Obesity: Exploring neural pathophysiological pathways and improving diagnostic strategies

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Obesity:
Exploring neural pathophysiological pathways
and improving diagnostic strategies

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Ter nagedachtenis aan Henriette Delemarre-van de Waal

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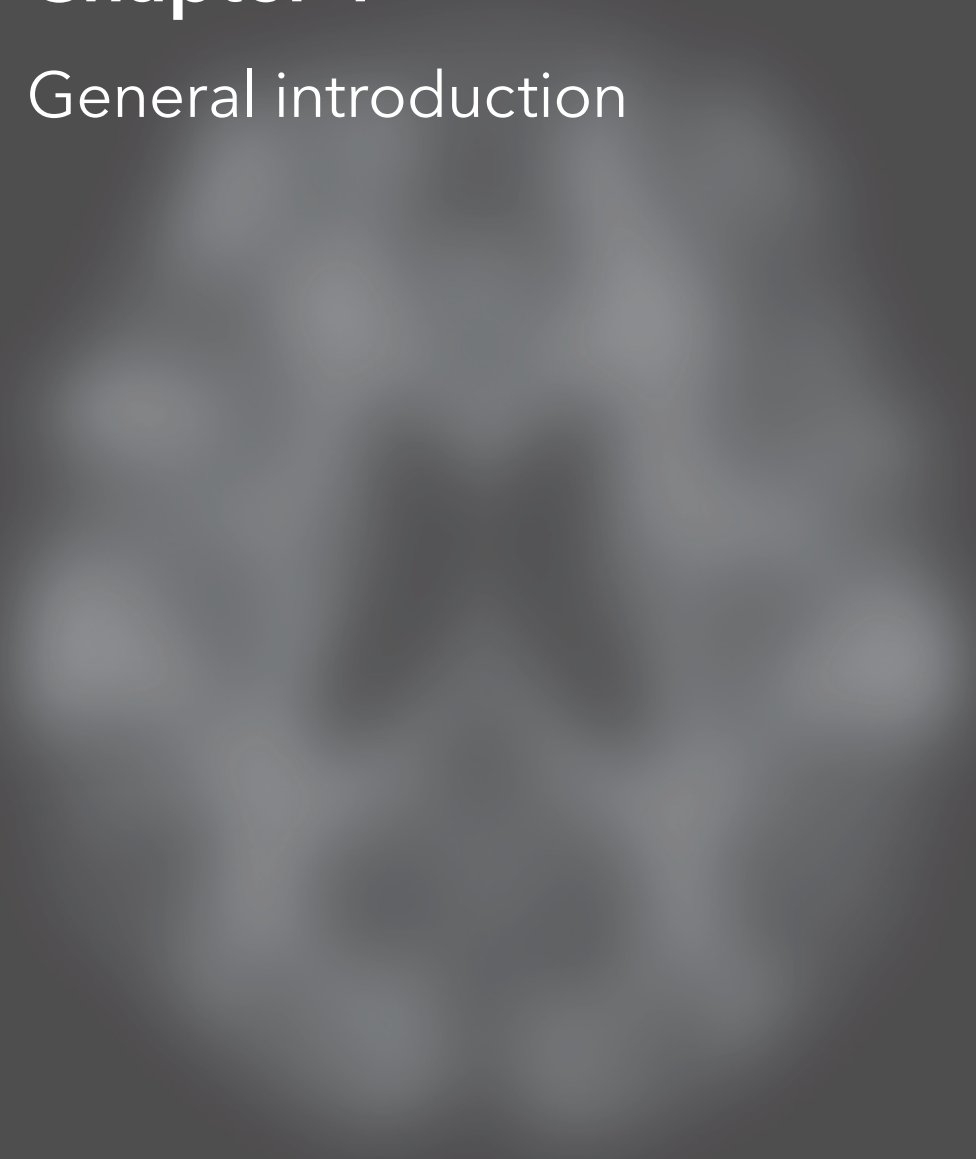
Part I

Chapter 1

General introduction



GENERAL INTRODUCTION

Globally, the number of overweight and obese adults has risen dramatically in the last three decades(1). This rise is accompanied by a rapid increase in childhood overweight and obesity, with prevalence rising in this period in both developed and developing countries(2,3). In clinical practice, subjects are usually defined according to body mass index (BMI). BMI is calculated by dividing the weight of a subject by its length in meters squared. Adults are considered overweight when BMI exceeds 25 kg/m^2 and are classified as severely overweight or obese when BMI is over 30 kg/m^2. For children, age and sex specific standard deviation curves are available, corresponding with the 25 and 30 kg/m^2 limits used in adults(4), which adjust for differences in body composition for children and adolescents at different ages. In the Netherlands, in 2016, 50,2% of adults (>20 years of age) were overweight of which 14,5% were obese(5). The most recent national growth study, performed in 2009, showed that approximately 14% of children in the Netherlands are classified as overweight and 2% of children are classified as obese(6). In both adults and children, these increases cause a rise in overweight and obesity associated diseases such as type 2 diabetes, metabolic syndrome, hepatic steatosis or psychological diseases, like depression(7-12). In turn, this leads to major rises in healthcare expenses. Globally, the annual costs of obesity are estimated to be 2 trillion US dollars(13). Therefore, reducing obesity prevalence and timely diagnosing obesity associated diseases are of the utmost importance. Recent meta-analysis on the success of treatment programs for obesity, however, show that the effect of lifestyle interventions are still limited(14, 15). This lack of success has been posed to result from an insufficient understanding of the complex endocrine, genetic, metabolic, neurological, psychological and societal factors known to be associated with obesity. Extending the knowledge on how these factors influence feeding behaviour and metabolism is crucial for further development of therapy. The second part of this thesis will be dedicated in investigating the associations of brain structure and function with obesity, feeding behaviour and genetics.

In recent years, an additional topic of debate, specifically in children, is the extensiveness of the diagnostic work-up of overweight and obese children. On the one hand, when and how to screen for underlying pathology such as hormonal, syndromic or monogenic diseases is an important task of clinicians. Assessment of growth, pubertal development and bone age are the cornerstone of this diagnostic workup. However, accelerated growth, puberty and bone age can be caused by obesity itself and can be difficult to discriminate from illnesses. On the other hand, clinicians are faced with the question when and how to screen for co-morbidities in overweight and obese children. The discussion is focussed on finding paediatric patients at high risk of developing co-

morbidity. The third part of this thesis will focus on tests that are used in the diagnostic work-up in overweight and obese children.

Neurological basis of feeding behaviour

In recent years, scientists have increasingly focussed on the role that the brain plays in feeding behaviour as a model for understanding obesity. From a perspective of feeding being needed to maintain energy balance, we now know that the hypothalamus and brainstem are key areas involved in maintaining this balance(16,17). These areas receive hormonal and neural input on energy balance from the digestive system (e.g. ghrelin, leptin and vagal nerve), endocrine organs such as the pancreas (e.g. insulin) and adipose tissue (i.e. leptin), and forward information to various cortical and subcortical brain structures, thereby signalling when energy is needed to maintain energy storage by inducing a feeling of hunger, and signalling when enough energy is taken in to maintain balance by inducing a feeling of satiety(17-19). Feeding behaviour, however, is not only driven by energetic demands, but also by the rewarding properties of foods. Seeing, smelling, or even thinking about food can trigger brain areas involved in reward and salience processing, resulting in an intensive drive to eat, even when there is no energetic demand. This is the result of activity in a complex of brain structures that ultimately makes us experience pleasure when we eat something palatable and, in sudden situations, make us feel hungry when we are, energetically speaking, satiated(20-25).

Central in reward processing, is dopamine release by neurons originating from the ventral tegmental area into subcortical and cortical brain structures, such as the nucleus accumbens, amygdala and medial prefrontal cortex(16). These neurons release dopamine when a person anticipates on receiving a rewarding stimulus, such as food. This anticipatory state can, in the case of food, be triggered by stimuli, such as seeing or smelling palatable foods, and leads to food seeking behaviour. When said food is taken in this again leads to dopamine release. Under normal conditions, these reactions are more pronounced in a hungry, than in a satiated state(21,22) and are under the influence of hormonal feedback from the digestive system through direct as well as indirect signalling via the hypothalamus (16,18,19,26). In addition, various cortical structures, mainly in the frontal lobe, are involved in exerting control over food intake, by interacting with the dopaminergic reward system and thereby controlling food intake(19,27-31). Furthermore, the visual cortex plays an important role in this system, since it is involved in signalling stimuli and, in collaboration with frontal areas, determines whether it draws the attention of an individual(32). In recent years, these brain systems have been increasingly investigated to find if they differ in persons with obesity.

Behavioural and neural aspects of obesity

Neuropsychological data have shown that people with obesity differ from their peers in a variety of traits. For instance, subjects with obesity show a reduced responsiveness to satiety signalling, an increased responsiveness to the rewarding value of food and perform poorer on tests of executive function (i.e. a collective term for self-regulatory processes such as inhibition, working memory and attention)**,** possibly reflecting a lack of control over feeding behaviour(30,33,34). This, combined with the rise of MRI as a modality to very precisely investigate both structure and function of the brain, led to an increase of imaging studies on the brains of obese subjects.

It was shown that people with obesity, children as well as adults, differ in volume and cortical thickness in areas known to be involved in reward and salience processing as well as executive function(30, 35-39). With the rise of functional magnetic resonance imaging (fMRI), which measures the blood oxygen level dependent (BOLD) signal, a derivative of brain activity, it was shown that people with obesity differ in activation, after seeing, smelling or tasting food cues, in areas implicated in reward and salience processing (e.g. nucleus accumbens, medial prefrontal cortex and amygdala), hunger and satiety signalling (e.g. the hypothalamus) and areas implicated in exerting control over behaviour (e.g. the dorsolateral prefrontal cortex and anterior cingulate cortex)(27-29, 40-43). Little is known, however, on said structural and functional differences in the brain, and how these relate to other factors related to obesity, such as behaviour and genetics. Furthermore, fMRI research recently evolved from investigating activation, to research on between structure communications, by means of connectivity analysis(44-48). This allows for the connectivity of networks of brain structures to be evaluated, as a proxy of communication. Although some knowledge has been assembled using connectivity analysis in obese adults, very little is known on connectivity differences in younger subjects with obesity. Therefore, part II of this thesis aims to investigate:

- Whether (variations in) brain volume and thickness correlate with executive function, appetitive and reward related behaviours and genetics in obese and lean subjects.
- Whether adolescents with and without obesity differ in resting state brain connectivity, with a specific focus on areas involved in hunger and satiety signalling, executive function and reward and salience processing.

Diagnostic work-up of overweight paediatric patients in clinical practice

The diagnostic work-up of overweight paediatric patients has been the subject of debate for years. The main goal of the diagnostic work-up is early identification of co-morbidity, such as impaired glucose tolerance, type 2 diabetes, hypertension and dyslipidemia and identification of underlying pathology. As underlying causes and co-morbidity of obesity, are relatively rare at young age, discussions on cost effectiveness of extensive

diagnostic protocols are ongoing. Therefore, further research in identification of patients at high risk for co-morbidity and underlying causes of obesity is warranted.

Diagnosing underlying pathology causing obesity has proven challenging. Assessment of growth, puberty and bone age are the cornerstone of the diagnostic work-up to find endocrine, syndromic or genetic causes of obesity. Children that are obese without underlying pathology, however, also show alterations in growth, puberty and bone age. On average, they show accelerated growth in height, earlier timing of puberty and advanced bone age(49-55). In this, they differ from endocrine causes of obesity, such as Cushing's disease and hypothyroidism, which usually show blunted growth in height(56,57). In contrast, it is much harder to use these parameters to differentiate lifestyle obesity from obesity caused by monogenic (e.g. MC4R mutations) and syndromic (e.g. overgrowth syndromes) causes of obesity, since they can also show accelerated height growth and bone age advancement(58). The problem of differentiating lifestyle obesity from other underlying causes of obesity with accelerated maturation might stem from the lack of understanding of the pathophysiological mechanism driving bone age advancement and accelerated growth. Therefore, unravelling these mechanisms is highly beneficial in the clinical practice.

Already in childhood, some children with obesity are affected by diseases caused by excess weight, such as glucose derailment (e.g. type 2 diabetes), hypertension, dyslipidaemia and fatty liver disease(7,10-12,59). Although incidences of diseases such as type 2 diabetes and hypertension are low and symptoms are mild compared to adults, they indicate an increased risk of developing serious co-morbidity in adulthood(10,60). For instance, presence of prediabetes (i.e. a relatively mild rise in glucose levels) at young age, was shown to be associated with a high chance of developing diabetes in early adulthood, especially when BMI increased(60). The combination of relatively low incidence of co-morbidity in overweight paediatric patients, combined with the high volume of young overweight subjects and severe consequences of co-morbidity at young age for adult life pose the challenge of finding a diagnostic strategy that is highly sensitive and specific, thereby being cost-effective.

Given the challenges in clinical practice considering the diagnostic workup over overweight and obese paediatric patients, the aims of part III of this thesis are:

- To investigate what drives accelerated growth and concomitant bone age advancement in childhood obesity.
- To investigate which parameters, available in everyday practice, predict the presence of impaired glucose tolerance in overweight and obese paediatric patients.

OUTLINE OF THE THESIS

Part I General introduction
Chapter 1 gives a brief overview of the topics investigated in this thesis.

Part II Behavioural, genetic and neural aspects of obesity
Chapter 2 describes the relationship between brain structure, appetitive traits and executive function in lean and obese adolescents.

Chapter 3 reports on differences in resting state activity between lean and obese adolescents.

Chapter 4 descibes the investigation of the relationship of the FTO risk-allele, rs9939609A, and volumes of reward related brain structures is reported.

Part III Diagnostic workup of overweight paediatric patients in clinical practice
Chapter 5 describes the relationship of various endocrine parameters with advanced bone age in obese children and adolescents.

Chapter 6 reports on markers for improving predictability of impaired glucose tolerance in overweight and obese children and adolescents.

Part IV General discussion and summary
Chapter 7 gives a general discussion on the results of the studies included in this thesis and provides perspectives for future research.

Chapter 8 summary

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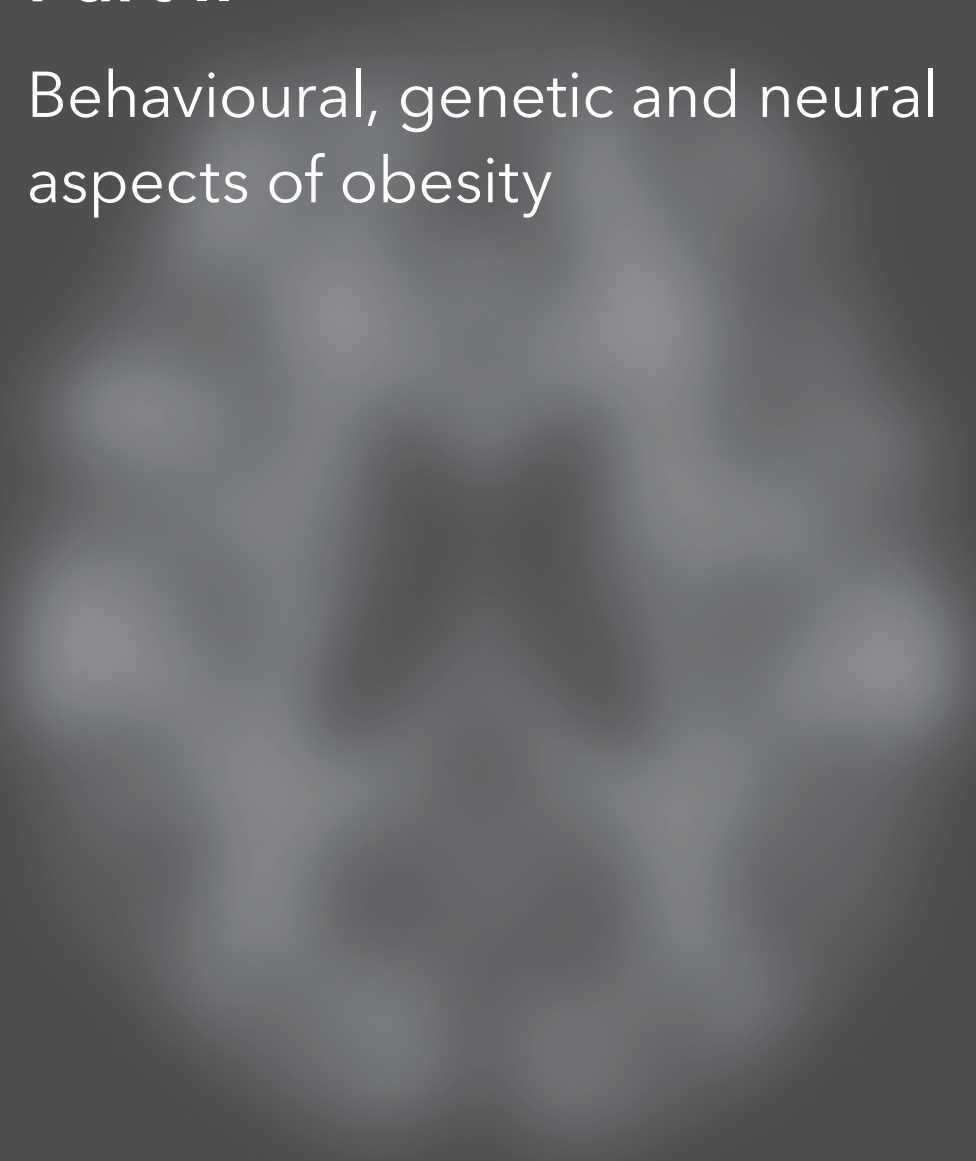
Part II

Behavioural, genetic and neural aspects of obesity



Chapter 2

Brain structure, executive function and appetitive traits in adolescent obesity

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ABSTRACT

Objective

Children with obesity show differences in brain structure, executive function and appetitive traits when compared to lean peers. Results of imaging studies, however, have been contradictory. Therefore, we investigate whether childhood obesity is associated with differences in brain structure and whether differences associate with executive function and appetitive traits.

Methods

A cross-sectional case-control study among 23 obese and 19 lean control subjects, aged 12-16 years, was conducted. Brain structures were measured by MRI using Voxel Based Morphometry, cortical thickness and subcortical volumes. Appetitive traits were measured by the Child Eating Behaviour Questionnaire and executive function by a Stop Signal Task and a Choice Delay Task. Associations between brain structures and appetitive traits or executive function tests were investigated using linear regression analysis.

Results

Obese adolescents had larger volumes of the pallidum; 1.78 mL (SE 0.03, p=0.014), when compared to controls; 1.65 mL (SE 0.02). In the obese group, increased pallidal volume was positively associated with the ability to delay reward in the Choice Delay Task (p=0.012).

Conclusion

The positive association of pallidal volumes and Choice Delay Task found in obese adolescents supports the hypothesis that the pallidum plays an important role in executive dysfunction described in obese children.

INTRODUCTION

A global rise in childhood obesity was seen in the last decades(1), leading to adverse outcomes, such as type II diabetes and metabolic syndrome(2). Early intervention in children is key to prevent adult disease. Obesity treatment, however, often fails because complex mechanisms in satiety, craving and reward systems play a role. Unravelling these mechanisms is currently the subject of research.

Neuroimaging studies investigating brain structure in childhood obesity suggest that overweight and obese children and adolescents differ from lean peers in subcortical reward related regions. Reduced(3), as well as increased(4) hippocampal volumes, increased size of the pallidum(3), and reduced volume of thalamus(5) have been described. Furthermore, in literature on obesity in adults, increased volume of the amygdala(6), thalamus(7), caudate nucleus(7), reduced volume of the putamen(8) and increased(6) as well as reduced(9) hippocampal volumes have been described in relation with a higher BMI. Additionally, in childhood obesity, as well as in literature on adult obesity, reductions in volume and cortical thickness, in regions involved in executive function (a collective name for self-regulatory processes) and reward processing, have been reported(4, 7, 9-11). Recent research suggests that reductions in brain volume and cortical thickness found in obese children might be caused by presence and duration of the metabolic syndrome(11).

Previous research on the behaviour of children with obesity has shown that they exhibit differences in executive function when compared to lean peers. More specifically, children that are overweight or obese are posed to have deficits in inhibitory capacity, possibly indicating they have problems to refrain from eating(12). Furthermore, they are reported to have problems delaying gratification(12). In other words, they tend to choose small, immediate rewards, such as high-calorie foods, over larger delayed rewards, such as a healthier weight. Moreover, childhood obesity is frequently associated with differences in appetitive traits when compared to lean peers(13). Research in this field shows that children with obesity are less sensitive to satiety signals, have increased responsiveness to food cues and find food more rewarding than lean peers(13). However, knowledge on structural neurological correlates possibly underlying this behaviour, is limited.

Although some neuroimaging studies have included a measure of executive function in their design, very few studies have specifically investigated inhibitory control, and none have specifically investigated delay of gratification. One study found that volumes of the dorsolateral prefrontal cortex were negatively correlated with inhibition measured by a Stroop task in lean but not in obese children(4). Another study found that reduced grey matter volume in the orbitofrontal cortex correlated with increased disinhibition in lean, but not obese children, as measured by an eating questionnaire(10). Moreover, to

our knowledge, the relationship between appetitive traits and brain structure has never been investigated in obese children.

In conclusion, there is limited knowledge on brain structure in adolescent obesity and the results of previous studies are contradictory. Furthermore, little is known about the correlation between brain structure and behaviour associated with childhood obesity. Therefore, we conducted a study to further elucidate this matter using MRI. The aim is to study whether adolescents with obesity differ in brain structures related with executive function and reward processing and whether differences, if found, associate with executive function and appetitive traits in obese and lean children.

METHODS

Study population

25 adolescents with obesity and 19 lean controls, as defined by the IOTF criteria(14), all of Caucasian origin, aged 12-16 years, were included in the study. Subjects with obesity were recruited from our outpatient obesity clinic, control subjects were recruited from their peers and by local advertising. The aim was to have comparable groups on age, sex and education level. We excluded subjects with obesity caused by endocrine disorders (e.g. hypothyroidism) subjects with type II diabetes and subjects that used psychofarmaca (e.g. methylphenidate), or reported IQ <80. Furthermore, we excluded participants with MRI contra-indications; e.g. irremovable metal objects. The study protocol, P10.105, was approved by the medical ethics committee of the Leiden University Medical Centre. The study was registered in the Dutch Trial Register (www.trialregister.nl) with number NTR2531. All participants and their parents provided written informed consent.

Procedure

Subjects visited the hospital on two separate occasions. On the first visit, both neuropsychological tasks and a dummy scan session were performed. Parents were asked to fill out the CEBQ. Height and weight were measured using a calibrated stadiometer and scale. During the second visit the MRI scan was made as part of a scan protocol which also included an fMRI study (to be reported elsewhere). The mean amount of time between the first and second visit was 11 days (range 1-65 days).

MRI acquisition and pre-processing

MRI acquisition

Participants were scanned on a 3T MRI scanner using a 32-channel head coil (Philips Healthcare, Best, The Netherlands). Acquisition parameters were a repetition time (TR) of 9.8 ms; echo time (TE) 4.6 ms; AP field of view (FOV) 224 x 224 mm; measured voxel size 1.20 mm isotropic; flip angle 8°. Total scan duration was 5 min.

MRI pre-processing

The analysis of MRI scans was performed with FSL 5.0 (FMRIB Software Library)(15) and Freesurfer(16). FSL-VBM was used to assess cortical thickness using a voxel-based morphometry analysis. As a first step, images were brain extracted using the Brain Extraction Tool. Subsequently, FAST4 (FMRIB's Automated Segmentation Tool) was used to carry out tissue-type segmentation. In the next step of the analysis grey matter partial volume images were aligned to standard space using FLIRT (FMRIB's Linear Image Registration Tool), followed by nonlinear registration. Averaging these images resulted in a study-specific template. The native grey matter images were then non-linearly re-registered to this template. Correction for local contraction or expansion was applied by dividing partial volume images by the Jacobian of the warp field. The segmented modulated images were then smoothed with a 3 mm isotropic Gaussian kernel. Ultimately, a voxel-wise general linear model was applied with non-parametric testing, using a correction for multiple comparisons across space (False Discovery Rate: 5%).

To specifically assess subtler differences in cortical thickness possibly missed by VBM analysis a cortical reconstruction and segmentation in frontal and limbic regions of interest was performed using the Freesurfer analysis suite in conjunction with the Desikan-Killiany atlas. The Freesurfer suite is an extensively validated software tool for performing automated cortical morphometric measurements on T1-weighted anatomical MRI-scans. The orbitofrontal cortex, anterior cingulate cortex, insular cortex, frontal pole and superior, medial and inferior frontal gyrus were the regions of interest for our analysis. If Freesurfer provided sub parcelations of regions of interest; these were averaged and then averaged between hemispheres.

FIRST (FMRIB's Integrated Registration and Segmentation Tool) was applied to assess volumes of the subcortical twin structures amygdala, caudate nucleus, hippocampus, nucleus accumbens, pallidum, putamen and thalamus. This tool first registers all images to MNI152 templates, followed by fitting models for all different structures to the images. Finally, it performs boundary correction for volumetric outputs. FSLstats was used to calculate these volumetric outputs. Volumes of twin structures were averaged between hemispheres.

