Genetische Mechanismen der Gewichtsregulation: genomweite Ansätze und Kandidatengenstudien

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Genetic mechanisms of body weight regulation: Genome-wide approaches and candidate gene studies

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AAR	adiposity rebound
ACTH	adrenocorticotropin
ADHD	attention-deficit/hyperactivity disorder
AGA	Arbeitsgemeinschaft Adipositas im Kindes- und Jugendalter
AgRP/AGRP	agouti-related protein
AGTL1	angiotensin receptor-like 1
AN	anorexia nervosa
BDNF	brain derived neurotrophic factor
BMI	body mass index
BN	bulimia nervosa
CDKN2A/B	cyclin-dependent kinase inhibitor 2A/B
CHD	coronary heart disease
cM	centi Morgan
CNTF	ciliary neurotrophic factor
DAG	Deutsche Adipositasgesellschaft
db-/-	diabetes mouse
DGAT1/2	diacylglycerol acyltransferase 1 and 2
DGI	Diabetes Genetics Initiative
dHPLC	denaturing high pressure liquid chromatography
DNA	deoxyribonucleic acid
ECSP	extreme concordant sibpair approach
ENPP1	ectonucleotide pyrophosphatase/phosphodiesterase
FTO	fat mass and obesity-associated
GAD2	glutamate decarboxylase 2
GALN	galanin
GHSR	growth hormone secretagogue receptor
GIST	genotype IBD sharing test
GLUT4	glucose transporter 4
GWAS	genome wide association study
HBSC	Health Behaviour in School-aged Children
IBD	identical by descent
INSIG2	insulin induced gene 2
KCTD15	potassium channel tetramerisation domain
GNPDA2	glucosamine-6-phosphate deaminase 2
kg	kilogram
KiGGS	Kinder- und Jugendgesundheitssurvey

LD	linkage disequilibrium
LOD	logarithm of the odds
m	meter
MAF	minor allele frequency
MAF	v-maf musculoaponeurotic fibrosarcoma oncogene
MAPK	mitogen-activated protein kinase
MC4R/MC1R	melanocortin 4 receptor/ melanocortin 1 receptor
MLB LOD	maximum likelihood binomial logarithm of the odd
MSH	melanocyte stimulation hormone
MTCH2	mitochondrial carrier homolog 2
MZ	monozygotic
NEGR1	neuronal growth regulator 1
NHANES	Nutrition Examination Survey
NIDDM	non insulin dependent diabetes mellitus
NPC1	Niemann-Pick disease, type C1 precursor
NT4	neurotrphin 4
NTKR2	neurotrophic tyrosine kinase, receptor, type 2
ob-/-ob/ob	obese mouse
PC/PCSK	prohormonconvertase
PCT	percentile
POMC	pro-opiomelanocortin
PPARγ	peroxisome proliferator-activated receptor gamma
PTER	phosphotriesterase related
RCT	randomised controlled trial
ROC	receiver operating characteristic
SDS	standard deviation score
SH2B1	SH2B adaptor protein 1
SLC16A14	solute carrier family 16, member 14
SNP	single nucleotide polymorphism
SSCP	single strand confirmation polymorphism
TBC1D1	TBC1 (tre-2/USP6, BUB2, cdc16) domain family
TDT	transmission/disequilibrium test
TG	triglycerides
TMEM18	transmembrane protein 18
TRKB	tyrosine kinase B
UCP2/3	uncoupling protein 2 and 3
UTR	untranslated region
VHA	ventromedial hypothalamic area

WAGR	wilms' tumor, aniridia, genitourinary anomalies, and mental retardation
WHO	World Health Organization
WTCCC	Welcome Trust Case Control Consortium

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1. Introduction

Obesity has reached epidemic proportions in many countries around the world (WHO, 1998). It is a major health risk for adults and increasingly for children in developed countries. Overweight and obesity in childhood represent a major risk factor for several common disorders including type 2 diabetes mellitus (NIDDM), coronary heart disease (CHD), and cancer. The socio-economic impact of obesity is considerable. While environmental factors are contributory, there is major evidence for genetic factors underlying severe obesity (Hebebrand et al., 2001).

1.1 Obesity

1.1.1 Definition and classification of obesity

Obesity is defined as an excessive accumulation of body fat. The proportion of fat mass to body mass (body mass = lean mass + fat mass) of an individual is increased. The body mass index (BMI) is used to define and classify obesity. It is used as a measure for relative weight that is largely adjusted for body height (Watson et al., 1979).

$$\text{Body mass index (BMI)} = \text{body weight (kg)} / \text{body height (m}^2\text{)}$$

In the mean BMI correlates well with the amount of body fat (Dietz et al., 1998) and is therefore, a good measure for obesity. In adults, weight classes are defined by BMI (Table 1).

Table 1: Weight classes for adults (WHO, 1998)

weight class	BMI in kg/m^2
underweight	< 18.5
normal weight	18.5 – 24.9
Overweight	≥ 25.0
pre-obese	25.0 – 29.9
obesity	≥ 30.0
obesity class I	30.0 – 34.9
obesity class II	35.0 – 39.9
obesity class III	≥ 40.0

Obesity in adults is defined as a BMI ≥ 30 kg/m² whereas extreme obesity is defined by a BMI ≥ 40 kg/m² (WHO 1998). However, the BMI has limitations. Despite the high correlation of BMI with fat mass, Body fat can for instance be overestimated in individuals with a high

muscle mass and can be underestimated in individuals with a reduced lean body mass – for example in elderly people.

In childhood and adolescence fat mass and lean body mass show stronger relative fluctuations than in adulthood (Gray 1989). Hence, percentile curves representing age and gender matched BMI distributions are used to define different weight classes for children and adolescents (Figure 1; Hebebrand et al., 1994).

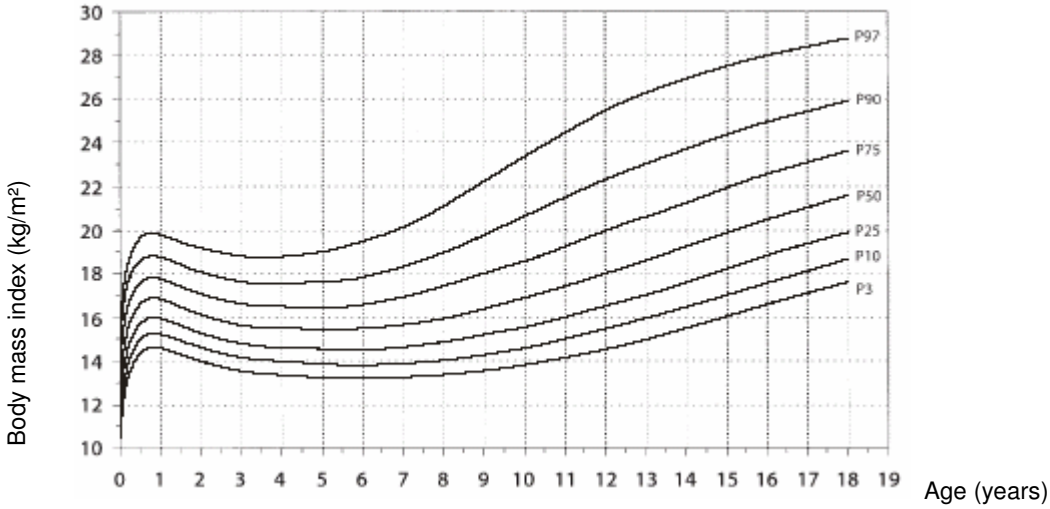

Figure 1: Example of BMI percentiles for boys at the age of 0-18 years (Kromeyer-Hauschild et al., 2001)

In childhood and adolescence the 90th and 97th percentile are used for definition of overweight and obesity in Germany (Kromeyer-Hauschild et al., 2001; http://www.mybmi.de, http://www.a-g-a.de). Additionally, the standard deviation score (SDS) can be used to quantify the degree of BMI deviation of an individual from the mean BMI in an age and gender matched normal population (Cole and Green, 1992). Calculations of SDS values for instance allow comparisons of BMI values within a study group of extremely obese children and adolescents.

1.1.2 Prevalence of obesity

During the last two decades, the prevalence of obesity increased worldwide. Obesity is becoming a major health problem, especially in developed countries and advanced developing countries. The cross-national survey “Health Behaviour in School-aged Children (HBSC)” was conducted in collaboration with the WHO and is based on nationally representative school-surveys of adolescents in Europe, Israel, and the USA in 1997-1998. This study showed that the prevalence of obesity (BMI ≥95th percentile) in 15 year old boys and girls is highest in the USA (13.9 % and 15.1 %) and lowest in Lithuania (0.8 % and 2.1 %; Lissau et al., 2004). The study reference standard was based on the 29.242 observations from all 15 participating countries. Cut-off points for overweight and obesity were determined

by BMI values at or above the 85th and 95th BMI percentiles for defined age groups. In this study, 15 year old boys and girls from Germany met the expected average value: 14.2 and 14.8 % of boys and girls were found to be overweight (BMI ≥85th percentile) whereas 5.4 % and 5.1 % were obese (BMI ≥95th percentile). Comparing the results from the 1999-2002 National Health and Nutrition Examination Survey (NHANES) with the results from NHANES II (1976-1980) and III (1988-1994), the prevalence of early onset obesity for different age and gender groups increased in the United States in the last 25 years from 2.3 to 3.3 fold (Ebbeling et al., 2002).

In 2007, the German Health Interview and Examination Survey for Children and Adolescents (KiGGS) provided the first representative national data on overweight and obesity in children and adolescents (Kurth et al., 2007). Between ages 3 and 17, 15% of the participants exceeded the 90th BMI percentile and were thus overweight, 6.3% exceeded the 97th BMI percentile and thus fulfilled the definition of obesity. Kurth et al. (2007) based their cut-offs for overweight and obesity on a joint analysis of non-representative samples (Kromeyer-Hauschild et al., 2001). A second national survey will be needed to truly investigate secular trends for overweight and obesity. Kurth et al reported that children were at a higher risk of being overweight or obese if they had a lower socioeconomic status, a migration background, or overweight mothers. No clear differences were detected between boys and girls or between East and West Germany.

1.1.3 Causes of obesity

Obesity is a complex multifactorial disease that occurs as a result of a combination of genetic, environmental and psychological factors. Obesity is seemingly caused by a positive energy balance that persists for a prolonged time period.

Altogether, the worldwide increase in obesity prevalence rates in the last decades presumably results from changes in life style which is reflected in decreased physical activity, and increased energy intake (Ebbeling et al., 2002). A detailed analysis of data from the United States revealed that just a part of the population responded with an increase in body weight to these changes in life style (Troiano et al., 1998). The 3rd, 10th and 50th BMI percentiles have remained nearly stable over time, whereas BMI values constituting the 90th and 97th percentiles are clearly increasing. An individual genetic predisposition to increased body weight in response to altered environmental factors is assumed to be the cause of this increase. During human evolution, when food was not easily available, individuals who had a very efficient system to utilize and store energy presumably had an advantage in survival. From an evolutionary point of view a genetic predisposition to obesity might have been advantageous in periods of decreased food availability. As a consequence, the human genome is presumably enriched with genetic variations that favour the storage of energy and

diminish energy expenditure (“thrifty genotype hypothesis”, Neel 1962). Today’s availability of cheap high-caloric food and the sedentary lifestyle result in a maladaptation of the physiological mechanisms formerly increasing chances of survival during famines (Hebebrand et al., 2000).

Taken together, the alteration of environmental factors in combination with a genetic predisposition for obesity can be viewed as an explanation for the epidemic increase of overweight and obesity.

1.1.4 Therapy

Following the guidelines of the German Society of Obesity (*DAG, Deutsche Adipositasgesellschaft*) the indication for obesity therapy in adults is given, if one of the following criteria is met:

1. BMI $\geq$ 30 kg/m^2
2. BMI between 25 and 29.9 kg/m^2 and additionally one of the following
    - health problems related to overweight (e.g. NIDDM, hypertension)
    - visceral body fat distribution
    - diseases aggravated by overweight
    - substantial psychosocial complaint

The indication for obesity therapy in children and adolescents is illustrated in figure 2. In light of the obesity epidemic and its dire health consequences the need for successful programs for treatment of obesity in childhood and adolescence is large. In contrast to adults, a growing child can achieve a BMI reduction by maintaining weight; a reduction in SDS-scores for BMI can even be accomplished if BMI increases over time. Children, adolescents and their parents need to learn that treatment effects in terms of weight loss are usually rather small and that ongoing efforts are required to prevent further or renewed weight gain.

As of today, only a very limited number of randomised controlled trials (RCT) of lifestyle interventions have been completed (Summerbell et al., 2005). In their Cochrane analysis based on 18 RCTs with 975 participants aged < 18 years, Summerbell et al. (2005) concluded that the respective studies were very small and included only homogeneous, motivated groups in hospital settings. As such, common evidence was limited and no direct conclusions for treatment of childhood obesity could be drawn.

In many conventional treatment studies the assessment of potential medium and long-term side effects has been ignored. Frequently, data on only successful completers are presented (e.g. percentage of subjects who have lost 5% or 10% of their body weight) at the end of a relatively short treatment episode. However, treatment of children and adolescents should entail that data on adverse outcomes be reported in detail; obviously medium and long-term

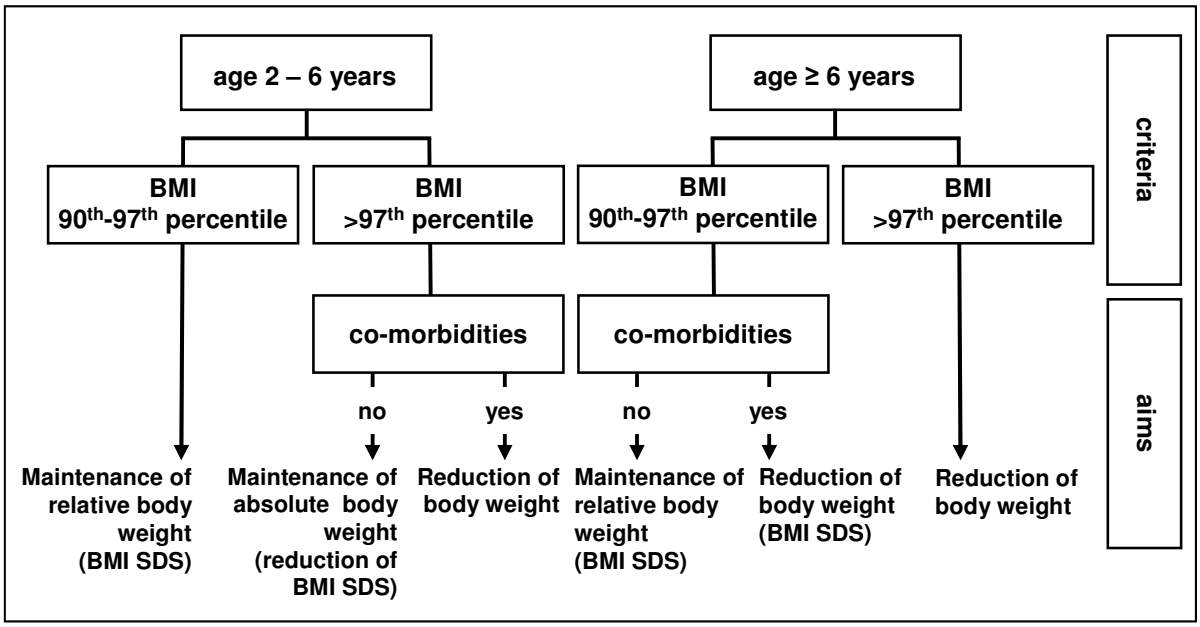

Figure 2: Criteria and aims for obesity therapy in children and adolescents, given by the guidelines of the task force obesity in childhood and adolescence (*AGA, Arbeitsgemeinschaft Adipositas im Kindes- und Jugendalter*)

follow-ups should be attempted. Conventional obesity treatment can result in transient curtailment of height growth velocity (Epstein et al., 1990). Sensible weight loss practices do not entail an elevated risk for the development of eating disorders (Butryn et al., 2005). Dieting has been associated with increased weight gain (Stice et al., 1999; Field et al., 2003). Elevated mortality rates have been associated with weight cycling in adults (Jeffery 1996; Wannamethee et al., 2002). However, this association has been attributed to disadvantageous lifestyle factors and pre-existing disease. Wannamethee et al. (2002) concluded that weight loss and weight fluctuation (cycling) does not directly increase the risk of death. However, Soerensen et al. (2005) revealed that intentional weight loss was linked to increased mortality. Obviously, the health effects of weight loss are complex and more research is needed. This holds particularly true for children and adolescents.

Currently available pharmacological interventions do not produce permanent changes in metabolism or behaviour. Hence, lifelong medication might be indicated. The two anti-obesity drugs (Sibutramine, Orlistat) currently available in Germany produce only modest weight losses, ranging from 3 to 7 % of initial body weight (Glazer 2001; van Gaal et al., 2005; Pi-Sunyer et al., 2006). Irrespective of the type of drug, long-term treatment will most likely be required, if the weight loss is to be maintained.

2. State of the art

2.1. Formal genetic findings

Body weight is a complex multifactorial phenotype which is determined by a combination of genetic factors and environmental conditions. Environmental conditions may be further divided into behavioural, cultural, and socioeconomic factors (Hebebrand and Remschmidt, 1995). The genetic influence on body weight regulation has been demonstrated by numerous twin, adoption, and family studies.

There is a general consensus that parental obesity is by far the strongest risk factor for childhood and adolescent obesity. The risk is influenced by the degree of parental obesity (Whitaker et al., 2004) and is further elevated if both parents are obese (Reilly et al., 2005). According to a number of studies, offspring BMI is somewhat more strongly correlated with maternal than paternal BMI (Magnusson and Rasmussen, 2002), suggesting intrauterine influences, imprinting effects and/or a rearing effect. Formal genetic studies have led to the conclusion that the strong predictive value of parental BMI mainly stems from genetic rather than environmental factors (Maes et al., 1997).

Twin studies (Maes et al., 1997; reviewed in: Hebebrand et al., 2000) have reported the most consistent and highest heritability estimates in the range of 0.6 to 0.9 for explained variance of BMI. These heritability estimates apply to both twins reared together and apart. While the majority of studies were conducted in twins reared together, some of which included thousands of twin pairs, only single studies with small sample sizes exist for twins reared apart. Additionally, a substantial number of twins reared apart were not separated immediately after birth. Except for this newborn period where the influence of the intrauterine environment is strong, age does not seem to affect heritability estimates of body weight to a substantial degree. For example, a heritability of body weight of 0.4 was calculated for the newborn period (Vlietinck et al., 1989). Similar findings exist for other anthropometric measurements such as body height where a smaller correlation was observed in the infant than in the childhood period in monozygotic (MZ) twins. Thus, higher heritability of body weight and BMI in e.g. school -age children may mirror a larger impact of genetic factors. Possibly, the heritability of BMI is maximal (≈ 0.9) during late childhood and adolescence (Pietilainen et al., 1999).

However, most adoption and family studies have reported considerably lower heritability estimates of BMI in the range of 0.25 to 0.7 (Maes et al., 1997; Hebebrand et al., 2004). The difference in heritability estimates may be related to age effects which are better controlled for in twin studies. Moreover, twin studies are more valid if non-additive genetic factors play a larger role in body weight regulation. For an adequate interpretation of the heritability

estimates it is noteworthy to point out that both direct and indirect genetic effects are subsumed under the genetic component. If for example both infant twins of a MZ pair are frequently irritable due to a biologically driven hunger (direct genetic effect), frequent feedings by the caretaker ensue (indirect genetic effect); even if the twins are separated at birth, the respective caretakers can be expected to respond similarly.

Another interesting and important aspect of formal genetic studies has been the observation that non-shared environment explains considerably more variance of the quantitative phenotype (BMI) than shared environment. In the large twin study of Stunkard and coworkers (Stunkard et al., 1990), which encompassed adult twin pairs reared together or apart, shared environment did not explain variance at all; instead non-shared environment totally explained the environmental component, estimated at 30%. Accordingly, only genetic factors would account for a familial loading with obesity. However, more recent studies indicate that the shared environment might play a more substantial role after all (Allison et al., 1996; Plomin et al., 1997); past research may have underestimated common environmental effects on BMI because the designs lacked the power or ability to detect them. Finally, the environment of modern day societies (easy access to a large variety of cheap and tasty foods, a life style promoting physical inactivity) is quite similar for basically all children, irrespective of the family in which they grow up.

The complexity of the genetic basis of obesity emerges from different sources (Hebebrand et al., 2004): Metabolic phenotypes including resting energy expenditure are partially under genetic control (Bosy-Westphal et al., 2008). Behavioural genetic research has convincingly demonstrated that approximately 50% of the variance of diverse complex quantitative behaviours is genetically determined (Plomin et al., 1997). Both macronutrient intake (Reed et al., 1997) and physical activity levels (Perusse et al., 1989) have been shown to be genetically co-determined. Restrained eating, drive for thinness and other eating behaviours show heritability estimates in the range of 20 to 55% (Hebebrand et al., 2004). It appears that television viewing may have an - albeit small - heritable component (Plomin et al., 1990).

Because the gene pool of a population cannot have changed within the past generation, environmental changes affecting both energy intake and expenditure are assumed to underlie the obesity epidemic (Taubes 1998). These changes are presumed to have a major impact because according to the thrifty genotype hypothesis (Neel et al., 1998) many common genotypes render humans obesity prone: Gene variants facilitating energy deposition as fat have accumulated over time in different species to enhance survival during periods of famine.

Epigenetic phenomena have also been invoked to contribute to the obesity epidemic. Indeed, it is conceivable that modern-day living might affect methylation patterns of specific genes,

which in turn increases the risk of obesity. In line with these considerations young monozygous twins are epigenetically indistinguishable from each other during the early years of life, whereas remarkable differences in their overall content and genomic distribution of 5-methylcytosine DNA and histone acetylation with an effect on gene-expression become evident with increasing age (Fraga et al., 2005). Such environmentally induced changes could have an influence on BMI.

2.2 Candidate gene studies

Almost all known metabolic pathways involved in body weight regulation were discovered in rodents. Most of the monogenic forms of human obesity were detected in mice - either as spontaneous mutations or in knockout models. Although only a small part of all obese animal models is in concordance with a monogenic inheritance, they give an important insight in the complex endocrine and metabolic pathways involved in body weight regulation. Two approaches are commonly used to investigate genetic mechanisms involved in body weight regulation. In candidate gene approaches, one selectively explores genes with a known role in metabolism based on prior information such as animal models. Genome-wide linkage and association analyses as the second approach will be introduced in a later chapter.

Association studies on genetic variants in candidate genes for obesity serve to assess correlations between genetic variants at a polymorphic site and an investigated phenotype. Such variants can either be directly involved in disease predisposition or indirectly involved through linkage disequilibrium with pathogenic variants in close vicinity.

In classical genetic association studies one usually compares genotype or allele frequencies in a group of cases and a group of controls. These studies are often criticized as positive association can result from factors different from the genetic variation leading to false-positive findings. One of the most frequently discussed factors is population stratification. Individuals with and without the investigated phenotype may possibly derive from different population subgroups which might also differ in allele frequencies (Lander and Schork, 1994). Analysing a mixed sample of different subpopulations may then result in a (false) positive finding. Although careful selections of case and control groups may help to reduce this problem, positive genetic association studies should at least be confirmed in a second sample. Family-based samples are appropriate for confirmation as they are not influenced by underlying stratification effects when statistical tests like the Transmissions-Disequilibrium Test (TDT) are applied

The TDT (Spielman et al., 1993) is a test for association in the presence of linkage. Typically, TDT study groups comprise so called trios or triads - one affected index patient and both biological parents. The analysis makes use of heterozygous parents. For each trio, one may

count the number of times an allele was transmitted and not transmitted from the parents to the child. In the case of no linkage or no association, the ratio of allele transmissions to allele non-transmissions is expected to be 1. Significant deviations from 1 indicate linkage and association to the investigated phenotype (Ott et al., 1989). However, one disadvantage that is common to all genetic association studies including TDT studies lies in the fact that it cannot be discriminated between associations due to linkage disequilibrium (LD) between alleles on the same haplotype and associations due to the functional variant itself. The following paragraphs summarize different genetic forms of obesity.

2.2.1 Dominant forms of monogenic obesity

In humans, one of the first reported forms of dominant monogenic obesity is due to the mutated peroxisome proliferative activated receptor gamma 2 gene (*PPARγ2*), an important determinant of adipogenesis. Ristow et al. (1998) described four subjects of German origin with a mutation (Pro115Gln) in the N terminus of the nuclear hormone receptor. This mutation leads to a receptor, which interferes with a negative regulatory site in the molecule. All subjects with the mutant allele were markedly obese, with BMI values ranging from 37.9 to 47.3 kg/m^2. Functional studies showed that a Pro115Gln mutation in *PPARγ2* leads to an acceleration of the differentiation of adipocytes. The Pro115Gln mutation is seemingly exceedingly rare, as subsequent studies did not find the variant in further study groups of obese und normal weight individuals (Clément et al., 2000, Hamer et al., 2002).

In humans and animals, one of the best investigated forms of dominant obesity is represented by (partial) deficiency in the melanocortin-4-receptor (MC4R). The MC4R is a hypothalamic receptor and target of the anorexigenic neuropetide alpha-melanocyte-stimulating hormone (α-MSH). Huszar et al. (1997) were able to show that the inactivation of the *Mc4r* in mice resulted in a maturity-onset obesity syndrome associated with hyperphagia, hyperinsulinemia and hyperglycinemia. Male adult *Mc4r*$^{-/-}$ deficient mice are on average 50% heavier than matched wild type controls. The body weight of heterozygous carriers ranged between homozygous mutation carriers and wild type controls.

