EDTA tubes were delivered to the clinical laboratory of the VUmc, for direct assess-

ment of clinical chemistry and complete blood count. Four collection tubes (1 large EDTA, 1 large heparin, 1 large P800 and 1 large serum) were centrifuged, after which plasma (and buffy coat and red cells from the EDTA tubes) was harvested, aliquoted (0.5 ml) and stored at -80° Celsius. A PAX-gene RNA tube was stored at -20° C, after a minimum of 2 hours at room temperature.

TABLE 1
Overview of blood tubes collected in this study

Vacutainer	Volume	Determination / storage
Heparin	3 ml	Chemistry
EDTA	2 x 4 ml	Complete blood count
Heparin	6 ml	Aliquoted and stored at -80° C
EDTA	6 ml	Aliquoted and stored at -80° C
Serum (no additives)	8.5 ml	Aliquoted and stored at -80° C
P800	8.5 ml	Aliquoted and stored at -80° C
PAX-gene RNA	2,5 ml	Stored at -20° C

Lunch meal Participants were presented a standardized varied choice meal, that consisted of white and multigrain bread, a mixed green salad, orange juice, Dutch cheese, fresh meats, margarine, mayonnaise, peanut butter, jam, cake, a chocolate muffin, a banana and an apple [22]. Co-twins were seated at two separate tables, each on the other side of the room. They could eat as much as they wanted and were not informed that their consumption of food was being monitored. Food items were coded with the corresponding NEVO-code, similarly as in Part 1 of this thesis [13].

Dietary recall assessments At the end of the test visit, participants were provided with detailed oral and written instructions for the collection of dietary intake data during the weeks following the test visit. In support of later dietary recall assessments, portion sizes were estimated at the end of the test visit, using a table scale and extensive tableware. Participants indicated which kind of tableware they were accustomed of using at home. After filling these cups/glasses with water, the weights (in grams) of these amounts were written down. In addition, participants were provided with a photobook, comprising pictures of varying food types in 4-6 different portion sizes. In the 2-3 weeks following the test visit, participants were contacted by the research physician by 3 unannounced telephone calls to list all foods and drinks they had consumed during the previous 24 hours. Intakes were recalled from 2 weekdays and one Sunday, using the validated Unites States Department of Agriculture multiple-pass method. Food items were coded with the corresponding NEVO-code [13] by an experienced dietician.

Accelerometry Finally, participants were provided with an Actigraph GT3X+ accelerometer (Actigraph LLC, Pensacola, FL, US) [32] and accompanying oral and written instructions. They were asked to wear the accelerometer attached to an elastic belt on the right hip for all waking hours during a 7-day period, except during water-based activities. Participant started wearing the device on the first Saturday after the test visit. Following the 7-day wear period for the accelerometer, participants completed the short version of the International Physical Activity Questionnaire [33], which examines the amount of time spent during the previous weeks in 3 different intensity levels of physical activity, i.e. vigorous activity, moderate activity and walking. Both accelerometer and completed questionnaire were returned to the research physician by mail.

PART 3 – GENETIC FACTORS AND FOOD INTAKE REGULATION

The third and last part of this thesis aimed at investigating whether genetic susceptibility to obesity is related to differences in food intake, physical activity and brain reward responsiveness to food.

RATIONALE OF STUDY DESIGN

Examination of the causal relation between traits is, besides through informative twin designs, also possible by testing direct effects of genetic factors that predispose to a trait. The basic principle of this method is that the direction of causation is always from the genetic predisposition to the trait of interest, and not vice versa.

Based on this reasoning, the so-called four corner model has been proposed [34, 35], in which individuals with either a high or low genetic susceptibility to a trait are selected to have either high or low observed levels of that trait (Figure 4). For example, both offspring from hypertensive parents (i.e. high genetic susceptibility) and offspring from normotensive parents (i.e. low genetic susceptibility) could be selected, who themselves have either low or high blood pressure levels. The expectation is that variables that are associated with blood pressure in offspring irrespective of parental blood pressure are secondary to high blood pressure. In contrast, variables that are associated with high blood pressure only in the hypertensive offspring of hypertensive parents, but not in the hypertensive offspring of normotensive parents, could be a causal link between genetic risk and high blood pressure.
The increasing availability of DNA data for a large number of individuals registered in the NTR [2], makes it possible to define genetic predisposition of an individual not on parental characteristics (as in the previous example), but on direct effects of specific genes. More specifically, the recently identified obesity-associated single nucleotide polymorphisms (SNPs) [36] together can be aggregated into a polygenic risk score (GRS),

which can be used as an individual's measure of genetic risk to obesity [37]. Importantly, whereas in the original design genetic predisposition was still influenced by shared familial environmental factors, such as socio-economic status (shared between parent and offspring), the use of measured GRS to define genetic predisposition eliminates such confounding by environmental factors.

In Chapter 7 and 8 we used an adapted four corners design that selects individuals with either a low or high GRS for obesity based on 77 recently established obesity-SNPs, and, within each group, either low or high measured BMI values. By doing so, we aimed to investigate whether genetic susceptibility to obesity is related with alterations in food intake and physical activity (Chapter 7) and brain reward responsiveness to food (Chapter 8) and, further, to examine whether these traits are causal or secondary to obesity.

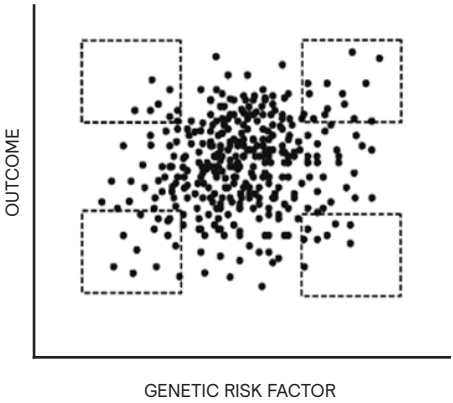

FIGURE 4
Four corner epidemiological study design as used in Part 3, in which individuals are selected based on either high or low values of a genetic risk factor and either high or low values on the outcome variable.

STUDY POPULATION

The selection of participants in this study was done in two phases, of which a flow chart is presented in Chapter 7. First, all individuals with available data on 1) measured BMI and 2) genome wide SNPs were identified from the Netherlands Twin Registry, which resulted in a sample of 11,495 individuals. Measurements of BMI and genotyping were done as part of earlier NTR studies and international genome-wide associations studies [3-5]. For every participant, a genetic risk score (GRS) for obesity was calculated. Because this selection phase started before the latest discovery of obesity-associated SNPs in 2015 [36], the initial calculation of the GRS was based on the 32 SNPs reported by Speliotes et al. in 2010 [5]. After the discovery of novel obesity-loci [36], we optimized our GRS by using calculations based on 77 known obesity SNPs found in individuals of European descent. Calculation of GRS was done by

summing the BMI-increasing alleles after weighting the alleles by their effect size. Before running a second selection phase, individuals were excluded if they 1) fell outside the age range of 18-75 years or 2) were part of the same family as another individual in the sample. To this end, spouses and parents of registered twins were first selected followed by randomly chosen siblings or members of twin pairs.

In a second phase, individuals were selected with either low or high GRS and either low or high measured BMI. To this end, a scatter diagram was constructed by plotting GRS on one axis and BMI on the other axis [34] (for an example see Figure 4). The initial creation of four corners was done by using cut-off values that corresponded with the lower and upper 20% of the distribution of BMI (i.e. ≤ 21.5 kg/m^2 and ≥ 27.9 kg/m^2) and the lower and upper 20% of the distribution of GRS (in either the 32-SNP or 77-SNP GRS). These initial cut-offs resulted in a number of individuals that was not expected to result in our final sample size of 60 participants (considering a participation rate of 30%). Therefore, the criteria for inclusion were broadened by using the lower and upper 25% of the BMI distribution (i.e. ≤ 22 kg/m^2 and ≥ 27 kg/m^2) instead. Finally, because of reported sex differences in the regulation of body weight, only female pairs were included to increase homogeneity of the study population.

Thus, a total of 248 females who were registered as active participants in the NTR were invited for participation by a detailed information letter. In a similar way as in Part 2, participants were contacted by telephone. In total 113 women (46%) were unwilling to participate, mostly because of lack of time. Of the remaining women, 46 (19%) were excluded due to the following: recent body weight change (n=15), co-morbidities or medication (n=23), pregnancy (n=4) or MRI contra-indications (n=4). Another 29 individuals (12%) could not be contacted by telephone due to loss of follow-up. Thus, 60 women (24%) were included in this study: 16 women with low GRS/low BMI, 12 with low GRS/high BMI, 15 with high GRS/low BMI and 17 with high GRS/high BMI.

DATA COLLECTION

The collection of data in Part 3 was done in a similar way as described above for Part 2, with the one exception that, during the test visit, participants were examined per individual, contrary to twins, who were examined in pairs.

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Intrauterine environment and food intake

PART 1

Lower birth weight is associated with alterations in dietary intake in adolescents independent of genetic factors: a twin study

III

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ABSTRACT

BACKGROUND & AIMS

Lower birth weight is associated with an increased risk of cardiovascular and metabolic disease. These associations may, at least in part, be explained by alterations in dietary intake in later life. The aim of this study is to examine whether lower birth weight is associated with alterations in dietary intake in later life, and whether this association is due to intrauterine environmental or genetic factors.

METHODS

In this observational study birth weight and dietary intake were investigated in 78 dizygotic (DZ) and 94 monozygotic (MZ) adolescent same-sex twin subjects. Birth weight was obtained from the mothers. Dietary intake was assessed by two-day dietary records.

RESULTS

In the total group of twins, lower birth weight was associated with higher intake of saturated fat after adjustment for current weight (1.2 per cent of total energy intake (E%) per kg increase in birth weight, $P<0.01$). Intra-pair analysis in all twin pairs demonstrated that twins with the lower birth weight had a 115 kcal higher total energy intake and a 0.7 E% higher saturated fat intake compared to their co-twins with the higher birth weight ($P<0.05$). Intra-pair differences in birth weight were negatively associated with differences in energy intake and differences in intake of saturated fat after adjustment for differences in current weight ($P=0.07$ and $P<0.05$, respectively). Intra-pair differences in birth weight were positively associated with intra-pair differences in intake of dietary fibres ($P<0.05$). These intra-pair differences and associations were similar for DZ and MZ twins (P for difference >0.6).

CONCLUSIONS

Lower birth weight was related with higher intake of energy and saturated fat within twin pairs, and these associations were independent of zygosity, suggesting that the association between birth weight and alterations in dietary intake in later life are explained by intrauterine environmental rather than genetic factors.

INTRODUCTION

In the last twenty years, many epidemiologic studies have shown that lower birth weight, a measure of reduced foetal growth, is associated with increased incidence of hypertension, type 2 diabetes and cardiovascular disease [1-4]. Several studies in singletons suggested that the association between lower birth weight and the increased risk to develop metabolic and cardiovascular disease may, at least in part, be explained by changes in dietary intake [5-8].

The origin of the possible association between birth weight and dietary intake in later life is not completely understood. The leading hypothesis proposes the programming of dietary preferences in reaction to a poor intrauterine environment. Such adaptive programming would be favourable if nutrition remained insufficient after birth. However, if nutrient availability becomes abundant, maladaptive consequences, such as obesity and type 2 diabetes, may occur [9]. This hypothesis is supported by two studies demonstrating that early prenatal exposure to undernutrition during the Dutch famine is associated with higher energy intake and a favour for diets rich in fat in later life [10, 11].

An alternative explanation states that the association between birth weight and dietary intake arises from pleiotropic genetic factors [12, 13]. In other words, the genotype responsible for the intake of an unhealthy diet may itself cause reduced foetal growth in utero. Such a genetic effect cannot be ruled out by the Dutch famine studies since these studies might have been influenced by selection bias. During the Dutch famine, the number of conceptions was about 50% lower than the pre-famine level and perinatal mortality as well as mortality in the first year after birth were higher in those who were born during the famine [14]. Thus, if women with specific dietary intake conceived more often and/or if their children survived more often, a genetic effect on dietary intake would cause these children to eat more or differently in later life.

If genetic factors are responsible, improving the intrauterine environment will not likely influence dietary intake in later life. If the association between birth weight and dietary intake is due to an intrauterine environmental factor, and if this factor is amenable to intervention, improving the intrauterine environment may be used to improve dietary intake and reduce the risk of adverse consequences in later life.

Twin studies offer a unique opportunity to distinguish between environmental and genetic influences [15]. Differences within dizygotic twin pairs can be a function of both genetic and non-genetic factors, whereas differences within monozygotic pairs are nearly always caused by non-genetic factors [16]. If genetic factors do not play a major role in the association between birth weight and dietary intake, one would expect that *both* for dizygotic and for monozygotic twins, the twin with the lower birth weight from each pair will also have the unhealthiest dietary intake compared to the co-twin with the higher birth weight.

If, however, genetic factors do play a role, this association would hold true only for dizygotic twins, and not for monozygotic twins.

The aim of this twin study is to investigate whether lower birth weight is associated with dietary intake in later life, and whether, based on the comparison of the association in monozygotic and dizygotic pairs, the association is due to intrauterine environmental or genetic factors (Figure 1).

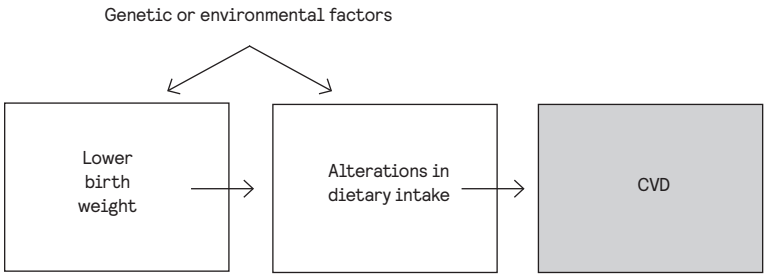

FIGURE 1
The postulated relations among birth weight, alterations in dietary intake and metabolic and cardiovascular disease. The aim of the study is to investigate whether the previously observed association between lower birth weight and alterations in dietary intake is influenced by genetic or environmental factors. CVD, cardiovascular disease

MATERIALS AND METHODS

PARTICIPANTS

Between 1985 and 1990, 160 adolescent (age 13 to 22 years) twin pairs and their parents took part in a study on cardiovascular risk factors [17-22]. All twins were still living were their parents. Details of the study have been described previously [19]. Parents of offspring underwent assessment for cardiovascular risk factors and responded to a large number of inventories. A survey on birth weight and gestational age was sent to the mothers a few weeks ahead of their visit to our department, allowing them to obtain these data from birth certificates. After visits to the department, including blood draws for zygosity assessment, data on dietary intake were collected in 120 twin pairs and their parents. The previously collected data were now analysed since it was only recently that Dutch hunger winter studies suggested an effect of the intrauterine environment on dietary intake in later life.

A flow chart of the study population selection and final study sample is presented in Figure 2. Data from opposite-sex dizygotic twin pairs (n=17) were excluded because of sex differences within a pair on birth weight. Data from eight twin pairs were not used because of missing information from one or both co-twins on either birth weight or dietary intake. Data from another 9 twin pairs were excluded from analysis because information written in the dietary records was too vague or unreadable to make a proper interpretation of foods actually

consumed. Thus, data of 39 dizygotic and 47 monozygotic twin pairs was available for analysis. The study was approved by an institutional review committee and all subjects gave informed consent.

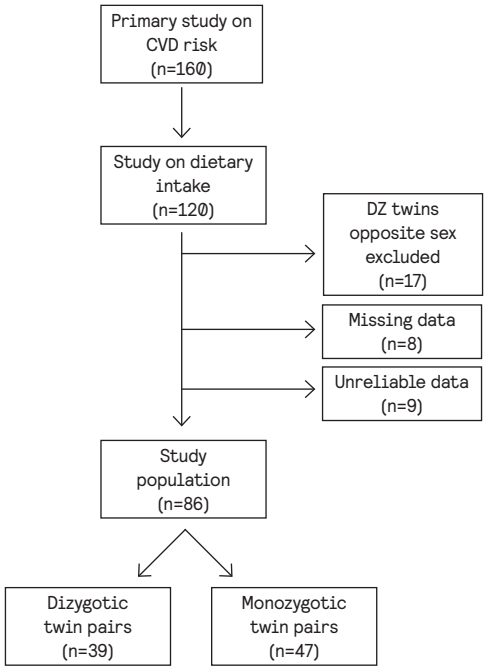

FIGURE 2
Flow chart of the study population. CVD, cardiovascular disease; DZ, dizygotic

MEASUREMENTS

Height and weight measurements and body mass index (BMI; in kg/m^2) calculations were done in a standardized way. Dietary intake was assessed using a two-day dietary record on one weekday and one weekend day. Dietary records and detailed written instructions were given to the participating families on the day of the study visit. In addition, oral instructions were given by trained dieticians. For the sake of clarification each dietary record contained an example of a completed record for one day. Parents were asked about preparation of dinner in a detailed manner. Within three weeks after returning the food records participants were contacted by telephone in case data were missing or unclear. Data were coded by two clinical dieticians who were not aware of the birth weight of the participants. Coding was done using a dietary analysis program based on the Dutch Food Composition Database (NEVO) [23]. Food products that were missing from this database were evaluated and matched to similar products in the database. Daily energy intake was expressed as kilocalories (kcal) and intakes of protein, carbohydrates, total fat and saturated fat were expressed as percentages of total energy intake (E%). Intake of dietary fibres was expressed as grams per 1000 kcal of total energy intake.

STATISTICAL METHODS

In the total group of twins linear regression analysis was used to examine the influence of birth weight on dietary intake after adjustment for age, sex and current weight [6, 8, 20]. This analysis was performed in Stata 13 including family ID as a cluster variable to account for non-independence of family members. An interaction analysis was performed to investigate whether sex, zygosity, current weight or current BMI influenced the associations between birth weight and dietary intake by introducing a product term of these variables and birth weight into the regression model. We compared twins with the lower birth weight from each pair with their co-twins with the higher birth weight [20, 21]. For this intra-pair analysis, a paired t-test was used [24]. To investigate the influence of intrauterine environmental or genetic factors, we compared the differences within twin pairs between dizygotic and monozygotic twin pairs, using independent samples t-tests.

As a first intra-pair analysis the comparison of dietary intake between twins with the lower and the higher birth weight is simple and illustrative. However, twin pairs that differ 1 gram in birth weight are not differentiated from twin pairs differing many hundreds of grams in birth weight. As a further analysis, linear regression analysis was used to analyse whether intra-pair differences in birth weight influenced intra-pair differences in dietary intake after adjustment for differences in current weight in dizygotic and monozygotic twins [21, 22]. An interaction analysis was performed to investigate whether sex, zygosity, gestational age, differences in current weight or differences in current BMI influenced the associations between intra-pair differences in birth weight and intra-pair differences in dietary intake.

To check the validity of reported energy intake across groups we calculated the ratio of energy intake to predicted basal metabolic rate of all individuals [25]. Predicted basal metabolic rate was calculated using the Schofield equations based on age, gender and weight [26]. A ratio of energy intake to basal metabolic rate lower than 1.34 was suggested to reflect underreporting [25]. There were no significant differences in underreporting between co-twins with lower birth weight and co-twins with the higher birth weight (P=0.24).

Results are expressed as mean (standard deviation) or regression coefficient (95% confidence intervals). A two-tailed P-value < 0.05 was considered to indicate statistical significance. IBM SPSS Statistics for Windows (version 20.0) was used for analysis of the data, except the first regression analysis in all twins, which was performed in Stata 13.

RESULTS

ASSOCIATION OF BIRTH WEIGHT WITH DIETARY INTAKE

In the total group of twins, birth weight was negatively associated with intake of saturated fat after adjustment for age, sex and current weight

(*P*=0.005; Table 1). Analyses without adjustment for current weight or after adjustment for current BMI instead of current weight, showed similar results (data not shown).

TABLE 1
Association between birth weight and macronutrient intake in the total group of twins (n=172)

	Beta (95% CI)	*P*
Energy (kcal)	-98 (-284.5 to 88.2)	0.3
Protein (E%)	-0.2 (-1.1 to 0.7)	0.6
Carbohydrates (E%)	1.3 (-0.5 to 3.2)	0.1
Total fat (E%)	-1.1 (-2.7 to 0.5)	0.2
Saturated fat (E%)	-1.2 (-2.1 to -0.4)	0.005
Fibres (g/1000 kcal)	0.2 (-0.7 to 1.2)	0.6

Data represent betas (95% confidence interval) per kg birth weight after adjustment for age, sex and current weight and including family ID as a cluster variable. E%, percentage of total energy intake

INTRA-PAIR DIFFERENCES

The differences in birth weight between the co-twins with the lower birth weight and those with the higher birth weight from each pair were similar for dizygotic and monozygotic twin pairs (363 g and 291 g, respectively; *P* for the difference, 0.2; Table 2). Co-twins with the lower birth weight were shorter in later life than their co-twins with the higher birth weight. Co-twins with the lower birth weight had a significantly lower body weight at adolescent age than their co-twins with the higher birth weight (*P*=0.03). BMI in later life did not differ between co-twins with the lower and co-twins with the higher birth weight.

In all twins, co-twins with the lower birth weight from each pair had a total energy intake that was 115 kcal higher than their co-twins with the higher birth weight (Figure 3A, left panel, *P*=0.04). To investigate whether this difference was influenced by intrauterine environmental and/or genetic factors, we compared dizygotic and monozygotic twin pairs. This difference in total energy intake was not different between dizygotic and monozygotic twin pairs (Figure 3A, right panel, 147 kcal vs. 88 kcal respectively, *P* for difference between dizygotic and monozygotic twin pairs, 0.6).

Furthermore, co-twins with the lower birth weight from each pair had an energy adjusted intake of saturated fat that was 0.7 E% higher compared to their co-twins with the higher birth weight (Figure 3B, left panel, *P*<0.05). This difference in intake of saturated fat was similar in dizygotic and monozygotic twin pairs (Figure 3B, right panel, 0.6 E% vs. 0.8 E% respectively, *P* for difference between dizygotic and monozygotic twin pairs, 0.8). No significant differences were found between co-twins with the lower and co-twins with the higher birth weight in intake of other macronutrients (Table 2).

TABLE 2
Clinical characteristics and intake of total energy and macronutrients in the co-twins with the lower and co-twins with the higher birth weight in all twin pairs and dizygotic and monozygotic twin pairs separately

	All Twin Pairs n=86			Dizygotic Twin Pairs n=39			Monozygotic Twin Pairs n=47		
Clinical characteristics	Co-twins with lower birth weight	Co-twins with higher birth weight	*P*	Co-twins with lower birth weight	Co-twins with higher birth weight	*P*	Co-twins with lower birth weight	Co-twins with higher birth weight	*P*
N (male)	86 (41)	86 (41)	-	39 (19)	39 (19)	-	47 (22)	47 (22)	-
Birth weight (g)	2311 ± 521	2635 ± 515	< 0.001	2325 ± 501	2688 ± 550	< 0.001	2300 ± 542	2591 ± 486	< 0.001
GA (weeks)	37.3 ± 2.8	37.3 ± 2.8	-	37.2 ± 2.7	37.2 ± 2.7	-	37.5 ± 2.9	37.5 ± 2.9	-
Age (years)	16.7 ± 2.0	16.7 ± 2.0	-	17.3 ± 1.9	17.3 ± 1.9	-	16.3 ± 2.0	16.3 ± 2.0	-
Current height (cm)	171.8 ± 8.7	173.2 ± 9.3	0.007	173.0 ± 7.3	175.3 ± 8.7	0.03	170.9 ± 9.7	171.5 ± 9.6	< 0.05
Current weight (kg)	58.8 ± 9.1	60.2 ± 8.7	0.03	60.6 ± 8.0	62.6 ± 8.4	0.1	57.3 ± 9.7	58.1 ± 8.4	0.2
BMI (kg/m^2)	19.9 ± 2.2	20.0 ± 2.1	0.4	20.2 ± 2.0	20.4 ± 2.0	0.6	19.6 ± 2.4	19.7 ± 2.1	0.4
Nutrient intake									
Energy (kcal)	2639 ± 703	2524 ± 743	0.04	2726 ± 714	2579 ± 639	0.1	2567 ± 693	2479 ± 824	0.2
Protein (E%)	15.0 ± 2.7	15.4 ± 3.2	0.2	14.7 ± 2.6	15.2 ± 2.8	0.3	15.2 ± 2.9	15.6 ± 3.4	0.4
Carbohydrates (E%)	49.1 ± 5.3	49.4 ± 5.6	0.5	49.4 ± 5.6	49.3 ± 6.5	1.0	48.8 ± 5.1	49.6 ± 4.9	0.3
Total fat (E%)	35.6 ± 5.0	34.8 ± 5.1	0.2	35.6 ± 5.3	35.0 ± 5.4	0.5	35.6 ± 4.8	34.6 ± 4.8	0.2
Saturated fat (E%)	14.8 ± 2.7	14.1 ± 3.0	< 0.05	14.6 ± 2.7	14.0 ± 2.8	0.2	15.0 ± 2.8	14.2 ± 3.2	0.1
Fibres (g/1000 kcal)	23.8 ± 8.5	24.4 ± 8.6	0.4	24.1 ± 9.7	25.3 ± 10.0	0.4	23.5 ± 7.6	23.6 ± 7.3	0.8

Data represent means (± SD). A paired t-test was used to calculate the differences between co-twins with the lower birth weight and co-twins with the higher birth weight.. E%, percentage of total energy intake; GA, gestational age

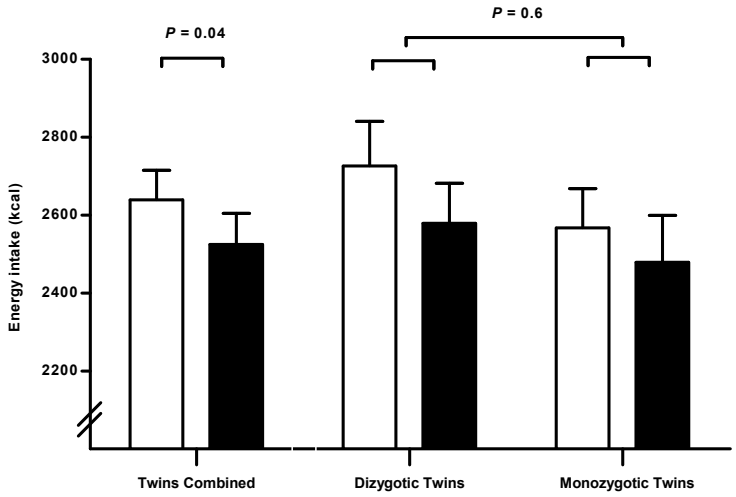

FIGURE 3A
Mean and SEM of total energy intake (kcal) in co-twins with the lower birth weight (white bars) and co-twins with the higher birth weight (black bars) from each pair in all twins and dizygotic and monozygotic twins separately. *P*-values are given for comparisons in intake within twin pairs as well as comparisons in differences between dizygotic and monozygotic twin pairs, and are calculated using the paired t-test.

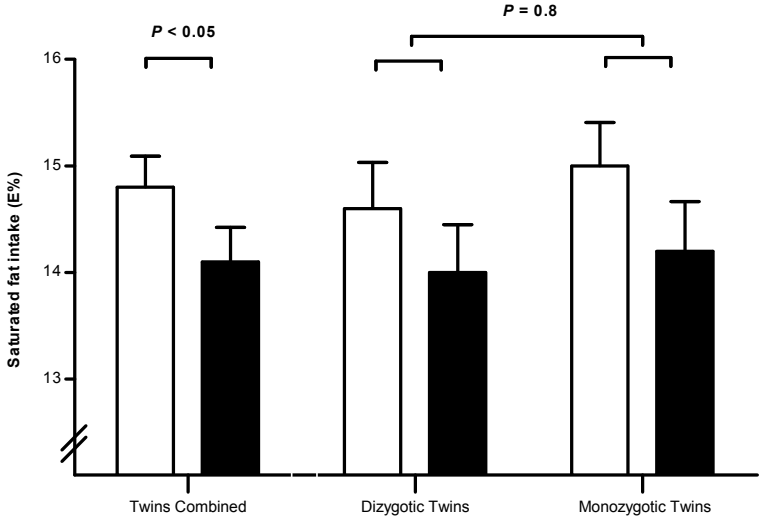

FIGURE 3B
Mean and SEM of saturated fat intake (E%) in co-twins with the lower birth weight (white bars) and co-twins with the higher birth weight (black bars) from each pair in all twins and dizygotic and monozygotic twins separately. *P*-values are given for comparisons in intake within twin pairs as well as comparisons in differences between dizygotic and monozygotic twin pairs, and are calculated using the paired t-test.

INTRA-PAIR ASSOCIATIONS

To further explore the relation between birth weight and dietary intake, we determined the associations between intra-pair differences in birth weight and intra-pair differences in dietary intake. Table 3 shows that intra-pair differences in birth weight tended to be negatively associated with intra-pair differences in total energy intake in all twin pairs, after adjustment for differences in current weight (*P*=0.07). The larger the difference in birth weight, the higher the total energy intake in the twin with the lower birth weight compared to the co-twin with the higher birth weight. To investigate whether this association was influenced by intrauterine environmental and/or genetic factors, we compared dizygotic and monozygotic twin pairs. This association was similar in dizygotic and monozygotic twin pairs (β: -238, [95% confidence interval: -662 to 185] kcal per kg birth weight vs. -265 [-643 to 113] kcal per kg birth weight respectively, *P* for the difference between dizygotic and monozygotic twins, 0.9; Table 3).

Furthermore, intra-pair differences in birth weight were negatively associated with intra-pair differences in intake of total fat and saturated fat in all twin pairs, after adjustment for differences in current weight (for total fat *P*=0.06; for saturated fat *P*=0.04). Again, these associations were similar in dizygotic and monozygotic twin pairs (for total fat β: -2.8 [-6.7 to 1.2] vs. -3.0 [-7.6 to 1.7] respectively, *P* for the difference, 0.9; for saturated fat β: -1.6 [-3.8 to 0.6] vs. -2.2 [-5.0 to 0.6] respectively, *P* for the difference, 0.9; Table 3).

Additionally, intra-pair differences in birth weight were positively associated with intra-pair differences in intake of dietary fibres (*P*= 0.04). The larger the difference in birth weight, the lower the intake of dietary fibres in the twin with the lower birth weight compared to the co-twin with the higher birth weight. Again, these associations were similar in dizygotic and monozygotic twin pairs (β: 2.0 [0.3 to 3.6] vs. 1.8 [-1.5 to 5.2] respectively, *P* for difference between dizygotic and monozygotic twin pairs, 0.9; Table 3).

Analyses without adjustment for differences in current weight or after adjustment for differences in BMI instead of differences in current weight, resulted in similar outcomes (data not shown).

INTERACTION ANALYSES

Interaction analysis indicated that the association between birth weight and dietary intake in the total group was not significantly modified by zygosity, gestational age, current weight or current BMI (data not shown). The association between birth weight and energy intake in the total group was stronger in boys (*P* for interaction <0.05). This effect modification by sex, however, was not found in the further intra-pair analyses of the association between birth weight and energy intake nor in the analyses of the association between birth weight and intake of other macronutrients (in the total group and intra-pair analyses). Further interaction analyses indicated that the association between

intra-pair differences in birth weight and differences in energy intake was weaker in twin pairs with larger differences in current weight (*P* for interaction <0.01), but effect modification by current weight was not found in the total group analyses nor in intra-pair analyses of the associations between differences in birth weight and differences in intake of other macronutrients. The associations between intra-pair differences in birth weight and intra-pair differences in dietary intake were not significantly influenced by zygosity or differences in BMI (data not shown).

TABLE 3
Association between intra-pair difference in birth weight and intra-pair difference in macronutrient intake in all twin pairs and dizygotic and monozygotic twin pairs separately

	All Twin Pairs n=86		**Dizygotic Twin Pairs n=39**		**Monozygotic Twin Pairs n=47**		***P* for difference between DZ and MZ**
	Beta (95% CI)	*P*	Beta (95% CI)	*P*	Beta (95% CI)	*P*	
Energy (kcal)	−249 (−522 to 25)	0.07	−238 (−662 to 185)	0.3	−265 (−643 to 113)	0.2	0.9
Protein (E%)	0.3 (−1.3 to 1.8)	0.7	−0.2 (−1.9 to 1.5)	0.8	0.9 (−1.7 to 3.6)	0.5	0.7
Carbohydrates (E%)	1.8 (−1.0 to 4.7)	0.2	1.9 (−2.8 to 6.5)	0.4	1.8 (−1.9 to 5.5)	0.3	0.9
Total fat (E%)	−2.8 (−5.8 to 0.1)	0.06	−2.8 (−6.7 to 1.2)	0.2	−3.0 (−7.6 to 1.7)	0.2	0.9
Saturated fat (E%)	−1.8 (−3.5 to −0.1)	0.04	−1.6 (−3.8 to 0.6)	0.1	−2.2 (−5.0 to 0.6)	0.1	0.9
Fibres (g/1000 kcal)	1.9 (0.1 to 3.7)	0.04	2.0 (0.3 to 3.6)	0.02	1.8 (−1.5 to 5.2)	0.3	0.9

Data represent betas (95% confidence interval) per kg birth weight after adjustment for differences in current weight. In the last column *P*-values are given for differences in intra-pair associations between DZ and MZ twin pairs, tested with interaction analysis using zygosity as interaction term. DZ, dizygotic; MZ, monozygotic: E%, percentage of total energy intake

DISCUSSION

In line with previous studies in singletons [5, 6, 8] we found in intra-pair analyses in an adolescent twin population that lower birth weight was related to higher total energy intake, higher intake of (saturated) fat and lower intake of dietary fibres. These intra-pair analyses in same sex twin pairs eliminate effects of multiple confounding factors such as sex, gestational age and maternal factors like smoking en social class. In addition, with intra-pair analyses in monozygotic twins also confounding by genetic factors is removed. The size and direction of the intra-pair differences and associations were similar in monozygotic and dizygotic twin pairs. This similarity in the intra-pair differences and intra-pair associations between monozygotic and dizygotic twin pairs suggests that the relation of birth weight with these alterations in dietary intake in later life is independent of genetic factors. These data are compatible with the hypothesis that the association between lower birth weight and alterations in dietary intake is due to intrauterine environmental factors.

Although studies in individuals that were exposed to famine in utero may have been influenced by selection [14], our findings are in line with

the results of two studies demonstrating that prenatal exposure to famine is associated with higher intake of total energy and fat in adulthood [10, 11]. More evidence for the importance of non-genetic factors comes from experimental studies in animals by showing that manipulation of the intrauterine environment can influence eating habits of the offspring [27-29]. Taken together, the results of these studies in combination with our twin study demonstrate that the origin of the association between birth weight and dietary intake lies in the intra uterine environment rather than in the genes of the foetus. These findings suggest that improving the intrauterine environment may positively influence dietary intake in later life.