All segmentations performed were visually checked to determine if segmentation was performed correctly. In two male obese subjects segmentation was not performed correctly. Therefore, they were excluded from further analyses, leaving 23 obese and 19 lean subjects.

Metabolic syndrome parameters

Presence of metabolic syndrome was added to our analysis, since it was shown that it might be a confounder in the relationship between obesity and structural differences(11). There are various definitions of metabolic syndrome. We used the IDF definition of ≥ 3 of the following parameters: increased waist circumference, lowered HDL-cholesterol, increased levels of triglycerides, hypertension or elevated fasting glucose(17). Additionally, we used HOMA-IR (> 3.4)(18) as a metabolic syndrome parameter instead of fasting glucose, since it has been posed to better correlate with presence of type II diabetes. To assess these parameters we used age and sex specific cut-offs for waist circumference sds (> p95)(19), HDL-cholesterol (< p5)(20), triglycerides (> p95)(20) and tension(21).

Neuropsychological evaluation

Child Eating Behaviour Questionnaire (CEBQ)

The CEBQ is a widely used questionnaire to evaluate various eating behaviour traits in children(22). We used the Dutch translation of this questionnaire(23). We used the 'enjoyment of food', 'food responsiveness', 'emotional overeating', 'desire to drink' and 'satiety responsiveness' subscales, frequently reported altered in childhood obesity (13), in this study.

Neuropsychological tasks

Stop Signal Task: Inhibitory control was tested using a Stop Signal Task (SST). This task tests the ability of a subject to withhold a pre potent response and is extensively used to measure inhibitory control(24). In this task a square is presented on either the left or right side of the screen of a laptop computer. The subject has to indicate whether this so-called target was projected on the left or right side of the screen by pushing the right or left shift-button as fast as possible. In 25% of the trials the target is followed by an auditory signal. In these trials subjects have to refrain from reacting to the target. The delay between appearance of the target and onset of the auditory signal varies as a result of successful or unsuccessful inhibition. The standard delay is 250 ms and after successful inhibition of the response the delay is increased by 50 ms. After unsuccessful inhibition, the delay period is decreased by 50 ms. The mean stop signal reaction time (SSRT) is the measure of interest in this study. SSRT is calculated by: "mean reaction time" - "mean stop signal delay". Longer SSRT's indicate that participants have poorer response inhibition.

Choice Delay Task: We investigated the ability to delay gratification with a Choice Delay Task (CDT). In this task participants are asked to choose twenty times between either one point or two points by clicking one of these choices on a screen. When they chose one point they received the point immediately, when they chose two points there was a delay of 30 sec. Subjects were told that with these points, they could get a gift and that a higher score increased the variety and size of gifts they could choose from. The number of large rewards was used for analysis. The task was preceded by five 'practice rounds' to familiarize participants with the extent of the delay when choosing the large reward.

Statistical analysis

All analyses were performed using SPSS 20.0.0. Student's t-tests and chi square tests were used to explore the baseline characteristics. Mann-Whitney U tests were used to analyse between group differences in task- and questionnaire-based measures and are displayed as median and interquartile range. Between group differences in cortical thickness and subcortical volumes were analysed using general linear modelling, correcting for age and sex, and are displayed as means with standard error. We regarded the comparison significantly different if the p-value was smaller than 0.05. We report on data uncorrected for multiple comparison as well as False Discovery Rate(FDR)-corrected p-values. Furthermore, we investigated the relationship between found volumetric differences and metabolic syndrome parameters by linear regression analysis using single metabolic syndrome parameters, as well as metabolic syndrome as a whole, as an extra independent. Additionally, linear regression modelling was used to assess whether structural differences between lean and obese children associate with task- and-questionnaire based measures. Task- and questionnaire-based measures were entered as dependent variables, sex, age and cortical thickness/subcortical volumes as independents.

RESULTS

Baseline characteristics

Table 1 shows the baseline characteristics of the study groups and metabolic syndrome parameters in the obese group. There were no significant differences between groups on the confounding factors age, sex and education level.

Table 1 Baseline characteristics

		Lean (n = 19)	Obese (n = 23)	p-value
Female n (%)		9 (47)	14 (61)	ns
Age in years (SE)		14.5 (0.4)	15 (0.2)	ns
Education level n (%)	**Lower**	12 (63)	18 (78)	ns
	Middle	3 (16)	1 (4)	
	Higher	4 (21)	4 (17)	
BMI sds (SE)		-0.08 (0.2)	3.54 (0.1)	<0.001
Hypertension (%)			9 (39)	
HOMA-IR > 3.4 (%)		-	8 (35)	-
Waist circumference > p95 (%)		-	16 (70)	-
HDL-cholesterol < p5 (%)		-	5 (22)	-
Triglycerides > p95 (%)		-	7 (30)	-
Average number of metabolic syndrome parameters per subject (SE)		-	1.96 (0.3)	-
Presence of metabolic syndrome (%)		-	10 (43)	-

Table 1. Abbreviations: ns: not significant; BMI: Body Mass Index; HOMA-IR: homeostatic model assessment of insulin resistance; HDL: High-Density Lipoprotein.

Neuropsychological tasks and questionnaires

The results of the between group comparison of neuropsychological tasks and questionnaires are shown in Table 2. Obese compared to lean children showed marginally lower scores on the CEBQ subscale 'satiety responsiveness' and significantly increased scores on the subscales 'food responsiveness' and 'emotional overeating' and had marginally higher scores on 'enjoyment of food'. Furthermore, obese subjects performed marginally lower on the choice delay task.

Table 2 Results of neuropsychological tasks and questionnaires

	Lean (n = 19)	Obese (n = 23)	p-value
Task-based measures			
Mean stop signal reaction time ms (IQR)	256 (199-305)	253 (221-300)	0.90
Choice delay task number of large rewards (IQR)	14 (7-20)	10 (5-12)	0.07
Questionnaire measures			
CEBQ Satiety responsiveness (IQR)	2.6 (2.2-2.9)	2.3 (1.6-2.7)	0.063
CEBQ Food responsiveness (IQR)	1.8 (1.6-2.2)	3.3 (2.4-3.7)	< 0.001
CEBQ Enjoyment of food (IQR)	3.3 (3.0-3.6)	3.7 (3.2-3.9)	0.06
CEBQ Emotional Overeating (IQR)	1.6 (1.3-2.0)	3.0 (2.0-3.6)	<0.001
CEBQ Desire to Drink (IQR)	2.3 (1.7-2.8)	3.0 (2.0-3.4)	0.17

Table 2. Data presented are median with interquartile range. Abbreviations: IQR: interquartile range, CEBQ: Child Eating Behavior Questionnaire.

Analysis of between group differences in brain structure

Voxel based morphometry showed no between group differences in cortical thickness. The between group analysis of cortical thickness in regions of interest showed no differences between groups (Table 3). Comparison of subcortical volumes showed that obese compared to lean subjects had larger pallidal volumes corrected for age, gender and FDR. Furthermore, at an uncorrected threshold, they had larger amygdalar volumes.

Table 3 Cortical thickness and subcortical volumes in lean and obese adolescents

	Lean (n=19)	Obese (n=23)	p-value	p-value FDR corrected
Cortical thickness(mm)				
Orbitofrontal cortex (SE)	2.90 (0.02)	2.83 (0.04)	0.21	0.49
Anterior cingulate cortex (SE)	3.20 (0.05)	3.17 (0.03)	0.78	0.97
Frontal pole (SE)	2.96 (0.06)	3.00 (0.07)	0.97	0.97
Inferior frontal gyrus (SE)	2.93 (0.03)	2.92 (0.04)	0.86	0.97
Middle frontal gyrus (SE)	2.75 (0.02)	2.71 (0.04)	0.61	0.85
Superior frontal gyrus (SE)	3.18 (0.03)	3.12 (0.04)	0.41	0.72
Insular cortex (SE)	3.25 (0.03)	3.23 (0.04)	0.57	0.85
Subcortical volumes(mL)				
Amygdala (SE)	1.24 (0.03)	1.35 (0.04)	0.03	0.21
Hippocampus (SE)	3.91 (0.06)	3.89 (0.09)	0.93	0.97
Nucleus Accumbens m (SE)	0.50 (0.02)	0.54 (0.02)	0.12	0.42
Caudate Nucleus (SE)	4.02 (0.09)	3.90 (0.07)	0.31	0.62
Putamen (SE)	5.01 (0.09)	5.24 (0.10)	0.19	0.49
Pallidum (SE)	1.65 (0.02)	1.78 (0.03)	0.001	0.014
Thalamus (SE)	8.03(0.14)	8.25 (0.14)	0.11	0.42

Table 3. P-values presented are derived from general linear modeling corrected for age and sex. Abbreviations: SE: standard error, ns: not significant,, FDR: false discovery rate.

Relationship of pallidal and amgydalar volumes and metabolic syndrome parameters

There was no relationship between presence of metabolic syndrome parameters or metabolic syndrome diagnosis and amygdalar or pallidal volumes in the obese group.

Analysis of pallidal and amygdalar volumes with appetitive traits and executive function

Analysis of the association between pallidal volumes and behavioural measures revealed a significant association between pallidal volumes and the number of large rewards in the Choice Delay Task in the obese group (p=0.012, adjusted for age and sex, Figure 1). We did not find this association in lean participants. Furthermore, we found a marginally significant negative relation between pallidal volumes and Stop Signal Reaction Time (p=0.055, adjusted for age and sex). So, increased pallidal volumes associated with better inhibitory control and better ability to delay gratification within the obese group. These results were independent of the presence of metabolic syndrome. No associations were found between pallidum volume and appetitive traits or between the amygdala volume and any of the behavioural measures.

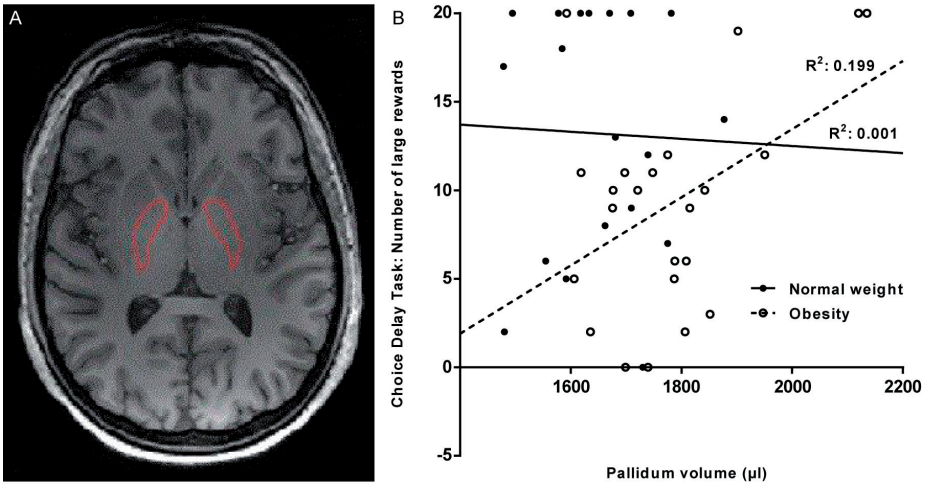

Figure 1

A: Example of the pallidum ROI in an obese participant; B: scatterplot of the relationship between pallidum volume and choice delay task performance in obese and lean subjects.

DISCUSSION

The results of this study show increased volume of the pallidum and amygdala in obese compared to lean adolescents, corrected for age, sex and FDR. Additionally, pallidal volumes were positively associated with inhibitory control and the ability to delay gratification. We did not find any differences between groups in the analyses of cortical regions, nor did we find an association between the neural structures and the metabolic syndrome.

The finding of increased pallidal volumes in obese compared to lean children is in accordance with recent findings in a group of 6-8 year old Mexican children(3). Furthermore, functional neuroimaging studies have shown that activation in the pallidum by high-calorie food images predicted BMI(25) and that reward sensitivity is associated with activation in the pallidum to appetizing food images(26). The ventral pallidum has been identified as possibly the most important structure in generating 'liking'-signals ('liking' is defined as an objective behavioural or neural hedonic reaction to a stimulus(27)). Therefore, our finding of increased pallidal volume might signal an abundance of endocannabinoid and opioid neurons responsible for 'liking', possibly contributing to increased intake of palatable food. Interestingly, we did not find an association with possible behavioural correlates of 'liking' as measured by the CEBQ (e.g. enjoyment of food), but with measures of executive function. Although it has been suggested that differences in the pallidum in obese compared to lean children probably signal differences in reward processing(3), the pallidum is well known for being involved in inhibitory control by inhibiting activity of various reward structures via GABA-ergic inhibition(28). Furthermore, a recent study poses that neurons originating from the globus pallidus (i.e. dorsal pallidum) might directly influence frontal cortical areas implicated in inhibitory control(29), showing another possible mechanism how the pallidum might influence executive function. In conclusion, our data suggest an important role of the pallidum in the executive dysfunction described in childhood obesity.

The increased amygdalar volume found in obese compared to lean adolescents in this study is novel in childhood obesity. Increased size of the amygdala in adult obesity has been previously reported by our group(6). We have to stress, however, that this has to be replicated because a false positive result due to multiple comparison cannot be excluded. In the functional neuroimaging literature, the amygdala has been frequently implicated in food processing. For example, the amygdala is activated when processing food compared to non-food cues in healthy weight adolescents(30), and activation is positively correlated with reward sensitivity(26). Therefore, we pose that the increased amygdalar volume found in obese compared to lean adolescents might indicate an increased reward sensitivity, leading to increased intake of highly palatable foods. It has to be noted, however, that amygdalar volume did not associate with any of the predefined behavioural measures.

Another interesting finding of this study is the lack of difference between groups in any of the predefined cortical regions. This is in contrast with some studies, that found reductions in the orbitofrontal cortex(4, 10, 11) and anterior cingulate cortex(11). This result is possibly explained by the relatively low prevalence of metabolic syndrome in our sample (< 50%), since recent studies suggest that reductions in cortical thickness might be caused by the harmful influence of the metabolic syndrome on the brain(11). Another possible explanation is that our sample is relatively young compared to most

cohorts describing cortical differences in obese compared to lean subjects. Therefore, the exposure of the obese adolescents in our cohort to adverse effects of metabolic syndrome parameters might have been too short to lead to cortical differences.

There are strengths to consider in regard to this study. An important strength of our study is that the subjects in our cohort were equal on educational level, which ensures that differences found are not confounded by possible differences in intellectual capabilities. Furthermore, we corrected our findings on brain differences for multiple comparisons, showing that the differences found in pallidal volume are robust.

A possible limitation of the study is the choice to measure appetitive traits by means of a questionnaire instead of by a food laboratory measure. We argue, however, that within this age group, known food laboratory measures of behavioural traits are highly influenced by adolescents exhibiting socially desirable behaviour. Furthermore, we did not assess metabolic syndrome parameters in the lean group, making it impossible to correct the brain differences found between groups for this possibly confounding factor. However, in the regression model, exploring the effect of variance in pallidal volumes, we did correct for presence of metabolic syndrome parameters and did not find an effect.

In conclusion, the results of this study show increased pallidal and amygdalar volumes in early adolescent obesity. Furthermore, we have shown an association between pallidal volumes and executive function in the obese group, indicating an important role of the pallidum in executive dysfunction described in childhood obesity.

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Chapter 3

Differences in functional brain connectivity between adolescents with and without obesity in a fed condition

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ABSTRACT

Objective

Adolescents with obesity differ from their lean peers in executive function, salience and reward processing. For these functions, it was shown that adults with obesity differ in functional brain connectivity with the corresponding salience and executive control networks, and with specific brain structures such as the amygdala and hypothalamus, even in an unstimulated state. Here, we investigate whether functional brain connectivity in adolescents with obesity differs from their peers in these areas.

Methods

Seventeen adolescents with and 15 without obesity between the age of 12-16 years, matched for age, sex, and education were included in the study. Resting state fMRI was performed in a fed condition. Whole brain connectivity with the salience network, default mode network and executive control was assessed. Additionally, seed based analysis was used to investigate connectivity of the amygdala, pallidum and hypothalamus.

Results

Compared to lean subjects, subjects with obesity had increased connectivity of the salience network with the occipital pole and reduced overall connectivity in the executive control network, specifically within the lateral occipital cortex.

Conclusion

In an unstimulated and fed state, connectivity of visual processing areas networks involved in executive control and salience processing are altered in adolescents with obesity.

INTRODUCTION

Obesity at young age is a global problem (1, 2), leading to significant morbidity in early life, including hypertension, dyslipidaemia and glucose metabolism abnormalities (3). The complex, multifactorial nature of obesity makes it hard to treat. Over the last decades, genetic, social, neural and behavioural factors have been shown to have a complex interplay in the pathogenesis of obesity. In paediatric and adolescent populations, behavioural as well as neural correlates of obesity have been identified. One of the key behavioural aspects of this group is that they tend to eat in the absence of hunger, leading to overconsumption of food (4, 5). In this respect, impaired executive functions, such as inhibitory control and delaying gratification (6), increased sensitivity to the rewarding value of food (7), and decreased responsiveness to satiety signalling (8) have been posed to underlie overconsumption.

Various differences in cerebral structure and function between youngsters with and without obesity have been described: Reduced volume and thickness of the anterior cingulate and orbitofrontal cortex (9), areas implicated in self-regulatory processes, were found in children with obesity. In contrast, volumes of areas related to reward processing, such as the pallidum, were larger (9, 10). On functional level, areas associated with reward processing, such as the amygdala, were more active while viewing food-related items in children with obesity than in lean children (11-13). Reward structures in the limbic system remained responsive to stimulation with food pictures after a meal in children with obesity, but not in lean children (11). This latter finding implicates that activity of the limbic system is continuously altered in children with obesity, even in a fed state and when not triggered by food stimuli.

During fasting a stronger functional connectivity, determined with resting state fMRI, between the hypothalamus and the medial prefrontal cortex (14), dorsal striatum (14) and insula (15) was found in adults with obesity. Moreover, in persons with obesity, specific alterations in the default mode network have been described: Connectivity of the posterior cingulate gyrus, lateral inferior parietal cortex and precuneus was increased, whereas connectivity in the anterior cingulate cortex was decreased (16, 17). The default mode network is active during wakeful rest and when reflecting on one's emotions and envisioning the future, and is mainly inactive when a person is actively engaging in an activity (18). In addition to alterations in the default mode network, increased network connectivity in the salience network was found (19), a network involved in integration of homeostatic and salient signals. These changes in both default mode and salience network indicate that, in adults with obesity, brain areas involved in reward- and salience processing, as well as areas involved in self-regulation are altered in a non-food-triggered state. Although data on resting state connectivity in children and adolescents

with obesity could aid in understanding the behaviour of this group, these data are limited, warranting a more extensive exploration.

To investigate potential changes in functional brain connectivity in the homeostatic and limbic system, and in the executive, salience and default mode network at rest in youngsters, we performed resting state fMRI in a case control study design, comparing adolescents with and without obesity, in a satiated state.

METHODS

Study population

Subjects with obesity, all Caucasian, were recruited from the obesity clinic in the Willem-Alexander Children's Hospital, according to the criteria stated by the International Obesity Task Force (20). Lean control subjects were recruited from lean peers of the obese subjects, and via local advertising. We included subjects with obesity between the age of 12-16 years. The lean control group was matched on age, sex and education level. The educational system in the Netherlands from the age of 12 onward is subdivided into lower ('VMBO'), middle ('HAVO') or higher education ('VWO') (21). Exclusion criteria were endocrine disorders, use of medication possibly affecting brain activity, mental retardation and wearing of irremovable metal objects potentially disturbing the fMRI signal. The medical ethics committee of the Leiden University Medical Center approved the study (P10.105) and all children and their parents provided written informed consent. The trial was registered under number NTR2531 (www.trialregister.nl).

Procedure

Subjects visited our centre on two separate occasions. During the first visit, weight and height were obtained using a calibrated scale and stadiometer. Subjects were familiarized with MRI and MRI noises to reduce anxiety by use of a mock scanner. During the second visit, the fMRI scan was made in a satiated state, within two hours after breakfast. Hunger scores were obtained using a 9-point Likert scale.

MRI acquisition

All subjects were scanned on a Philips Achieva 3.0 T scanner with an eight-channel SENSE head coil (Philips Healthcare, Best, The Netherlands). The resting state scans were acquired during a 7 min scan using a T2*-weighted gradient-echo echo-planar imaging (EPI) scan (acquisition parameters: EPI factor 35, repetition time (TR) 2.2 s, echo time (TE) 30 ms, flip angle 80°, Field of View (FOV) 220 x 220 mm, measured voxelsize 2.75 mm isotropic, slice gap 0.275 mm). A high resolution T1-weighted MRI-scan (TR 9.8 ms; TE 4.6 ms; AP FOV 224 x 224 mm; measured voxel size 1.20 mm isotropic; flip angle 8°) and

a high resolution T2*-weighted MRI-scan (TR of 2.2 s; a TE 30 ms; AP FOV 220 x 220 mm; measured voxel size 2 mm isotropic; flip angle 80°) were made for registration purposes.

MRI pre-processing and statistical analysis

Datasets were pre-processed and analysed using FSL version 5.0.9 (FMRIB's Software Library, Oxford University) (22). Anatomical images and high-resolution functional images were brain-extracted using FSL's BET-tool (23). The functional datasets were corrected for head motion using FSL's MCFLIRT-tool (24) and smoothed using a 5 mm full-width-half-maximum kernel. Registration matrices were computed for linear and nonlinear registration of the functional data to MNI-space via each subject's respective high-resolution functional and anatomical images (25). Secondary motion artefact removal was performed using ICA-AROMA (0.3 beta), a data-driven method that employs independent component analysis and a pre-trained classifier working on a robust set of theoretically motivated features (26). The resulting denoised functional datasets were brain-extracted with the FSL-BET tool and registered to MNI-space using the previously computed registration matrices. Low frequencies were removed using a highpass filter with a 0.01 Hz cut-off value.

Statistics and processing

Differences in functional connectivity between adolescents with and without obesity, were evaluated for the default mode network, executive control network and salience network using the templates published by Smith et al (27). Analyses of connectivity of the hypothalamus, amygdala and pallidum were based on anatomically defined seed regions. The hypothalamus was chosen as a region of interest (ROI), given its central role in hunger and satiety sensing and the differential connectivity shown in adult obese subjects (12). The bilateral amygdala was chosen as a region of interest, given the differential activation and connectivity in previous fMRI studies in subjects with obesity (12, 15). Furthermore, the pallidum was chosen, since we recently showed that subjects with obesity compared to lean subjects had larger pallidum volume and that pallidum volume correlated with executive function (10).

The binary seed ROI were manually created on the MNI (Montreal Neurological Institute) template. The hypothalamus ROI was defined using two spheres with a radius of 2 mm at MNI coordinates: x = ±4, y = -2, z = -12, as previously identified (28) and used (29) by Küllmann et al. The bilateral pallidum ROI was defined by two spheres (which were slightly asymmetrical between left and right) with a radius of 3 mm at MNI coordinates x= -18, y= -4, z = -2 (left) and x = 20, y= -4, z= -2 (right). The bilateral amygdala ROI was defined by two (also slightly asymmetrical) spheres with a radius of 4 mm at MNI coordinates x = -24, y = -4, z = -18 (left) and x = 26, y = -4, z = -18 (right).

Connectivity analysis were performed using FSL's *dual regression* tool (30). Using this tool, subject-specific connectivity maps were generated for each network template and seed region. Each network template or binary seed region was evaluated in a separate model that also included two nuisance regressors based on conservative CSF and WM templates. In the first analysis, voxel wise Z-value measures of network connectivity were derived from the standardized parameter estimates that were produced during stage 2 of the dual regression procedure. Using these Z-value maps, the mean within-network Z-value was computed for each network by averaging the values of the voxels within the network template, resulting in a measure of within-network connectivity per participant for each network. These measures were subsequently compared between groups using an independent samples Mann-Whitney- U-test.

In a second analysis, the subject-level connectivity maps for each seed and network of interest, that resulted from stage 2 of the dual regression procedure, were tested for group-level differences, by means of a nonparametric two-sample whole brain T-test as implemented in FSL's *randomise* tool (31) at a family wise error (FWE) corrected p-value of 0.05.

RESULTS

Baseline characteristics

A total of 37 subjects, 19 with obesity and 18 normal weight controls, were included in the study. The baseline characteristics of the cohort are shown in Table 1. There were no significant differences in sex, age and education level. Furthermore, there was no significant difference in reported hunger. Five subjects were excluded from the analysis for various reasons. Two male participants of the normal weight group were excluded due to scanner related artefacts on their T1 weighted scans. One female participant from the group with obesity was excluded due to excessive motion. One female participant in the normal weight group had an unexpectedly large artefact due to a remnant of a dental brace. One male subject with obesity had unexpected symptoms of claustrophobia, which led to abortion of the study protocol. Therefore, a total of 32 subjects (17 with obesity and 15 lean) were included in the analysis.