In humans, the first families in which heterozygous mutations in the *melanocortin-4-receptor gene* (*MC4R*) were associated with dominantly inherited obesity were reported in 1998 and 1999 (Vaisse et al., 1998; Yeo et al., 1998; Hinney et al., 1999). Until now, several missense, frameshift- and nonsense mutations leading to haploinsufficiency of the receptor have been detected (Vaisse et al., 1998; Vaisse et al., 2000; Yeo et al., 1998; Hinney et al., 1999, 2003, 2006; Farooqi et al., 2000; Jacobson et al., 2002; Stutzmann et al.,, 2008). Hinney et al. (2003) screened the *MC4R* in 808 extremely obese children and adolescents and 327 underweight or normal-weight controls. A total of 15 obese patients carried at least one functionally relevant mutation (frequency of 1.86 %), whereas no functionally relevant

mutations were found in normal weight controls. Carriers of the mutations leading to haploinsufficiency or dominant negative effects were extremely obese, but no obvious clinical or endocrinologic abnormalities were found. Until today, more than 80 non-synonymous mutations in the *MC4R* are known and further classified based on their functional relevance in pharmacological *in vitro* assays. Most of these mutations lead to a partial or complete loss of function of the receptor (Ho et al., 1999; Vaisse et al., 2000; Farooqi et al., 2000, 2003; Kobayashi et al., 2002; Hinney et al., 2003); some are reported to lead to dominant negative effects (Biebermann et al., 2003; Tarnow et al.,, 2008). It was expected that missense mutations would always result in obesity but surprisingly some carriers were normal weight (Sina et al., 1999; Farooqi et al., 2000; Vaisse et al., 2000; Dubern et al., 2001; Hinney et al, 2006). As approximately two percent of extremely obese children and adolescents carry functionally relevant mutations in the *MC4R* (Hinney et al., 1999), common obesity can only partially be explained via this known major gene effect.

Dempfle et al. (2004) estimated the quantitative effect of *MC4R* mutations on BMI in a sample of 25 pedigrees with segregating mutations observed in relatives of extremely obese index patients. The observed effect on current BMI suggested that mutations in the *MC4R* gene are relevant for the development of obesity. The effect was about twice as strong in females than in males and corresponds to a mean difference in current BMI of approximately 9.5 kg/m^2 for females and 4.0 kg/m^2 for males in the age range 30–40 years. In conclusion, heterozygous male mutation carriers are on average 15 – 20 kg heavier whereas female mutation carriers are on average 30 kg heavier than their family members without *MC4R* mutations.

The pathways downstream of the MC4R are also involved in body weight regulation. Several lines of evidence indicate an involvement of brain derived neurotrophic factor (BDNF) in body weight regulation and activity: (i) heterozygous *Bdnf* knockout mice (*Bdnf+/-*) are hyperphagic, obese, and hyperactive (Kernie et al., 2000); (ii) central infusion of Bdnf leads to severe, dose-dependent appetite suppression and weight loss in rats (Pelleymounter et al., 1995); (iii) Bdnf infusion into the brain suppresses the hyperphagia and excessive weight gain observed on higher-fat diets in mice with deficient Mc4r signalling (Xu et al., 2003). Additionally, *Bdnf+/-* mice are also obese and show an increase in body weight similar to that seen in heterozygous melanocortin-4-receptor deficient (*Mc4r*$^{+/-}$) mice. MC4R signalling controls BDNF expression in the ventromedial hypothalamic area (VHA) and supports the hypothesis that BDNF is an important effector through which MC4R signalling controls energy balance (Xu et al., 2003). Han et al. (2008) showed that among patients with the WAGR (Wilms' tumor, aniridia, genitourinary anomalies, and mental retardation) syndrome, *BDNF* haploinsufficiency is associated with lower levels of serum BDNF and with early onset obesity. Very recently, Thorleiffson et al. (2009) identified *BDNF* as a candidate gene for

obesity and related traits in a GWAS. Therefore, *BDNF* should also be discussed as a polygene for obesity.

The BDNF receptor (TRKB) is also relevant in body weight regulation. Mouse mutants expressing TrkB at a severely reduced amount showed hyperphagia and excessive weight gain on high-fat diet (Kernie et al., 2000). Yeo et al. (2004) described an 8-year-old boy with severe obesity and a complex developmental syndrome, who was heterozygous for a Tyr722Cys substitution in the TRKB. The mutated TRKB led to markedly impaired receptor autophosphorylation and to reduced signalling to the MAP kinase. Mutations of *NTRK2*, the gene encoding TRKB, seem to result in a unique human syndrome including hyperphagia and obesity. The associated impairment in memory, learning and nociception seen in the proband reflects the crucial role of TRKB in the human nervous system (Yeo et al., 2004). Again, mutations in this gene seem to be rare and cannot explain the genetic basis of obesity in the general population.

2.2.2 Recessive forms of monogenic obesity

The two most well known forms of monogenic obesity are caused by recessively inherited mutations in the leptin gene (Zhang et al., 1994) and the leptin receptor gene (Tartaglia et al., 1995, Chen et al., 1996). The *obese* mouse ($ob^{-/-}$) produces a non-functional protein (leptin) whereas the *diabetes* mouse ($db^{-/-}$) produces a defect leptin receptor. The phenotype of both mice is nearly identical. Both show early onset obesity, hyperphagia and neuroendocrinological abnormalities. In contrast to the $ob^{-/-}$ mouse for which a central injection of leptin reverses the phenotype, the phenotype of the $db^{-/-}$ mouse is not affected by such an intervention.

The first monogenic human obesity syndrome reported was congenital leptin deficiency. Montague et al. (1997) reported two severely obese children who were members of the same highly consanguineous pedigree of Pakistanian origin. Both children had very low serum leptin levels despite their markedly elevated fat mass and in both a homozygous deletion leading to a frame-shift mutation in the coding region of the leptin gene was found. Both children were severely hyperphagic, showed aggressive behaviour when denied food, and developed severe obesity. The leptin deficient children were treated daily with injections of human recombinant leptin (Farooqi 1999, 2002). The treatment showed beneficial effects on appetite, fat mass, hyperinsulinaemia, and hyperlipidemia. The food intake of the children decreased substantially and parents reported a near normalization of eating behaviour (Farooqi 1999, 2002).

Leptin receptor deficient subjects show a phenotype that is similar to the phenotype of leptin deficient subjects. Correspondingly, patients with leptin receptor deficiency showed severe hyperphagia resulting in early onset obesity and aggressive behaviour when food was

denied. First, Clement et al. (1998) reported data from a patient with a leptin receptor deficiency with both parents being members of a consanguineous pedigree of Kabilian origin. The deficiency was caused by a homozygous mutation in the leptin receptor gene resulting in a truncated leptin receptor lacking both the transmembrane and the intracellular domains. Farooqi et al. (2007) examined the presence of leptin receptor mutations systematically in a sample of 300 subjects with early onset obesity and hyperphagia including 90 probands from consanguineous families. They detected seven homozygous and one compound heterozygous mutation carrier. Affected subjects were characterized by hyperphagia, severe obesity, alterations in immune function, and delayed puberty due to hypogonadotropic hypogonadism. Serum leptin levels were within the range predicted by the elevated fat mass in these subjects. These results indicated that leptin is an important physiological regulator of several endocrine functions in humans. As seven of the mutation carriers were from consanguineous families, this sample is unlikely to reflect the prevalence of leptin receptor mutations in the general population.

Two additional genes encoding peptides of the leptinergic-melanocortinergic pathway are involved in monogenic obesity: Pro-opiomelanocortin (POMC) is produced by hypothalamic neurons of the arcuate nucleus. Studies in animal models elucidated a central role of α-MSH in the regulation of food intake by activation of the brain Mc4r. Cleaved by prohormone convertases POMC serves to build peptides like anorexigenic α-MSH, ß-MSH and ACTH. The dual role of α-MSH in regulating food intake (Fan et al., 1997) and the identification of mutant alleles at the α-MSH receptor 1 locus (*MC1R*) producing hair pigmentation phenotypes (Robbins et al., 1993) predicted that the phenotype of POMC deficiency would include obesity and alteration in pigmentation, in addition to ACTH deficiency. In line with these findings, Krude et al. (1998) reported two obese German children with early onset obesity, pale skin, red hair, and adrenal insufficiency due to ACTH deficiency. A second study on early onset obese, red-haired children revealed homozygosity or compound heterozygosity for mutations in the *POMC* gene for three additional children.

Prohormone-Convertase-I/3 (PCSK1), one of the peptides cleaving POMC, is also related to monogenic obesity: There are three known cases of prohormone-convertase-I-deficiency. O'Rahilly et al. (1995) described a female with severe childhood obesity, abnormal glucose homeostasis, low plasma insulin, but elevated levels of proinsulin, hypogonadotropic hypogonadism, and hypocortisolaemia associated with elevated levels of POMC. This patient was found to be compound heterozygous for a missense mutation (Gly593Arg), which causes failure of propeptide cleavage of PCSK1 and its retention in the ER, and was also heterozygous for +4A>C in the splice donor site of intron 5, resulting in exon skipping, a frameshift, and the introduction of a premature stop codon in the catalytic domain (Jackson et al., 1997). The second patient, identified by the same group (Jackson et al., 2003) was

compound heterozygous for a nonsense mutation (Glu250stop) which truncates the protein in the catalytic domain and an in-frame deletion (Ala213del). Farooqi et al. (2007) identified a boy homozygous for a novel missense mutation (Ser307Leu) with severe, early-onset obesity. As with the previous two patients, this child had obesity and persistent diarrhea. The patient showed markedly increased food intake, confirming that hyperphagia makes a major contribution to the obesity seen in this syndrome.

Recently, Benzinou et al. (2008) assessed the contribution of *PCSK1* to polygenic obesity and genotyped single nucleotide polymorphisms (SNPs) in the genomic region comprising *PCSK1* in a total of 13,659 individuals of European ancestry from eight independent case-control or family-based cohorts. The nonsynonymous variants Asn221Asp, and haplotype Gln665Glu-Ser690Thr were consistently associated with obesity in adults and children. Functional analyses showed a significant impairment of the Asn221Asp-mutant PCSK1 protein catalytic activity. Studies by independent investigators will show if this finding can be confirmed.

2.2.3 Polygenic obesity

The first validated polygene for human obesity was discovered by a family based association study (Geller et al., 2004). Subsequent polygenes were identified in larger samples and validated in population based cohorts exploiting more cost effective large scaled genotyping methods in combination with new statistical approaches.

The most common MC4R missense variant Val103Ile (rs2229616) was initially reported to be similarly distributed between obese and non-obese individuals. These results were in line with functional studies, as no functional implications of the variant could be shown. Geller et al. (2004) initially performed a TDT in 520 obesity trios (extremely obese index patient with both parents) and observed a lower transmission rate of the Ile103 allele (p= 0.017). Based on the unexpected finding, two large case-control studies were performed and their data were combined with those from 12 published studies resulting in a total of 7,713 individuals. The meta analysis provided evidence for a negative association of the Ile103 allele with obesity (p=0.03). Carriers of the Ile103 variant were on average 1.5 kg (0.5 BMI units) leaner than non-carriers. Heid et al. (2005) confirmed this result in an extended study group (N > 8000 individuals) while Young et al. (2007) conducted a meta-analysis encompassing 29,563 individuals. Xiang et al. (2006) showed *in vitro* that the human MC4R harbouring 103Ile has a normal endogenous agonist ligand affinity and normal receptor expression at the cell surface. However, they also observed that the Ile103-MC4R possesses a modest but statistically significant 2-fold decrease in antagonist AGRP potency, which is consistent with the initial finding of negative association to obesity. Further support of the genomic region comprising the *MC4R* comes from a meta analysis of data of genome-wide association studies (GWA)

data of 16,876 individuals (Loos et al., 2008). The strongest association signal after *FTO* (see below) was observed for the variant rs17782313 which maps 188 kb downstream of *MC4R*. For further confirmation and initial characterization of the locus more than 90,000 individuals were genotyped. The per allele effect in 60,352 adults was 0.05 Z-score units (0.22 kg/m^2) and 0.13 Z-score units in 5,988 children. The localisation of the associated SNP and patterns of phenotypic associations are consistent with effects mediated through altered MC4R function. In sum, *MC4R* seems to entail both loss and gain of function and represents the first identified polygene for body weight regulation.

The first GWA based on approx. 100,000 SNPs analyzed in families of the Framingham cohort, identified an association of a SNP in the proximity of the insulin-induced gene 2 (*INSIG2;* rs7566605) with obesity. Approximately 10% of the analysed individuals harboured the CC genotype that, according to this study, predisposes to obesity (Herbert et. al., 2006). Several attempts to replicate the *INSIG2* finding have been or are currently being undertaken. Both confirmations (Lyon et. al., 2007) as well as negative findings (Dina et. al., 2007; Loos et. al., 2007; Rosskopf et. al., 2007) have been reported. Data have been compiled for a large-scaled meta-analysis that underscored the role of the *INSIG2* SNP in (extreme) obesity (Heid et al., *submitted*). Interestingly, it was recently reported that CC homozygotes for the relevant *INSIG2* SNP (rs7566605) lost less weight in a one-year lifestyle intervention program, than individuals with the two other genotypes. This finding further supports a role of this polymorphism in weight regulation (Reinehr et al., 2007).

(FTO): A genome wide association study for type 2 diabetes susceptibility genes identified a common variant in the *fat mass and obesity associated gene (FTO)* that showed a BMI-mediated association to NIDDM. After correction for BMI the NIDDM-effect vanished, so *FTO* seemed to be more relevant for obesity. The BMI-related association was replicated in 13 samples with 38,759 participants. The 16% of adults who are homozygous for the risk allele weighed about 3 kg (≤ 0.8 BMI units) more than average and had a 1.67-fold increased risk of obesity when compared with those not inheriting a risk allele (Frayling et al., 2007). Two additional GWAs also found *FTO* to be associated with obesity. Whereas Scuteri et al. (2007) investigated the genetically isolated population of Sardinia (N=4,617), Hinney et al. (2007) performed the first GWA for extreme early onset obesity (N=942) and found markers in *FTO* to be significantly related to obesity even after correcting for genome wide multiple testing. Additionally, Dina et al. (2007) detected the same effect in 8,000 French individuals. Since then, the association of first intron variants of *FTO* and increased BMI has been confirmed in many studies (e.g. Sladek et al., 2007; Kring et al., 2008; Qi et al., 2008). As the functional impact of *FTO* on body weight regulation is still unclear, the latest studies focus on more specialised phenotypes in smaller samples to figure out the possible function: Andreasen et al. (2008) showed that low physical activity might accenuate the effect of the

FTO rs9939609 on body fat accumulation. Tschritter et al. (2007) found that *FTO*-SNPs seem to be associated with cerebrocortical insulin resistance in humans. Wahlen et al. (2008) detected an association of rs9939609 to fat cell lipolysis. Klöting et al. (2008) revealed a potential inverse relationship between obesity and *FTO* gene expression in visceral adipose tissue in humans. The investigation of these more specialized phenotypes might help to narrow down the functional implications of *FTO* on obesity by stimulating ideas for functional *in vitro* and *in vivo* studies. Currently, *FTO* is one of the most promising and most consistently supported findings in obesity genetics.

2.3 Genome-wide approaches

2.3.1 Genome-wide linkage analysis

Linkage is the association of gene loci on the same chromosomal region. Linked genes and markers are inherited together. Besides other factors, the physical distance between genetic loci is important for linkage: the smaller this distance on a chromosome the less likely two genetic loci will be separated by a meiotic recombination event called crossing-over (Morgan, 1911). The relationship between recombination frequency and chromosomal genetic distance is defined by the relative unit Morgan with one centi Morgan (1 cM) being roughly equivalent to a recombination frequency of one percent. On the other hand, a group of alleles, which is inherited preferentially together, is called a haplotype. Close linkage between marker- and disease locus can lead to an allelic phenomenon called linkage disequilibrium (LD). LD is detected whenever the observed frequencies of haplotypes in a population deviate from haplotype frequencies predicted by the product of individual genetic marker allele frequencies of each haplotype assuming allelic independence.

Classical linkage studies are parametric and model-based, so that usually some prior knowledge of the mode of inheritance, the allele frequencies and the penetrance is necessary. These analyses were very successful for the identification of genetic loci causing Mendelian monogenic disorders like Huntington´s disease (Walker, 2007). For classical linkage analyses up to 500 multiallelic DNA markers (micro satellites; di-, tri-, or tetranucleotid repeats) with an average distance of up to 10 cM were investigated in large multi-generational pedigrees including affected and non-affected members. Patterns of co-segregation with the phenotype were analysed (Hebebrand et al., 2001). A measure of linkage is the so called LOD (logarithm of the odds) score (Morton 1955). The LOD score summarizes evidence in the sample comparing the hypothesis of linkage vs. no linkage between a marker (or gene) locus and a disease locus. Evidence for linkage is given if the maximum LOD exceeds a certain threshold which is e.g. dependent on the size of the investigated genome or number of investigated markers. Chromosomal regions surrounding

markers with significant or increased LOD scores are often called candidate regions. Fine mapping with denser marker sets (smaller average distance) is used to narrow down such regions.

Most genome scans for complex traits are non-parametric linkage studies in which the whole genome is systematically analyzed for phenotype related chromosomal regions. Usually an (affected) sib pair approach is applied requiring the sampling of an index patient, of at least one affected sib and ideally both biological parents. The method is based on the idea that the phenotype similarities investigated in sib pairs results from the same underlying and thus shared genotype. A major advantage of methods like the extreme concordant sib pair approach (ECSP; Risch and Zhang, 1995) is that, compared to classical linkage analyses, they do not require knowledge of the mode of inheritance (Lander and Krugylak, 1995; Risch and Zang, 1995). Thus, the intention of genome scans is to identify chromosomal regions that are observed more frequently in the sibs than would be expected by Mendelian inheritance. A common measure to describe the genetic similarity between sibs is the number of alleles shared identical by descent (IBD; Fishman et al., 1978). However, given the lack of clear patterns of inheritance coupled with small genetic effect sizes and the multiple genetic and environmental factors that influence complex traits, the utility of genome scans to identify candidate regions or even candidate genes for complex disorders like obesity is still questioned. Such genome scans are further complicated by the fact that instead of a single test for linkage, one must conduct multiple tests across the entire genome. As a consequence, Lander and Krugylak (1995) have argued that a LOD score ≥3.3 may be viewed as evidence for linkage whereas a LOD score ≥1.9 but <3.3 should be quoted as evidence for suggestive linkage that needs further support.

Until today more that 40 conventional genome scans pertaining to obesity and related phenotypes have been performed. Genome scans for obesity and related traits (Adeyemo et al., 2003; Atwood et al., 2002; Bell et al., 2004; Chen et al., 2004, 2005; Deng et al., 2002; Feitosa et al., 2002; Hager et al., 1998; Hanson et al., 1998; Hsueh et al., 2001; Hunt et al., 2001; Iwasaki et al., 2003; Kissebah et al., 2000; Lee et al., 1999; Lembertas et al., 1997; Lindsay et al., 2001; Meyre et al., 2004; Moslehi et al., 2003; Norris et al., 2005; Ohman et al., 2000; Palmer et al., 2003; Perola et al., 2001; Platte et al., 2003; Price et al., 2002; Reed et al., 1996; Saar et al., 2003; Stone et al., 2002; Van der Kallen et al., 2000; Watanabe et al., 2000; Wu et al., 2002; Zhu et al., 2002) have come up with some consistent regions. Especially chromosomes 1p, 3q, 6q, 11q and 16q showed overlapping evidence for linkage.

However, the general comparability of genome scans from different research groups is often limited by e.g. differences in ethnic groups and/or phenotypes (e.g. obesity related phenotypes like fat mass and plasma leptin level) studied. It has to be kept in mind, that

genome scans are appropriate to identify major gene loci i.e. those with a large penetrance, whereas genetic loci with small effects or rare mutations with reduced penetrance can not be detected. Recently, Saunders et al. (2007) performed a meta analysis on 37 published genome-wide linkage scans containing data on over 31,000 individuals from more than 10,000 families. They found suggestive evidence for linkage to increased BMI at chromosomes 13q13.2-q33.1 and 12q23-q24.3 in the pooled analysis and suggestive evidence for chromosome 11q13.3-22.3. Interestingly, the *FTO* locus at 16q12.2 also showed nominal evidence for linkage. Despite having substantial statistical power even for smaller genetic effects, Saunders et al. did not identify specific loci for increased BMI or obesity. Several reasons may have contributed to this finding. First of all one may argue that effect sizes of genes that influence body weight are even smaller than those that the study was powered for. Other reasons might be substantial locus and allelic heterogeneity or variable dependence of genetic factors on environmental factors.

Until today, three genes contributing to observed linkage peaks for obesity related phenotypes have been identifies: (1) neurotransmitter transporter *SLC6A14* (*solute carrier family 6 member 14*) on chromosome Xq23-24 maps to a linkage peak in Finnish sib pairs (Öhman et al., 2000; Suviolahti et al., 2003). Durand et al. (2004) confirmed the finding in 1,267 obese French adult cases and 649 lean control French subjects, whereas Brönner et al. (*in preparation*), found no association in up to 700 German obesity trios. (2) The second gene is *GAD2* (*glutamate decarboxylase 2*) for which a haplotype comprising three SNPs located in the linkage region on chromosome 10p was found to predispose to obesity (Boutin et al., 2003). The haplotype may contribute to a peak region described by Hager et al. (1998) which was confirmed by our own genome-wide scan (Saar et al., 2003). However, this finding could not be confirmed in larger samples of children and adolescents contributing to the linkage peak (Saar et al., 2003) or independent obese adults and obesity families (Swarbrick et al., 2005; Groves et al., 2006). (3) The third gene, ectonucleotide pyrophosphatase / phosphodiesterase 1 (*ENPP*1) on chromosome 6q16.3-q24.2 was found to be associated with childhood obesity (Meyre et al., 2005). The *Genotype IBD Sharing Test* (GIST) suggested that the obesity-associated *ENPP1* risk haplotype contributed to the observed linkage on chromosome 6q with childhood obesity. The same group investigated the predictive value of *ENPP1* SNPs with regard to the risk to develop obesity and/or type 2 diabetes in a large French cohort. They found no association of the risk haplotype with adult obesity and NIDDM. However, they detected nominal evidence of an association between the Lys121Gln polymorphism and both severe adult obesity at baseline and the risk of NIDDM in participants with a family history for this disease (Meyre et al., 2007). The recent analysis of McAteer et al. (2008) consisted of 30 studies comprising 15,801 case and 26,241

control subjects and revealed that *ENPP1* variant Gln121 increases the risk for NIDDM under a recessive model of inheritance.

It is possible that more than one gene leads to the described linkage peaks. For single mutations, SNPs and haplotypes, future discoveries will potentially reveal to what extent this is the case (Hebebrand et al., 2001). Especially in the case of human obesity, it seems possible that some of the peaks actually represent the joint interacting effect of several SNPs or even several haplotypes (at more than one locus). As all three genes (*SLC6A14, GAD2, ENPP1*) did not light up in the available GWAs for obesity and related phenotypes, it is probably more likely that they have no major impact on general obesity.

2.3.2. Genome-wide association studies

Within the last three years the number of genetic association studies using large numbers of genetic markers (up to 1,000,000) to search for genetic variation underlying common diseases like diabetes, cardiovascular disease and cancer has increased dramatically. SNPs (frequency >1%) are common and occur on average once every 1,000 base pairs (International HapMap Project, 2007). GWA studies rely on the assumption that LD enables one SNP to act as a surrogate marker for association to other sequence variants in the same region (Freimer and Sabatti, 2007). Depending on the used genotyping platform, GWA studies differ in terms of the number and criteria for SNP selection. Some used SNPs evenly distributed across the genome (Affymetrix 500k; e.g. Frayling et al., 2007), whereas others selected SNPs to capture most of the common variation given the data of the International HapMap Project (Illumina; e.g. Sladek et al., 2007)

By genotyping a large number of SNPs, there is a good chance that at least one SNP will be in LD with common functional variant(s) relevant for the investigated phenotype. Genome wide association studies represent a major step forward in the study of common genetic variation in complex diseases. The thousands of densely spaced SNPs genotyped using high-throughput genotyping arrays provide means for a comprehensive evaluation of common genetic variation unbiased by candidate gene hypotheses (Dupuis and O´Donnell, 2007). Until today, several GWAs revealed previously unknown gene-disease associations, e.g. *FTO* and obesity (Sladek et al., 2007; Frayling et al., 2007) or *CDKN2A/B* and CHD and NIDDM (Saxena et al., 2007; Scott et al., 2007; Zeggini et al., 2007; McPherson et al., 2007).

Three recent GWAS have successfully identified a total of 17 new loci for obesity (Thorleiffson et al., 2008; Willer et al., 2009; Meyre et al., 2009). In the study of Thorleiffson et al. (2008) 305,846 SNPs were genotyped in 25,344 Icelandic, 2,998 Dutch, 1,890 European American and 1,160 African American subjects. The results were combined with previously published data of the Diabetes Genetics Initiative (DGI) based on 3,024 Scandinavians. In eleven chromosomal regions a total of 29 variants (some of these are in

high LD), reached genome-wide significance. The known candidate genes *FTO* and *MC4R* were reconfirmed; furthermore, the two obesity candidate genes *BDNF* and *SH2B1* were identified.

In parallel a meta-analysis of 15 GWAS for BMI (n = 32,387) was performed by the GIANT (Genetic Investigation of ANthropometric Traits) consortium based on approximately 2.4 million SNPs (Willer et al., 2009). The top 35 signals were followed up in 14 additional cohorts (59,082 probands). A strong confirmation was detected for *FTO* and *MC4R*. Additionally, six new loci were identified: *TMEM18, KCTD15, GNPDA2, SH2B1, MTCH2,* and *NEGR1*. Together, the six newly discovered loci account for 0.40% and in combination with *FTO* and *MC4R* for a total of 0.84% of the BMI variance. Subsequently, the combined impact of these loci on BMI was estimated: Individuals with 13 or more obesity-predisposing alleles across the eight loci were in average 1.46 kg/m^2 (equivalent to 3.7–4.7 kg for an adult 160–180 cm in height) heavier than those individuals with less than 3 of these alleles (Willer et al., 2009)

Most recently, 38 SNPs of a GWAS based on 1,380 Europeans with early-onset obesity and morbidly obese adult individuals and 1,416 age-matched normal-weight controls showed strong association with obesity and were further evaluated in 14,186 European individuals (Meyre et al., 2009). In addition to *FTO* and *MC4R*, significant association with obesity was detected for three new risk loci in (*NPC1, MAF*, and *PTER*).Additionally, candidate genes were analyzed in the GWAS data set. Nevertheless, a number of limitations must be considered when interpreting such studies in which multiple genetic markers are tested and when small to moderate effects are expected for most common genetic variations. Typical multiple testing procedures, such as Bonferroni and permutation testing, are used to limit the probability of false discovery. However, such error control becomes very conservative as the number of statistical tests increases, eventually preventing the discovery of true associations. Even with a less conservative approach to correct for multiple testing to find genes with small contributions to the phenotype remains a challenge. Alternative data mining techniques and other novel statistical approaches will be required to identify important interactions without excessively increasing the number of statistical tests performed.

