Obesity and Food Reward Regulation by the Brain

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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).

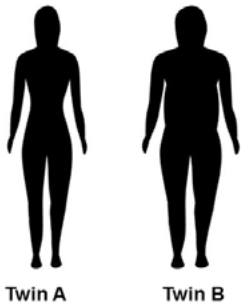

FIGURE 1
Monozygotic twin intra-pair differences model as used in of Part 2, comprising monozygotic twins with intra-pair BMI discordance.

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.

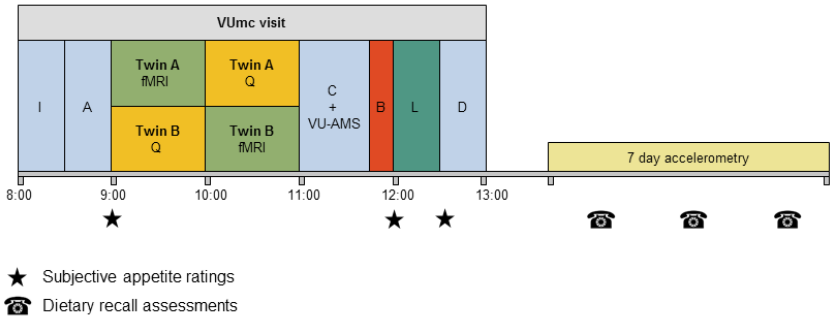

FIGURE 2
Overview of study procedures followed in Part 2 and 3. I, informed consents and standardized interview; A, anthropometry; fMRI, functional magnetic resonance imaging; Q, questionnaires; C, indirect calorimetry; VU-AMS, VU University ambulatory monitoring system; B, blood draw; L, lunch meal; 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.

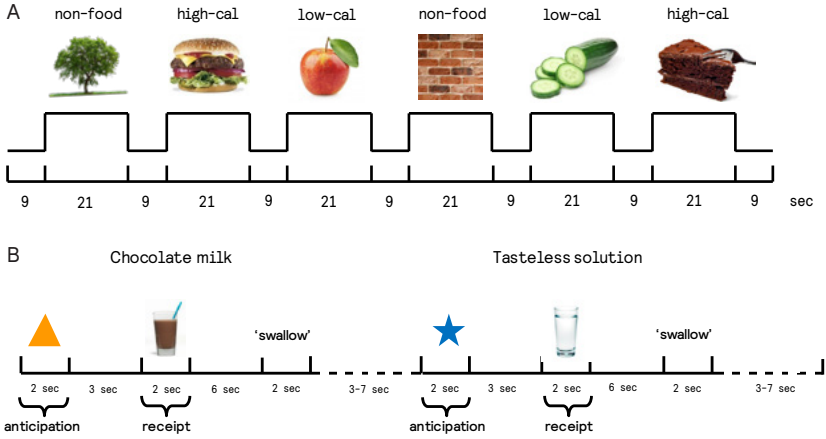

FIGURE 3
Example of timing of picture presentation during the food picture fMRI paradigm (A) and example of timing of cue presentation and stimuli delivery during the chocolate milk fMRI paradigm (B), as used in Part 2 and 3. Cal, calorie; sec, seconds.

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.

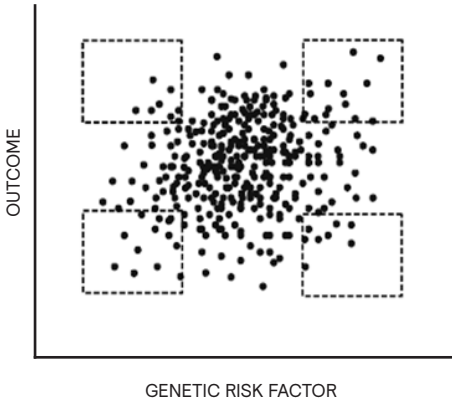

FIGURE 4
Four corner epidemiological study design as used in Part 3, in which individuals are selected based on either high or low values of a genetic risk factor and either high or low values on the outcome variable.

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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