The molecular mechanisms underlying this programming of appetite are not clear. Several studies have emphasised the important role of leptin in the programming of appetite. Intrauterine growth restricted offspring from undernourished rat dams developed hyperphagia, hyperleptinaemia and obesity in adult life [28], findings that are thought to be induced by peripheral and central leptin resistance [30, 31]. More recently, it has been suggested that the programming of appetite in rats may be reversible by the treatment of leptin injections in a late phase of developmental plasticity [32]. Another hypothesis is that maternal undernutrition leads to an altered set point of the hypothalamic-pituitary-adrenal (HPA) axis in the foetus which causes an increase in its circulating glucocorticoids [33]. As the administration of glucocorticoids in healthy men has shown to stimulate food intake [34], these higher levels in intrauterine growth restricted children could ultimately lead to altered dietary intake , as observed in our study. Unfortunately, in our study, leptin and cortisol levels are not available. Regardless the underlying pathophysiological route, evidence is growing for the involvement of epigenetics in the programming of metabolic disease, suggesting that alterations in dietary intake may be caused by epigenetic changes in the offspring DNA.

It could be proposed that the association between birth weight and dietary intake is due to differences in physical activity [10, 11]. However, epidemiological studies demonstrated that birth weight was not related to physical activity [35, 36] and adjustment for physical activity in a previous study did not influence the results on macronutrient intake [8]. Furthermore, the association between lower birth weight and altered dietary intake may be influenced by a higher basal metabolic rate in individuals born with a lower birth weight [37, 38]. Although we do not have data on basal metabolic rate in our sample of twins, we did perform our analyses with adjustments for age, current weight and/or BMI, factors that mostly determine basal metabolic rate.

Some potential limitations regarding the methodology of this study can be taken into consideration. Underreporting of unhealthy foods is a disadvantage in most methods of dietary intake assessment. We cannot exclude the possibility that underreporting influenced the results of our study. However, we expect that this underreporting would have

resulted in an underestimation of the true effect of birth weight on dietary intake. An advantage of dietary records in our study is that they do not depend on memory as compared to food frequency questionnaires and 24 hours recalls, and thus allow qualitative measurements of the amounts and types of foods at the time they are actually consumed.

It has been suggested that early life nutrition plays an important role in food preference and the development of obesity in later life [39, 40]. Similar to previous studies investigating the relation of birth weight with metabolic and cardiovascular risk [5-8], we did not use data on postnatal feeding. Considering the relevance of this issue, however, future research in this field should try to take into account the influence of early life nutrition on dietary intake in later life.

It could be argued that birth weight in twins are a poor model for differences in birth weight in singletons since intrauterine growth in twins is different from that in singletons [41]. However, the association between birth weight and saturated fat intake in the total group of our twin cohort was similar to the association in singletons in previous studies [6]. In addition, birth weight in twins has been associated with many variables that have been related to birth weight in singletons, such as blood pressure, atherogenic profile, sympathetic activity and type 2 diabetes [20-22, 42]. Although intrauterine growth in twins may be different from that in singletons, the associations between birth weight and cardiovascular risk factors in twins suggest that birth weight in twins is relevant for the development of cardiovascular disease, and that differences in birth weight in twins can be used as a model for differences in birth weight in singletons.

In our study, there was an interaction between birth weight and sex in the total group analysis, such that the association between birth weight and energy intake was stronger in men than in women. This interaction, however, was not present in the intra-pair analysis nor in all other (intra-pair) associations between birth weight and macronutrients. Previous studies did not find this interaction between birth weight and sex for energy intake. However, two singletons studies did show stronger effects of birth weight on fat intake in boys than in girls [5, 6].

The effects we found in this study may seem small and, similar to previous studies [7, 11], there were no differences in current BMI between subjects with lower and subjects with higher birth weight . However, results from animal and observational studies consistently show that even minor improvements in dietary habits reduces risk on cardiovascular disease [43, 44]. Replacement of 1 E% from saturated fatty acids with polyunsaturated fatty acids lowers LDL cholesterol and is likely to produce a reduction in CVD incidence of 2-3% [43]. Furthermore, dietary fibres carry out a protecting effect on CVD risk by enhancing signals of satiety through a bulking effect, thereby controlling total caloric energy intake. Consuming an additional 7 g/day of total fibres lowers the risk of CVD with 9% [44]. Thus, the alterations we observed in our study may have considerable health effects when persisted throughout life.

Therefore, various health organizations highlight the importance of a fibre-rich diet with a total energy content that does not exceed energy expenditure and a limited intake of saturated fatty acids, for instance through the replacement with polyunsaturated fatty acids [45-47].

In summary, we found a higher intake of total energy and saturated fat in the twins with the lower birth weight from each pair compared to their co-twins with the higher birth weight. Also, negative associations were found between intra-pair differences in birth weight and differences in intake of total fat and saturated fat. We found a positive association between intra-pair differences in birth weight and differences in intake of dietary fibres. These differences and associations were similar in dizygotic and monozygotic twins, suggesting that intrauterine rather than genetic factors are responsible for the association between birth weight and dietary intake in later life. Future studies are needed to investigate which specific intrauterine environmental factors are responsible for the association between lower birth weight and alterations in dietary intake. If these intrauterine environmental factors are amenable to intervention, future studies should explore whether improving the intrauterine environment may be used to improve dietary intake and reduce risk of adverse consequences in later life.

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Environmental factors and food intake regulation

PART 2

Physical activity and dietary intake in BMI discordant identical twins

IV

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ABSTRACT

OBJECTIVE

Despite the latest discovery of obesity associated genes, the rapid rise in global obesity suggests a major role for environmental factors. We investigated the influence of environmental factors on physical activity and dietary intake independent of genetic effects.

METHODS

We studied sixteen female monozygotic twins aged 48.8 ± 9.8 years (range 37-70) with a mean BMI discordance of 3.96 ± 2.1 kg/m^2 (range 0.7-8.2). We determined physical activity using 7-day accelerometry, and dietary intake using 3-day 24-hour recalls.

RESULTS

Heavier co-twins were generally less physically active (mean activity counts x 1000 per day ± SD; 505.5 ± 155.1 vs. 579.6 ± 185.4, P=0.047) and tended to spend 6.1 min/day less in moderate to vigorous physical activity than leaner co-twins (P=0.09). Energy intake did not significantly differ within pairs. Total fat intake (E%; P=0.03), in specific monounsaturated fat (P<0.01) and polyunsaturated fat (P=0.08), was higher in the heavier co-twins.

CONCLUSIONS

After eliminating genetic effects, higher BMI is associated with lower overall and moderate to vigorous physical activity and higher intake of total fat, although the direction of causality cannot be determined. Future identification of the environmental factors responsible for these findings might contribute to developing new strategies in managing obesity.

INTRODUCTION

The global rise in overweight and obesity is a major health concern because of the increased risk for chronic diseases like type 2 diabetes, cardiovascular disease (CVD) and cancer [1]. Causes of the obesity epidemic are suggested to be of both environmental and genetic origin. Currently, genes are being identified that predispose to the development of obesity [2]. However, since genetic variation has not changed substantially in the past 30 years, genes alone cannot explain the recent increase in obesity rates, suggesting a major role for a changing environment.

At the individual level, body weight increases if energy intake exceeds energy expenditure. Although dietary intake and physical activity are known as lifestyle factors, exposure to these factors has shown to be under genetic and environmental control. Studies show that an individual's genotype influences the level of exposure to a certain lifestyle factor (gene-environment correlation) [3-5], but also the way an individual responds to this lifestyle factor in terms of body weight gain (gene-environment interaction) [6,7].

In addition to these genetic effects, lifestyle behaviours are also influenced by environmental factors. Identifying environmental factors that influence lifestyle and are amenable for intervention offers a possibility to develop strategies for the prevention and treatment of obesity. To disentangle the effects of the environment from the effects of genes and reduce the impact of gene-environment interaction and gene-environment correlation, we eliminated the influence of genetic factors by using the special design of 'clonal controls', rare monozygotic twins discordant for body mass index (BMI) [8,9]. Monozygotic twins are identical in their genomic sequence, therefore differences in BMI between co-twins must arise from differences in individual-specific environmental factors. Our aim is to investigate whether lifestyle factors such as physical activity, sedentary behaviour and dietary intake are associated with BMI when genetic factors are eliminated.

METHODS

SUBJECTS

All participants are registered with the Netherlands Twin Register (NTR) [10], which comprises twins and their family members who took part in longitudinal survey studies between 1991-2009 and/or in the NTR biobank project between 2004-2008 [11,12]. BMI data were available for 2775 monozygotic twin pairs [13]. The selection of twins is shown in Figure 1. Twin pairs were selected for the current study if measured BMI difference was ≥ 3 kg/m^2 between co-twins at the biobank project. If subjects also had available survey data, twin pairs were selected if BMI difference ≥ 3 kg/m^2 at ≥ 1 survey (most recent), if BMI difference ≥

2 kg/m² at ≥ 3 surveys, or if BMI difference ≥ 2 kg/m² at the most recent survey. Only female pairs were included for homogeneity purposes.

Fifty-four pairs met the first selection criteria, were invited by letter and contacted by telephone to check further eligibility. Fourteen pairs (26%) were unwilling to participate mostly because of lack of time. Twenty-one pairs (39%) were excluded because of pregnancy (n=2), history of eating disorder (n=4), presence of diabetes mellitus (n=1), serious heart disease (n=1), neurological illness (n=3) or reported BMI difference < 2 kg/m² due to recent weight change (n=7). Because the subjects also participated in an MRI study, another 3 twin pairs were excluded because of MRI contra-indications. Finally, 2 pairs could not be contacted due to loss of follow-up. Thus, 16 female, weight-stable (< 5% weight change in previous 3 months) monozygotic pairs (31%) were included in this study. Zygosity of the twins was determined as described previously [12]. One pair was part of a monozygotic triplet. All twins lived apart from their co-twin, except for one pair that had lived in the same household since birth. The study was approved by the ethics committee of the VU University Medical Centre and was performed in accordance with the Helsinki Declaration. All subjects provided written informed consent.

CLINICAL AND BIOCHEMICAL ASSESSMENTS

All measurements were done by an experienced research physician and dietician during a 5-hour test visit in our research clinic and during the month following this visit. Subjects arrived after a12-hour overnight fast. Information on socio-demographics and health status was collected by a short oral and standardized interview. Weight, height and waist and hip circumferences were measured without shoes and wearing light clothing only. For the assessment of body composition, bio-electrical impedance analysis (Maltron BF-906 body fat analyser, Maltron Ltd, Essex, UK) was used. Blood pressure was measured in supine position (Dinamap Pro 100, GE Medical Systems Information Technologies, Inc., Milwaukee, Wisconsin). Heart rate was measured using a 3-lead electrocardiogram (VU University ambulatory monitoring system, VU-AMS) [14]. Venous blood samples were drawn for the assessment of glucose, HbA1c, total cholesterol, high-density lipoprotein cholesterol and triglycerides. Low-density lipoprotein cholesterol was calculated from the Friedewald formula. All biochemical assessments were done at the clinical chemistry laboratory of the VU University Medical Centre. Indirect calorimetry was used to estimate resting energy expenditure, while participants remained in supine position with eyes closed for 15 minutes (Vmax Encore n29; Viasys Healthcare, Houten, Netherlands). Participants completed the 36-item Short Form Health Survey to estimate overall mental and physical health status [15] and the Centre for Epidemiologic Studies-Depression (CES-D) questionnaire to screen for depressive symptoms. A CES-D score of 16 or greater identified subjects at risk for clinical depression [16].

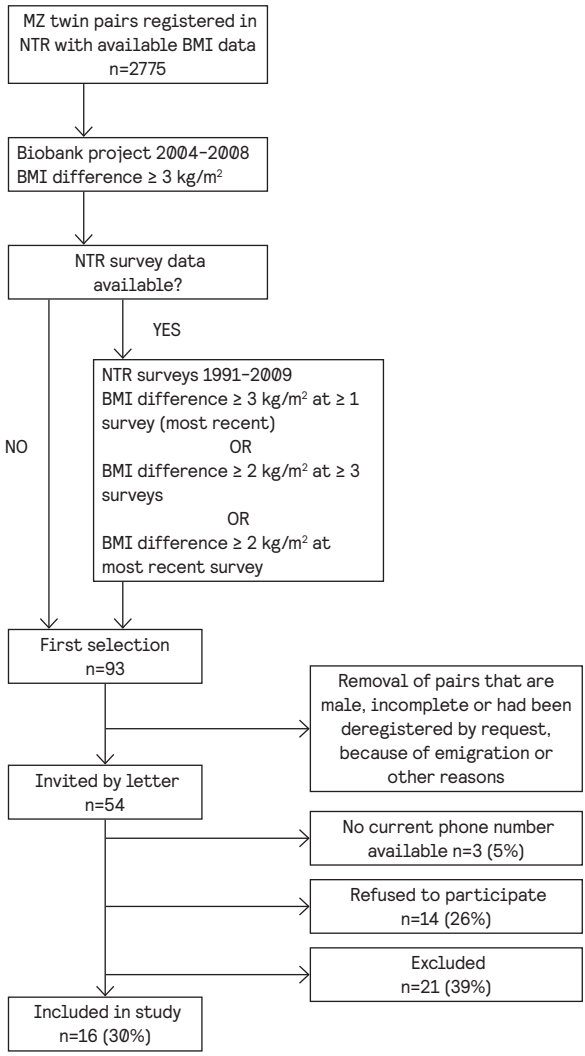

FIGURE 1
Flow chart of the study population. All numbers in the figure represent numbers of monozygotic twin pairs.

PHYSICAL ACTIVITY

Physical activity was measured using two methods.

Accelerometry Subjects received an Actigraph GT3X+ accelerometer (Actigraph LLC, Pensacola, FL, US) [17], and wore the accelerometer attached to an elastic belt on the right hip for all waking hours during a 7-day period, except during water-based activities. Every participant started wearing the device on a Saturday. Recorded data were analysed using Actilife software (version 6.10.2). Non-wear time was defined and excluded if there were 60 consecutive minutes with zero counts, with allowance of 2 minutes with counts between 0-100. Wear time was considered acceptable when there was a minimum of 4 days of

at least 10 hours of wear time per day. Existing cut-points were used to define sedentary (<100 counts/minute), light (100-2019 counts/minute), moderate (2020-5998 counts/minute) and vigorous (>5999 counts/minute) intensity activity [18].

Questionnaire Following the 7-day wear period for the accelerometer, participants completed the short version of the International Physical Activity Questionnaire (IPAQ-SF) [19]. The IPAQ-SF assesses three types of physical activity over the previous week, including vigorous activity, moderate activity and walking. According to the IPAQ-SF scoring manual [19] outliers (i.e. cases in which the sum of walking, moderate and vigorous was greater than 960 min) were excluded. Also, each intensity domain (walking, moderate, vigorous) exceeding 180 min per day was truncated at a duration of 180 min per day. Total physical activity was calculated by multiplying time spent in each intensity domain by their estimated intensity in METs. One MET represents the energy expended while sitting quietly at rest. The MET intensities used were vigorous (8 METs), moderate (4 METs) and walking (3.3 METs).

DIETARY INTAKE

Dietary intake data was collected through 24-hour recalls on 2 weekdays and 1 Sunday by unannounced telephone calls during the month following the test visit [20], using the validated Unites States Department of Agriculture (USDA) five-step multiple-pass method [21]. A food portion size photo book, a table scale type KERN FCE 6 K2 ® and extensive tableware were used for portion-size estimation. All recalls were conducted by one research physician who was instructed by an experienced nutritionist. Food items were coded with the corresponding NEVO-code (Dutch Food Composition Table) [22] by a dietician blinded to BMI status of the subjects. Portion sizes were entered in gram weights. Food consumption and nutrient intake were determined using the NEVO database [22] and included mean intake of total energy (kcal); total fat, saturated fatty acids, mono- and polyunsaturated fatty acids, protein, carbohydrate, alcohol (E%) and dietary fibre (g/1000 kcal). We also determined mean intake of micronutrients calcium, iron, vitamin A, folate, vitamin B12, C and D.

STATISTICAL ANALYSIS

All data preparation and analysis were conducted using IBM SPSS Statistics for Windows (version 20, IBM Corp., 2011, Armonk, NY). Results are expressed as mean ± SD for data with a normal distribution. Differences between the leaner and heavier co-twins were tested with paired t-tests for continuous variables [23], McNemar tests for dichotomous variables and Wilcoxon signed-ranks tests for ordinal data. Since IPAQ-SF data are non-normally distributed results are expressed as median and interquartile ranges [19], and differences were tested with Wilcoxon signed-ranks test. In the total group of twins (n=32) linear

regression analysis was used to examine whether BMI and body fatness were associated with physical activity and dietary intake. To account for non-independence of family members, these analyses were done in Stata 13, including family ID as a cluster variable.

RESULTS

CLINICAL CHARACTERISTICS

Clinical and biochemical characteristics of the leaner and heavier co-twins are presented in Table 1. Selected twins had a mean age of 48.8 ± 9.8 years (range 37-70). As a result of the selection criteria, co-twins differed significantly in weight, BMI, waist-hip ratio and body fat percentage. Mean BMI difference was 3.96 kg/m^2 (range 0.7-8.2) during the test visit.

Resting energy expenditure was higher in the heavier than in the leaner co-twins. However, relative to lean body mass, resting energy expenditure was similar. Without exception the cardiovascular and metabolic risk factors were less favourable in the heavier than in the leaner co-twins, but, except for HDL-cholesterol and total/HDL-cholesterol ratio, these differences were not statistically significant. There were no differences between the co-twins in socio-demographic variables, including smoking, marital status, menopausal status, general physical and mental health and symptoms of depression (Table 2).

TABLE 1
Clinical and biochemical characteristics of leaner and heavier co-twins

	Leaner co-twins (n=16)	**Heavier co-twins (n=16)**	***P*-value**
Age (y)	49.8 ± 9.8	49.8 ± 9.8	-
Height (m)	1.68 ± 0.04	1.68 ± 0.05	0.6
Weight (kg)	68.9 ± 9.2	80.5 ± 11.0	< 0.001
BMI (kg/m²)	24.4 ± 3.1	28.4 ± 3.5	< 0.001
Waist-hip ratio	0.80 ± 0.1	0.84 ± 0.1	0.02
Body fat (%)	32.0 ± 6.1	37.8 ± 6.1	< 0.001
Systolic RR (mmHg) supine	119.4 ± 21.5	126.5 ± 20.6	0.09
Diastolic RR (mmHg) supine	66.6 ± 12.3	70.3 ± 6.1	0.3
Heart rate at rest (bpm)	59.7 ± 7.3	62.7 ± 8.2	0.3
REE (kcal/day)	1564.1 ± 144.3	1701.9 ± 236.3	0.01
REE/LBM (kcal/kg)	33.8 ± 2.8	34.3 ± 2.8	0.5
Glucose (mmol/L)	4.7 ± 0.3	4.8 ± 0.3	0.5
HbA1c (mmol/mol)	36.3 ± 2.6	36.7 ± 2.6	0.3
Total cholesterol (mmol/L)	5.2 ± 1.1	5.3 ± 1.2	0.8
HDL cholesterol (mmol/L)	2.0 ± 0.4	1.7 ± 0.4	0.05
LDL cholesterol (mmol/L)	2.9 ± 1.0	3.2 ± 1.2	0.3
Ratio total / HDL cholesterol	2.7 ± 0.6	3.2 ± 1.0	0.01
Triglycerides (mmol/L)	0.8 ± 0.2	0.9 ± 0.3	0.1

Mean ± SD; REE, resting energy expenditure; REE/LBM, resting energy expenditure divided by lean body mass; HbA1c, glycated haemoglobin; HDL, high-density lipoprotein; LDL, low-density lipoprotein

TABLE 2
Socio-demographic characteristics of leaner and heavier co-twins

	Leaner co-twins (n=16)	Heavier co-twins (n=16)	*P*-value
Education			0.3
Only secondary (%)	5 (31.2)	5 (31.2)	
Vocational (%)	7 (43.8)	10 (62.5)	
Higher or academic (%)	4 (25.0)	1 (6.2)	
Work			0.3
Employed (%)	14 (87.5)	12 (75.0)	
Unemployed (%)	2 (12.5)	2 (12.5)	
Retired (%)	0 (0)	2 (12.5)	
Marital status			0.3
Unmarried (%)	4 (25.0)	1 (6.2)	
Married (%)	11 (68.8)	14 (87.5)	
Divorced or widower (%)	1 (6.2)	1 (6.2)	
Smoking status			0.3
Current smoker (%)	4 (25)	3 (18.8)	
Non-smoker (%)	12 (75)	13 (81.2)	
Menopausal status			0.7
Premenopausal (%)	7 (43.8)	6 (37.5)	
Postmenopausal (%)	6 (37.5)	5 (31.2)	
Unknown (%)	3 (18.8)	5 (31.2)	
Symptoms of depression			
CES-D score mean ± SD	7.1 ± 7.7	6.4 ± 7.1	0.8
CES-D score > 16 (%)	2 (12.5)	3 (18.8)	0.5
Health status			
SF-36 Physical Health	50.7 ± 8.8	51.7 ± 7.2	0.6
SF-36 Mental Health	52.6 ± 6.1	54.7 ± 7.2	0.3

N (%); Mean ± SD; CES-D, Centre for Epidemiologic Studies-Depression; SF-36, Short Form 36-item Health Survey

PHYSICAL ACTIVITY

Median duration of accelerometer monitoring was 7 days with mean duration of 14.9 hours (SD ± 0.9) per day. All participants had acceptable wear time duration and no differences existed in wear time between leaner and heavier co-twins (15.0 ± 0.89 vs. 14.7 ± 0.9; P=0.2). Heavier co-twins had 74100 lower overall activity counts (P<0.05) and 957 fewer step counts (P=0.05) per day than their leaner co-twins (Table 3). Linear regression analyses in the total group of twins (n=32) showed that activity counts correlated negatively with BMI (r=-0.2, P=0.07), fat percentage (r=-0.3, P=0.03) and fat mass (r=-0.3, P=0.03). Step counts correlated negatively with BMI (r=-0.3, P=0.055), fat percentage (r=-0.4, P<0.05) and fat mass (r=-0.4, P<0.05). In total, fifteen out of 16 (93.7%) leaner co-twins and eleven out of 16 (68.7%) heavier co-twins carried out at least 150 minutes per week of moderate to vigorous physical activity (MVPA) (P=0.2). Mean time spent in MVPA was 6.1 minutes per day less in heavier co-twins as compared to leaner co-twins (P=0.09).

Different results were obtained with self-reported physical activity measurements. Completed IPAQ-SF's from five leaner co-twins had to be excluded from the analyses because of outliers, following the IPAQ-SF scoring manual. Median (interquartile range, IQR) total physical activity was 4638 (IQR, 2719-6497) MET/min/week in leaner co-twins and 2853 (IQR, 2234-4788) MET/min/week in heavier co-twins (*P*=0.6). Also, no significant differences were found in median walking, moderate activity or vigorous activity as measured with IPAQ-SF between heavier and leaner co-twins (walking 1386 (IQR, 421-1386) vs. 2657 (IQR, 792-8316) MET/min/week, *P*=0.5; moderate activity 460 (IQR, 210-760) vs. 480 (IQR, 0-1920) MET/min/week, *P*=0.1; vigorous activity 0 (IQR, 0-1060) vs. 480 (IQR, 0-1920) MET/min/week, *P*=0.09).

TABLE 3
Physical activity of leaner and heavier co-twins measured by 7 day accelerometry

	Leaner co-twins (n=16)	Heavier co-twins (n=16)	*P*-value
Sedentary (min/day)	643.8 ± 37.5	656.2 ± 58.6	0.3
Light activity (min/day)	217.5 ± 54.8	195.8 ± 47.7	0.1
MVPA (min/day)	38.5 ± 17.4	32.4 ± 16.7	0.09
Sedentary (%)	71.8 ± 5.8	74.3 ± 6.0	0.1
Light activity (%)	24.0 ± 5.0	22.1 ± 4.9	0.2
MVPA activity (%)	4.2 ± 1.7	3.6 ± 1.8	0.09
Activity count (x 1000 per day)	579.6 ± 185.4	505.5 ± 155.1	< 0.05
Steps (steps/per day)	8294.8 ± 2708.7	7338.3 ± 2573.7	0.05

Mean ± SD; MVPA, moderate to vigorous physical activity

DIETARY INTAKE

Mean daily energy intake did not differ between the co-twins (Table 4). However, mean total fat intake was 4.4 E% higher in the heavier than in the leaner co-twins (*P*=0.03). More specific, monounsaturated fat intake was higher (*P*<0.01) and polyunsaturated fat intake tended to be higher (*P*=0.08) in heavier than leaner co-twins, while no differences were found for saturated fat intake. Leaner co-twins had a higher alcohol intake than heavier counterparts. Micronutrient analyses showed that leaner relative to heavier co-twins had a higher intake of iron, in specific, non-heme iron. Heavier co-twins had a higher intake of fats, oils and savoury sauces as compared to leaner co-twins. Linear regression analyses in the total group of twins (n=32) showed that energy intake was not associated with BMI. However, total fat intake was positively associated with BMI (r=0.3, *P*<0.05) and fat mass (r=0.3, *P*<0.05).

TABLE 4
Dietary intake for leaner and heavier co-twins as estimated by 3 day 24-hour recalls

	Leaner co-twins (n=16)	Heavier co-twins (n=16)	*P*-value
Total energy (kcal)	1971.4 ± 496.8	1912.8 ± 443.6	0.7
Macronutrients			
Carbohydrates (E%)	47.0 ± 5.0	45.5 ± 5.7	0.4
Protein (E%)	15.3 ± 3.1	15.2 ± 2.9	0.9
Total fat (E%)	31.2 ± 4.1	35.6 ± 6.7	0.03
Saturated fatty acids (E%)	12.3 ± 1.7	13.1 ± 2.8	0.4
Monounsaturated fatty acids (E%)	10.0 ± 1.7	12.1 ± 2.9	< 0.01
Polyunsaturated fatty acids (E%)	5.9 ± 2.1	7.1 ± 2.1	0.08
Dietary fibre (g/1000 kcal)	9.8 ± 1.6	9.2 ± 2.9	0.5
Alcohol (E%)[a]	3.1 (0.2 – 6.4)	0.1 (0 – 3.1)	< 0.01
Micronutrients			
Calcium (mg)	1110.9 ± 349.9	957.5 ± 419.2	0.1
Total iron (mg)	10.6 ± 3.0	8.6 ± 2.3	< 0.01
Iron heme (mg)	0.6 ± 0.5	0.7 ± 0.4	0.8
Iron non-heme (mg)	9.9 ± 3.1	7.9 ± 2.3	< 0.01
Vitamin A (µg)	1866.3 ± 1912.7	1745.6 ± 1158.4	0.8
Folate (µg)	212.5 ± 61.7	201.5 ± 60.0	0.4
Vitamin B12 (µg)	3.6 ± 1.7	4.1 ± 1.7	0.5
Vitamin C (mg)	85.3 ± 44.1	87.4 ± 54.0	0.8
Vitamin D (µg)	2.4 ± 1.3	2.4 ± 1.2	0.9
Food groups			
Fruits and vegetables (g)	319.6 ± 93.3	305.4 ± 155.4	0.7
Dairy products and cheese (g)	436.8 ± 265.1	327.2 ± 184.0	0.1
Bread, potato and grain products (g)	317.5 ± 130.4	363.3 ± 121.4	0.2
Meat (g)	95.5 ± 59.2	91.7 ± 33.8	0.8
Fish (g)[a]	0 (0 – 87.8)	0 (0 – 75.9)	0.4
Nuts, seeds, crisps and snacks (g)[a]	36.8 (0 – 72.0)	50.3 (19.4 – 104.8)	0.2
Sugar, sweets and pastries (g)	81.8 ± 38.6	83.9 ± 48.9	0.9
Fats, oils and savoury sauces (g)	34.8 ± 20.4	58.1 ± 29.9	< 0.05

Mean ± SD unless otherwise specified; E%, percentage of total energy intake. [a]Median and interquartile range

DISCUSSION

We found that, within rare monozygotic twins discordant for BMI, heavier co-twins had a lower level of total physical activity and a trend towards less time spent in moderate to vigorous (MVPA) than their leaner co-twins. There were no differences in energy intake, but heavier co-twins had a higher intake of fats, oils and savoury sauces resulting in higher macronutrient intake of total fat, in specific, monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA), as compared to their leaner co-twins. Leaner co-twins had an increased intake of alcohol and total iron than their heavier counterparts. These analyses in monozygotic twins with identical genetic backgrounds allow the elimination of confounding by genetic factors and strongly reduce confounding by gene-environment interaction and correlation.

Thus, the differences we observed must have emerged from differences in exposure to environmental factors.

Our observations are in line with previous studies investigating lifestyle factors in monozygotic twins discordant for obesity [24, 25]. A preference for fatty foods was found in obese versus lean co-twins after qualitative recall of food consumption patterns [24]. Apparently, this acquired preference was already present at adolescence before onset of BMI discordance, suggesting a causal role in the development of obesity [24]. Due to the qualitative nature of the data, however, the fatty foods preferred by the obese co-twins in the previous study could not be subdivided into proportions of saturated and unsaturated fats. In our more quantitative data analyses we showed that the intake of, in specific, MUFA was higher in heavier versus leaner co-twins. The effect of MUFA on CVD risk is controversial, as previous meta-analyses of cohort studies showed inconsistent results [26, 27]. However, subgroup analyses found a significant beneficial effect on CVD of MUFA derived from vegetable oils rather than animal products [28]. Olive oil is suggested to be the driver of the beneficial health effects of the Mediterranean diet [29]. We were unable to identify the exact food sources of MUFA in our study. However, the higher intake of fats, oils and savoury sauces might give a part of the explanation, since this food group mainly contains fat products derived from plants (e.g. margarine, oils and table sauces). Also the concomitant higher intake of PUFA in the heavier co-twins suggests that the MUFA are supplied by vegetables rather than animal products.

Our results are in contrast to a previous study that observed lower rather than higher MUFA and PUFA intakes in the obese compared to non-obese monozygotic co-twins [30]. An explanation for this discrepancy might be that in the previous study fat intake was avoided more actively in obese subjects, while in our study participants had primarily declared not to be on weight loss diets. Another option is that relative to 24-h recalls, food diaries as used in the previous study are more susceptible to underreporting of unhealthy foods, such as fats, since participants may influence their food intake when they are aware all consumed foods must be recorded.

Similar to our study, no differences were found in total energy intake between lean and obese co-twins in a study using food diaries in monozygotic discordant twins [30]. It has been suggested that overweight and obese subjects underreport their energy intake during dietary surveys [31]. The previous twin study confirmed this underreporting by comparing data from food diaries with doubly labelled water assessments as a measure for true total energy expenditure [30]. The USDA five-step multiple-pass method we used in our study is a valuable method for quantitative dietary intake assessments as compared to, for example, food frequency questionnaires and food diaries [20]. Nevertheless, we cannot exclude the possibility that obesity-related underreporting affected our results.

Current physical activity guidelines recommend at least 150 minutes per week of MVPA to reduce risk for many chronic diseases [32]. Our data show that 93.7% of leaner and 68.7% of heavier co-twins get sufficient physical activity to meet this requirement, which are similar proportions as in the general population where 7 of 10 individuals reach this demand [33]. The clinical relevance of our observations in MVPA remains open to question since the influence of MVPA on body weight is controversial [34]. Several accelerometer studies showed inverse associations between MVPA and risk of obesity [35, 36], while other longitudinal studies failed to detect an association between self-reported leisure time exercise behaviour and BMI [37, 38]. The discrepancies in these observations might be a result of different definitions of physical activity and different test methods being used. Accelerometers measure motion without distinguishing between voluntary leisure time exercise behaviour and other aspects of physical activity such as household activities and walking or cycling to work. Thus, the higher MVPA we observed in the leaner versus heavier co-twins in our study represents activity performed in all domains of activity during a day rather than just exercise behaviour.

The lower total physical activity and time spent in MVPA in heavier versus leaner co-twins we observed is in line with two previous studies investigating monozygotic twins discordant for BMI [25, 30]. Similar to our results, obese versus lean co-twins showed lower accelerometer activity counts [25] and less reported high-intensity activity [30]. Another study failed to detect differences in physical activity among BMI discordant monozygotic co-twins [39]. This study, however, used retrospective interviews rather than objective assessment tools such as accelerometry. Taken together, the results of two previous studies [25, 30] in combination with our results demonstrate that the origin of the association between lower physical activity and higher BMI lies (at least in part) in the exposure to unique environmental factors, independent of genotype.

It could be hypothesized that these unique environmental factors could act through the genome by causing epigenetic differences [40]. Alterations in DNA methylation and histone modification before and after birth may influence the development of disease without changing the DNA sequence. A recently published study, however, found no significant differences in gene expression of BMI-associated loci between BMI discordant monozygotic twins [13]. However, the assessments were performed in peripheral blood only, leaving role for an epigenetic influence on regulatory pathways underlying BMI discordance through other tissues.

Because of the cross-sectional nature of our study no conclusion can be drawn whether the reduced physical activity resulted in higher BMI or whether the increased weight led to decreased physical activity. However, a previous retrospective monozygotic twin study on leisure time activity observed that the physically inactive co-twin at adolescence had a higher risk of becoming obese compared to their

physically active co-twin in adulthood [25], suggesting a causal role for physical inactivity in the development of obesity.

Our final study sample comprised 2 twin pairs that were not strictly BMI discordant during the clinical assessments (BMI differences of 0.71 and 1.02 kg/m^2). This is possibly because subjects guessed their BMI incorrectly at the moment of screening. Post hoc analyses after excluding these 2 pairs did not influence the results in terms of effect sizes, although the decrease in power obviously resulted in less statistical significance of our findings.

We acknowledge that a sample of 16 twin pairs may seem relatively small. However, since body weight is a highly heritable trait, a mean BMI discordance of almost 4 kg/m^2 as seen in our study sample between monozygotic twins is very rare [13]. The sample size should be appreciated in light of this design, which involves monozygotic twins discordant for BMI, but perfectly matched with respect to age, gender and genetic background. This design in combination with the accuracy of the phenotypic measures is optimal with respect to power.

Strengths of our study are the use of accelerometers to objectively measure physical activity and the use of the USDA five-step multiple-pass method to assess dietary intake. Although underreporting remains a subject of concern as in all dietary surveys, the 24-hour recall method, in our opinion, is the next best thing by not interfering with actual dietary behaviour and keeping respondent burden low.

In summary, we demonstrated that higher BMI is associated with a lower total physical activity and a higher intake of total fat, in specific MUFA and PUFA. Our finding that these associations were observed within monozygotic twin pairs with an identical genetic background implicates that these associations are independent of genetic factors. Thus, exposure to unique environmental factors is responsible for the more health-compromising lifestyle factors observed in individuals with a higher BMI. However, it cannot be determined whether the lower physical activity and higher fat intake are a cause or a consequence of the increased BMI. Future identification of the underlying unshared environmental factors responsible for our findings, for instance by qualitative in-depth interviews of BMI discordant monozygotic pairs, may provide starting points towards developing new strategies in the management of obesity.

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Brain reward responses to food stimuli among female monozygotic twins discordant for BMI

V

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ABSTRACT

OBJECTIVE

Obese individuals are characterized by altered brain reward responses to food. Despite the latest discovery of obesity-associated genes, the contribution of environmental and genetic factors to brain reward responsiveness to food remains largely unclear.

METHODS

Sixteen female monozygotic twin pairs with a mean BMI discordance of 3.96 ± 2.1 kg/m^2 were selected from the Netherlands Twin Register to undergo functional MRI scanning while watching high- and low-calorie food and non-food pictures and during the anticipation and receipt of chocolate milk. In addition, appetite ratings, eating behaviour and food intake were assessed using visual analogue scales, validated questionnaires and an ad libitum lunch.