Table 1 baseline characteristics

		Lean (n = 15)	Obese (n = 17)	p-value
Sex female (%)		7 (47)	9 (56)	0.73
Age (sds)		15.0 (1.5)	15.0 (1.2)	0.92
BMI (sds)		18.6 (1.9)	35.1 (5.8)	<0.001
BMI-sds (sds)		-0.2 (0.8)	3.5 (0.5)	<0.001
Education level	**Lower (%)**	11 (73)	13 (76)	0.36
	Middle (%)	3 (20)	1 (6)	
	Higher (%)	1 (7)	3 (18)	
Hungerscale prescan (sds)		2.8 (2.1)	2.4 (1.6)	0.53

Table 1. *Abbreviations: BMI: body mass index, sds: standard deviation score.*

Functional connectivity of the predefined seeds and networks

Functional connectivity, within network as well as whole brain connectivity of the network, was evaluated of the default mode network, the executive control network and the salience network. Additionally, connectivity was evaluated with three anatomically defined seed regions: the hypothalamus, the bilateral amygdala, and the bilateral pallidum.

Description default mode network

For the default mode network, for entire group; with and without obesity combined, whole brain connectivity maps are shown in Figure 1A. Positive connectivity was found of the default mode network with frontal regions including the frontal pole, the superior frontal gyrus, orbitofrontal cortex and anterior cingulate cortex, in the thalamus, caudate nucleus and nucleus accumbens, and a posterior cluster, mainly covering the precuneous, lateral occipital cortex and occipital pole. Negative connectivity was found with the insular cortex, the primary and supplementary motor cortices and two smaller clusters in the lateral occipital cortex. No differences were found between lean subjects and those with obesity, neither in whole brain connectivity of this network nor in connectivity within this network.

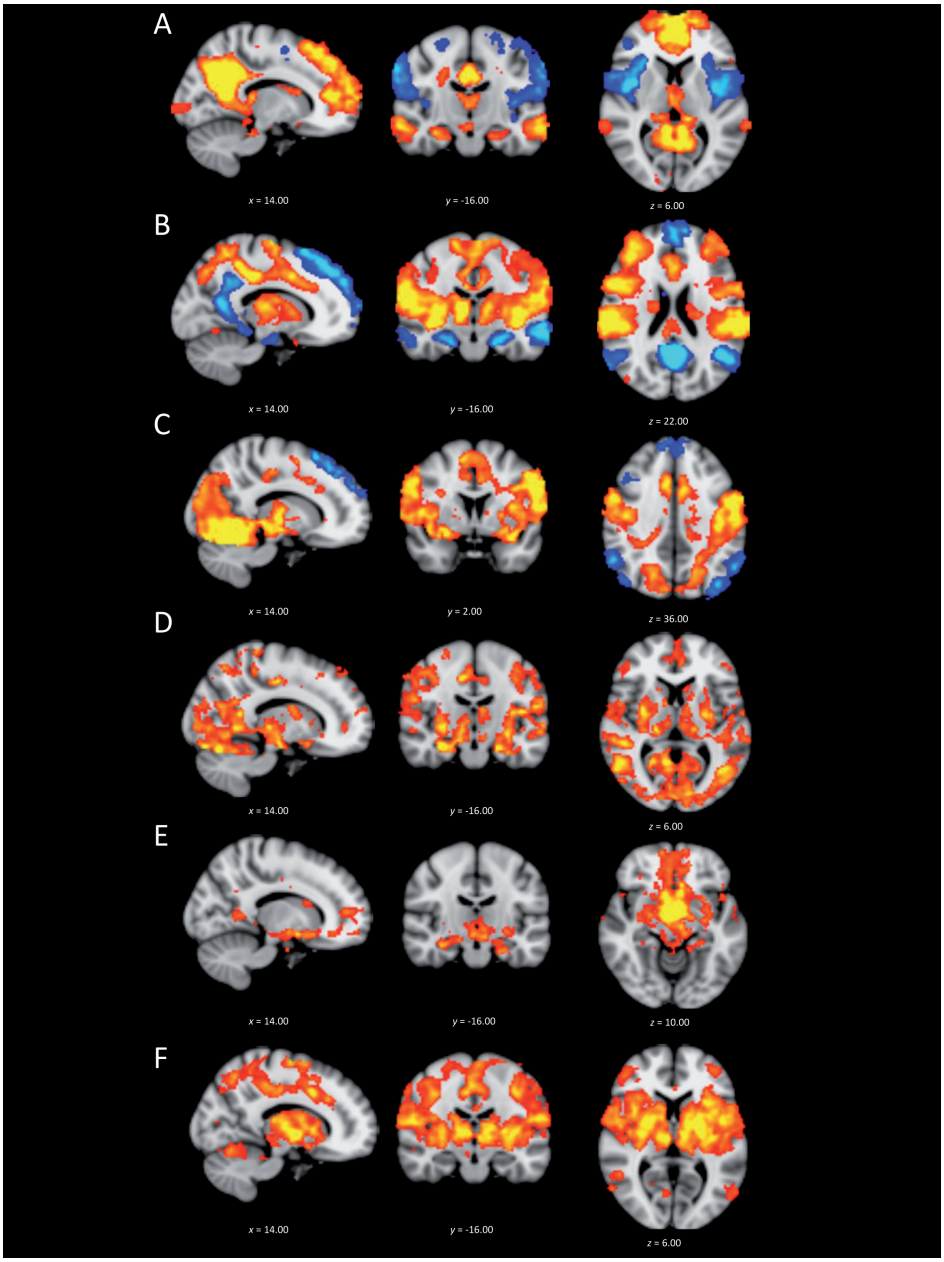

Figure 1. *Connectivity of the predefined seeds and networks in the cohort, from left to right in the X, Y and Z axis. A: Default Mode Network, B: Executive Control Network, C: Salience Network, D: Amygdala, E: Hypothalamus, F: Pallidum. Positive connectivity is thresholded at Z-score > 4 and shown in yellow/red; negative connectivity is thresholded at Z-score < -4 and shown in blue.*

Description executive control network

Whole group connectivity maps for the executive control network are shown in Figure 1B. Positive connectivity was found with frontal structures including the frontal pole and anterior cingulate cortex. In addition, positive connectivity was found with the insular cortex and in the putamen and caudate nucleus. Positive connectivity was also found with posterior areas, including the middle temporal gyrus, superior parietal lobule, lateral precuneal cortex and lateral occipital cortex. Negative connectivity of the network was found with the superior frontal gyrus and orbitofrontal cortex, and posterior clusters comprising the posterior cingulate gyrus, medial precuneus and lateral occipital cortex. Subjects with obesity showed reduced connectivity within the executive control network (Table 2). More specifically, dual regression analysis showed that adolescents with obesity had reduced connectivity within the executive control network in two adjacent clusters in the right lateral occipital cortex (Figure 2A, Table 3).

Table 2 Analysis of within network connectivity strength

	Lean (n = 15)	Obese (n = 17)	p-value
Salience Network	1.22 (0.73-1.61)	1.05 (0.78-1.53)	Ns
Default Mode Network	1.53 (1.04-1.70)	1.37 (1.15-1.60)	Ns
Executive Control Network	1.57 (1.25-1.38)	1.33 (1.08-1.54)	0.044

Table 2. *Comparison of the median of Z-scores(with interquartile range) between lean and obese participants. Abbreviations: ns: not significant.*

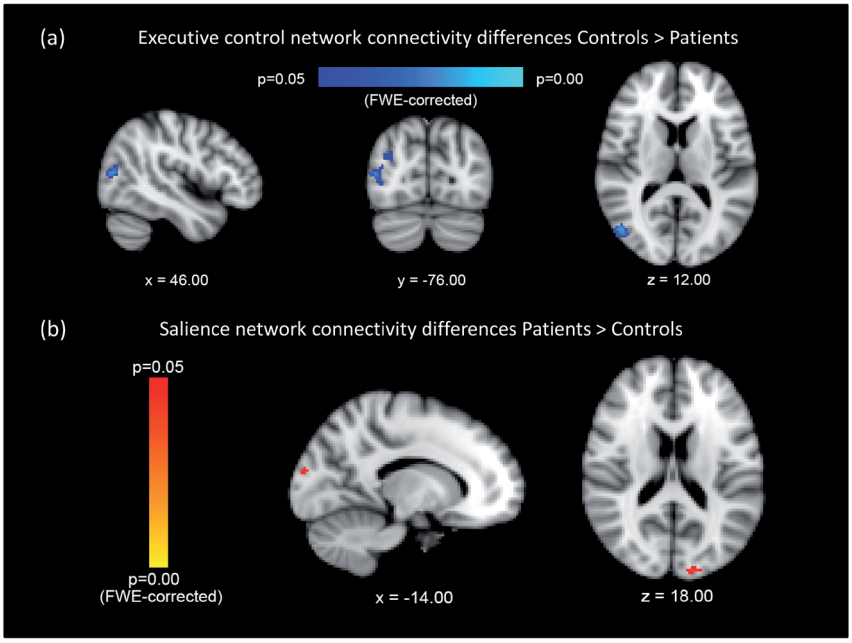

Figure 2. *Regions differing in connectivity between lean and obese subjects. A: the Executive Control Network; B: the Salience Network*

Table 3 Regions exhibiting significant connectivity with predefined networks and seeds

	Hemisphere	X	Y	Z	Cluster size in voxels	T-value	p-value
Lean > obese							
Executive control network							
LOC inferior division	R	48	-72	12	99	5.67	0.017
LOC superior division	R	34	-80	30	85	4.49	0.033
Obese > lean							
Salience network							
Occipital pole	L	-12	-94	18	31	4.96	0.042

Table 3. *regions of significant connectivity. X, Y and Z represent the MNI-coordinates of the voxel of maximal T-value of the cluster. T-value represents the maximal T-value of the cluster. Abbreviations: R: right, L: left. LOC: lateral occipital cortex.*

Description salience network

Salience network whole group connectivity maps are shown in Figure 1C. The network had positive connectivity with lateral clusters, including the insula, pre- and postcentral gyri, and posteriorly with the lateral occipital cortex, occipital pole and the lingual gyrus. Furthermore, positive connectivity with subcortical structures was found with the amygdala, putamen, thalamus, pallidum and brain stem. Negative connectivity was found with the frontal pole and superior frontal gyrus, and an occipitally with the lateral occipital cortex. No differences were found analysing within network connectivity between subjects with and without obesity. Whole brain connectivity analysis showed increased connectivity of the salience network with a cluster in the left occipital pole in subjects with obesity (Figure 2B, Table 3).

Amygdala

In whole group analysis, the bilateral amygdala was positively connected to frontal regions including the frontal pole, anterior cingulate cortex, inferior and middle frontal gyrus and orbitofrontal cortex (Figure 1D). Within the basal ganglia it was connected with the putamen, hippocampus and thalamus. In addition, connectivity was found with the middle temporal gyrus, the lingual gyrus, and two large areas in the occipital lobe covering the occipital pole and the lateral occipital cortex. There were no differences in connectivity between subjects with and without obesity for these structures.

Hypothalamus

Connectivity mapping in of the medial hypothalamus in the entire group (Figure 1E) showed connectivity to the anterior cingulate, paracingulate gyrus, nucleus accumbens, pallidum and brainstem. There were no differences in connectivity between subjects with and without obesity for these structures.

Pallidum

The pallidum whole group connectivity map (Figure 1F) showed connectivity with frontal regions including the frontal pole, anterior cingulate cortex, orbitofrontal cortex and the inferior, medial and superior frontal gyri, the insular cortex, putamen, thalamus and amygdala. Further connections included the lingual and supramarginal gyrus and superior parietal lobule. There were no differences in connectivity between subjects with and without obesity for these structures.

DISCUSSION

In this study we show that, in a fed state, adolescents with obesity have lower resting state connectivity within the executive control network in the lateral occipital cortex, and have higher resting state connectivity of the salience network with the occipital pole. We did not find any differences in resting state connectivity of the amygdala, pallidum, hypothalamus, and default mode network in whole brain analysis.

Our finding of adolescents with obesity showing lower within network connectivity in the executive control network, specifically in the lateral occipital cortex is of interest. The lateral occipital cortex is a structure involved in higher level visual processing, object recognition and visual attention (32), and has gained attention as an area involved in obesity pathophysiology. Obesity has been associated with reductions in volume and cortical thickness in the lateral occipital cortex (33-35). Although in normal weight subjects this area is activated by visual stimuli of high calorie foods (36) in subjects with obesity the lateral occipital cortex shows hypo-activity in response to food pictures (37). Furthermore, while viewing rewarding food and non-food items, subjects with obesity showed reduced connectivity in a frontal occipital network including the lateral occipital cortex (38). In addition, during this task, the lateral occipital cortex was connected to fewer nodes and connections were weaker in subjects with obesity compared with lean subjects (39). It has been suggested that rewarding stimuli influence reward processing by recruiting more attentional resources towards these rewarding cues in people with obesity compared with lean people (39) with frontal brain regions exerting less control over these processes (40). Considering this hypothesis, it is of interest that a meta-analysis of studies on attention deficit hyperactivity disorder specifically found

lower activation in the lateral occipital cortex during executive function tasks (41), showing that this brain area is indeed hypoactive during executive functions in subjects with known deficits in executive function. Our results are in agreement with these data, and extend the literature by showing that, even at young age, obese subjects show decreased connectivity within a network involved in executive function, specifically in the lateral occipital cortex, even in an unstimulated state and in the absence of hunger. This poses the question whether this finding signals a constant vulnerability to food cues drawing attention and concomitantly triggering executive areas insufficiently, even when satiated, ultimately leading to overconsumption. To further investigate this, study designs aimed at investigating connectivity during executive function combined with food cues are needed. Furthermore, it would be interesting to apply subliminal food cues in the designs of these studies to investigate how food cues that obese subjects perceive without being aware (e.g. extensive food cues in the public domain) influence this connectivity.

In addition to our findings of altered connectivity in the executive control network, obese subjects showed increased connectivity between the salience network and the occipital pole compared with lean participants. The salience network is a network that integrates salient with homeostatic signals; and alterations within this network have repeatedly been associated with obesity in adults, but also in children with Prader Willi syndrome (19, 40, 42). Previously it was shown that within the salience network, connectivity of the putamen was increased in subjects with obesity (19). In this paper it was proposed that this might signal an imbalance between autonomic and reward processes. Children with Prader Willi syndrome showed a generally increased connectivity within the salience network, indicating widespread alterations in the processing of internal as well as external food cues (42). However, this is a very specific group of subjects suffering from obesity with very specific feeding behaviour. Therefore, generalizations of these findings to other subjects with non-syndromic obesity, should be made with caution. Most interestingly, and in line with our current findings, are recent data comparing connectivity of adults with and without obesity during a visual task with high-calorie, low-calorie and non-food pictures (40). It was shown that the connectivity of the salience network with a temporal-visual network, which includes the occipital pole, was increased in subjects with obesity. It did, however, not vary with caloric content of the food pictures, possibly indicating a more general preferential processing of visual food cues in subjects with obesity(40). Our data complement these findings by showing that, already at young age and in a fed- and resting state, obesity leads to an increased connectivity between a primary visual area and the salience network. It can be argued that this indicates a constantly present vulnerability to visual food cues being processed with priority.

We did not find differences in connectivity neither in the seed-based analysis of the amygdala, hypothalamus or pallidum, nor in default mode network connectivity between groups. However, it has been shown that in adults with obesity, connectivity in the default mode network, hypothalamus and amygdala are altered (14-17). It is likely that these apparent differences are caused by differences in the level of brain development between the adolescent and a fully developed, adult brain. Moreover, our participants were scanned in a fed state, whilst other studies were almost exclusively performed in a fasting state. Possibly, these structures and networks show more pronounced differences in connectivity in obese subjects during fasting.

A particular strength of the study is the matching on education level, since it reduces bias possibly introduced by differences in intellectual capabilities. A limitation of this study is the limited sample size, introducing type-2 errors, although this is usually the case in these studies as they are labour intensive. Furthermore, we were specifically interested in connectivity during the absence of hunger, so the results are limited by the fact that it only investigated subjects in a fed state. To gain insights in connectivity differences related to hunger, a fasting study could provide complementary insights. Additionally, we did not prospectively acquire data on metabolic parameters in our cohort, making it impossible to investigate whether our differences might be correlated with these parameters.

CONCLUSION

This study shows that, at young age, obesity is associated with a decreased network connectivity within the executive control network, specifically in the lateral occipital cortex, and increased connectivity of the salience network in the occipital pole. This shows that even at rest and while satiated visual areas are differentially connected to areas implicated in salience- and reward processing and executive control in adolescents with obesity, possibly reflecting a constant vulnerability to food cues.

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Chapter 4

Association between the fat mass and obesity-associated gene risk allele, rs9939609A, and reward-related brain structures

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ABSTRACT

Objective

Recently, the fat mass and obesity-associated gene (*FTO*) has been identified as a genetic risk factor for developing obesity. The underlying mechanisms remain speculative. Recently, SNPs within *FTO* have been associated with brain atrophy in frontal and occipital regions, suggesting that *FTO* might affect body weight through cerebral pathways. Behavioural studies suggested a relationship between *FTO* and the reward-related behavioural traits. We therefore investigated the relationship between the *FTO* risk-allele rs9939609A and volumes of reward-related brain structures.

Design and Methods

492 Dutch individuals (56% males, age: 70 to 82 years) participating in the PROSPER-study underwent a 3D-T1-w MRI to assess the volumes of reward-related brain structures (e.g. amygdala, nucleus accumbens) and of grey matter and white matter. Linear regression analysis was performed to test for the association of subcortical and cortical structures with rs9939609A.

Results

rs9939609A is associated with lower volumes of the nucleus accumbens (p=0.03) and trended towards lower cortical grey matter volumes (p=0.08). This association is independent of gender, age, and BMI, FDR corrected.

Conclusion

The *FTO* risk-allele is associated with lower nucleus accumbens volumes, suggesting that the higher body weight of risk-allele carriers might be due to changes within reward-related brain structures.

INTRODUCTION

For several years it has been known that alleles within *FTO* are associated with increased BMI. In 2007, Frayling et al. reported a risk allele, rs9939609A, within *FTO*, which was strongly associated with increased BMI. The per-A allele odds ratio for obesity was 1.31 (95% CI 1.23-1.39; $P = 6 \times 10^{-16}$) and for overweight 1.19 (95% CI 1.13-1.24; $P = 2 \times 10^{-17}$), compared to the T allele(1). In the following years, numerous studies confirmed the association between this and other single nucleotide polymorphisms (SNPs) within the *FTO* gene and BMI in various populations(2-4).

The *FTO* gene encodes a 2-oxoglutarate-dependent nucleic acid demethylase(5). Cell models have shown that overexpression of FTO reduces ghrelin mRNA N6-methyladenosine and concomitantly increases ghrelin peptide levels. As such, it appears to play an important role in hypothalamic signalling of homeostatic hunger and satiation by influencing ghrelin production(6, 7). Furthermore, intronic variants within *FTO* have been shown to regulate expression of *IRX3* in the hypothalamus, a gene well known for its role in the regulation of body mass and body composition(8). These findings partially explain the earlier reported changes in eating behaviour such as impaired satiety responsiveness and increased food intake in carriers of risk alleles(9-12).

Studies investigating the association between *FTO* and feeding behaviour traits, however, have also shown an association between *FTO* risk alleles and traits such as loss of eating control, emotional control, self-regulation and symptoms of ADHD. These traits have been found to be under the influence of reward-related brain structures(13-15). This poses the question whether *FTO* also influences reward-related brain structures.

The association between BMI and brain volume has been well described, especially in regions associated with the regulation of taste, reward, and eating behaviour(16-25), such as the hippocampus(22, 24), amygdala(20, 24), thalamus(22), (orbito)frontal structures(18, 21-23), and putamen(21). Moreover, a recent study indicated that *FTO* SNPs were also associated with regional brain volume changes using a tensor-based morphometric approach(17).

The major aim of the present study was to investigate whether the *FTO* risk allele, rs9939609A, is associated with specific volume differences in the reward-system.

METHODS

Study population

Data were drawn from the nested MRI sub-study of the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER). Of the 1,095 eligible Dutch participants for PROSPER, 554 were randomly selected for MRI. Of these 554 subjects, 492 (56% males) had a suc-

cessful MRI (i.e. successful high resolution T1-w MRI scan, without excessive artefacts) and *FTO* gene analysis. Subjects undergoing an MRI exam did not differ in terms of age, gender, medical history and cardiovascular risk profile from the entire group.

PROSPER is a double-blind, randomized, placebo-controlled trial aimed at assessing the effect of pravastatin therapy on vascular events in subjects with vascular disease or at risk of vascular disease (26) in the elderly. Inclusion criteria for PROSPER were: age 70 to 82 years; total cholesterol 4.0-9.0 mmol/L; or stroke, transient ischemic attack, myocardial infarction, arterial surgery, or amputation for vascular disease >6 months before study entry; or ≥1 of the following risk factors for vascular disease: current smoker, hypertension (currently receiving drug treatment), or known diabetes mellitus or fasting blood glucose >7 mmol/L. (24).

All data including MRI were recorded at baseline of PROSPER. The mean BMI at the time of intake was 26.8 kg/m^2 (standard deviation=3.6). Systolic and diastolic blood pressure were recorded in a sitting position using a fully automatic electronic sphygmomanometer (Omron M4, Kyoto, Japan). None of the participants had been diagnosed with Minimal Cognitive Impairment (MCI) or Alzheimers Disease (AD) prior to the first visit as was established by extensive questioning of their medical history. Furthermore, subjects underwent a Mini Mental State Examination (MMSE)(27), and were excluded from PROSPER if MMSE <24 at baseline. All participants gave written informed consent.

MRI acquisition

All analyses in this study were based on 3D-T1-weighted gradient-echo MRI scans obtained at 1.5T (Philips Medical Systems, Best, The Netherlands). Acquisition parameters were: repetition time (TR) = 30 msec; echo time (TE) = 4.6 msec; flip angle = 30°; slice thickness = 1.5 mm; 120 slices; no interslice gap; field of view (FOV) = 220 x 220 mm, and a matrix size of 256 x 256.

MRI post-processing and analysis

All MRI scans were analysed using different tools of FSL (FMRIB Software Library)(28, 29). Whole brain volume, grey and white matter volumes were calculated using the FSL- SIENAX tool (Structural Image Evaluation, using Normalization, of Atrophy). SIENAX starts by extracting brain and skull images from the single whole-head input data. The brain image is then affine-registered to MNI152 space, using the skull image to determine the registration scaling. This is done in order to obtain the volumetric scaling factor, to be used as a normalization for head size. Tissue-type segmentation with partial volume estimation is performed to calculate the total volume of brain tissue, including separate estimates of grey matter and white matter volumes.

To assess local differences in cortical thickness, FSL-VBM, a voxel-based morphometry analysis was performed. First, structural images were brain-extracted using the Brain

Extraction Tool. Subsequently, tissue-type segmentation was carried out using FAST4 (FMRIB's Automated Segmentation Tool). The resulting gray matter partial volume images were aligned to MNI152 standard space using the affine registration tool FLIRT (FMRIB's Linear Image Registration Tool), followed by nonlinear registration. The resulting images were averaged to create a study-specific template, to which the native gray matter images were non-linearly re-registered. To correct for local expansion or contraction, the registered partial volume images were modulated by dividing them by the Jacobian of the warp field. The modulated segmented images were smoothed with an isotropic Gaussian kernel with a sigma of 3 mm. Finally, a voxel-wise general linear model was applied using permutation-based non-parametric testing, correcting for multiple comparisons across space (False Discovery Rate: 5%).

To determine the volume of the brain stem and the volumes of the subcortical twin structures nucleus accumbens, amygdala, caudate nucleus, hippocampus, pallidum, putamen and thalamus, FMRIB's Integrated Registration and Segmentation Tool (FIRST) was used. FIRST starts by registering all images to MNI152 templates. It then fits models for all different structures (meshes) to the images, and finally applies boundary correction for the volumetric output.

Genetic analysis

The genotyping of the *FTO* rs9939609 polymorphism [intronic nucleotide substitution T>A; reported global minor allele frequency 0.34, [http://www.ncbi.nlm.nih.gov/projects/SNP] was carried out using real-time PCR with TaqMan SNP Genotyping Assays from Applied Biosystems (Foster City, California, USA) at the Tufts University in Boston. The minor allele frequency in our cohort was 0.38, which is in accordance with various European populations reported previously(1, 3, 30). Genotyping success rate for the entire cohort was 99.1%. χ^2-testing was performed to see whether our population was within Hardy-Weinberg equilibrium for rs9939609.

Statistical analysis of baseline data

If not otherwise stated, data are presented as mean with standard error (SE). Medical history was established via a questionnaire prior to the first visit. Chi-square tests were used to test for differences in gender, history of hypertension, history of vascular disease, history of myocardial infarction (MI) or history of diabetes mellitus (DM) between groups.

Statistical analysis of relationship between genotype and reward related brain structures

For all continuous variables regression analysis was used. A linear regression model was used to assess the association of various brain tissue volume measurements with TT, TA and AA subgroups. In model 1, we corrected for age and gender. To exclude the effects

of BMI on brain structures, we additionally added BMI as a covariate (model 2). We corrected for multiple comparisons using a False Discovery Rate approach for both models. For all statistical analyses, SPSS software for windows (version 20.0.0.1; SPSS) was used.