Dupuis and O´Donnell (2007) summarized the major requirements for GWA-based studies on common diseases: first, large samples with sufficient power to detect small to moderate effects will be required; GWAs performed on small samples will only be useful for generation of hypotheses. Second, for discovery of true associations it is likely of importance that the samples are ethnically homogeneous. While homogeneity may not ensure the generalizability of the association to other populations, it improves the odds that genetic effects are detectable in the test population and reduces the risk of false positive association

due to population stratification. Third, the implementation of well-defined, reproducible, phenotypes will be crucial to clearly distinguish signal from noise in genetic association studies. And fourth, as statistical evidence alone cannot distinguish between “causal” variants and non-functional variants in LD with the true causal mutations, the evidence for causality should include demonstration of functional significance for the genetic variant. As the last task requires substantial time and effort, it is more likely that causality of variants will be investigated in studies subsequent to the initial publications (Dupuis and O´Donnell, 2007).

2.4 Aims of the study

An overall objective of this thesis was to study the genetic mechanisms of human body weight regulation. Specific aims were:

- **To use genome-wide approaches to identify chromosomal regions/candidate genes/genetic variants involved in body weight regulation**
    - Identification of chromosomal regions involved in the etiology of early onset obesity using linkage analyses in 89 families of German origin with two or more extremely obese children (Saar et al., 2003, *publication 1*)
    - Identification of genetic variants involved in body weight regulation using a genome-wide association study (GWA) for extreme, early onset obesity (Hinney et al., 2007, *publication 2*)
- **To investigate candidate genes for obesity**
    - Analyses of the *brain-derived neurotrophic factor precursor gene* (*BDNF*) as a candidate gene for body weight regulation and physical activity (Friedel et al., 2005, *publication 3*).
    - Analysis of the involvement of two single nucleotide polymorphisms (SNPs) of the *insulin-responsive glucose transporter 4 gene* (*GLUT4*) in samples from different weight extremes (Friedel et al., 2002, *publication 4*)
    - Investigation of the *diacylglycerol O-acyltransferase homolog 2 gene* (*DGAT2*) as a positional and functional candidate gene for early onset obesity on chromosome 11q13 (Friedel et al., 2007, *publication 5*)
- **To discuss the influence of functionally relevant *MC4R*-variants on weight loss during a lifestyle intervention program (Reinehr et al., 2009**, publication 6**)**
- **To discuss the perspectives of molecular genetic research in human obesity (Hebebrand et al., 2003**, *publication 7***)**

3.1 Publication I

Saar K, Geller F, Rüschendorf F, Reis A, Friedel S, Schäuble N, Nürnberg P, Siegfried W, Goldschmidt HP, Schäfer H, Ziegler A, Remschmidt H, Hinney A, Hebebrand J. **Genome scan for childhood and adolescent obesity in German families**. Pediatrics. 2003;111(2):321-7.

The aim of this study was to detect chromosomal regions/candidate genes that are involved in the aetiology of early onset obesity via a genome-wide linkage study. The genome scan was based on 89 families with 2 or more obese children. A total of 369 individuals were initially genotyped for 437 microsatellite markers. A second sample of 76 families was genotyped using microsatellite markers that localize to regions for which maximum likelihood binomial logarithm of the odd (MLB LOD) scores on use of the concordant sibling pair approach exceeded 0.7 in the first sample.
Regions with MLB LOD scores >0.7 were detected on chromosomes 1p32.3-p33, 2q37.1-q37.3, 4q21, 8p22, 9p21.3, 10p11.23, 11q11-q13.1, 14q24-ter, and 19p13-q12 in sample 1; MLB LOD scores on chromosomes 8p and 19q exceeded 1.5. In the second sample, MLB LOD scores of 0.68 and 0.71 were observed for chromosomes 10p11.23 and 11q13, respectively.
We consider that several of the peaks identified in other conventional genome scans for obesity were overlapping with signals in this scan as promising for ongoing pursuits to identify relevant genes.

Own contribution:

- Establishment of a database to collect and manage the data of all published genome-wide linkage studies for obesity and related phenotypes
- Comparison of our linkage data with all scans collected in the database
- *in silico* analyses of peak regions and identification of potential candidate genes for obesity
- interpretation and implementation of these results in the manuscript

Genome Scan for Childhood and Adolescent Obesity in German Families

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ABSTRACT. *Objective.* **Several genome scans have been performed for adult obesity. Because single formal genetic studies suggest a higher heritability of body weight in adolescence and because genes that influence body weight in adulthood might not be the same as those that are relevant in childhood and adolescence, we performed a whole genome scan.**

Methods. **The genome scan was based on 89 families with 2 or more obese children (sample 1). The mean age of the index patients was 13.63 ± 2.75 years. A total of 369 individuals were initially genotyped for 437 microsatellite markers. A second sample of 76 families was genotyped using microsatellite markers that localize to regions for which maximum likelihood binomial logarithm of the odd (MLB LOD) scores on use of the concordant sibling pair approach exceeded 0.7 in sample 1.**

Results. **The regions with MLB LOD scores >0.7 were on chromosomes 1p32.3-p33, 2q37.1-q37.3, 4q21, 8p22, 9p21.3, 10p11.23, 11q11-q13.1, 14q24-ter, and 19p13-q12 in sample 1; MLB LOD scores on chromosomes 8p and 19q exceeded 1.5. In sample 2, MLB LOD scores of 0.68 and 0.71 were observed for chromosomes 10p11.23 and 11q13, respectively.**

Conclusion. **We consider that several of the peaks identified in other scans also gave a signal in this scan as promising for ongoing pursuits to identify relevant genes. The genetic basis of childhood and adolescent obesity might not differ that much from adult obesity.** ***Pediatrics*** **2003;111:321–327;** ***linkage analysis, BMI, body weight.***

ABBREVIATIONS. BMI, body mass index; ECSP, extremely concordant sibling pair; MLB LOD, maximum likelihood binomial logarithm of the odd; PCR, polymerase chain reaction.

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The number of whole genome scans for obesity and obesity-related phenotypes has rapidly increased after publication of the initial scan pertaining to a search for genes that influence percentage body fat in Pima Indians in 1997.[1] The ethnically diverse populations include Pima Indians[1–4]; Mexican Americans[5–7]; European and African Americans[8–11]; French Canadians[12,13]; Old Order Amish[14]; and Europeans from France,[15] Finland,[16–18] the Netherlands,[19] and Sweden.[17] Several different chromosomal regions have been identified in these whole genome scans, some of which have been confirmed in independent whole genome or regional studies, including linkage to chromosomes 2p,[7,15] 7q,[11,14,20–25] 10p and q,[9,15,26,27] and 20q.[9,28]

One of the highest heritability estimates for body mass index (BMI; kg/m^2) has been determined in a twin study based on adolescents.[29] These findings suggest that heritability might even be higher at this age than in adulthood, for which estimates derived from twin studies typically range in the magnitude of 0.6 to 0.8.[30,31]

Human obesity as a result of rare single gene mutations such as in the leptin[32,33] and leptin receptor genes[34] typically manifests early in life. Nonsense, frameshift, and functionally relevant missense mutations in the melanocortin-4 receptor gene, which occur with a frequency of 2% to 4% among extremely obese adolescents and adults, are often associated with (extreme) obesity during childhood.[35–41] It has been estimated that only 40% of the genes that influence BMI at age 20 continue to do so at ages 40 and 60.[42] Nevertheless, obesity, in particular extreme obesity in adolescence, commonly persists in adulthood,[43] the risk being even higher when at least 1 parent is also obese.[44]

Currently, no published whole genome scan for obesity has been based on children or adolescents as index patients. In light of the potentially stronger genetic determination of childhood and adolescent BMI and the possibility of age-dependent genetic influences on body weight, genome-wide scans based on children and adolescents are of obvious interest. Furthermore, scans based on young probands entail the advantage that the parents can be readily ascertained, thus enabling more accurate determination of the identity by descent status. Because the typical complications of obesity, including non–insulin-dependent diabetes and hypertension, have not fully become manifest at adolescence, these com-

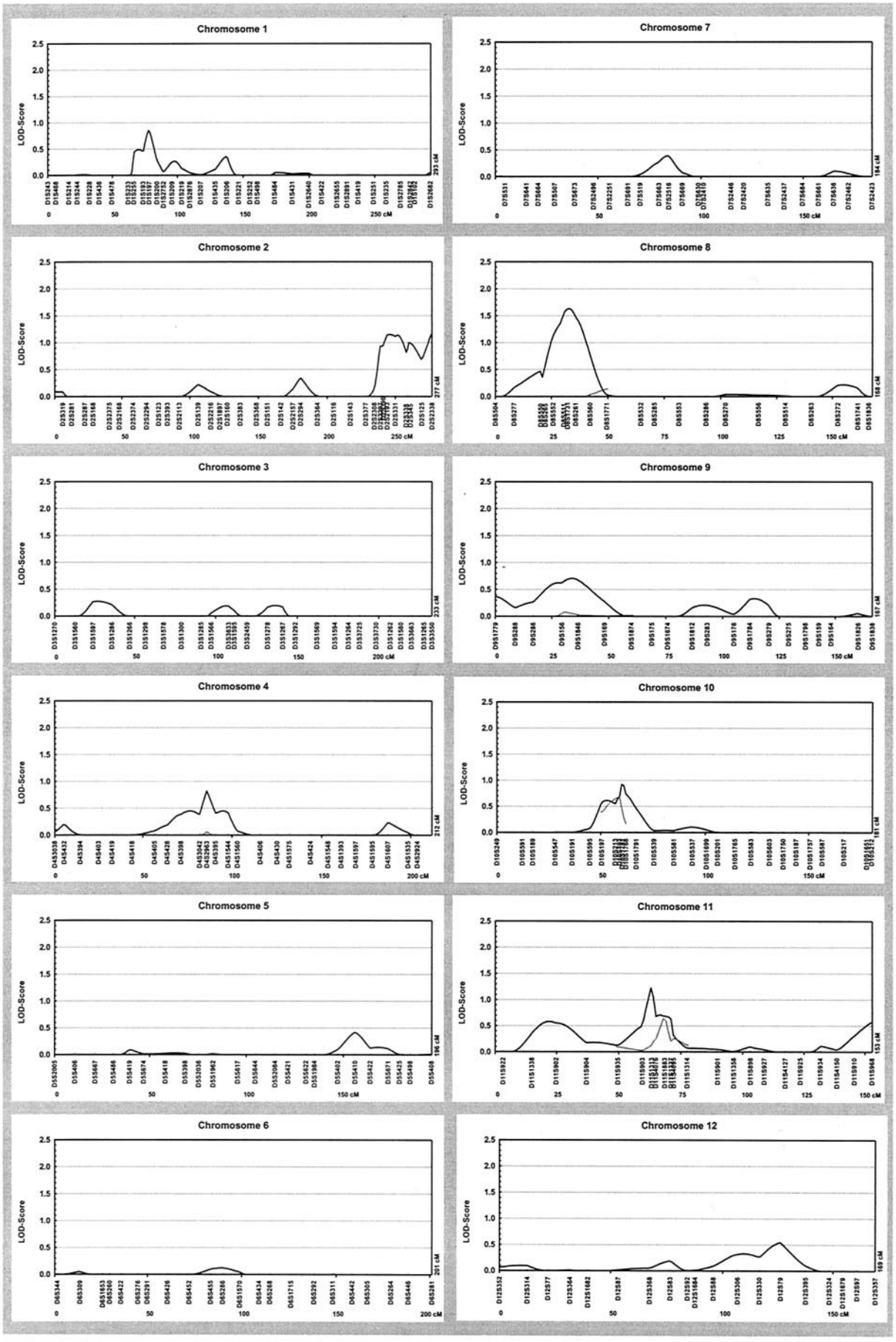

Fig 1. MLB LOD scores for chromosomes 1 to 12 (A) and 13 to 22 and X (B) based on a genome scan of 89 families with 2 or more obese children (black lines) and MLB LOD scores obtained in an additional sample of 76 families in regions of interest (gray lines).

B

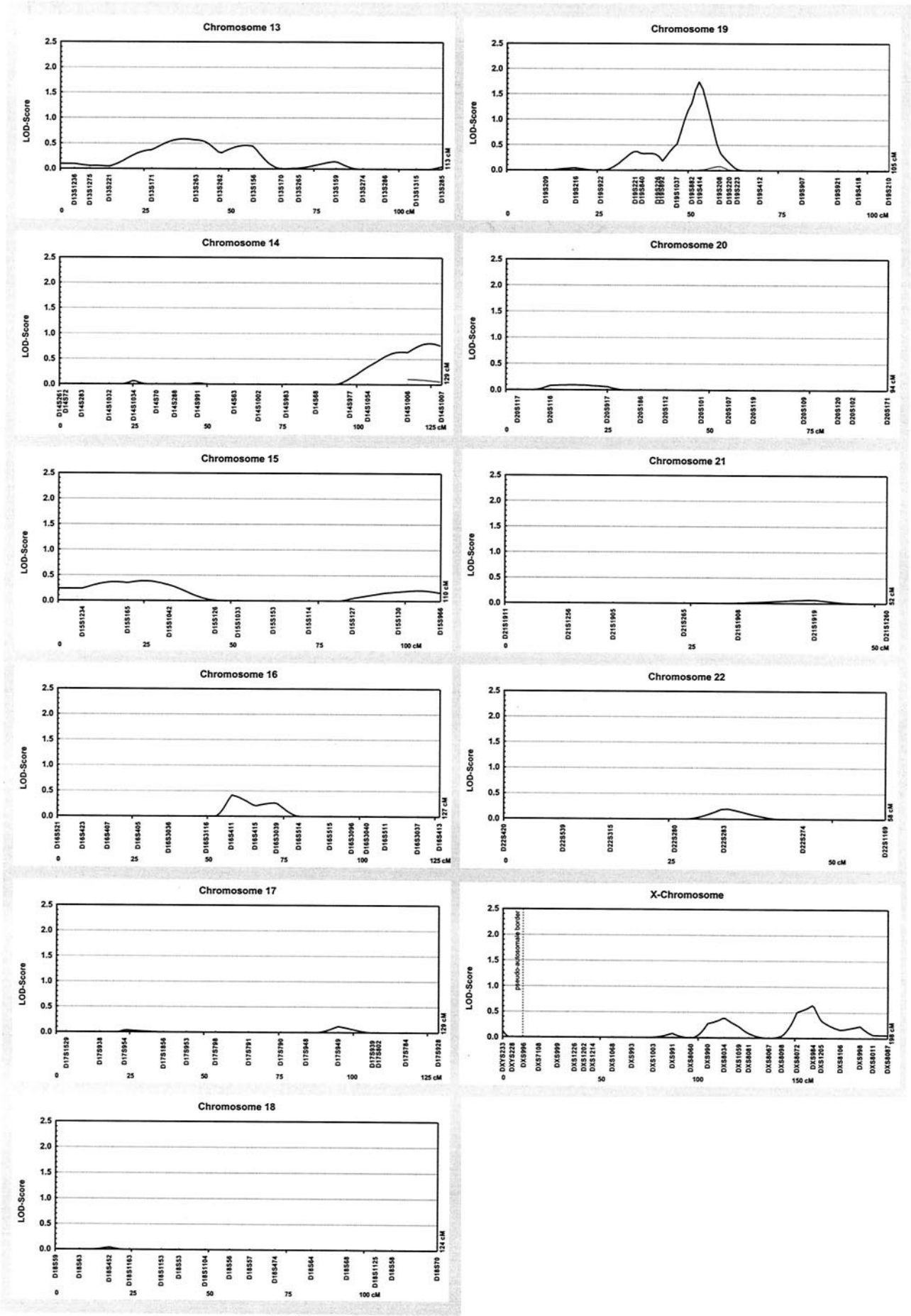

Fig 1. *Continued.*

plications cannot be co-assessed in a scan based on young siblings. Particularly, a scan based on adolescents is complicated by the fact that several obesity-related traits are influenced by pubertal status; serum leptin levels represent a good and well-characterized example[45] of this phenomenon.

The standard definitions for obesity based on absolute BMI[46] cannot be applied to children and adolescents. In our molecular genetic studies, we have used BMI centiles based on the representative German National Nutrition Survey[47] to define the degree of obesity of our index patients in the age range 5 to 22 years and their siblings (eg,[26,38,39,48]). Different centiles have been used to define overweight and obesity in children and adolescents, including the 85th and 95th[49] and the 90th and 97th[50] centiles, respectively. On the basis of the extremely concordant sibling pair approach (ECSP[51]) we have ascertained obese index patients and their siblings via a BMI ≥95th for 1 sibling and ≥90th centile for the other(s). Under consideration of the parameters estimated in previous segregation analyses for obesity, we have shown that the ECSP is better suited than the extreme discordant sibling pair approach to detect linkage.[52] On use of the ECSP approach, we have confirmed linkage of obesity to chromosome 10p based on a regional scan encompassing 93 families with 2 or more obese offspring.[26]

In this study, we present for the first time results of a whole genome scan based on 89 young obese affected sibling pairs. The 452 microsatellite markers were spaced at an average distance of 8.4 cM and included markers for fine mapping; maximum likelihood binomial logarithm of the odd (MLB LOD) scores[53] were calculated to determine linkage on the basis of the ECSP approach. We were subsequently able to genotype an additional 76 families with 2 young obese offspring in chromosomal regions of interest identified in the first group.

METHODS

Ascertainment of obese index patients was performed at 3 German hospitals (Klinik Hochried, Murnau; Adipositas Rehabilitationszentrum Insula, Berchtesgaden; and Spessart Klinik, Bad Orb) that specialize in the inpatient treatment of extremely obese children and adolescents. Families that were willing to participate were included when 1) at least 1 offspring had an age- and gender-specific BMI centile ≥95, 2) at least 1 sibling had an age- and gender-specific BMI centile ≥90, and 3) the DNA of both biological parents was available. There were 77, 11, and 1 families with 2, 3, and 4 obese children, respectively. Accordingly, the total number of individuals genotyped for the whole genome scan was $n = 369$ (sample 1). During genotyping of sample 1, an additional 76 families (sample 2) were recruited as part of an ongoing ascertainment to enable a future genome scan based on 300 families. Sample 2 also fulfilled the aforementioned 3 criteria. Again, the majority of these families ($n = 68$) had 2 obese offspring; 6, 1, and 1 had 3, 4, or 5 obese siblings, respectively. These families (sample 2) were genotyped for those markers that contributed to peak regions identified in sample 1 as defined by a MLB LOD >0.70. Finally, families of samples 1 and 2 were genotyped for 15 additional markers that localize within the identified peak regions. These markers were chosen by applying informativity criteria. Descriptive statistics for both samples are presented in Tables 1 and 2. In single families in samples 1 and 2, an obese offspring was aged ≥18 years: the oldest index patient (22 years) had an 18-year-old sibling; 18 siblings of index patients who were younger than 17 years were older than 22 years. Age- and gender-adjusted BMI centiles were calculated from the large and representative German National Nutrition Survey.[47] Written informed consent was given by all participants; in the case of minors, consent was given by their parents. This study was approved by the Ethics Committee of the University of Marburg.

TABLE 1. Descriptive Statistics Based on 89 Families With at Least 1 ECSP Used to Perform a Whole Genome Scan (Sample 1)

	Mean	Minimum	Maximum	SD
Obese index patients ($n = 89$)*				
Age	13.63	7.59	22.05	2.75
BMI	32.53	24.22	56.68	6.36
BMI centile	99.30	95.00	100.00	1.23
Obese sibs ($n = 102$)†				
Age	14.97	6.71	34.63	5.11
BMI	28.85	19.77	51.99	5.59
BMI centile	97.47	90.00	100.00	2.84
Fathers ($n = 89$)				
Age	44.65	33.62	59.63	6.01
BMI	31.20	20.94	49.33	5.57
BMI centile	82.62	2.00	100.00	23.47
Mothers ($n = 89$)				
Age	41.62	30.95	61.97	5.32
BMI	31.15	21.91	48.13	6.35
BMI centile	86.41	34.00	100.00	17.91

SD indicates standard deviation.
* Females: $n = 52$ (58%).
† Females: $n = 53$ (52%).

TABLE 2. Descriptive Statistics Based on 76 Families With at Least 1 ECSP (Sample 2) Used in an Attempt to Confirm Linkage to Regions With an MLB LOD score >0.70 as Detected in Sample 1

	Mean	Minimum	Maximum	SD
Obese index patients ($n = 76$)*				
Age	13.25	5.10	18.18	2.30
BMI	31.76	22.62	49.96	5.12
BMI centile	99.28	90.00	100.00	1.55
Obese sibs ($n = 87$)†				
Age	15.26	7.26	29.38	5.35
BMI	28.23	19.61	50.02	5.14
BMI centile	97.28	90.00	100.00	2.79
Fathers ($n = 76$)				
Age	44.75	30.42	62.01	5.91
BMI	30.57	22.62	56.16	5.34
BMI centile	81.08	10.00	100.00	23.28
Mothers ($n = 76$)				
Age	41.62	30.69	52.42	5.11
BMI	31.26	19.38	47.47	6.29
BMI centile	86.04	7.00	100.00	20.11

* Females: $n = 45$ (59%).
† Females: $n = 45$ (52%).

Genotyping

DNA was isolated from peripheral white blood cells using standard protocols.[48] The Gene Mapping Centre panel of 372 highly polymorphic microsatellite markers with an average distance of 9.9 cM and an average heterozygosity of 0.78 was selected from the final Généthon linkage map as previously described.[54] In brief, markers were amplified on microtiter plates in single reactions on Tetrad polymerase chain reaction (PCR) machines (MJ Research Biozym, Hessisch Oldendorf, Germany). All pre- and post-PCR pipetting steps were performed using robotic devices. PCR product pools were separated on ABI377XL (Applied Biosystems [ABI], Darmstadt, Germany) sequencers and on MegaBace sequencers (Pharmacia Amersham, Freiburg, Germany), respectively. Semiautomated genotyping was performed using the Genescan and Genotyper (ABI) software and the genetic profiler in the case of MegaBace data. Instrument allele calling was checked manually. All genotypes were subject to an automatic Mendelian check using the Linkrun routine (T. F. Wienker, unpublished), which in turn calls the program Unknown v5.20 from the Linkage Package.[55] All allele sizes were standardized to known Centre

d'Etude Polymorphisme Humain control individuals. Sixty-five additional markers were typed where the total information content[56] was below 0.6 in our samples. Statistical analyses included Crimap to minimize false double recombinants. Peak regions from sample 1 with MLB LOD scores >0.70 were then typed with 2 additional flanking markers on each side of the peak, encompassing 10 cM on either side of the peak loci on chromosomes 1, 2, 4, 8, 9, 10, 11, 14, and 19.

Statistical Analysis

We conducted model-free linkage analysis because the mode of inheritance is unknown for obesity. Multipoint LOD score analysis was performed using the MLB statistics as implemented in MLBGH, Version 1.0.[53] This test statistic is based on the binomial distribution of parental alleles among extremely concordant offspring and accounts for multiple sibships in a natural way.

Gender-averaged map distances (cM) from the Généthon map were transformed to recombination fractions and vice versa using Haldane's map function. X chromosomal calculations were conducted with Genehunter, version 1.3.[56]

RESULTS

The whole genome scan performed with sample 1 based on all 452 markers did not reveal a MLB LOD score ≥2. Two peaks on chromosomes 8p and 19q surpassed a MLB LOD score of 1.5. MLB LOD scores of 2 additional peaks on chromosomes 2q and 11q were ≥1.0. Figure 1 demonstrates that 2 peaks on chromosomes 10p and 11q out of the total of 9 peak regions, for which sample 2 was also genotyped, revealed a MLB LOD score ≥0.5. In addition, Table 3 gives an overview of all 21 markers with 2-point LOD scores ≥0.70 in the first sample. These markers primarily contribute to the reported multipoint LOD scores.

DISCUSSION

To our knowledge, this study represents the first genome scan for adolescent obesity; the mean ages of the index patients and their siblings range between 13 and 15 years (Tables 1 and 2). Only single offspring were older than 18 years; in all sibships, 1 sibling was aged ≤18 years. Because the majority of the index patients had BMIs above the maximal BMI observed in the age- and gender-matched population-based reference group,[47] it seems reasonable to assume that the onset of obesity dated before age 10 in most of the index patients and their siblings. We had hypothesized that a genome scan based on childhood- and adolescent-onset obesity has a greater potential to detect relevant chromosomal regions than a scan based on obese adult sibling pairs. This hypothesis stemmed from findings indicating a potentially higher genetic load in childhood and adolescent obesity.[29] Furthermore, such young sibling pairs are more homogeneous with respect to age at onset, thus potentially limiting a major source of heterogeneity. Finally, and in contrast to most genome scans based on adult probands, we ascertained both parents of all of our young sibling pairs so that the parental phase was primarily used as source of information instead of allele frequencies.

Recently, Altmüller et al[57] reviewed 101 published genome scans for complex disorders. They pointed out that most of the analyzed studies were not able to detect "significant" linkage according to the Lander and Kruglyak criteria.[58] The results of our genome scan based on only 89 families fall within this category. Despite our failure to detect suggestive evidence for linkage according to the strict Lander and Kruglyak criteria,[58] the following aspects need to be considered:

First, for 77 of our 89 families, only 2 obese offspring were ascertained. The advantage of a genome scan based on mostly single and independent sibling pairs is that the respective results can be considered more representative of families with obese offspring in a given population than a scan that includes a mixture of both small and large or only large sibships. However, because heterogeneity of obesity is evident, the reliance on single sibling pairs entails the disadvantage that a hypothetical major gene operative in a limited number of families cannot lead to a high LOD score in a small sample. For identifying such major genes, large pedigrees with several affected family members should be sampled.