RESULTS

In the overall group, visual and taste stimuli elicited significant activation in regions of interest (ROIs) implicated in reward, i.e. amygdala, insula, striatum and orbitofrontal cortex. However, when comparing leaner and heavier co-twins no statistically significant differences in ROI-activations were observed after family wise error correction. Heavier versus leaner co-twins reported higher feelings of hunger (P=0.02), cravings for sweet food (P=0.04), body dissatisfaction (P<0.05) and a trend towards more emotional eating (P=0.1), whereas caloric intake was not significantly different between groups (P=0.3).

CONCLUSION

Our results suggest that inherited rather than environmental factors are largely responsible for the obesity-related altered brain responsiveness to food. Future studies should elucidate the genetic variants underlying the susceptibility to reward dysfunction and obesity.

INTRODUCTION

Increasing evidence suggests that altered brain reward responses to food stimuli promote excessive eating, making people prone to the development of obesity [1, 2]. In studies using functional magnetic resonance imaging (fMRI), we and others demonstrated that obese compared to lean individuals have higher activity in reward-related areas, such as the insula, striatum and amygdala when watching palatable food images or cues that predict palatable food receipt [3-6], as well as less activation in response to the actual receipt of palatable food [7, 8]. Increased activity to food cues in obese individuals may reflect higher craving for food, while decreased activation to actual consumption may reflect a reward deficit leading to compensatory overeating [9].

Body weight regulation is known to be influenced by a multitude of genetic and environmental factors and their interactions [10]. Results from twin and adoption studies suggest that 40-70% of inter-individual variability in BMI is explained by genetic factors whereas the shared environment of family members, such as living in the same household, has only a limited impact [11, 12]. Previous neuroimaging studies observed altered brain responses to food stimuli in individuals with rare monogenic forms of hyperphagia and obesity [13, 14], and in carriers of risk alleles of genes associated with common obesity, such as the *FTO*-gene [15, 16]. These findings indicate that altered reward function in the brain is a feature of the genetic predisposition to excessive eating and weight gain.

In addition to heredity, environmental factors play an important role in body weight regulation and obesity development, as evidenced by the rapid increase in obesity prevalence during a time period in which gene pools of populations remained relatively stable. Further evidence for a role for the environment comes from monozygotic twins which, despite identical genetic backgrounds, can differ in body weight and dietary intake [17, 18]. In contrast to genetic factors, the influence of environmental factors on brain reward responsiveness to food has not been investigated. Although a recent fMRI study investigated brain responsiveness to food in monozygotic twins [19], the focus of this study was on the degree of *similarity* within the twins, which provides a measure of genetic influences, whereas focusing on intra-pair *differences* allows for the investigation of unique environmental influences. Since monozygotic twins are genetically identical, all differences between the twins must be ascribed to unique environmental factors.

Therefore, in the present study we used a special design of monozygotic twins discordant for BMI to investigate the influence of environmental factors on individual differences in brain reward responsiveness to visual food cues and to the anticipation and receipt of a palatable food stimulus as measured with fMRI.

METHODS

SUBJECTS

The selection of participants from the Netherlands Twin Registry [20] was done as described in detail previously [17]. In short, out of 2775 monozygotic twin pairs, 54 female monozygotic twin pairs were selected as having a BMI discordance of ≥ 2 kg/m^2 based on previously measured BMI [21]. Only females were selected because of earlier reported sex-differences in food-related brain activations and larger responses in females compared to males [3]. Twin pairs were invited by letter and contacted by telephone to check their willingness and eligibility. Inclusion criteria comprised age range 18-75 years, stable body weight (<5% reported change during the previous 3 months) and normoglycemia as defined by fasting glucose <7.0 mmol/L on the day of the test visit. Exclusion criteria were current diabetes mellitus, serious heart, liver or renal disease, malignancies, uncontrolled thyroid disease, neurological or psychiatric disease including eating disorders and depression (assessed by the Centre for Epidemiologic Studies Depression Scale [22]), pregnancy or breast feeding, MRI contra-indications, alcohol or drug abuse and the use of glucose-lowering drugs or psychoactive medication. Fourteen twin pairs were unwilling to participate mostly because of reported lack of time. Twenty-one twin pairs were excluded due to exclusion criteria as published previously [17].

Thus, 16 female monozygotic twin pairs were willing and eligible to participate. Zygosity assessments were based on DNA genotyping performed on Affymetrix 6.0 [20]. One pair was part of a monozygotic triplet. The study protocol was approved by the medical ethics committee of the VU University Medical Centre and performed in accordance with the Helsinki Declaration. All subjects provided written informed consent.

MEASURES

Clinical assessments Participants were asked to consume their regular meals the day prior, but to refrain from eating or drinking for 12 hours and performance of heavy exercise for 24 hours preceding their test visit. Both co-twins of a pair arrived at the research clinic between 8 and 10 AM on the same day. Information on socio-demographics and health was collected using oral interviews. Anthropometric data were measured in a standardized manner as described previously [17].

Questionnaires Before the scanning session participants were asked to rate their feelings of appetite on a Likert scale ranging from 0 ('not at all' or 'nothing at all') to 10 ('extremely' or 'a lot') [5,23]. Participants were asked the questions1) How hungry are you now? 2) How full are you now? 3) How much could you eat right now? 4) How much is your desire right now to eat something sweet / savoury / fat? The Dutch Eating Behaviour Questionnaire (DEBQ) [24], a 33-item validated tool to assess

eating behaviour, was used to assess emotional, external and restrained eating. The Eating Disorder Inventory (EDI) version 2 [25] was used to assess 3 psychological aspects relevant for eating disorders (i.e. drive for thinness, bulimia and body dissatisfaction). In these analyses we used the untransformed scoring system, with ratings from one to six [26].

Food stimuli ratings After the scanning session participants viewed all food pictures that were presented during the fMRI session and rated each picture on how attractive the food in the picture appeared to them at that moment on a Likert scale ranging from 1 ('not at all') to 7 ('extremely'). On a similar scale participants rated the attractiveness of the taste of chocolate milk and tasteless solution used in the fMRI experiment.

Ad libitum lunch meal At the end of the test visit participants were presented a standardized varied choice meal [5, 8]. The meal consisted of white and multigrain bread, a mixed green salad, orange juice, Dutch cheese, fresh meats, margarine, mayonnaise, peanut butter, jam, cake, a chocolate muffin, a banana and an apple. Twins were seated at two separate tables, each on the other side of the room. They could eat as much as they wanted and were not informed that their consumption of food was being monitored. Food items were coded with the corresponding NEVO-code (Dutch Food Composition Table) [27]. Intake of energy (kcal) and percentages of kcal derived from total fat, saturated fat, unsaturated fat, protein and carbohydrates was determined.

IMAGING PARADIGMS

Imaging paradigms used in the current study were described in detail previously [5, 6, 8, 28].

Food pictures Pictures were presented in 3 runs comprising 6 blocks each: 2 blocks of high-calorie (HC) food (e.g. chocolate cake, ice-cream, pizza, and hamburgers), 2 blocks of low-calorie (LC) food (e.g. apples, broccoli, tomatoes and green salads) and 2 blocks of non-food items (e.g. trees, flowers, rocks and bricks) (Figure 1a). Within each block 7 pictures were presented for 2.5 sec each, separated by a 0.5 sec blank screen. Participants were instructed to attentively watch each picture. One hour after the scanning session a recognition test was performed. The recognition test consisted of 20 pictures of which subjects needed to identify those 10 pictures that were previously shown in the scanner.

Palatable food stimuli Each fMRI run included 64 trials. Chocolate milk (Chocomel; 86 kcal, 2.7 g fat, 11.8 g sugar per 100 ml) was used as a palatable food stimulus. A tasteless solution was used as a neutral stimulus, designed to mimic the natural taste of saliva [7]. During each trial an image was presented (either an orange triangle or a blue star) that signalled the delivery of either 0.4 ml chocolate milk or tasteless

solution (Figure 1b). Images were presented for 2 sec (i.e. anticipation) in random order, followed by 3 sec of blank screen with a fixation cross and 2 sec of stimulus delivery (i.e. receipt). Participants were instructed to keep the solution in their mouth during 6 sec until the sign 'swallow' appeared. The next trial was started after a jitter of 3-7 sec. In 40% of the events, the cue was not followed by a stimulus delivery [4].

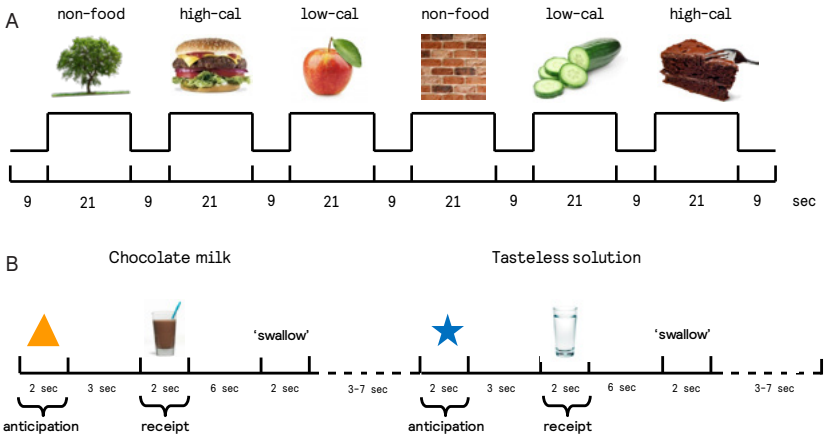

FIGURE 1
Example of timing of picture presentation during the food picture fMRI paradigm (A) and example of timing of cue presentation and stimuli delivery during the chocolate milk fMRI paradigm (B). Cal, calorie

IMAGE ACQUISITION

Imaging data were acquired using a 3.0 Tesla GE Signa HDxt scanner (General Electric, Milwaukee, WI, USA). For structural imaging, T1 weighted scans were acquired using a 3D fast spoiled gradient-echo sequence. For the functional data, a $T2^*$ weighted gradient echo-planar imaging sequence was used (repetition time/echo time = 2160/30 msec, flip angle 80°, slice thickness 3 mm, matrix size 64 x 64, 211 x 211 mm^2 field of view, voxel size 3 x 3 x 3 mm, 40 slices).

DATA ANALYSIS

Clinical data Clinical and behavioural data were analysed using IBM SPSS Statistics (version 20, IBM Corp., 2011, Armonk, NY). Results are expressed as mean ± SD. Differences between the leaner and heavier co-twins were tested with paired sample t-tests for continuous variables [29], McNemar tests for dichotomous variables and Wilcoxon signed-ranks tests for ordinal data.

Imaging data Data were pre-processed using SPM8 software (Wellcome Trust Centre for Neuroimaging, London, UK) run within Mat-

lab R2012a (Mathworks, Inc.). Due to obvious artefacts resulting from a metal implant in the spinal cord, the data set of one woman (and her twin sister in case of paired analyses) was excluded from further fMRI analyses. Of the remaining data, the origin of each volume was aligned to the anterior commissure. Functional images were realigned to the first volume and slice-time corrected to the onset of the middle slice. After co-registration to T1 scans, volumes were normalized into standard Montreal Neurological institute (MNI) space. Volumes were resliced into 3 x 3 x 3 mm voxels and spatially smoothed using a 8 mm full width at half maximum Gaussian kernel. The functional data were passed through a high-pass filter (cut-off 128 s). No data set showed within-run head movement of >2.5 mm in translation or >2.5° in rotation.

Block-design BOLD-responses were analysed within the context of the general linear model. At the first level, for each participant contrast images were generated for 1) watching food vs. non-food pictures, 2) watching high-calorie vs. non-food pictures, 3) anticipating chocolate milk vs. baseline, and 4) chocolate milk receipt vs. baseline. Baseline was defined as the jittered time between trials, excluding the first 3 sec. To specifically assess the effect of anticipating and receiving a palatable taste stimulus as opposed to anticipating and receiving a taste stimulus in general, contrasts were also generated for 5) anticipation of chocolate milk vs. tasteless solution and 6) receipt of chocolate milk vs. tasteless solution.

Based on previous studies on food reward and motivation [3, 4] we selected the amygdala, insula, caudate nucleus, putamen and orbitofrontal cortex (OFC) as our a priori regions of interest (ROIs). We defined functional ROIs specific to our tasks and contrasts based on the orthogonal main effects of all participants in this study [30, 31]. To this end, contrasts of all participants were entered in a one-sample t-test and, for each contrast, a statistical map was calculated. An implicit anatomical mask containing our bilateral ROIs (created with the Wake Forest University (WFU) toolbox, Winston-Salem, NC, USA) was used to visualize brain activation in our a priori anatomical ROIs only. Statistical maps of the one-sample t-tests were thresholded at $P<0.001$ uncorrected. Montreal Neurological Institute (MNI) coordinates of significantly activated peak voxels were used to create contrast-specific ROIs, by using spheres around the peaks with a radius of 10 mm (or 5 mm for amygdala). Group differences in contrast-specific ROI activations between leaner and heavier co-twins were examined with paired t-test in SPM using a threshold of $P<0.05$ family wise error (FWE) corrected for small volume. In addition to ROI-analyses, results are reported of regions not of our a priori interest when $P<0.05$ FWE whole brain corrected.

RESULTS

CLINICAL CHARACTERISTICS

We included 16 female monozygotic twin pairs with a mean BMI difference of 3.96 ± 2.1 kg/m² (range 0.7-8.2) and a mean age of 48.8 ± 9.8 (Table 1). After excluding the twin pair comprising the participant with imaging artefacts, the mean BMI discordance was 4.2 ± 1.9 kg/m² (range 1.0-8.2). Without exception, metabolic risk factors were less favourable in the heavier than in the leaner co-twins, although only lower HDL-cholesterol and higher HDL/total cholesterol ratio in the heavier co-twins were statically significant. All subjects had normal fasting glucose levels.

TABLE 1
Characteristics of leaner and heavier co-twins

	Leaner co-twins (n=16)	Heavier co-twins (n=16)	*P*-value
Age (y)	49.8 ± 9.8	49.8 ± 9.8	-
Weight (kg)	68.9 ± 9.2	80.5 ± 11.0	< 0.001
BMI (kg/m²)	24.4 ± 3.1	28.4 ± 3.5	< 0.001
Waist-to-hip ratio	0.80 ± 0.1	0.84 ± 0.1	< 0.05
Percentage body fat (%)	32.0 ± 6.1	37.8 ± 6.1	< 0.001
Fasting glucose (mmol/L)	4.7 ± 0.3	4.8 ± 0.3	0.5
HbA1c (mmol/mol)	36.3 ± 2.6	36.7 ± 2.6	0.3
Total cholesterol (mmol/L)	5.2 ± 1.1	5.3 ± 1.2	0.8
HDL cholesterol (mmol/L)	2.0 ± 0.4	1.7 ± 0.4	0.05
LDL cholesterol (mmol/L)	2.9 ± 1.0	3.2 ± 1.2	0.3
Ratio total / HDL cholesterol	2.7 ± 0.6	3.2 ± 1.0	0.01
Triglycerides (mmol/L)	0.8 ± 0.2	0.9 ± 0.3	0.1

Mean ± SD, all biochemical assessments are done in the fasted state. HbA1c, glycated haemoglobin; HDL, high-density lipoprotein; LDL, low-density lipoprotein

Subjects used the following medication: thyroid hormone replacement medicines (n=5, in 3 twin pairs), oral contraceptives (n=4, in 3 twin pairs), antihypertensive medication (n=9, in 7 twin pairs) and statins (n=8, in 5 twin pairs). Leaner and heavier co-twins were comparable for self-reported daily smoking (*P*=0.5), handedness (*P*=1.0) and menopausal status (*P*=0.7). Of the included women, 6 were daily smokers: in 2 pairs both co-twins smoked and in 2 pairs only the leaner co-twin smoked. Two women were left handed: 1 leaner and 1 heavier co-twin in different pairs. Thirteen women were premenopausal (defined as having a regular menstrual cycle): 7 leaner and 6 heavier co-twins in 7 pairs. In premenopausal women we initially aimed to perform all scans during the follicular phase [32] defined as day 1–12 counting forward from the start of the menstruation. However, since both co-twins of a pair were scanned on the same day, this was not always feasible. Nevertheless, no significant group differences were present in menstrual cycle phase, with 3 women scanned during the follicular phase in each group (*P*=0.3).

BEHAVIOURAL MEASURES

Questionnaires Heavier co-twins reported higher feelings of hunger (P=0.02) and desire to eat something sweet (P=0.04) as compared to the leaner co-twins prior to the scanning session (Figure 2), while there was a trend in desire to eat something savory (P=0.08) and something high in fat (P=0.06). Heavier co-twins tended to score higher on emotional eating (P=0.1 Figure 3a), and significantly scored higher on body dissatisfaction (P<0.05 Figure 3b) than leaner co-twins.

Food stimuli ratings Participants in both groups rated the low-calorie food pictures as more appealing than the high-calorie food pictures (5.3 ± 0.9 vs. 3.8 ± 0.6; P<0.001 in leaner co-twins; and 4.8 ± 0.8 vs. 4.0 ± 1.0; P<0.05 in heavier co-twins). Both groups rated the chocolate milk and tasteless solution as equally appealing (4.9 ± 1.2 vs. 5.1 ± 1.4; P=0.7 in leaner co-twins; 5.3 ± 1.8 vs. 4.9 ± 1.3; P=0.3 in heavier co-twins). Furthermore, leaner and heavier co-twins performed similarly on the image recognition test after the scan (P=0.9), with mean percentages of images correctly recognized of 83.8 ± 8.7 vs. 84.1 ± 12.3, respectively.

Ad libitum lunch meal Total energy intake during the lunch meal following the scanning session was not significantly different between heavier and leaner co-twins, (815 ± 212 vs. 763 ± 188 kcal respectively, P=0.3). There were also no differences in macronutrients intake between the groups (data not shown).

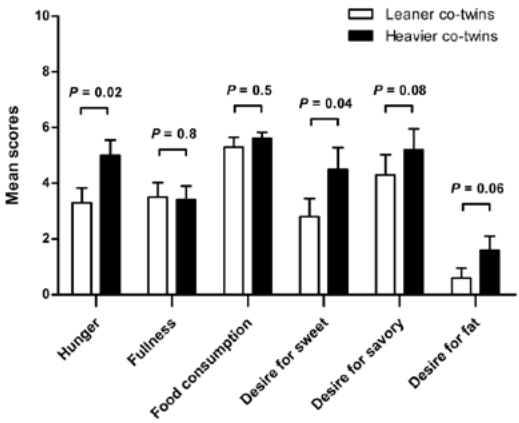

FIGURE 2
Mean ± SEM hunger and appetite ratings of leaner and heavier co-twins prior to the scanning session on a scale from 1 to 10 for the questions 1) How hungry are you? 2) How full are you? 3) How much food could you eat right now? 4) How strong is your desire right now to eat something sweet / savory / fat?

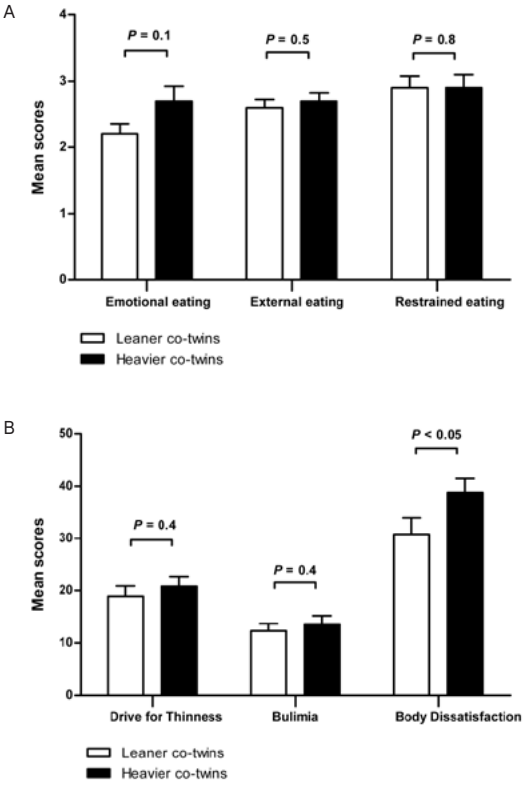

FIGURE 3
Mean scores ± SEM of leaner and heavier co-twins on A) emotional, external and restraint eating, and B) drive for thinness, bulimia and body dissatisfaction

BRAIN RESPONSES TO FOOD PICTURES

In the overall group of women, we observed a significant main effect of watching food vs. non-food pictures within our a priori ROIs, in particular the left amygdala and bilateral orbitofrontal cortex (OFC) (Table 2 and Figure 4). Watching high-calorie vs. non-food pictures resulted in activation of bilateral amygdala, bilateral OFC, bilateral caudate nucleus and left insula. Main effects of tasks in other regions of the brain ($P<0.05$ FWE whole brain corrected) are presented in Supplementary Table 1.

When comparing groups for mean activation in the contrast-specific ROI's, no significant differences were observed between leaner and heavier co-twins in watching food vs. non-food pictures or high-calorie vs. non-food pictures (FWE corrected for small volume). Post hoc exploration at a more lenient threshold of $P<0.001$ in a priori anatomical ROIs also revealed no significant differences between leaner and heavier co-twins. Additional analyses were performed using anatomical ROIs based on the ALL atlas included in the WFU Pickatlas toolbox. Again no significant differences in ROI activation between leaner and heavier co-twins were found.

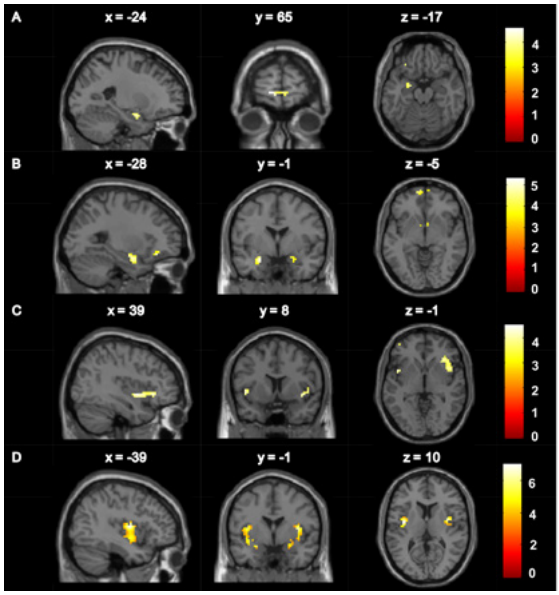

FIGURE 4
Main activations in a priori anatomical ROIs in the total group of participants with threshold P<0.001 uncorrected for the contrasts A) watching food vs. non-food pictures, B) watching high-calorie vs. non-food pictures, C) anticipation of chocolate milk vs. baseline, and D) receipt of chocolate milk vs. baseline. Colour bar represents T-value

BRAIN RESPONSES TO ANTICIPATION AND RECEIPT OF PALATABLE FOOD

In the overall group of women, we observed a significant main effect of chocolate milk anticipation vs. baseline in bilateral insula and bilateral OFC (Table 2 and Figure 4). The receipt of chocolate milk vs. baseline significantly activated bilateral insula and bilateral amygdala. Main effects of task in other regions of the brain (P<0.05 FWE whole brain corrected) are presented in Supplementary Table 1. When contrasted to the tasteless solution, no main effects of chocolate milk anticipation or receipt were observed in our ROIs.

When comparing groups for mean activation in the contrast-specific ROI's, no significant differences were observed between leaner and heavier co-twins for the anticipation of chocolate milk vs. baseline or the receipt of chocolate milk vs. baseline (FWE corrected for small volume). Post hoc exploration of group differences at a more lenient threshold of P<0.001 in a priori anatomical ROIs revealed that heavier vs. leaner co-twins had lower activation during anticipation of chocolate milk vs. baseline in the left OFC (MNI -27 35 -11, T=3.8 cluster size k=2 P=0.0009). In contrast, heavier vs. leaner co-twins had higher activation to the receipt of chocolate milk vs. baseline in the left insula (MNI -36 11 7, T=4.2 k=4 P=0.0004). Additional analyses using anatomical ROIs based on the ALL atlas included in the WFU Pickatlas toolbox did not reveal significant differences in ROI activation between leaner and heavier co-twins.

TABLE 2
Main effects of tasks in ROIs

				MNI			
	Side	**k**	**T**	**x**	**y**	**z**	***P*-value**
Food vs. non-food pictures	L	24	4.6	-12	65	-2	3.9×10^{-5}
OFC	R		3.9	6	65	-2	2.7×10^{-4}
	L	1	3.7	-30	32	-17	4.1×10^{-4}
Amygdala	L	15	4.2	-24	-1	-20	1.0×10^{-4}
High-calorie vs. non-food pictures							
Amygdala	L	36	5.3	-24	-1	-20	4.8×10^{-6}
	R	8	4.1	21	-4	-17	1.5×10^{-4}
OFC	L	13	5.0	-27	32	-14	1.3×10^{-5}
		24	4.9	-12	65	-2	1.7×10^{-5}
	R		3.8	3	65	-2	3.5×10^{-4}
Insula	L	3	4.1	-36	5	-14	1.3×10^{-4}
		7	4.0	-36	-7	4	1.9×10^{-4}
Caudate nucleus	L	4	3.9	-9	14	-2	2.9×10^{-4}
		1	3.5	-6	5	-5	7.1×10^{-4}
	R	2	3.7	6	5	-5	3.9×10^{-4}
Anticipation chocolate milk vs. baseline							
Insula	R	111	4.5	39	2	-5	4.8×10^{-5}
OFC	R		4.4	36	26	-8	7.1×10^{-5}
Insula	R		4.3	42	11	-5	8.2×10^{-5}
		2	3.4	36	20	13	9.1×10^{-4}
	L	7	4.3	-45	8	-2	7.9×10^{-5}
		1	3.5	-36	-10	-8	8.0×10^{-4}
		1	3.4	-27	26	-5	9.0×10^{-4}
OFC	L	1	3.5	-42	53	-2	8.1×10^{-4}
Receipt chocolate milk vs. baseline							
Insula	L	96	7.0	-39	-4	10	4.2×10^{-8}
	L		5.5	-36	-4	-8	2.7×10^{-6}
	R	56	6.2	39	-1	10	4.1×10^{-7}
	R		4.6	39	-1	-2	3.9×10^{-5}
Amygdala	L	15	5.7	-24	-1	-17	1.6×10^{-6}
	R	16	4.5	27	-1	-14	5.2×10^{-5}

Montreal Neurological Institute (MNI) coordinates of peak voxels activated in a priori anatomical ROIs in the total group of participants with threshold P<0.001 uncorrected. Reported *P*-values are uncorrected. K, cluster size; T, T-statistic; OFC, orbitofrontal cortex; L, left; R, right.

DISCUSSION

We used a unique design of monozygotic twins discordant for BMI to examine the influence of unique environmental factors on obesity-related alterations in brain reward responses to food. In the overall group of females we observed significant main effects of our fMRI experiments, i.e. watching (high-calorie) food pictures and the anticipation and receipt of a palatable food stimulus, in brain regions implicated in reward and motivation, such as the insula, amygdala, caudate nucleus and orbitofrontal cortex (OFC). However, when comparing heavier and leaner co-twins in activation of these regions of interest (ROIs), we observed no statistically significant differences between the groups.

These findings are of interest since in previous studies in unrelated individuals we [5, 6, 8, 28] and others [3, 4, 33, 34] observed that obese relative to lean individuals have increased reward region responsiveness to palatable food images or cues that predict palatable food receipt, and decreased striatal activation to the consumption of a palatable food. The lack of these associations in our monozygotic twin design suggests that the previously observed associations between brain reward responses and obesity in unrelated individuals can be explained by genetic factors. This aligns with findings of two previous studies in twins. First, evidence for a substantial genetic influence on food reward was provided by a classical twin study showing that 75% of variability in food cue responsiveness, as examined with validated questionnaires, was explained by genetic factors [35]. More recently, a study in monozygotic twins reported greater similarity *within* twin pairs than *between* twin pairs in brain responses to visual food cues as measured with fMRI [19], indicating an important role of inherited factors in the brain's appetite regulation.

This aligns with emerging evidence that genetic variants associated with obesity are involved in the regulation of reward and appetite by the central nervous system [36]. Recently identified obesity-related loci have shown high expression not only in the hypothalamus, which is a key site for the central regulation of appetite, but also in the limbic system, which regulates reward, learning and motivation [37]. Furthermore, neuroimaging studies have demonstrated altered brain reward responses to food stimuli in patients with monogenic forms of obesity and in individuals with common risk alleles of obesity-associated genes (such as *FTO* and *MC4R*) [14-16]. Although many other obesity-loci are suggested to act through the brain [37], their underlying mechanism are yet to be investigated, for instance through the promising large scale collaborations on genetic variants and brain function [38].

An additional finding of our study was that actual food intake was similar in leaner and heavier co-twins during the ad libitum lunch meal, which echoes the results of the previous fMRI study in monozygotic twins in which a similar test meal was used [19]. The results suggest that consistent inherited influences impact on actual food intake when

eating to satiety, possibly mediated by reward responsiveness in the brain. However, since subjects had lunch simultaneously and in the presence of the research physician, we cannot exclude the possibility that the co-twins influenced each other's eating behaviour or that bias resulted due to social desirability.

This study is novel in that it investigates the influence of unique environmental factors on obesity-related brain reward responsiveness to food in a unique design of monozygotic twins discordant for BMI, thereby allowing for the control of genetic influences. However, there were some limitations that should be noted. First, we had only a moderate sample size because it is difficult to find discordance for BMI in MZ twins, and nearly impossible to find pairs that are extremely discordant, i.e. one being obese and the other of normal weight. The low sample size and relatively modest difference in BMI may have resulted in a power being too low to detect significant differences between leaner and heavier co-twins, particularly in our behavioural measures, such as the questionnaires and food intake during the choice lunch meal. However, in previous studies from our group [5, 6, 8, 28] using identical techniques and similar sample sizes, we were able to detect significant differences between lean and obese unrelated individuals, similar to other investigations [3, 4, 7, 33, 34]. Compared to these previous studies, our unique design of rare monozygotic twins highly discordant for BMI but ultimately matched for confounding factors such as age, sex, shared environmental factors and genetic background should have enhanced the power of our study for investigating unique environmental influences on brain reward responses to food. With the caution that the absence of group differences within the classical inference framework does not prove equivalence between groups, we tentatively interpret the absence of group differences as support for a substantial role of genetic factors on food reward regulation by the brain.

Inherent to the absence of significant differences in food reward responsiveness, the question arises which factors do explain the BMI discordance between the monozygotic twins which we investigated. First, the important role of the homeostatic regulation of feeding mediated by the hypothalamus and brainstem should not be disregarded. Since visualization of the hypothalamus is, however, hampered by its location in the brain [39], we did not include the hypothalamus as ROI in our analyses. Secondly, the differences in BMI within pairs may have resulted from observed differences in eating behaviours, such as emotional eating in our current study, and disinhibition and restraint in previous studies [40, 41]. Further, differences in body weight may be ascribed to differences in physical activity. As previously published, we [17] and others [42, 43] observed significantly lower physical activity, in particular moderate-to-vigorous intensity activity in heavier compared to leaner co-twins of monozygotic twin pairs. Together, these findings suggest that the influence of unique environmental factors on body weight may be mainly through differences in homeostatic feeding pathways and

deviant behaviours in eating behaviour styles and physical activity, rather than through an altered reward function in the brain.

Together, our findings suggest that heritable traits have a substantial influence on reward region responses to palatable food stimuli, since no differences were observed within monozygotic twin pairs that are highly discordant for BMI. This implies that individuals that are genetically determined to increased reward responsiveness to food cues are at considerable greater risk in an environment in which the availability of energy dense palatable foods is abundant. Future studies are needed to identify the genetic variants underlying altered food reward observed in obese individuals, which may provide new clues in the development of treatment options against obesity.

SUPPLEMENTARY TABLE 1
Main effects of tasks whole brain

	Side	k	T	MNI			*P*-value
				x	y	z	
Food vs. non-food pictures							
Inferior and middle occipital gyrus	L	466	13.6	-39	-73	-11	5.1×10^{-10}
Inferior temporal, inferior occipital and fusiform gyrus	R	289	9.6	51	-64	-11	2.8×10^{-6}
Precuneus	L	30	7.5	-27	-61	52	4.9×10^{-4}
Precuneus	R	36	7.5	27	-70	34	5.1×10^{-4}
Middle occipital gyrus	R	35	6.5	36	-82	7	0.004
Posterior cingulate	L	8	6.0	-6	-52	25	0.015
Superior parietal gyrus	R	4	5.9	30	-58	55	0.020
High-calorie vs. non-food pictures							
Inferior and middle occipital gyrus	L	658	11.5	-39	-73	-11	3.6×10^{-8}
Inferior temporal and middle occipital gyrus	R	583	11.5	51	-64	-11	3.7×10^{-8}
Precuneus	L	50	7.4	-27	-61	52	6.0×10^{-4}
Superior parietal gyrus	R	26	7.1	30	-58	55	0.001
Precuneus	L	11	6.4	-6	-52	19	0.005
Anticipation chocolate milk vs. baseline							
Middle temporal, middle occipital and fusiform gyrus	R/L	2323	16.2	33	-58	-17	5.1×10^{-12}
Inferior parietal gyrus	L	50	7.5	-30	-55	43	5.3×10^{-4}
Inferior parietal gyrus	R	29	7.0	36	-58	49	0.002
Precentral gyrus	R	11	6.5	45	-1	40	0.006
Superior temporal gyrus	R	16	6.3	45	-46	10	0.010
Lingual gyrus	L	3	5.8	-12	-70	4	0.029
Inferior frontal gyrus	R	3	5.7	45	8	25	0.037
Receipt chocolate milk vs. baseline							
Cerebellum	L	123	9.0	-21	-67	-26	1.2×10^{-5}
Supramarginal gyrus	R	91	8.1	60	-16	25	9.8×10^{-5}
Precentral gyrus	R	48	7.2	57	5	22	7.0×10^{-4}
Insula	L	17	7.0	-39	-4	10	0.001
Middle temporal gyrus	L	24	6.7	-48	-55	4	0.002
Cerebellum	R	27	6.4	21	-67	-26	0.004
Insula	R	10	6.2	39	-1	10	0.008
Cerebellum	R	2	5.9	15	-73	-38	0.014
Precentral gyrus	L	4	5.9	-60	5	22	0.015
Amygdala	L	1	5.7	-24	-1	-17	0.024
Precentral gyrus	L	1	5.7	-57	2	28	0.024
Cerebellum	L	1	5.6	-15	-76	-38	0.033
Insula	L	1	5.5	-36	-4	-8	0.035
Rolandic operculum	R	1	5.4	51	5	13	0.048

Montreal Neurological Institute (MNI) coordinates of peak voxels activated in the total group of participants with threshold $P<0.05$ FWE whole brain corrected. Reported *P*-values are FWE whole brain corrected. K, cluster size; T, T-statistic; L, left; R, right.

