

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/m², 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 heavier 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 obesity⁸. 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.

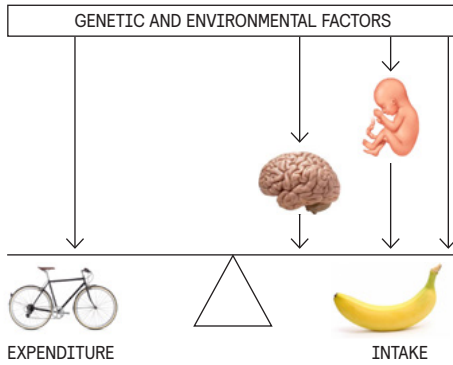


FIGURE 1
 The balance between energy expenditure (derived by basal metabolism and physical activity) and energy intake determines the amount of energy stored as fat. The balance can be influenced by physical activity, food reward regulation by the brain, and intrauterine effects. Each of these effects 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 inaccurate¹⁵ and, furthermore, that the long-term health effects of breastfeeding remain to be found¹⁶.

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-suppressing neuropeptides¹⁷. Furthermore, studies suggested a lower functioning of the leptin-mediated feedback loop between peripheral fat storages and the hypothalamus, possibly resulting from central leptin resistance¹⁸. 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 humans¹⁹.

Regardless the route of programming, evidence is growing that the intrauterine environment may exert its effects on health in later life through epigenetic mechanisms²⁰. Epigenetics refers to all modifications to genes other than changes in the DNA sequence itself, including DNA methylation and histone modifications²¹. Indeed, research in both animals and humans have reported altered methylation patterns of genes involved in appetite regulation in subjects exposed to intrauterine undernutrition²².

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 foods²³. 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 obesity²⁴. Bluntly, when comparing obese and lean subjects, previous neuroimaging studies observed increased reward responses to pictures of palatable food²⁵, whereas a lower striatal response was found in response to the actual receipt of a palatable taste stimulus²⁶. This altered reward functioning is thought to induce a) higher food craving and b) compensatory overeating, comparable to behaviours seen in drug addiction²⁷.

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 questionnaires²⁸, and b) brain reward responsiveness to visual food cues as measured with fMRI²⁹. 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 brain³⁰. 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 system⁸. These brain sites include the hypothalamus and limbic system, brain areas that play a pivotal role in the regulation of appetite and reward²⁴. 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 score³¹, 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 children³², altered food-cue responses in homeostatic and reward areas³³⁻³⁵, increased nucleus accumbens size and responsiveness to food advertisements in children³⁶, grey matter deficits in the prefrontal cortex³⁷ and altered functional connectivity in resting state networks,

including the salience network³⁸. 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 fMRI⁴¹. 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 network⁴². 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 participants⁴³⁻⁴⁵. 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 relation⁴⁷. This co-twin control model has been used to test a wide variety of associations, including the relation between smoking and lung cancer⁴⁸ and the effect of exercise behaviour on well-being⁴⁹. 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 SNPs⁸ 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 risk⁵⁴. 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 compensation⁵⁷, although evidence that support this theory is scarce⁵⁸. 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 encountered⁵⁹. 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 obesity⁶³. 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 underreport 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 method⁶⁴. 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 intake⁶⁵. 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 composition⁶⁶. 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 others⁶⁷. For instance, total energy intake was shown to be higher when participants consumed diets relatively high in fat than when they ate lower fat diets⁶⁸. Furthermore, the intake of sugar-sweetened beverages has consistently shown to be associated with long-term weight gain, even in a direct dose-response relationship⁶⁹. 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 manner⁷². 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 assessments⁷³. 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 health⁷⁴. 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 control⁷⁵. Negative effects of MUFAs and PUFAs have been reported before, since higher dietary fat intake provides concomitant higher caloric intakes⁷⁶. 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 frequencies⁷⁷, fried food⁷⁸ and sugar-sweetened beverages⁷⁹ 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 score⁸⁰. 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 BMI⁸³. 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 activity⁸⁴. 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 tissue⁸⁵, 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 gain⁸⁶. 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 level⁶¹. However, the relative contribution of physical activity is under considerable debate in the literature⁸⁷. 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 changed⁸⁸. On the other hand, scientists 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 century⁸⁴. 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 to 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 counts⁹² and less reported high-intensity activity⁶⁵ in heavier versus leaner co-twins. Another study failed to detect differences within MZ twins⁹³, 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 adolescents⁹⁴. 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 activity⁹¹. Moreover, evidence for the suggestion of a reverse causal relation was found by a recent study using Mendelian randomization⁹⁶. This technique aims at testing causality between traits, by using measured genetic variants as instrumental variables⁴⁵. 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 accelerometers⁹⁶. 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 muscularity⁹⁷. 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/m² and obesity if BMI >30 kg/m²) 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/m²)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 disappeared⁹⁹. 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 coefficient 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 studies²⁵. Since BMI is highly heritable but also variable in time¹⁰⁰, 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/m², comprised 2 twin pairs that were not strictly BMI discordant during the test visit (BMI differences of 0.71 and 1.02 kg/m²). 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 polygenic 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 outcomes³¹, such as mechanisms involved in food intake regulation. Previous studies already reported significant associations between polygenic obesity risk scores and satiety responsiveness in children³² 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 studies 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 obesity⁸. 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 homeostasis¹⁰⁴, physical activity levels¹⁰⁵, eating behaviours¹⁰⁶ and even brain reward responsiveness to food cues²⁵, 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 men²⁵, thereby optimizing power), 2) clinical (females are more likely to become obese than men¹⁰⁷, 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 pairs¹⁰⁰, 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 normal¹⁰⁹. 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 effects¹¹⁰. Apparently, leptin acts more like a starvation hormone rather than a satiety hormone¹¹¹, 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 defend 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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