Second, despite the low MLB LOD scores, it is of interest to observe that some of the peaks localize to the same or close to chromosomal regions that have previously been detected in other scans based on adult populations of European origin (Table 4). Thus, previously identified peaks on chromosomes 1p,[19] 2q,[15] 8q,[9] 10p,[15] 11q,[9] and 14q[2] also gave a peak signal in the current scan. The moderate size of our peaks is in line with considerations that substantially larger sample sizes are needed to replicate previous findings with LOD scores that fulfill the Lander and Kruglyak criteria.[58] In addition, it is worthwhile to point out that with the exception of our peaks on chromosome 14q and 19p, all of the other peaks with a MLB ≥0.70 have previously been identified in 2 of the scans based on extremely obese probands of European origin.[9,15] Because in both of these studies extremely obese adult index patients were ascer-

TABLE 3. Two-Point MLB LOD Scores >0.70 in Sample 1 and Corresponding LOD Scores in Sample 2 (if Available)

Chromosome	Position (cM)*	Marker	LOD MLB Sample 1	LOD MLB Sample 2
2	180.7	D2S294	1.13	—
2	240.2	D2S396	1.29	0.00
2	244.0	D2S2193	0.83	0.00
2	250.0	D2S331	1.30	0.00
2	259.4	D2S345	0.76	—
2	269.5	D2S125	0.77	—
2	277.0	D2S2338	1.36	—
4	86.0	D4S2963	0.70	1.01
8	20.9	D8S265	0.75	0.00
8	29.5	D8S511	0.91	—
8	35.8	D8S261	1.02	0.06
9	29.5	D9S156	0.73	0.01
9	36.5	D9S1846	0.77	0.00
10	50.5	D10S197	2.24	0.22
10	60.3	D10S1781	0.94	0.22
11	24.7	D11S902	0.89	—
11	63.2	D11S1313	1.65	0.15
11	64.9	D11S4076	0.80	0.53
14	117.1	D14S1006	0.77	0.16
14	128.6	D14S1007	0.75	0.41
16	57.8	D16S411	1.16	—
19	35.5	D19S221	0.88	—
19	53.2	D19S414	1.97	0.00

* Location (cM) according to *Genethon* from *NCBI* (www.ncbi.nlm.nih.gov/).

TABLE 4. Chromosomal Regions Identified Both in the Current and in Previous Genome Scans

Peak Region in Current Screen	Reference	Chromosomal Region/Location	Marker(s)	LOD Score/*P*	Phenotype
1p32.3–1p33	van der Kallen et al[19]	1p31.1† (91cM–103 cM)	D1S3728-D1S1665	LOD = 1.8	Plasma leptin concentration
		1p31.1† (91 cM–103 cM)	D1S3728-D1S1665	LOD = 0.5	BMI
2q37.1–2q37.3	Hager et al[15]	2q37.1† (241 cM)*	D2S206	LOD = 1.6	BMI
	Feitosa et al[11]	2q37.3 (241 cM)	D2S1279	LOD = 1.5, *P* = .0048	BMI
	Lee et al[9]	2q36.2† (232 cM)*	D2S439	*P* = .0045§	% fat
	Norman et al[1]	2q36.1†–2q37.1† (227–237 cM)*	D2S1363-D2S427	*P* = .0123	%fat
	Norman et al[2]	2q36.1–2q37.1† (227–237 cM)*	D2S1363-D2S427	LOD = 1.0	%fat
8p22	Lee et al[9]	8p21‡ (43 cM)*	D8S560	*P* = .0239§	%fat
10p11.23	Hager et al[15]	10p12.1† (52–54 cM)*	D10S197‖–D10S611	LOD = 4.85	BMI
11q11–11q13.1	Norman et al[1]	11q13.4† (76 cM)*	D11S2371	*P* = 0.0163	%fat
14q32	Norman et al[2]	14q32.12† (106 cM)*	D14S617	LOD = 0.8	%fat

* Location (cM) according to *Marshfield map* from *NCBI* (www.ncbi.nlm.nih.gov/).
† Chromosomal region according to *Golden path August 2001* (genome.cse.ucsc.edu/).
‡ Chromosomal region according to *NCBI.*
§ See also Erratum.
‖ Peak marker in our screen.

tained, it is possible that several of these also had a childhood onset of their obesity.

Finally, despite the finding that study groups several times the size of the original sample need to be analyzed to confirm reliably the linkage regions,[59] 2 of our peaks (chromosome 10p and 11q) also showed up—albeit weakly—in our second sample. The chromosome 10p linkage currently can be considered one of the most consistent findings in obesity scans.[15,26,27] Promising candidate genes localized within the chromosome 10 and 11 regions of interest include glutamic acid decarboxylase 2 (chromosome 10) and angiotensin receptor-like 1, ciliary neurotrophic factor, galanin, and uncoupling proteins 2 and 3 (www.ensembl.org/). Previously, we had not found evidence for association of a null allele of the ciliary neurotrophic factor gene with obesity.[60] Furthermore, despite a positive association study pertaining to an uncoupling protein 2 promoter polymorphism,[61] we were not able to replicate this finding; we also did not find evidence for linkage based on the families investigated in the current study.[62]

CONCLUSION

Whereas our scan did not reveal MLB LOD scores ≥2, we nevertheless consider that several of the previously identified peaks also gave a signal in this scan as promising for ongoing pursuits to identify genes within the respective chromosomal regions.

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3.2 Publication II

Hinney A, Nguyen TT, Scherag A, Friedel S, Brönner G, Müller TD, Grallert H, Illig T, Wichmann HE, Rief W, Schäfer H, Hebebrand J. **Genome wide association (GWA) study for early onset extreme obesity supports the role of fat mass and obesity associated gene (*FTO*) variants.** PLoS ONE. 2007;2(12):e1361.

We aimed to to detect chromosomal regions/candidate genes that are involved in the aetiology of early onset obesity via a genome-wide association study.
We performed a GWA (Affymetrix, Genome-Wide Human SNP Array 5.0) for early onset extreme obesity based on 487 extremely obese young German individuals and 442 healthy lean German controls. We aimed to identify and subsequently confirm the 15 SNPs (minor allele frequency > or =10%) with the lowest p-values of the GWA by four genetic models: additive, recessive, dominant and allelic. Six single nucleotide polymorphisms (SNPs) in *FTO* within one linkage disequilibrium (LD) block including the GWA SNP rendering the lowest p-value (rs1121980; log-additive model: nominal p = 1.13 x 10(-7), corrected p = 0.0494; odds ratio (OR)(CT) 1.67, 95% confidence interval (CI) 1.22-2.27; OR(TT) 2.76, 95% CI 1.88-4.03) belonged to the 15 SNPs showing the strongest evidence for association with obesity. For confirmation we genotyped 11 of these SNPs in the 644 independent families. For both *FTO* SNPs the initial association was confirmed. However, none of the nine non-*FTO* SNPs revealed significant transmission disequilibrium. Additionally, similar to the approach of Scuteri et al. (2007), we re-analysed specific candidate genes for obesity in our data.
Our GWA for extreme early onset obesity substantiates that variation in *FTO* strongly contributes to early onset obesity. This is a further proof of concept for GWA to detect genes relevant for highly complex phenotypes. We concurrently show that nine additional SNPs with initially low p-values in the GWA were not confirmed in our family study, thus suggesting that of the best 15 SNPs in the GWA only the *FTO* SNPs represent true positive findings.

Own contribution:

- defining a strategy for and selection of specific candidate gene for obesity
- re-analyses and summarizing the of GWA data for the selected candidate genes
- interpretation and implementation of the results in the manuscript

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PLoS one

Genome Wide Association (GWA) Study for Early Onset Extreme Obesity Supports the Role of Fat Mass and Obesity Associated Gene *(FTO)* Variants

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Background. **Obesity is a major health problem. Although heritability is substantial, genetic mechanisms predisposing to obesity are not very well understood. We have performed a genome wide association study (GWA) for early onset (extreme) obesity.** ***Methodology/Principal Findings.*** **a) GWA (Genome-Wide Human SNP Array 5.0 comprising 440,794 single nucleotide polymorphisms) for early onset extreme obesity based on 487 extremely obese young German individuals and 442 healthy lean German controls; b) confirmatory analyses on 644 independent families with at least one obese offspring and both parents. We aimed to identify and subsequently confirm the 15 SNPs (minor allele frequency ≥10%) with the lowest p-values of the GWA by four genetic models: additive, recessive, dominant and allelic. Six single nucleotide polymorphisms (SNPs) in *FTO* (fat mass and obesity associated gene) within one linkage disequilibrium (LD) block including the GWA SNP rendering the lowest p-value (rs1121980; log-additive model: nominal $p = 1.13 \times 10^{-7}$, corrected p = 0.0494; odds ratio $(OR)_{CT}$ 1.67, 95% confidence interval (CI) 1.22–2.27; OR_{TT} 2.76, 95% CI 1.88–4.03) belonged to the 15 SNPs showing the strongest evidence for association with obesity. For confirmation we genotyped 11 of these in the 644 independent families (of the six *FTO* SNPs we chose only two representing the LD bock). For both *FTO* SNPs the initial association was confirmed (both Bonferroni corrected p<0.01). However, none of the nine non-*FTO* SNPs revealed significant transmission disequilibrium.** ***Conclusions/Significance.*** **Our GWA for extreme early onset obesity substantiates that variation in *FTO* strongly contributes to early onset obesity. This is a further proof of concept for GWA to detect genes relevant for highly complex phenotypes. We concurrently show that nine additional SNPs with initially low p-values in the GWA were not confirmed in our family study, thus suggesting that of the best 15 SNPs in the GWA only the *FTO* SNPs represent true positive findings.**

Citation: Hinney A, Nguyen TT, Scherag A, Friedel S, Brönner G, et al (2007) Genome Wide Association (GWA) Study for Early Onset Extreme Obesity Supports the Role of Fat Mass and Obesity Associated Gene *(FTO)* Variants. PLoS ONE 2(12): e1361. doi:10.1371/journal.pone.0001361

INTRODUCTION

The advent of genome wide association studies (GWAs) already has had a major impact on the identification of polygenes involved in human body weight regulation [1]. However, a GWA based on obese cases and lean controls has not yet been described. GWA have recently proven extremely powerful for the detection of genes/SNPs for different complex disorders [2,3]. The progress has been particularly impressive for type 2 diabetes mellitus (T2DM) [4–7]. *FTO* was one of the genes picked up in GWA studies for T2DM [5,6], adjustment for BMI revealed that this effect was solely based on this quantitative phenotype [5]. We performed a GWA (Genome-Wide Human SNP Array 5.0; Affymetrix) on patient samples stemming from both ends of the BMI distribution and subsequently aimed to confirm the 15 GWA SNPs with minor allele frequency (MAF) ≥10% rendering the lowest p-values determined upon analysis of four genetic models (additive, recessive, dominant and allelic) in an independent family-based study.

RESULTS

The GWA was analysed for the four genetic models additive, recessive, dominant and allelic. By sorting all analysed SNPs with a MAF ≥10% by minimal nominal p-values a list was derived for the best 15 SNPs (Table 1). Six SNPs (rs1121980, rs9939973, rs7193144, rs9940128, rs8050136, rs9939609, pair-wise r^2 range 0.88–1) in *FTO* were among these 15 best SNPs of the scan (see Table 1); all six SNPs localize to the same linkage disequilibrium (LD) block. *FTO*-SNP rs1121980 rendered the lowest nominal p-value of 1.13×10^{-7}; this SNP was the only SNP that survived correction for multiple testing (corrected p = 0.0494; Table 1). The log-additive OR for the rs1121980 risk T-allele was 1.66 (95% CI 1.37–2.01); the odds ratios for heterozygotes (CT) and homozygotes (TT) were estimated at 1.67 (95% CI 1.22–2.27) and 2.76 (95% CI 1.88–4.03), respectively. Frequencies of the T-allele in cases and controls were 0.53 and 0.41 (Table 1).

Eleven of the best 15 markers were subsequently genotyped in 644 independent obesity families based on at least one young

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Table 1. Top 15 SNPs associated with early onset extreme obesity from the Genome-Wide Human SNP Array 5.0 (lowest nominal p-values across four genetic models) and their confirmation using family-based association studies for the risk allele derived from the GWA data

chromo-some	nearest gene or cDNA[1]	SNP	GWA 500K case-control approach						confirmation-family-based association study[2]	
			dbSNP alleles (bold: obesity risk related)	minor allele frequency cases/ controls [%]	genotype distribution cases[%]/ controls[%]			odds ratio (95% CI) under best genetic model for minor allele	p-value ($x10^{-6}$) (corrected empirical p-value)	risk allele (more frequently transmitted)	p-value (one-sided) (Bonferroni corrected p-value)
16	*FTO*	rs1121980	C/**T**	53.0/40.7	104 [21.5]/ 153 [34.6]	247 [51.0]/ 218 [49.3]	133 [27.5]/ 71 [16.1]	1.660 (1.369;2.017) log-additive	0.113 (0.0494)	T	0.0001 (0.0011)
16	*FTO*	rs9939973	**A**/G	52.7/40.7	104 [21.5]/ 153 [34.6]	250 [51.7]/ 218 [49.3]	130 [26.9]/ 71 [16.1]	1.644 (1.355;1.999) log-additive	0.211 (0.0875)	A	0.0003 (0.003)
16	*FTO*	rs7193144	**C**/T	50.1/38.7	121 [25.0]/ 165 [37.4]	241 [49.8]/ 211 [47.8]	122 [25.2]/ 65 [14.7]	1.593 (1.318;1.925) allelic	0.765 (0.2802)	-[4]	-
16	*FTO*	rs9940128	**A**/G	52.1/40.7	106 [21.8]/ 153 [34.6]	254 [52.3]/ 218 [49.3]	126 [25.9]/ 71 [16.1]	1.606 (1.324;1.954) log-additive	0.772 (0.2831)	-[4]	-
20	*C20orf75*	rs6076920	C/**G**	14.9/8.3	347 [71.4]/ 375 [84.8]	133 [27.4]/ 61 [13.8]	6 [1.2]/ 6 [1.4]	2.242 (1.601;3.157) dominant	0.86 (0.3081)	C	0.850 (1)
20	*none*	SNP_A-1967967[5]	**A**/G	14.5/7.7	348 [71.5]/ 375 [84.8]	137 [28.1]/ 66 [14.9]	2 [0.4]/ 1 [0.2]	2.236 (1.596;3.148) dominant	0.94 (0.3321)	G[3]	0.9742 (1)
16	*FTO*	rs8050136	**A**/C	50.0/38.7	123 [25.3]/ 165 [37.3]	240 [49.4]/ 212 [48.0]	123 [25.3]/ 65 [14.7]	1.585 (1.312;1.915) allelic	0.976 (0.3433)	-[4]	-
14	*TSHR*	rs3783950	C/**G**	43.7/54.9	151 [31.5]/ 88 [20.2]	239 [49.8]/ 216 [49.7]	90 [18.8]/ 131 [30.1]	0.635 (0.526;0.767) allelic	1.38 (0.4541)	G	0.132 (1)
4	*BC041448*	rs2969001	C/**G**	38.1/27.6	194 [39.8]/ 240 [54.3]	215 [44.1]/ 160 [36.2]	78 [16.0]/ 42 [9.5]	1.614 (1.320;1.974) allelic	1.60 (0.5044)	G	0.108 (1)
16	*FTO*	rs9939609	A/**T**	49.7/38.7	123 [25.4]/ 164 [37.1]	241 [49.8]/ 214 [48.4]	120 [24.8]/ 64 [14.5]	1.565 (1.295;1.892) allelic	1.94 (0.5681)	-[4]	-
4	*none*	rs619819	C/**G**	30.4/39.4	248 [51.0]/ 157 [35.5]	181 [37.2]/ 222 [50.2]	57 [11.7]/ 63 [14.3]	0.529 (0.402;0.694) dominant	1.96 (0.5717)	C	0.566 (1)
4	*none*	rs2172478	**A**/G	30.6/21.2	234 [48.1]/ 281 [63.6]	207 [42.6]/ 135 [30.5]	45 [9.3]/ 26 [5.9]	1.880 (1.433;2.467) dominant	2.33 (0.6348)	A	0.292 (1)
20	*PCSK2*	rs16998603	**A**/G	14.4/7.9	346[71.8]/ 371 [84.7]	133 [27.6]/ 65 [14.8]	3 [0.6]/ 2 [0.5]	2.177 (1.552;3.068) dominant	2.37 (0.6427)	G[3]	0.878 (1)
6	*HLA-DQA2*	rs9276431	**C**/T	35.5/45.9	203 [41.9]/ 121 [27.4]	218 [45.0]/ 236 [53.4]	63 [13.0]/ 85 [19.2]	0.522 (0.392;0.694) dominant	3.45 (0.7697)	T[3]	0.967 (1)
4	*none*	rs10008032	**C**/T	44.9/53.2	157 [32.4]/ 84 [19.0]	221 [45.6]/ 246 [55.7]	107 [22.1]/ 112 [25.3]	0.490 (0.357;0.671) dominant	3.58 (0.7825)	T	0.591 (1)

[1]genes or transcripts according to the UCSC Genome Bioinformatics (http://genome.ucsc.edu/): ***FTO***: fat mass and obesity associated; ***C20orf75***: hypothetical protein LOC164312; ***TSHR***: thyroid stimulating hormone receptor isoform 1; ***BC041448***: Homo sapiens cDNA clone IMAGE:5170949, partial cds; ***PCSK2***: Homo sapiens cDNA FLJ34186 fis; ***HLA-DQA2***: major histocompatibility complex, class II, DQ; none: no gene 250 kb up- or downstream of the SNP,

[2]FBATs were all evaluated under additive genetic model (as it is e.g. unknown if a dominant model is also appropriate for the analysis of the family-based data),

[3]SNPs showed evidence for a deviation from HWE in founders ($p \leq 0.05$),

[4]not genotyped due to strong linkage disequilibrium to other *FTO* SNPs,

[5]rs41492957according to NCBI Build 36.2

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obese index patient; of the six positive *FTO* SNPs belonging to the same LD block, we genotyped only the two SNPs with the lowest p-values. Confirmation of the initial finding using FBAT (family-based association test) was detected for the two *FTO* SNPs (both Bonferroni corrected $p \leq 0.01$). However, none of the risk alleles of the nine other SNPs showed evidence for association in the independent families.

Additionally, similar to the approach of Scuteri et al. [8], we analysed specific candidate genes (coding sequence plus approximately 50 kb flanking the 5′ and 3′ regions, respectively). We chose those candidate genes delineated in the current version of the *Obesity Gene Map Database* (http://obesitygene.pbrc.edu/; [9]), for which two or more independent positive associations to obesity have been reported in addition to those genes listed in the Database to harbour mutations leading to monogenic forms of obesity. In Table S1 (Supporting Information), for each of these genes, the SNP with the lowest p-value and the respective genetic model are shown. Overall, among the 745 SNPs tested 75 (12) had a p-value below 0.05 (0.01). We re-assessed the original publications for the markers in Table S1 (Supporting Information) which had a nominal p-value below 0.005. For three SNPs of the previous publications (*ESR1*: rs2234693; rs9340799 and *LDLR* rs688) HapMap data were available. Hence, we checked for linkage disequilibrium by D' and r^2. For *ESR1* both previously investigated SNPs (rs2234693; rs9340799 [10,11]) were approximately 16 kb apart from the 500k SNP rs712221. D' was 0.321 and 0.809, respectively, r^2 was 0.103 and 0.378, respectively. For *LDLR* the previous SNP (rs688 [12,13]) was just 48 bp apart from the 500k SNP rs1799898. D' was 1 and r^2 was 0.144. However, the allele frequencies of the original SNPs and the closest GWA SNPs were quite different (e.g. rs1799898 MAF approximately 12% versus a MAF of 45% in rs688), complicating statements dealing with whether or not both markers tag the same disease related haplotype. Clearly, this possibility requires further attention.

DISCUSSION

Here we show by a GWA including early onset extremely obese cases (mean BMI Zscore 4.63 ± 2.27) and healthy underweight controls (mean BMI Zscore -1.38 ± 0.35; BMI$<15^{th}$ age percentile) that variation in *FTO* strongly contributes to the development of early onset obesity. Recently, the *FTO* gene was found to be associated with T2DM as based on two GWAs [5,6]. However, after adjusting for BMI the T2DM association vanished indicating that *FTO* explains variation of body weight. Confirmation in 13 samples with 38,759 individuals and a meta-analysis showed that the A-allele of the variant rs9939609 is associated with a 31% increased risk to develop obesity [5]. These results were independently supported in 8,000 individuals from different populations [14] and in a GWA for obesity-related traits in an epidemiological cohort [8]. The best SNP rs1421085 of the study of Dina et al. [14] showed a nominal $p = 3.46 \times 10^{-7}$ (log-additive OR for the risk allele 1.69, 95% CI 1.38–2.06) in a case-control sample which also comprised obese German children. For our best SNP rs1121980, which is located 8.3 kb upstream of rs1421085 (pairwise $r^2 = 0.90$ in CEU HAPMAP; both within intron 1), we found similar estimated genetic effect sizes. As effect sizes for the best markers derived from GWA data sets are usually overestimated [15], our GWA data is an example that this will not always be the case.

Given the relatively small sample size used in our GWA, this investigation nevertheless revealed a single SNP in *FTO* that remained significant after a proper control of the type I error. The *FTO* SNPs have previously been shown to be relevant for obesity in both children and adults [5,8,14]. To determine, if the finding is present in all children or only among the older teenagers we did a median split for age within the case group and explored the relationship of each subgroup in comparison to controls as well as to each other (data not shown). The effect is valid in both subgroups and there is no difference between the subgroups. Frayling et al. reported that the association is relevant by the age of 7 and persists into the pre-pubertal period and beyond [5]. Only a meta-analysis addressing developmental aspects will be able to pinpoint, if the effect of the *FTO* variants is more relevant for children or for adults.

Confirmation of the 11 SNPs genotyped in 644 independent obesity families succeeded only for the two *FTO* SNPs (Table 1). Hence, our data suggest that of the best 15 SNPs of the GWA only the *FTO* SNPs represent true positive findings. This is in accordance with a population-based GWA for body weight that also merely resulted in the initial confirmation of only one candidate gene [8].

Our data pertaining to the candidate gene analyses (Table S1) are not readily comparable with the previous publications, as for instance the number of analysed individuals was quite low for some of the previous reports. We restricted our analyses to genes listed in the *Obesity Gene Map Database* (http://obesitygene.pbrc.edu/; [9]) with at least two confirmations; the quality of the original reports varied considerably and for some of the genes different SNPs/variants had been analysed. Hence, we suggest that the candidate genes with SNPs resulting in nominal p-values below 0.005 in our scan should be followed up in subsequent studies.

In general, this report is another proof of concept in favour of GWAs contributing to the investigation of common variation in complex phenotypes.

METHODS

Participants

487 extremely obese children and adolescents ('cases') were recruited in hospitals specialized for the inpatient treatment of extreme obesity (Table 2; mean BMI Z score: 4.63 ± 2.27) while 442 healthy lean individuals ('controls') were ascertained at the University of Marburg (Table 2). We relied on older healthy underweight controls to substantially reduce the probability of their becoming overweight and to increase power [e.g. 16]. Based on self-reported questionnaire data on body-weight course, 78% of the lean controls reported having had a below average body weight at age 15, which is similar to the mean age of our obese cases. Thus, our control group mainly comprises individuals who presumably also were in the lower body weight range during adolescence. Details on power considerations are provided in the Supporting Information (Text S1). Written informed consent was given by all participants and in case of minors their parents. The study was approved by the Ethics Committees of the Universities of Marburg and Essen and conducted in accordance with the guidelines of *The Declaration of Helsinki*.

Genotyping

Genotyping was performed on the Genome-Wide Human SNP Array 5.0 (http://www.affymetrix.com/) at the Affymetrix Services Lab (California, USA). 440,794 genotypes of 929 individuals (Dynamic Model algorithm call rate>86%) were called by the BRLMM-P algorithm. For the replication of 11 SNPs genotyping was performed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis of allele-dependent primer extension products as described elsewhere [17].

Table 2. Description of samples genotyped with the Genome-Wide Human SNP Array 5.0 and the obesity families used for confirmation of the 11 best SNPs

population		status	n total (female)	age [years] (mean; SD)	BMI [m/kg^2] (mean; SD)	BMI Zscore (mean; SD)	Waist-hip ratio (mean; SD)
GWA							
	German extremely obese children and adolescents	cases	487 (278)	14.38 (3.74)	33.40 (6.81)	4.63 (2.27)	0.90 (0.11)
	German lean subjects	controls	442 (271)	26.07 (5.79)	18.31 (1.10)	−1.38 (0.35)	0.77 (0.06)
confirmation family-based association study							
	German extremely obese children and adolescents	index patients	644 (363)	13.55 (2.91)	31.92 (5.96)	4.20 (2.01)	0.90 (0.09)
	German obese children and adolescents	siblings	337 (181)	14.91 (5.09)	27.86 (5.24)	2.80 (1.62)	0.88 (0.09)
	parents of the obese children and adolescents	parents	1288 (644)	42.99 (5.92)	30.44 (6.26)	1.69 (1.83)	0.91 (0.11)

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Statistical Methods

For the GWA data, SNPs with a call rate<95%, departure from Hardy-Weinberg equilibrium in the control group (exact test p<0.01), or with minor allele frequency below 10 percent were excluded from the final analysis (151,503 excluded; 289,291 retained; see Supporting Information; Text S1 and Table S2). The statistical analyses followed the procedure of Sladek et al. [4]. Details are described in the Supporting Information (Text S1). All reported nominal p-values of the GWA are two-sided and asymptotic. In addition, empirical p-values corrected for genome-wide testing and maximization across genetic models are provided. We used a genome-wide significance level of .05 (two-sided). For the confirmation study, both nominal one-sided and Bonferroni corrected (11 tests) p-values are presented for the risk alleles identified in the GWA.