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Overweight is associated with lower resting state functional connectivity in females after eliminating genetic effects: a twin study

VI

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ABSTRACT

Obesity is related to altered functional connectivity of resting state brain networks that are involved in reward and motivation. It is unknown to what extent these associations reflect genetic confounding and whether the obesity-related connectivity changes are associated with differences in dietary intake. In this study, resting state functional MRI was performed after an overnight fast in 16 female monozygotic twin pairs (aged 48.8 ± 9.8 years) with a mean BMI discordance of 3.96 ± 2.1 kg/m^2 (range 0.7-8.2). Functional connectivity of the salience, basal ganglia, default mode and anterior cingulate - orbitofrontal cortex networks was examined by independent component analysis. Dietary intake was assessed using 3-day 24-hour recalls. Results revealed that within the basal ganglia network, heavier versus leaner co-twins have decreased functional connectivity strength in bilateral putamen (P<0.05, FWE-corrected). There were no differences in connectivity in the other networks examined. In the overall group, lower functional connectivity strength in the left putamen was correlated with higher intake of total fat (P<0.01). We conclude that, after eliminating genetic effects, overweight is associated with lower resting state functional connectivity in bilateral putamen in the basal ganglia network. The association between lower putamen connectivity and higher fat intake suggests an important role of the putamen in appetitive mechanisms. The cross-sectional nature of our study cannot discriminate cause and consequence, but our findings are compatible with an effect of lower putamen connectivity on increased BMI and associated higher fat intake.

INTRODUCTION

Obesity is a major public health problem due to its pandemic occurrence and its association with the prevalence of diabetes, cardiovascular disease and musculoskeletal disorders [1]. Causes of obesity are a mixture of genetic and environmental factors and its interactions [2]. Heritability estimates have indicated that 40-70% of inter-individual variation in BMI is explained by genetic factors [3], and recent meta-analyses of genome-wide association studies have identified 97 genetic loci associated with obesity [4,5].

The brain has shown to be an important regulator of food intake and it is hypothesized that excessive eating in obese individuals is due to dysfunction of the reward system in the brain [6,7]. We and other investigators using task-based functional magnetic resonance imaging (fMRI) demonstrated that obese compared to lean individuals have altered brain reward region responses to food stimuli [8-12].

Previous studies also reported obesity-related alterations in spontaneous brain activity when no task was performed and participants were at rest [13-16]. This so-called resting state fMRI allows for the investigation of brain networks that comprise spatially distinct but functionally connected brain areas [17]. The degree of functional connectivity provides information on the integrity of these resting state networks [18]. These resting state networks are commonly identified using methods such as Independent Component Analysis (ICA) [19]. Such methods determine connectivity between two or more groups, within the spatially distinct networks. It does not indicate connectivity between location A and B, for which a seed-based analysis is more appropriate. Advantages of ICA over seed-based analyses included the data-driven approach instead of predefined seeds, networks are less sensitive to noise and motion, and the ICA networks converge largely with results from task-related activation studies.

Most previous resting state studies on obesity focussed on networks implicated in reward and attention, i.e. the salience, basal ganglia and default mode network (DMN). The salience network comprises the insula and anterior cingulate cortex (ACC) and integrates sensory, emotional and cognitive information which contributes to decision making [20]. The basal ganglia network comprises subcortical structures such as the caudate nucleus and putamen and is involved in movement, cognition and reward-related motivation [21]. The DMN comprises the precuneus and posterior cingulate cortex and is active during resting, awake conditions but deactivates during the performance of a task [22].

Comparable to BMI variation, the inter-individual variation in functional connectivity of resting state networks has shown to be in part under genetic control, as recently demonstrated by a classical twin study [23]. More evidence for a genetic contribution to network connectivity comes from studies in individuals with rare genetic mutations, such as Prader-Willi syndrome [24], and individuals that carry risk alleles

of the *FTO*-gene [25], the obesity-associated gene with the largest effect on BMI in common obesity.

Together, these studies suggest an overlap between genetic factors that influence BMI and genetic factors that influence functional connectivity of resting state networks. Such an overlap could reflect causal effects of connectivity on body weight, but is also compatible with the situation in which genetic factors exert their effects independently on body weight and on resting state networks, i.e. through pleiotropy. In that case, the association between obesity and altered resting state connectivity in previous observational studies may have resulted not from a true causal relationship, but rather, from genetic confounding. Consequently, this would indicate that interventions aimed at improving the integrity of the brain reward system would not contribute to a reduced risk of obesity.

Discordant monozygotic twins provide a unique opportunity to investigate the association between BMI and resting state functional connectivity independent of genetic effects, since these twins are genetically identical [26-28]. If, within discordant twin pairs, differences in resting state functional connectivity exist, this is supportive for a causal relationship between resting state connectivity and obesity. Accordingly, we studied BMI-discordant monozygotic twins on resting state functional connectivity of the salience network, basal ganglia network and DMN. In addition, we studied a network comprising the anterior cingulate cortex (ACC) and orbitofrontal cortex (OFC), as this network has shown involvement in gustation, reward and response inhibition [29]. A secondary aim was to investigate whether alterations in connectivity within these networks are related to differences in dietary intake, which has not been investigated previously.

MATERIALS AND METHODS

PARTICIPANTS

In the present study we included 16 female monozygotic twin pairs with a previously measured BMI discordance. Subjects were selected from the Netherlands Twins Registry, as described in detail previously [30]. In short, out of 2775 monozygotic twin pairs 54 pairs were selected based on relatively large intra-pair differences (≥ 2 kg/m^2) in previously measured BMI [31]. We selected only females to achieve a study population that is homogeneous with respect to gender, considering earlier reported gender-related differences in brain responses to food cues, with females showing higher activations than men [32]. Sixteen pairs were willing to participate and fulfilled our inclusion criteria, i.e. no history of metabolic, neurological or psychiatric disease including eating disorders and depression (as assessed with the Centre for Epidemiologic Studies Depression Scale [33]), no drug dependence, no MRI contra-indications (metal implants or claustrophobia), no pregnancy

or recent weight change (>5% self-reported weight change in the previous 3 months).

The study was approved by the ethics committee of the VU University Medical Centre and was performed in accordance with the Helsinki Declaration. All subjects provided written informed consent.

CLINICAL ASSESSMENTS

Both co-twins of a single pair arrived at the test visit between 8:00 and 10:00 AM after an overnight fast. Weight, height, waist-to-hip ratio and body composition were measured in a standardized manner as described in detail previously [30]. Handedness was assessed using a validated questionnaire [34]. Venous blood samples were drawn for the assessment of fasting glucose and lipid spectrum. Before the scanning session feelings of appetite and hunger were scored on a visual analogue scale (VAS) that ranged from 0 ('not at all') to 10 ('extremely'). Participants were asked the questions 1) How hungry are you now? 2) How full are you now? 3) How much could you eat right now? 4) How much is your desire right now to eat something sweet / savoury / fat?

DIETARY INTAKE

During the weeks following the test visit dietary intake was examined using the validated Unites States Department of Agriculture (USDA) five-step multiple-pass 24-hour recalls method by unannounced telephone calls on two weekdays and one Sunday, as described previously [30, 35]. To reduce misreporting, we additionally used a food portion size photo book, a table scale and extensive tableware as an aid in portion-size estimation. Food items were coded and analysed using the Dutch Food Composition Table (NEVO) [36]. We calculated daily intake of total energy (kcal), percentages of kcals (E%) derived from protein, carbohydrates and total fat, and percentages of total fat (%) derived from saturated, mono- and polyunsaturated fatty acids.

DATA ACQUISITION

Magnetic resonance imaging data were obtained using a 3.0 Tesla GE Signa HDxt scanner (General Electric, Milwaukee, WI, USA). For structural imaging, T1 weighted scans were acquired using a 3D fast spoiled gradient-echo sequence. For the resting state scan an echo-planar imaging sequence (repetition time/echo time = 1800/35 msec, flip angle 80°, slice thickness 3 mm, matrix size 64 x 64, voxel size 3 x 3 x 3 mm, 34 slices) was used to acquire 202 images. During the resting state scan participants were instructed to keep their eyes closed and not fall asleep.

DATA ANALYSIS

Clinical Data Clinical data were analysed using IBM SPSS Statistics (version 20, IBM Corp., 2011, Armonk, NY). Results are expressed as mean ± SD, unless otherwise stated. Differences between the leaner

and heavier co-twins were tested with paired t-tests for continuous variables [37], McNemar tests for dichotomous variables and Wilcoxon signed-ranks tests for ordinal data.

Imaging data Imaging data were preprocessed using SPM8 software (Wellcome Trust Centre for Neuroimaging, London, UK) run within Matlab R2012a (Mathworks, Inc.). A first inspection of the data revealed that the images of one participant had artefacts due to a metal implant in the spinal cord. The data of this participant (and her twin sister in case of paired analyses) was excluded from further imaging analyses. Of the remaining subjects, the origin of each imaging volume was aligned to the anterior commissure. The first two images of functional time series were discarded for steady-state magnetization. Functional images of each subject were then slice time corrected and realigned to the mean image to correct for head motion. No subject had more than the maximum allowed displacement of 3 mm in translation and 2,5° in rotation. Following this step, all data were co-registered with the structural scan and segmented to be spatially normalized to the standard Montreal Neurological institute (MNI) template. Finally, images were smoothed using an 8 mm full-width at half maximum Gaussian kernel.

We used the Group ICA of fMRI Toolbox (GIFT) (http://icatb.sourceforge.net) [38] on preprocessed images to identify 25 spatially independent resting state components. The minimum description length criterion [39], implemented in GIFT, indicated that the optimal number of components in our data set was 25. Data from all subjects were concatenated and the aggregated data set reduced using principal component analysis. Independent group components were estimated using the Infomax algorithm [40]. Consistency of the derived networks were analysed using the ICASSO software, implemented in GIFT.

Identification of the salience network, basal ganglia network, DMN and ACC-OFC network was performed in two steps. First, networks were visually identified through comparison with previous literature based on their spatial configuration [19, 29, 41]. Next, the 25 independent components were spatially correlated with masks of the intrinsic connectivity networks (ICN) previously described by Laird et al. (http://www.brainmap.org/icns) [29]. Components with the highest correlation with Laird's ICN 4, ICN 3, ICN 13 or ICN 2 were identified as the salience network, basal ganglia network, DMN and ACC-OFC network, respectively.

Since ICA identifies aggregate component spatial maps of the whole group, it is not capable of capturing connectivity strength at a subject-level. Therefore, we used GICA-based back-reconstruction, as implemented in the GIFT software, to regress back the time-course and spatial distribution of our networks of interest to the subjects' own fMRI scan. With back-reconstructing, every voxel of each spatial component is given a value that quantifies the relationship between that voxel and the time-course of the component. Finally, each subject com-

ponent image and time course was converted to Z-values, to obtain voxel values that are comparable across subjects.

For group comparisons Z-value images for each component were entered into a second level analysis model in SPM8. One sample t-tests, with a threshold of P<0.05 family-wise error (FWE) whole brain corrected, were performed on each network to visualize the network and to create a mask of each network containing all brain regions that contributed to the network, which served as a region of interest in further analyses. Group differences between leaner and heavier co-twins were investigated using paired t-tests [37]. An explicit mask of the network-specific group map was used to investigate results within brain regions that contributed to the network only. Results were considered statistically significant when $P<0.05$ FWE-corrected at the cluster level.

To correlate resting state functional connectivity with dietary intake, Z values of individual component maps were extracted using MarsBaR (MRC Cognition and Brain Sciences Unit, Cambridge, UK) for each anatomical brain region (WFU Pickatlas) that showed significant group differences between leaner and heavier co-twins. Linear regression analyses in the total group of twins were performed in Stata13, using family ID as a cluster variable to correct for non-independence of family members.

RESULTS

CLINICAL CHARACTERISTICS

Clinical characteristics of the 16 included twin pairs are presented in Table 1. Twin pairs had a mean age of 48.8 ± 9.8 years, ranging from 37 to70 years. The selection of discordant twin pairs resulted in expected significant differences between co-twins in weight, BMI, waist-hip ratio and body fat percentage. The mean BMI discordance was 3.96 kg/m^2 and ranged from 0.7 to 8.2 kg/m^2. After excluding the twin pair comprising the participant with imaging artefacts, the mean BMI discordance was 4.2 ± 1.9 kg/m^2 (range 1.0 - 8.2). There were no significant differences in biochemical assessments except for HDL-cholesterol and total/HDL-cholesterol ratio, which were less favourable in the heavier versus the leaner co-twins. VAS-scores on hunger and appetite showed that the heavier co-twins had stronger feelings of hunger ($P<0.05$) and a stronger desire to eat something sweet ($P<0.05$) as compared to the leaner co-twins prior to the scanning session. Leaner and heavier co-twins were comparable for self-reported daily smoking ($P=0.5$), handedness ($P=1.0$) and menopausal status ($P=0.7$). Of the included women, 6 were daily smokers: in 2 pairs both co-twins smoked and in 2 pairs only the leaner co-twin smoked. Two women were left handed (1 leaner and 1 heavier co-twin in different pairs). Thirteen women were premenopausal (defined as having a regular menstrual cycle): 7 leaner and 6 heavier co-twins in 7 pairs. In premenopausal women we initial-

ly aimed to perform all scans during the follicular phase, defined as day 1–12 counting forward from the start of the menstruation. However, since both co-twins of a pair were scanned on the same day, this was not always feasible. Nevertheless, no significant group differences were present in menstrual cycle phase, with 3 women being scanned during the follicular phase in each group (*P*=0.3).

TABLE 1
Clinical characteristics

	Leaner co-twins (n=16)	Heavier co-twins (n=16)	*P*-value
Age (y)	49.8 ± 9.8	49.8 ± 9.8	-
Weight (kg)	68.9 ± 9.2	80.5 ± 11.0	< 0.001
BMI (kg/m²)	24.4 ± 3.1	28.4 ± 3.5	< 0.001
Waist-to-hip ratio	0.80 ± 0.1	0.84 ± 0.1	< 0.05
Body fat (%)	32.0 ± 6.1	37.8 ± 6.1	< 0.001
Glucose (mmol/L)	4.7 ± 0.3	4.8 ± 0.3	0.5
Total cholesterol (mmol/L)	5.2 ± 1.1	5.3 ± 1.2	0.8
HDL cholesterol (mmol/L)	2.0 ± 0.4	1.7 ± 0.4	0.05
LDL cholesterol (mmol/L)	2.9 ± 1.0	3.2 ± 1.2	0.3
Ratio total / HDL cholesterol	2.7 ± 0.6	3.2 ± 1.0	0.01
Triglycerides (mmol/L)	0.8 ± 0.2	0.9 ± 0.3	0.1
VAS-scores			
Hunger	3.3 ± 2.1	5.0 ± 2.2	< 0.05
Fullness	3.5 ± 2.1	3.4 ± 2.0	0.8
Prospective food consumption	5.3 ± 1.4	5.6 ± 0.9	0.5
Desire for sweet food	2.8 ± 2.6	4.5 ± 3.1	< 0.05
Desire for savoury food	4.3 ± 2.9	5.2 ± 3.0	0.08
Desire for high fat food	0.6 ± 1.4	1.6 ± 2.0	0.06

Mean ± SD, all biochemical assessments are done in the fasted state. HDL, high-density lipoprotein; LDL, low-density lipoprotein; VAS, visual analogue scale.

RESTING STATE NETWORKS IDENTIFICATION

Components with the highest correlation with intrinsic connectivity networks previously described [29] were identified as the salience network (correlation coefficient, r=0.41), basal ganglia network (r=0.58), DMN (r=0.54) and ACC-OFC network (r=0.57). Selected components based on spatial correlation matched our initially selected components based on visual inspection. The identified independent components are visualized with threshold P<0.05 whole brain FWE corrected and presented in Figure 1. Brain regions that contributed to the networks are listed in detail in Table 2.

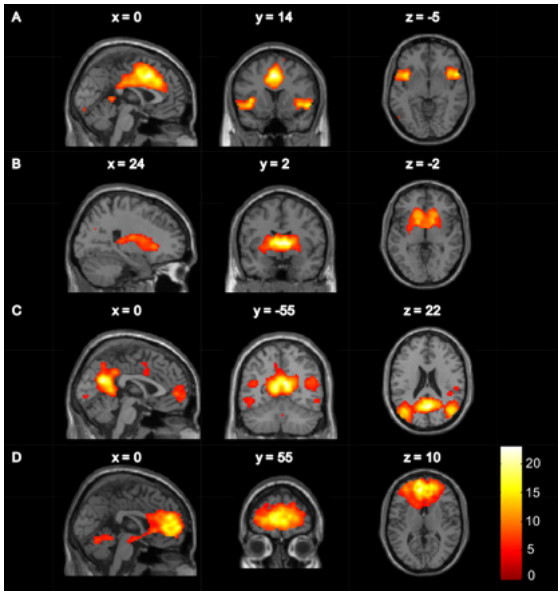
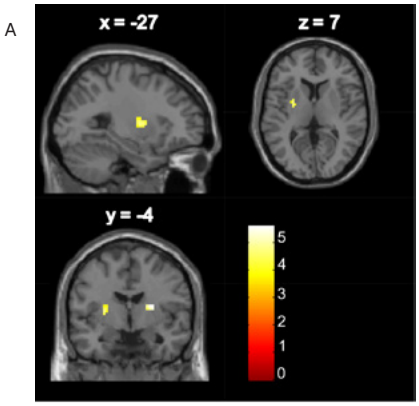


FIGURE 1
Spatial maps of the resting state networks of interest: A) salience network, B) basal ganglia network, C) default mode network, D) anterior cingulate cortex – orbitofrontal cortex network. Colour bar represents t statistics. Maps were visualized using a threshold of *P*<0.05 whole-brain family-wise error corrected.

GROUP DIFFERENCES

In the basal ganglia network, we observed significantly lower functional connectivity strength in the heavier as compared to the leaner co-twins in 2 different independent clusters. The fist cluster was found in the left putamen (MNI coordinates peak voxel (mm): x=-27, y=-4, z=7, cluster size 23 voxels, T-value 4.69, *P*=0.017 FWE-corrected on cluster level). The second cluster was located in the right putamen (MNI coordinates peak voxel (mm): x=24, y=-4, z=13, cluster size 21 voxels, T-value 5.61, *P*=0.022 FWE-corrected on cluster level) (Figure 2). In the other networks no statistically significant differences were observed between the groups.

We added an analysis to investigate whether the observed difference in functional connectivity in the bilateral putamen was explained by the difference in individual hunger and appetite scores by performing an additional regression analysis between VAS-scores of hunger and appetite and mean connectivity strength in all individuals (n=31). No significant associations were observed.



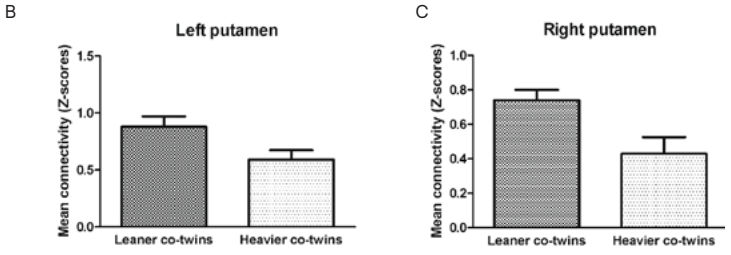


FIGURE 2
Decreased functional connectivity strength in heavier versus leaner co-twins in left and right putamen of the basal ganglia network. Left putamen: MNI −27 −4 7, cluster size 23 voxels, T-value 4.69, *P*=0.017 FWE-corrected on cluster level. Right putamen: MNI 24 −4 13, cluster size 21 voxels, T-value 5.61, *P*=0.022 FWE-corrected on cluster level. Colour bar represents t statistics. A) difference in connectivity strength of heavier < leaner co-twins presented in sagittal, coronal and axial plane, B) means and SEM of functional connectivity strength (in Z-scores) in anatomically located left putamen, and C) right putamen (after extracting data using MarsBaR).

RELATION BETWEEN FUNCTIONAL CONNECTIVITY AND DIETARY INTAKE

As we have previously published [30], dietary intake assessments showed that heavier compared to leaner co-twins had a higher intake of total fat (35.6 ± 6.7 versus 31.2 ± 4.1 E%, *P*<0.05). Intake of total energy (kcal), and relative intake of other macronutrients (E%) and types of fatty acids (% of total fat) did not differ between groups. Linear regression analysis in the total group of twins revealed that lower functional connectivity strength in the left putamen within the basal ganglia network was associated with higher intake of total fat (r=-0.37 *P*<0.01), see Figure 3. After adjustment for BMI this association remained significant (r=-0.32 *P*=0.02). We performed an additional regression analysis to investigate whether the variability of left putamen connectivity within pairs correlated with variability of intra-pair differences in fat intake. No such intra-pair association was observed.

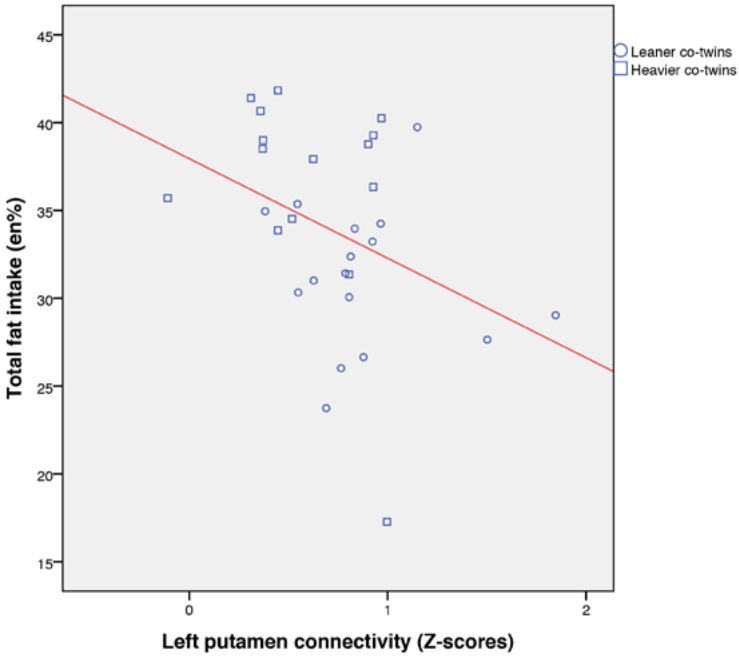


FIGURE 3
Relationship between functional connectivity strength in the left putamen within the basal ganglia network (in Z-scores) and intake of total fat (in percentages of total energy intake).

TABLE 2
Brain regions contributing to resting state networks of interest

	Side	k	T	MNI coordinates (mm)		
				x	y	z
Salience network						
Anterior and middle cingulate cortex	L/R	2366	23.02	-3	11	40
Insula	R	299	16.02	54	14	-5
Insula	L	288	15.79	-42	11	-5
Middle frontal gyrus	R	192	13.43	33	44	22
Inferior parietal lobe	R	468	13.09	66	-31	31
(Pre)cuneus	R	163	12.19	15	-70	34
Cuneus	L	161	10.91	-12	-70	22
Posterior cingulate cortex	L	38	9.64	-3	-46	4
Inferior frontal gyrus	L	48	9.50	-51	5	28
Inferior frontal gyrus	R	77	9.27	51	8	31
Middle occipital gyrus	L	37	8.69	-45	-70	-14
Lingual gyrus	R	12	7.50	18	-28	-11
Cerebellum	L	34	7.31	-36	-61	-26
Superior frontal gyrus	L	26	7.11	-21	2	61
Inferior parietal lobe	L	11	6.97	-48	-31	25
Middle occipital gyrus	L	9	6.69	-45	-79	19
Inferior temporal gyrus	R	3	6.57	54	-55	-11
Middle frontal gyrus	L	7	6.43	-36	-4	49
Middle occipital gyrus	R	1	6.02	42	-76	22
Superior parietal gyrus	R	2	6.00	42	-46	61
Inferior parietal lobe	L	1	5.99	-51	-46	25
Thalamus	R	1	5.92	6	-13	10
Basal ganglia network						
Caudate, putamen, thalamus	L/R	2442	26.22	9	2	7
Cerebellum	R	40	9.48	0	-58	-29
Precuneus	L	24	8.77	-21	-76	22
Middle cingulate cortex	R	16	7.46	3	-37	49
Cerebellum	R	22	7.28	12	-46	-26
Superior occipital gyrus	R	4	6.82	27	-70	28
Superior parietal lobe	L	6	6.76	-24	-70	43
Insula	L	5	6.44	-39	-4	-11
Inferior parietal lobe	L	3	6.37	-39	-58	40
Calcarine gyrus	L	1	6.22	0	-79	10
Lingual gyrus	R	1	6.07	15	-73	-11
Middle temporal gyrus	R	1	5.94	39	-64	28
Default mode network						
Precuneus, posterior cingulate cortex	L/R	4030	27.26	9	-55	16
Middle and superior frontal gyrus	R	832	15.01	27	29	43
Middle and superior frontal gyrus	L	833	14.49	-24	29	43
Orbitofrontal cortex	L	254	10.92	-6	62	-5
Middle temporal gyrus	R	58	9.59	57	-10	-20
Middle occipital and fusiform gyrus	L	83	9.06	-6	-91	-2
Calcarine gyrus	R	61	8.43	15	-94	-2
Postcentral gyrus	R	79	8.09	54	-25	25
Insula	L	13	7.68	-42	-10	-2
Inferior parietal lobe	R	11	7.53	45	-37	22
Insula	R	33	7.31	45	-1	-2
Caudate	R	9	6.92	6	5	10

Cerebellum	R	6	6.70	27	-61	-32
Inferior temporal gyrus	R	14	6.70	54	-55	-8
Cerebellum	R	1	6.16	3	-55	-32
Precentral gyrys	L	4	6.14	-48	2	31
Inferior parietal lobe	R	1	5.92	36	-40	43
ACC-OFC network						
Anterior cingulate cortex, orbitofrontal cortex, medial frontal gyrus	L/R	4952	27.92	-6	47	10
Cerebellum	R	222	10.51	-9	-67	-14
Precuneus	L	33	10.38	-9	-55	19
Pre- and postcentral gyrus	R	159	9.54	45	-31	55
Precuneus	R	3	7.03	12	-58	58
Insula	R	16	6.95	36	-22	13
Rolandic operculum	L	8	6.59	-39	-31	16
Cerebellum	R	1	6.13	12	-52	-35
Precuneus	L	1	6.10	-15	-46	4

Brain regions as identified by the Wake Forest University (WFU) Pickatlas. Montreal Neurological Institute (MNI) coordinates and T-values are presented of the peak voxel within each cluster. Results are $P<0.05$ whole-brain family-wise error corrected. K, cluster size; ACC, anterior cingulate cortex; OFC, orbitofrontal cortex.

DISCUSSION

Using a unique design of monozygotic twin pairs highly discordant for BMI, we investigated whether the association between altered functional connectivity of reward-related resting state networks and obesity could reflect a causal relationship or simply resulted from genetic confounding. We observed that, in the basal ganglia network, heavier co-twins had lower functional connectivity in bilateral putamen as compared to leaner co-twins. The fact that these differences were observed in monozygotic twins with identical genetic backgrounds implies that the relation between altered functional connectivity and obesity is not influenced by genetic confounding [27, 28]. Inherent to our twin design is that the observed lower putamen connectivity in heavier females resulted from environmental factors, which have the benefit of being potentially modifiable and, thereby, offering opportunities for future research on obesity prevention and treatment strategies. We, furthermore, observed that lower functional connectivity of the putamen was associated with higher intake of total fat, a finding that was not done before.

Our findings are in line with some but not all previous studies investigating obesity-related alterations in resting state connectivity in singletons [13-16, 42, 43]. In correspondence with our results are two studies that also reported lower functional connectivity strength in obese versus lean individuals in brain regions implicated in food reward and taste processing, in specific the anterior cingulate cortex (ACC) and insula [13], and the inter-regional connectivity between the amygdala and the ventromedial prefrontal cortex (PFC) [43]. However, despite this similarity in direction of the observed effects, there are also differences between our study and previous reports. In the particular study that used independent component analysis (ICA) similar to our approach [13], the observed differences were found in the DMN and temporal lobe network, rather than in the basal ganglia network. In fact, this study observed no BMI-related differences in the basal ganglia network, similar to a ICA-based resting state study performed a year later [14]. Interestingly, the latter study did observe differences in connectivity in the putamen, although this was done when the salience network was investigated. Specifically, in this study the obese individuals showed increased connectivity in the putamen relative to lean individuals [14], which contrasts our observations of decreased putamen connectivity in the heavier co-twins. Two other following studies reported BMI-related alterations in putamen connectivity in singletons [16, 42]. In both studies obese individuals had higher putamen connectivity compared to lean individuals.

Several explanations could be put forward for the discrepancy in findings between our study and previous studies in singletons. First, since monozygotic twins are identical in their genome, the lack of differences in our monozygotic twin design in functional connectivity of,

for instance, the DMN and salience network, suggests that positive findings in previous studies in unrelated individuals may be explained by genetic factors. Further, as monozygotic twins are also identical in age, gender and shared environment, the use of our monozygotic twin model allows for the elimination of confounding by all such factors. Thus, whereas previous studies may have been influenced by factors that exert their effect independently on both resting state network connectivity and on body weight, the observations in our current study are independent of such confounding [27, 28]. Second, given the different connectivity patterns in the putamen when studied in either the basal ganglia network or the salience network [14], it might be speculated that the degree of connectivity in the putamen depends on the specific network involved. The salience network typically comprises the insula and anterior cingulate cortex (ACC) [20, 29], brain regions that showed to be *highly activated* in response to watching palatable food pictures or cues that predicted the delivery of a palatable food stimulus [32] in task-based fMRI studies comparing obese and lean individuals. In contrast, the basal ganglia network comprises the dorsal striatum, which has repeatedly been related to *decreased* brain activations in response to palatable food receipt in previous task-based fMRI studies [9, 12]. Although some might say that resting state fMRI and task-based fMRI evaluate different aspects of brain functioning, numerous studies have demonstrated that brain regions that are co-activated during a task tend to be positively correlated during rest [44, 45]. Thus, it is tempting to speculate that, when implicated in the salience network, the putamen in obese versus lean individuals shows *higher* connectivity [14], whereas, when implicated in the basal ganglia network, the putamen in obese individuals shows *lower* connectivity. However, this explanation is speculative and it would be interesting to investigate this theory in ICA-based studies further. Furthermore, differences in results between our study and previous studies could be ascribed to differences in participant characteristics and study designs which have shown to influence the regulation of brain reward, e.g. gender [32], scheduled time of the scanning session [46] and fasting duration [32]. Specifically, whereas previous studies investigated males [42] or both males and females [13, 14, 43], performed scans throughout the day [14, 42] after only short fasts [14, 42], in our study we investigated only females, performed all scans in the early morning after an overnight fast. Finally, the discrepant study outcomes may have resulted from the use of different analytical approaches [17]. In contrast to some previous studies using seed-based analyses [15, 16, 42, 43], analyses in the current study were done using ICA, which has the benefit of not having a priori assumptions about regions of interest, thereby reducing the influence of selection bias.

The lower functional connectivity in the putamen in heavier versus lean individuals was related to a higher intake of total fat. This observation extends findings of a recent resting state study that found a link between dorsal striatum functional connectivity and cravings

for palatable food, as assessed with ratings of pictures of appetizing foods [47]. Our finding of higher fat intake is consistent with the proposed hypothesis of an obesity-related hypo-functioning reward system [48,49], which postulates that obese individuals have reduced reward system activation during actual food consumption, which induces compensatory overeating of highly rewarding foods. Evidence for this theory comprises studies that observed reduced striatal response to the receipt of a palatable milkshake in obese versus lean individuals, as observed with task-based fMRI [9,12], and reduced striatal dopamine receptor availability in morbid obesity, as assessed with positron emission tomography [50]. Thus, in line with this reasoning, the lower functional connectivity we observed in bilateral putamen might reflect a dysfunction of the reward system, which may result in increased intake of palatable high-fat foods to compensate for a lack of reward.

Although we have successfully eliminated genetic confounding, the cross sectional nature of our study does not allow us to determine whether lower functional connectivity of the putamen, and its associated increased fat intake, is causal to obesity development or, rather, results as a consequence of it. Support for a causal effect of altered connectivity on obesity comes from a previous resting state study which showed that altered functional connectivity between the dorsal striatum and the somatosensory cortex predicted future weight gain [47]. In the context of these findings, the altered functional connectivity of the putamen observed in our study may be explained as a vulnerability factor that might lead to weight gain, possibly through mediating effects of an acquired increased preference for high fat foods. However, our findings are also compatible with the hypothesis that impaired connectivity in the brain results as a consequence of high-fat diet consumption, for instance through inflammatory processes in neurons and glia, as observed previously in a rodent model [51]. In line with this finding, an observational MRI study using diffusion tensor imaging reported an inverse correlation between systemic inflammation and integrity of brain structures involved in food reward [52]. Future studies on resting state, ideally using longitudinal data in genetically informative designs, should elucidate whether altered network connectivity is mainly a cause or consequence of obesity and high fat intake.

A further limitation of our study was a relatively small sample size, although similar and even smaller sample sizes were used in previous studies [13,14,16,43].The power of our study, however, should be evaluated within the context of the study design, i.e. monozygotic twins being highly matched for possible confounding factors such as age, gender and genetic background, but, in the same time, ultimately discordant for BMI, which together enhance the power of this study to a great extent [26,53].

In conclusion, our study extends evidence for altered functional connectivity of brain regions involved in food reward in overweight and obesity. We demonstrated that, when genetic factors are elimi-

nated, overweight is associated with lower functional connectivity of bilateral putamen within the basal ganglia network. Furthermore, the lower connectivity in the left putamen showed a link with subsequent higher intake of total fat, suggesting that the putamen is involved in the regulation of appetite and food preferences. Since monozygotic twins have identical genomes, the observed alterations in functional connectivity of the putamen in females with higher BMI should be ascribed to unique environmental factors that exert their influence on the brain either direct or indirect, e.g. through epigenetic mechanisms [54]. Identification of the environmental factors that are responsible for the observed alterations in brain network connectivity may contribute to developing new strategies in the management of obesity, such as pharmacotherapies or neuromodulation techniques.

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Genetic factors and food intake regulation

PART 3

Physical activity and food intake in females with low and high genetic risk to obesity

VII

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ABSTRACT

OBJECTIVE

Observational studies cannot determine whether deviant physical activity, dietary intake and eating behaviour are a cause or consequence of obesity.

METHODS

In an attempt to seperate cause and effect, we selected 60 women (age 45.8 ± 6.9 years, BMI range 17.4 – 43.9 kg/m^2) with either a low or high genetic risk score (GRS) for obesity and either a low or high BMI from 11495 people in the Netherlands Twin Register. GRS was based on 77 obesity-associated single nucleotide polymorphisms. We measured physical activity, dietary intake and eating behaviour using 7-day accelerometry, 3-day 24-hour recalls and questionnaires.

RESULTS

Women with high BMI had fewer step counts, more sedentary behaviour and more emotional and restrained eating than women with low BMI, irrespective of GRS. Women with high GRS and high BMI had a higher intake of (animal) protein than women with high GRS but low BMI. In women with low GRS protein intake of low and high BMI groups was similar.

CONCLUSIONS

Unfavourable changes in the balance between sedentary behaviour and light intensity physical activity, emotional and restrained eating are likely secondary to increased BMI. In contrast, higher intake of (animal) protein may be a component of the genetic predisposition to obesity.