RESULTS

Genetic testing

The *FTO* rs9939609 polymorphism's minor allele (A) frequency (MAF) was 0.38. The *FTO* TT variant was found in 185 of the 492 subjects (38%), the TA variant was found in 236 of the 492 subjects (48%), and the AA variant was found in 71 subjects (14%). Hardy-Weinberg equilibrium (HWE) testing using a χ^2-test showed that rs9939609 was in HWE (HWE = 0.09, $P = 0.760$).

Table 1 shows all demographic data for the various *FTO* genotypes. No difference in age and gender was found between groups. Subjects homozygous for the *FTO* risk allele A demonstrated a significant higher BMI, corrected for age and gender, compared with subjects with the T allele. No difference in either systolic nor diastolic blood pressure was found between groups. There was also no difference in history of hypertension, vascular disease, MI or DM between the three groups.

Table 1. Baseline characteristics

	FTO rs9939609TA polymorphism			
	TT (n=185)	**TA (n=236)**	**AA (N=71)**	**P-value**
Gender female n(%)	82 (44)	107 (45)	29 (41)	0.80
Age (years)	74.6 (0.2)	74.4 (0.2)	74.4 (0.4)	0.77
BMI (kg/m2)	26.6 (0.3)	26.6 (0.2)	27.9 (0.5)	0.015
Systolic BP (mmHg)	157.2 (1.6)	157.4 (1.3)	159.9 (2.8)	0.65
Diastolic BP (mmHg)	85.5 (0.8)	85.9 (0.7)	87.1 (1.3)	0.55
History of vascular disease n(%)	75 (40)	97 (41)	35 (49)	0.41
History of MI n(%)	23 (12)	29 (12)	7 (10)	0.83
History of hypertension n(%)	108 (58)	151 (64)	47 (66)	0.38
History of DM n(%)	31 (17)	47 (20)	7 (10)	0.14

Table 1: Abbreviations: MI= Myocardial infarction, DM= Diabetes Mellitus, BMI=Body Mass Index, BP=Blood pressure.

Relationship reward-related brain structures and FTO

Table 2 shows the volumetric data for all *FTO* mutation variants. Of all subcortical structures the nucleus accumbens demonstrated a gradual 12% decrease in volume from TT, 0.59 ml, to AA, 0.52 ml (p = 0.04 adjusted for age and sex, and FDR corrected for multiple comparisons). This association remained significant when additionally adjusting for BMI (p = 0.03). No subcortical volume changes between the *FTO* groups were found for the hippocampus, amygdala, caudate nucleus, pallidum, thalamus, and putamen.

Furthermore, we found a small (1.2%) decline in the volume of the cortical gray matter from TT to AA, which trended towards significance (p = 0.08, corrected for age, sex and BMI, and FDR corrected for multiple comparisons). To assess regional differences in cortical thickness in contrast to overall volumetric changes, VBM (voxel-based morphometry) analysis was performed; this did not reveal any significant focal cortical differences among groups. No difference between groups was found for white matter volume reduction.

Table 2. Volumes and association of different brain volumes with FTO.

	FTO rs9939609TA polymorphism			Model 1	model 2
	TT (n=185)	TA (n=236)	AA (N=71)	P-value	P-value
Grey matter (ml)	469 (4)	464 (4)	463 (7)	0.012	0.017
White matter (ml)	604 (4)	607 (5)	605 (4)	0.73	0.86
Hippocampus (ml)	4.68 (0.04)	4.58 (0.04)	4.69 (0.06)	0.48	0.37
Amygdala (ml)	2.02 (0.02)	1.97 (0.02)	2.05 (0.04)	0.90	0.75
Nucleus caudatus (ml)	3.79 (0.04)	3.74 (0.04)	3.77 (0.06)	0.55	0.58
Nuc. accumbens (ml)	0.59 (0.01)	0.55 (0.01)	0.52 (0.02)	0.004	0.003
Pallidum (ml)	1.93 (0.03)	1.94 (0.03)	1.95 (0.05)	0.81	0.88
Thalamus (ml)	8.10 (0.05)	8.04 (0.05)	8.12 (0.09)	0.62	0.53
Putamen (ml)	5.24 (0.04)	5.18 (0.04)	5.34 (0.08)	0.56	0.54

Table 2: Data are presented as Mean (SE). MODEL 1: Linear regression correcting for gender and age; MODEL 2 Linear regression correcting for gender. age and BMI.

DISCUSSION

The results of this study show that the risk allele rs9939609A of the *FTO* gene is associated with a significantly lower volume of the nucleus accumbens and trended towards

a smaller cortical grey matter volume. This association is independent of gender, age and BMI in this cohort. Although other studies have shown global grey matter volume differences with the *FTO* gene or BMI, to our knowledge, this is the first study to identify specific brain structures within the reward system that are affected by the *FTO* gene. No association between *FTO* and other subcortical brain structures was found.

Very little is known about the relationship between the *FTO* gene and the nucleus accumbens. There is, however, more known about the role that FTO plays in hypothalamic signalling. Research in chickens, mice and rats has shown expression of FTO throughout the brain with high expression in the hypothalamus (5, 31-34). The hypothalamus has an influence on the nucleus accumbens through dopaminergic projections via the ventral tegmental area.

The nucleus accumbens is a brain structure with a central role in the reward circuitry. It has a role in motivation-related behaviour, reward, and the craving of various substances including food(35, 36), and has a regulating role in dopaminergic signalling to the output-structures of the reward-system(37). The activity of the hypothalamus - nucleus accumbens pathway has recently been shown to be up-regulated by ghrelin and down-regulated by leptin, hormones known to be important for the regulation of homeostatic hunger(36). A study by Karra et al. revealed that healthy subjects with a normal weight with genotype AA at SNP rs9939609 have attenuated post-prandial ghrelin suppression (7) compared to subjects with a normal weight with genotype TT at SNP rs9939609. Furthermore, this study showed that the response to hedonic food pictures was differentially modulated between AA and TT phenotypes in the nucleus accumbens(7). In addition it was shown that knockout of *FTO* in mice impairs dopamine receptor type 2 and 3-dependent control of neuronal activity and behavioural responses(38).

Another possible mechanism through which *FTO* might have an influence on reward signalling is by regulating the expression of *IRX3*. This gene has been implicated in regulating body weight and is expressed in the hypothalamus, amygdala and caudate nucleus, all of which connect with the nucleus accumbens(8).

Our data, showing a lower nucleus accumbens volume in people with the *FTO* AA genotype, support the hypothesis that subjects with the *FTO* AA gene have attenuated regulation of dopaminergic projections to the nucleus accumbens and through that to the output-structures of the reward-system leading to the previously mentioned differences in feeding behaviour between *FTO* TT and *FTO* AA.

Our study also showed a trend line effect of the *FTO* polymorphism on the total volume of grey matter. The relationship between SNPs within the *FTO* gene and brain volume deficits has been previously reported in populations of elderly subjects (17) as well as in adolescents(39). It has been suggested that the effect of FTO on brain volume deficits probably originates in developmental stages of life, given that it is already apparent in adolescence. It was suggested that there might be an inverse relationship between FTO

and brain tissue compared to the relationship between FTO and adipose tissue(39). The findings of our study support these results, showing a trend line association between the presence of the *FTO* risk-allele and increased cortical brain atrophy (39). Since it was previously shown that BMI was not associated with lower nucleus accumbens volumes(22, 24), our finding that *FTO* is specifically associated with lower nucleus accumbens volumes indicates that the underlying cerebral process associated with BMI and brain atrophy is different.

However, our data do not support the results of a recent study by Cole et al.(40) showing that only BMI, and not *FTO*, was associated with brain atrophy. In this study younger subjects with a different SNP (rs3751812) were included(40). Still, our data unequivocally show that the association between the *FTO* polymorphism and brain volume deficits is independent of BMI.

The major limitation of this study is that we included elderly subjects with an increased cardiovascular risk profile. Though we did not find differences in cardiovascular risk profiles between the *FTO* polymorphism groups, our data may not be generalizable to a healthy population.

CONCLUSION

In conclusion, the results of our study support previous research stating that *FTO* is associated with brain volume deficits as well as studies stating a role for FTO in dopaminergic signalling within the reward-system. Further research should focus on functional imaging of the reward system and the relationship with *FTO* to determine whether the differences in reward-related brain structures are a result of altered hypothalamic signalling or an independent effect of FTO on reward-related brain structures.

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Part III

Diagnostic workup of overweight paediatric patients in clinical practice

Chapter 5

Determinants of advanced bone age in childhood obesity

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ABSTRACT

Background

Childhood obesity is associated with advanced bone age (BA). Previous studies suggest that androgens, oestrogens, sex hormone binding globulin and insulin are responsible for this phenomenon, but results are contradictory and might be biased by confounders. We aim to elucidate this matter by applying a multivariate approach.

Method

We performed a correlation analysis of BA standard deviation score (SDS) with age and sex specific SDS for androgens, oestrogens, and with indicators of insulin secretion derived from oral glucose tolerance testing, in a group of obese children. A multivariate analysis was performed to investigate which parameters were independently predictive of BA SDS.

Results

In this cohort (n=101; mean age 10.9 yrs; mean BA 11.8 yrs; mean BMI SDS 3.3), BMI SDS was significantly correlated to BA SDS ($r=0.55$, $p< 0.001$). In a regression analysis in the total cohort ($B=0.27$, $p<0.001$), as well as in females ($B=0.34$, $p=0.042$), males ($B=0.31$, $p=0.006$) and pubertal children ($B=0.32$, $p=0.046$), DHEAS showed a positive, independent association with BA SDS. No association with indicators of insulin secretion was found.

Conclusion

BMI SDS is highly correlated to BA SDS in obese children. Increased DHEAS has a central role in advanced bone age in obese children.

INTRODUCTION

The worldwide increase in overweight and obese children has led to significant morbidity, including type 2 diabetes, cardiovascular diseases, fatty liver disease, impaired development and psychological problems (1). Furthermore, children with excess weight have been reported to have accelerated sexual maturation and linear growth, often accompanied by an advanced bone age (BA), and a decreased pubertal growth spurt, compared to normal weight children (2-4). The mechanism driving this BA advancement, however, has remained unclear.

Various alterations in hormone levels have been proposed to be responsible for this phenomenon, such as androgens (5-8), oestrogens (5-7, 9) and sex hormone binding globulin (SHBG) (7). Furthermore, two recent studies have indicated that increasing insulin resistance and insulin secretion are associated with BA advancement (10, 11). These studies, however, vary widely in study design and outcome parameters investigated, and led to contradictory results. For example, some studies evaluated the difference between BA and calendar age (CA) (8, 10) whereas others assessed the ratio between these parameters (11), while an age-adjusted indicator would theoretically be superior. Furthermore, some studies included prepubertal children only (6, 10), while others also included pubertal children (5, 7). Additionally, most studies reported on androgen and oestrogen levels as absolute levels, although these vary significantly with age and pubertal staging, making age an important potential confounder in association studies. Finally, various factors are expected to be mutually dependent.

Therefore, we investigated the multivariate relationship between BA standard deviation score (SDS) for age and sex versus age- and sex-adjusted serum concentrations of serum androgens, oestradiol, SHBG (expressed as SDS) and indicators of insulin secretion in a cohort of prepubertal and pubertal obese children.

METHODS

Study cohort

Obese children visiting our obesity clinic between January 2012 and July 2015, in whom BA assessment and an oral glucose tolerance test (OGTT) were performed, were included into this retrospective cohort study. The OGTT, BA assessment and endocrine measurements were, at that time, part of an extensive diagnostic package which we performed as standard care for all obese children. The aims of this diagnostic approach were a) early detection of glucose metabolism abnormalities and other complications of obesity such as polycystic ovary syndrome (PCOS) and b) detection of endocrine or genetic causes of obesity. Exclusion criteria for this study were endocrine disorders (e.g.

hypothyroidism), syndromes known to affect insulin sensitivity or increased skeletal maturation BA (e.g. Bardet-Biedl syndrome or overgrowth syndromes), medication affecting insulin sensitivity or skeletal maturation (e.g. metformin or methylphenidate), missing fasting insulin or unreliable OGTT data (e.g. due to vomiting or problems with i.v. catheter) and missing BA SDS (e.g. outside age reference range of BoneXpert). In this cohort, patients with marked hyperphagia and early onset obesity (onset of obesity < 5 years of age) were tested for genetic causes of obesity by means of a genetic panel developed at the University Medical Centre in Utrecht. It tests for 53 genes known to cause monogenic obesity. Patients with genetic defects indicating monogenic obesity were included in this study, since there is no reason to assume that their BA is affected in any other way than in other obese children. The results for these patients are shown with specific symbols in the figures. The study was approved by the medical ethics committee of the Leiden University Medical Center and conducted within the terms of declaration of Helsinki. Since all participants received standard of care only, subject consent was waived.

Anthropometric data and definitions

At the first visit, height and weight were measured using a stadiometer and calibrated scale, respectively. Obesity and BMI SDS were determined using the International Obesity Taskforce criteria (12). Height SDS was determined based on the Dutch nation-wide growth study performed in 2009 (13). Modified Tanner staging (14) was performed to determine pubertal stage (Tanner stage >G1 in males or >B1 in females were scored as pubertal).

BA evaluation

We used BoneXpert to determine BA and BA SDS on a radiograph of the left hand (15). BoneXpert is a fully automated system based on an extensive database, which determines the Greulich and Pyle BA by analysing 15 bones of the left hand and wrist (15, 16). BoneXpert is validated to determine BA and associated SDS in males of 2.5-17 years old and in females of 2-15 years for different ethnicities (15, 16). We used Caucasian as standard reference, since our cohort was largely Caucasian and the non-Caucasian participants were of north African and middle eastern descent, for which BoneXpert does not provide ethnicity-specific SDS. The radiographs were made on the date of the first visit, or during the visit for oral glucose tolerance testing.

Oral Glucose Tolerance Test procedure

OGTT was performed after an overnight fast with a minimum of 10 hours. A standardized dose of oral glucose of 1.75 gram/kg, with a maximum of 75 gram, was administered at the beginning of the test. An intravenous catheter was used to collect the blood samples

at t = 0, 30, 60, and 120 minutes. These samples were analysed for insulin and glucose concentrations. An extra sample to measure concentrations of oestradiol (E2), testosterone (T), androstenedione (Adione), dehydroepiandrosterone sulphate (DHEAS), and sex hormone binding globuline (SHBG) was obtained at t=0.

Laboratory measurements

Blood samples were analysed in the clinical laboratory of the Leiden University Medical Center (LUMC, the Netherlands). Immulite 2000 XPi (Siemens Healthcare Diagnostics, Tarrytown NY, USA) immunoassays were used to determine the serum concentrations of insulin (mU/l), SHBG (nmol/l), and DHEAS (umol/l). T (nmol/l) was analysed by immunoassay (ECLIA) on a Roche Modular E170 immunoanalyser, Adione (nmol/l) was analysed using a radioimmunoassay of Beckman Coulter (formerly DSL, Woerden, the Netherlands). Glucose was analysed in serum using a hexokinase method on Roche Modular P800 chemistry analyser. Two different but compatible methods, the automated ECLIA assay of Roche and the Orion ultra-sensitive RIA, were used to measure the E2 levels (pmol/l). Concentrations of the Orion RIA method were converted to ECLIA by a conversion factor. Due to the retrospective nature of this study, using data obtained in standard care, no mass spectrometry measurements were available for estradiol and testosterone. The Roche testosterone (generation 2) and estradiol generation 2 assays are state of the art immunoassays with limits of detection of 0.09 nmol/l and 18.4 pmol/l respectively. Both assays have been standardized against international reference methods (ID-GCMS). The Orion ultrasensitive RIA for estradiol had a similar limit of detection with excellent correlation in comparison with the Roche assay.

Concentrations of measured outcomes under the detection limit were defined as the mean between the lower detection limit of the test and zero. The Homeostatic Model Assessment of insulin resistance (HOMA-IR) was calculated using the formula: T0 glucose (mmol/l) x T0 insulin (mU/l) / 22.5 (17). We calculated the area under the curve for insulin levels during the OGTT using the trapezoid method**.**

Conversion of serum steroid levels to standard deviation scores

In order to estimate the possible influence of serum SHBG, E2 and steroid levels on BA advance, we converted patients' serum levels to SDS, based on published reference values using the same assays. Since the age distribution is skewed, for each age interval separate SD values were calculated above and below the mean. Values for +1 SD and -1 SD were estimated by dividing the difference between P97.5 and P50 and the difference between P50 and P2.5 by 1.96, respectively, as previously described (18). For DHEAS and SHBG we used the age and sex specific centiles provided by Elmlinger et al. (19).

Reference data for T and E2 were derived from the Caliper database (20). For these parameters, we calculated SDS only for children ≥9.0 years of age, since the reference

values for children below 9 years of age were largely below the detection limit. For children ≥9.0 years with plasma concentrations below the detection limit we imputed the data by dividing the lower detection limit by 2. We used the data from the Caliper database to directly calculate 1SD and -1SD scores in different age groups.

For DHEAS, SHBG, T and E2 smoothed-fit lines of the -1SD, P50 and 1SD data points were created, providing an equation to calculate age- and sex-adjusted SDS for these hormones: (serum concentration – age specific P50)/(age specific -1 or +1SD); SDS(X)=([X]-P50)/SD. There were no reference data applicable for our assay for serum insulin and Adione, so these concentrations in our patients could not be expressed as SDS. The results of smooth-fitting and plots of SDS scores in our cohort are summarized in the supplementary figures; SHBG SDS (supplementary figure 1), DHEAS SDS (supplementary figure 2), T SDS (supplementary figure 3), E2 SDS (supplementary figure 4).

Statistical analysis

All analyses were performed using IBM 23.0 SPSS Statistics. We performed analyses for the total cohort, as well as for subgroups based on sex and puberty. Normality was tested using the Kolmogorov-Smirnov and the Shapiro-Wilk Test ($p > 0.05$ was considered normally distributed). We report on mean and SD and median with interquartile range (IQR) for Gaussian and non-Gaussian distributed data, respectively. For the various SD-scores we investigated whether they significantly differed from zero using one-sample t-tests or a one sample Kolmogorov-Smirnov test. Correlation analyses were performed using Pearson and Spearman correlations depending on normality.

First, we performed correlation analyses exploring the possible effect of age and BMI SDS on the various parameters possibly influencing BA. We then investigated the correlation of these parameters with BA SDS. In both analyses, we report on significant correlations ($p<0.05$).

As a last step, we investigated which parameters were independently associated with BA SDS using backward regression analyses, using the pairwise exclusion option in SPSS. In a model with BA SDS as the dependent variable we entered age, sex, DHEAS SDS, SHBG SDS, fasting insulin, HOMA-IR and AUC insulin and investigated independent relationships to BA SDS in the total cohort and subgroups split on sex and puberty. E2 SDS and T SDS were only entered in the model for the pubertal subgroup, since they were unavailable for most prepubertal subjects. We tested the assumptions of each model by checking the independence of the residuals (Durbin-Watson test), inspecting their homogeneity (inspection of the scatterplot) and testing their normality (Kolmogorov-Smirnov test > 0.05).

RESULTS

Study cohort characteristics

Out of the 184 children that visited the Willem-Alexander Children's Hospital 101 children met the inclusion criteria for this study. Figure 1 summarizes the reasons for exclusion of the remaining subjects. Baseline characteristics are presented in table 1. The cohort had a mean age of 10.9 years and a mean BA of 11.8 years resulting in a mean BA SDS of 1.2; 57 % of the children were pubertal, and 47% female. Mean height SDS was 0.6 and mean BMI SDS 3.3. Mean BA SDS and DHEAS SDS were increased (both $p<0.001$), while T SDS and SHBG SDS were decreased versus age references ($p= 0.032$ and 0.003, respectively).

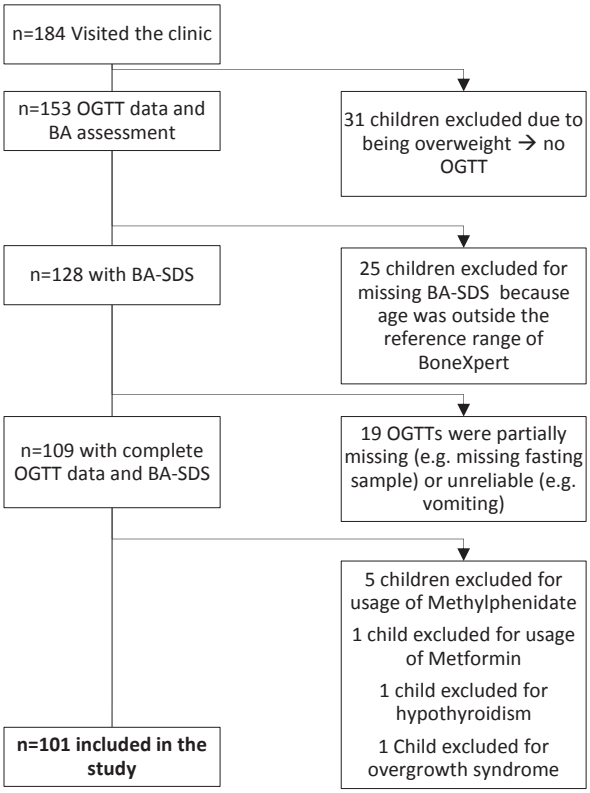

Figure 1. *Flowchart with reasons of exclusion.*

Table 1. Baseline characteristics, expressed as mean (standard deviation) unless otherwise stated

	Total cohort		Subgroups by sex				Subgroups by puberty			
	n	(n=101)	n	Female (n=47)	n	Male (n=54)	n	Prepubertal (n=43)	n	Pubertal (n=57)
Age, yrs	101	10.9 (3.1)	47	10.3 (2.9)	54	11.4 (3.1)	43	8.4 (1.9)	57	12.8 (2.3)
BA, yrs	101	11.8 (2.1)	47	10.9 (2.9)	54	12.6 (2.9)	43	9.6 (2.0)	57	13.5 (2.5)
BA SDS	101	1.2 (1.1)*	47	0.8 (1.2)*	54	1.4 (1.0)*	43	1.6 (1.1)*	57	0.8 (1.1)*
Caucasian[a]	101	64 (63.4)	47	27 (57.4)	54	37 (68.5)	43	19 (44.2)	57	44 (77.2)
Height SDS	101	0.6 (1.0)*	47	0.4 (1.0)*	54	0.7 (1.1)*	43	0.8 (1.0)*	57	0.4 (1.0)*
BMI SDS	101	3.4 (0.6)*	47	3.1 (0.5)*	54	3.6 (0.7)*	43	3.6 (0.7)*	57	3.2 (0.5)*
Fasting insulin mU/l[b]	101	12 (6/18)	47	11 (6/19)	54	12 (7/16)	43	7 (4/11)	57	16 (10/26)
HOMA-IR[b]	101	2.1 (1.3/3.7)	47	2.1 (1.3/3.9)	54	2.3 (1.3/3.5)	43	1.3 (0,8/2.0)	57	2.4 (2.1/4.8)
AUC insulin mU/l[b]	94	305 (202/468)	43	267 (186/ 454)	51	309 (210/482)	40	211 (159/397)	53	362 (238/544)
Oestradiol SDS	50	0.0 (1.4)	21	-0.2 (1.1)	29	0.1 (1.5)		-	39	0.0 (1.3)
Testosterone SDS[b]	64	-0.6 (-1.2/0.3)*	28	-0.7 (-2.7/1.6)	36	-0.6(-1.1/0.0)*		-	51	-0.7 (-1.3/0.4)*
DHEAS SDS[b]	96	0.4 (0.0/1.0)*	45	0.3 (-0.8/0.7)	51	0.4 (0.1/1.0)*	41	0.4 (-0.3/1.1)	54	0.3 (0.0/0.9)*
***SHBG SDS**[b]	96	-1.9 (-2.4/-1.1)*	46	-1.9 (-1.2/-2.2)*	50	-1.9 (-2.4/-1.1)*	40	-1.5 (-2.2/-0.7)*	55	-2.0 (-2.4/-1.4)*
Androstenedione nmol/l[b]	100	2.25 (1.30/3.60)	47	2.40 (1.20/4.10)	53	2.20 (1.30/3.30)	42	1.25 (0.78/2.23)	57	3.00 (2.15/4.50)

Table 1. *Data on oestradiol SDS and testosterone SDS are on age group ≥ 9 years.* [a] *n (%)* [b] *Median (interquartile range) * SDS p < 0.05. Abbreviations: SDS, standard deviation score; BA, bone age; BMI, body mass index; HOMA-IR, homeostatic model assessment for insulin-resistance; AUC, area under the curve; DHEAS, dehydroepiandrosterone sulphate; SHBG, sex hormone binding globulin.*

In five subjects of the final cohort, a genetic mutation was found. Two male subjects showed a heterozygous *MC4R* mutation (Cys293Tyr and Ile251Leu) and one male subject showed a heterozygous mutation in *WDPCP* (Leu379Ser). Furthermore, one female subject showed a heterozygous, mutation in *BBS7* (Gln365Leu), while another female subject was found to have two heterozygous variants in *CEP290* (Ile1059fs) and *MKKS* (Ala242Ser).

Correlation between outcome parameters and age or BMI SDS

The correlation analysis of the outcome parameters with age and BMI SDS are presented in table 2 and scatterplots are shown in Figure 2. The data are presented as Pearson correlation or Spearman's ρ, where applicable. BMI SDS was negatively correlated with age in prepubertal children and positively in pubertal children, showing a U-shape over the whole age range (Fig. 2A).