Confirmation

The 11 of the 15 best SNPs (ranked by p-value, irrespective of the genetic model; see Table 1) were genotyped in 644 independent obesity families comprising 644 extremely obese children and adolescents (index patients) and both of their biological parents; additionally in 297 families obese sibs were also included (for details see Table 2). As none of the 11 SNPs showed strong evidence for a recessive genetic model in the GWA, it was decided to restrict the family-based association testing to the additive model for each of the SNPs (FBAT additive) in order to reduce the amount of multiple testing.

Candidate gene analyses

Within the GWA data we analysed genes previously suggested to be involved in body weight regulation. We examined 745 SNPs (located within the gene and approximately 50kb 5′and 50kb 3′to the gene) in 47 candidate genes (single gene mutations with an obesity phenotype and candidate genes associated with obesity in at least two independent studies as shown in the *Obesity Gene Map Database*; http://obesitygene.pbrc.edu/) and determined the number of SNPs with p-values ≤ 0.05 (0.01). In addition, we provided information on the SNP with the lowest p-value among all tested genetic models for the respective candidate gene in Table S1 (Supporting Information). For markers which had a nominal p-value below 0.005 we re-assessed the original publications in order to figure out if the marker in our GWA scan matches the information provided in the original publications.

SUPPORTING INFORMATION

Table S1 Analyses of obesity candidate genes (according to the *human obesity gene map*: the 2005 update: Rankinen et al., 2006) in the GWA approach
Found at: doi:10.1371/journal.pone.0001361.s001 (0.16 MB DOC)

Table S2 Genotyping and quality control
Found at: doi:10.1371/journal.pone.0001361.s002 (0.08 MB DOC)

Text S1 Genotyping and quality control; Additional information on statistical analyses; References for the Supporting Information.
Found at: doi:10.1371/journal.pone.0001361.s003 (0.04 MB DOC)

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Author Contributions

Conceived and designed the experiments: JH AH. Performed the experiments: SF GB TM HG. Analyzed the data: JH AS TI TN HS HW AH. Contributed reagents/materials/analysis tools: WR AS TN HS. Wrote the paper: JH AS SF AH.

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3.3 Publication III

Friedel S, Horro FF, Wermter AK, Geller F, Dempfle A, Reichwald K, Smidt J, Brönner G, Konrad K, Herpertz-Dahlmann B, Warnke A, Hemminger U, Linder M, Kiefl H, Goldschmidt HP, Siegfried W, Remschmidt H, Hinney A, Hebebrand J. **Mutation screen of the brain derived neurotrophic factor gene (BDNF): identification of several genetic variants and association studies in patients with obesity, eating disorders, and attention-deficit/hyperactivity disorder**. Am J Med Genet B Neuropsychiatr Genet. 2005;132B:96-9.

We investigated the role of *BDNF* as a functional candidate gene in obesity, eating disorders, and attention-deficit/hyperactivity disorder (ADHD). A mutation screen via single stranded confirmation polymorphism (SSCP) and denaturing high pressure liquid chromatography (DHPLC) of the translated region of BDNF in 183 extremely obese children and adolescents and 187 underweight students was performed. Additionally, we genotyped two common polymorphisms (rs6265: p.Val66Met; c.-46C>T) in 118 patients with anorexia nervosa, 80 patients with bulimia nervosa, 88 patients with ADHD, and 96 normal weight controls. Three rare variants (c.5C>T: p.Ile2Thr; c.273G>A; c.*137A>G) and the known polymorphism (p.Val66Met) were identified. A role of the Ile2 allele in the aetiology of obesity cannot be excluded. We found no association between p.Val66Met and the additionally genotyped variant c.-46C>T and obesity, ADHD or eating disorders.

Own contribution:

- selection of the candidate gene and study design (with Hebebrand J and Hinney A)
- assay design and mutation screen
- genotyping of detected variants
- data evaluation, interpretation and writing of the manuscript

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Mutation Screen of the Brain Derived Neurotrophic Factor Gene (*BDNF*): Identification of Several Genetic Variants and Association Studies in Patients With Obesity, Eating Disorders, and Attention-Deficit/Hyperactivity Disorder

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Several lines of evidence indicate an involvement of brain derived neurotrophic factor (BDNF) in body weight regulation and activity: heterozygous *Bdnf* knockout mice (*Bdnf*$^{+/-}$) are hyperphagic, obese, and hyperactive; furthermore, central infusion of BDNF leads to severe, dose-dependent appetite suppression and weight loss in rats. We searched for the role of *BDNF* variants in obesity, eating disorders, and attention-deficit/hyperactivity disorder (ADHD). A mutation screen (SSCP and DHPLC) of the translated region of *BDNF* in 183 extremely obese children and adolescents and 187 underweight students was performed. Additionally, we genotyped two common polymorphisms (rs6265: p.V66M; c.−46C > T) in 118 patients with anorexia nervosa, 80 patients with bulimia nervosa, 88 patients with ADHD, and 96 normal weight controls. Three rare variants (c.5C > T: p.T2I; c.273G > A; c.*137A > G) and the known polymorphism (p.V66M) were identified. A role of the I2 allele in the etiology of obesity cannot be excluded. We found no association between p.V66M or the additionally genotyped variant c.−46C > T and obesity, ADHD or eating disorders. This article contains supplementary material, which may be viewed at the American Journal of Medical Genetics website at http://www.interscience.wiley.com/jpages/0148-7299:1/suppmat/index.html.

KEY WORDS: weight regulation; BMI; anorexia nervosa; bulimia nervosa

INTRODUCTION

BDNF plays a key role in regulating neuronal survival during the development of the central nervous system, differentiation and maintenance of the phenotype of mature neurons [Maisonpierre et al., 1991], and prevents neuronal death [Tuszynski et al., 1994]. Human *BDNF* is localized on chromosome 11p14.1 (http://genome.ucsc.edu/, Freeze July 2003) and encodes a 247 amino acid (aa) preprotein that is proteolytically cleaved to form the 120 aa mature protein [Darling et al., 1983], which is 100% conserved between mouse, rat, pig, and humans [Maisonpierre et al., 1991]. Human *BDNF* consists of five alternatively used 5′ exons and one major 3′ exon. Alternative splicing of the 5′ exons results in six different transcripts leading to three preproprotein isoforms (a, b and c) that differ in the length of their signal peptide (http://www.ncbi.nlm.nih.gov/LocusLink, April 2004). Isoforms b and c contain additional N-terminal aa compared to isoform a.

Several lines of evidence indicate an involvement of genetic factors in the etiology of the complex and multifactorial disorders obesity, anorexia nervosa (AN), bulimia nervosa (BN), and attention-deficit/hyperactivity disorder (ADHD). We propose a role of *BDNF* in the development of these disorders for the following reasons: (i) obesity and eating disorders: heterozygous *Bdnf* knockout mice (*Bdnf*$^{+/-}$) are obese and develop hyperphagia [Kernie et al., 2000]. Their increase in body weight is similar to that seen in heterozygous melanocortin-4-receptor deficient (*Mc4r*$^{+/-}$) mice, a well-known model for human obesity [e.g., Huszar et al., 1997; Hinney et al., 2003]. BDNF is expressed at high levels in the ventromedial hypothalamus (VMH), where it is regulated by nutritional state and by MC4R signaling [Xu et al., 2003]. Bilateral lesions of the VMH entail hyperphagia and obesity [Shimizu et al., 1987]. Central infusion of BDNF leads to severe, dose-dependent appetite suppression, weight loss, and increase in hypothalamic 5-hydroxy-indoleacetic acid (5-HIAA) and serotonin in rats, implying an anorexigenic function of BDNF [Pelleymounter et al., 1995].

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A recent study revealed a strong association of the M66 allele of *BDNF* with obsessive-compulsive disorder [OCD; Hall et al., 2003], which is quite common in patients with eating disorders, suggesting that there may be a common genetic predisposition to both OCD and AN [Halmi et al., 1991]. Ribases et al. [2003] recently detected an association of the M66 variant in the region encoding BDNF proprotein [Momose et al., 2002] to AN (restricting type) and to a low minimum body mass index (MBMI). Additionally, it was shown that female patients with eating disorders (ED) have decreased levels of serum BDNF compared to healthy normal weight controls [Nakazato et al., 2003]. (ii) *ADHD*: a relationship between ADHD and BDNF has already been hypothesized [Tsai, 2003]. Conditional deletion of *Bdnf* in postnatal mice brain leads to hyperactivity after exposure to stressors [Rios et al., 2001]. A recent study revealed evidence for the involvement of the M66 variant (rs6265) of BDNF in poor verbal episodic memory [Egan et al., 2003]. Therefore, the M66 allele might be relevant in ADHD. BDNF affects central nervous system myelination [Cellerino et al., 1997]; central dysmyelination has been found in patients with ADHD [Overmeyer et al., 2001]. Neurochemical and behavioral analysis of heterozygous $Bdnf^{+/-}$ mice revealed that a partial impairment of BDNF expression causes physiological disturbances which were associated with impaired impulse control, manifested as exaggerated aggressiveness, and excessive appetite/food intake [Lyons et al., 1999].

Therefore, we hypothesize that gain of function mutations could predispose to AN, whereas loss of function mutations could be expected to result in obesity and ADHD. The M66 variant is of particular interest, because it is the only known frequent non-conservative polymorphism in the *BDNF* gene. Furthermore, the M66 variant affects intracellular processing and secretion of the mature protein [Egan et al., 2003].

MATERIALS AND METHODS

In order to assess an involvement of *BDNF* in weight regulation, we screened the translated region of *BDNF* in 183 extremely obese children and adolescents and 187 healthy underweight students (initial screening sample). We identified two new variants and three known SNPs, which enabled us to perform association studies.

Study Subjects

We screened 183 extremely obese children and adolescents and 187 healthy underweight students. The mean BMI percentile of the 183 obese probands exceeded the 99th BMI-percentile, the BMI of the underweight students was below the 15th percentile, as previously determined in a representative German population sample [Hebebrand et al., 1996]. For association studies, we used the initial screening-sample and samples of patients with ADHD, AN, BN, and normal weight controls (see the online Table II at http://www.interscience.wiley.com/jpages/0148-7299:1/suppmat/index.html). Patients with ED or with ADHD fulfilled DSM-IV criteria [APA, 1994]. Written informed consent was given by all participants and, in the case of minors, their parents. This study was approved by the Ethics Committee of the University of Marburg.

PCR, DHPLC, and SSCP

Six transcript variants encoding three preproprotein isoforms have been described for this gene. The nomenclature of the described polymorphisms is according to den Dunnen and Antonarakis [2001] and in relation to transcript variant 1 encoding isoform a (Acc. No. NM_001709). Variant c.−46C > T was earlier described as 270C > T [Kunugi et al., 2001]. We screened the translated region of human *BDNF* in two overlapping fragments A and B (see the online Table III at http://www.interscience.wiley.com/jpages/0148-7299/suppmat/index.html). For PCR amplification primers were placed so that potential splice site variants could be detected. Mutation screen on fragment A was performed with denaturing high performance liquid chromatography (DHPLC) analysis on Transgenomic WAVE® system [Transgenomic, Cheshire, UK; Oefner and Underhill, 1998]. All chromatograms were compared with chromatograms of sequenced wild-type samples. PCR amplicons that showed a peak appearance different to the wild-type pattern were sequenced (Seq Lab, Göttingen, Germany). To detect mutations in fragment B, the PCR-products were digested and standard nonisotopic single-strand conformation polymorphism analyses (SSCP) were performed at room temperature and at 4°C [Hinney et al., 1999]. The sensitivities for DHPLC have been described to be approximately 95% [Ellis et al., 2000] and about 97% for SSCP by using two temperatures, respectively [Salazar et al., 2002].

SNPs

PCR products of all SNPs were run on ethidium bromide-stained 2.5% agarose gels. Positive controls for the variant alleles were run on each gel. For validity of the genotypes, allele determinations were rated independently by at least two experienced individuals. Discrepancies were resolved unambiguously either by reaching consensus or by retyping (see the online Table IV at http://www.interscience.wiley.com/jpages/0148-7299:1/suppmat/index.html).

Statistics

Differences in genotype frequencies were investigated using the Cochran-Armitage trend test. Pearson's χ^2-tests were carried out to investigate differences in allele frequencies. Initially, obese children and adolescents were compared with underweight students. Our latter analyses tested each of the groups AN, BN, and ADHD separately against normal weight students. We did not correct for the multiple tests we performed for the different groups at the two loci. Therefore, all reported *P* values are nominal.

RESULTS

By sequencing PCR products showing an aberrant SSCP or DHPLC-pattern we identified three rare variants (c.5C > T; c.273G > A; c.*137A > G) in addition to the common missense mutation p.V66M. In the study groups comprising 183 extremely obese children and adolescents and 187 healthy underweight students each rare variant was observed only once: (a) the novel silent variant c.273G > A in codon 91 of the region coding for the proprotein was discovered by DHPLC. An extremely obese male (age 16.2 years, BMI 50.4 kg/m^2, BMI ≥ 99th percentile) was heterozygous for this variant. (b) A known variant c.5C > T (rs8192466), previously detected in a patient with idiopathic congenital central hypoventilation syndrome [CCHS; Weese-Mayer et al., 2002] leading to the non-conservative non-synonymous aa change p.T2I was detected in a single extremely obese boy (age 11.1 years, BMI 40.4 kg/m^2, BMI ≥ 99th percentile) by DHPLC. The allele coding for I2 was transmitted by the overweight mother (BMI 28.7 kg/m^2); the overweight sib had inherited the wild type allele. (c) One of the underweight controls (age 24.1 years, BMI 19.7 kg/m^2, 6th BMI percentile) was heterozygous for a novel 3′UTR variant c.*137A > G, detected by SSCP.

We analyzed two common polymorphisms (V66M in the 5′ pro-region; SNP c.−46C > T in one of the 5′UTR exons, Kunugi et al., 2001) in the initial study groups and additionally in 118 patients with AN, 80 patients with BN, 88 patients with

TABLE I. Genotype Distribution of p.V66M (Isoform a, rs6265) in the Translated Region and SNP c.−46 > T in the Untranslated Region of *BDNF*

	Obese children and adolescens, n (%)	Underweight controls, n (%)	Normal weight controls, n (%)	Patients with AN, n (%)	Patients with BN, n (%)	Patients with ADHD, n (%)
V66M						
n	183	187	96	118	80	83
GG	114 (62.3)	110 (58.8)	62 (64.6)	81 (68.6)	51 (63.8)	56 (67.5)
GA	57 (31.1)	71 (38.0)	33 (34.4)	32 (27.1)	27 (33.7)	24 (28.9)
AA	12 (6.6)	6 (3.2)	1 (1.0)	5 (4.3)	2 (2.5)	3 (3.6)
c.−46 > T						
n	182	185	82	118	80	86
CC	158 (86.8)	164 (88.7)	75 (91.5)	103 (87.3)	65 (81.3)	75 (87.2)
CT	24 (13.2)	20 (10.8)	7 (8.5)	15 (12.7)	13 (16.2)	10 (11.6)
TT	0 (0)	1 (0.5)	0 (0)	0 (0)	2 (2.5)	1 (1.2)

AN, anorexia nervosa; BN, bulimia nervosa; ADHD, attention-deficit/hyperactivity disorder.
Genotype-frequencies did not differ from Hardy–Weinberg equilibrium.

ADHD, and 96 normal weight controls. Genotype frequencies did not differ from Hardy–Weinberg equilibrium. Association studies revealed no significant differences in genotype or allele distributions between extremely obese children and adolescents and underweight controls, as well as between AN, ADHD, and normal weight students; all nominal P values were >0.2 (Table I). We detected a trend towards association for the −46T allele in 80 patients with BN compared to 82 normal weight controls (nominal $P = 0.06$ for the genotypes and nominal $P = 0.03$ for the alleles). At the nominal significance level of 5%, for the given sample sizes and the observed allele frequencies in controls, our study had a power of 80% to detect a 10% increase in M66 frequency between underweight controls and obese children and adolescents; for the comparisons involving normal weight controls the respective power was about 60%. For c.−46C > T, we had a power of 80% to detect a twofold increase in −46T allele frequency between underweight controls and obese children and adolescents; for the comparisons involving normal weight controls the respective power was about 40%.

DISCUSSION

We screened the translated main exon of *BDNF* for mutations in a total of 370 German obese and underweight individuals. Three variants were identified apart from the previously known SNP p.V66M (rs6265): (i) we found the previously detected non-conservative amino acid substitution p.T2I [Weese-Mayer et al., 2002] in a single extremely obese male who inherited the mutation from his obese mother. Amino acid position 2 of isoform a is equivalent to position 10 in isoform b and position 17 in isoform c of the BDNF preproprotein. The threonin at this position is conserved between all species, of which a sequence has been deposited into public databases, including mouse, rat, pig, various bears, kangaroo, chicken, carp, platy fish, and zebra fish [Weese-Mayer et al., 2002]. It is unclear whether the I2 variant affects the mode of action of the signal peptide. If it results in a loss of function, the mutation could very well be relevant for obesity; the body weights of the extremely obese carrier (BMI 40.4 kg/m^2) of the I2 variant and his overweight mother are in the expected range as based on the phenotype of *Bdnf*$^{+/-}$ mice that show a significant weight increase in males (50%) and females (27%) compared to wild type littermates. The weight of the index mutation carrier has increased by approximately 50 kg in the last 3 years after loss of 15 kg in an inpatient weight reduction program. Initially, the p.T2I has been described in a dysphagic patient affected by CCHS with a BMI of 16 [Weese-Mayer et al., 2002; Weese-Mayer, personal communication]. His heterozygous father has a BMI of 26 and does not show symptoms of CCHS, but of the associated autonomic nervous system dysfunction [ANSD; Weese-Mayer et al., 2002; Weese-Mayer, personal communication]. This is not readily compatible with a putative role of I2 in the development of obesity. Nevertheless, I2 could be involved in the etiology of obesity because being affected with CCHS or ANSD could explain why obesity does not ensue in these two heterozygotes. ANSD and CCHS are severe syndromes accompanied by oesophageal dysmotility, gastroesophageal reflux, and dysphagia. Until further functional studies are carried out, it is unclear what effect the mutation at I2 might have on the mode of action of the signal peptide and how it may relate to the clinical condition of obesity. (ii) The novel variant c.273G > A was detected once in an extremely obese male. We assume that there is no major effect because this mutation is silent. (iii) The 3′UTR variant c.*137A > G was detected in one underweight control (BMI 19.7 kg/m^2), an influence on the mode of action of BDNF is unlikely. (iv) We did not detect an association between obesity, AN or ADHD and SNP p.V66M or c.−46C > T in the genomic region of *BDNF*. For BN, we found a trend towards an association with −46T. We were not able to follow-up on this result due to our limited number of BN cases and the trend needs to be judged in the context of the multiple tests we performed. Apart from a false positive result, two different mechanisms could explain this finding: First, the c.−46C > T variant is in linkage disequilibrium with a yet unknown variant or an unknown susceptibility gene directly involved in the etiology of BN. Alternatively, this variant itself entails an increased risk that may result from an alteration in the translation efficacy [Shintani et al., 1992]. No data are available as to potential functional consequences of this variant. Our results were not in line with Ribases et al. [2003], who reported an association of the M66-allele with AN in a Spanish sample. Some of our data on patients with AN or BN and controls have been included in a recent meta-analysis pertaining to the polymorphisms V66M and c.−46C > T. The meta-analysis showed that the M66 variant is strongly associated to all ED subtypes and that the −270C (−46T) BDNF variant has an effect on BN and age at onset of weight loss [Ribases et al., 2004].

In conclusion, our results do not suggest a large role of genetic variation of *BDNF* in AN, BN, ADHD, or obesity; possibly the I2 variant plays a role in obesity. To exclude moderate effects of the two investigated polymorphisms larger samples need to be assessed.

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3.4 Publication IV

Friedel S, Antwerpen B, Hoch A, Vogel C, Grassl W, Geller F, Hebebrand J, Hinney A. **Glucose transporter 4 gene: association studies pertaining to alleles of two polymorphisms in extremely obese children and adolescents and in normal and underweight controls**. Ann N Y Acad Sci.2002;967:554-7.

The human insulin-responsive glucose transporter 4 gene (GLUT4) has been related to non-insulin-dependent diabetes mellitus (NIDDM) in several studies. Obesity is commonly found in patients with NIDDM. Hence, genes involved in NIDDM might also be relevant for obesity. We have analyzed 212 extremely obese children and adolescents, 82 normal-weight students, and 94 underweight students for two single nucleotide polymorphisms in the vicinity of the *GLUT4* by polymerase chain reaction with subsequent restriction fragment length polymorphism analyses (PCR-RFLP) or single-strand conformation polymorphism analyses (SSCP). Allele and genotype distributions were similar in all study groups (all p values > 0.05). Hence, we did not detect association of any of the analyzed SNP alleles in the *GLUT4* to different weight extremes, so these seem not to be involved in weight regulation in our study groups.

Own contribution:

- genotyping of detected variants (in cooperation with Antwerpen B, Hoch A, Vogel C)
- data evaluation, interpretation and writing the manuscript

Glucose Transporter 4 Gene

Association Studies Pertaining to Alleles of Two Polymorphisms in Extremely Obese Children and Adolescents and in Normal and Underweight Controls

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ABSTRACT: The human insulin-responsive glucose transporter 4 gene (*GLUT4*) has been related to non-insulin-dependent diabetes mellitus (NIDDM) in several studies. Obesity is commonly found in patients with NIDDM. Hence, genes involved in NIDDM might also be relevant for obesity. We have analyzed 212 extremely obese children and adolescents, 82 normal-weight students, and 94 underweight students for two single nucleotide polymorphisms (SNPs: promoter –30G/A; exon 4a: silent 2061T/C) in the vicinity of the *GLUT4* by polymerase chain reaction with subsequent restriction fragment length polymorphism analyses (PCR-RFLP) or single-strand conformation polymorphism analyses (SSCP). Allele and genotype distributions were similar in all study groups (all p values > 0.05). Hence, we did not detect association of any of the analyzed SNP alleles in the *GLUT4* to different weight extremes, so these seem not to be involved in weight regulation in our study groups.

KEYWORDS: *GLUT4*; weight regulation

INTRODUCTION

Obesity is a multifactorial disease that is influenced by both environmental and genetic factors. Patients with non-insulin-dependent diabetes mellitus (NIDDM) are often obese, so genes involved in NIDDM might also be relevant for obesity. The human insulin-responsive glucose transporter 4 gene (*GLUT4*) has been analyzed in several studies pertaining to NIDDM.[1] Additionally, two recent genome-wide scans for phenotypes related to diet and the metabolic syndrome identified a region on chromosome 17p13 that harbors the *GLUT4*.[2,3]

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Additional evidence implicates *GLUT4* in body weight regulation: *GLUT4* knockout mice have greatly reduced fat depots.[4] Female mice overexpressing *GLUT4* (transgenic) have increased adipose cell size and tissue weight.[5] *GLUT4* promoter activity is increased in obese compared to lean rats.[6]

MATERIALS AND METHODS

Study Subjects

Briefly, blood samples were collected[7] from 212 extremely obese German children and adolescents (mean BMI: 32.8 ± 6.41 kg/m^2; mean age: 14 ± 2.48 years), 82 normal-weight students (mean BMI: 21.84 ± 1.05 kg/m^2; mean age: 24.8 ± 2.62 years), and 94 underweight students (mean BMI: 18.51 ± 1.15 kg/m^2; mean age: 25.35 ± 3.76 years). Sixty-seven percent of the obese children and adolescents had an age- and gender-specific BMI $\geq$ 99th percentile, as previously determined in a representative German population sample.[8] The BMI of the underweight students was below the 15th percentile and between the 40th and 60th percentile for the normal-weight students.

Written informed consent was given by all participants and, in the case of minors, their parents. This study was approved by the Ethics Committee of the University of Marburg.

Molecular Analyses

We investigated two single nucleotide polymorphisms (SNPs) in the vicinity of *GLUT4*: one SNP in the promoter region (−30G/A) and another one in exon 4a (silent Asp-130).[9–11]

For the promoter (−30G/A) SNP, we performed standard polymerase chain reaction (PCR) and subsequent restriction fragment length analysis (RFLP) with *Bam HI*. PCR primers were as follows: 5′-GGGCTTCTCGCGTCTTTT-3′ (forward) and 5′-TGAAAGAACCGATCCTGGAG-3′ (reverse). The amplicon (189 bp) with the A-allele was digested by *Bam HI* (124 bp/65 bp). PCR-RFLP products were run on ethidium bromide–stained 2.5% agarose gels. Positive controls were run on each gel.

To detect the alleles of the SNP in exon 4a, standard nonisotopic single-strand conformation polymorphism analysis (SSCP) (15% acrylamide gel run at 600 V for 2.5 h at ambient temperature and subsequent silver staining) was performed. Primers were 5′-AAAGAGGAAGGGAGCCACTG-3′ (forward) and 5′-GTGCCCGTGAG-TACCTGAGT-3′ (reverse). The amplified segment was 203 bp in length.

Statistics

Pearson's χ^2 test (asymptotic, two-sided) and Cochran-Armitage's trend test (exact, two-sided) were used to investigate for association.

TABLE 1. Genotype and allele distributions of a single nucleotide polymorphism (–30G/A) in the promoter region of *GLUT4*

Study group	Genotypes			Alleles	
	GG (%)	GA (%)	AA (%)	G (%)	A (%)
Extremely obese children and adolescents ($n = 212$)	40 (18.9)	92 (43.4)	80 (37.7)	172 (40.6)	252 (59.4)
Underweight students ($n = 94$)	13 (13.8)	39 (41.5)	42 (44.7)	65 (34.6)	123 (65.4)
Normal-weight students ($n = 82$)	15 (18.3)	32 (39.0)	35 (42.7)	62 (37.8)	102 (62.2)

NOTE: Genotype frequencies are not different from Hardy-Weinberg equilibrium.