INTRODUCTION

The on-going rise in obesity prevalence has been attributed to an increased availability of high calorie energy dense food and a decreased physical activity level [1]. Experimental studies manipulating energy intake or physical activity have confirmed the existence of robust causal effects of each of these factors on body mass index (BMI) [2,3], yet it remains difficult to establish the relative contribution of dietary intake and physical activity patterns in the development of obesity in the population. Observational studies in population based samples can establish the extent of the association between physical activity, diet and BMI but they cannot rule out that BMI itself is causative of changes in physical activity level or diet [4].

In an attempt to separate cause and effect, the so-called four corners epidemiological model has been proposed [5-7]. In this model, participants with either a high or low genetic susceptibility to a trait of interest are selected to have either high or low observed levels of that trait. For instance, offspring from hypertensive parents could be selected that have either low or high blood pressure levels themselves, and a similar selection of either low or high blood pressure can be made in offspring of normotensive parents [5]. The expectation is that variables that are associated with blood pressure in offspring irrespective of the genetic risk for blood pressure are secondary to high blood pressure or largely influenced by environmental determinants that operate independent of the familial risk for blood pressure. In contrast, variables that are associated with high blood pressure *only* in the hypertensive offspring of hypertensive parents, but not in the hypertensive offspring of normotensive parents, could be part of a causal pathway leading from genetic risk to high blood pressure. This four corners model has for instance been applied to show that defective microvascular function may be a causal factor leading to high blood pressure and is not simply a mere consequence of it [6].

An important assumption in the original four corners design is that parents expressing a high level of a risk factor do so because of an underlying heritable factor. When applied to parents with high BMI this need not be the case. Shared environmental factors related to socioeconomic position or cultural and neighbourhood characteristics could also lead to high BMI in both parents. This would interfere with the primary reasoning in the four corners design. Fortunately, thanks to the recent progress in gene discovery based on genome wide association studies (GWAS) [8,9], we can now express the genetic risk for BMI independent of the family environmental background by using genetic risk scores (GRS) based on known markers of causal alleles. We therefore propose an adapted four corners design that selects individuals with either high or low BMI values, and, within each group, either low or high GRS based on recently established single nucleotide polymorphisms (SNPs) [8,9].

The aims of this study were to investigate whether physical activity, sedentary behaviour, dietary intake and eating behaviour are associated with GRS for obesity and, thus exert a direct effect on BMI or, rather, reflect a consequence of increased BMI itself.

METHODS

PARTICIPANTS

All participants eligible for inclusion in this study are registered in the Netherlands Twin Register (NTR) [10], which consists of twins and their family members taking part in longitudinal survey studies every 2-3 years. The sampling frame for the current study were all participants who had undergone BMI measurements as part of earlier studies [10, 11] and genotyping as part of GWAS projects [8, 12]. DNA was extracted from either blood or buccal cell samples using the QIAamp DNA Blood Maxi (QIAGEN, Dusseldorf, Germany). Genotyping of single nucleotide polymorphisms (SNPs) was done as described in more detail previously [10]. For each participant a genetic risk score (GRS) for obesity was calculated using 77 known obesity SNPs from the most recent meta-analysis of GWAS in adults [9]. The 77 loci were used that reached genome-wide significance for BMI (P<5 x 10-8) in the European analysis [9]. A weighted GRS was calculated by summing the BMI-increasing alleles after weighting the alleles by the effect size reported for that locus (Supplementary Table 1).

The total sample with both BMI and GRS data available was 11495 participants (Figure 1). Before running a second selection phase, individuals were excluded if they 1) had no weight and/or height recorded during the latest NTR biobank study [11], necessary for calculating recent BMI, 2) were part of monozygotic twins or triplets, or 3) were part of the same family as another individual in the sample. The removal of all but one member of a same family was done to create a sample of genetically unrelated individuals. To this end, spouses and parents of registered twins were first selected followed by randomly chosen siblings or members of twin pairs. Figure 2 displays the distributions of GRS and BMI of the population at this point during the selection phase. Then, a scatter diagram was constructed by plotting BMI on one axis and GRS on the other axis (Figure 3). Four corners were created by using cut-off points that corresponded to the lower and upper 25% of the distribution of BMI (i.e. ≤ 22 kg/m^2 and ≥ 27 kg/m^2) and the lower and upper 20% of the distribution of GRS (i.e. ≤ 0.90 and ≥ 1.03). The creation of four corners resulted in women with low GRS low BMI, low GRS high BMI, high GRS low BMI and high GRS high BMI. Finally, because of earlier reported gender differences in responses to food cues, with females showing higher activations than men [13] we excluded all male participants from this final sample.

In total 248 women received an invitation letter and were contact-

ed by telephone to check further eligibility for participation and provide more information when needed (Figure 1). Hundred and thirteen women were unwilling to participate because of lack of time or travel distance being too long. Of the remaining, 15 women with recent body weight change (>5% change in previous 3 months) were excluded. Another 31 women were excluded because of comorbidities or use of weight-affecting medication (Figure 1). Because the subjects also participated in an MRI study, 4 women were excluded because of MRI contra-indications. Thus, a final sample of 60 women was included in this study, in specific: women with low GRS/low BMI (n=16), low GRS/high BMI (n=12), high GRS/low BMI (n=15) and high GRS/high BMI (n=17). The study was approved by the VU University Medical Centre ethics committee and performed in accordance with the Helsinki Declaration. All women provided written informed consent.

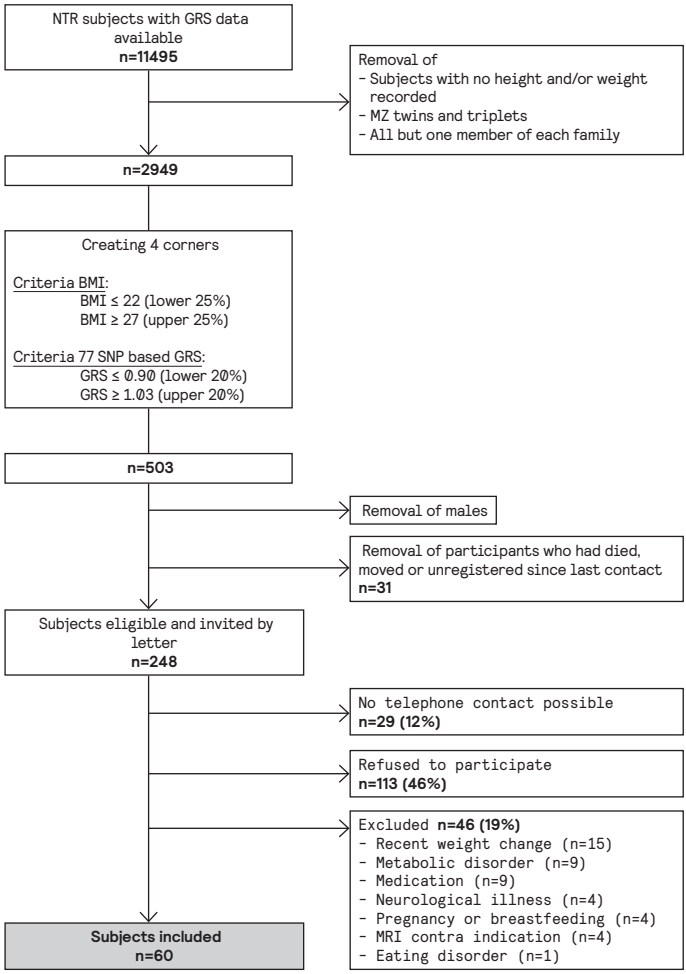

FIGURE 1
Flow chart of the study population. NTR, Netherlands Twin Registry; BMI, body mass index; GRS, genetic risk score; MZ, monozygotic; MRI, magnetic resonance imaging.

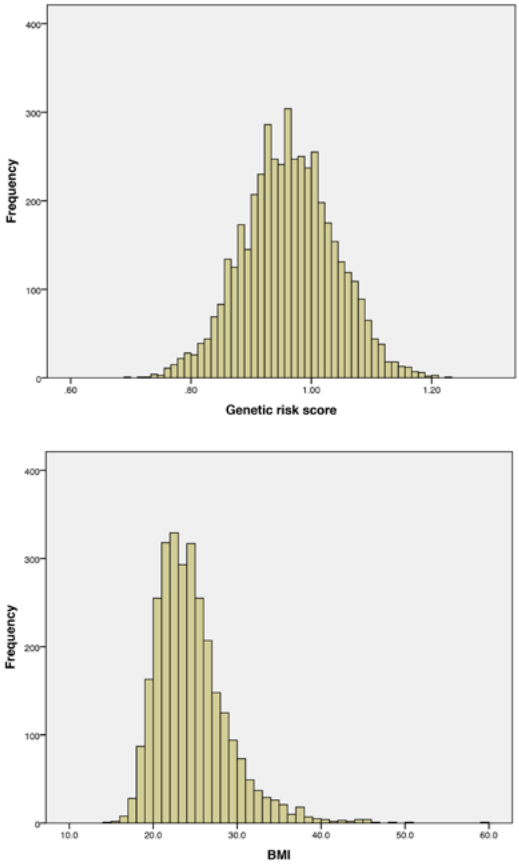

FIGURE 2
Histograms showing the distributions of genetic risk score (A) and BMI (B) of the larger population before the four corners were created.

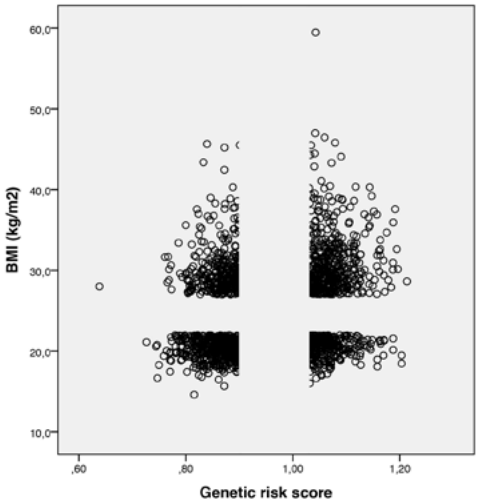

FIGURE 3
Representation of a scatter diagram of women with genetic risk score (GRS) on the horizontal axis and measured BMI on the vertical axis. The creation of four corners is based on the lower and upper 25% of BMI distribution and lower and upper 20% of GRS distribution.

MEASUREMENTS

Clinical and biochemical measurements All measurements were done during a visit in our research clinic and during the month following this visit, as described in more detail previously [14]. Participants arrived between 8 and 10 AM at the clinic after a 12-hour overnight fast. Information on health and socio-demographics was collected by a short oral and standardized interview and using two questionnaires on physical and mental health [15, 16]. Anthropometrics were measured with participants wearing no shoes and light clothes only. Body composition was measured using bio-electrical impedance analysis (Maltron BF-906 body fat analyser, Maltron Ltd, Essex, UK). Blood samples were drawn and analysed in the clinical chemistry laboratory of the VU University Medical Centre for the assessment of glucose and lipid spectrum. We used indirect calorimetry for the assessment of resting energy expenditure, as assessed during 15 minutes while participants were lying at rest with their eyes closed.

Physical activity, dietary intake and eating behaviour Physical activity was measured by 7-day accelerometry using Actigraph GT3X+ devices during all waking hours in the week following the test visit [17]. All participants started wearing the device on a Saturday. Recorded data was analysed using Actilife software (version 6.10.2). Non-wear time was defined and excluded if there were 60 consecutive minutes with zero counts, with allowance of 2 minutes with counts between 0-100 counts/min. The amount of wear time was considered acceptable when there was a minimum of 4 days of at least 10 hours of wear time per day. Existing cut-points were used to define sedentary (<100 counts/minute), light (100-2019 counts/minute), moderate (2020-5998 counts/minute) and vigorous (>5999 counts/minute) intensity activity [18].

Dietary intake was estimated in two ways. First, at the end of the visit participants were offered a test meal of which they could eat as much as they wanted [19]. The meal comprised white and multigrain bread, a mixed green salad, orange juice, Dutch cheese, fresh meats, margarine, mayonnaise, peanut butter, jam, cake, a chocolate muffin, a banana and an apple. Participants were unaware that their food consumption was monitored afterwards. Further, to estimate dietary intake of participants in their natural environment we used 24-hour recalls during unannounced telephone calls on two weekdays and one Sunday in the weeks following the visit [20]. Food items were coded by a skilled nutritionist who was blinded for BMI and GRS of the participant. Intake of total energy (kcal) and macronutrients (E%) during the test meal and recall assessments were analysed using the Dutch Food Composition Database (NEVO) [21].

Eating behaviour was assessed with the Dutch Eating Behaviour Questionnaire (DEBQ), a 33-item standardized and validated tool to measure eating behaviour on the scales emotional (13 items), external (10 items) and restrained eating (10 items) [22]. Answers ranged from

one (never) to five (often). Twelve questions had the extra option 'not applicable', in which case this question was excluded from the analysis. Finally, The Eating Disorder Inventory (EDI) was administered to screen for symptoms of eating disorders [23].

STATISTICAL ANALYSIS

Results of continuous variables with a normal distribution are presented as mean ± SD. Differences in these variables among the four corners were tested using one-way ANOVA and, if the overall ANOVA was $P<0.05$, Tukey's post hoc analyses, similar to previous studies using a four corners design [6,7,24,25]. Not normally distributed data are expressed as median and interquartile ranges, and group differences were tested using the non-parametric Kruskal-Wallis test. Chi-square test was used for analyses of categorical variables. All analyses were conducted using IBM SPSS Statistics (version 20, IBM Corp., 2011, Armonk, NY).

To illustrate the interpretation of the data in the four corners approach [5], the expected patterns are presented in Figure 4. In a first pattern (Figure 4A) participants with high BMI have higher values on the variable of interest than participants with low BMI, irrespective of GRS. In that case, the variable of interest likely reflects a consequence of high BMI rather than being a causal intermediate between the genetic risk for obesity and increased BMI. In a second pattern (Figure 4B) participants with high BMI have higher outcomes than participants with low BMI when GRS is high. When GRS is low, differences are much smaller or absent. This pattern likely reflects a causal pathway where the genetic predisposition to obesity acted on BMI in part through an effect on the variable of interest.

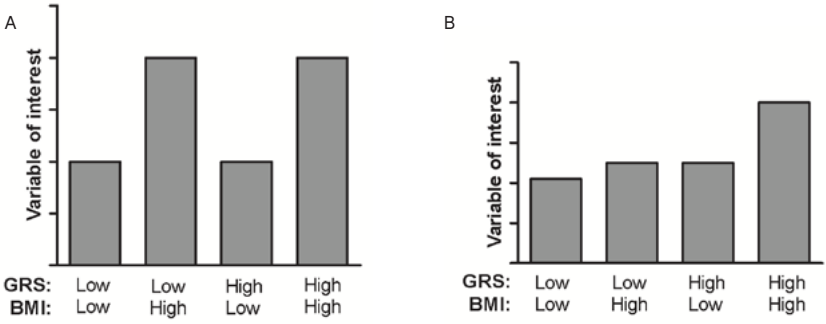

FIGURE 4
Possible patterns of data by the four corners model. Figure A shows the pattern in which participants with high BMI have higher levels of a variable than participants with low BMI, irrespective of GRS. This pattern likely reflects a consequence of high BMI. In figure B participants with high BMI have higher levels of a variable *only* when GRS is high. When GRS is low, the difference between participants with low BMI and participants with high BMI is absent (or, at least, much smaller). This pattern reflects a feature of genetic predisposition to increased BMI.

RESULTS

CLINICAL CHARACTERISTICS

There were no differences between the four groups in social-demographic variables (Table 1). The selection of four corners resulted in expected differences among groups in GRS and BMI (Table 2). Body weight, waist-to-hip ratio and body fat percentage were significantly higher in women with high BMI, irrespective of GRS. Resting energy expenditure was similar among groups after accounting for differences in body mass. In keeping with a causal effect of BMI on these risk factors, women with high BMI had more unfavourable outcomes on blood pressure, HDL cholesterol and total/HDL cholesterol ratio irrespective of GRS, although for blood pressure this difference was not statistically significant.

PHYSICAL ACTIVITY

All but one woman had acceptable wear time duration of the accelerometer and could be included in the analysis. Wear time duration was similar among the four groups (Table 3). Women with high BMI had fewer step counts than women with low BMI, irrespective of GRS. For total activity counts, this pattern was similar (P=0.05). Specification of accelerometer data showed that women with high BMI spent more time in sedentary behaviour (Figure 5A) and less time in light activity as compared to women with low BMI, irrespective of GRS.

DIETARY INTAKE

Dietary intake during the ad libitum test meal was not different between the groups (data not shown). The 24-hour recall assessments showed no statistically significant group differences in intake of total energy or fat (Table 3). However, when GRS was high, women with high BMI had higher intake of protein, in specific animal protein, than women with low BMI (P<0.05) (Figure 5B). When GRS was low, this difference was not present.

EATING BEHAVIOUR

Women with high BMI scored significantly higher on the DEBQ on the items emotional eating and restrained eating, irrespective of GRS (Figure 5C). The EDI revealed that high BMI was associated with a higher drive for thinness, more frequent symptoms of bulimia and more experienced body dissatisfaction (Table 3). These higher scores were also irrespective of GRS.

TABLE 1
Socio-demographic characteristics

	Low GRS Low BMI	Low GRS High BMI	High GRS Low BMI	High GRS High BMI	*P*-value
	n=16	n=12	n=15	n=17	
Food vs. non-food pictures					
Only secondary (%)	2 (12.5)	2 (16.7)	2 (13.3)	2 (11.8)	1.0
Vocational (%)	8 (50)	6 (50)	6 (40)	9 (52.9)	
Higher or academic (%)	6 (12.5)	4 (33.3)	7 (46.7)	6 (35.3)	
Work					
Employed (%)	15 (93.8)	11 (91.7)	14 (93.3)	17 (100)	0.7
Unemployed (%)	1 (6.2)	1 (8.3)	1 (6.7)	0 (0)	
Marital status					
Single (%)	1 (6.2)	1 (8.3)	1 (6.7)	2 (11.8)	0.8
Married (%)	12 (75)	11 (91.7)	13 (86.7)	13 (76.5)	
Divorced or widower (%)	3 (18.8)	0 (0)	1 (6.7)	2 (11.8)	
Smoking status					
Current smoker (%)	4 (25)	1 (8.3)	2 (13.3)	0 (0)	0.4
Previous smoker (%)	4 (25)	5 (41.7)	5 (33.3)	5 (29.4)	
No smoker (%)	8 (50)	6 (50)	8 (53.3)	12 (70.6)	
Menopausal status					
Premenopausal (%)	11 (68.8)	7 (58.3)	11 (73.3)	9 (52.9)	0.4
Postmenopausal (%)	2 (12.5)	2 (16.7)	3 (20)	5 (29.4)	
Unknown (%)	3 (18.8)	3 (25)	1 (6.7)	3 (17.6)	
Symptoms of depression					
CES-D score	5.9 ± 6.2	6.2 ± 4.1	5.0 ± 7.3	5.9 ± 5.5	1.0
CES-D score > 16 (%)	1 (6.3)	0 (0)	2 (13.3)	1 (5.9)	0.6
Health status					
SF-36 Physical Health	56.3 ± 2.8	52.2 ± 9.9	51.7 ± 10.2	53.8 ± 4.6	0.3
SF-36 Mental Health	52.8 ± 7.6	51.8 ± 9.1	53.6 ± 7.7	52.8 ± 4.1	0.9
Medication use					
Oral contraceptives	3 (18.8)	4 (33.3)	1 (6.7)	2 (11.8)	0.3
Antihypertensive medication	1 (6.3)	1 (8.3)	0 (0)	2 (11.8)	0.6
Statins	0 (0)	0 (0)	1 (6.7)	1 (6.7)	0.6
Alcohol use					
Not at all	1 (6.2)	3 (25)	4 (26.7)	5 (29.4)	0.2
Not daily	12 (75)	6 (50)	11 (73.3)	11 (64.7)	
Daily	3 (18.8)	3 (25)	0 (0)	1 (5.9)	

N (%) or mean ± SD; GRS, genetic risk score; CES-D, Centre for Epidemiologic Studies-Depression scale; SF-36, Short Form 36-item Health Survey

TABLE 2
Clinical and biochemical characteristics

	Low GRS Low BMI (1)	Low GRS High BMI (2)	High GRS Low BMI (3)	High GRS High BMI (4)	*P*-value
	n=16	n=12	n=15	n=17	
Age (y)	44.8 ± 6.3	47.0 ± 7.3	44.3 ± 7.4	47.1 ± 6.9	0.6
GRS	0.87 ± 0.05	0.87 ± 0.05	1.06 ± 0.05	1.05 ± 0.06	<0.001*
Weight (kg)	61.2 ± 6.6	89.7 ± 10.9	60.5 ± 4.8	92.7 ± 13.2	<0.001†
BMI (kg/m²)	20.9 ± 0.9	31.6 ± 3.7	20.4 ± 1.3	32.8 ± 4.8	<0.001†
Waist circumference (cm)	73.9 ± 4.7	97.3 ± 8.6	73.0 ± 3.0	102.8 ± 14.0	<0.001†
Waist-hip ratio	0.78 ± 0.04	0.87 ± 0.05	0.78 ± 0.03	0.87 ± 0.06	<0.001†
Body fat (%)	27.8 ± 3.1	42.7 ± 2.9	27.1 ± 3.4	44.6 ± 4.7	<0.001†
Systolic RR (mmHg)	116.3 ± 14.7	129.1 ± 16.9	116.0 ± 12.1	123.2 ± 17.7	0.1
REE (kcal/day)	1466.8 ± 217.8	1626.2 ± 193.7	1445.7 ± 129.8	1687.0 ± 256.0	0.004‡
REE/LBM (kcal/kg)	33.3 ± 3.8	29.9 ± 4.9	32.9 ± 3.0	32.8 ± 5.9	0.2
Glucose (mmol/L)	4.8 ± 0.4	4.8 ± 0.5	5.0 ± 0.4	5.1 ± 0.6	0.1
Total chol (mmol/L)	4.9 ± 0.8	5.2 ± 0.8	4.9 ± 1.2	5.2 ± 1.3	0.9
HDL chol (mmol/L)	1.9 ± 0.3	1.4 ± 0.5	1.9 ± 0.3	1.5 ± 0.6	0.008§
LDL chol (mmol/L)	2.8 ± 0.6	3.5 ± 0.9	2.7 ± 1.0	3.1 ± 1.0	0.1
Ratio total / HDL chol	2.8 ± 0.8	4.0 ± 1.0	2.6 ± 0.9	4.0 ± 1.9	0.003‖
Triglycerides (mmol/L)	1.0 ± 0.5	1.1 ± 0.5	0.9 ± 0.6	1.2 ± 0.6	0.4

Mean ± SD; GRS, genetic risk score; REE, resting energy expenditure; REE/LBM, resting energy expenditure divided by lean body mass; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

*$P<0.001$ for comparing corner 3>1; 4>1; 3>2; 4>2; †$P<0.001$ for comparing corner 2>1; 2>3; 4>1; 4>3; ‡$P<0.05$ for comparing corner 4>1; 4>3; §$P<0.05$ for comparing corner 3>2; ‖$P<0.05$ for comparing corner 2>3; 4>1; 4>3.

TABLE 3
Physical activity, dietary intake and eating behaviour

	Low GRS Low BMI (1)	Low GRS High BMI (2)	High GRS Low BMI (3)	High GRS High BMI (4)	*P*-value
Physical activity					
Wear time (h/day)	14.7 ± 1.3	14.8 ± 1.1	14.4 ± 1.2	14.9 ± 0.8	0.6
Activity counts (x 1000/day)	561.8 ± 99.3	437.4 ± 128.6	592.1 ± 165.0	530.0 ± 158.9	0.05
Steps (steps/day)	7327.3 ± 1194.2	5935.0 ± 2153.9	8719.3 ± 2318.3	7448.8 ± 2471.8	<0.05*
Sedentary (min/day)	636.0 ± 87.7	682.3 ± 44.2	600.7 ± 67.5	653.7 ± 52.8	<0.05*
Light activity (min/day)	209.7 ± 38.3	176.6 ± 40.4	221.9 ± 48.9	201.2 ± 39.7	0.06
MVPA (min/day)	36.9 ± 12.4	28.8 ± 13.0	40.5 ± 15.9	36.6 ± 20.0	0.3
Sedentary (%)	71.9 ± 5.1	77.0 ± 4.6	69.6 ± 5.8	73.4 ± 5.0	<0.01*
Light activity (%)	23.9 ± 4.6	19.8 ± 3.6	25.7 ± 5.0	22.6 ± 4.2	0.01*
MVPA activity (%)	4.3 ± 1.7	3.2 ± 1.3	4.7 ± 1.6	4.1 ± 2.1	0.2
Dietary intake					
Energy (kcal)	2067 ± 529	1762 ± 447	1837 ± 484	1830 ± 438	0.3
Carbohydrates (E%)	44.6 ± 6.3	46.2 ± 6.8	44.2 ± 7.0	43.2 ± 6.0	0.7
Protein (E%)	14.4 ± 3.2	15.7 ± 2.9	15.4 ± 2.1	18.2 ± 3.4	<0.01†
Vegetable protein (E%)	6.0 ± 1.6	6.0 ± 1.0	6.7 ± 1.6	6.3 1.2	0.4
Animal protein (E%)	8.4 ± 3.0	9.8 ± 2.8	8.6 ± 3.0	11.9 ± 3.6	<0.01†
Total fat (E%)	36.7 ± 5.0	33.3 ± 5.7	36.0 ± 5.7	34.5 ± 6.3	0.4
Saturated fat (E%)	13.8 ± 3.1	13.0 ± 2.9	13.6 ± 3.6	13.0 ± 3.1	0.9
Monounsaturated fat (E%)	12.9 ± 2.5	11.2 ± 2.0	12.3 ± 2.0	11.7 ± 2.8	0.3
Polyunsaturated fat (E%)	6.5 ± 1.5	5.9 ± 1.4	6.9 ± 2.0	6.4 ± 2.1	0.6
Eating Behaviour					
Emotional eating	1.9 ± 0.7	2.6 ± 0.5	1.8 ± 0.5	2.4 ± 0.8	<0.01‡
External eating	2.9 ± 0.6	2.9 ± 0.3	2.8 ± 0.4	3.1 ± 0.7	0.6
Restrained eating	1.8 ± 0.9	3.1 ± 1.0	2.3 ± 0.9	3.2 ± 0.8	<0.001§
Symptoms of Eating Disorder					
Drive for Thinness	11.9 ± 2.9	21.2 ± 7.8	12.5 ± 4.1	20.9 ± 5.3	<0.001‖
Bulimia	8.9 ± 2.2	11.8 ± 3.1	8.1 ± 1.2	12.4 ± 4.0	<0.001#
Body Dissatisfaction	19.9 ± 7.0	35.3 ± 12.0	20.4 ± 6.8	38.6 ± 8.7	<0.001‖

Mean ± SD; GRS, genetic risk score; MVPA, moderate to vigorous physical activity; E%, percentage of total energy intake. *P<0.05 for comparing corner 3>2; †P<0.05 for comparing corner 4>1; 4>3; ‡P<0.05 for comparing corner 2>3; 4>3; §P<0.05 for comparing corner 2>1; 4>1: 4>3; ‖P<0.001 for comparing corner 2>1; 4>1; 2>3; 4>3; #P<0.01 for comparing corner 4>1; 2>3; 4>3.

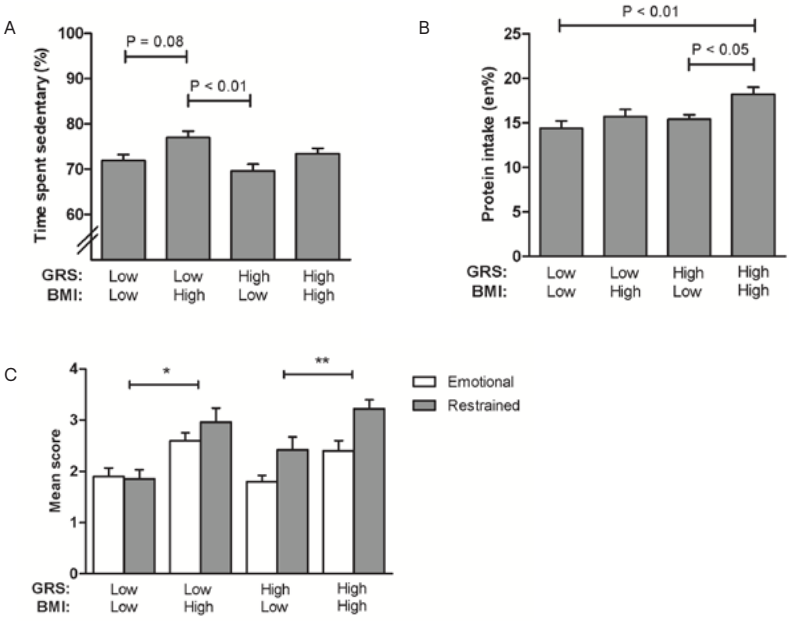

FIGURE 5
Differences between the women from the four corners in time spent in sedentary behaviour (A), intake of protein (B) and emotional and restrained eating scores on the Dutch Eating Behaviour Questionnaire (C). Data represent means ± standard error of mean. E%, percentage of total energy intake. *P<0.01 for restrained eating. **P<0.05 for emotional and restrained eating.

DISCUSSION

We used a special, previously established four corners study design in an attempt to investigate whether deviant physical activity, sedentary behaviour, dietary intake and eating behaviour exert a causal effect on BMI or merely reflect a consequence of increased BMI itself [5-7]. We improved the original four corners design by selecting participants not on parental characteristics but on their observed genetic risk score (GRS), thus preventing confounding by shared environmental factors inherent in the original parent-offspring four corners model.

Our accelerometer data showed that women with higher BMI had lower overall step counts than women with low BMI, irrespective of GRS for obesity. Specification of the data revealed that this difference was explained by an unfavourable imbalance of increased sedentary behaviour and decreased light intensity physical activity, rather than lower engagement in moderate to vigorous physical activity (MVPA), since differences in the latter were not observed. Our finding that this imbalance between sedentary behaviour and light intensity physical activity was observed regardless of GRS for obesity, suggests that these deviant behaviours have no causal contribution to the genetic predisposition to obesity but, rather, reflect a consequence of increased BMI itself. This clue for a reverse causal relation is in line with a recent study using Mendelian randomization to demonstrate that adiposity causes an increase in sedentary behaviour in children [26]. However, neither study can rule out the possibility of bidirectional causality, i.e. a vicious circle of increased physical inactivity due to obesity that in turn further advances adiposity by negatively impacting on the energy balance.

The results of our study provide support for an important role of environmental factors in kick-starting this vicious circle. In our design, women in the low GRS/high BMI corner are selected to be at an above-average environmental risk for high BMI. This environmental risk is clearly transferred to their daily engagements in light intensity physical activity and sedentary behaviour. This is in accordance with results from twin studies in which we [14] and others [27, 28] demonstrated that lower physical activity levels in overweight individuals are explained by adverse environmental factors, although these observations mostly concerned changes in (moderate to vigorous) physical activity rather than sedentary behaviour. Together, these studies suggest that a small change in weight, induced by environmental factors, might subsequently initiate a cycle of physical inactivity resulting in further weight gain that further increases physical inactivity, and so on.

During the test meal and 24-hour recall assessments, we did not observe differences in intake of total energy or fat between the groups. Since underreporting is a well-known issue in self-reported dietary surveys, especially in women with higher BMI [29], we cannot exclude the possibility that true differences in these variables remained undetected in our study. However, using 24-hour dietary recalls, we did observe a

higher intake of protein, in specific animal protein, in women with high BMI, *only* if GRS for obesity was high. This pattern would not be expected if increased animal protein intake was secondary to high BMI but, instead, suggests that the intake of animal protein contributes to the genetic predisposition to obesity. This conflicts with existing practices of high-protein diets being used as a tool to lose body weight after obesity has already developed [30]. However, this beneficial effect of protein consumption has been debated, especially when the protein sources are considered [31, 32]. Whereas vegetable proteins have been suggested to lower risk of obesity, the intake of animal protein appears to have a deleterious effect on BMI [31, 33]. Possible mechanisms are higher levels of insulin and insulin-like growth factor 1 (IGF-1), and a concomitant higher intake of fat and total calories, inherent to the consumption of meat products [34].

Since the GRS we used in our study is based on SNPs of multiple genes, it is uncertain which specific gene is responsible for the higher protein intake we observed in high BMI women. Genes suggested to influence appetite and food intake are those involved in leptin-melanocortin signalling and include *POMC, MC4R, BDNF* and *FTO* [35], although *FTO* has also been suggested to function beyond the regulation of food intake [36]. More studies are needed to investigate which genes are responsible for specific preferences in macronutrient intake.

The higher emotional and restraint eating seen in individuals with high BMI are consistent with previous literature [37]. Emotional eating refers to eating in response to emotional states such as sadness or anxiety, whereas restrained eating refers to a tendency to cognitively restrict food intake in order to avoid weight gain. Paradoxically, dietary restraint could cause weight gain through periods of disinhibition and bingeing, and often coincides with emotional eating [38]. The pattern of our data, i.e. higher emotional and restraint eating in women with high BMI, *irrespective* of GRS, suggests that abnormal eating behaviour occurs *as a result* of increased BMI, rather than being a genetically etiological component of it. This finding is in contrast to a previous study which observed an association between a 32 SNP based GRS for obesity and emotional eating, thereby suggesting a mediating link between genetic variation and obesity [39]. However, this study , in contrast to our current study, could not rule out the influence of confounding by BMI itself.

A weakness of our study was a relatively modest sample size, which may have caused our study to have insufficient statistical power to detect small differences in e.g. MVPA and emotional eating between individuals with low GRS and individuals with high GRS. Also, the upper BMI cut-off point of ≥ 27 kg/m^2 resulted in the inclusion of overweight and not just obese individuals, which might have affected the sensitivity to detect differences between the groups with low BMI and high BMI. For these reasons we used a lenient nominal alpha of 0.05 throughout. Unfortunately, this does not just increase power but also

increases the risk of type 1 errors. Our findings, therefore, must be considered exploratory and clearly need confirmation in independent studies. On the other hand, the power of our study should be evaluated with respect to the study design, i.e. a unique selection of individuals with extreme genotypes and phenotypes from a cohort of >11 thousand individuals. Using a GRS rather than parental history information to index genetic predisposition enabled us to remove confounding by shared environmental factors from the original four corner design. Further, relative to previous studies using risk scores based on single SNPs or smaller amounts of genetic variants, we enhanced power to detect GRS-related effects by using a weighted GRS based on 77 recently detected obesity loci [9]. Nevertheless, future research may benefit from larger sample sizes and risk scores comprising more obesity-related SNPs, to optimize power of detecting genetic effects on behavioural traits underlying obesity.

Although we cannot exclude the possibility that our data are biased due to underreporting or under-eating for the duration of data collection [29], misreporting is an obstacle in all nutritional surveys and the 24-hour recall method is not more susceptible to this issue than other methods. Rather, the 24-hour recall method has shown to be valid in estimating the 'usual diet' while providing a low participant burden [40]. A final limitation is that our study sample was restricted to women, which makes it difficult to generalize our findings to the male population.

In summary, using a special four corners approach, we suggested that higher levels of sedentary behaviour and emotional and restrained eating likely occur after the development of overweight, which could favour the initiation of a vicious circle of more weight gain and physical inactivity and abnormal eating behaviour. In contrast, a higher intake of (animal) protein might be a causal component of the genetic predisposition to obesity. This study provides ground to call for further interventional studies characterizing the effects of (animal) protein intake on BMI.