The insulin parameters in the total cohort as well as in subgroups according to sex and puberty showed a positive correlation with age. T SDS showed a significant negative correlation with age (ρ = -0.35) in males. In the prepubertal subgroup, SHBG SDS

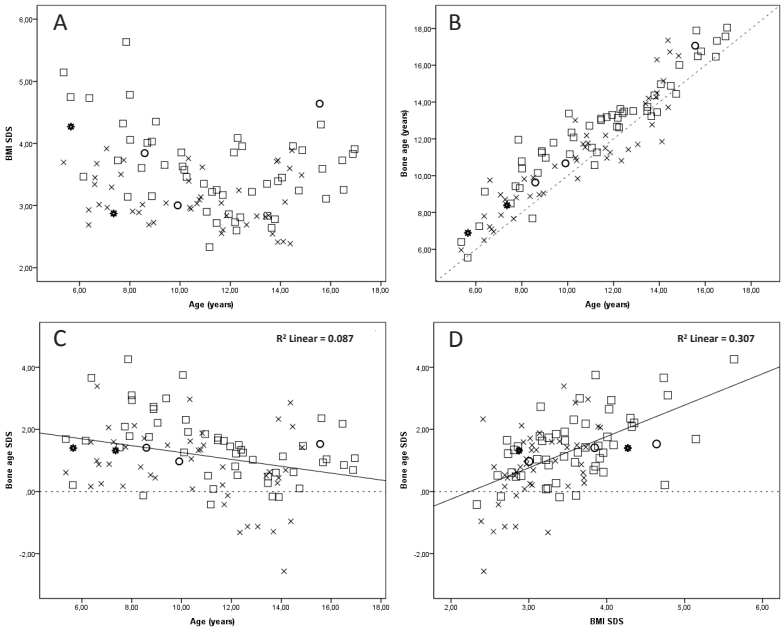

Figure 2. *A: association between BMI SDS and age (years); B: association between bone age (years) and chronological age (years); C: association between bone age SDS and age; D: association between bone age SDS and BMI SDS. Abbreviations: SDS: standard deviation score; BMI: body mass index; R^2: Coefficient of deviation. Squares represent males, x represent females, bold circles represents males with monogenetic obesity, bold stars represent females with monogenetic obesity.*

Table 2. Correlations between outcome parameters and age or BMI SDS

		BMI-SDS[a]	Fasting insulin	HOMA-IR	Insulin AUC	Oestradiol SDS_a	Testosterone SDS	DHEAS SDS	SHBG SDS
Age	**Total cohort**	-0.22*	0.65***	0.65***	0.48***	0.19	-0.17	0.05	-0.12
	Female	-0.26	0.68***	0.69***	0.58***	0.29	-0.09	0.00	0.02
	Male	-0.35**	0.63***	0.62***	0.40**	0.15	-0.35*	0.03	-0.20
	Prepubertal	-0.36*	0.59***	0.61***	0.53***	-	-	0.35*	-0.38*
	Pubertal	0.28 *	0.33*	0.30*	0.24	0.28	-0.10	-0.03	0.19
BMI SDS	**Total cohort**	-	-0.08	-0.10	0.11	0.19	0.14	0.20	-0.17
	Female	-	-0.11	0.11	0.06	0.28	0.36	0.22	-0.15
	Male	-	-0.13	-0.15	0.08	0.13	-0.16	0.07	-0.14
	Prepubertal	-	-0.19	-0.25	-0.05	-	-	0.13	-0.08
	Pubertal	-	0.28*	0.28*	0.42**	0.23	0.07	0.27	-0.39**

Table 2. *Correlations are shown as Spearman rho's unless otherwise stated. Correlations for oestradiol SDS and testosterone SDS are calculated on age group ≥ 9 years. Abbreviations: BMI, body mass index; HOMA-IR, homeostatic model assessment of insulin resistance; insulin AUC, area under the curve of insulin during oral glucose tolerance test; SDS, standard deviation score; DHEAS, dihydroepiandrosterone sulphate; SHBG, sex hormone binding globulin.* [a] *Pearson correlation; *p < 0.05; **p <0.01; *** p<0.001.*

negatively correlated with age (ρ = -0.38), in contrast to a positive correlation with age (ρ = 0.35) for DHEAS SDS.

Fasting insulin (ρ = 0.28), HOMA-IR (ρ = 0.28) and AUC insulin (ρ = 0.42) showed a significant positive correlation with BMI SDS in the pubertal subgroup. BMI SDS showed a trend toward a positive correlation with DHEAS SDS in the whole cohort and pubertal subgroup. In contrast, there was a trend toward a negative correlation with SHBG SDS in the whole cohort, which reached statistical significance in the pubertal subgroup.

Correlation between BA SDS versus clinical and biochemical parameters

Figure 2B shows that in the great majority of patients BA is advanced. As shown in table 3 and Fig 2C, BA SDS is relatively more advanced in young children: there was a significant negative correlation between BA SDS and age in the total cohort (*r* = -0.29) as well as in subgroups split on sex (female r = -0.31, male r = -0.41). BMI SDS showed a strongly significant correlation with BA SDS in the total cohort (ρ = 0.55) (figure 2D), as well as in female (ρ = 0.49), male (ρ = 0.55), prepubertal (ρ = 0.52) and pubertal (ρ = 0.51) subgroups.

Correlations between BA SDS and biochemical variables are presented in table 3. In females, T SDS showed a positive correlation with BA SDS (ρ = 0.44). In the total cohort, as well as in the male and both puberty subgroups, DHEAS SDS showed a positive correlation with BA SDS. SHBG SDS was negatively associated with BA SDS, particularly in pubertal children (ρ = -0.31). The insulin parameters and E2 SDS did not show significant correlations with BA SDS in the total cohort, nor in any subgroup.

Table 3. Correlation between bone age SDS and clinical and biochemical variables

		Sex		Puberty	
	Total cohort	**Female**	**Male**	**Prepubertal**	**Pubertal**
Age in years[a]	-0.29**	-0.31*	-0.41**	-0.15	-0.07
BMI SDS[a]	0.55 ***	0.49***	0.55***	0.52***	0.51***
Fasting insulin	-0.14	-0.22	-0.15	0.02	0.09
HOMA-IR	-0.14	-0.21	-0.14	-0.03	0.12
AUC insulin	0.07	-0.06	0.13	0.13	0.22
Oestradiol SDS[a]	0.14	0.13	0.13	-	0.10
Testosterone SDS	0.24	0.44*	-0.06	-	0.18
DHEAS SDS	0.29**	0.18	0.33*	0.32*	0.28*
SHBG SDS	-0.17	-0.10	-0.22	-0.24	-0.31*

Table 3. *Correlations are expressed as Spearman's rho (p-value) unless otherwise stated. [a] Pearson correlate (p-value). Correlations for oestradiol SDS and testosterone SDS are calculated on age group ≥ 9 years. Abbreviations: SDS, standard deviation score; BA, bone age; BMI, body mass index; HOMA-IR, homeostatic model assessment for insulin-resistance; AUC, area under the curve; DHEAS, dehydroepiandrosterone sulphate; SHBG, sex hormone binding globulin. *p < 0.05; **p <0.01; *** p<0.001.*

Regression analysis for BA SDS

The results of backward regression analysis are summarized in table 4. In the total cohort, backward regression analysis resulted in a model including sex, DHEAS SDS and age, explaining 27% of the total variance in BA SDS (overall fit of the regression model F=10.55, p<0.001). In the female subgroup we found a model explaining 21% of the vari-

Table 4. Backward regression analysis of bone age SDS

		Coefficiënt	CI 95%	R^2	p-value
Total cohort (n=88)	Constant	2.17	1.39/2.96		<0.001
	Sex male	0.62	0.18/1.06		0.006
	DHEAS SDS	0.27	0.09/0.44		<0.001
	Age	-0.13	-0.20/-0.06		0.036
	Model			0.27	<0.001
Female (n=52)	Constant	2.68	1.25/4.11		0.001
	DHEAS SDS	0.34	0.01/0.66		0.042
	SHBG SDS	0.29	-0.04/0.62		0.086
	Age	-0.14	-0.26/-0.02		0.024
	Model			0.21	0.030
Male (n=46)	Constant	2.81	1.80/3.81		<0.001
	DHEAS SDS	0.31	0.09/0.52		0.006
	Age	-0.14	-0.22/-0.05		0.002
	Model			0.30	<0.001
Prepubertal (n=36)	Constant	2.26	1.17/4.02		0.001
	Sex male	0.98	0.30/1.67		0.006
	SHBG SDS	-0.41	-0.76/-0.09		0.013
	Age	-0.27	-0.46/-0.07		0.009
	Model			0.31	0.006
Pubertal (n=37)	Constant	0.70	0.32/1.08		0.001
	DHEAS SDS	0.43	0.01/0.85		0.046
	Model			0.11	0.046

Table 4. *Backward linear regression analysis of bone age SDS. Variables included in all model: age, fasting insulin, HOMA-IR, AUC-insulin, DHEAS SDS, SHBG SDS. In the total cohort and pubertal subgroups, sex was added as an independent variable. In the pubertal subgroup only, oestradiol SDS and testosterone SDS were added as independent variables. Abbreviations: HOMA-IR, homeostatic model assessment of insulin-resistance; SHBG, sex hormone binding globulin; DHEAS, Dihydroepiandrosterone sulphate; SDS, standard deviation score.*

ance in BA SDS including DHEAS SDS, SHBG SDS and age (F=3.33, p=0.030). In contrast, in males the model did not include SHBG SDS, but only contained DHEAS SDS and age (F=9.50, p<0.001), explaining 30% of the variance. For the subgroups split on puberty, regression analysis showed a model explaining 31% of variance in BA SDS, including sex, SHBG SDS and age in prepubertal subjects (F=4.88, p=0.006) and a model explaining 11% of variance only including DHEAS SDS (F=4.27, p=0.046) in pubertal subjects.

DISCUSSION

The results of this study show that the mechanisms driving BA advancement in obese children are complex. In multiple regression analysis we have shown that DHEAS levels positively associate with BA SDS and SHBG levels negatively. Results are variable, however, across subgroups according to sex and pubertal status. Furthermore, we were able to explain only a limited percentage of the variance in BA SDS (with a maximum of 31% in prepubertal children), indicating that some factors driving bone age advancement were not included in this analysis.

As expected, in this cohort of obese children, mean height SDS was above average for the population, and BA was advanced compared to chronological age (CA). Furthermore, BA SDS and BMI SDS were strongly correlated. This is in line with studies reporting advanced linear growth and skeletal maturation in children with excess weight (2-4, 21). In addition, we have confirmed previous studies (3, 7, 22) showing that obese children have high DHEAS levels compared to a reference population. Our observation that DHEAS SDS is associated with BA SDS, especially in pubertal children, independent of various confounders, is in accordance with the results of a study by Sopher et al., which showed that, in a group of obese children, the highest tertile of the ratio between BA and CA was associated with high DHEAS levels (6). These authors posed that high DHEAS levels indicate high levels of androgens, leading to increased levels of E2 by peripheral conversion, which in turn leads to advanced bone maturation. The absence of an association between E2 and BA SDS in our cohort might be caused by the fact that our E2 assay lacks sensitivity in the lower ranges. Consistent with this explanation is a study by Klein et al. showing that E2 levels correlated with bone age in obese and lean children when using a more sensitive assay (5). Alternatively, it has been suggested that the production of E2 takes place at tissue level (6), so that no rise in circulating E2 levels can be detected, thereby explaining the lack of association between E2 and BA SDS in our cohort. Furthermore, our findings are in line with the work of DeSalvo et al. who showed that, in a cohort of children with premature adrenarche, the subgroup of children with BA advancement > 2 years had higher BMI and higher DHEAS levels than the subgroup of children with BA advancement < 1 year. This might suggest an overlap between the

pathophysiological mechanisms leading to BA advancement in patients with premature adrenarche and patients with obesity (23).

Although the pathophysiological mechanism remains uncertain, the results of our study show an independent association between DHEAS SDS and BA SDS in the total cohort as well as in males, females and pubertal children, indicating a central role for DHEAS in the BA advancement found in obese children. The scientific implications of the results of our study are that insulin is an unlikely cause of bone advancement in obese children, while DHEAS secretion can now be viewed as at least one of the intermediary factors. A possible clinical implication of our findings could be that it would be useful to measure DHEAS in obese children with substantial bone age advancement and/or increased statural growth. If available, it would also be useful to measure serum oestradiol with an ultrasensitive assay. In case of high concentrations, these could be accepted as causes of the clinical phenotype, so that the clinician can consider abstaining from further diagnostic workup of the patient.

Our finding of decreased plasma SHBG levels in obese children compared to reference intervals, based on lean children, is in accordance with previous reports (3, 7, 23) and has been reported to be caused by hyperinsulinemia, related to insulin resistance and low grade inflammation (24). Using sensitive E2 assays, it was also shown that obese adolescents have increased E2 levels, combined with decreased SHBG levels, possibly resulting in high levels of free E2 (23), which in turn might lead to increased bone maturation (24).

In addition to the generally decreased SHBG in obese children, we found a negative association in the regression analysis of SHBG SDS with BA SDS in prepubertal children. Decreased SHBG is associated with the increase of andrenal androgens during puberty (25), which in there turn, can stimulate bone maturation by locally increasing oestrogen levels via expression of aromatase (26). In contrast, a trend toward a positive association between SHBG SDS and BA SDS was found in regression analysis in the female subgroup, possibly reflecting increased gonadal oestrogen production during puberty, stimulating SHBG in girls. This association, however, did not reach significance ($p=0.086$), possibly because it is obscured by lack of assay sensitivity or the combination of the results of two oestrogen immunoassays.

It is of interest that we did not find an association between any of the insulin parameters with bone age advancement, neither in correlation analyses, nor in regression analyses. In the literature, contradictory results on the association between hyperinsulinemia and advanced bone age have been reported. No association between insulin resistance and the ratio between BA and CA was found in prepubertal children in a study by Sopher et al. (6) whereas Klein et al. found an association between insulin levels and the top tertile of this ratio in a cohort aged 3-18 years (5). Furthermore, Pinhas-Hamiel et al. showed that overweight children aged 4-13 years with a fasting insulin > 30 mU/l had a 6.8 fold increased risk of falling into the top tertile of the ratio between BA and CA,

independent of degree of obesity (11). Lee et al. investigated the relation between insulin resistance and bone age in prepubertal obese children and found an independent, positive correlation between HOMA-IR and the difference between BA and CA using multiple regression analysis (10). None of these three studies, however, corrected for the possible confounding effects of androgens and oestrogens, which might bias these results, and the outcome parameter of bone advance was not adjusted for age and sex. Furthermore, there was considerable variability in ethnicity between studies, which might in part explain the differences in outcome. In addition, the positive association between insulin secretion and age could lead to bias too. Another possible explanation for the lack of association between bone age SDS and insulin parameters in this cohort might be that a large number of the subjects in this cohort is already insulin resistant. Possibly the effects of insulin on bone age are more pronounced in children in the early stages of developing insulin resistance.

The finding of independent effects of sex in the multiple regression analysis is remarkable. It suggests that male and female subjects are differentially affected by increased BMI in their advanced bone maturation. This is in agreement with the findings of Crocker et al. who have recently shown that pubertal development is differentially affected in obese male and female subjects. They showed that, in female subjects, progressive Tanner staging correlated with advanced BA, while in boys BA advancement was independent of testicular development. Furthermore, insulin resistance correlated positively with breast development in girls while it was negatively correlated with testicular size in boys (27). This underlines the sexual dimorphism in the way obesity affects maturation.

As shown in our regression models, the maximum percentage of variance explained by a model was 31%, suggesting that factors not included in this study might contribute to BA advancement. It has been suggested that leptin (28) and IGF-1 (8) might contribute to bone age advancement in obesity, although recent work by Sopher et al. showed no association between these parameters and BA advancement (6). Future studies in larger cohorts should include these parameters to clarify the role these factors play in this matter.

A major strength of our study is the use of an automated method for BA assessment, which results in a reduced inter-subject, and an absent inter-observer variance (29). The use of BoneXpert also enabled the calculation of a reliable BA SDS from a representative population reference. Furthermore, where possible, we used age and sex specific SDS to investigate the relationship between hormone levels and BA SDS, thereby correcting for variance in these hormones caused by age and sex. Furthermore, we corrected for multiple confounders using regression analysis, which makes a causal relationship between the observed factors associated with advanced BA more plausible.

A limitation of our study is the fact that BoneXpert only supports the BA assessment of boys between 2.5 – 17 years and girls between 2.0 – 15 years (15, 16). However, older ad-

olescents have usually reached near adult height by this age, and we pose that therefore they are a clinically less relevant study group. Secondly, BoneXpert contains reference data for standard deviation scores of Caucasian, Asian, Hispanic, or Afro-Americans (16) but not from children of Turkish or Moroccan background. Therefore, we used Caucasian references as the standard for all children. The majority of the cohort, however, is Caucasian. A third limitation is that the assays for oestradiol has a limited sensitivity, which might obscure its association with BA SDS in prepubertal children. Finally, due to the large number of potential confounders included in the regression models, our sample size was too small to investigate sex effects separately in the prepubertal and pubertal age group. In addition, the small sample size in some subgroups (e.g. prepubertal), may have led to false negative results in the multiple regression analysis. Future research should therefore include larger cohorts, allowing for adjusting for multiple confounders in the regression analysis. Furthermore, longitudinal designs could help to gain additional insights into the mechanisms driving accelerated bone maturation in obesity. In addition, future studies would benefit from age and sex specific SDS for Adione and insulin, and should include leptin and IGF-1.

In conclusion, using multiple regression analysis, we have shown that increased DHEAS levels, reflecting adrenal androgen production, play a central role in BA advancement in obese children and adolescents and that decreased SHBG levels may further contribute to this phenomenon, though this finding needs further investigation.

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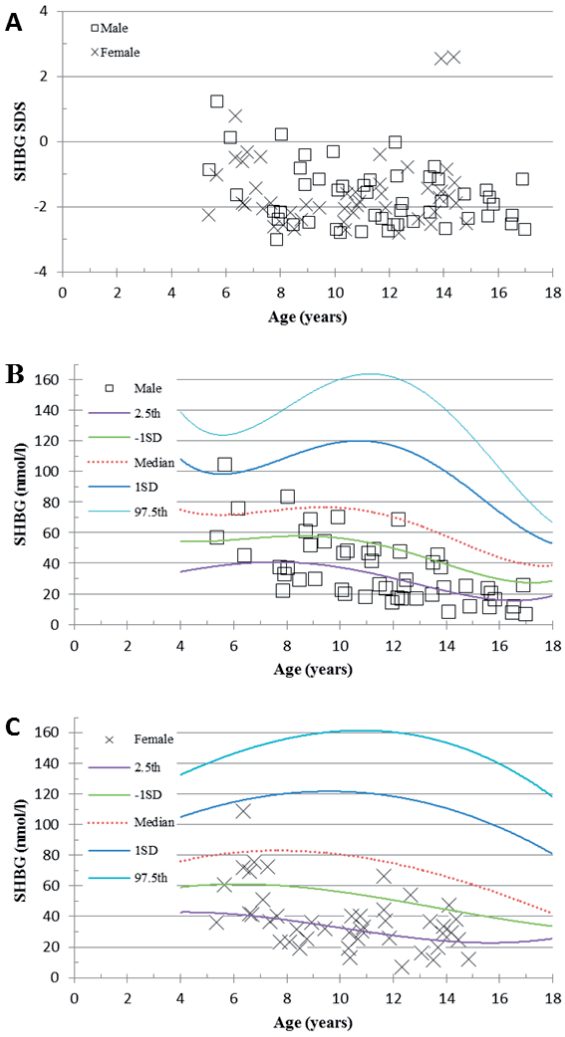
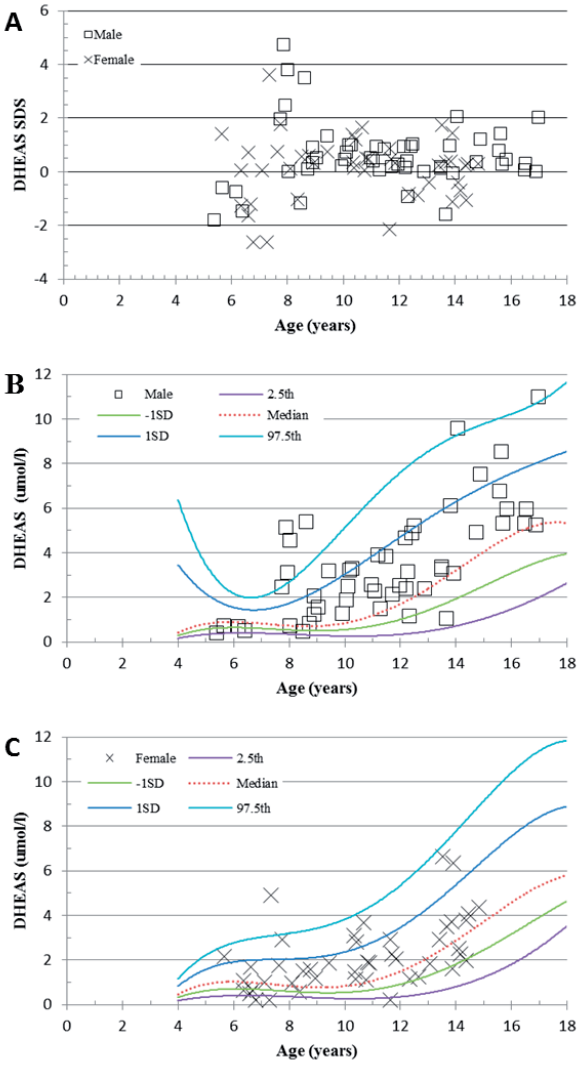
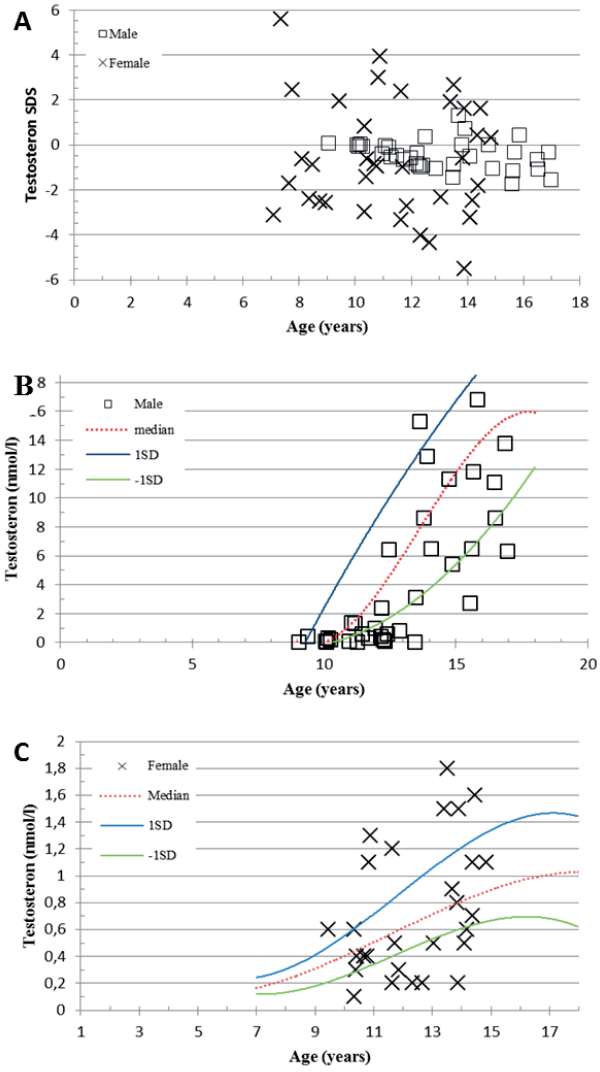
SUPLEMENTARY FIGURES



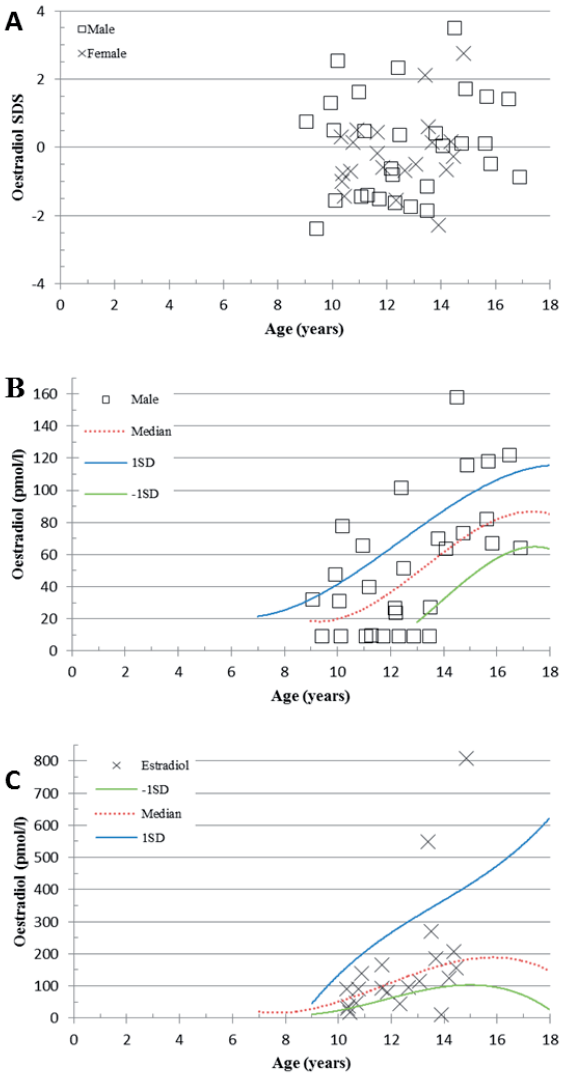
Supplementary figure 1. *A: scatterplot of SHBG SDS versus age. B: SHBG (nmol/l) versus age in males plotted on the age reference range. C: SHBG (nmol/l) versus age in females, plotted on the age reference range. Abbreviations: SDS, standard deviation score; SHBG, sex hormone binging globulin. The coloured lines represent references values of normal weight children from Elmlinger et. al. (19).*



Supplementary figure 2. *A: scatterplot of DHEAS SDS versus age. B: DHEAS (µmol/l) versus age in males plotted on the age reference range. C: DHEAS (µmol/l) versus age in females, plotted on the age reference range. Abbreviations: SDS, standard deviation score; DHEAS, dehydroepiandrosterone sulphate. The coloured lines represent references values of normal weight children from Elmlinger et. al. [19].*



Supplementary figure 3. *A: scatterplot of testosterone SDS versus age. B: testesterone (nmol/l) versus age in males plotted on mean ± 1SD lines based on a reference population. C: testosterone (nmol/l) versus age in females plotted on mean ± 1SD lines based on a reference population. Abbreviations: SDS, standard deviation score. The coloured lines represent references values of normal weight children from Konforte et. al. [20].*



Supplementary figure 4. *A: scatterplot of oestradiol SDS versus age. B: oestradiol (pmol/l) versus age in males plotted on mean ± 1SD lines based on a reference population . C: oestradiol (pmol/l) versus age in females plotted on mean ± 1SD lines based on a reference population . Abbreviations: SDS, standard deviation score. The coloured lines represent references values of normal weight children from Konfronte et. al. [20].*

Chapter 6

High predictability of impaired glucose tolerance by combining cardiometabolic screening parameters in obese children

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ABSTRACT

Background

There is debate on which overweight and obese children should be screened for the presence of impaired glucose tolerance (IGT) by oral glucose tolerance testing (OGTT).