TABLE 2. Genotype and allele distributions of a single nucleotide polymorphism (silent Asp-130) in exon 4a of *GLUT4*

Study group	Genotypes			Alleles	
	TT (%)	TC (%)	CC (%)	WT	Mut
Extremely obese children and adolescents ($n = 212$)	24 (11.3)	99 (46.7)	89 (42.0)	147 (34.7)	277 (65.3)
Underweight students ($n = 94$)	7 (7.5)	49 (52.1)	38 (40.4)	63 (33.5)	125 (66.5)
Normal-weight students ($n = 82$)	11 (13.4)	35 (42.7)	36 (43.9)	57 (34.8)	107 (65.2)

NOTE: Genotype frequencies are not different from Hardy-Weinberg equilibrium.

RESULTS

The genotype and allele distributions of both SNPs are given in TABLES 1 and 2. The genotype frequencies were not different from Hardy-Weinberg equilibrium. No significant differences in genotype and allele distributions were found between extremely obese children and adolescents, underweight students, and normal-weight students. All nominal p values were >0.2.

CONCLUSIONS

This study analyzed a possible association of body weight with two SNPs in the insulin-stimulated *GLUT4* in a German Caucasian population. We investigated a total of 388 probands: 212 extremely obese children and adolescents, 94 underweight students, and 82 normal-weight students.

We did not detect association of any of the analyzed SNP alleles in the vicinity of *GLUT4* to different weight categories. Hence, the analyzed polymorphisms are not involved in weight regulation in our study groups. Our results do not exclude the occurrence of relevant mutations in the *GLUT4*.

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3.5 Publication V

Friedel S, Reichwald K, Scherag A, Brumm H, Wermter AK, Fries HR, Koberwitz K, Wabitsch M, Meitinger T, Platzer M, Biebermann H, Hinney A, Hebebrand J. **Mutation screen and association studies in the diacylglycerol O-acyltransferase homolog 2 gene (DGAT2), a positional candidate gene for early onset obesity on chromosome 11q13**. BMC Genet. 2007;8:17.

The aim of this study was to investigate the role of DGAT2 as a positional and functional candidate gene in obesity. We performed a mutation screen in 93 extremely obese children and adolescents and 94 healthy underweight controls. Association studies were performed in samples of up to 361 extremely obese children and adolescents and 445 healthy underweight and normal weight controls. Additionally, we tested for linkage and performed family based association studies at four common variants in the 165 families of our initial genome scan.

The mutation screen revealed 15 DNA variants, four of which were non-synonymous (p.Val82Ala, p.Arg297Gln, p.Gly318Ser and p.Leu385Val) and ten variants were synonymous exchanges. Additionally, the small biallelic trinucleotide repeat rs3841596 was identified. None of the case control and family based association studies showed an association of investigated variants or haplotypes in the genomic region of *DGAT2*.

In conclusion, our results do not support the hypothesis of an important role of common genetic variation in *DGAT2* for the development of obesity in our sample. Anyhow, such an effect might be conferred by the less common variants or the detected, rare non-synonymous variants.

Own contribution:

- selection of the candidate gene and study design (with Hebebrand J and Hinney A)
- assay design and mutation screen
- genotyping of detected variants
- data evaluation, interpretation and writing the manuscript

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Research article

Mutation screen and association studies in the Diacylglycerol O-acyltransferase homolog 2 gene (*DGAT2*), a positional candidate gene for early onset obesity on chromosome 11q13

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Abstract

Background: *DGAT2* is a promising candidate gene for obesity because of its function as a key enzyme in fat metabolism and because of its localization on chromosome 11q13, a linkage region for extreme early onset obesity detected in our sample.

We performed a mutation screen in 93 extremely obese children and adolescents and 94 healthy underweight controls. Association studies were performed in samples of up to 361 extremely obese children and adolescents and 445 healthy underweight and normal weight controls. Additionally, we tested for linkage and performed family based association studies at four common variants in the 165 families of our initial genome scan.

Results: The mutation screen revealed 15 DNA variants, four of which were coding non-synonymous exchanges: p.Val82Ala, p.Arg297Gln, p.Gly318Ser and p.Leu385Val. Ten variants were synonymous: c.-9447A > G, c.-584C > G, c.-140C > T, c.-30C > T, IVS2-3C > G, c.812A > G, c.920T > C, IVS7+23C > T, IVS7+73C > T and *22C > T. Additionally, the small biallelic trinucleotide repeat rs3841596 was identified. None of the case control and family based association studies showed an association of investigated variants or haplotypes in the genomic region of *DGAT2*.

Conclusion: In conclusion, our results do not support the hypothesis of an important role of common genetic variation in *DGAT2* for the development of obesity in our sample. Anyhow, if there is an influence of genetic variation in *DGAT2* on body weight regulation, it might either be conferred by the less common variants (MAF < 0.1) or the detected, rare non-synonymous variants.

Background

Obesity has become a major public health problem in industrialized countries and its prevalence is still increasing worldwide [1]. Estimates from twin studies attribute up to 80% of human body weight variation to genetic factors [2] and positional candidate gene analyses in linkage peak regions identified in genome wide scans for obesity have been suggested as a means to detect obesity associated genes [i.e. [3-7]]. Examples for positional candidate gene association findings pertain to (a) *SLC6A14* on chromosome (chr.) Xq24 [3] which was confirmed by Durand et al. [4] and (b) *GAD2* on chr. 10p12 [5] which was confirmed by the same group [6]. In contrast, Swarbrick et al. [7] found no evidence for a relationship between the three *GAD2* SNPs and obesity in a sample comprising 2,359 individuals.

A genome wide scan for obesity based on 89 German families, comprising extremely obese children and adolescents and both of their parents and at least one obese sib, identified nine regions with maximum likelihood binomial logarithm of the odd (MLB LOD) scores > 0.7; in an independent confirmation sample of 76 obesity families MLB LOD scores of 0.68 and 0.71 were observed for chromosomes 10p11.23 and 11q13, respectively [8].

The hypothesis of a susceptibility gene for obesity and related phenotypes on chromosome 11q13 was additionally supported by independent linkage studies for BMI and obesity related phenotypes [9-12]. Further support was obtained from chromosomal regions homologous to human chromosome 11q13 in rodents in which quantitative trait loci (QTL) for obesity related phenotypes such as leptin level [13] and BMI [14] were identified. Taken together, there is evidence for a candidate gene for obesity in this chromosomal region.

In earlier studies, we investigated different promising candidate genes on chr.11q, but none of them contributed to the linkage peak [15-17]. Diacylglycerol-O-acyltransferase homolog 2 (*DGAT2*), another potential candidate gene, is also located on chr. 11q13. DGAT2 is a key enzyme in fat metabolism [18,19]. It is responsible for the synthesis of triglycerides and catalyzes the reaction that joins diacylglycerol covalently to long chain fatty acyl-CoAs. It was hypothesized that leptin regulates adipocyte size by altering expression patterns of Diacylglycerol O-acyltransferase 1 (DGAT1) and its functional homolog DGAT2 via the CNS to determine the levels of triglyceride synthesis [20]. The deduced 387-amino acid human DGAT2 protein contains at least one transmembrane domain, three potential N-linked glycosylation sites, six potential phosphorylation sites, and a putative glycerol phospholipid domain found in acyltransferases [18]. Although functionally related, *DGAT2* shares no sequence homology with the members of the *DGAT1* family. The gene was identified via homology search with fungal DGAT subsequent to the finding that *Dgat1* knockout mice (*Dgat1*$^{-/-}$) were viable and still able to synthesize triglycerides [18,19,21].

Dgat2 knockout mice (*Dgat2*$^{-/-}$) are lipopenic, their total carcass triglyceride content was reduced by 93% [22]. In contrast to *Dgat1*$^{-/-}$ mice, where *Dgat2* is able to compensate the role of Dgat1 in triglyceride synthesis, *Dgat1* was unable to compensate for the absence of Dgat2 in *Dgat2*$^{-/-}$ mice. *Dgat2*$^{-/-}$ mice die in the early postnatal period, apparently from abnormalities in energy homeostasis and from impaired permeability barrier function in the skin. The results indicate that Dgat2 is the major enzyme of triglyceride synthesis in mice [22].

Based on both positional as well as on functional arguments, we hypothesized that genetic variations in *DGAT2* might alter triglyceride synthesizing activity of the protein in humans. Genetic variations leading to a gain of function of DGAT2 may thus be associated with obesity, whereas variations entailing a reduced function could be relevant in underweight.

Results

Gene structure

To include all potentially relevant exons of *DGAT2*, its structure was analyzed both *in silico* and experimentally. Visual inspection of ESTs assembled to the *DGAT2* locus in the UCSC genome browser identified two ESTs (BF979495, BF979677) which seemed to harbour alternative/additional exons. The sequences of both ESTs overlap by 200 bp and form a transcript of 1,238 bp. Alignment of this mRNA to genomic DNA revealed the presence of an alternative first noncoding exon of human *DGAT2*, while exons2–8 are as defined by AB048286 (suppl. table 1). Sequencing of EST BF979677 revealed the presence of an alternative internal exon which is located between exon1 and exon2 as defined in AB048286. Furthermore, by RT-PCR in human adipocyte mRNA, a transcript was identified that comprised 7exons in which exon1 and exons3–8 are as defined by AB048286 while exon 2 is missing. In sum, three alternatively spliced transcripts of the human *DGAT2* gene were identified. Including the two previously reported mRNAs (AB048286, ENST00000228027) there are at least five different mRNAs transcribed from this locus [see additional file 1].

Mutation screen

Screening was performed in the coding region, the predicted promoter region and in the identified non-coding 5' exon. The mutation screen in ten fragments comprised 3,079 bps and revealed 15 (14 novel) DNA variants, four of which are coding non-synonymous exchanges:

Table 1: Summary of DGAT2 variants detected in the coding region, the predicted promoter region and a 5'non-coding exon: 15 (14 novel) identified and 2 previously described (rs1017713 and rs3060), minor allele frequency among all successfully genotyped individuals and results of the case control association studies with cases (extremely obese children and adolescents) and controls (normal- or underweight healthy individuals)

variant		region	Study group[1]	minor allele frequency n (%)		p-value[2]
				cases	controls	
g.-9447 A > G		exon 01	2	29 (8.06)	33 (8.82)	0.79
c.-584C > G		promoter	1	0 (0)	1 (0.53)	nd.
c.-140C > T		5'UTR/exon 1	3	2 (0.28)	9 (1.01)	0.13
c.-30C > T		5'UTR/exon 1	1	0 (0)	2 (1.06)	nd.
c.475T > C	p.Val82Ala	exon 2	1	1 (0.54)	0 (0)	nd.
g.IVS1+212T > C	rs1017713	exon 2	2	25 (7.65)	26 (7.06)	0.77
g.IVS2-3C > G		intron 2	1	0 (0)	1 (0.53)	nd.
c.812A > G	p.Thr194Thr	exon 5	1	1 (0.54)	0 (0)	nd.
c.920T > C	p.Ser230Ser	exon 6	1	1 (0.54)	0 (0)	nd.
c.1020G > A	p.Arg297Gln	exon 7	1	0 (0)	2 (1.06)	nd.
c.1492G > A	p.Gly318Ser	exon 7	1	0 (0)	2 (1.06)	nd.
g.IVS7+23C > T		intron 7	1	1 (0.54)	0 (0)	nd.
g.IVS7+73C > T		intron 7	1	2 (1.08)	0 (0)	nd.
g.IVS7+164(TAG)2–3	rs3841596	intron 7	2	24 (6.67)	28 (7.61)	0.67
c.1383C > G	p.Leu385Val	exon 8	1	0 (0)	1 (0.53)	nd.
g.*19T > C	rs3060	3'UTR/exon 8	2	27 (7.50)	27 (7.76)	1
g.*22C > T		3'UTR/exon 8	1	1 (0.54)	0 (0)	nd.

Hardy Weinberg equilibrium was fulfilled (all exact $p > > 0.20$). [1]for descriptions of study groups see Methods; [2]Fisher's exact test, two-sided.

p.Val82Ala, p.Arg297Gln, p.Gly318Ser and p.Leu385Val whereas ten variants are synonymous c.-9447A > G, c.-584C > G, c.-140C > T, c.-30C > T, IVS2–3C > G, c.812A > G, c.920T > C, IVS7+23C > T, IVS7+73C > T and *22C > T (see also table 1). Additionally, a small known biallelic trinucleotide repeat (IVS7+164(TAG)2–3 = rs3841596) located in intron 7 was identified.

Case control association studies

Minor allele frequencies (MAF) of the variants were estimated in sample 1. Most of the variants were rare and it was thus decided to genotype only the more frequent variations rs3841596, rs1017713 and rs3060 in sample 2. Variant -140C > T, located 5' to the translation start, was genotyped in sample 3 which includes sample 2 but is larger and therefore has an improved power (see table 1). Given the sample sizes, the study had a statistical power of more than 80% to detect allelic differences between the respective case and control groups of e.g. 0.17 and 0.1 in MAFs. Genotype distribution in all study samples did not differ from Hardy-Weinberg equilibrium. No significant differences in genotype or allele distributions were found in samples 2 and 3, all nominal p-values were >> 0.05 (see table 1).

Family based association studies

To investigate the contribution of *DGAT2* polymorphisms to the linkage peak on chromosome 11q13 [8] SNPs -9447A > G and -140C > T, as well as two additional known variants (rs1017713 (IVS1+212T > C) and rs3060 (*19T > C)) were genotyped in the families contributing to the genome scan peak (sample 4). Neither single marker family based association analyses (PDT) in all 165 families nor in the 48 families contributing to the linkage peak on 11q13, revealed significant evidence for allelic associations (all p-values > > 0.05). Consistent with this finding, subsequent haplotype analyses using FAMHAP did not indicate an associated haplotype (best nominal p-value 0.5 with the zhaomax allcombi option).

Discussion

The linkage scan in 89 families revealed the highest LOD at D11S1313. Subsequent fine-mapping in 76 independent families revealed a combined peak region at position 67.8 – 69.1 Mb (approximately 68.55 - 68.01 cM, UCSC, hg16) between D11S1337 and D11S4095 [, *unpublished data*]. *DGAT2* is located at 75 Mb and thus close to this peak region. In light of the small sample size, which leads to considerable stochastic variation in the location estimate of linkage peaks [23] and combined with its important role in fat metabolism *DGAT2* is a very plausible positional and functional candidate gene for obesity in our sample.

A mutation screen in the coding region of the gene, the predicted promoter sequence and a 5' non-coding exon (altogether 3,079 bp) revealed 15 genetic variants, 14 of which were novel. Twelve of the variants were rare (MAF

= 1%) and would thus have a too low statistical power to allow for a comparison in a case control association analysis. Nonetheless, these rare variants might have an impact on the phenotype. Four coding non-synonymous variants were detected: p.Val82Ala occurred once in an extremely obese male, whereas p.Arg297Gln, p.Gly318Ser and p.Leu385Val were detected in underweight controls. [1] The conservative amino acid (aa) exchange p.Val82Ala is located in a predicted transmembrane domain of the DGAT2 protein [18]. This position is situated within an area highly conserved among the selected species with Val82 being unchanged for more than 1 billion years of evolution. While this non-synonymous variant seemingly does not affect the predicted transmembrane domain (aa 73 to aa 95), altered function may be the consequence as already postulated for other genes [24]. Moreover, for the very same aa substitution positioned within a transmembrane domain (TM) an inactivating variant in TM2 of the monocarboxylate transporter 8 [25] as well as an activating variant in TM1 of the lutropin receptor [26] had been described. Therefore although Val82Ala is a conservative exchange it has been shown that a Valin to Alanin substitution is able to materially affect membrane protein functions in both an activating as well as in an inactivating manner. Hence, assuming that a gain of function might well lead to obesity, it is reasonable to consider the Valin to Alanin substitution in DGAT2 as a potential cause for the patient's remarkably increased BMI (see table 2). [2] Arg297Gln is a non conservative amino acid exchange. In contrast to arginine, glutamine has an amide-side group that is able to form hydrogen bonds, which might influence protein structure. However, positioned in a region of little evolutionary conservation characterised by a difference in amino acid sequence length between mammals and plants and a non-conservative amino acid exchange between these kingdoms (basic polar arginine in mammals vs. neutral unpolar methionine in plants) an exchange of the wt arginine vs. also polar but neutral glutamine does not suggest a functional consequence of this substitution. [3] The substitution of glycine to serine at position 318 is also non-conservative. During evolution persisted at this position a neutral unpolar amino acid; therefore an exchange by polar serine may be functionally relevant. However, several amino acids flanking position 318 show little conservation; therefore the patient's remarkably low BMI as consequence of this amino acid substitution seems rather speculative. [4] The exchange of leucine to valine at position 385 is conservative. The non reactive aliphatic side chains of leucine and valine that are important for hydrophobic bonds within the protein are not affected. Functional studies of these variants in DGAT2 have to be performed to clarify the effect of the detected variants on body weight regulation.

There is no indication that the rare synonymous variants might have an effect on body weight regulation. Variant c.-584C > G in the putative promoter region is located in a potential binding site for the transcription factor ARP-1 (COUP-TF II), which might participate in regulation of lipid metabolism and cholesterol synthesis [27] and is assumed to negatively influence PPARα gene transcription [28]. Two variants were detected in untranslated regions (-30C > T in the 5'UTR and *22C > T in the 3'UTR). These variants may influence mRNA stability, but as they are rare, we assumed that they have no major effect on common obesity under a "common disease common variant"-perspective given that the estimated MAF of each variant was 1/186 = 0.54% (95% confidence interval 0.014%...2.96%). The intronic variants IVS2–3C > G, IVS7+23C > T and IVS7+73C > T are also rare and neither affect any consensus splice site nor do they introduce cryptic splice sites. None of the case control and family based association studies showed an association of investigated variants or haplotypes in the genomic region of *DGAT2*.

Starting off with a mutation screen of the coding sequence and the 5'flanking region we were investigating both case control samples and independent samples with families contributing to a linkage peak. However, due to insufficient statistical power to explore the less common variants (MAF < 0.1), our study design only allows evaluation of common variants.

In conclusion, our results do not support the hypothesis of an important role of common genetic variation in *DGAT2* for the development of obesity in our sample. One may thus speculate that if there is an influence of genetic variation in *DGAT2* on body weight regulation, it might either be the less common synonymous or non-coding variants that play an important role.

Methods

Study subjects

The ascertainment strategy for the extremely obese and underweight study groups was previously described in detail [29]. Briefly, extremely obese German index patients were ascertained at German hospitals specialized in inpatient treatment of extreme obesity in children and adolescents. All index patients had an age- and gender-specific BMI ≥90th percentile as previously determined in a representative German population sample [30]. The BMIs of the underweight students were below the 15th percentile whereas normal weight controls had BMIs between the 40th and the 60th age- and gender-specific percentile. Mean BMI and age and the respective standard deviations are provided below. Written informed consent was given by all participants and, in the case of minors, their parents. This study was approved by the Ethics Committee of the University of Marburg.

Table 2: Phenotypic characteristics (gender, age, BMI, BMI-SDS) of heterozygous carriers of infrequent variants detected in the genomic region of *DGAT2*

Mutation	Gender	Age [years]	BMI [kg/m²]	BMI-SDS*
p.V82A	male	12	29.30	3.6
p.R297Q	female	26	16.9	-1.3
	female	23	17.0	-1.6
p.G318S	male	25	19.6	-1.2
	male	20	17.9	-1.9
p.L385V	male	23	20.4	-0.9
c.812A > G (T194T)	female	12	31.4	4.6
c.920T > C (S230S)	male	13	31.5	3.7
-584C > G	female	34	18.4	-1.3
-30C > T	male	24	19.6	-1.4
IVS2–3C > G	male	27	19.5	-1.5
IVS7+23C > T	female	14	35.9	5.4
	female	16	33.8	5.2
IVS7+73C > T	male	12	29.3	3.3
*22C > T	male	21	33.8	4.7

All individuals are heterozygous carries of these variants. * Estimates based on Hebebrand et al., 1996 (53)

The coding exons of *DGAT2*, the predicted promoter region and an additionall non-coding 5' exon were screened in a 'screening sample' (sample 1) comprising 93 extremely obese children and adolescent cases (48.4 % females, mean BMI 34.4 ± 5.0 kg/m²; mean age 14.1 ± 2.0 yrs) and 94 healthy underweight controls (36.2 % females, mean BMI 18.5 ± 1.2 kg/m²; mean age 25.5 ± 4.0 yrs). Identified sequence variants were genotyped in sample 2, comprising both the initial groups (sample 1) and additional 87 cases (51.7 % females, mean BMI 36.9 ± 7.0 kg/m²; mean age 14.6 ± 2.8 yrs) as well as 93 healthy underweight controls (52.7 % females, mean BMI 18.3 ± 1.0 kg/m²; mean age 25.7 ± 3.8 yrs). Finally, in order to increase the power to detect association for one variant (-140C > T), sample 2 was further extended (sample 3). Sample 3 comprised a total of 361 extremely obese cases (53.2 % females, mean BMI 34.7 ± 6.3 kg/m²; mean age 14.4 ± 2.6 yrs) and a total of 445 control subjects comprising 278 underweight students (50.7 % females, mean BMI 18.2 ± 1.1 kg/m²; mean age 25.0 ± 3.7 yrs) and 167 normal weight controls (60.5 % females, mean BMI 21.8 ± 1.1 kg/m²; mean age 24.6 ± 2.4 yrs).

To investigate the potential genetic effects of variants in *DGAT2* on body weight regulation; SNPs rs1017713, rs3060, -9447A > G and -140C > T were genotyped in a family based association analysis, the respective markers were also genotyped in the 165 genome scan families (sample 4) described previously [8] to test for linkage. Sample 4 is independent of samples 1–3. The aim of our study was the investigation of associations of *common DGAT2* variants with extreme early-onset obesity.

Promoter prediction and evaluation of gene structure

Promoter sequence was predicted by PromoterInspector, Mammalian Promoter Prediction Software from Genomatix, [31]. Analyses were based on human genome assemblies hg15 and hg16 [32] and the corresponding ENSEMBL genome browser [33]. cDNA clone sequences of Unigene cluster Hs.334305 representative for the human *DGAT2* gene were downloaded from NCBI [34] and assembled using GAP4 [35]. *DGAT2* transcripts were aligned to human genomic sequence using Sim4 [36]. Two known human mRNAs mapped to the *DGAT2* locus in genome assemblies hg15 and hg16. One of these, AB048286 (2,439 bp) formed the basis for RefSeq entry NM_032564, the annotation status of which was provisional. The second mRNA AL834287 (2,347 bp) was 92 bp shorter at its 5'end than AB048286. Nonetheless, both transcripts harbour 8 exons; and as defined by AB048286, the human *DGAT2* at chr. 11q13.5 covers 32,766 bp with a coding region (CDS) of 1167 bp extending from exon1 to exon8. In the corresponding Ensembl genome browser [33] there were also two transcripts assigned to the *DGAT2* locus (ENST00000289503, 1,545 bp; ENST00000228027, 2,238 bp). The former entry harboured 8 exons as found in AB048286 while the latter contained only 7 exons, i.e. exon5 was missing which indicated the presence of at least one alternatively spliced *DGAT2* transcript.

Sequencing

Human cDNA clone BF979958 was obtained from RZPD [37] and cultured by standard methods [38]. Sequencing was performed using vector primers and BigDye Termina-

tor Cycle Sequencing v2.0 kit (Applied Biosystems, Weiterstadt, Germany). Sequencing reactions were electrophoresed on ABI 3700 automated sequencers. Base calling was performed using phred [39,40]. Sequence assembly was done using phrap [41]. Trace files were inspected visually in GAP4. RT-PCR: Primers located in exons 1 and 8 of *DGAT2* as defined by reference sequence NM_032564 were used in a nested PCR approach (PCR I: 1F [ACCCTCATAGCCGCCTACTC], 1R [AGGTTAGCTGAGCCACCCAG]; PCR II: 2F [CTCATAGCCGCCTACTCC], 2R [CTAGAACAGGGCAAGCTGGA]) on human multiple tissue cDNA (Clontech, Heidelberg, Germany) or adipocyte mRNA [42]. Omniscript RT Kit (QIAGEN, Hilden, Germany) was used for reverse transcription. PCR products were cloned into pCR2.1-TOPO (Invitrogen, Karlsruhe, Germany). Sequencing of recombinant clones, sequence assembly, trace file inspection and alignment to genomic sequence was done as described above.

Mutation screen

A mutation screen was performed in the 8 coding exons of human *DGAT2* and also in the predicted promoter region and a non-coding 5' exon. For PCR amplification, primers corresponding to intron sequences were used in order to detect potential splice site variants [for PCR primers see additional file 2]. Mutation screens of exon 6 and 8 were performed using denaturing high performance liquid chromatography (dHPLC) analysis on a Transgenomic WAVE® system [Transgenomic, Cheshire, UK;]. The optimal melting temperatures for separation of homo- and heteroduplices were deduced from the melting temperature of the PCR-amplicon using WAVEmaker software, version 4.0 (Transgenomic, Cheshire, UK). All chromatograms were compared with chromatograms of sequenced wild-type samples. PCR amplicons showing a peak appearance different to the wild-type pattern were sequenced (SeqLab, Göttingen, Germany). To detect mutations in exons 1–5, 7, the promoter region and the non-coding 5' exon standard nonisotopic single-strand conformation polymorphism analyses (SSCP) was performed [44]. 15% acrylamide gels (Q-BIOgene, Heidelberg, Germany; 37.5:1) were run at 600 V for 16 h at 4°C and for 5.5 h at ambient temperature; all gels were silver stained. The sensitivity of dHPLC has been described to be approximately 95% [45] and that of SSCP about 97% when using two temperatures [46]. All SSCP patterns were compared with patterns of sequenced wild-type samples. Samples that showed a pattern different from that of the wild-types were re-sequenced (Seq Lab, Göttingen, Germany). The nomenclature of the described variants follows den Dunnen and Antonarakis [47] and NM_032564.