SUPPLEMENTARY TABLE 1
SNPs included in the polygenic risk score

Chr	Nearest gene	SNP name	Alleles Effect / Other	Effect allele frequency	GWAS β	Variance explained
16	*FTO*	rs1558902	A/T	0.415	0.082	0.325%
18	*MC4R*	rs6567160	C/T	0.236	0.056	0.111%
2	*TMEM18*	rs13021737	G/A	0.828	0.060	0.103%
4	*GNPDA2*	rs10938397	G/A	0.434	0.040	0.079%
1	*SEC16B*	rs543874	G/A	0.193	0.048	0.072%
6	*TFAP2B*	rs2207139	G/A	0.177	0.045	0.058%
11	*BDNF*	rs11030104	A/G	0.792	0.041	0.056%
1	*NEGR1*	rs3101336	C/T	0.613	0.033	0.053%
12	*BCDIN3D*	rs7138803	A/G	0.384	0.032	0.047%
2	*ADCY3*	rs10182181	G/A	0.462	0.031	0.047%
16	*ATP2A1*	rs3888190	A/C	0.403	0.031	0.046%
3	*ETV5*	rs1516725	C/T	0.872	0.045	0.045%
19	*QPCTL*	rs2287019	C/T	0.804	0.036	0.041%
16	*GPRC5B*	rs12446632	G/A	0.865	0.040	0.038%
19	*ZC3H4*	rs3810291	A/G	0.666	0.028	0.036%
11	*MTCH2*	rs3817334	T/C	0.407	0.026	0.033%
1	*GNAT2*	rs17024393	C/T	0.040	0.066	0.033%
15	*MAP2K5*	rs16951275	T/C	0.784	0.031	0.033%
5	*POC5*	rs2112347	T/G	0.629	0.026	0.032%
4	*SLC39A8*	rs13107325	T/C	0.072	0.048	0.030%
7	*PMS2L11*	rs2245368	C/T	0.180	0.032	0.030%
1	*FPGT-TNNI3K*	rs12566985	G/A	0.446	0.024	0.029%
13	*MTIF3*	rs12016871	T/C	0.203	0.030	0.029%
3	*RASA2*	rs16851483	T/G	0.066	0.048	0.029%
3	*CADM2*	rs13078960	G/T	0.196	0.030	0.028%
14	*NRXN3*	rs7141420	T/C	0.527	0.024	0.028%
9	*LINGO2*	rs10968576	G/A	0.320	0.025	0.027%
13	*OLFM4*	rs12429545	A/G	0.133	0.033	0.026%
1	*AGBL4*	rs657452	A/G	0.394	0.023	0.025%
4	*SCARB2*	rs17001654	G/C	0.153	0.031	0.024%
11	*CADM1*	rs12286929	G/A	0.523	0.022	0.023%
1	*PTBP2*	rs11165643	T/C	0.583	0.022	0.023%
14	*STXBP6*	rs10132280	C/A	0.682	0.023	0.023%
10	*TCF7L2*	rs7903146	C/T	0.713	0.023	0.022%
2	*FLJ30838*	rs1016287	T/C	0.287	0.023	0.021%
8	*HNF4G*	rs17405819	T/C	0.700	0.022	0.021%
4	*HHIP*	rs11727676	T/C	0.910	0.036	0.021%
10	*HIF1AN*	rs17094222	C/T	0.211	0.025	0.021%
1	*FUBP1*	rs12401738	A/G	0.352	0.021	0.020%
7	*HIP1*	rs1167827	G/A	0.553	0.020	0.020%
11	*TRIM66*	rs4256980	G/C	0.646	0.021	0.020%
16	*NLRC3*	rs758747	T/C	0.265	0.023	0.020%
14	*PRKD1*	rs12885454	C/A	0.642	0.021	0.020%
14	*PRKD1*	rs11847697	T/C	0.042	0.049	0.019%
3	*FHIT*	rs2365389	C/T	0.582	0.020	0.019%
6	*C6orf106*	rs205262	G/A	0.273	0.022	0.019%
2	*ERBB4*	rs7599312	G/A	0.724	0.022	0.019%
1	*NAV1*	rs2820292	C/A	0.555	0.020	0.019%
16	*SBK1*	rs2650492	A/G	0.303	0.021	0.018%

9	*TLR4*	rs1928295	T/C	0.548	0.019	0.018%
2	*KCNK3*	rs11126666	A/G	0.283	0.021	0.017%
16	*KAT8*	rs9925964	A/G	0.620	0.019	0.017%
19	*TOMM40*	rs2075650	A/G	0.848	0.026	0.017%
12	*CLIP1*	rs11057405	G/A	0.901	0.031	0.017%
3	*RARB*	rs6804842	G/A	0.575	0.019	0.017%
6	*PARK2*	rs13191362	A/G	0.879	0.028	0.016%
3	*GBE1*	rs3849570	A/C	0.359	0.019	0.016%
17	*RPTOR*	rs12940622	G/A	0.575	0.018	0.016%
17	*RABEP1*	rs1000940	G/A	0.320	0.019	0.016%
9	*C9orf93*	rs4740619	T/C	0.542	0.018	0.016%
2	*LRP1B*	rs2121279	T/C	0.152	0.025	0.015%
10	*NT5C2*	rs11191560	C/T	0.089	0.031	0.015%
15	*DMXL2*	rs3736485	A/G	0.454	0.018	0.015%
10	*GRID1*	rs7899106	G/A	0.052	0.040	0.015%
6	*FOXO3*	rs9400239	C/T	0.688	0.019	0.015%
9	*LMX1B*	rs10733682	A/G	0.478	0.017	0.015%
1	*ELAVL4*	rs11583200	C/T	0.396	0.018	0.015%
6	*TDRG1*	rs2033529	G/A	0.293	0.019	0.015%
2	*EHBP1*	rs11688816	G/A	0.525	0.017	0.015%
2	*UBE2E3*	rs1528435	T/C	0.631	0.018	0.015%
11	*HSD17B12*	rs2176598	T/C	0.251	0.020	0.015%
19	*KCTD15*	rs29941	G/A	0.669	0.018	0.015%
18	*GRP*	rs7243357	T/G	0.812	0.022	0.014%
19	*PGPEP1*	rs17724992	A/G	0.746	0.019	0.014%
9	*EPB41L4B*	rs6477694	C/T	0.365	0.017	0.014%
8	*RALYL*	rs2033732	C/T	0.747	0.019	0.014%
18	*C18orf8*	rs1808579	C/T	0.534	0.017	0.014%

Chr, chromosome; SNP, single nucleotide polymorphism. Alleles, effect allele frequency, effect size and explained variance are values from meta-analysis in European males and females by Locke et al.

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Polygenic risk to obesity and alterations in fMRI brain reward system responses to food in females

VIII

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ABSTRACT

Obesity has been linked to altered brain reward responsiveness to food, although the causal direction of these associations remain unclear. Body weight is highly heritable and genome wide association studies have successfully identified obesity-associated single nucleotide polymorphisms (SNPs). Effects of these variants are small, but can be aggregated in a polygenic risk score (GRS). We aimed to investigate whether altered food reward responses are explained by a GRS comprised of 77 obesity-associated SNPs or reflect a consequence of increased BMI itself. Using a four corner model, we studied 60 females (BMI range 17.4 – 43.9 kg/m^2) who were selected to have either a low or high GRS for obesity and low or high measured BMI, from a sample of ~11,000 people. Functional MRI scans were obtained during the presentation of low-calorie, high-calorie and non-food pictures, and during the anticipation and receipt of chocolate milk. Individuals with high versus low GRS had greater activation in the right orbitofrontal cortex during chocolate milk anticipation, irrespective of current BMI. In response to chocolate milk consumption, individuals with high versus low BMI had greater activation in bilateral amygdala, irrespective of GRS. No effects of GRS, BMI or their interactions were observed on activations to watching (high-calorie) food pictures. Our findings support the notion that genetic predisposition to obesity may impact on weight through increased reward responsiveness to anticipatory food cues. Increased BMI itself may lead to increased valuation of palatable food receipt which may lead to overeating and weight gain in a food-abundant environment.

INTRODUCTION

The excessive eating in obesity is suggested to result from food intake driven not by nutritional needs, but by the rewarding properties of highly palatable energy-dense foods that override homeostatic signals of the body [1]. In previous functional MRI (fMRI) studies we [2,3] and others [4-6] demonstrated that obese compared to lean individuals have increased responses to appealing food pictures and to cues that signal the delivery of palatable food [7] in brain regions mediating reward and motivation, such as the insula, striatum and orbitofrontal cortex (OFC). Results from prospective studies [8,9], demonstrating that this hyper-responsiveness to food predicts future weight gain, suggest a causal role in the development of obesity. Additionally, a lower striatal response to the actual consumption of a palatable food has been observed in obese versus lean individuals, as assessed with fMRI [10,11]. Evidence from animal experiments [12] and longitudinal studies in humans [13] suggests that this reduced striatal response to food consumption results from habitual overeating of energy dense palatable food.

Body weight is highly heritable [14] and genome wide association studies (GWAS) have successfully identified polymorphisms that contribute to common obesity [15,16]. Several findings suggest that these loci may be implicated in the regulation of food reward and appetite by the brain. Rare monogenic mutations that cause severe early-onset hyperphagia and obesity have shown to involve pathways in the brain that regulate appetite, energy balance and reward [17,18]. Furthermore, genes that are repeatedly linked to common obesity (such as *FTO* and *MC4R*) are highly expressed in the hypothalamus [15] and are associated with altered reward responsiveness to food cues [19-21].

However, the pathways by which other recently detected single nucleotide polymorphisms (SNPs) influence BMI are mostly unknown. In addition, the effects of individual SNPs on BMI are small, and studies investigating single SNPs may consequently have a limited ability to detect subtle differences in brain reward responses to food. Combining the effects of multiple genetic variants in a polygenic risk score (GRS) [22] may be more useful to study alterations in brain reward system functioning caused by genetic variation. Therefore, in the present study we used a 77 SNPs GRS to identify individuals to be at either low or high genetic risk for obesity and investigate whether this difference in genetic susceptibility to obesity is related to differences in brain reward responsiveness to food, which could act as an intermediate causal step in the development of obesity.

Further classification of this study sample into individuals with either a low or high current BMI provides the opportunity to investigate whether altered brain reward responsiveness to food reflects a cause or, rather, a consequence of increased BMI. Specifically, alterations in brain reward responsiveness to food that are related to current BMI irrespective of GRS for obesity are more likely to be secondary to high

BMI. In contrast, alterations in brain reward responsiveness to food that are related to GRS for obesity may be an etiological factor contributing to genetic susceptibility to obesity. This approach is based on the previously established 'four corners epidemiological model' [23-25] which, in a similar fashion, demonstrated that defective angiogenesis may be an etiological component in the genetic predisposition to high blood pressure, rather than being a consequence of increased blood pressure as such [23]. In fact, we improved the four corners design by using measured GRS instead of parental characteristics to define genetic predisposition, as in the original design. By doing so, we excluded the influence of confounding effects of familial environmental factors, which still explained part of the familial predisposition to the trait of interest in earlier studies.

We hypothesized that increased responsiveness to anticipatory food cues in brain regions mediating reward and motivation is a feature of the genetic predisposition to obesity, whereas a decreased striatal response to consuming a palatable food stimulus may, in contrast, result as a consequence of increased BMI.

SUBJECTS AND METHODS

SUBJECTS

Participants were recruited from the Netherlands Twin Register (NTR), comprising twins and their family members for longitudinal survey studies on health and disease [26, 27]. For the current study, participants were selected in two phases (for a flow chart, see Figure 1 in Chapter 7). In the first phase, all registered individuals were selected who had undergone DNA extraction and genotyping as part of previous genome-wide association studies (GWAS) [15, 28]. Genotyping was done as described in detail previously [27]. For each of these 11,495 individuals, a polygenic risk score (GRS) for obesity was calculated using 77 known obesity SNPs in individuals of European descent from the most recent meta-analysis of GWAS in adults [16]. The 77 loci were used that reached genome-wide significance for BMI ($P<5 \times 10^{-8}$) in the European analysis in males and females. A summed weighted GRS was calculated by summing the BMI-increasing alleles after weighting the alleles by their effect sizes [22]. Before running a second selection phase, individuals were excluded if they 1) had no weight and/or height recorded during the latest NTR biobank study [29], necessary for calculating recent BMI, 2) were part of monozygotic twins or triplets, or 3) were part of the same family as another individual in the sample. The removal of all but one member of a same family was done to create a sample of genetically unrelated individuals. To this end, spouses and parents of registered twins were first selected followed by randomly chosen siblings or members of twin pairs.

For the second selection phase the sample was then divided into four corners using cut-offs that corresponded to the lower and upper 25% of

the distribution of BMI (i.e. ≤ 22 kg/m^2 and ≥ 27 kg/m^2) and the lower and upper 20% of the distribution of GRS (i.e. ≤ 0.90 and ≥ 1.03). This resulted in four groups of individuals identified as having either low GRS/low BMI, low GRS/high BMI, high GRS/low BMI or high GRS/high BMI. Finally, because of earlier reported gender differences in responses to food cues, with females showing higher activations than men [4], we excluded all male participants from this final sample.

Thus, after confirming whether individuals were still registered as active participants in the NTR, a total of 248 letters were sent with detailed information and an invitation for participation in the study. Through telephone contact more information was provided when needed, and exclusion criteria were checked, i.e. the presence of diabetes mellitus, serious heart, liver or renal disease, malignancies, uncontrolled thyroid disease, neurological or psychiatric disease including eating disorders and depression [30], pregnancy or lactation, alcohol or drug abuse, the use of glucose-lowering drugs or psychoactive medication, history of bariatric surgery, recent weight change (>5% reported weight change in previous 3 months) and MRI contra-indications. In total 113 women were unwilling to participate because of lack of time or travel time being too long. Of the remaining women, 46 were excluded due to recent body weight change (n=15), co-morbidities or medication (n=23), pregnancy (n=4) or MRI contra-indications (n=4). Another 29 individuals could not be contacted by telephone due to loss of follow-up information. Thus, 60 women were included in this study: 16 women with low GRS/low BMI, 12 with low GRS/high BMI, 15 with high GRS/low BMI and 17 with high GRS/high BMI. The study was approved by the VU University Medical Centre ethics committee and performed in accordance with the Helsinki Declaration. All participants provided written informed consent.

MEASURES

Clinical assessments Participants arrived at the clinic between 8:00 and 10:00 AM after an overnight fast. Information on health and socio-demographics was collected using standardized oral interviews. Handedness was assessed using a validated questionnaire [31]. Weight, height and waist- and hip circumference were measured in a standardized manner, as described in detail previously [32]. BMI (kg/m^2) was calculated to reflect body fatness. Body composition was measured using bio-electrical impedance analyses. Venous blood samples were drawn for the assessment of fasting glucose and lipid spectrum.

Hunger and appetite Before the scanning session participants were asked to rate their feelings of appetite and hunger on a Likert scale ranging from 0 ('not at all') to 10 ('extremely'). Participants were asked the questions 1) How hungry are you now? 2) How full are you now? 3) How much could you eat right now? 4) How much is your desire right now to eat something sweet / savoury / fat right now?

Food stimuli ratings After the scanning session participants viewed all 84 food pictures that were presented during the fMRI scan and rated each picture on how palatable the food in the picture appeared to them at that moment on a 7-point Likert scale ranging from 1 ('not at all') to 7 ('extremely'). On a similar scale participants rated the palatability of the receipt of the chocolate milk and tasteless solution used in the fMRI paradigm.

TASK PARADIGMS

Both imaging paradigms used in this study were described in detail previously [2, 3, 10].

Food picture paradigm Blood-oxygen level dependent (BOLD) signal was measured during the presentation of 42 pictures of high-calorie food (e.g. pizza, French fries, ice cream, donuts), 42 pictures of low-calorie food (e.g. fruit salads, an apple, green vegetable salads, a cucumber) and 42 pictures of non-food items (trees, bricks, stones), all pictures being matched for colour, shape and size (see Figure 1A in Chapter 5). Using a block-design, 7 same-category pictures were presented in each block for 2.5 sec per picture, separated by a 0.5 sec blank screen. At the beginning of the scanning session participants were instructed to watch each picture attentively. To test their concentration during the task, one hour after the scan a recognition test was performed comprising 20 pictures of which participants needed to identify the10 pictures that were previously presented in the scanner.

Chocolate milk paradigm In a second fMRI task, BOLD signal was measured during the anticipation and delivery of chocolate milk (Chocomel, 86 kcal, 2.7 g fat, 11.8 g sugar per 100 ml) and a tasteless solution (see Figure 1B in Chapter 5). The tasteless solution was used as a neutral stimulus, designed to mimic the natural taste of saliva and consisted of 2.5 mM NaHCO3 and 25 mM KCL [11]. Two images were presented (an orange triangle or a blue star) that signalled the delivery of either 0.4 ml chocolate milk or tasteless solution, respectively. Images were presented for 2 sec (i.e. anticipation time) in random order, followed by 3 sec of grey screen with a fixation cross and 2 sec of stimulus delivery (i.e. receipt). Participants were instructed to keep the solution in their mouth during 6 sec until the sign 'swallow' appeared. The next trial was started after a jitter of 3-7 sec (i.e. baseline period). In 40% of the events, the cue was not followed by a stimulus delivery [7]. Tastes were delivered using programmable syringe pumps and Vygon syringes that were inserted into the participant's mouth.

IMAGING ACQUISITION AND ANALYSIS

Imaging data were acquired using a 3.0 Tesla GE Signa HDxt scanner (General Electric, Milwaukee, WI, USA). For structural imaging, T1 weighted scans were acquired using a 3D fast spoiled gradient-echo

sequence. For the functional data, a T2* weighted gradient echo-planar imaging sequence was used (repetition time/echo time = 2160/30 msec, flip angle 80°, slice thickness 3 mm, matrix size 64 x 64, 211 x 211 mm^2 field of view, voxel size 3 x 3 x 3 mm, 40 slices).

Pre-processing was done using SPM8 software (Wellcome Trust Centre for Neuroimaging, London, UK) run within Matlab R2012a (Mathworks, Inc.). The origin of each imaging volume was aligned to the anterior commissure. Functional images were realigned to the first volume to correct for head motion, and slice-time corrected to the onset of the middle slice. Further, data were co-registered to the structural scan and segmented to be spatially normalized to the standard Montreal Neurological institute (MNI) template. Finally, images were spatially smoothed using an 8 mm full-width at half maximum Gaussian kernel. The functional data were passed through a high-pass filter (cut-off 128 sec) to remove low-frequency artefacts. No data set showed within-run head movement of more than 3 mm (translation) or >2.5° (rotation).

Block-design BOLD-responses were analysed within the context of the general linear model, as implemented in SPM8. At the first level, for each participant statistical contrasts were generated for comparing brain activations to 1) watching food vs. non-food pictures, 2) watching high-calorie vs. non-food pictures, 3) anticipating chocolate milk vs. baseline, and 4) chocolate milk receipt vs. baseline. Baseline was defined as the jittered time between trials, with removal of the first 3 sec [33]. To specifically assess the effect of anticipating and receiving a palatable taste stimulus as opposed to anticipating and receiving a taste stimulus in general, contrasts were also generated for 5) anticipation of chocolate milk vs. tasteless solution and 6) receipt of chocolate milk vs. tasteless solution.

STATISTICS

Clinical data Clinical and behavioural data were analysed using IBM SPSS Statistics (version 20, IBM Corp., 2011, Armonk, NY). Results are expressed as mean ± sd. Differences between the groups were tested using a 2 by 2 factorial ANOVA. *P*-values were calculated and reported for separate effects of GRS and BMI, and for the interaction between the two factors [24]. Chi-square test was used for the analyses of categorical variables. Palatability ratings of high-calorie and low-calorie food pictures were analysed using a mixed between-group (2 by 2 factors) within-group (2 food categories) ANOVA [5]. A similar analysis was used to compare taste ratings of chocolate milk and tasteless solution among the four groups.

Imaging data Based on our focus on brain regions involved in reward and motivation [4], we selected the amygdala, insula, putamen, caudate nucleus and orbitofrontal cortex (OFC) as our a priori regions of interest (ROI). We defined and localized ROIs specific to our tasks and

contrasts based on the orthogonal main effects of all participants in this study [34, 35]. To this end, contrasts of all participants were entered in a one-sample t-test and, for each contrast, a statistical map was calculated. To visualize brain activation in our anatomical ROIs only, we used an implicit anatomical mask that contained our bilateral ROIs, created with the WFU-Pick atlas toolbox (Wake Forest University, Winston-Salem, NC, USA). Using this implicit mask, we computed the statistical map of the one-sample T-test thresholded at $P<0.05$ family-wise error (FWE) whole brain corrected. Montreal Neurological Institute (MNI) coordinates of significantly activated peak voxels were used to create contrast-specific ROIs, by using spheres around the peaks with a radius of 10 mm (for insula, putamen, caudate nucleus and OFC) or 5 mm (for amygdala). Group comparisons were performed using a 2 by 2 factorial ANOVA design in SPSS [25], after extracting the individual raw beta-weights of each created ROI, using MarsBaR (MRC Cognition and Brain Sciences Unit, Cambridge, UK). *P*-values were calculated and reported for separate effects of GRS and BMI, and for the interaction between the two factors. A nominal *P*-value of 0.05 was set for these GRS and BMI effects.

RESULTS

CLINICAL CHARACTERISTICS

The four groups were comparable for age, handedness, daily smoking and menopausal status, as presented in Table 1. The proportion of premenopausal women that was scanned in the follicular phase of their menstrual cycle [36] was similar between groups. As expected, selection of participants from the four corners resulted in significant differences among groups in GRS and BMI ($P<0.001$). In keeping with a causal effect of BMI on these risk factors, women with high BMI had more unfavourable outcomes on HDL cholesterol and total/HDL cholesterol ratio irrespective of GRS. For fasting glucose a near significant ($P=0.057$) effect of GRS was observed irrespective of current BMI. Self-reported race was Caucasian (n=59) and Indo-Surinamese (n=1). However, projection of the genome-wide SNP data on a genome reference dataset [37] identified two individuals with a non-European ancestry, of which one matched the person identified through self-report.

BEHAVIOURAL RESULTS

Mean ratings of hunger and appetite prior to the scanning session were not affected by BMI, GRS or their interactions (Table 2). Figure 1A shows the mean palatability ratings of the high-calorie and low-calorie food pictures in the four groups. ANOVA revealed that there was no group by category interaction ($F[1, 56]=0.142$ $P=0.7$). Participants in all groups rated the low-calorie food pictures as more palatable than the high-calorie food pictures ($F[1, 56]=81.679$ $P<0.001$). Similarly,

no group by category interaction was observed for the ratings of chocolate milk and tasteless solution ($F[1, 55]=0.359$ $P=0.9$) (Figure 1B). Participants rated the taste stimuli as equally palatable ($F[1, 55]=0.196$ $P=0.7$). Furthermore, participants performed similarly on the image recognition test performed after the scan ($P>0.4$ for BMI, GRS and interaction), with mean percentages ± SD of images correctly recognized of 85.9 ± 10.0, 84.2 ± 6.0, 85.3 ± 7.9 and 86.8 ± 6.8 for the participants with low GRS/low BMI, low GRS/high BMI, high GRS/low BMI and high GRS/high BMI, respectively.

BRAIN RESPONSES TO FOOD PICTURES

In the overall group of participants, we observed a significant main effect for the contrast watching food vs. non-food pictures within our a priori anatomical ROIs, in particular the left amygdala, left OFC and bilateral insula (Table 3). Watching high-calorie vs. non-food pictures resulted in more activation of the left amygdala and left insula. Main effects of tasks in other regions of the brain are presented in Supplementary Table 1.

After extracting the mean activation of the task-specific ROIs created with MarsBaR, group comparisons showed no significant effects of GRS, BMI or its interactions on the mean activation in the left amygdala, OFC and bilateral insula to watching food vs. non-food pictures (Table 3), as analysed with factorial ANOVA. Similarly, no significant effects of BMI, GRS or its interactions were observed in the mean activation of the left amygdala and insula to watching high-calorie vs. non-food pictures.

BRAIN RESPONSES TO ANTICIPATION AND RECEIPT OF PALATABLE FOOD

Due to a technical problem during the scanning session, one participant (with low GRS/low BMI) could not complete the fMRI chocolate milk task. In the remaining group of women, we observed a significant main effect of chocolate milk anticipation vs. baseline in bilateral OFC (Table 3). The receipt of chocolate milk vs. baseline significantly activated bilateral amygdala, insula and right putamen. Main effects of tasks in other regions of the brain are presented in Supplementary Table 1. When contrasted to the tasteless solution, no main effects of chocolate milk receipt or anticipation were observed.

When comparing groups for mean activation in the task-specific ROI's, factorial ANOVA revealed that women with high GRS as compared to women with low GRS had significantly higher activation associated with the anticipation of chocolate milk vs. baseline in the right OFC, irrespective of BMI ($P=0.04$) (Table 3 and Figure 2A and B). The receipt of chocolate milk vs. baseline elicited higher activation in women with high BMI as compared to women with low BMI in bilateral amygdala, irrespective of GRS ($P<0.05$) (Figure 2C and D). No significant interactions between GRS and BMI were observed.

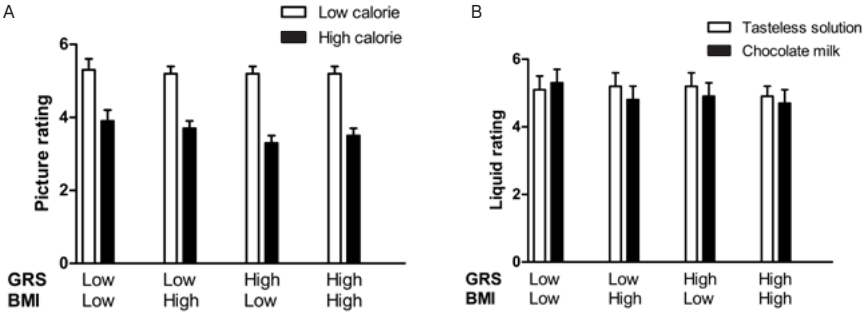

FIGURE 1 Appeal ratings of functional MRI stimuli
Mean scores ± SEM of self-reported appeal of low-calorie and high-calorie food pictures (A) and chocolate milk and tasteless solution (B) used in the functional MRI paradigms in women with low GRS/low BMI (n=16), low GRS/high BMI (n=12), high GRS/low BMI (n=15) and high GRS/high BMI (n=17). Data are analysed using mixed between-group (2 by 2 factors) within-group (2 food categories) ANOVA.

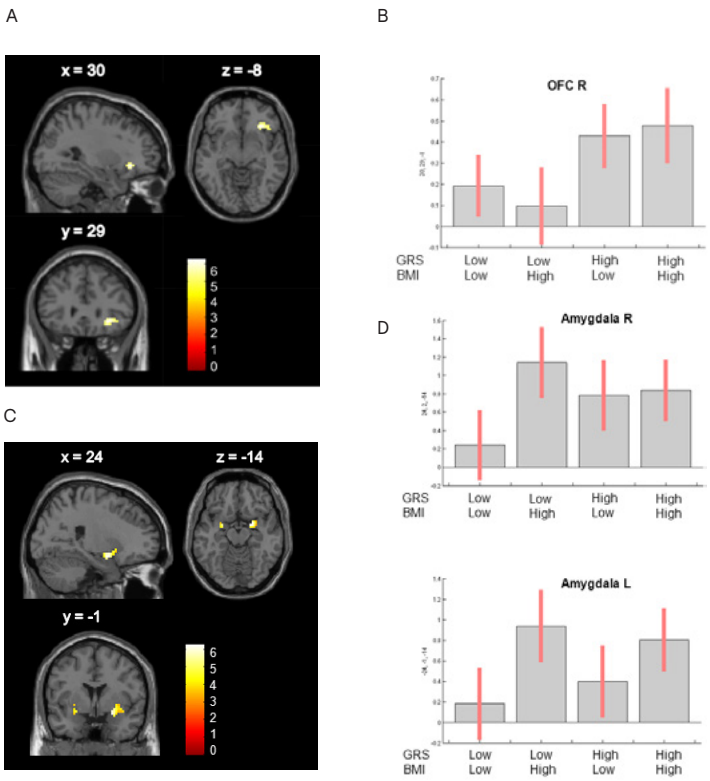

FIGURE 2 Main effects of functional MRI tasks and group differences among the four corners
Sagittal, axial and coronal slices showing the main effect of task (A) and group differences (B) in activation in response to the anticipation of chocolate milk vs. baseline in the right orbitofrontal cortex (OFC) (MNI 30 29 −8). Main effect of task (C) and group differences (D) in activation in response to chocolate milk receipt vs. baseline in the left amygdala (MNI −24 −1 −14) and right amygdala (MNI 24 2 −14). Left side of axial and coronal slices is the left side of the brain. X, Y and Z are the MNI space coordinates. Colour bar represents t statistics. Bars in figure B and D represent mean and 90% confidence intervals of contrast estimates (effect size, in arbitrary units) for women with low GRS/low BMI (n=15), low GRS/high BMI (n=12), high GRS/low BMI (n=15) and high GRS/high BMI (n=17). GRS, polygenic risk score; OFC, orbitofrontal cortex; R, right; L, left.

DISCUSSION

We investigated brain reward responses to the viewing of food pictures and to the anticipation and consumption of a palatable food stimulus in women with either a low or a high genetic predisposition to obesity, based on a polygenic risk score (GRS) of the 77 recently identified obesity-associated single nucleotide polymorphisms (SNPs) in European individuals [16]. To differentiate genetic effects that predispose to obesity from effects that are secondary to a high BMI, we further divided the study population into women with either a low or a high current BMI. This so-called 'four corners epidemiological approach' has been established previously [23-25] and used to identify a variety of factors that mediate the link between parental predisposition to hypertension and high blood pressure in the offspring.

In the current study, we observed that the anticipation of chocolate milk receipt elicited increased activation in the right orbitofrontal cortex (OFC) in women with high GRS for obesity as compared to women with low GRS for obesity, irrespective of current BMI. This finding suggests that an elevated response to the anticipation of palatable food in brain areas implicated in reward is a feature of the genetic predisposition to obesity. This finding fits with evidence from an earlier prospective study which demonstrated that greater OFC activation when viewing appetizing food pictures predicts future weight gain [8, 9]. Moreover, a study that used parental overweight to identify adolescents as having either a high or low genetic risk to obesity, demonstrated that individuals with high obesity risk have greater activation of reward regions, including the OFC, in response to palatable food receipt than individuals with low obesity risk [38]. In the Stice et al. study, however, high-risk participants had a slightly higher BMI than low-risk participants which makes it difficult to infer a causal relationship regarding reward region responsiveness and BMI. Furthermore, the use of parental occurrence of overweight to define familial risk to obesity in the offspring does not fully exclude effects of shared environmental factors unlike measured GRS as was done in our current study.

Evidence is emerging that multiple genetic variants associated with common obesity exert their effects on weight through the regulation of appetite and reward in the brain [16, 18]. Besides the genetic determinant with the strongest effect on BMI, the *FTO* gene, an association with processes in the central nervous system has been found for *ELAVL4*, *GRID1*, *CADM2*, *NRXNR*, *NEGR1* and *SCG3* [16]. Other SNPs are located near genes that have clearly shown involvement in the leptin-melanocortin pathway in the hypothalamus (*MC4R*, *BDNF*, *POMC* and *SH2B1*), as observed by studying monogenic forms of obesity [17, 18]. Although the recently identified obesity-associated loci together explain only a small amount (2.7%) of BMI variation [16], the usefulness of a multilocus GRS in studying appetitive mechanisms has already been demonstrated previously [39-41]. Specifically, whereas the effects of a 28-locus GRS on BMI

was mediated by lower satiety responsiveness in children [39], two other studies suggested mediating effects of uncontrolled and emotional eating behaviour, using risk scores comprising 32 and 90 BMI-related loci, respectively [40, 41]. Similar to our study, these observations provide evidence that a combined weighted GRS has the potential to identify mechanisms involved in food reward and appetite that contribute to the genetic predisposition to obesity.

A second major finding of our current study was an elevated response in bilateral amygdala in response to the receipt of chocolate milk in women with high BMI compared to women with low BMI, irrespective of GRS for obesity. This finding suggests that an *increased* amygdala response to palatable food receipt is secondary to overeating and/or weight gain. This result is at odds with our expectation based on previous studies that weight gain induces a *decreased* striatal response to palatable food receipt [11, 13]. These discrepant observations may reflect the multi-faceted aetiology of obesity, in which different brain regions implicated in the experience of eating may exert different effects to the receipt of palatable food. Whereas a decreased striatal response to food receipt may reflect the experience of lower reward due to neuro-adaptive changes caused by habitual overeating [13], an increased amygdala response to food receipt may reflect elevated responsiveness of regions involved in reward-based learning [42]. The latter, also known as conditioning, has shown to occur in response to repeated intake of high-calorie palatable foods [43]. It should be noted, however, that the increased amygdala response in our study was observed in response to *food consumption*, whereas conditioning, by definition, elicits responses to *cues* that signal the delivery of the food. A possible role for conditioning is nonetheless supported by several previous studies in which consumption of a palatable food stimulus also elicited *increased* rather than *decreased* activation in obese versus lean individuals in regions implicated in reward, attention and gustation, including the amygdala [7, 38, 44]. Thus, we propose that the receipt of small amounts of palatable foods may have conditioning effects similar to visual food cues. Together, our results suggest that an increased valuation of palatable food receipt may occur secondary to overeating and/or weight gain, which may further enhance weight gain in an environment dominated by the supply of high-calorie palatable food.

Contrary to our expectations, we did not observe significant effects of BMI, GRS or its interactions on the viewing of food pictures, or specifically high-calorie food pictures. It may be argued that, given the food appeal ratings, watching high-calorie as opposed to low-calorie food pictures failed to elicit sufficiently rewarding effects. However, watching food pictures and high-calorie food pictures in the overall group of women was associated with robust BOLD activation in the a priori ROIs, indicating that our paradigm was effective. A possible explanation for the absence of significant group differences in the food picture paradigm is lack of power. Our sample size was based on the

findings of significant BMI-related effects in previous published studies that included similar or even smaller sample sizes with comparable group differences in BMI [2-5], although we cannot rule out that these studies were underpowered for the BMI-related effect themselves.

Perhaps more concern is warranted for the power of the GRS. The currently identified obesity SNPs explain only a small percentage of the variance in BMI [16]. However, we again note that previous studies have shown potential effectiveness of polygenic risk scores based on fewer obesity-SNPs [39, 41] and that the power in our study was further enhanced compared to these previous studies by selecting individuals with extreme genotypes from a very large base population (n>11,000). Nevertheless, future research using larger sample sizes and risk scores comprising more obesity-related SNPs are warranted, as these may be able to detect more significant genetic effects on reward responses to visual food cues.

A final limitation of our study is that, when analysing the data of our chocolate milk experiment, robust reward responses in the total group of women were observed when the receipt and anticipation of chocolate milk were contrasted against a baseline BOLD activation, but not relative to the tasteless solution. This implicates that the group differences we observed are associated with the receipt and anticipation of a taste stimulus in general, but not specifically a high-calorie palatable taste stimulus. The absence of a significant main effect of chocolate milk when contrasted to the tasteless solution is most likely the result of a rewarding effect of the latter stimulus, which was originally designed to act as a neutral comparator [11]. This fits with our results of the food appeal ratings of the taste stimuli after the scan, which did not differ between chocolate milk and tasteless solution.