Objective

Identify risk factors predictive of the presence of IGT.

Method

In a cohort of overweight children, who underwent OGTT, we determined the association of anthropometric and laboratory parameters with IGT and whether combining parameters improved the sensitivity of screening for IGT.

Results

Out of 145 patients, IGT was present in 11, of whom 2 had impaired fasting glucose (IFG). Elevated blood pressure (p=0.025) and elevated liver enzymes (p=0.003) were associated with IGT, whereas IFG was not (p=0.067), screening patients with either one of these parameters predicted IGT with a high sensitivity of 1.00, and a number needed to screen of 5.7.

Conclusion

Screening all patients with either IFG, presence of elevated blood pressure and elevated liver enzymes, significantly increases predictability of IGT compared to using IFG alone.

INTRODUCTION

The global prevalence of childhood obesity has risen dramatically in the last decades(1-3). In the Netherlands, the number of children classified as overweight has increased two- to three -fold, whereas the prevalence of obesity has even increased four- to six-fold in the last 30 years(4). This rise has been shown to be closely correlated with a rise in type 2 diabetes mellitus and metabolic syndrome(5-7). Identifying which children are at high risk for developing type 2 diabetes is crucial for determining the individual treatment strategy. The optimal strategy to screen for type 2 diabetes and prediabetes, however, remains the subject of debate among clinicians and healthcare workers.

Type 2 diabetes is preceded by a period of insulin resistance, ultimately resulting in the prediabetic phenotypes impaired fasting glucose (IFG), defined as a fasting glucose concentration ≥ 5.6 mmol/l(8) and impaired glucose tolerance (IGT), defined as a 2-hour glucose concentration ≥ 7.8 mmol/l(8) during oral glucose tolerance testing (OGTT) (9-11). Finding children with IGT is essential, since a considerable number of them progress into type 2 diabetes, especially when BMI increases over time(11,12). This warrants a more intensive treatment and follow up for these patients. The Dutch guideline, however, suggests to perform OGTT in patients with IFG(13), while recent research has shown that IFG correlates poorly with IGT(6, 9, 14-19).

The major reason for using IFG in the current guideline is that it is easier and more reproducible than IGT in children and adolescents(20). On the other hand, discussion is ongoing on the clinical value of fasting glucose in comparison to OGTT, because fasting plasma glucose measurement misses most of the cases with glucose dysregulation. In addition, children with discordant results on OGTT are still at high risk for developing type 2 diabetes(20). Furthermore, IGT is more predictive than IFG of future progression to type 2 diabetes(11). This underlines the importance of finding children with IGT.

Consequently, various parameters have been evaluated for their predictive value of developing IGT. A number of studies have indicated that the homeostatic model assessment of insulin resistance (HOMA-IR), the quantitative insulin sensitivity check index (QUICKI) and other surrogate measures of insulin resistance and sensitivity correlate with IGT(14,17,21,22) and that HOMA-IR is a more useful parameter than IFG(19). Lipid levels, that have also been shown to correlate with IGT(14,17), demonstrate a good sensitivity when used in a selective obese population(17) and are predictors of long term development of type 2 diabetes(23). Furthermore, elevated blood pressure in childhood has been shown to be associated with IGT(14) and development of type 2 diabetes in adulthood(23). Additionally HbA1c has been suggested to be predictive of IGT (24,25), but results are contradictory(24). Other parameters possibly predictive of IGT are elevated liver enzymes (e.g. AST and ALT), since they signal liver steatosis, which is associated with insulin resistance(26). Furthermore, IGT is highly prevalent in children

with non-alcoholic fatty liver disease(27), further underlining the possible predictive value of liver enzymes.

Although these parameters are promising for providing a better diagnostic approach for predicting IGT, these studies either fail to provide cut-off points, are performed in a selective population, or lack sufficient sensitivity. This makes it hard to implement this body of knowledge into everyday practice. The aim of this study is to evaluate the sensitivity, specificity, predictive values and numbers needed to screen in predicting IGT of diagnostic parameters using widely accepted cut-offs in a group of overweight and obese children, in whom OGTT was performed as a standard of care. Furthermore, we hypothesise that a combination of parameters could improve the sensitivity of the strategy for finding individuals at high risk of IGT.

PATIENTS AND METHODS

Patient characteristics

All patients visiting the obesity clinic of the Willem-Alexander Children's Hospital between January 2012 and July 2015 were analysed. Patients with obesity as defined by Cole(28) were all tested by OGTT as a standard of care. Patients who were classified as overweight were tested with OGTT if they had an increased risk of type 2 diabetes. This was defined based on the risk criteria as defined in the American Diabetes Association guideline (e.g having a family history of type 2 diabetes, acanthosis nigricans at first presentation, ethnicity other than Caucasian)(8). The medical ethics committee of the Leiden University Medical Centre approved the study. This study was conducted in accordance with the terms of the declaration of Helsinki.

Anthropometric measurements and definitions

A medical history was obtained, including information on ethnicity and family history of type 2 diabetes, at the first obesity clinic visit. Puberty stage was assessed according to Tanner(29). Height was measured to the nearest 0.1 cm with a stadiometer, and weight to the nearest 0.1 kg using a calibrated scale, with clothing and footwear removed except for underwear. Overweight and obesity class I where defined as described by Cole(28) and obesity class II and III and waist circumference SD-scores were classified using the data of the fifth Dutch national growth study(30). These BMI-classes are determined using age and sex specific curves, and correspond with an adult BMI of ≥ 25-29.9 kg/m^2 for overweight, ≥ 30-34.9 kg/m^2 for obesity class I, ≥ 35-39.9 kg/m^2 for obesity class II and ≥ 40 kg/m^{2}for class III. Blood pressure (BP) was measured in sitting position using an electronic sphygmomanometer (Dinamap Pro 300 V2, GE Medical Systems, Tampa, Florida, USA). The lowest BP value out of three consecutive measurements was

recorded. Elevated blood pressure was defined as diastolic or systolic blood pressure values exceeding the 95th percentile for age, sex and height using the cut-off values described by the National High Blood Pressure Education Program Working Group(31).

OGTT procedure

OGTT was performed after an overnight fast of at least 10 hours. Glucose dose was 1.75 g/kg with a maximum of 75 g. Blood samples were taken from an intravenous catheter at t=0, 30, 60 and 120 minutes to determine glucose concentrations. At t=0 an extra blood sample was taken for the analysis of lipids, insulin and liver enzymes.

Laboratory measurements and definitions

All laboratory analyses were conducted by the hospital laboratory of the Leiden University Medical Centre (LUMC Leiden, The Netherlands). Plasma glucose levels were determined using hexokinase method using Roche Modular Analytics P800 (Roche). Plasma insulin levels were measured with the use of a sandwich immunoassay using Immulite 2000 Xpi (Siemens Healthcare diagnostics). Impaired fasting glucose and impaired glucose tolerance were defined using the ADA criteria, i.e. IFG is fasting glucose ≥ 5.6 mmol/L and < 7.0 mmol/L and IGT is 2-hour glucose concentration ≥ 7.8 mmol/L and < 11.1 mmol/L during OGTT(8). Type 2 diabetes was defined as fasting plasma glucose ≥ 7.0 mmol/L or 2-hour plasma glucose ≥ 11.1 mmol/L. Glucose abnormalities were defined as having either IFG, IGT or type 2 diabetes. HOMA-IR was calculated by the following formula: glucose (mmol/L)* insulin (mU/L) / 22.5(32). We chose a cut-off of HOMA-IR ≥ 3,4 because this cut-off improved the prediction of IGT compared to using IFG in previous research(19, 33, 34). HbA1c was determined by ion exchange chromatography using Ultra^2 HbA1c affinity analyser (Trinity Biotech). HDL-cholesterol, total cholesterol and triglycerides were measured using enzymatic methods (Roche Modular Analytics P800, Roche) and LDL-cholesterol was calculated using Friedewald's formula (LDL-cholesterol = total cholesterol – HDL-cholesterol – 0.456 * triglycerides)(35). Elevated total cholesterol, LDL-cholesterol and triglycerides were defined as values exceeding the p95 for age and sex. For HDL-cholesterol a value below p5 for age and sex was considered abnormal(36). Dyslipidaemia was defined as having any abnormality in the lipid profile. ALT and AST were measured using the standard methods of the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC)(37) and gamma GT was analysed by the calorimetric enzymatic method (Roche Modular Analytics P800). Cut-off points were sex specific(37) as provided by the manufacturer. The variable 'elevated liver enzymes' was defined as elevated levels of either ALT, AST or gamma GT above the reference range.

Statistical analysis

All data were analysed using SPSS 20.0.0. Differences between normal glucose tolerant and impaired glucose tolerant individuals were explored using a Mann-Whitney U test, for continuous data. Fischer Exact test was used for nominal and ordinal data, unless otherwise stated. Parameters significantly associated with presence of IGT were compared with IFG considering sensitivity, specificity, number needed to screen in predicting IGT and percentage of cases of IGT missed. Furthermore, we evaluated the sensitivity, specificity, number needed to screen in predicting IGT and percentage of cases of IGT missed when taking the number of abnormal screening parameters in to account.

RESULTS

Characteristics of normal- and impaired glucose tolerant patients

A total of 181 patients visited the Willem-Alexander Children's Hospital obesity clinic between January 2012 and July 2015. In 24 patients OGTT was not performed because they were overweight and were classified as 'no increased risk of type 2 diabetes'. In 8 patients OGTT was not performed for other reasons (e.g. problems with catheter placement, OGTT recently performed in another hospital). Four patients did have an OGTT, but were excluded from the analysis (one because of a missing 2-hour glucose value, one was diagnosed with mature-onset diabetes of the young during follow-up, one was suspected of having an overgrowth syndrome and one was excluded because of unreliable outcome of OGTT due to vomiting after glucose ingestion). Therefore, a total of 145 patients were included in this study. 89 were of Dutch descent, 9 had a western background other than Dutch, 17 were of Moroccan origin, 5 were of Turkish origin and 27 had another non-western background. The baseline characteristics of the cohort and the comparison between NGT and IGT patients are shown in table 1. In total, 15 out of 145 subjects were diagnosed with glucose abnormalities. Of these 15 subjects, 6 were diagnosed with IFG, of whom two were also diagnosed with IGT. Based on the OGTT, 11 out of 15 subjects were diagnosed with IGT. None of the subjects were diagnosed with type 2 diabetes. IGT subjects had significantly higher numbers of elevated blood pressure (p=0.035) and elevated liver enzymes (p=0.003). Furthermore, subjects with IGT had higher triglyceride levels (p=0.014) and lower HDL-cholesterol levels (p=0.030). When considering the number of subjects exceeding the reference range, however, there was no between group difference for these parameters. All but 1 IGT subjects were pubertal. When considering pubertal subjects only, elevated blood pressure (p=0.028) and elevated liver enzymes (p=0.003) were still the only parameters significantly higher in IGT subjects.

Table 1 Characteristics of the cohort and comparison of NGT and IGT subjects.

		Total n=145	NGT n=134	IGT n=11	p-value
Sex female (%)		77 (53)	72 (54)	5 (46)	p>0.10
Age		11.6 (8.1-14.4)	11.5 (8.0-14.2)	14.3 (11.9-15.3)	0.048
Ethnicity Caucasian (%)		96 (66)	87 (65)	9 (82)	p>0.10
BMI SDS		3.3 (2.9-3.8)	3.3 (2.9-3.8)	3.8 (3.2-4.3)	p>0.10
Waist circumference SDS		3.1 (2.8-3.5)	3.1 (2.8-3.4)	2.9 (2.9-3.9)	p>0.10
Acanthosis nigricans (%)		58 (41)	52 (40)	6 (60)	p>0.10
BMI category (%)	Overweight	23 (16)	22 (16)	1 (9)	p>0.10
	Obesity class I	56 (38)	53 (40)	3 (27)	
	Obesity class II	36 (25)	34 (25)	2 (18)	
	Obesity class III	30 (21)	25 (19)	5 (46)	
Pubertal subjects (%)		85 (63)	76 (61)	10 (91)	0.097
OGTT Glucose mmol/l t=0		4.4 (4.2-4.8)	4.4 (4.2-4.8)	4.6 (4.2-5.3)	p>0.10
OGTT Glucose mmol/l t=30		7.4 (6.3-8.3)	7.3 (6.3-8.3)	7.9 (7.3-8.4)	p>0.10
OGTT Glucose mmol/l t=60		6.2 (5.2-7.5)	6.1 (5.2-7.2)	9.3 (7.8-10.7)	<0.001
OGTT Glucose mmol/l t=120		5.8 (5.2-6.6)	5.7 (5.1-6.4)	8.5 (8.1-9.6)	<0.001
IFG (%)		6 (4)	4 (3)	2 (18)	0.067
IGT (%)		11 (8)	na	na	na
Type 2 diabetes (%)		0 (0)	na	na	na
HbA1c mmol/mol		33 (32-35)	33 (32-35)	35 (32-36)	p>0.10
HbA1c > 40 mmol/mol (%)		1 (1)	1 (1)	0 (0.0)	p>0.10
HOMA-IR		2.3 (1.3-3.8)	2.1 (1.3-3.8)	2.9 (1.3-7.0)	p>0.10
HOMA-IR > 3.4 (%)		45 (32)	41 (31)	4 (40.0)	p>0.10
Systolic BP mmHg		115 (109-125)	115 (108-124)	131 (118-134)	0.007
Diastolic BP mmHg		69 (65-75)	67 (64-73)	72 (69-78)	0.025
Elevated BP (%)		88 (69)	33 (28)	7 (64)	0.035
AST U/L		26 (22-31)	25 (22-31)	34 (28-43)	0.007
Elevated AST (%)		27 (21)	21 (18)	6 (60)	0.006
ALT U/L		21 (15-27)	20 (15-26)	34 (26-65)	0.002
Elevated ALT (%)		15 (11)	11 (9)	4 (40)	0.015
Gamma GT U/L		16 (12-22)	15 (11-20)	24 (21-34)	0.001
Elevated Gamma GT (%)		4 (3)	3 (2)	1 (9)	p>0.10
Elevated liver enzymes (%)		31(23)	24 (19)	7 (64)	0.003
Triglycerides mmol/l		0.9 (0.7-1.2)	0.9 (0.7-1.1)	1.2 (1.0-1.8)	0.014
Triglycerides > p95 (%)		29 (21)	25 (20)	4 (36)	p>0.10
Cholesterol mmol/l		4.0 (3.6-4.6)	4.0 (3.6-4.5)	4.2 (3.5-5.6)	p>0.10
Cholesterol > p95 (%)		13 (9)	10 (8)	3 (27)	0.070
LDL-cholesterol mmol/l		2.4 (2.2-2.8)	2.4 (2.0-2.8)	2.6 (2.2-3.8)	p>0.10
LDL-cholesterol > p95 (%)		13 (10)	10 (8)	3 (27)	0.070
HDL-cholesterol mmol/l		1.2 (1.0-1.3)	1.2 (1.0-1.3)	1.0 (0.9-1.2)	0.030
HDL-cholesterol < p5 (%)		19 (13)	17 (13)	2 (18)	p>0.10
Dyslipidemia (%)		48 (35)	42 (33)	6 (55)	p>0.10

Table 1. *Percentages are valid percentages. Data are expressed as median with IQR unless otherwise stated. Abbreviations: BP: Blood Pressure, NGT: Normal Glucose Tolerance, IGT: Impaired Glucose Tolerance, BMI: Body Mass Index, SE: Standard Error, SDS: Standard Deviation Score, IFG: Impaired Fasting Glucose, IGT: Impaired Glucose Tolerance, OGTT: Oral Glucose Tolerance Test, na: not applicable.*

Relationship of predefined risk factors with IGT

In Table 2 the associations with IGT are compared for IFG, elevated blood pressure and elevated liver enzymes with regard to their sensitivity, specificity, number needed to screen and percentage of patients with IGT missed, when using this parameter to determine whether OGTT is necessary. The sensitivity of IFG was 0.18. The maximum sensitivity of a single parameter was 0.64 for elevated blood pressure and elevated liver enzymes. Analysing pubertal subjects only showed similar results for IFG (sensitivity 0.20, specificity 0.96), elevated blood pressure (sensitivity 0.70, specificity 0.66) and elevated liver enzymes (sensitivity 0.60, specificity 0.80).

Table 2 Predictability of IGT by single risk factors

	NGT (n=134)	IGT (n=11)	Sensitivity (95% CI)	Specificity (95% CI)	NNS	IGT missed n (%)
IFG (%)	4 (3)	2 (18)	0.18 (0.05-0.48)	0.97 (0.93-0.99)	3.0	9 (82)
Elevated BP (%)	33 (28)	7 (64)	0.64 (0.35-0.85)	0.75 (0.67-0.82)	5.7	4 (36)
Elevated liver enzymes (%)	24 (19)	7 (64)	0.64 (0.35-0.85)	0.82 (0.75-0.88)	4.4	4 (36)

Table 2 Percentages shown are valid percentages. Abbreviations: NGT: Normal Glucose Tolerance, IGT: Impaired Glucose Tolerance, IFG: impaired fasting glucose, NNS: number needed to screen, IGT missed: number of cases missed if this parameter was used to assess the necessity to perform OGTT, ns: not significant.

Analysis of the number of abnormal diagnostic parameters and the presence of IGT

All patients with IGT (n=11) had one or more abnormalities in the parameters IFG, elevated blood pressure and elevated liver enzymes. If all subjects with one or more abnormal diagnostic parameters were to be examined by OGTT this would result in a sensitivity of 1.00 (95% CI 0.74-1.00), a specificity of 0.56 (0.49-0.65), a number needed to screen of 5.7 and no cases of IGT missed (table 3). Only analysing those subjects with elevated blood pressure or elevated liver enzymes would result in a sensitivity of 0.91 (0.62-0.98) and a specificity of 0.64 (0.56-0.72) and a similar number needed to screen (5.8).

Table 3 Predictability of IGT by combining significant risk factors

Number of abnormal screening parameters [b]	NGT (n=134)	IGT (n=11)	p-value	Sensitivity (95% CI)	Specificity (95% CI)	NNS	IGT missed n (%)
0	82	0	<0.001	Na	na	13.2	0 (0)
≥1	52	11		1.00 (0.74–1.00)	0.56 (0.49-0.65)	5.7	0 (0)

Table 3 Abbreviations: NGT: Normal Glucose Tolerance, IGT: Impaired Glucose Tolerance, NNS: number needed to screen, IGT missed: number of cases missed if this parameter was used to assess the necessity to perform OGTT, na: not applicable. [b]number of abnormal screening parameters in IFG, elevated blood pressure and liver enzymes.

DISCUSSION

The results of the present study show that combining diagnostic parameters, available in every day practice, can significantly improve the screening strategy to identify subjects at high risk of IGT among obese children. Additionally, this study extends the evidence stating that IFG correlates poorly with IGT and is not a parameter with sufficient sensitivity to predict IGT. The percentage of cases of IGT missed and the number needed to screen using IFG as a parameter to assess the necessity to perform an OGTT, are high. Remarkably, our data show that elevated liver enzymes and elevated blood pressure were more useful in predicting IGT than IFG, considering sensitivity and number needed to screen (Table 3).

Combining the parameters IFG, elevated blood pressure and elevated liver enzymes and using a cut-off of 1 or more abnormal parameters, increased the sensitivity of finding patients with IGT to 1.00 with a number needed to screen of 5.7 (Table 3). Identifying patients with IGT is of the utmost importance, since they are at increased risk of developing type 2 diabetes (11, 12). Therefore, obesity treatment of patients with IGT, is more intensive and has a more frequent follow-up, as recommended in the current guideline(13). A major strength of the current study is that the results of this study are applicable to the general population of children with obesity, since all obese patients underwent OGTT without selection.

Implementing our model in practice could have various implications for healthcare. A major advantage of this approach is that it only uses parameters that are available in everyday practice. Furthermore, the number needed to screen is relatively low, combined with a high sensitivity, which makes it an attractive screening strategy. Although one might pose that implementing this strategy would strain healthcare budgets and manpower by an increasing number of OGTT's (e.g. a patient has to stay in the hospital or clinic for 2 hours), only performing OGTT's in patients with IFG has been proven insufficient by a vast body of previous evidence(6, 9, 14, 15, 17). In our population, applying the strategy of only testing subjects with IFG, elevated blood pressure and elevated liver enzymes would mean that approximately one third of our cohort would undergo an OGTT. This number would be substantially lower if only pubertal subjects would be screened, or only those with elevated blood pressure and/or elevated liver enzymes. In this cohort, however, applying either one of these strategies would result in cases of IGT missed, which is undesirable. It has to be noted, though, that only screening patients with elevated blood pressure and/or elevated liver enzymes would be interesting for clinical practice, since this would waive the necessity to perform fasting screening. Therefore, further research on these parameters, possibly combined with other non-fasting parameters, could have important implications for clinical practice. In summary

we provide a model for selecting patients at high risk of having IGT, with good sensitivity and an acceptable number needed to screen.

The finding that elevated liver enzymes predict IGT is of particular interest (Table 1). It is in line with research in adults, showing a correlation between elevated AST and ALT and prediabetes(38). It is in contrast, though, with a recent report concerning a population of Thai adolescents(18). However, this study was performed in a group of adolescents with obesity at high risk for co-morbidity, whereas we did not select the high-risk patients in our group defined as 'obese'. Elevated liver enzymes probably signal hepatic steatosis, a common complication in childhood obesity(9, 26). Recently it was shown that children with hepatic steatosis show a high prevalence of IGT(27). Furthermore, hepatic steatosis is associated with insulin resistance(9, 26) and metabolic syndrome(39), both of which are also associated with IGT, showing a possible shared causative origin. Our study adds to the literature that the presence of elevated liver enzymes is a highly predictive of IGT, especially when combined with IFG.

Another finding of interest is that there was only one subject with abnormal HbA1c levels in our cohort (Table 1). This might be the reason that we could not find a correlation between HbA1c levels and presence of IGT. In literature on this subject, prevalence is usually higher(25, 26, 40). These studies differed from our cohort in respect of ethnicity, since they were largely Korean(24, 40) and Hispanic(25). Furthermore, although it was previously shown in adults that the association of HbA1c with IGT is only modest, it is a highly sensitive parameter for detecting the presence of type 2 diabetes in children(41), correlates well with abnormal glucose indices as measured by continuous glucose monitoring(42) and has the advantage of easy measurement in daily practice. However, both HbA1c and OGTT are of separate added value as they identify different patient populations with likely different underlying insulin metabolism abnormalities, and are not sequential in their appearance(41).

There are limitations to consider in respect to this study. Although there was no selection of the patients classified as obese, we only included high-risk overweight patients which might have caused selection bias for the small overweight group. However, only 24 patients were excluded on a total of 181 based on the fact that they were at low-risk of type 2 diabetes and in the overweight class. Furthermore, some screening parameters were not included in this study that deserve further research. Thyroid-stimulating hormone, for example, has been shown to be associated with glucose metabolism abnormalities(43).