Genotyping

High-throughput genotyping for two additional intronic SNPs (rs1017713, rs3060,) as well as for variants -9447A > G and -140C > T entering the family based association studies was performed as described earlier [48] using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). For case control association studies, genotyping of SNPs -9447A > G and c.920T > C was perfomed via tetra-ARMS-PCR [49] [see additional file 3]. For all other SNPs [see additional file 3], PCR with subsequent diagnostic restriction fragment length polymorphism analyses (RFLP) was used. PCR products were run on ethidium bromide-stained 2.5% agarose gels. Positive controls for the variant alleles and a negative control (water) were run on each gel. To validate the genotypes, allele determinations were rated independently by at least two experienced individuals. Discrepancies were resolved unambiguously either by reaching consensus or by retyping. Missings were retyped twice. Genotyping success rate was above 99%. Genotyping of rs3841596, a biallelic trinucleotide repeat was carried out using fluorescence-based semi-automated technique on an automated DNA sequencing machine (LiCor 4200-2; MWG-Biotech, Ebersberg, FRG). Analyses and assignment of the marker alleles were done with ONE-Dscan Version 1.3 software (MWG-Biotech).

In silico evaluation of non-synonymous variants

To gain information about putative functional relevance of an amino acid substitution, public sequence database [34] was mined for full length mammalian and more distant related DGAT2 orthologs where particular attention was given to species surpassing oil production. These data were utilized to determine the evolutionary conservation of the DGAT2 amino acid sequence. Protein sequence alignment was carried out via Omiga (Oxford Molecular Ltd.). Transmembrane domains were predicted *in silico* [50].

Statistics

Associations in the case control sample were analyzed by Cochran-Armitage trend test for genotype frequencies and Fisher's exact test for alleles. Family based association analyses were performed using the pedigree transmission disequilibrium test [PDT;]. Analyses of linkage disequilibrium (LD) between the investigated polymorphisms as well as haplotype associations in the families were investigated by FAMHAP v16 [e.g.]. All reported p-values are nominal. Due to lack of p-values < 0.05 (see below), adjustment for multiple testing was considered unnecessary.

Abbreviations

BMI: body mass index

CNS: central nervous system

CNTF: ciliary neurotrophic factor

DGAT: diacylglycerol O-acyltransferase homolog

EST: expressed sequence tag

GAD2: glutamate decarboxylase 2

GAL: galanin

MAF: minor allele frequency

MLB LOD: maximum likelihood binomial logarithm of the odd

QTL: quantitative trait locus

SLC6A14: solute carrier family 6 member 14

SNP: single nucleotide polymorphism

TM: transmembrane domain

UCP: uncoupling protein

UTR: untranslated region

Authors' contributions

SF designed and carried out PCR and mutation screening, performed genotyping and drafted the manuscript. KR and MP performedpromoter prediction, evaluation of gene structure and resequencing. AS performed statistical analyses. HBr and HBi performed in silico evaluation of non-synonymous variants. AKW and RF participated in the study design and helped drafting the manuscript. KK and TM performed the high-throughput genotyping. MW contributed mRNA for sequencing analyses and participated in the study design. AH and JH conceived the study, and participated in its design and coordination and drafted the manuscript. All authors read and approved the final manuscript.

Additional material

Additional File 1

mRNAs transcribed from human DGAT2 *locus. list of mRNAs transcribed from human DGAT2 locus, including status, evidence and gene structure defined by mRNA*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2156-8-17-S1.doc]

Additional File 2

PCR primer for mutation screen in DGAT2. *list of PCR primer for mutation screen in DGAT2 exons*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2156-8-17-S2.doc]

Additional File 3

Genotyping information: Genotypes were generated via RFLP, tetra-arms PCR [3] and MALDI-TOF. Genotyping information for investigated SNPs including primer, genotyping methods and restriction enzymes
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2156-8-17-S3.doc]

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3.6 Publication VI

Reinehr T, Hebebrand J, Friedel S, Toschke AM, Brumm H, Biebermann H, Hinney A. **Lifestyle intervention in obese children with variations in the melanocortin 4 receptor gene.** Obesity (Silver Spring). 2009;17(2):382-9.

Since information on weight changes after lifestyle intervention in children with mutations in the melanocortin 4 receptor gene (*MC4R*) is scarce, we compared weight changes after lifestyle intervention between children with and without *MC4R* mutations.
514 overweight children, who presented to participate in a one-year lifestyle intervention based on exercise, behaviour and nutrition therapy were screened for *MC4R* mutations. For comparison, children with *MC4R* mutations leading to reduced receptor function were each randomly matched with 5 children of same age and gender without *MC4R* mutations. Changes of weight status were analyzed as change of body mass index standard deviation scores (BMI-SDS). Sixteen children (3.1%) harboured *MC4R* mutations leading to reduced receptor function and 17 (3.3%) children carried variations not leading to reduced receptor function. Children with and without *MC4R* mutations reduced their overweight at the end of intervention to a similar degree ($p=0.318$ between groups based on an intention-to-treat analysis). The maintenance of weight loss after intervention among children with *MC4R* mutations leading to reduced receptor function failed in contrast to children without such mutations ($p<0.001$ adjusted for BMI-SDS at baseline, age, and gender in an intention-to-treat analysis). In conclusion, children with *MC4R* mutations leading to reduced receptor function were able to lose weight in a lifestyle intervention but had much greater difficulties to maintain this weight loss supporting the impact of these mutations on weight status.

Own contribution:

- *MC4R* mutation screen
- *in silico* validation of functional relevance of detected mutations
- ascertainment of probands (in coop. with Reinehr T)

Lifestyle Intervention in Obese Children With Variations in the Melanocortin 4 Receptor Gene

Thomas Reinehr[1], Johannes Hebebrand[2], Susann Friedel[2], André M. Toschke[3], Harald Brumm[4], Heike Biebermann[4] and Anke Hinney[2]

Because information on weight changes after lifestyle intervention in children with mutations in the melanocortin 4 receptor (*MC4R*) gene is scarce, we compared weight changes after lifestyle intervention between children with and without *MC4R* variations. A group of 514 overweight children (aged 5–16 years), who presented to participate in a 1-year lifestyle intervention based on exercise, behavior, and nutrition therapy were screened for *MC4R* mutations. For comparison, children with *MC4R* mutations leading to reduced receptor function (group A) were each of them randomly matched with five children of same age and gender without *MC4R* mutations (group B). Changes of weight status were analyzed as change of BMI standard deviation scores (BMI-SDSs). Furthermore, 16 children (3.1%) harbored *MC4R* mutations leading to reduced receptor function, and 17 (3.3%) children carried variations not leading to reduced receptor function. Children with and without *MC4R* mutations reduced their overweight at the end of intervention to a similar degree ($P = 0.318$ between groups based on an intention-to-treat analysis). The maintenance of weight loss after intervention among children with *MC4R* mutations leading to reduced receptor function failed in contrast to children without such mutations ($P < 0.001$ adjusted for BMI-SDS at baseline, age, and gender in an intention-to-treat analysis). In conclusion, children with *MC4R* mutations leading to reduced receptor function were able to lose weight in a lifestyle intervention but had much greater difficulties to maintain this weight loss supporting the impact of these mutations on weight status.

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INTRODUCTION

Obesity has been recognized by the World Health Organization as one of the major global health problems, and its increasing prevalence calls for knowledge of the genetic factors influencing body weight regulation. Studies in mice and humans have pointed out the critical importance of the central melanocortinergic pathway in the control of energy homeostasis, in particular, the pivotal role of the melanocortin 4 receptor (*MC4R*) (1). Previous studies in humans have shown that the prevalence of functionally relevant *MC4R* mutations ranges from 0.5 to 5.8% in obese children and adolescents ascertained for molecular genetic studies (2–8). Most importantly, little is known about the impact of these mutations on the effect of a lifestyle intervention program. We hypothesized that children with *MC4R* mutations leading to reduced receptor function lose less weight than those without the mutations.

More than 90 different obesity-associated mutations in the *MC4R*, most of which are nonsynonymous mutations leading to either total or partial loss of function, have so far been reported (9,10). The phenotype of carriers with mutations leading to a reduced receptor function considerably varies in their effect on body weight (4,10–12). These mutations within the coding region are assumed to have a major effect on body weight averaging ~4.5 kg/m^2 and 9 kg/m^2 in adult males and females, respectively (13). Interestingly, two nonsynonymous polymorphisms, Val103Ile and Ile251Leu, which occur in 1–3% of the examined populations, respectively, are both associated with a slightly decreased BMI (14–18).

One of the studies of children with *MC4R* mutations leading to a reduced receptor function resulted in the definition of a "*MC4R* syndrome" which is characterized by early onset obesity, increased linear growth, body fat and fat free mass, increased bone mineral density as well as hyperphagia and hyperinsulinemia (5,11). However, several studies could not replicate these findings (4,12,19). Therefore, alterations of growth and hormone profile are controversially discussed.

The primary aim of this study was to compare the long-term degree of weight change between *MC4R* mutation carriers and noncarriers participating in a 1-year lifestyle intervention 1 year after end of intervention. Secondary aims included

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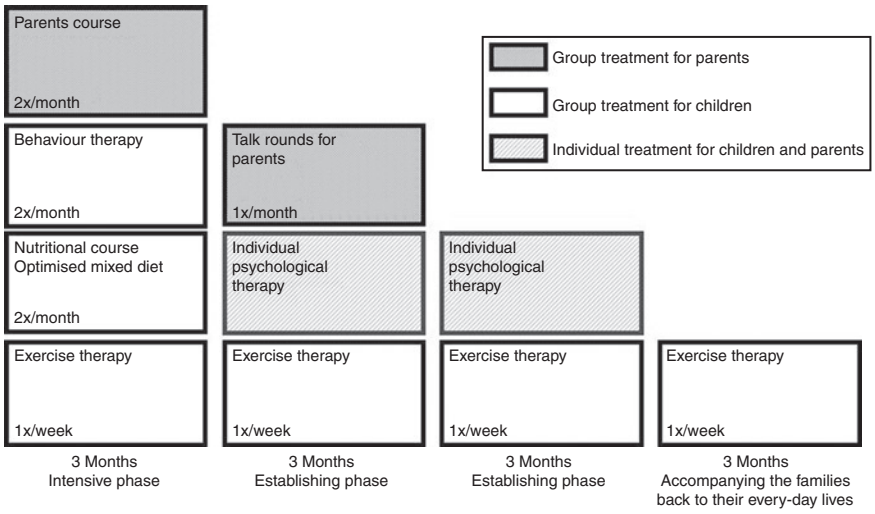

Figure 1 Structure of the lifestyle intervention "Obeldicks."

to explore the weight change at end of intervention and the anthropometrics, cardiovascular risk factors, and hormone profiles at baseline by individuals harboring and not harboring a *MC4R* mutation.

METHODS AND PROCEDURES

The local ethics committees of the Universities of Witten/Herdecke and of Duisburg-Essen approved this study. Written informed consent was obtained from all subjects and corresponding parents. The study was conducted in accordance with the guidelines of the Declaration of Helsinki.

We examined all 514 overweight children aged 5–16 years (mean age 10.7 ± 2.7 years, 44% male; median BMI 27.1 interquartile range 24.4–29.8 kg/m^2; median BMI-SDS 2.37 interquartile range 2.06–2.77) consecutively presenting to our outpatient obesity clinic to attend the 1-year outpatient lifestyle intervention program "Obeldicks" in the years 2001–2004. None of the children were on any medications or suffered from endocrine disorders including type 2 diabetes mellitus, familial hyperlipidemia, or syndromal disorders.

Body height was measured to the nearest centimeter using a rigid stadiometer. Weight was measured in underwear to the nearest 0.1 kg using a calibrated balance scale. The degree of overweight was quantified using Cole's least mean square method, which normalizes the BMI skewed distribution in childhood and expressed BMI as a standard deviation score (BMI-SDS) (20). German population–based reference data were used for height, weight, and BMI (21). Overweight was defined according to the International Obesity Task Force (22).

The blood pressure was measured according to the guidelines of the National High Blood Pressure Education Program (23). Systolic and diastolic blood pressure were measured twice at the right arm after a 10-min rest in the supine position using a calibrated sphygmomanometer and afterwards averaged.

The pubertal stage was determined according to Marshall and Tanner. The triceps and subscapularis skinfold thickness was measured in duplicate using a caliper and averaged to calculate the percentage of body fat using a skinfold thickness equation with the following formulas (24): boys: body fat % = 0.783 × (subscapularis skinfold thickness + triceps skinfold thickness in mm) + 1.6; girls: body fat % = 0.546 × (subscapularis skinfold thickness + triceps skinfold thickness in mm) + 9.7.

Fasting serum insulin, leptin, glucose, glycated hemoglobin, uric acid, triglyceride, total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, thyroid stimulating hormone, free T3, free T4, luteinizing hormone, follicle stimulating hormone, cortisol, total testosterone, dehydroepiandrosterone-sulfate, insulin like growth factor-I, and insulin like growth factor binding protein-3 concentrations were measured. An oral glucose-tolerance test was performed in all children ≥12 years. The children had been carefully instructed to fast over a period of at least 10 h. Serum hormone concentrations were determined by high-specific chemiluminescence immunoassays (Cortisol Immulite DPC Los Angeles, thyroid stimulating hormone Immulite DPC Los Angeles, free T3 Immulite DPC Los Angeles, free T4 Immulite DPC Los Angeles, Leptin DRG, Marburg, testosterone ADVIA, dehydroepiandrosterone-sulfate Immulite DPC Los Angeles, luteinizing hormone Immulite DPC Los Angeles, follicle stimulating hormone Immulite DPC Los Angeles, insulin like growth factor-I Immulite DPC Los Angeles, insulin like growth factor binding protein-3 Immulite DPC Los Angeles, and insulin Abbott). Intra- and inter-assay variations were <10% in all assays. Leptin was transformed into SDS (leptin-SDS) according to gender, pubertal stage, and degree of overweight (25). Serum fasting triglycerides, total cholesterol, high-density lipoprotein and low-density lipoprotein cholesterol, transaminases, uric acid, glycated hemoglobin, and glucose concentrations were determined by commercially available test kits (Roche Diagnostics, Mannheim, Germany; Boehringer, Mannheim, Germany; Ortho Clinical Diagnostics, Neckargemuend, Germany). Intra- and inter-assay variations of these variables were <5%. Homeostasis model assessment was used to detect the degree of insulin resistance (26): resistance (homeostasis model assessment) = (insulin (mU/l) × glucose (mmol/l))/22.5.

Genetic analyses

The complete coding region of *MC4R* was screened for mutations by dHPLC as described previously (6). We performed *in silico* analyses for nonsynonymous mutations by PolyPhen (http://genetics.bwh.harvard.edu/pph/index.html); for synonymous, nonsense, and frameshift mutations these analyses were impossible as PolyPhen analyses only nonsynonymous mutations. However, it can be assumed that nonsense and frameshift mutations are not compatible with a normal receptor function, so that these mutations can all be classified as leading to a "loss-of-function."

Functional characterization of MC4R mutations

All investigated novel mutant *MC4R*s of this study and the wild-type (WT) *MC4R* were cloned in a pcDps expression vector. For functional studies mutant and WT receptors were transiently transfected into COS-7 cells using Metafectene (Biontex, Munich, Germany) according to the manufactures protocol. After 48 h, cyclic adenosine monophsphate accumulation assays were determined by a nonradioactive cyclic adenosine monophosphate assay based on the AlphaScreen technology (Perkin Elmer Life Science, Boston, MA). To investigate agonist independent cell surface expression WT and mutants receptors

were N-terminally HA-tagged and cell surface ELISAs were performed. Cells were transfected in 48-well plates. Three days later cells were washed, fixated, and probed with anti-HA-biotin antibody (Roche, Grenzach-Wyhlen, Germany). Bound anti-HA antibody was detected by peroxidase-labeled streptavidin (Dianova, Hamburg, Germany).

Lifestyle intervention

To participate in the intervention program "Obeldicks," the overweight children had to prove their motivation by filling out a questionnaire concerning their eating and exercise habits and by attending exercise groups for overweight children regularly for at least 8 weeks (27). Only children who had filled out the questionnaires and who had participated in the exercise groups were included in the "Obeldicks" lifestyle intervention program.

The intervention program "Obeldicks" has been described in detail elsewhere (28,29). The intervention program "Obeldicks" was based on physical exercise, nutrition education, and behavior therapy including the individual psychological care of the child and his/her family. An interdisciplinary team of pediatricians, diet assistants, psychologists, and exercise physiologists was responsible for the training. The children were divided into groups according to their sex and age. The 1-year training program was divided into three phases (see **Figure 1**): In the intensive phase (3 months), the children took part in the nutritional course and in the eating behavior course in six group sessions, each lasting for 1.5 h. At the same time, the parents were invited to attend six parents' evenings. In the establishing phase (6 months), individual psychological family therapy was provided (30 min/month). In the last phase of the program (accompanying the families back to their everyday lives) (3 months), further individual care was possible, if and when necessary. The exercise therapy took place once a week during the whole year and consisted of ball games, jogging, trampoline jumping, and instructions in physical exercise as part of everyday life and in reduction of the amount of time spent watching television. The nutritional course was based on the prevention concept of the "Optimized mixed diet." Here, the present scientific recommendations were translated into food-based dietary guidelines also considering the dietary habits of children and families in Germany (28–30). In contrast to the present-day diet of children in Germany with a fat content of 38% of energy intake (E%), 13 E% proteins, and 49 E% carbohydrates including 14 E% sugar (31), the "Optimized mixed diet" was both fat and sugar reduced and contained 30 E% fat, 15 E% proteins, and 55 E% carbohydrates including 5 E% sugar. The children followed a "traffic-light system" (30) when selecting their food. In this system, the foods and drinks available in Germany were separated according to their fat and sugar contents into "red = stop," "orange = consider the amount," and "green = o.k. when hungry or thirsty." The traffic light system has been described in detail elsewhere (28–30). Three-day weighed dietary records demonstrated a reduction of the mean energy content of 1,459 kcal (s.d. 379) per day before intervention to a median of 1,250 kcal (s.d. 299) per day at the end of intervention and a reduction of E% fat from 36.3 (s.d. 5.0) to 30.4% (s.d. 7.1) (ref. 30).

Of the 514 overweight children, 240 (47%) dropped out in the motivation phase preceding the intervention and 44 (9%) in the first three months of the intervention period. The dropouts did not differ in age, gender, BMI-SDS, cardiovascular risk factors, or hormone profile from the children finishing the intervention. The 44 children who dropped out during the intervention period had the same mean BMI-SDS at last visit as compared to baseline. The reasons for dropout were a perceived lack of success in 41 children and disciplinary dismissal in three children. No child dropped out in the observation period after the end of intervention. The children and parents who hade completed the lifestyle intervention had participated in >95% of all sessions.

Statistical analysis

For comparison, children with *MC4R* mutations leading to reduced receptor function (group A) were each randomly matched with five children of same age and gender without *MC4R* mutations (group B). All calculations were carried out using Winstat for Exel and the statistical software package SAS version 9.1 (SAS Institute, Cary, NC). Normal distribution of variables was tested by Kolmogorov–Smirnov tests with Lilliefors correction. For the exploration we used Student's t tests for paired and unpaired observations, Mann–Whitney *U*-test, Wilcoxon test, and chi-square test as appropriate. For comparison of blood pressure, the values were adjusted for height.

To examine the BMI-SDS difference from baseline to 1 year after end of intervention between children of group A and B, we used a linear model with random intercepts in order to account for the correlated data structure (family as cluster variable). These models were adjusted for BMI-SDS at baseline, sex, and age. We followed an intention-to-treat approach by carrying the last observation forward for the children who aborted the intervention and no follow-up data were available. Significance was assumed for P values <0.05. Data are presented as mean and s.d. for normally distributed variables and as median and interquartile range for not normally distributed variables.

RESULTS

The clinical characteristics of the children with *MC4R* mutations are demonstrated in **Table 1**. In this table, we also summarized the functional *in silico* and published *in vitro* data.

Functional characterization was carried out for the novel investigated Ala244Val, Met281Val, and Gln307Stop mutant *MC4R*. WT and mutants were transiently transfected into COS-7 cells and signal transduction properties after [Nle4,d-Phe7]–α–melanocyte-stimulating hormone challenge as well as ligand-independent cell surface expression was examined. Cell surface expression and signal transduction properties of the Ala244Val mutant were comparable to the WT (**Table 2**). The Met281Val mutant showed a reduction in cell surface expression; EC50 values were slightly shifted to higher [Nle4,d-Phe7]–α–melanocyte-stimulating hormone concentrations; therefore, this receptor has to be classified as a partial loss of function. As expected the Gln307Stop mutant resulted in a complete loss of function (**Table 2**).

In five mutations, *in silico* and *in vitro,* data were inconsistent. Two mutations (Thr112Met and Ala175Thr) were *in silico* predicted to be "benign" but the published *in vitro* data showed that they lead to a reduced function (5,32). Additionally, our *in vitro* data demonstrated that the mutation that was predicted to be "benign" (Met281Val) lead to a reduced function (**Table 2**). Therefore, the children with these mutations were grouped to the children with mutations leading to reduced receptor function (group A). The mutation Pro48Ser was *in silico* predicted to be "possibly damaging" but the published *in vitro* data showed that this mutation is functionally similar to the WT receptor (33). Because our functional *in vitro* data showed that the new mutation Ala244Val predicted to be "possibly damaging" was similar to the WT (**Table 2**), this mutation was also defined as non functionally relevant.

In conclusion, we dealt with 16 children with nonsynonymous mutations leading to a reduced function (group A) and 17 (2.7%) children with *MC4R* variations that do not lead to a reduced receptor function as they are (i) synonymous (1× T5T), (ii) in the noncoding 3′ region (1× 15 C>T), (iii) functionally similar to the WT receptor as shown by *in vitro* analyses (1× Asn274Ser (18), 2× Pro48Ser (33), and 1× Ala244Val (**Table 2**)), (iv) lead to a slightly enhanced receptor function

Table 1 Clinical characteristics of the overweight children with mutations in the melanocortin 4 receptor (*MC4R*) gene

Number	Age (years)	Gender	Weight (kg) (SDS)	Height (cm) (SDS)	BMI (kg/m^2) (SDS)	Mutation (effect on amino acid level)	Functional relevance: *in silico* analyses[a]	Functional relevance: *in vitro* analyses
1	10.8	Female	83.2 (3.0)	151.0 (0.0)	36.5 (3.2)	None (T5T)	Analysis impossible	—
2	13.1	Male	78.2 (1.9)	168.7 (0.6)	27.5 (2.0)	None (15 C>T)	Analysis impossible	—
3	8.0	Male	48.2 (2.7)	134.8 (0.9)	26.5 (2.9)	[Tyr35Stop; 110A>T]	Analysis impossible	Loss of function (6)
4	13.3	Male	77.5 (2.1)	152.9 (–0.7)	33.2 (2.7)	[Tyr35Stop; 110A>T]	Analysis impossible	Loss of function (6)
5	10.0	Female	69.5 (3.0)	155.5 (2.1)	29.0 (2.6)	[Tyr35Stop; 110A>T]	Analysis impossible	Loss of function (6)
6	10.8	Female	56.5 (1.5)	155.0 (0.5)	23.5 (1.6)	[Tyr35Stop; 110A>T]	Analysis impossible	Loss of function (6)
7	9.1	Female	58.9 (2.6)	154.9 (2.4)	24.6 (2.1)	A: [Tyr35Stop; 110A>T]	A: analysis impossible	A: loss of function (6)
						B: Ile251Leu	B: possibly benign	B: like wild-type (9)
8[b]	14.2	Female	107.8 (3.5)	166.5 (0.5)	38.9 (3.3)	Pro48Ser	Possibly damaging	Like wild-type (33)
9[b]	15.0	Female	76.0 (1.8)	164.1 (–0.2)	28.2 (2.0)	Pro48Ser	Possibly damaging	Like wild-type (33)
10	9.4	Male	47.3 (1.8)	135.2 (–0.7)	25.9 (2.4)	Val103Ile	Possibly benign	Slightly enhanced function (18)
11	10.1	Male	50.9 (1.6)	152.7 (1.2)	21.8 (1.5)	Val103Ile	Possibly benign	Slightly enhanced function (18)
12	14.5	Female	87.4 (2.5)	168.1 (0.6)	30.9 (2.5)	Val103Ile	Possibly benign	Slightly enhanced function (18)
13	10.9	Male	69.4 (2.3)	156.4 (0.9)	28.4 (2.3)	Val103Ile	Possibly benign	Slightly enhanced function (18)
14	5.4	Female	35.4 (2.9)	122.4 (1.5)	23.6 (3.0)	Val103Ile	Possibly benign	Slightly enhanced function (18)
15	11.9	Male	76.1 (2.8)	153.0 (0.9)	32.5 (2.8)	Thr112Met	Possibly benign	Partially inactive (5,32)
16	13.0	Female	79.6 (2.4)	163.4 (0.6)	29.8 (2.4)	Thr112Met	Possibly benign	Partially inactive (5,32)
17[c]	9.9	Male	67.3 (2.8)	155.7 (2.1)	27.8 (2.5)	A: Ser127Leu	A: possibly damaging	A: reduced function (38)
						B: Val103Ile	B: possibly benign	B: slightly enhanced function (18)
18[c]	8.4	Female	54.6 (3.1)	143.0 (2.5)	26.7 (2.8)	A: Ser127Leu	A: possibly damaging	A: reduced function (38)
						B: Val103Ile	B: possibly benign	B: slightly enhanced function (18)
19	13.1	Female	78.0 (2.2)	169.0 (1.0)	27.3 (2.0)	A: Ser127Leu	A: possibly damaging	A: reduced function (38)
						B: Val103Ile	B: possibly benign	B: slightly enhanced function (18)
20	11.6	Female	105.0 (3.6)	170.5 (1.8)	36.1 (3.1)	Ala175Thr	Possibly benign	Reduced function (5)
21	12.9	Female	112.9 (3.8)	165.5 (0.9)	41.2 (3.4)	Leu211fsX216	Analysis impossible	Loss of function (18)
22	11.7	Female	120.0 (4.1)	167.4 (1.4)	42.8 (3.5)	Ala244Val	Possibly damaging	Like wild-type (see Table 2)
23	12.1	Male	69.0 (1.8)	165.7 (1.2)	25.1 (1.8)	Ile251Leu	Possibly benign	Like wild-type (9)
24	7.3	Female	43.0 (2.5)	131.1 (0.8)	25.0 (2.7)	Ile251Leu	Possibly benign	Like wild-type (9)
25	16.5	Male	79.5 (1.1)	159.7 (–2.5)	31.2 (2.3)	Ile251Leu	Possibly benign	Like wild-type (9)
26	14.1	Female	105.5 (3.4)	179.7 (2.3)	32.7 (2.7)	Ile251Leu	Possibly benign	Like wild-type (9)
27	12.6	Female	107.6 (3.6)	165.5 (0.5)	39.3 (3.3)	Ile251Leu	Possibly benign	Like wild-type (9)
28	13.5	Female	97.8 (3.2)	163.9 (0.3)	36.4 (3.1)	Ile251Leu	Possibly benign	Like wild-type (9)
29	12.6	Male	81.7 (2.0)	153.2 (–1.2)	34.8 (2.8)	Asn274Ser	Possibly benign	Like wild-type (18)

Table 1 Continued on next page

Table 1 (contunued)

Number	Age (years)	Gender	Weight (kg) (SDS)	Height (cm) (SDS)	BMI (kg/m^2) (SDS)	Mutation (effect on amino acid level)	Functional relevance: *in silico* analyses[a]	Functional relevance: *in vitro* analyses
30	14.5	Female	100.7 (2.7)	168.1 (0.2)	35.6 (2.8)	Met281Val	Possibly benign	Reduced function (see Table 2)
31	11.3	Male	61.7 (2.2)	145.7 (–0.1)	25.8 (2.5)	Arg305Gln	Possibly damaging	Reduced function (2)
32[d]	7.9	Male	56.1 (3.1)	133.5 (0.6)	31.5 (3.3)	Gln307Stop	Analysis impossible	Loss of function (see Table 2)
33[d]	8.3	Female	45.4 (2.1)	136.3 (0.6)	24.4 (2.3)	Gln307Stop	Analysis impossible	Loss of function (see Table 2)

SDS, standard deviation score.
[a]For nonsynonymous mutations *in silico* analysis were performed by PolyPhen (http://genetics.bwh.harvard.edu/pph/index.html); for synonymous, nonsense and frameshift mutations these analyse were impossible (PolyPhen determined that "this variant is predicted to be possibly damaging" (short in the Table "Possibly damaging") or that "this variant is predicted to be possibly benign" (short in the Table "Possibly benign")). [b,c,d]These patients are siblings.