Strengths of our study are the use of a GRS based on the most recent findings of obesity-related SNPs [16] and the use of the four corner epidemiological model [23-25] which, by selecting individuals on extreme phenotypes and genotypes, allows to separate effects that predispose to obesity from effects secondary to increased BMI. Furthermore, the use of a measured GRS rather than parental characteristics to express genetic risk allowed us to study genetic effects independent of family environmental background.

In conclusion, genetic predisposition to obesity may act, at least in part, through elevated reward responsiveness to cues that signal palatable food receipt. In addition, habitual overeating and increased BMI itself may result in higher valuation of palatable food, which may result in even more overeating if the availability of energy dense palatable food is high. Our findings imply that interventions should focus on discouraging the intake of energy-dense palatable food in individuals from early age to prevent the development of conditioning to cues linked to the intake of these food types, especially in individuals with high genetic risk to obesity.

TABLE 1
Clinical characteristics

	Low GRS Low BMI (n=16)	Low GRS High BMI (n=12)	High GRS Low BMI (n=15)	High GRS High BMI (n=17)	*P*-value by ANOVA		
					GRS	BMI	Interaction
Age (y)	44.8 ± 6.3	47.0 ± 7.3	44.3 ± 7.4	47.1 ± 6.9	0.935	0.171	0.874
GRS	0.87 ± 0.05	0.87 ± 0.05	1.06 ± 0.05	1.05 ± 0.06	< 0.001	0.779	0.764
Weight (kg)	61.2 ± 6.6	89.7 ± 10.9	60.5 ± 4.8	92.7 ± 13.2	0.642	< 0.001	0.459
BMI (kg/m²)	20.9 ± 0.9	31.6 ± 3.7	20.4 ± 1.3	32.8 ± 4.8	0.636	< 0.001	0.289
Waist-hip ratio	0.78 ± 0.04	0.87 ± 0.05	0.78 ± 0.03	0.87 ± 0.06	0.744	< 0.001	0.726
Body fat (%)	27.8 ± 3.1	42.7 ± 2.9	27.1 ± 3.4	44.6 ± 4.7	0.573	< 0.001	0.208
Fasting glucose (mmol/L)	4.8 ± 0.4	4.8 ± 0.5	5.0 ± 0.4	5.1 ± 0.6	0.057	0.193	0.787
Total cholesterol (mmol/L)	4.9 ± 0.8	5.2 ± 0.8	4.9 ± 1.2	5.2 ± 1.3	0.869	0.410	0.567
HDL cholesterol (mmol/L)	1.9 ± 0.3	1.4 ± 0.5	1.9 ± 0.3	1.5 ± 0.6	0.413	< 0.001	0.937
LDL cholesterol (mmol/L)	2.8 ± 0.6	3.5 ± 0.9	2.7 ± 1.0	3.1 ± 1.0	0.611	0.042	0.398
Ratio total / HDL cholesterol	2.8 ± 0.8	4.0 ± 1.0	2.6 ± 0.9	4.0 ± 1.9	0.524	< 0.001	0.916
					P-value by chi-square		
Daily smoking (%)	2 (12.5)	1 (8.3)	2 (13.3)	0 (0)		0.5	
Right handedness (%)	15 (93.8)	9 (75)	13 (86.7)	15 (88.2)		0.7	
Premenopausal status (%)	11 (68.8)	7 (58.3)	11 (73.3)	9 (52.9)		0.6	
Follicular menstrual phase (%)	5 (45.5)	2 (28.6)	4 (36.4)	3 (33.3)		0.8	
Oral contraceptives use (%)	3 (18.8)	4 (33.3)	1 (6.7)	2 (11.8)		0.3	

Mean ± SD or n (%). All biochemical assessments are done in the fasted state. GRS, polygenic risk score; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

TABLE 2
VAS-scores of hunger and appetite ratings

	Low GRS Low BMI (n=16)	Low GRS High BMI (n=12)	High GRS Low BMI (n=15)	High GRS High BMI (n=17)	*P*-value by ANOVA		
					GRS	BMI	Interaction
Hunger	5.0 ± 2.8	3.6 ± 1.9	4.0 ± 2.2	3.6 ± 2.2	0.417	0.138	0.412
Fullness	3.5 ± 1.8	3.5 ± 2.5	2.7 ± 1.9	2.9 ± 1.8	0.199	0.839	0.839
Prospective food consumption	5.8 ± 1.6	4.3 ± 1.7	5.3 ± 1.4	5.3 ± 1.9	0.537	0.099	0.091
Desire for sweet food	3.1 ± 2.8	2.4 ± 2.3	2.7 ± 2.4	3.2 ± 2.2	0.813	0.876	0.341
Desire for savory food	5.1 ± 3.0	3.1 ± 2.4	4.6 ± 2.1	4.1 ± 2.7	0.707	0.069	0.294
Desire for fat	2.0 ± 2.2	1.1 ± 1.6	1.7 ± 1.9	1.3 ± 1.7	0.900	0.191	0.579

Mean ± SD. GRS, polygenic risk score; VAS, visual analogue scale

TABLE 3
Main effects of tasks in regions of interest and group comparisons

			Main effect		MNI			*P*-value by ANOVA		
Contrast	Region	Side	k	T	x	y	z	GRS	BMI	Interaction
Food vs. non-food pictures	Amygdala	L	2	5.05	-21	-4	-17	0.631	0.727	0.320
	Insula	L	17	7.01	-36	5	-14	0.434	0.705	0.699
	OFC	L	9	5.41	-6	62	-5	0.670	0.965	0.701
	Insula	R	2	4.91	39	8	-14	1.000	0.626	0.455
High-calorie vs. non-food pictures	Amygdala	L	4	5.49	-21	-4	-17	0.276	0.957	0.838
	Insula	L	6	5.88	-36	5	-14	0.572	0.582	0.862
Anticipation choco vs. baseline	OFC	L	1	4.84	-48	17	-8	0.711	0.605	0.580
	OFC	R	27	6.15	30	29	-8	0.040	0.935	0.891
Receipt choco vs. baseline	Amygdala	L	2	5.29	-24	-1	-14	0.716	0.013	0.192
	Insula	L	15	6.31	-36	-7	10	0.619	0.461	0.372
	Amygdala	R	16	6.27	24	2	-14	0.309	0.049	0.059
	Insula	R	31	7.65	39	-1	13	0.448	0.779	0.340
	Putamen	R	16	5.42	27	5	-5	0.831	0.242	0.574

Montreal Neurological Institute (MNI) coordinates of peak voxels activated in the total group of participants with threshold $P<0.05$ FWE whole brain corrected and visualized with an anatomical mask of a priori regions of interest; and group comparisons by factorial ANOVA. K, cluster size; T, T-statistic; GRS, polygenic risk score; L, left; R, right; OFC, orbitofrontal cortex; choco, chocolate milk.

SUPPLEMENTARY TABLE 1
Main effects of tasks whole brain

	Side	k	T	MNI x	MNI y	MNI z
Food vs. non-food pictures						
Occipital cortex and fusiform gyrus	L	893	13.4	-42	-82	-2
Occipital cortex	R	736	12.1	39	-70	-11
Posterior cingulate cortex	L	175	8.8	-6	-52	22
Postcentral gyrus	L	74	7.2	-42	-28	34
Insula	L	26	7.0	-36	5	-14
Superior parietal lobe	R	31	6.4	21	-61	58
Superior parietal lobe	L	71	6.0	-24	-58	58
Superior parietal lobe	L	9	5.4	-6	62	-5
Amygdala	L	2	5.1	-21	-4	-17
Superior frontal lobe	L	1	5.0	-18	32	43
Superior frontal lobe	L	2	4.9	-12	38	43
Insula	R	3	4.9	39	8	-14
High-calorie vs. non-food pictures						
Occipital cortex	L	903	13.8	-42	-79	-2
Occipital cortex	R	774	13.2	42	-76	-2
Posterior cingulate cortex	L	101	8.4	-6	-52	22
Superior parietal lobe	R	45	6.4	24	-58	58
Postcentral gyrus	L	58	6.1	-45	-28	34
Postcentral gyrus	R	22	6.0	60	-19	28
Insula	L	10	5.9	-36	5	-14
Superior parietal lobe	L	43	5.8	-24	-58	58
Amygdala	L	4	5.5	-21	-4	-17
Middle occipital cortex	R	2	5.2	27	-70	31
Superior occipital cortex	L	2	4.9	-24	-70	31
Anticipation chocolate milk vs. baseline						
Occipital cortex and fusiform gyrus	L/R	6018	19.0	-39	-67	-11
Precentral gyrus	L	75	7.2	-51	-1	43
Precentral gyrus and inferior frontal gyrus	R	282	6.7	51	8	43
Orbitofrontal cortex	R	32	6.2	30	29	-8
Supplementary motor area	R	30	5.6	3	11	55
Inferior parietal lobe	R	22	5.4	48	-34	40
Inferior frontal gyrus	L	25	5.4	-45	8	28
Middle temporal gyrus	R	7	5.2	48	-22	-8
Inferior frontal gyrus	L	2	5.1	-24	26	-8
Superior temporal gyrus	L	3	5.0	-51	14	-11
Cerebellum	L	2	5.0	-6	-79	-35
Inferior frontal gyrus	L	1	4.8	-60	11	13
Receipt chocolate milk vs. baseline						
Pre- and postcentral gyrus	L	640	11.4	-60	-22	22
Postcentral gyrus, rolandic operculum and insula	R	746	11.3	60	-16	19
Cerebellum	R	100	8.1	24	-70	-23
Supplementary motor area	L/R	73	6.5	6	5	55
Insula	L	21	6.3	-36	-7	10
Cerebellum	L	83	6.3	-18	-67	-23
Amygdala and putamen	R	20	6.3	24	2	-14
Middle temporal gyrus	L	42	6.1	-54	-67	4
Cerebellum	L	7	5.4	-42	-61	-29
Temporal lobe	R	1	5.4	45	-22	-14

Cerebellum	L	7	5.3	−15	−76	−35
Amygdala	L	2	5.3	−24	−1	−14
Temporal lobe	R	2	5.2	48	−16	−17
Superior frontal lobe	L	2	5.1	−18	−1	67

Montreal Neurological Institute (MNI) coordinates of peak voxels activated in the total group of participants with threshold $P<0.05$ FWE whole brain corrected. K, cluster size; T, T-statistic; L, left; R, right

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Summary and General Discussion

IX

The overarching aim of this thesis was to investigate the contribution of genetic and environmental factors to food intake, physical activity and food reward regulation by the brain. Further, we aimed to disentangle whether the altered reward system functioning in individuals with obesity precedes overeating and weight gain or is secondary to overweight itself. The three parts of this thesis describe three different study designs that were used to investigate 1) the contribution of the intrauterine environment to food intake, and the contribution of 2) environmental factors and 3) genetic factors to food intake, physical activity and the regulation of food reward by the brain (Figure 1).

SUMMARY

PART 1 – INTRAUTERINE ENVIRONMENT AND FOOD INTAKE

Previous epidemiological studies have repeatedly demonstrated that intrauterine growth restriction is associated with increased cardiovascular risk in adult life [1-3]. A possible mechanism explaining this increased risk may be the adherence to an unhealthy diet, as observed in previous studies in singletons [4-7]. We investigated whether this observed relation between intrauterine growth restriction and unfavourable feeding preferences in later life is a result of intrauterine environmental factors, independent of confounding by genetic factors. Therefore, in Chapter 3 we analysed birth weight and food intake in 78 dizygotic (DZ, sharing on average 50% of their genes) and 94 monozygotic (MZ, sharing nearly 100% of their genes) adolescent same-sex twin subjects selected from the Netherlands Twin Register (NTR). Since differences within DZ twins are explained by both genetic and environmental factors, whereas differences within MZ are explained only by environmental factors, the comparison of intra-pair differences within DZ and MZ twins allows to separate environmental from genetic effects. We observed that co-twins born with lower birth weights had higher intake of total energy and saturated fat in later life than their co-twins born with higher birth weights. This observation was done in both DZ and MZ twins, which implies that the observed association between lower birth weight and unhealthier food intakes in later life results from a true intrauterine environmental influence, rather than from genetic confounding.

PART 2 – ENVIRONMENTAL FACTORS AND FOOD INTAKE REGULATION

In the second part of this thesis we investigated the role of environmental factors on food intake, physical activity and the regulation of food reward by the brain. Therefore, we studied 16 MZ female twin pairs with a rare, mean intra-pair difference in BMI of 3.96 kg/m^2, selected from the NTR. Chapter 4 describes the study in which we investigated physical activity using 7-day accelerometry, and dietary intake using

3-day 24-hour recalls. We observed that, when comparing the heavier and leaner co-twins of a pair, the heaver co-twin was on average less physically active, tended to perform less moderate to vigorous physical activity and ingested more total fat and mono- and polyunsaturated fatty acids, than the leaner co-twin of that pair. No differences in total energy intake were found. Since MZ twins are different only in their exposure to unique environmental factors, while being nearly identical in their shared environmental and genetic background, our observations in this study can only be explained by unique environmental factors impacting on lifestyle behaviours.

In Chapter 5 we investigated whether the differences in food intake within the BMI discordant female twin pairs could be explained by differences in food reward regulation by the brain. Therefore, we used functional MRI (fMRI) to measure brain activity in brain areas involved in reward and motivation (e.g. the insula, amygdala, striatum and orbitofrontal cortex) during two different fMRI tasks. First, we studied the reward response to visual food stimuli by measuring brain activity while participants watched full-colour pictures of high-calorie food (e.g. pizza and ice cream), low-calorie food (e.g. fruits and vegetables) and non-food items (e.g. plants and stones). Secondly, we studied the response to actual taste stimuli by measuring brain activity while participants anticipated or received a sip of chocolate milk or a tasteless solution in their mouth. Results of both fMRI tasks revealed that there were no significant differences in brain reward activity to either visual or taste stimuli between the leaner and heavier co-twins of the BMI discordant pairs. These findings suggest that the altered brain reward responses to food previously observed in obese versus non-obese singletons (rather than twins, as in our study) are largely explained by inherited factors. By excluding this influence of inherited factors by comparing genetically identical twins, the previously observed relation between obesity and alterations in food reward disappeared.

In addition to the above task-based fMRI, we measured brain activity in BMI discordant MZ twins while no tasks were performed and participants were at complete rest. By doing so, we investigated functional connectivity of so-called resting state brain networks involved in food reward and motivation. In Chapter 6 we observed that within the basal ganglia network, heavier versus leaner co-twins had lower functional connectivity strength in bilateral putamen, a brain area involved in reward-related motivation. The fact that this difference was found within MZ twins implies that the BMI-related alterations in putamen functional connectivity are independent of genetic confounding. Additional analysis in the overall group of females (thus, considering every female as an individual) revealed that lower functional connectivity strength in the left putamen correlated with higher intake of total fat, as measured with 3-day 24-hour recalls. Thus, Chapter 6 a) suggests a genetically-independent correlation between lower putamen connectivity and higher BMI, and b) adds to the idea that environmental fac-

tors can lower putamen connectivity leading to increased BMI through higher intake of fat.

PART 3 – GENETIC FACTORS AND FOOD INTAKE REGULATION

The final part of this thesis deals with the contribution of genetic effects. We aimed to investigate whether genetic susceptibility to obesity is associated with alterations in food intake, physical activity and regulation of food reward by the brain and, further, to examine whether these traits are causal or secondary to obesity. To this end, we selected 60 females from a total of >10.000 individuals registered in the NTR with available data on BMI and genetic risk for obesity, using calculated genetic risk scores based on 77 previously discovered single nucleotide polymorphisms (SNPs) associated with obesity [8]. Women were selected when having a) either a low or high genetic risk for obesity and, b) either a low or high measured BMI. This resulted in four corners of women with extreme measures of both genotype and phenotype [9,10].

In Chapter 7 we observed that, irrespective of genetic risk for obesity, women with high BMI had fewer step counts, more sedentary behaviour and more emotional and restrained eating (based on eating behaviour questionnaires) than women with low BMI. Since these unfavourable lifestyles correlate with participants' current BMI rather than their genetic susceptibility for obesity, we conclude that these lifestyles possibly develop secondary to increased BMI. Furthermore, we concluded that a higher intake of (animal) protein may lead to obesity only in women with a high genetic predisposition to obesity, since we observed higher (animal) protein intake in women with high BMI versus low BMI, but *only* if genetic risk to obesity was also high. If genetic risk to obesity was low, such difference in food intake was absent.

Finally, Chapter 8 describes the observed differences in brain activity in response to food stimuli in the four corners study. We observed that, irrespective of current BMI, females with high genetic obesity risk had greater fMRI brain activation in the right orbitofrontal cortex (OFC, involved in food reward) during chocolate milk anticipation than females with low genetic obesity risk. We concluded that these findings support the notion that genetic predisposition to obesity may impact on weight through increased reward responsiveness to anticipatory food cues. Another main finding was an elevated response in bilateral amygdala in response to the receipt of chocolate milk in women with high BMI compared to women with low BMI, irrespective of GRS for obesity. We concluded that increased BMI itself may also lead to increased valuation of palatable food receipt, which may induce even more overeating and weight gain.

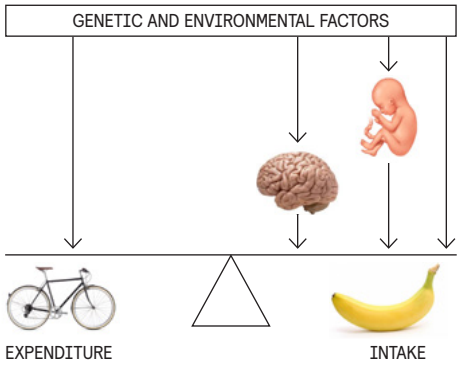

FIGURE 1
The balance between energy expenditure (derived by basal metabolism and physical activity) and energy intake determines the amount of energy stored as fat. The balance can be influenced by physical activity, food reward regulation by the brain, and intrauterine effects. Each of these effects in turn are influenced by genetic and environmental factors

GENERAL DISCUSSION

THE INTRAUTERINE ENVIRONMENT AND FOOD INTAKE

We repeated findings from previous studies [4-7] that low birth weight is related to unfavourable food intake in later life, in specific higher energy and saturated fat intake, which may put individuals at higher risk of cardiovascular disease (Chapter 3). Whereas results of previous studies may have suffered from genetic confounding [11], we excluded this possibility by finding similar intra-pair associations within MZ and DZ twin pairs, thereby eliminating genetic effects. In addition, we excluded possible confounding by maternal factors, such as socio-economic class and cigarette smoking. Our observations are of clinical interest, since our results imply that attempts at improving the intrauterine environment may actually have causal, beneficial effects on later food intake and subsequent health.

It might be argued that the association between the intrauterine environment and food intake is explained by differences in physical activity between co-twins at adolescence. However, an association between low birth weight and lower physical activity has not been found in previous studies [12]. Moreover, although in our study physical activity data were not available, a previous study observed that adjustment for physical activity did not influence the relation between birth weight and later food intake [7]. Furthermore, it should be noted, that we also cannot ascertain whether our observations were influenced by differences in post-natal feeding. That is, breastfeeding has shown to have, albeit small, protective effects on childhood obesity, compared to formula-feeding [13,14]. In other words, the results of our study may have been explained by the possibility that co-twins with lower birth weight received different amounts or sorts of feeding postnatally, than the co-

twins with higher birth weight. Similar to almost all previous studies on this topic, we cannot exclude this possibility since our observations were not adjusted for early life feeding. Research has shown that maternal recall of infant feeding is often inaccurate [15] and, furthermore, that the long-term health effects of breastfeeding remain to be found [16].

Studies in animals, particularly rodents, have tried to explain the mechanism of how intrauterine conditions may affect, or 'programme', food intake in later life. There is evidence for altered structure and function of hypothalamic areas involved in food intake regulation, such as an upregulation of appetite-stimulating and downregulation of appetite-supressing neuropeptides [17]. Furthermore, studies suggested a lower functioning of the leptin-mediated feedback loop between peripheral fat storages and the hypothalamus, possibly resulting from central leptin resistance [18]. Finally, a role for the hypothalamus-adrenal axis has been proposed, since increased levels of appetite-stimulating glucocorticoids were found in intrauterine growth restricted subjects in both animals and humans [19].

Regardless the route of programming, evidence is growing that the intrauterine environment may exert its effects on health in later life through epigenetic mechanisms [20]. Epigenetics refers to all modifications to genes other than changes in the DNA sequence itself, including DNA methylation and histone modifications [21]. Indeed, research in both animals and humans have reported altered methylation patterns of genes involved in appetite regulation in subjects exposed to intrauterine undernutrition [22].

In sum, our findings support the hypothesis of a causal link between poor intrauterine conditions and unfavourable food intake in later life, which increases susceptibility to adult disease. Identification of factors that comprise this poor intrauterine state might lead to finding possible targets for intervention.

FOOD REWARD REGULATION BY THE BRAIN: GENETIC AND ENVIRONMENTAL INFLUENCES

Obesity is characterized by food intake that is driven not by metabolic needs, but by the rewarding aspects of food consumption, which is particularly the case for palatable energy-dense foods [23]. This has led to research of the human brain reward system, and how dysregulation of this system might be seen in or lead to overeating and obesity [24]. Bluntly, when comparing obese and lean subjects, previous neuroimaging studies observed increased reward responses to pictures of palatable food [25], whereas a lower striatal response was found in response to the actual receipt of a palatable taste stimulus [26]. This altered reward functioning is thought to induce a) higher food craving and b) compensatory overeating, comparable to behaviours seen in drug addiction [27].

Using two genetically informative study designs (Part 2 and 3 of this thesis), we aimed to investigate the role of genetic and environmental factors in the observed reward dysregulation in obesity. Together,

the results of our studies provide evidence for a major role of genetic factors to altered brain reward system functioning in obesity. First, when comparing (genetically identical) MZ twins with rare intra-pair BMI discordance, we observed no differences in brain activation in either of the fMRI-tasks, i.e. watching palatable food pictures and the anticipation and receipt of chocolate milk (Chapter 5). Thus, after excluding the influence of genetic effects by studying differences within genetically identical twins, the association between reward dysregulation and obesity as previously observed in singletons disappeared. Importantly, this lack of difference was found despite the presence of adequate main effects of tasks in participants, i.e. all subjects had appropriate brain reward activation when viewing palatable food pictures and when anticipating and receiving chocolate milk (or, in other words, the tasks 'worked'). Secondly, we observed that females who were selected as having high genetic risk for obesity, based on a 77-SNP obesity risk score, had higher OFC activation in response to chocolate milk anticipation than females selected as having low genetic risk for obesity, regardless whether these females had normal or increased BMI (Chapter 8). These results suggests that the brain reward system acts as a vulnerability factor mediating the relation between genetic susceptibility and obesity.

Our findings are in line with many other observations. First, the important role of genes in the regulation of food reward emerges from previous twins studies that showed high similarity of MZ twins in a) food cue responsiveness as examined with questionnaires [28], and b) brain reward responsiveness to visual food cues as measured with fMRI [29]. Second, studies investigating individuals with rare monogenic forms of hyperphagia and obesity, such as leptin-deficiency and *MC4R* mutations, reported altered reward responses to food cues, suggesting involvement of these genes in the reward circuitry in the brain [30]. Third, many of the obesity-associated single nucleotide polymorphisms (SNPs), recently discovered by genome wide association studies (GWAS), are located in or nearby genes that are primarily expressed in the central nervous system [8]. These brain sites include the hypothalamus and limbic system, brain areas that play a pivotal role in the regulation of appetite and reward [24]. Finally, recent studies have been examining the influence of these identified obesity-associated genetic variants on brain reward system structure and function. To this end, effects of single SNPs were either studied individually, or after aggregating the effects of multiple SNPs into a polygenic risk score [31], similar to our current study. Thus far, these common genetic variants have been associated with a variety of measures reflecting altered reward system functioning, including lower satiety responsiveness in children [32], altered food-cue responses in homeostatic and reward areas [33-35], increased nucleus accumbens size and responsiveness to food advertisements in children [36], grey matter deficits in the prefrontal cortex [37] and altered functional connectivity in resting state networks,

including the salience network [38]. Taken together, our current results and previous observations provide evidence for a substantial role of genetic factors on altered reward system functioning in obesity. These factors may explain why certain people develop obesity in the current food abundant obesogenic environment, whereas others do not.

In addition to our above findings in task-related fMRI experiments, we performed fMRI analyses in the resting state (Chapter 6). Within the BMI discordant MZ twins, we found an association between higher BMI and lower functional connectivity strength in bilateral putamen within the basal ganglia network. This finding is consistent with the proposed hypothesis of an obesity-related hypo-functioning reward system [39, 40], which postulates that obese individuals have reduced reward system activation during food consumption, which induces compensatory overeating of highly rewarding foods. The fact that our observations were done within genetically identical twins, implies that the association between increased BMI and lower putamen connectivity can occur independent of genetic effects and, thus, results from the exposure to unique environmental factors. This seems in conflict with the earlier conclusions that reward dysregulation in obesity is mainly a result of genetic factors. However, it should be noted that there is an important difference between task-based fMRI and resting state fMRI [41]. That is, task-based fMRI measures blood-oxygen level dependent (BOLD) brain activations in response to an active task, whereas resting state fMRI measures the synchronisation of brain regions that are part of the same functionally-connected brain network [42]. In other words, whereas task-based fMRI investigates *activity within brain regions*, resting state fMRI investigates *connectivity between brain regions*. Thus, because the two techniques investigate two different aspects of brain functioning, it is possible that, depending on the nature of the underlying brain defect (i.e. brain activity or connectivity), genetic or environmental factors take centre stage in explaining the reward dysregulation in obesity.

FOOD REWARD REGULATION BY THE BRAIN AND OBESITY: TESTING CAUSALITY

Knowing whether an observed association between exposure and outcome arises from true causality is crucial for the implementation of treatment and prevention policies. True causality between exposure factor A and outcome factor B requires 1) that A causes B and not, in reverse, that B causes A (i.e. reverse causality), and 2) that the association between A and B is not explained by another (unknown) factor C influencing both A and B (i.e. confounding). While randomized controlled trials (RCTs) are the gold standard for testing causality, they are expensive, time consuming, and may be practically or ethically unfeasible. In addition, due to inevitable use of strict in- and exclusion criteria, results of RCTs may not always be generalizable to the population at large. Alternatively, cause-and-effect relations can be

studied in cross-sectional observational studies by making use of genetic information of study participants [43-45]. In this thesis we used two genetically informative study designs to test the causal nature of the association between reward dysregulation and obesity. In Part 2 we eliminated confounding factors using a discordant MZ twin model, whereas in Part 3 we aimed at testing the direction of causality using genetic risk scores in a four corners epidemiological model.

Discordant monozygotic twin model Causality testing using discordant MZ twins capitalizes on the fact that MZ twins are identical for many factors that may act as possible confounders in observational studies in singletons, such as pleiotropic genes (i.e. genes influencing two or more seemingly unrelated phenotypic traits) [43,46]. This is comparable with the use of randomization in RCTs, which aims at obtaining two groups that are similar for many different variables, but systematically differ in the exposure variable. In the discordant MZ twin model, MZ twin pairs are selected of which one co-twin has been environmentally exposed to a certain factor, whereas the other has not. If the co-twin with the exposure also shows higher measures of a possible outcome, then the association between the exposure and outcome is independent of confounding and, thus, reflects a true causal relation [47]. This co-twin control model has been used to test a wide variety of associations, including the relation between smoking and lung cancer [48] and the effect of exercise behaviour on well-being [49]. In Part 2 of this thesis we applied this design by investigating food reward regulation by the brain in MZ twins discordant for BMI. Using task-based fMRI we observed no differences in brain reward activation in response to food stimuli between leaner and heavier co-twins (Chapter 5). Following the reasoning of the co-twin control design, we concluded that the relation between reward dysregulation and obesity, as previously observed in singletons, is likely to be explained by genetic confounding.

In contrast, our fMRI measurements in the resting state showed that, within BMI discordant MZ twins, leaner co-twins had higher putamen functional connectivity in the basal ganglia network than heavier co-twins. Therefore, we concluded that the relation between BMI and resting state network connectivity is independent of genetic confounding. It should be noted, however, that we cannot ascertain that these results were unaffected by reverse causality or by unique environmental factors affecting both BMI and resting state network connectivity. In fact, ideal co-twin control studies are designed to have MZ twins discordant for the exposure variable, after which the relation with an outcome variable is measured. In other words, if we wanted to test the commonly proposed hypothesis that altered food reward regulation by the brain (i.e. exposure) causes overeating and obesity (i.e. outcome), we would have ideally selected MZ twins discordant for food reward regulation by the brain, instead of MZ twins discordant for BMI, as we have done in our current study. Needless to say,

this ideal strategy is rather difficult to realize because it would require scanning thousands of MZ twin pairs with MRI designs as used in this study. Therefore, MZ twins in our current study were, more feasibly, selected based on BMI discordance. BMI is usually available for large sample through existing biobanks in twin registries like the NTR and can even be reliably assessed by survey or interview. In keeping with this, many previous studies investigated MZ twins discordant for BMI to study the nature of correlates of BMI, although most studies indeed focussed on factors secondary to adiposity [50,51], rather than factors causing adiposity.

Nevertheless, regardless whether the discordance of twins is based on presumed *exposure* or *outcome* variable, investigating within-pair differences in MZ twins allows to falsify the hypothesis of causal relations between traits in epidemiological studies. That is, if the hypothesis reads that factor A causes B, then differences in A within MZ twins should be associated with differences in B. If, however, differences in A within MZ twins are not associated with differences in B, then the hypothesis of causality would be falsified. Particularly, in the latter case the apparent association between A and B in the singleton population would have been driven by genetic (or shared environmental) factors. When applying this principle to our own MZ twin study, we can conclude that 1) brain reward responses to food cues and BMI are not causally related, but explained by genetic factors, and 2) lower putamen connectivity and higher BMI could be causally related, independent of genetic confounding.

Four corners epidemiological model In order to investigate the direction of causality in cross-sectional observations, one could make valuable use of information on genetic predisposition to a trait provided by participants. The basic principle of this approach is that the direction of causality is always from the genetic predisposition to the trait of interest, and not vice versa. In an attempt to distinguish whether alterations in food reward regulation are a cause or consequence of obesity, we used an adapted version of the previously established four corners epidemiological model [9,10]. That is, we measured brain reward responsiveness to food cues in females selected as having either a high or low genetic risk to obesity (based on calculated polygenic risk scores using 77 obesity SNPs [8] and either a high or low measured BMI (Chapter 8). According to the four corners model, factors associated with BMI irrespective of genetic predisposition are more likely to be secondary to increased BMI, or to be largely influenced by environmental determinants that operate independent of the genetic risk to obesity. In contrast, factors that are associated with the genetic risk could be part of a causal pathway leading to obesity.

In our chocolate milk fMRI experiment, we observed that the anticipation of chocolate milk elicited greater brain activation in the OFC in individuals with high genetic risk versus low genetic risk for obesity.

Following the reasoning of the four corners model, this result suggests that a genetically mediated increased brain reward responsiveness to anticipatory food cues is causal to increased BMI. In contrast, we observed that the actual consumption of chocolate milk elicited greater brain activation in bilateral amygdala in individuals with high BMI versus low BMI, irrespective whether genetic risk to obesity was high or low. This suggests that higher reward valuation of palatable food receipt may develop secondary to increased BMI.

Our first observation fits with evidence from previous neuroimaging studies. First, numerous prospective studies demonstrated that individuals with greater response of reward regions (including the OFC) to high-calorie food images exhibit elevated future weight gain [52, 53]. Secondly, a study in which parental overweight was used to identify adolescents as having either a high or low genetic risk to obesity, demonstrated that individuals with high obesity risk have greater response of reward regions, including the OFC, to palatable food receipt than individuals with low obesity risk [54]. Taken together, the results of our current study and previous findings suggest that an initial higher reward-region response to food cues is a genetic vulnerability factor for elevated food intake and weight gain.

Our second observation, i.e. higher amygdala response to the receipt of chocolate milk in higher BMI females irrespective of genetic obesity risk, was at odds with our expectations based on previous findings by others. When focusing primarily on experiments in animals and prospective studies in humans (rather than cross-sectional studies, which cannot make inferences on causality), evidence is growing that overeating and weight gain results in lower striatal activity in response to palatable food receipt [55, 56]. Theorists have proposed that this reward deficit during food consummation may lead to even more overeating by means of compensation [57], although evidence that support this theory is scarce [58]. Therefore, our observation of higher (instead of lower) amygdala response to chocolate milk receipt in higher BMI females was somewhat unexpected. However, our results do find support by another etiological model that has been proposed by theorists, i.e. the incentive sensitization model. This model posits that repeated intake of high-calorie palatable foods results in an elevated responsivity of regions involved in incentive valuation (including the amygdala) to cues that are associated with palatable food intake via conditioning, which prompts craving and overeating when these cues are encountered [59]. In order to use this model as support for our results we must, however, assume that the receipt of small amounts of palatable foods, as in our chocolate milk experiment, may have conditioning effects similar to visual food cues. Indeed, previous cross-sectional studies observed elevated reward-region activation (including the amygdala) not only in response to visual stimuli, but also to taste stimuli in obese versus lean subjects [40, 54, 60] which might suggest that taste stimuli may act as conditioning cues that signal the delivery of palatable foods.

Thus, we propose that overeating and weight gain may, independent of genetic risk to obesity, lead to elevated valuation of palatable taste stimuli which may act as possible cues signalling even more palatable food intake, resulting in higher craving. A final remark should be made, however, since the four corner design does not discriminate between factors that are secondary to increased BMI and factors that are causal to increased BMI but largely influenced by environmental exposures, independent of genetic predisposition to obesity. Therefore, whereas the elevated amygdala response to chocolate milk receipt is suggestive to be a result of increased BMI, this cannot be actually proven by our current study, and needs conformation from longitudinal studies.

Taken together, the results of our fMRI experiments in both discordant MZ twins study and four corners study provide support for the hypothesis that an initial genetic vulnerability to an increased reward response to food may induce higher food cravings, elevated intake of high-calorie palatable food and subsequent weight gain. Subsequently, obesity itself may lead these individuals to develop higher valuation of palatable food cues which may lead to even more overeating and weight gain in an food cue-abundant, obesogenic environment.

FOOD INTAKE AND PHYSICAL ACTIVITY

At the population level, the rise in obesity rates during the last decades can be explained by the emergence of an obesogenic environment, i.e. an environment in which palatable, high-calorie foods are easily accessed and sedentary behaviour is heavily promoted [61]. However, although everyone is exposed to this hazardous environment, not everyone becomes obese. In fact, obesity rates can highly vary between ethnic groups. Therefore, the way an individual responds to the obesity-promoting environment is highly determined by genetic factors. Indeed, twin studies estimated heritability of BMI between 40% and 70% [62]. Using the genetically informative study designs from Part 2 and Part 3 of this thesis (i.e. BMI discordant MZ twin pairs and the four corner design), we aimed to investigate the contribution of environmental and genetic effects on the most important BMI effectors, i.e. food intake and physical activity. In addition, an attempt was made to disentangle cause and effect among these effectors and BMI.