Future research is needed to confirm whether our findings can be replicated in patient groups with a different profile on age, country or ethnicity. If our findings are confirmed, this model is suitable for implementation in daily practice and could have consequences for current guidelines on screening for co-morbidity obese children is.

In conclusion, this paper provides a new method of screening for IGT, with high sensitivity and a low number needed to screen compared to using IFG alone. Our data show that in patients with 1 or more abnormal diagnostic parameters, i.e. IFG, elevated blood pressure and elevated liver enzymes, there is a high risk of IGT and therefore these patients should be investigated by OGTT. This model is easy to use, simple and feasible for implementation in current clinical practice.

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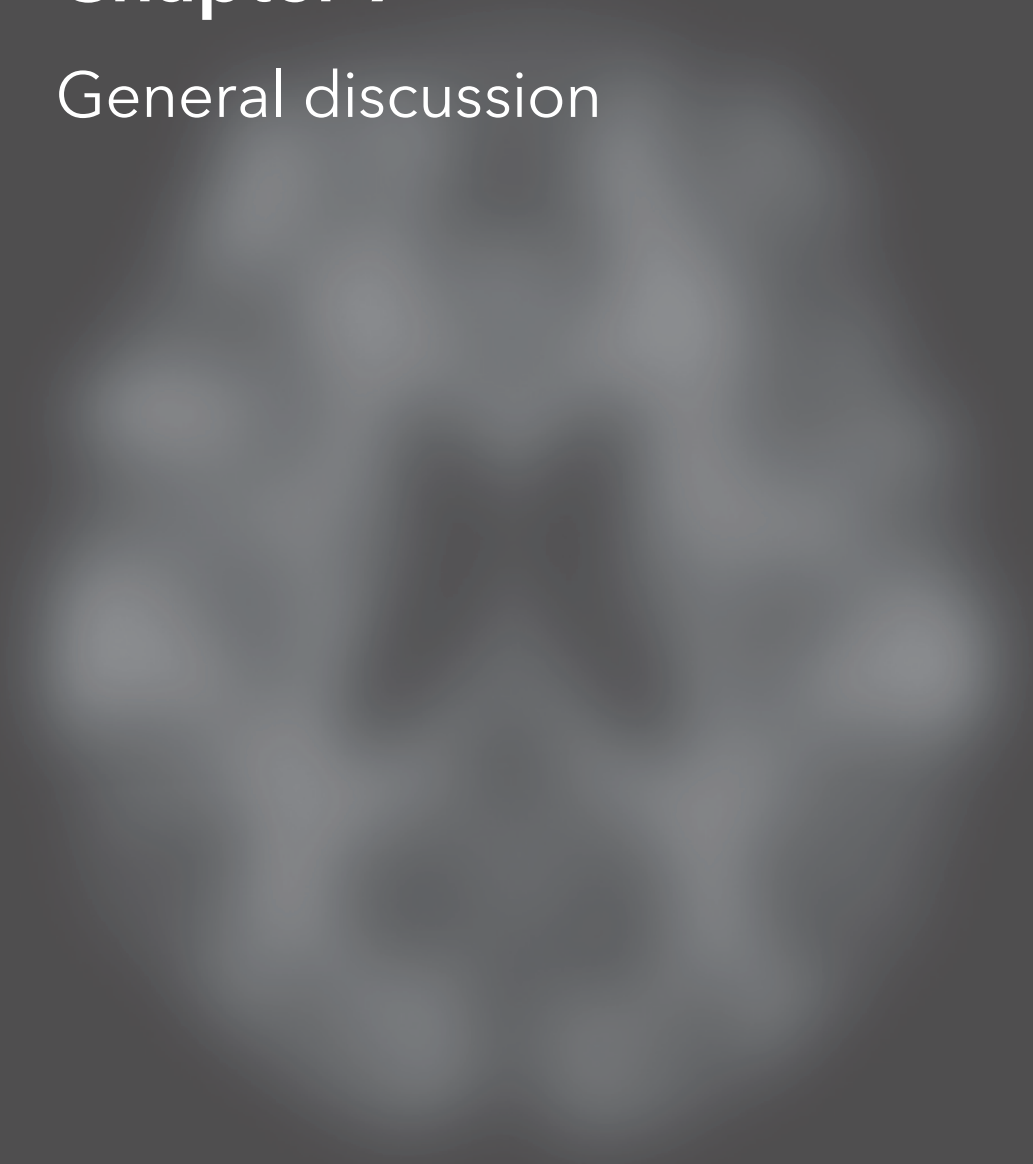
Part IV

Chapter 7

General discussion



This thesis addresses two important issues in obesity research. Part II reports on the investigations in the neural aspects of obesity, specifically their interactions with behaviour and genetics, to gain insight in the pathological mechanism leading to obesity, while the third part explores ways to improve the diagnostic strategy in children with obesity.

BEHAVIOURAL AND NEURAL ASPECTS OF OBESITY

Understanding the complex pathophysiology of obesity is the key in developing successful therapies. Considering the limited success of current treatment, further understanding of patterns of feeding behaviour in obese people is needed to improve on current treatment strategies(1). In the last two decades, a substantial number of brain areas have been shown to be altered in size, thickness or activation in this population. The understanding of how these differences influence behaviour is at this moment lacking. Although vast progress is made, there are still a lot of dots that need connecting.

Feeding behaviour and weight gain in childhood are amongst the most important predictors of future eating behaviour and adult weight status. Therefore, understanding of feeding behaviour at young age is key to the development of prevention and treatment strategies(2). In this respect, executive functioning is an important area of interest. Executive function is a collective name for self-regulatory processes(3), of which inhibitory control and the ability to delay gratification are most often described as being impaired in children with obesity(4-6). More specifically, impaired executive function negatively influences the outcome of treatment(7). Furthermore, children with obesity tend to show increased responsiveness to overeating, decreased satiety responsiveness and were shown to eat triggered by emotion(8).

Adolescent obesity, brain structure and executive function

In **chapter 2** we described the relationship between differences in brain structure and aspects of behaviour and showed that the pallidum is significantly larger in adolescents with obesity, and that pallidum size correlated with executive function in obese adolescents. This finding is in line with findings that pallidum size is increased in children with obesity(9) and that visual food stimuli activate the pallidum to a greater extent in subjects with obesity, than in lean subjects(10). It was suggested that this is a sign of increased reward processing, while we did not find a relationship between the pallidum and reward driven appetitive behaviours, but with executive function. The dorsal part of the pallidum has extensive inhibitory GABA-ergic projections to various structures related to reward processing(11) and projects toward various frontal areas involved in exerting control over behaviour(12). This shows that different parts of the pallidum

might have different roles in the pathophysiology of obesity. In children and adolescents with ADHD, a condition known for impairment of executive function, amplitude of the BOLD-signal was increased in the globus pallidus, during rest(13). This might suggest that this is a compensatory mechanism for hypofunction of other executive areas. This is in line with our explanation of the larger pallidum size we found in obese adolescents. However, future research on the exact mechanisms and role of the pallidum in obesity pathophysiology is warranted.

It would be interesting to design a study on pallidum BOLD response including high and low calorie food items. Furthermore, more intricate designs involving testing of executive function during fMRI could aid in specifically finding the domains of executive function in which the pallidum is involved. This research should not only focus on BOLD-response, but should also include connectivity analysis, to gain inside in the communication with other brain structures and networks during executive function tasks. As a first step, we analysed resting state connectivity differences in obese and lean adolescents **(chapter 3),** but did not find any differences in resting state connectivity of the pallidum between these groups. There is a variety of reasons that could explain the lack of differences. The first being that the study was performed in a resting state only, posing the question whether differences in pallidum connectivity could arise only when actively engaged in either executive function or when challenged by food cues. Furthermore, the participants were satiated while the study was performed, possibly suppressing connectivity that is more pronounced when hungry. The results of our resting state study, however, are in line with research in children with ADHD, which did not show any alteration in functional connectivity of the globus pallidus during rest when compared to non-ADHD control subjects(13). These findings indicate that differences in pallidum function and connectivity probably arise when actively engaging in a task.

Resting brain connectivity in adolescent obesity

Although between lean and obese adolescents no difference in connectivity of the pallidum was found, we did find differences in resting state connectivity. In **chapter 3** we investigate differences in resting state connectivity between lean and obese adolescents, of the default mode network, executive control network and salience network as well as connectivity differences of brain structures involved in hunger and satiation processing (the hypothalamus), reward processing (the amygdala) and executive function (the pallidum), while in a fed condition. The fed condition was chosen since children and adolescents with obesity tend to eat in the absence of hunger, thereby exceeding caloric demands(14,15). This analysis showed differences in the executive control network, a network involved in exerting control over behaviour. The executive control network showed lower connectivity in obese compared to lean adolescents specifically in the lateral occipital cortex, possibly indicating that visual food cues trigger areas involved

in controlling feeding behaviour to a lesser extent. Furthermore, we found higher connectivity of the occipital pole with the salience network in obese, indicating that salient food signals might be processed with high priority, as was also suggested by recent work in obese adults(16). Previously, it was shown that the salience network showed increased within network connectivity in both adults with obesity and in subjects with Prader Willi syndrome(17, 18). This was explained as an imbalance between autonomic processes, such as hunger and satiety signalling, and reward processing. The results of **chapter 3** indicate that, even at rest, the brain of obese adolescents is programmed to prioritise salient food cues, thereby overriding satiety signalling, while simultaneously triggering areas involved in exerting control over food intake to a lesser extent than in lean subjects. In line with our findings, it was recently shown that fed adolescents with obesity show decreased resting state connectivity between the insula, part of the salience network, and the dorsolateral prefrontal cortex, a key area in cognitive control over food intake(19). This indicates that, in adolescent obesity, there is an imbalance between salience processing and the executive control over these processes, providing a framework for the possible mechanism that explains why adolescents with obesity eat in the absence of hunger.

Still, a lot of questions remain unanswered. Differences in resting state connectivity do not tell us how they affect behaviour. Therefore, future work should focus on investigating processing of food cues using connectivity analysis to find what happens when visual information is forwarded to the salience and executive control network. Furthermore, it would be of interest to repeat our investigation in a state of hunger, to explore if there are different mechanisms in a non-satiated state that might contribute to obesity. Most importantly, the question we must ask is whether we can influence this brain activity and ultimately improve treatment outcome. Regarding this question, it is of interest that executive function training, using inhibitory control and working memory tasks, was able to help children lose more weight and maintain weight loss(20). Combining these trainings with longitudinal fMRI data could further aid to the understanding of changes in executive function present in children with obesity. All in all, **chapter 2 and 3** provide valuable new insights in executive dysfunction of adolescents with obesity.

Looking at the behavioural data presented in **chapter 2,** one of the most remarkable aspects is the variability of the data. Some adolescents with obesity performed markedly worse on executive function testing, while others performed at or above the average of their lean peers. In the latter group, other mechanisms than impaired executive function, seem to cause alternative feeding patterns ultimately leading to obesity. This is in line with the experience of healthcare workers, who see a marked difference in reaction to treatment between those who suffer from, for example, binge eating and children who have a sedentary lifestyle. More carefully defining different behavioural

phenotypes leading to obesity could aid in future research and ultimately aid treatment outcome.

FTO and the reward system

Another matter of concern is that we do not fully understand the underlying cause of differences in brain structure and function. This knowledge can be crucial in prevention and treatment strategies. It has been put forward that structural brain changes are primarily caused by metabolic derangement(21, 22), thereby suggesting that 'nurture' is the major driver of alterations in brain structure and ultimately function. In this regard, however, it should be noted that overwhelming progress was made in understanding how genetics relate to our body composition in the last two decades. Genetic studies in body composition, led to the identification of various genes that are associated with BMI. Some rare mutations, such as mutations in the melanocortin-4-receptor gene, have been shown to be associated with marked hyperphagia and strongly increased weight(23), while more common variants, such as the fat-mass-and-obesity-associated gene (*FTO)* showed smaller effects, but affect a much larger part of the population(24), hereby showing that 'nature' also adds to the equation.

The FTO gene was identified in one of the first genome wide association studies on body composition(24). In a western population, people homozygous for the A risk allele on rs9939609 were on average 3 kg heavier, and people heterozygous for this allele were on average 1.7 kg heavier than people who were homozygous for the wildtype T allele. With 16% of the population in this study being homozygous for the risk allele, and 47% being heterozygous for this allele, these effects, although small, affect a large portion of the population. Therefore, understanding the mechanisms through which *FTO* influences body weight, is crucial.

It was previously shown that *FTO* encodes a 2-oxoglutarate-dependent nucleic acid demethylase(25) and that FTO-gene overexpression leads to increased production of ghrelin, a hunger inducing hormone produced in the stomach, thereby influencing the endocrine communication to the hypothalamus, which is the centre of hunger and satiety signalling(26). These factors only partially explain behavioural patterns associated with the risk allele. They do, however, not explain the relationship of FTO with emotional eating and loss of eating control(27-29), suggesting that higher brain functions were affected as well. In **chapter 4,** the relationship between the FTO-gene risk allele and brain structure was described. Our data show that the FTO risk allele is associated with changes in the dopaminergic reward system. More specifically, the FTO-gene was associated with a smaller volume of the nucleus accumbens, independent of BMI. This suggests that *FTO* does not only affect hunger and satiety signalling but also reward processing. Moreover, it was shown that nucleus accumbens responses to hedonic food pictures were different between risk allele and non-risk allele carriers(30) and that

knockout of *FTO* dysregulated dopamine receptor-dependent control of neuronal activity in the nucleus accumbens(31).

More recent reports on *FTO* have shown that, even in childhood, FTO-genotype is related to appetitive behaviours. It was shown that not only decreased satiety responsiveness, but also increased food responsiveness modulated the relationship between FTO risk allele carriers and BMI. This further underlines that *FTO* alters reward signalling, even at young age(32). Moreover, it was shown that children with AA genotype have increased nucleus accumbens activity while viewing food commercials, suggesting increased responsiveness to rewarding food cues(33). Interestingly, in contrast to the results reported in **chapter 4** this latter study found higher volume of the nucleus accumbens in children, suggesting that *FTO* might influence the rate of neural development at young age, leading to atrophy at later age. Recent fMRI studies in adults have suggested that *FTO* does not only influence reward processing, but also mediates salience processing of food cues and activity of frontal regions involved in exerting control over food intake(34, 35). In summary, combined with the results presented in this thesis, the current literature shows that *FTO* influences brain areas involved in various aspects of feeding behaviour, not only hunger and satiety signalling.

The question remains how pathophysiological knowledge on *FTO* can improve current treatment. Given the differential patterns in feeding behaviour of risk allele carriers shown in previous work, one can argue that, in the future, it would be particularly interesting to learn how differences in *FTO* genotype influences treatment outcome in different treatment regiments. Then, ultimately, with declining costs of genetic testing, genotyping *FTO* could aid in treatment selection for people burdened with obesity.

DIAGNOSTIC WORKUP OF OVERWEIGHT PAEDIATRIC PATIENTS IN CLINICAL PRACTICE

Bone age advancement in obese paediatric patients

Clinicians involved in the diagnostic workup of children with obesity are frequently encountered with the challenge of assessing whether obesity is caused by unhealthy feeding habits solely, or whether underlying pathology contributes to obesity. Endocrine, syndromic and genetic conditions have been shown to influence bodyweight(23, 36-38). Prevalence of these condition, however, is low. Assessment of growth, puberty and bone age have a central place in the diagnostic workup for finding underlying pathology in obese children. The most challenging aspect of the workup in children with obesity is that bone age and growth in height are often advanced without underlying pathology(39-44). Former research has shown various possible factors that influence bone age maturation, including androgens, oestrogens and insulin(39-44). These stud-

ies, however, show variable results, possibly due to the variability in these hormones throughout childhood. Therefore, **chapter 5,** of this thesis describes the relationship between androgens, oestrogens and parameters of insulin resistance with bone age. This study improved on former research by using age and sex specific SDS scores for androgens, oestrogens and bone age. It was shown using multiple regression analysis that increased levels of dehydroepiandrosteron-sulphate (DHEAS) are independently associated with bone age advancement. This suggests that increased production of androgens by the adrenal gland plays a central role in the advanced bone age and concomitant accelerated growth in height. This is in line with a study performed in the past(45). The pathophysiological mechanism through which increased DHEAS levels influence bone age, are likely to be indirect. A plausible mechanism is that increased production of DHEA, of which DHEAS is the inactive derivative, causes higher peripheral conversion to oestradiol, thereby accelerating bone maturation(45).

It has to be noted that the model derived from multiple regression analysis only explained a maximum of 31% of the variance in bone age SDS, indicating that other factors are involved in bone age advancement. Of the factors included in this study, it is of interest that oestrogen, testosterone and parameters of insulin resistance were not related to bone age, while other studies suggest a relationship(42-46). This could be due to the, although widely used in clinical practice, insensitive assays for oestradiol and testosterone used and the fact that we were unable to calculate SDS scores for the insulin parameters. Alternatively, other factors could contribute to advanced bone age, such as leptin and IGF-1(42, 47), although some research suggests that there is no relationship(45). Again, calculation of age and sex specific SDS scores could aid in clarifying this matter.

An interesting secondary result of this study was that the small number of children with suspected monogenic obesity did not seem to differ in bone age SDS or accelerated height, possibly suggesting that similar mechanisms induce advancements in growth and bone maturation. This group, however, was too small to draw conclusions considering the precise mechanisms that underlie bone age advancement in these children.

In conclusion, the results of this part of the thesis suggest that in obese children with accelerated growth in height, combined with advanced bone age and high levels of DHEAS, preferably expressed as SDS, clinicians can consider abstaining from further diagnostic workup. The results of this study suggest that if a patient has increased height and/or bone age SDS, but lacks high levels DHEAS, further investigations are warranted. However, to make this applicable in clinical practice further research should determine reference ranges for DHEAS SDS in relation to bone age SDS.

Predicting impaired glucose tolerance

There is an ongoing debate among clinicians considering the diagnostic work-up of co-morbidity screening in overweight and obese children. This discussion is caused by the need to diagnose children with a high risk of co-morbidity, such as patients with impaired glucose tolerance, on the one hand, and the low diagnostic yield and high costs of performing an extensive diagnostic work-up in all obese children, on the other hand. The reason that identifying glucose derailment early, specifically impaired glucose tolerance, is deemed so crucial, is that it increases the chance of developing type 2 diabetes early in life(48, 49). Therefore, current guidelines recommend intensive treatment and stricter follow up of children with impaired glucose tolerance(50). The problem with identifying children with impaired glucose tolerance is that it is diagnosed via oral glucose tolerance testing (OGTT), which is an invasive and time consuming procedure compared to taking a single blood sample in a fasted condition. Therefore, the current Dutch guideline prescribes assessing fasting glucose as a first step of the diagnostic process(50). If fasting glucose is >5.6 mmol/L, further diagnostics, such as OGTT, should be considered. A vast body of research, however, suggests that the strategy of testing fasting glucose to assess whether further diagnostics are needed misses most cases of impaired glucose tolerance(51-57). Therefore, **chapter 6** was dedicated to investigating whether a combination of simple and cheap parameters, available in everyday practice, could improve the sensitivity of the diagnostic approach. The results indicate that combining fasting glucose with the presence of hypertension and elevation in liver enzymes could significantly improve the sensitivity of finding impaired glucose tolerance, with an acceptable number needed to treat of 5.7. If this finding can be replicated in a second cohort, we would advocate to adjust current guidelines, suggesting to screen for patients at risk for DM with fasting glucose, blood pressure and liver enzymes to evaluate whether they are at increased risk for glucose derailment.

Of specific interest is the finding that a combination of the presence of hypertension and elevated liver enzymes also improved sensitivity, since they are parameters that do not require fasted blood sampling. Development of a diagnostic strategy that waives the need for fasted sampling could improve healthcare logistics, and improve the convenience of patients. Further improvement on this strategy could be found by adding new parameters. In this respect, developments in diabetic adults are of particular interest. In recent years, parameters of systemic inflammation, such as high sensitive C-reactive protein and interleukin 6 have been shown to correlate with type II diabetes in young adult men(58). Given that obese children show signs of systemic inflammation(59), it would be interesting to investigate if they improve the predictive model.

Furthermore, taking individual feeding patterns could be promising for future diagnostic strategies. Recently, for example, it was shown that binge eating can significantly impair insulin sensitivity in healthy young adults(60), showing that individual feeding

patterns, independent of the quantity of calories consumed, might play a role in metabolic derailment found in obesity. To date, however, this has not been investigated in obese children.

In conclusion, screening patients with either elevated blood pressure, elevated liver enzymes and/or impaired fasting can significantly improve the sensitivity of finding children with impaired glucose tolerance and thereby improve the diagnostic strategy, compared to the current guideline.

CONCLUSIONS AND FUTURE RECOMMENDATIONS

This thesis describes investigations into two aspects of obesity research. The first part is dedicated to investigating neural pathophysiological mechanisms contributing to obesity. In this section, it was shown that pallidum size is increased in adolescent obesity and that higher pallidum size is linked to better ability to delay gratification and better inhibitory response in adolescents with obesity (**chapter 2).** Furthermore, in chapter 3, it was shown that resting state connectivity within the executive control network in the lateral occipital gyrus was decreased in obese participants, possibly showing that visual cues might not trigger executive control areas to the same extend as in their lean pears. These data also showed increased connectivity between the primary visual fields and the salience network in adolescent obesity, suggesting that the brain of these youngsters is continuously programmed to forward visual food cues with preference. The obesity associated gene, *FTO*, is associated with nucleus accumbens volume in **chapter 4** showing that the FTO gene is also involved in reward processing, in addition to hypothalamic processing of satiety and hunger.

The second part of this thesis is dedicated to improving the diagnostic strategy of children and adolescents with obesity. **Chapter 5** presents detailed and extensive data on the correlation of various endocrine measures with bone age advancement and shows that DHEAS is a key component in the pathophysiological mechanism of advanced bone age. Finally, the results of **Chapter 6** show that performing oral glucose tolerance testing in all children with either elevated blood pressure, elevated liver enzymes or impaired fasting glucose significantly improves the sensitivity of the diagnostic strategy to find impaired glucose tolerance, compared to the current strategy.

Considering the results of this thesis, future research should focus on investigating the role of the pallidum in executive dysfunction, specifically by applying fMRI-task designs testing various domains of executive function, preferably in subgroups based on feeding patterns and executive dysfunction. Furthermore, task based connectivity studies are needed to improve knowledge on how different areas of the occipital lobe interact with executive function and salience signalling. Considering the new knowledge on the role

FTO plays in brain signalling, future studies should define groups of obese subjects with specific feeding patterns associated with presence of the *FTO* risk allele, and investigate BOLD-response and connectivity differences during tasks that test reward responsivity.

To gain more insight in the pathophysiology of advanced bone age, it is important that future studies include additional parameters and use ultrasensitive assays to detect variance in hormonal levels, since some hormonal levels are undetectable by assays available in clinical practice. Furthermore, it is crucial to calculate age and sex specific SDS to overcome the challenge of hormonal variance throughout childhood and adolescence. Research on diagnostic strategies to detect glucose derailment early, should investigate predictive models including multiple parameters. Furthermore, investigation of feeding patterns, as well as measures of systemic inflammation provide interesting targets for future research.

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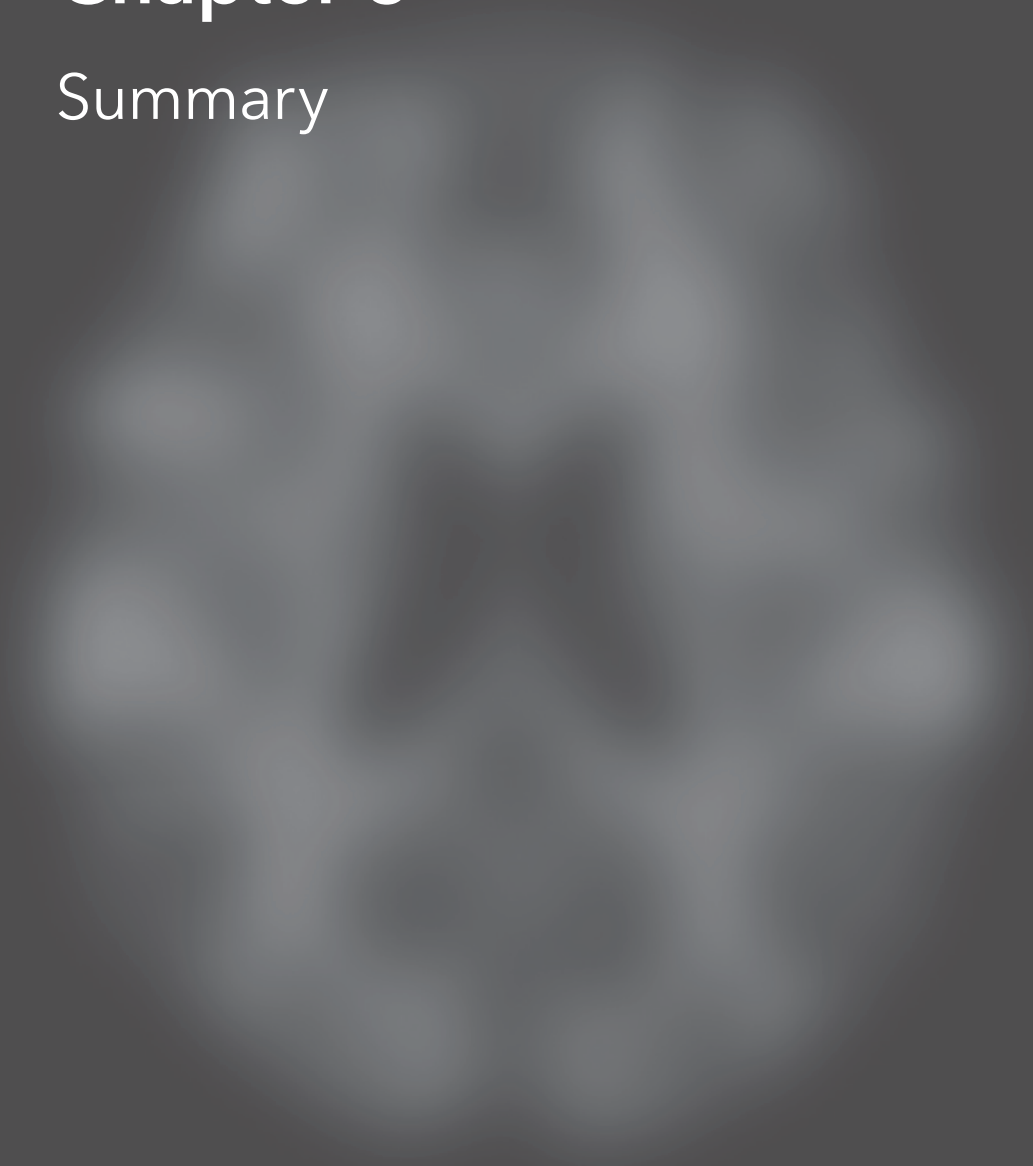
Chapter 8

Summary



ENGLISH SUMMARY

The aim of this thesis is to gain insight in two important aspects of human obesity. First, it aims to investigate structural and functional differences between subjects with and without obesity, specifically the relationship between these differences and behaviour and genetics. Secondly, it investigates the diagnostic strategy for finding causes and consequences of obesity in children, aiming to improve this strategy.

Chapter 1 provides an introduction into the research described in this thesis.

Chapter 2 reports on structural differences in subcortical and cortical brain structures involved in reward behaviour and executive function (brain functions involved in self-regulation) in adolescents with and without obesity and the relationship of these differences with behaviour. 44 adolescents (25 with obesity and 19 without obesity, aged 12-16 years) underwent an MRI-scan of their brain. The volume of various subcortical brain structures, involved in reward behaviour, as well as cortical thickness of structures involved in executive function, were measured. Furthermore, eating behaviour was investigated using a parental questionnaire (the Child Eating Behaviour Questionnaire) and executive functions were assessed using two tasks. The first was a Stop-Signal-Task, in which the ability to inhibit a impulsive response is tested. The second task was a Choice Delay Task, with which the ability to choose a larger, delayed reward over a smaller direct reward was tested. On both tasks it was shown before that children with obesity score lower than their lean peers. The results of this part of the thesis showed larger pallidum size in children with obesity ($p=0.014$, FDR corrected) and a larger amygdala in the uncorrected analysis ($p=0.03$). Specifically in the group of subjects with obesity, a positive association was found between pallidum size and the results of the Choice Delay Task ($p=0.012$). Furthermore, a marginally significant negative correlation between results of the Stop Signal Task and pallidum volume ($p=0.055$) were found. Given that a lower score on the Stop Signal Task means that the ability to inhibit responses is better, the latter result also indicated that larger pallidum volume was associated with better task performance. The pallidum has many functions. For this research it is interesting to know that the pallidum has an inhibitory influence on other reward related structures and is directly connected to several frontal lobe structures involved in executive function. The results of our research suggest that the overall larger pallidum size in these adolescents might be an adaptive mechanism in patients with obesity, aiming to control uninhibited feeding behaviour. In adolescents with obesity and larger pallidum volume it appears, given the results of neuropsychological testing, that this mechanism has developed more successfully. Further research, for instance using fMRI, could help to gain more insight in the specific mechanism through which the pallidum influences executive function.

Chapter 3 describes the findings of research dedicated to exploring differences in functional brain connectivity between adolescents with and without obesity. In connectivity research, the degree of synchronous activity of a brain structure or brain network with other brain structures or networks is investigated. This is used as a proxy of communication between brain structures and/or networks and is usually measured either while performing a task or in rest. In this chapter, the connectivity of 32 adolescents (17 with and 15 without obesity, 12-16 years in age) are investigated in rest, while satiated. These investigations were on the connectivity of three brain structures: the amygdala (involved in reward processing), the pallidum (involved in executive function) and the hypothalamus (involved in hunger and satiety signalling). In addition, the connectivity of three networks was explored: the default mode network (mainly active during wakeful rest), the executive control network (involved in executive function) and the salience network (active when salient events occur in ones environment). In this study it was shown that adolescents with obesity have less functional connectivity in the executive control network of a part of the occipital lobe (the lateral occipital cortex) with the rest of the executive control network. This might suggest that this part of the brain, when triggered by visual food stimuli, activates the rest of the executive control network to a lesser extent. Furthermore, in adolescents with obesity, another part of the occipital lobe, the occipital pole, had increased connectivity with the salience network. This indicates that even in a resting state, the brain of these adolescents is programmed to process visual (food) cues with priority and give more attention to them. In conclusion, in adolescents with obesity, there appears to be a disturbed balance, even in rest and when satiated, with the brain being in a constant state of visual information being regarded more salient and triggering the part of the brain that exerts control over ones actions to a lesser extent.

Variants in the FTO gene have been associated with higher body weight in a wide variety of populations. Studies investigating the pathophysiological mechanism have thus far shown that these variants are associated with alterations in hunger and satiety signalling. There are, however, indications that FTO variants also affect subcortical and cortical signalling. This led to the investigations described in **chapter 4** in which the relationship between the FTO risk allele, RS9939609A, and reward related brain structures was studied in a group of 492 elderly participants, in which MRI and FTO genotyping were performed. It was shown that subjects homozygous for the risk allele A had significantly smaller volume of the nucleus accumbens those homozygous for the wild type T allele. This relationship was independent of BMI. Hereby it was shown that FTO does not only affect hunger and satiety signalling, but also effects reward related behaviour.

The results of investigation in to the pathophysiological mechanism driving advanced bone age in children with obesity are described in **chapter 5.** In many children with obesity, growth in height as well as bone age are advanced. In this patient category, it

is challenging for clinicians to determine whether these advancements can be contributed solely to obesity, or whether an endocrine or genetic cause should be considered. Therefore, more knowledge on the pathophysiological mechanism driving bone age advancement is necessary. In a total of 101 children with obesity, bone age was determined using an X-ray of the wrist and extensive endocrine testing was performed. The results showed that BMI SDS is, as expected, strongly correlated with bone age SDS. In multiple regression analysis, it was shown that dehydroepiandrosteron sulphate (DHEAS) SDS correlated independently with bone age SDS in the total cohort, as well as in subgroups based on sex and pubertal status. A possible pathophysiological explanation for the relationship between the concentration of DHEAS and advanced bone age is that DHEAS signals high levels of DHEA, which is converted at tissue level to oestrogens, thereby contributing to faster maturation of the bone. This research suggests that in paediatric patients with obesity and an advanced bone age and isolated rise in DHEAS, clinician can consider refraining from further diagnostic testing.

Chapter 6 is dedicated to early detection of impaired glucose tolerance and diabetes in children who are overweight or obese. The current Dutch CBO guideline recommends that children who are overweight or obese should be evaluated by an oral glucose tolerance test (OGTT) to rule out impaired glucose tolerance (IGT; two hour glucose in OGTT ≥ 7,8 mmol/L but < 11,1 mmol/L) or diabetes (two hour glucose in OGTT ≥ 11,1 mmol/L) if they have impaired fasting glucose (fasting glucose ≥ 5,6 mmol/L but < 7,0 mmol/L). A substantial amount of evidence has shown that this strategy misses a significant number of cases of IGT and diabetes. Therefore, we investigated whether combining diagnostic parameters, available in everyday practice, could improve the sensitivity of detecting patients with glucose derailment. In this study, a total of 145 overweight or obese paediatric patients the anthropometric data, fasting blood sample and OGTT data were analysed. It was shown that when all children with either elevated blood pressure, elevated liver enzymes or impaired fasting glucose were tested by OGTT sensitivity for detecting IGT would increase from 0.18 to 1.00. Given the fact that IGT in childhood, particularly in patients with ongoing increase in BMI, is predictive of diabetes in (young) adulthood, we advise, if our findings can be replicated in an independent cohort, to adjust the CBO guideline.

Chapter 7 contains the general discussion and conclusion of this thesis. It also gives suggestions for future research in the neuropathophysiology of obesity as well as the diagnostic strategy for children with obesity.

Nederlandse samenvatting

NEDERLANDSE SAMENVATTING

Het doel van dit proefschrift is om inzicht te vergaren in twee belangrijke aspecten van obesitas. Ten eerste heeft het als doel te onderzoeken wat de structurele en functionele verschillen in het brein zijn tussen mensen met en zonder obesitas, in het bijzonder de relatie tussen deze verschillen met genetica en gedrag. Ten tweede onderzoekt het de diagnostische strategie voor het vinden van oorzaken en gevolgen van obesitas bij kinderen, om zodoende deze strategie te optimaliseren.

Hoofdstuk 1 Bevat een introductie met betrekking tot de in dit proefschrift beschreven onderzoeken.

Hoofdstuk 2 rapporteert over de structurele verschillen in subcorticale en corticale breinstructuren die betrokken zijn bij beloningsgedrag en executieve functies (breinfuncties waarmee controle over gedrag wordt uitgeoefend) in adolescenten met en zonder obesitas en de relatie van deze structuren met gedrag. 44 adolescenten (25 met obesitas en 19 slanke controle proefpersonen, 12-16 jaar) kregen een MRI-scan van het brein, waarbij de volumina van een aantal subcorticale structuren, betrokken bij beloningsgedrag, werden gemeten. Verder werd de dikte van de hersenschors gemeten van een aantal corticale structuren die betrokken zijn bij controle over gedrag. Daarnaast werd eetgedrag op verschillende aspecten gescoord met een vragenlijst die door de ouders van de deelnemers werd ingevuld (de Child Eating Behaviour Questionaire; CEBQ) en werden de executieve functies getest met een tweetal taken. De eerste betrof een stop-signaal-taak, waarbij het vermogen tot remming van een impulsieve respons wordt getest. De tweede betrof een uitgestelde-beloning-taak, waarmee het vermogen om een grote, uitgestelde beloning te verkiezen boven een kleine, directe beloning wordt getest. Zowel van responsinhibitie als van het vermogen om beloning uit te stellen is eerder aangetoond dat kinderen en adolescenten met obesitas hier lager op scoren. De resultaten van dit onderzoek toonden dat het pallidum, een subcorticale hersenstructuur betrokken bij beloningsgedrag, van adolescenten met obesitas significant groter is ($p=0.014$, FDR gecorrigeerd) en dat de amygdala, een subcorticale hersenstructuur betrokken bij beloningsgedrag en angst, in de ongecorrigeerde analyse groter was in deze groep ($p=0.03$). Specifiek in de groep proefpersonen met obesitas werd voor het pallidum een positieve associatie gevonden met de uitkomst van de uitgestelde-beloning-taak; in deze groep was een groter volume van het pallidum gecorreleerd met een hogere score op de uitgestelde beloning taak ($p=0.012$). Daarnaast was er een negatieve correlatie tussen de uitkomst van de stop-signaal-taak en het volume van het pallidum met een trend naar significantie ($p=0.055$). Omdat een lage score op de stop-signaal-taak een betere responsinhibitie inhoud, was er ook hier sprake van dat een groter volume van het pallidum geassocieerd is met een betere uitkomst van de taak. Het pallidum heeft vele functies. Een van de voor dit onderzoek interessante functies is dat

het een remmend effect heeft op andere structuren van het beloningssysteem en ook directe verbindingen heeft met diverse structuren van de frontaalkwab, die betrokken zijn bij de controle van het gedrag. De uitkomsten van dit onderzoek suggereren dat het algeheel grotere volume van het pallidum bij adolescenten met obesitas mogelijk een adaptatie is, als contramechanisme tegen ontremd eten. Bij adolescenten met obesitas bij wie het pallidum volume groter is, lijkt, in neuropsychologisch onderzoek, dit corrigerend mechanisme beter te zijn ontwikkeld. Verder onderzoek, bijvoorbeeld met functionele MRI, zal in de toekomst moeten uitwijzen wat het specifieke mechanisme is waarmee het pallidum executieve functies beïnvloed.

Hoofdstuk 3 beschrijft de bevindingen van onderzoek naar verschillen in connectiviteit in het brein tussen adolescenten met en zonder obesitas. Bij connectiviteitsonderzoek wordt van een breinstructuur, of netwerk van breinstructuren, de mate waarin activiteit synchroon met andere structuren optreedt bepaald. Hieruit wordt communicatie tussen breinstructuren of netwerken, bij het uitvoeren van een taak, of juist in rust, afgeleid. In dit onderzoek werden 32 adolescenten (17 met obesitas en 15 slanke controle proefpersonen, 12-16 jaar) gescand in rust, kort na het ontbijt. Er werd gekeken naar de connectiviteit van 3 hersenstructuren namelijk: de amygdala (belangrijk bij beloningsgedrag), het pallidum (betrokken bij executieve functies) en de hypothalamus (betrokken bij het signaleren van honger en verzadiging). Daarnaast werd de connectiviteit bepaald van 3 netwerken aan breinstructuren, namelijk het default mode netwerk (vooral actief wanneer men niet met een specifieke taak bezig is), het executieve controle netwerk (betrokken bij controle over gedrag) en het salience netwerk (actief bij het signaleren van opvallende gebeurtenissen). In dit onderzoek werd aangetoond dat een deel van de occipitaalkwab (de visuele hersenschors) binnen het executieve controle netwerk, minder connectiviteit vertoonde met de rest van het netwerk bij adolescenten met obesitas. Dit suggereert mogelijk dat visuele informatie (zoals het zien van voeding), die via de visuele hersenschors binnenkomt, de gebieden die gaan over controle van gedrag minder activeren bij adolescenten met obesitas. Daarnaast werd gezien dat een ander deel van de occipitaalkwab sterkere connectiviteit had met het salience netwerk in de groep adolescenten met obesitas. Dit doet vermoeden dat het brein van deze adolescenten, zelfs in rust en terwijl een proefpersoon wel gevoed is, vatbaar is om visuele prikkels, zoals visuele voedselprikkels, extra aandacht te geven. Concluderend lijkt het erop dat, zelfs in rust en terwijl er geen honger wordt ervaren, het brein van adolescenten met obesitas geprogrammeerd is om visuele voedselprikkels meer aandacht te geven en over het binnen komen van die prikkels minder controle uit te oefenen.

In **Hoofdstuk 4** wordt de relatie beschreven tussen een gen waarvan is aangetoond dat varianten in dit gen leiden tot een hoger gewicht (het, fat-mass-and-obesity-associated gen of FTO-gen) en volumes van het beloningssysteem in het brein. Eerder werd in een aantal studies aangetoond dat het FTO-gen invloed had op het signaleren

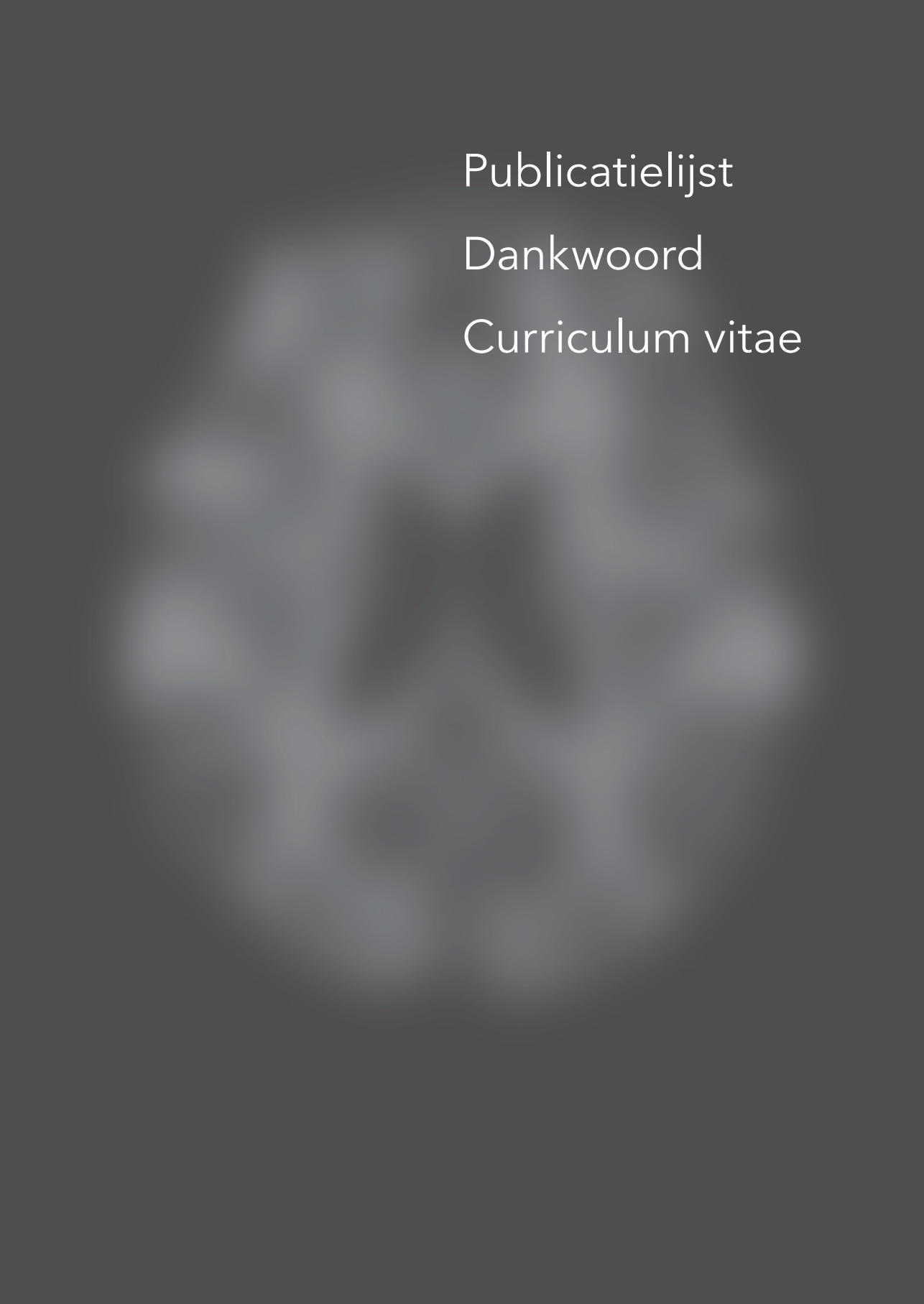
van honger en verzadiging. In het in dit proefschrift beschreven onderzoek werd in een groep van 492 ouderen, waarbij zowel een MRI was gemaakt als genotypering van FTO had plaatsgevonden, aangetoond dat mensen met twee A risicoallelen op positie RS9939609 een significant kleinere nucleus accumbens, een subcorticale hersenkern met een centrale rol in beloningsgedrag, hebben dan mensen met twee T wild type allelen. Deze relatie was onafhankelijk van BMI. Hiermee werd aangetoond dat niet alleen de signalering van honger en verzadiging wordt beïnvloed door FTO, maar dat waarschijnlijk ook beloningsgedrag wordt beïnvloed door FTO.

De resultaten van het onderzoek naar het pathofysiologische mechanisme achter voorlopende botleeftijd bij kinderen met obesitas zijn beschreven in **hoofdstuk 5**. Bij veel kinderen met obesitas is sprake van voorlopende lengtegroei ontwikkeling gecombineerd met voorlopende skeletleeftijd. Voor clinici is het moeilijk om bij kinderen met obesitas, die zowel voorlopen in lengtegroei als in skeletleeftijd te bepalen of dit het gevolg is van obesitas, of dat er ook een endocriene of genetische oorzaak overwogen moet worden. Daarom is meer inzicht in de pathofysiologie van dit verschijnsel wenselijk. In totaal werd bij 101 kinderen met obesitas de skeletleeftijd bepaald met een röntgenfoto en werd uitgebreid bloedonderzoek verricht. Er werd aangetoond dat BMI SDS sterk gecorreleerd is met skeletleeftijd SDS. In multipele regressieanalyse werd aangetoond dat dehydroepiandrosteron sulfaat (DHEAS) SDS in het totale cohort, maar ook in de subgroepen met vrouwelijke, mannelijke en pubertaire patiënten onafhankelijk positief gecorreleerd is met skeletleeftijd SDS. Een mogelijke pathofysiologische verklaring voor de relatie tussen hogere concentraties DHEAS en voorlopende skelet is dat hoge concentratie DHEAS een indirect gevolg zijn van een hoge concentratie dehydroepiandrosteron (DHEA), wat op weefselniveau in de botten wordt omgezet naar oestrogenen, die weer zorgen voor een snellere uitrijping van het bot. Dit onderzoek suggereert dat bij patiënten met obesitas en een voorlopende botleeftijd, met bij initieel onderzoek alleen een verhoogd DHEAS, wellicht afgezien kan worden van verdere diagnostiek.

In **hoofdstuk 6** worden de resultaten beschreven van onderzoek naar vroege detectie van verminderde glucosetolerantie (Engels: impaired glucose tolerance, afgekort IGT) van kinderen met overgewicht en obesitas. De huidige CBO richtlijn adviseert bij kinderen met overgewicht en obesitas aanvullend onderzoek middels een orale glucose tolerantie test (OGTT) te doen naar IGT (twee-uurs glucose in OGTT ≥ 7,8 mmol/L maar < 11,1 mmol/L) en diabetes (twee-uurs glucose in OGTT ≥ 11,1 mmol/L) bij patiënten die een verhoogd nuchter glucose hebben (nuchter glucose ≥ 5,6 mmol/L maar < 7,0 mmol/L). Een omvangrijke hoeveelheid literatuur laat zien dat met deze strategie veel gevallen van (vroege) glucoseontregeling worden gemist. Derhalve werd onderzocht of het combineren van gegevens, die bij pediatrische patiënten met obesitas standaard gescreend worden, een betere detectie van glucose ontregeling oplevert. In totaal

werden van 145 pediatrische patiënten met overgewicht en obesitas de antropometrische gegevens, gegevens van nuchtere screening en orale glucose tolerantie testen geanalyseerd. Hierbij werd gevonden dat wanneer alle kinderen met een verhoogde bloeddruk boven de p95 of verhoogde levertransaminasen of verhoogde nuchtere glucose waarden onderzocht zouden worden middels OGTT, de sensitiviteit voor het vinden van IGT zou stijgen van 0,18 naar 1,00. Gezien het feit dat aanwezigheid van IGT op de kinderleeftijd, zeker als BMI stijgt, voorspellend is voor diabetes mellitus op (jong) volwassenleeftijd, adviseren wij, indien deze resultaten in een onafhankelijk cohort gerepliceerd kunnen worden, de richtlijn aan te passen en alle kinderen met verhoogde bloeddruk, of verhoogde levertransaminasen of verhoogd nuchter glucose te onderzoeken middels OGTT.

Hoofdstuk 7 bevat een algemene discussie en conclusie van dit proefschrift. Ook schetst het de toekomstperspectieven voor het onderzoek naar de neurologische pathofysiologie van obesitas en de diagnostische strategie van kinderen met obesitas.



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CURRICULUM VITAE

Cornelis Jan de Groot was born on April 19th 1984 in Gorinchem. After he completed secondary school at the Oude Hoven Lyceum in Gorinchem he started his medical training in 2002 at the Vrije Universiteit Amsterdam. During the last period of his internships he focussed on paediatrics and obtained his medical degree in 2009. After graduation, he started working as a paediatric resident at the Tergooi Hospital in Blaricum in 2009 and subsequently at the Leiden University Medical Center in 2011. In May 2012 he started as a PhD candidate at the Willem-Alexander Children's Hospital of the Leiden University Medical Center under the supervision of prof. dr. Henriette Delemarre-van de Waal, prof. dr. Frans Walther and dr. Jeroen van der Grond. After the demise of prof dr. Henriette Delemarre-van de Waal at February 13th 2014 and the emigration of prof. dr. Frans Walther, prof. dr. Edmond Rings and dr. Erica van den Akker were added to his supervision team. This thesis describes the research performed during his period as a PhD candidate.

In May 2016 Cornelis Jan started his clinical training in paediatrics at the Willem-Alexander Children's Hospital under supervision of dr. W.J.W. Kollen till November 2017 and under the supervision of dr. R.G.M. Bredius from November 2017 onward. Currently he is continuing his training at the Groene Hart Hospital in Gouda under the supervision of dr. Kramer-van Driel.

Cornelis Jan married Marit van Meegen on May 31st 2013. They have two daughters: Sara, born July 14th 2014 and Noor, born November 11th 2017.