Food intake Remarkably, in neither of our studies we observed that differences in BMI were associated with differences in energy intake. Under the assumption that we had sufficient power for these analyses, these null findings mean that either all BMI differences among study participants are due to differences in energy expenditure or, more plausibly, that measured energy intake did not match habitual energy intake. Underreporting of habitual food intake is a common problem in nutrition research, especially in females and individuals with over-

weight or obesity [63]. People underreport food intake because of social desirability, errors in portion size estimation and simply because they forget what they have eaten. In addition, study participants may undereat during the time of data collection, which may also have affected our data. Ideally, recognition of study participants who underreport is done by simultaneous measurement of total energy expenditure, assessed by the doubly-labelled water method [64]. This technique is, however, expensive and not always feasible in larger study samples. Therefore, we cannot ascertain whether the lack of differences in energy intake between low and high BMI females in our studies resulted from underreporting. This suggestion is supported by findings from a previous study in BMI discordant MZ twins which used doubly labelled water to check the validity of using food diaries for measuring food intake [65]. This study observed more underreporting in obese versus lean co-twins, which highlights the problem of underreporting also in twin populations.

Since many years, nutrition research has focussed on the question whether the occurrence of overweight is a simple result of the equation between energy in and energy out, or whether certain macronutrients are more likely to induce weight gain than others. Studies comparing long-term weight loss effects of dietary regimens that are low in energy but have different compositions of fat, carbohydrates and protein, demonstrated that the amount of weight loss by obese individuals is independent of diet macronutrient composition [66]. These findings suggest that macronutrients do not impact on body weight, and that obesity is a simple result of calorie excess. However, in situations when food can be accessed freely without energy restrictions (i.e. ad libitum), certain macronutrients have shown to drive up food intake more than others [67]. For instance, total energy intake was shown to be higher when participants consumed diets relatively high in fat than when they ate lower fat diets [68]. Furthermore, the intake of sugar-sweetened beverages has consistently shown to be associated with long-term weight gain, even in a direct dose-response relationship [69]. While there is an ongoing debate in nutrition research about the relative importance of fat versus sugar [70, 71], evidence is clear that a combined intake of both sugar and fat (which is often considered as highly palatable) drives up food intake in an addiction-like manner [72]. Eventually, this sort of eating, driven by the hedonic aspects of palatable food beyond metabolic requirements, may lead to energy excess and weight gain. Thus, whereas the amount of energy stored as fat is a direct result of energy in and energy out, the total caloric intake is highly influenced by the relative contribution of, especially, combined fat and sugar.

In contrast to our null findings in energy intake, we did observe differences in macronutrient intakes between females with high BMI versus low BMI in both our study designs. In specific, within discordant MZ twins, heavier co-twins had higher intake of total fat and mono- (MUFAs) and polyunsaturated fatty acids (PUFAs) than leaner co-twins

(Chapter 4). These analyses in genetically identical twins eliminate genetic confounding and, thus, are compatible with a causal effect of elevated intake of total fat, MUFAs and PUFAs on BMI. This observation is in line with a previous study in BMI discordant MZ twins, which reported a higher recalled preference for fatty foods in the obese versus the leaner co-twin, as measured with qualitative recall assessments [73]. The finding that this food preference was already present *before* onset of BMI discordance, is again compatible with a causal role for higher fat intake to weight gain, independent of genetic effects. It should be noted, however, that in our current study the observed higher fat intake in heavier versus leaner co-twins was mostly explained by higher intake of MUFAs and PUFAs, which are fatty acids often known for their supposed protective (rather than deleterious) effects on cardiovascular health [74]. MUFAs and PUFAs are highly found in vegetable oils, such as olive oil, which are central to the Mediterranean diet and are suggested to have a positive influence on weight control [75]. Negative effects of MUFAs and PUFAs have been reported before, since higher dietary fat intake provides concomitant higher caloric intakes [76]. Thus, the relationship between a diet rich in MUFAs and PUFAs and weight control has not been fully addressed. Nevertheless, our results support the existence of a causal effect of intake of fat, MUFAs and PUFAs on overweight, independent of genetic confounding.

In addition to these environmentally-induced changes in macronutrient intakes, we found support for an interaction between genetic predisposition and macronutrient intake in our four corner study (Chapter 7). In specific, females with high BMI had elevated intake of protein, particularly protein derived from animal products, compared to females with low BMI, however *only* when genetic risk to obesity was high. If genetic risk to obesity was low, this association was absent. This pattern would not be expected if increased (animal) protein intake was secondary to high BMI but, instead, suggests that the intake of (animal) protein modifies the relation between genetic risk and obesity development. These results are in line with previous studies investigating interactions between genetic obesity risk and food intake. For example, interactions of genetic risk with meal frequencies [77], fried food [78] and sugar-sweetened beverages [79] have been described. Remarkably, one study also found that the association between increased body fat mass and higher intake of protein, and in particular animal protein, was stronger when genetic risk for obesity was high than when genetic risk for obesity was low, based on a 16-SNP obesity genetic risk score [80]. Although protein consumption has been thought to protect against overweight by enhancing satiation, the beneficial effect of protein intake is debated, mainly when its source is considered [81, 82]. Whereas vegetable proteins may have protective effects, animal protein has shown to be associated with higher BMI [83]. Thus, the results of our current study and previous studies suggest that elevated intake of protein, in specific animal protein, may amplify the effect of genetic risk factors for obesity.

Physical activity On the other side of energy balance, there is energy expenditure. Energy is expended mainly through resting energy expenditure (i.e. the amount of energy necessary to fuel the body at rest) and physical activity [84]. Resting energy expenditure is proportional to the amount of lean body mass. Since adiposity is accompanied by an increase in, not only fat mass, but also lean body mass tissue [85], obese individuals have higher absolute rates of resting energy expenditure than lean individuals. Indeed, in our studies we observed higher resting energy expenditure in females with higher BMI, as measured with indirect calorimetry. However, after correction for lean body mass, resting energy expenditure was similar among groups. Therefore, differences in BMI among females were not expected to be explained by differences in resting energy expenditure. Indeed, research has shown little evidence that a low metabolism plays a significant role in weight gain [86]. Thus, the most important contributor to energy expenditure is not energy expended in rest but, instead, through physical activity. The epidemic of obesity has been attributed to both increased food intake and decreased physical activity level [61]. However, the relative contribution of physical activity is under considerable debate in the literature [87]. Some researchers claim that the increased amount of energy intake is sufficient to explain the obesity epidemic, since in the last 30-40 years (in which obesity rates escalated) physical activity levels have little changed [88]. On the other hand, scientist declare that there is still an important influence of the decreased physical activity level induced by industrialization and urbanization, which emerged in the first half of the 20th century [84]. On the level of body weight, experimental studies manipulating physical activity levels have confirmed the existence of robust causal effects on BMI [89, 90]. However, it remains difficult to establish the relative contribution of physical activity patterns in the development of overweight and obesity in the population at large. Although observational studies in population based samples can establish the extent of the association between physical activity and BMI, they cannot rule out confounding by genetic factors and reverse causation (i.e. that BMI itself is causative to changes in physical activity) [91]. Therefore, using the genetically informative study designs from Part 2 and Part 3 of this thesis, we investigated whether physical activity is associated with BMI independent of genetic factors. In addition, an attempt was made do disentangle cause and effect in this relationship.

Together, both our studies show that higher BMI is associated with less physical activity, independent of genetic effects. In specific, heavier versus leaner co-twins of genetically identical twin pairs had 1) fewer mean accelerometer activity counts per day, and 2) a trend towards less time spent in moderate-to-vigorous physical activity (MVPA) (Chapter 4). These findings imply that the relation between increased BMI and lower physical activity, in specific, MVPA, is independent of genetic confounding. Furthermore, results of our four corner study showed that females with high BMI had 1) fewer daily step counts, and 2) more

time spent in sedentary behaviour, than females with low BMI, irrespective of their genetic risk to obesity (Chapter 7). According to the reasoning of the four corner design, these findings imply that lower physical activity, in specific more sedentary behaviour, is secondary to increased BMI itself or, alternatively, a causal factor to BMI largely influenced by environmental exposures, independent of genetic predisposition. Thus, taken together, both studies provide support for a reverse causal relation, where BMI affects physical activity instead of vice-versa.

The results of our MZ twin study are in line with some but not all previous studies investigating BMI discordant MZ twins [65, 92, 93]. Two studies also observed lower accelerometer counts [92] and less reported high-intensity activity [65] in heavier versus leaner co-twins. Another study failed to detect differences within MZ twins [93], however, this study used retrospective interviews which, rather than accelerometry, have limited reliability and validity to measure physical activity. Remarkably, another recent NTR twin study on cross-sectional and longitudinal data also found no evidence for a causal relation between exercise behaviour and BMI in adolescents [94]. This is not a full replication, because exercise and MVPA reflect different hallmarks of physical activity. Whereas exercise behaviour signifies regular voluntary activity performed in leisure time and in structured settings (such as team sports and visiting health clubs), moderate to vigorous physical activity comprises all activities that require 3 to 6 times higher amounts of efforts (i.e. metabolic equivalents, METs) compared to quietly sitting (such as team sports and visiting health clubs, but also bicycling, hiking, gardening and carrying heavy loads) [95].

In our four corner study we found a further clue for a reverse causal relationship between high BMI and lower physical activity, specifically that higher BMI induces an unfavourable imbalance of increased sedentary behaviour and decreased light intensity physical activity. This finding is supported by a previous longitudinal study which observed that sedentary time did not predict BMI, whereas BMI did predict sedentary time, at follow up, after adjustment for baseline physical activity [91]. Moreover, evidence for the suggestion of a reverse causal relation was found by a recent study using Mendelian randomization [96]. This technique aims at testing causality between traits, by using measured genetic variants as instrumental variables [45]. More specifically, a genetic variant that influences an exposure variable (i.e. sedentary behaviour) should also predict an outcome variable (i.e. BMI) if exposure and outcome variable are causally related. The authors concluded that higher childhood adiposity may cause lower physical activity levels, including higher sedentary behaviour, as measured with accelerometers [96]. However, they also acknowledged their inability to test, in reverse, whether low physical activity causes weight gain, due to the fact that genetic scores for physical activity were not available in the study. Therefore, as the authors also declared, results of this study

should be interpreted with caution. Nevertheless, results of our current study and previous studies suggest that increased BMI induces lower physical activity, in specific more sedentary behaviour, which pushes people at even greater risk of energy excess and further weight gain in a food abundant environment.

METHODOLOGICAL CONSIDERATIONS

Body mass index to reflect adiposity In all our study designs, we used BMI (i.e. body weight in kilograms divided by the square of the body height in meters) as an indicator of body fatness. BMI is an easy, inexpensive and non-invasive surrogate of body fatness, which enables data collection in large populations and at different time points, such as in the Netherlands Twin Registry. On the other hand, because BMI measures excess weight and not excess fat, BMI does not differentiate between fat mass and muscle mass, nor does it provide information on the distribution of fat. Therefore, the degree of how well BMI represents body fatness depends on factors such as sex, age and muscularity [97]. In our studies we mainly investigated women only, who were all in their adulthood and of whom no one was a highly-trained athlete. Also, we observed that our measures of BMI fairly correlated with measures of body fat mass, as assessed with bio-impedance analysis. Thus, we conclude that BMI reflected body fatness in our study to a reasonable extent.

Furthermore, the definitions of overweight and obesity based on BMI cut-offs (i.e. overweight if BMI 25-30 kg/m^2 and obesity if BMI >30 kg/m^2) were questioned several years ago, after the publication of a study which observed lower all-cause mortality rates in subjects with overweight or mild obesity as compared to subjects with normal body weight (i.e. BMI 18.5-25 kg/m^2) [98]. These findings were tackled, however, by a more recent study which excluded smokers and people with serious illnesses from the analyses, after which the seemingly paradoxical association disappeared [99]. Thus, the standard BMI cut-offs as used in our current study have shown its valid use for defining who is overweight and who is not.

Sample sizes In part 1 of this thesis we found evidence for an intrauterine environmental effect on the association between birth weight and higher energy and saturated fat intake in later life. It should be noted, however, that evidence for an intrauterine environmental effect does not exclude the possibility of a genetic effect. That is, confidence intervals of our correlation efficient were wide and, more specific, ranged from positive to negative values in both MZ and DZ twins. For example, the intra-pair association between birth weight and energy intake within DZ twins (-238 kcal per kg birth weight) ranged from -662 to 185, whereas the association within MZ twins (-265 kcal per kg birth weight) ranged from -643 to 113. In other words, our study could not exclude the possibility that the association in DZ twins was, for example, neg-

ative, while the association in MZ twins was absent, which would be indicative for a genetic effect. To completely rule out the possibility of a genetic influence, detailed food intake recording is needed in very large twin cohorts , which is a cost-intensive undertaking with large logistic challenges.

The sample sizes in Part 2 and 3 of this thesis were determined based on previous fMRI studies using identical techniques and comparable BMI differences among study groups as expected in our current studies [25]. Since BMI is highly heritable but also variable in time [100], identifying MZ twins with consistent BMI discordance is difficult. This resulted in a final study sample that, despite a rare mean intra-pair BMI difference of 3.96 kg/m^2, comprised 2 twin pairs that were not strictly BMI discordant during the test visit (BMI differences of 0.71 and 1.02 kg/m^2). Post hoc analyses after excluding these 2 pairs did not influence our results in terms of effect sizes, although statistical significance decreased (Chapter 4). We acknowledge that, although our sample sizes are common in neuroimaging research, our studies may have been underpowered to detect significant differences in other variables, such as behavioural measures (using questionnaires) and food intake. However, with respect to our discordant MZ twin study, power should be evaluated within the context of the study design, i.e. monozygotic twins being highly matched for possible confounding factors such as age, gender and genetic background, but, in the same time, ultimately discordant for BMI, which together enhance the power of this study to a great extent [46, 101]. With respect to our four corner study, we enhanced power for detecting effects of BMI and genetic risk to obesity by selecting individuals from a very large base population (>10.000 individuals) based on extreme values of both genotype and phenotype.

The use of a polygenic risk score It could be argued that the clinical use of obesity-associated genetic variants in predicting disease is limited, since the identified obesity-associated SNPs together explain only a small amount of BMI variation (i.e. 2.7% opposed to the heritability of 40-70% estimated by twin studies) [8, 62]. However, aggregating information from multiple SNPs into a polygenetic risk score, in particular after effect size weighting, has shown to be a useful tool for examining the cumulative effect of genetic variants on phenotypic outcomes [31], such as mechanisms involved in food intake regulation. Previous studies already reported significant associations between polygenetic obesity risk scores and satiety responsiveness in children [32] and different types of eating behaviour [102, 103]. Thereby, these studies provided evidence for the utility of a combined weighted genetic obesity risk score for identifying mechanisms involved in food intake regulation. We emphasize that, whereas previous studied used genetic risk scores based on ~32 SNPs, we enhanced power for examining genetic effects by using genetic risk scores based on 77 obesity-related SNPs, as identified in the most recent GWAS on obesity [8]. In fact, by using a four corner design

in which individuals were selected from a larger base population with extreme genotypes only, we enhanced power even further.

Generalization to males In Part 2 and Part 3 of this thesis, our examinations were done in females only, which limits the ability to generalize our findings to the general population. Our main reason to exclude males was to create a study sample that was homogeneous in the most desirable and feasible manner. Earlier studies reported gender-related differences in energy homeostasis [104], physical activity levels [105], eating behaviours [106] and even brain reward responsiveness to food cues [25], suggesting that the inclusion of both males and females would have resulted in higher inter-individual variation and possibly less power to detect significant effects, in particular after stratification for gender. Our reasons to include females instead of males were 1) methodological (females showed higher food cue BOLD responsiveness than men [25], thereby optimizing power), 2) clinical (females are more likely to become obese than men [107], thereby enhancing the clinical relevance of our findings) and 3) logistical (with respect to Part 2, BMI discordance within MZ twins is more common in female than male twin pairs [100], thereby facilitating participant enrolment). As a result, however, generalization of our findings in Part 2 and 3 to the male population should be done with caution.

CLINICAL IMPLICATIONS AND FUTURE RESEARCH

The main findings of this thesis suggest that genetic vulnerability may explain why certain people are more likely to respond to palatable high-calorie food cues in terms of overeating and subsequent weight gain, whereas others do not. This support for an important genetic influence on food reward regulation by the brain is of clinical importance in several ways.

First, further identification of genes involved in food intake may unravel pathways that lead to overweight and obesity, which may contribute to the development of new therapeutic strategies against obesity, as has been demonstrated previously [30, 108]. For example, studies in monogenic obesity revealed that in leptin-deficient individuals, which are characterized by hyperphagia and morbid obesity, replacement of the hormone leptin reduced food intake and body weight back to normal [109]. Unfortunately, this replacement therapy has not shown to be effective in common obesity, i.e. in which not a single gene with a large defect is responsible but, rather, multiple genes with much more subtle effects [110]. Apparently, leptin acts more like a starvation hormone rather than a satiety hormone [111], since lower leptin levels have shown to induce elevated food intake, whereas administration of extra leptin does not decrease food intake a contrary way. Thus, although treatment is already available for patients with monogenic obesity, future studies are needed to identify alternative routes between genetic susceptibility and food intake, thereby providing the possibility of developing new therapeutics against common obesity.

Second, our findings may suggest a role for personalized treatment, meaning that obese individuals may be selected for treatment (such as medication, cognitive therapies or surgery) based on their genetic susceptibility to obesity. Although this is already common practise in certain fields of cancer treatment, the predictive value of individual polygenetic risk scores to common obesity is still very poor [31], which hampers its utility for personalized medicine. This limited predictive value is mainly due small effect sizes of individual obesity-SNPs which, together, explain only several percentages of BMI variation [8]. This missing heritability has been ascribed to hitherto undefined genetic influences, epigenetic differences and gene-environment interactions.

From the important role of genetic variants we should not conclude that we are fully determined by our genes. In fact, since obesity rates escalated during a time in which genes remained relatively stable, evidence is clear for a major role of environmental factors [61]. Fortunately, unlike genetic factors, environmental factors are often amenable for intervention, thereby offering possibilities in combating obesity through changing the environment. These changes include reducing the presence of cues to palatable high-calorie food (such as advertisements on television and billboards), decreasing the availability of these foods in places that once did not sell food (such as gas stations, pharmacies and public transport) and reducing portion sizes in restaurants. Instead, intake of healthy food, i.e. low in energy but high in nutritional value and fibres (such as vegetables), should be promoted and made available for more people, for instance by lowering its prices. By changing the environment, individuals (in particular those with high genetic susceptibility) may become less exposed to cues promoting high-caloric eating beyond metabolic needs and subsequent weight gain.

From an energy balance point of view, prevention of obesity would be far more effective than obtaining weight loss once obesity is present [84]. This is because the body more easily adapts to a state of positive energy balance than negative energy balance, in other words, the body tries to defends itself for future weight loss [112]. Since with weight loss comes loss of muscle mass and subsequent loss of resting energy expenditure, a person requires substantial and permanent change of behaviour to maintain substantial weight loss [84]. Unsurprisingly, not many people are able to maintain their body weight after having lost weight following energy restriction [113]. However, research has shown that individuals who combined their diet interventions with increased physical activity levels were more likely to maintain their lower body weight than individuals who did not change their activity pattern [114]. Thus, as supported by our findings in this thesis, both food intake and physical activity are important for reaching and maintaining a healthy body weight.

Obesity is a complex and multifactorial disease, which implies that many more factors are involved than we could have investigated in this thesis. Most importantly, due to well-known practical difficulties asso-

ciated with the use of fMRI, we were not able to examine differences in functioning of the hypothalamus, which is known to be a key player in the regulation of homeostatic food intake [115,116]. Further, in addition to altered functioning of the subcortical brain reward system, obesity is characterized by lower ability to inhibit food cravings through (frontal) cortical functioning [117]. Our fMRI experiments were not designed to test this cognitive functioning and we did not examine the role of inhibitory control in this thesis. Finally, evidence is emerging for an important influence of factors such as stress [118], sleep patterns [119] and the human gut microbiome [120], which may even exert their effect on body weight through altering the brain reward system. Thus, future studies may focus on these important effectors on body weight, which may contribute to weight gain and obesity development. For making inferences on causality, studies in population based samples should focus on longitudinal data collected in genetically informative subjects, ideally including twins, thereby ruling out the influence of genetic confounding and reverse causation.

CONCLUSION

To conclude, findings of this thesis are supportive for a substantial influence of genetic effects on altered reward responsiveness to palatable, high-calorie food cues, which promotes eating beyond metabolic needs and, subsequently, puts people at increased risk of overweight and its associated disease, such as type 2 diabetes mellitus and cardiovascular disease. Changing the environment by reducing the presence of cues that promote such food intake could halt the ongoing obesity epidemic, which now also emerges in low-income countries.

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Nederlandse Samenvatting
Dankwoord

X

NEDERLANDSE SAMENVATTING

INLEIDING

Wereldwijd neemt het aantal mensen met overgewicht steeds verder toe. In Nederland heeft ruim de helft van de huidige bevolking overgewicht, en 14% heeft een ernstige vorm van overgewicht, obesitas. Men spreekt van overgewicht bij een body mass index (BMI) van meer dan 25 kg/m^2 en van obesitas bij een BMI van meer dan 30 kg/m^2. De BMI is te berekenen door het gewicht te delen door de lengte in het kwadraat. Overgewicht kan leiden tot gezondheidsproblemen als suikerziekte (diabetes type 2), verhoogd cholesterolgehalte, hoge bloeddruk en slaapstoornissen. Hiermee ontstaat een verhoogd risico op hart- en vaatziekten, gewrichtsslijtage (artrose) en sommige vormen van kanker.

Overgewicht ontstaat, zeer eenvoudig gezegd, als de hoeveelheid energie-inname (in de vorm van eten en drinken) de hoeveelheid energieverbruik (in de vorm van beweging) overschrijdt. Het lichaam slaat de overtollige energie dan op als vet waardoor het gewicht toeneemt. Waarom de ene persoon in verhouding tot zijn of haar energieverbruik te veel energie inneemt en de ander niet is echter nog niet volledig duidelijk. Onderzoek heeft aangetoond dat de hersenen een belangrijke rol spelen in het reguleren van de energiebalans onder andere door het beïnvloeden van het eetgedrag. Bij deze regulering van eetgedrag spelen prikkels van buiten een belangrijke rol. Prikkels die aanzetten tot eten zijn bijvoorbeeld aantrekkelijke afbeeldingen en geuren van lekker voedsel. De hersenen reageren op deze prikkels door gebieden te activeren die betrokken zijn bij het gevoel van beloning. Om de functie van de hersenen in beeld te brengen kan gebruik worden gemaakt van magnetic resonance imaging (MRI). Eerdere MRI onderzoeken hebben aangetoond dat mensen met overgewicht andere reacties hebben in deze beloningsgebieden op voedselprikkels dan mensen zonder overgewicht. Zo blijken mensen met overgewicht een sterker beloningssignaal af te geven op het zien van lekker eten, maar een zwakker signaal op het daadwerkelijk ontvangen van lekker eten, vergeleken met mensen zonder overgewicht. Deze uitkomsten suggereren dat mensen met overgewicht geneigd zijn om eerder maar ook meer van iets te eten om een zelfde gevoel van beloning te ervaren dan mensen zonder overgewicht. Hierdoor ontstaat meer inname van voedsel, wat bij een gelijk blijvend energieverbruik leidt tot nog meer energieopslag en dus gewichtstoename.

Zeer waarschijnlijk wordt de werking van deze hersengebieden beïnvloed door zowel erfelijke (genetische) factoren als factoren uit de omgeving. Het is echter niet duidelijk in welke mate deze beide factoren een rol spelen. Ook is het niet duidelijk of de veranderingen in hersenfuncties in mensen met overgewicht een oorzaak of juist een gevolg zijn van het overgewicht. Deze vragen zijn van belang, omdat kennis van onderliggende oorzaken van overgewicht cruciaal is voor

de ontwikkeling van nieuwe middelen en manieren om overgewicht te voorkomen en te behandelen.

DOEL VAN DIT PROEFSCHRIFT

Met het huidige proefschrift hebben we inzicht proberen te krijgen in de invloed van omgevingsfactoren en genetische factoren op 1) de beloningsgebieden in de hersenen, 2) voedsel inname en 3) lichaamsbeweging. Daarnaast hebben we onderzocht in hoeverre de veranderingen in de beloningsgebieden van mensen met overgewicht een oorzaak of juist een gevolg zijn van overgewicht. Om deze vragen te beantwoorden hebben we drie verschillende onderzoeken uitgevoerd met proefpersonen van het Nederlands Tweelingenregister, die elk in een apart deel van dit proefschrift beschreven worden. Deze drie delen behandelen achtereenvolgens de invloed van omgevingsfactoren in de baarmoeder, de invloed van omgevingsfactoren in het algemeen en de invloed van genetische factoren.

DEEL 1

Grote epidemiologische onderzoeken hebben aangetoond dat personen met een laag geboortegewicht meer kans hebben op hart- en vaatziekten in het latere leven. Eén van de mechanismen die dit verband zouden verklaren is dat personen met een laag geboortegewicht een ongezondere voedselinname hebben. Dit verband is door eerdere onderzoeken al aangetoond. Over het algemeen wordt aangenomen dat dit verband wordt veroorzaakt door een verminderde groei in de baarmoeder, waardoor organen minder goed worden aangelegd en er op latere leeftijd ziektes ontstaan. Volgens deze hypothese zal verbetering van de omstandigheden in de baarmoeder leiden tot betere aanleg van organen en dus minder ziekten in het latere leven. Echter, een alternatieve hypothese is dat het verband verklaard wordt door genetische factoren die samenhangen met zowel een laag geboortegewicht als de ontwikkeling van hart- en vaatziekten. Anders gezegd, het genotype voor hart- en vaatziekten heeft ook invloed op het geboortegewicht. Als deze alternatieve hypothese juist is, zal verbetering van de groei in de baarmoeder niet leiden tot minder hart- en vaatziekten. Hetzelfde geldt voor het verband tussen geboortegewicht en een ongezondere voedselinname in het latere leven.

Om onderscheid te maken tussen beide hypothesen analyseerden we een groep twee-eiige en eeneiige tweelingparen. We onderzochten of de helft van het tweelingpaar met het laagste geboortegewicht ook degene was met een ongezondere voedselinname dan de andere helft van het tweelingpaar. Twee-eiige tweelingen zijn grofweg voor 50% gelijk in hun genen, terwijl eeneiige tweelingen 100% genetisch gelijk zijn. De verschillen tussen twee-eiige tweelingen kunnen dus verklaard worden door zowel genetische factoren als omgevingsfactoren, terwijl de verschillen tussen eeneiige tweelingen alleen verklaard kunnen worden door verschillen in omgeving. Dus, door te kijken naar ver-

schillen binnen eeneiige tweelingparen wordt als het ware de invloed van genetische factoren geëlimineerd.

In ons onderzoek was de tweelinghelft met het laagste geboortegewicht gemiddeld ook degene die het meeste (ongezonde) verzadigde vet at op latere leeftijd. Deze uitkomst vonden we in zowel de twee-eiige als de eeneiige tweelingparen. Omdat met de observatie in eeneiige tweelingparen de invloed van genetische factoren is geëlimineerd, kunnen we concluderen dat het verband tussen laag geboortegewicht en meer inname van (verzadigd) vet onafhankelijk is van genetische invloeden. Blijkbaar speelt hier de omgeving die het geboortegewicht beïnvloedt een belangrijke rol. Verbetering van de groei in de baarmoeder zou dus inderdaad een positief effect kunnen hebben op de voedsel inname later in het leven.

DEEL 2

Onderzoek van eeneiige tweelingparen biedt een uitgelezen kans om ook de invloed van andere omgevingsfactoren te bestuderen. Omdat eeneiige tweelingen namelijk voor bijna 100% genetisch gelijk, kunnen alle verschillen binnen een paar alleen verklaard worden door verschillen in de omgeving. Wij onderzochten 16 unieke en speciaal geselecteerde eeneiige tweelingparen met een verschil in BMI om de invloed van omgevingsfactoren op overgewicht te bestuderen. Als de omgeving een belangrijke rol zou spelen bij de eerder beschreven veranderde beloningsgebieden in personen met overgewicht, dan zouden wij in ons onderzoek ook een duidelijk verschil in beloningsgebieden moeten vinden tussen de lichtere en zwaardere helft van de eeneiige tweelingparen. In ons onderzoek gebruikten we functionele MRI om de hersenactiviteit van beloningsgebieden te meten van de proefpersonen gedurende 2 verschillende taken met 'lekkere' voedselprikkels, namelijk het zien van voedselplaatjes en het krijgen van slokjes chocolademelk. Daarnaast verzamelden we gegevens van onder andere voedselinname en lichaamsbeweging in hun thuissituatie door middel van gestructureerde telefoongesprekken en bewegingsmeters.

Het gemiddelde verschil in BMI tussen de lichtere en zwaardere tweelinghelft van de onderzochte paren bedroeg uiteindelijk 4 kg/m^2, wat overeenkomt met bijna 12 kg in lichaamsgewicht. Ondanks dit grote verschil ontdekten wij tussen de lichtere en zwaardere helft van de tweelingen geen verschil in hersenactiviteit in beloningsgebieden tijdens het zien van voedselplaatjes of het krijgen van chocolademelk. Deze uitkomst suggereert dat er weinig invloed is van omgevingsverschillen op de veranderde hersenfuncties, maar juist wel van genetische factoren. Anders gezegd, de veranderde hersenreacties op voedselprikkels in mensen met overgewicht worden waarschijnlijk veroorzaakt door een genetische aanleg hiertoe. Deze bevindingen worden ondersteund door resultaten van andere recente onderzoeken.

Onze metingen van voedselinname en lichaamsbeweging lieten zien dat zwaardere personen vergeleken met hun lichtere tweeling-

helft gemiddeld meer vet aten en minder aan middelmatige tot stevige lichaamsbeweging deden. Bij deze bevindingen lijken omgevingsverschillen dus wel een belangrijke rol te spelen. Een verschil in totale hoeveelheid energie-inname (gemeten in kilocalorieën) hebben we niet ontdekt tussen de lichtere en zwaardere helften van de tweelingen. Een mogelijke verklaring hiervan is dat, zoals ook in andere voedingsonderzoeken steeds weer wordt ervaren, het exact meten van de hoeveelheid ingenomen voedsel erg moeilijk is.

DEEL 3

In dit laatste deel hebben we de invloed van genetische factoren op de beloningsreacties op voedselprikkels onderzocht. Hiervoor hebben we proefpersonen geselecteerd met ofwel een hoge ofwel een lage genetische risicoscore voor het hebben van overgewicht. Deze score is gebaseerd op het hebben van zogenoemde genetische variaties die, zoals gebleken uit eerdere zeer grote onderzoeken, sterk zijn geassocieerd met overgewicht en obesitas. Personen met veel van deze obesitas-variaties hebben meer risico op het krijgen van overgewicht dan mensen met weinig van deze obesitas-variaties. Van een groot deel van de proefpersonen in het Nederlands Tweelingenregister is de hoeveelheid aanwezige obesitas-variaties bekend. De sterkte van meerdere obesitas-variaties tezamen kan samengevat worden in een zogenaamde obesitas genetische risicoscore. Van de totale groep ingeschreven proefpersonen met beschikbare risicoscores (zo'n 10.000) hebben wij 60 individuele personen geselecteerd die ofwel een hoge ofwel een lage obesitas risicoscore hebben. Dus, door de beloningsreacties op voedselprikkels tussen beide groepen te vergelijken (door middel van de fMRI onderzoeken exact zo uitgevoerd als in deel 2 van dit proefschrift), kunnen we de bijdrage van genetische factoren onderzoeken.

In dit onderzoek hebben we zowel personen met een laag BMI als personen met een hoog BMI onderzocht. Hiermee is een oorzakelijke, genetische invloed te onderscheiden van een invloed die mogelijk het gevolg is van een hoog BMI zelf. Dus, als we veranderde hersenreacties zouden zien in personen met een hoog BMI, onafhankelijk van hun genetische risicoscore voor obesitas, dan pleit dit meer voor een effect als gevolg van een hoog BMI. Daarentegen, als de veranderde hersenreacties samengaan met een verhoogde genetische risicoscore voor obesitas, onafhankelijk van de huidige BMI, dan pleit dit meer voor een oorzakelijk, genetische invloed op de hersenreacties.

Ook in dit onderzoek hebben proefpersonen meerdere testen ondergaan, exact zoals in het onderzoek bij deel 2.

Personen met een hoog genetisch risico voor obesitas hadden een sterkere hersenactiviteit in een gebied dat betrokken is bij beloning (de zogenaamde orbitofrontale cortex) dan personen met een laag genetisch risico voor obesitas, tijdens het wachten op een slokje chocolademelk. Deze bevinding was onafhankelijk van het huidige BMI van de deelnemers, wat doet vermoeden dat het effect van veranderde

hersenreacties daadwerkelijk (via de genen) oorzaak is van een hoog BMI, en niet andersom. Anders gezegd, de ontdekte obesitas-variaties lijken het BMI te kunnen beïnvloeden door middel van een toegenomen beloningsgevoel en verlangen naar lekker eten of drinken, waardoor een grotere inname van dit eten of drinken kan ontstaan.

Tijdens het daadwerkelijk ontvangen van de chocolademelk werd een verhoogde activiteit in een ander beloningsgebied (de zogenaamde amygdala) gemeten in personen met een hoog BMI vergeleken met een laag BMI. Deze bevinding duidt meer op een effect dat een gevolg kan zijn van een hoog BMI, en suggereert dat er ook veranderingen in de hersenen kunnen optreden na het ontstaan van obesitas, waardoor als het ware een vicieuze cirkel ontstaat. Met andere woorden, personen met overgewicht gaan lekker voedsel nog meer waarderen waardoor mogelijk nog grotere voedselinname en gewichtstoename ontstaat.

Metingen van voedselinname lieten zien dat personen met een hoog genetisch risico voor obesitas meer dierlijke eiwitten aten dan personen met een laag genetisch risico. Mogelijk draagt inname van dierlijk eiwit dus via bepaalde genen bij aan overgewicht. Daarentegen vonden we geen duidelijke invloed van genetische factoren op veranderde lichaamsbeweging. Wel zagen we heel duidelijk dat zwaardere personen vaker inactief zijn (stilzitten) dan lichtere personen. Het is uit ons onderzoek niet duidelijk op te maken of deze inactiviteit een oorzaak of gevolg is van overgewicht.

CONCLUSIE

De resultaten van dit proefschrift tonen aan dat genetische factoren een erg belangrijke rol spelen bij veranderde hersenreacties in beloningsgebieden op voedsel-gerelateerde prikkels van buitenaf. Hierdoor ontstaat er een toegenomen verlangen en hunkering naar lekker, maar vaak ongezond eten, waardoor mensen met een erfelijke aanleg hiertoe geneigd zijn om vaker en meer te eten en daardoor aan te komen in gewicht. Met overgewicht hebben mensen een verhoogd risico op het krijgen van diverse ziektes, zoals suikerziekte (type 2 diabetes) en hart- en vaatziekten. Om de toename van obesitas in de wereld een halt toe te roepen, is het belangrijk om verleidelijke prikkels van buitenaf, zoals aantrekkelijke voedselreclames, en de makkelijke beschikbaarheid van ongezond eten, sterk te verminderen.