Table 2 Functional characterization of mutant *MC4R*

	cAMP accumulation			
		NDP–α–MSH		Cell surface expression
Construct	Basal cAMP (nmol/l)	E_{max} cAMP (nmol/l)	EC_{50} (nmol/l)	(% of WT-MC4R)
MC4R-WT	12.8 ± 0.25	344 ± 76	0.41 ± 0.01	100
A244V	8.65 ± 1.05	408 ± 143	0.9 ± 0.1	98 ± 25
M281V	7.8 ± 3.5	153 ± 6	1.3 ± 0.3	52 ± 15
Q307X	n.d.	n.d.	n.d.	n.d.

n.d., Not determinable with sufficient accuracy. COS-7 cells were transfected with the wild-type or MC4R mutants. EC_{50} and E_{max} values were obtained from concentration-response curves (from 0.01 to 100 nmol/l NDP–α–MSH), using the computer program GraphPad Prism. Data are indicated as means ± s.e.m. of two independent experiments performed in triplicates. Cell surface expression experiments were performed twice in quadruplicates with GFP transfected cells as negative control. Values are gives as percentage of wild-type *MC4R* expression.
cAMP, cyclic adenosine monophosphate; *MC4R*, melanocortin 4 receptor; NDP–α–MSH, [Nle4,d-Phe7]–α–melanocyte-stimulating hormone; WT, wild-type.

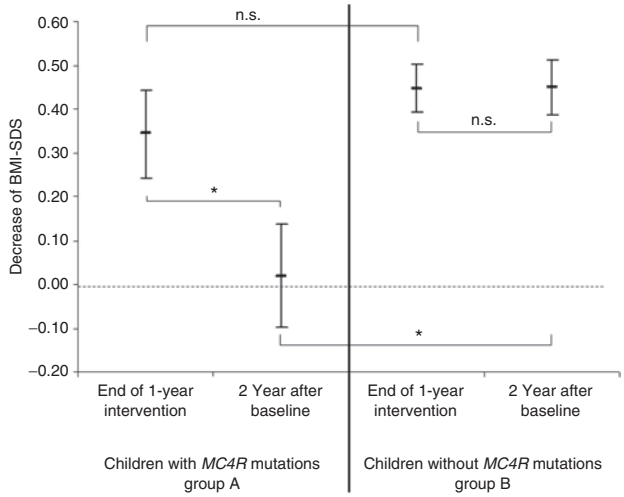

Figure 2 Decrease of BMI-SDS (standard deviation score) (mean and s.e.m.) in nine children with melanocortin 4 receptor (*MC4R*) gene mutations that lead to a reduced receptor function (group A) and 46 age- and gender-matched children without *MC4R* mutations (group B) at the end of a 1-year lifestyle intervention and 1 year after end of intervention compared to baseline BMI-SDS, *$P < 0.05$. n.s., nonsignificant.

(5× Val103Ile), or (v) presumably lead to a slightly enhanced receptor function (6× Ile251Leu) (14–18).

A total of 9 (56%) out of the 16 children of group A started the lifestyle intervention, while seven children of group A dropped out the motivation phase. This frequency was similar as compared to the 481 children without *MC4R* mutations (52% participation) and to the 80 age- and gender-matched children without *MC4R* mutations (group B): A total of 46 children (58%) of group B started the intervention, while 34 children aborted in the motivation phase, and one child (11%) of group A and five children (11%) of group B aborted the intervention.

To test our primary hypothesis, we followed an intention-to-treat approach and carried the last observation forward for children who aborted the intervention. In the follow-up, the nine children of group A did not reduce their overweight 1 year after the end of the 1-year intervention as compared to the children of group B in a linear model with random intercepts considering the family cluster variable and adjusting for BMI-SDS at baseline, age, and gender ($P < 0.001$ for mean BMI-SDS change; Figure 2).

In contrast to the long-term findings, the nine children of group A, who underwent the intervention, reduced their overweight at the end of intervention to a similar extent as compared to the age- and gender-matched children without *MC4R* mutations (group B) based on an intention-to-treat approach in a linear model considering the family as random effect and adjusting for BMI-SDS at baseline, age, and gender ($P = 0.318$ for mean BMI-SDS change between groups; Figure 2).

The children with functionally relevant *MC4R* mutations (group A) did not significantly differ from the randomly selected

matched controls (group B) in respect to any anthropometrical marker, insulin levels, or insulin resistance index homeostasis model assessment at baseline apart from higher-weight SDS, waist circumference, and leptin concentrations in the children of group A (Table 3). However, SDS-leptin and waist-to-hip ratio did not differ significantly between the children with and without the *MC4R* mutations leading to reduced receptor function. The children of group A und B did not differ significantly in respect to any cardiovascular risk factor such as blood pressure, fasting triglycerides, total cholesterol, high-density lipoprotein and low-density lipoprotein cholesterol, transaminases, uric acid, and glucose, as well as glucose levels at 2 h in the oral glucose-tolerance test. Furthermore, glycated hemoglobin, thyroid stimulating hormone, free T3, free T4, cortisol, insulin like growth factor-I, insulin like growth factor binding protein-3, luteinizing hormone, follicle stimulating hormone, testosterone, and dehydroepiandrosterone-sulfate concentrations did not differ significantly between the children of group A and B.

Furthermore, 4 (25%) of the children with *MC4R* mutations leading to reduced receptor function were siblings of two families, while 14 (2.9%) of the 481 children without *MC4R* mutations were siblings of seven families ($P < 0.001$), and two of the nine children of group A who participated in the lifestyle intervention were siblings.

Comparing the 17 overweight children with *MC4R* variations that do not lead to a reduced receptor function to the overweight children with the WT receptor demonstrated no significant differences in any anthropometrical measurements, cardiovascular risk factors, and hormone profile at baseline, as well as degree of overweight reduction at the end of intervention and at the 1-year follow-up (data not shown). The overweight children with *MC4R* variations that do not lead to a reduced receptor function demonstrated a significant ($P = 0.028$) higher degree of overweight reduction at the 1-year follow-up after the end of intervention (mean decrease in BMI-SDS 0.38 ± 0.17) as compared to the children with *MC4R* variations that do not lead to a reduced receptor function (group A), while the children did not differ significantly at the end of intervention ($P = 0.716$).

Separating the children by gender or pubertal stage did not reveal different findings in respect to any anthropometrical measurement, cardiovascular risk factor, or hormone profile (data not shown).

Table 3 Baseline anthropometrical data, fasting insulin, insulin resistance index HOMA, and leptin in overweight children with *MC4R* mutations that lead to a reduced receptor function (group A) and age- and gender-matched overweight children without *MC4R* mutations (group B)

	MC4R mutations	No *MC4R* mutation
Number	16	80
Age (years)	10.9 ± 2.2	10.9 ± 2.2
Gender	38% Male	38% Male
Pubertal stage	50% Prepubertal	50% Prepubertal
Height (cm)	154.2 ± 13.2	152.3 ± 12.3
Height-SDS	0.77 ± 1.05	0.43 ± 1.07
Weight (kg)	70.5 ± 18.8	68.1 ± 16.3
Weight-SDS	2.47 ± 0.60	2.16 ± 0.64*
BMI (kg/m^2)	29.3 ± 4.7	28.5 ± 4.3
BMI-SDS	2.48 ± 0.51	2.37 ± 0.46
Triceps skinfold (cm)	34 (28–37)[a]	33 (29–36)[a]
Subscapularis skinfold (cm)	33 (30–36)[a]	33 (28–38)[a]
Percentage body fat (%)	48 (45–57)[a]	48 (39–53)[a]
Waist circumference (cm)	99 ± 18	88 ± 12*
Hip circumference (cm)	101 ± 19	98 ± 13
Waist-to-hip ratio	0.97 ± 0.09	0.91 ± 0.12
Fasting insulin (mU/l)	17 ± 11	18 ± 10
HOMA	3.8 ± 3.1	3.9 ± 2.3
Leptin (ng/ml)	58 ± 9	44 ± 19*
Leptin-SDS	1.0 ± 1.2	1.0 ± 1.5

Data as mean and s.d.
HOMA, homeostasis model assessment; *MC4R*, melanocortin 4 receptor; SDS, standard deviation score.
[a]Data as median and interquartile range since variable was not normally distributed, none of the variables differed significantly except *(P <0.05 derived from t-test for unpaired observation).

DISCUSSION

This is the first report of a long-term response to a 1-year lifestyle intervention of *MC4R* variation carriers compared to noncarriers. We identified 16 (3% of all screened subjects) children with eight different *MC4R* mutations leading to a reduced receptor function. In concordance with a small previous report comprising four children with *MC4R* mutations (34), our nine children with *MC4R* mutations that lead to a reduced receptor function, who had participated in a lifestyle intervention, decreased their overweight at the end of the intervention. However, 1 year after the end of the intervention, children with these *MC4R* mutations demonstrated a similar degree of overweight as at baseline, while children without these mutations had sustained their degree of weight loss. These findings support an impact of *MC4R* mutations on weight status. Because carriers of the *MC4R* mutations can lose body weight but have difficulties to maintain this weight loss, very long lifestyle interventions seem to be necessary for these children.

The novel *MC4R* mutations were functionally characterized in this study (Table 2). The nonsense mutation Gln307Stop resulted, expectedly, in a complete loss of function due to the premature stop codon which led to a truncated receptor that lacks the C-terminal part. The Met281Val mutation is located at the beginning of transmenbrane domain 7. This position is highly conserved throughout species (35). The exchange of a methionine residue at amino acid position 281 for a valine residue thus results in a partial loss of function. Position Ala244 is as well highly conserved (35). The mutation of alanine 244 to glutamic acid was reported to result in a partial loss of function (6,18). In this study we examined the exchange of Ala244Val and found no functional differences to the WT MC4R in cell surface expression and for signal transduction properties after [Nle4,d-Phe7]–α–melanocyte-stimulating hormone challenge.

This might be due to the slight steric differences between alanine and valine at the beginning of transmenbrane domain 6. Hence, there are presumably no conformational changes, which are more likely if alanine 244 is exchanged to glutamate.

Children with *MC4R* mutations did not differ significantly in their pubertal stage, cardiovascular risk factor, or hormone profile from obese children without *MC4R* mutations matched for age and gender. The higher leptin concentrations in children with *MC4R* mutations that lead to a reduced receptor function can be explained by the slightly higher degree of overweight, as SDS-leptin adjusted for BMI did not differ between children with and without these *MC4R* mutations. Therefore, we were unable to identify any clinical or laboratory characteristic pointing toward *MC4R* mutations. However, one-fourth of children with *MC4R* mutations that lead to a reduced receptor function were siblings, potentially suggesting that the presence of a relevant *MC4R* mutation should be considered if sibs are referred to obesity units.

We did not find a significant difference between height, percent body fat, and insulin levels between children with and without *MC4R* mutations in contrast to the inital report of Farooqi and colleagues who described the "*MC4R* syndrome" (5). These discrepancies may be explained at least in part by different ways of body composition measurement. The dual-energy X-ray absorptiometry method in the original report represents the gold standard in contrast to determinate percent body fat based on skinfold measurement. Furthermore, there was a trend toward greater height in the children with *MC4R* mutations in our study. It could be argued that our sample size was too small to detect significant differences. However, in concordance with our findings several other studies have not replicated the "*MC4R* syndrome" (4,19,34,36). Because we matched the children with *MC4R* mutations to children without these mutations according to age and gender, which might also explain partially the differences between our study and the report of Farooqi and Keogh (5) due to growth, hormone levels, and body fat depending on these factors. Furthermore, a different age range may explain different findings because Farooqi and Keogh observed that hyperinsulinemia of obese subjects with *MC4R* deficiency seems to decline with age (5). In concordance with our findings, analyses of homozygous loss of function mutations suggest that in humans the *MC4R* does not mediate the effect of leptin on linear growth and other endocrine axes (37). In addition, complete *MC4R* deficiency is not a cause of relative hyperinsulinemia (37).

This study has a few potential limitations. First, this was an observation study and not a randomized clinical trial. Therefore, the findings have to be interpreted cautiously. Second, the study was clinically based and the observed prevalence of *MC4R* mutations might rather represent the frequency that can be observed among obese children asking for medical advice than the frequency among obese children in the population. However, there is no evidence that obese children/families with *MC4R* mutations differ from obese children/families in behavior in general or in behavior regarding contact to health services. Third, one-fourth of the children with *MC4R* mutations that lead to a reduced receptor functions were siblings. Family eating and exercise behaviors likely also influence the response to a lifestyle intervention. However, we have adjusted our analyses for relatives.

In summary, overweight children with *MC4R* mutations that lead to a reduced receptor function were significantly more frequently siblings, while they did not differ in respect to any anthropometrical marker, hormone profile, or cardiovascular risk factor as compared to obese children without these *MC4R* mutations. Children with *MC4R* mutations were able to lose weight in a lifestyle intervention program. However, the children with *MC4R* mutations that lead to a reduced receptor function had much greater difficulties to maintain this weight loss as compared to children without *MC4R* mutations supporting the impact of these mutations on weight status.

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DISCLOSURE

The authors declared no conflict of interest.

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3.7 Publication VII

Hebebrand J, Friedel S, Schäuble N, Geller F, Hinney A. **Perspectives: molecular genetic research in human obesity.** Obes Rev. 2003;4(3):139-46.

Within the past decade the molecular basis of single forms of monogenic obesity has been elucidated. With the exception of functionally relevant mutations in the *MC4R*, which occur in approximately 2-4% of extremely obese individuals, all other currently known monogenic forms are rare and additionally associated with distinct endocrinological abnormalities. A large number of association studies have been performed in 'normal' obesity. Whereas many associations have been reported, it is largely unclear which of these represent true positive findings. More than 30 genome scans pertaining to obesity and related phenotypes have been performed; specific chromosomal peak regions have been identified in different scans. We review the current status and discuss relevant issues related to phenotyping, association and linkage studies. We recommend that the procedure via which a consensus is reached as to what constitutes a true positive association finding requires formalization

Own contribution:

- Establishment of a database to collect and manage the data of all published genome-wide linkage studies for obesity and related phenotypes (in cooperation with Schäuble N)
- Comparison of our linkage data with all scans collected in the database
- Graphical analyses and comparison of linkage data (Figure1)
- Writing the paragraph “Genome scans”

Perspectives: molecular genetic research in human obesity

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Summary

Within the past decade the molecular basis of single forms of monogenic obesity has been elucidated. With the exception of functionally relevant mutations in the melanocortin-4 receptor gene, which occur in approximately 2–4% of extremely obese individuals, all other currently known monogenic forms are rare and additionally associated with distinct endocrinological abnormalities. A large number of association studies have been performed in 'normal' obesity. Whereas many associations have been reported, it is largely unclear which of these represent true positive findings. Over 20 genome scans pertaining to obesity and related phenotypes have been performed; specific chromosomal peak regions have been identified in different scans. We review the current status and discuss relevant issues related to phenotyping, association and linkage studies. We recommend that the procedure via which a consensus is reached as to what constitutes a true positive association finding requires formalization.

Keywords: Ascertainment, BMI, candidate gene, genome scan, phenotyping

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Introduction

In order to allow a fruitful discussion of future perspectives to be pursued in molecular genetic studies in obesity, we review the current 'main stream' approaches to detect mutations and polymorphisms predisposing to the development of obesity and related phenotypes. We proceed by critically reflecting on these approaches to dissect both their strengths and weaknesses. Finally, from a subjective point of view we discuss complementary or alternative strategies which we believe will broaden our potential of understanding the genetic mechanisms involved in body weight regulation. The better our understanding of the genetic mechanisms and the underlying pathways becomes, the more we stand a chance of addressing genotype–genotype and genotype–environment interactions.

Phenotyping

Apart from body mass index (BMI; kg m^{-2}), percent body fat as assessed by, for example, bioelectrical impedance analysis, different skin fold measurements, waist to hip circumferences and/or other more sophisticated measurements are frequently used as additional phenotypes in molecular genetic studies. Other variables correlated with both BMI and percent body fat are also frequently co-determined. These include, for example, serum adiponectin and leptin levels.

This large number of phenotypes tends to obscure the important fact that current phenotyping cannot address the major phenotypes energy intake and expenditure in sufficiently large samples. If these phenotypes were not black boxes, we would presumably be able to considerably better address the underlying genetic mechanisms. Because only a small positive energy balance is required to develop obesity over a prolonged period of time (1), measurements of energy intake and expenditure would have to be very sensitive. They would also have to precisely take into account the current fat-free mass as the major determinant of energy expenditure. Thus, whereas anthropometric and endocrinological variables are undoubtedly relevant, it appears highly probable that the current phenotyping schemes may

be missing out on other genetically based phenotypes that contribute to the development of an energy imbalance. In particular, behavioural and sensory phenotypes are currently not being analysed extensively in molecular genetic studies. This presumably stems from the fact that in medical terms obesity is frequently perceived as a metabolic disorder; this perception has led to a self-perpetuating mechanism in that the belief in a metabolic aetiology has facilitated access of clinicians and scientists into the field with a corresponding background who then use their expertise to study the phenotype. Because behavioural phenotypes have already been deemed as important in the premolecular genetic era, it seems that 'endocrinologists' based on their training have more readily been able to initiate molecular genetic studies than behavioural scientists. Undoubtedly, part of the problem also stems from the fact that behavioural phenotypes are frequently more difficult to address than endocrinological parameters.

Behavioural phenotypes are often viewed critically. However, it is frequently forgotten that even the most precise measurements of fat mass or serum parameters merely represent a momentary glimpse into a complex organism which is subject to developmental change. This is particularly true for the genetic analysis of children and adolescents, in whom the transitions made during puberty clearly underscore the problems inherent to an over-reliance on absolute anthropometric and endocrinological parameters. Along the same lines, a longitudinal twin study (2) has revealed that the genetic factors that influence BMI at age 20 only partially overlap with those relevant at age 40. Irrespective of these considerations, the rather low reliability rates for measurement of, for example, waist and hip circumference in extremely obese subjects need to be reflected upon critically. In addition, even serum parameters are subject to circadian rhythms and short-term alterations of eating behaviour, which, if not properly accounted for, are likely to diminish the chance of detecting relevant alleles.

The relative negligence of addressing behavioural phenotypes is becoming more and more apparent in light of the importance of central mechanisms in body weight regulation. Most of our current knowledge pertains to hypothalamic pathways. However, other brain regions have emerged and will continue to do so (3). As such, the implication of a specific region entails questions as to what behavioural and/or sensory phenotypes are related to it and if these might be relevant for obesity. For example, recent advances in understanding olfaction and gustatory sensation (4,5) lead to the question as to if and how genetic variability in sensation of smell and taste has potential implications for eating behaviour and obesity. The brain reward system in which the nucleus accumbens play a prominent role is also a system that could have an influence on body weight regulation (6). Such considerations can also be extended to the candidate gene level. Thus, melanin concentrating hormone (MCH) is exclusively expressed in neurones of the lateral hypothalamic area that project to widespread brain regions including the olfactory bulb, anterior olfactory nucleus, neocortex and amygdala (7). A melanin concentrating hormone receptor 1 (MCHR1) antagonist not only has been shown to induce weight loss but also has both an anti-depressant and anxiolytic effect (8). MCHR1 knock-out mice not only have an altered body weight but also display hyperactivity (9,10). Conceivably, feeding-related functions of MCH include appetite, arousal and anxiety, food-searching behaviour, olfaction, regulation of energy balance, swallowing and mastication. In general, pathways involved in energy intake and expenditure can overlap with those relevant for mood regulation, anxiety, activity and cognition. This is underscored by the findings in several transgenic animal models with an altered body weight in which effects are also apparent for these phenotypes [e.g. $Npy^{-/-}$ mice (11), $Cb1^{-/-}$ mice (12,13), $5\text{-}Ht2cr^{-/-}$ mice (14)].

This brief discussion serves to illustrate the fact that we could profit from an inclusion of behavioural and sensory phenotypes. Behavioural phenotypes generally show heritability estimates in the range of 0.5 (15) and are thus in a range similar to those for many anthropometric and endocrinological phenotypes relevant for body weight (16); as has repeatedly been shown in formal genetic studies pertaining to BMI (16,17), the environmental component is largely explained by non-shared environment (15). Behavioural phenotypes which warrant consideration for genetic analyses of body weight regulation include eating behaviour (e.g. binge eating, restrained eating), activity, stress, mood and anxiety. It is granted that the assessment of these phenotypes is complicated; we argue that narrowing the focus on assessment of their (assumed) impact on energy intake and expenditure might, nevertheless, prove valuable for unveiling the molecular mechanisms involved in body weight regulation. More research is required to address if and how these behavioural phenotypes have an influence on body weight. Depression which in prospective studies conducted in children and adolescents has been shown to predict a higher BMI (18,19) may serve as an example.

Furthermore, the delineation of taste and dietary preferences should prove valuable, too. The very recent positional cloning of the quantitative trait locus (QTL) underlying sensitivity to phenylthiocarbamide (5) raises the question if this QTL also has an impact on dietary preferences and potentially on body weight.

Quantity or quality?

It is commonly assumed that the better specific subgroups are delineated at the phenotypical level, the more homogeneous the underlying genetic factors will turn out to be.

Such a precise phenotyping might actually be a prerequisite for identifying genes in the first place. However, it is evident that not all conceivable phenotypes of potential relevance in body weight regulation can be addressed in every individual who is willing to participate in a molecular genetic study. Both the endurance of the proband and the costs for phenotyping represent a limiting factor. These limitations necessitate diversity in phenotypical approaches. We recommend that behavioural phenotypes are better integrated into current assessment schemes. Alternatively, study groups well characterized for single behavioural phenotypes or for psychiatric disorders can be used to get further insight into the significance of alleles once their involvement in weight regulation has been demonstrated unambiguously. As such, we need to convince researchers pursuing behavioural and psychiatric genetic studies to at least obtain a measurement of body height and weight in the respective probands to allow calculation of BMI.

In light of the multitude of potentially relevant mechanisms underlying body weight regulation, it is debatable if the probands whose DNA samples are used for genome scans and mutation screens really need to be that well phenotypically characterized. Future research findings undoubtedly have the potential to necessitate inclusion of a phenotype that was originally not considered as being within the spectrum of relevant phenotypes. As a consequence, expensively ascertained samples will not be able to deal with such a novel phenotype, unless the probands are recontacted. Thus, it might currently make more sense to first identify genes relevant in weight regulation in a very large but phenotypically not too well-characterized study group to then proceed to test the effect of specific alleles in samples well characterized for specific behavioural, physiological and endocrinological phenotypes. This strategy would also at least partially circumvent the problem of testing several different phenotypes within a single sample (multiple testing). For BMI alone, several different peaks have been reported in genome scans, some of which have been confirmed in different studies (20–40; Fig. 1). Evidently, for the identification of the relevant genes in these peak regions, no further phenotypical information is required.

If we perceive obesity as a multifactorial disorder, in which polygenic effects account for a large proportion of the total genetic effect, we need to perform our sampling accordingly. Thus, to initially detect and subsequently confirm alleles associated with relative risks below 1.5, we need samples encompassing thousands of probands.

In conclusion, we definitely need exceedingly large samples. If financial resources permit a top notch phenotypical assessment of thousands of probands, this obviously represents a superb solution. To deal with developmental aspects, such a sample should optimally be assessed longitudinally. However, if financial resources are limited, the research team has to decide between the extremes of high quality phenotyping in a limited number of probands and a simple phenotypical assessment of a large number of probands. Researchers in other fields, particularly those involved in behavioural and/or psychiatric genetic studies, should be encouraged to include simple anthropometric measurements, thus potentially allowing the assessment of an allele which in prior stud-

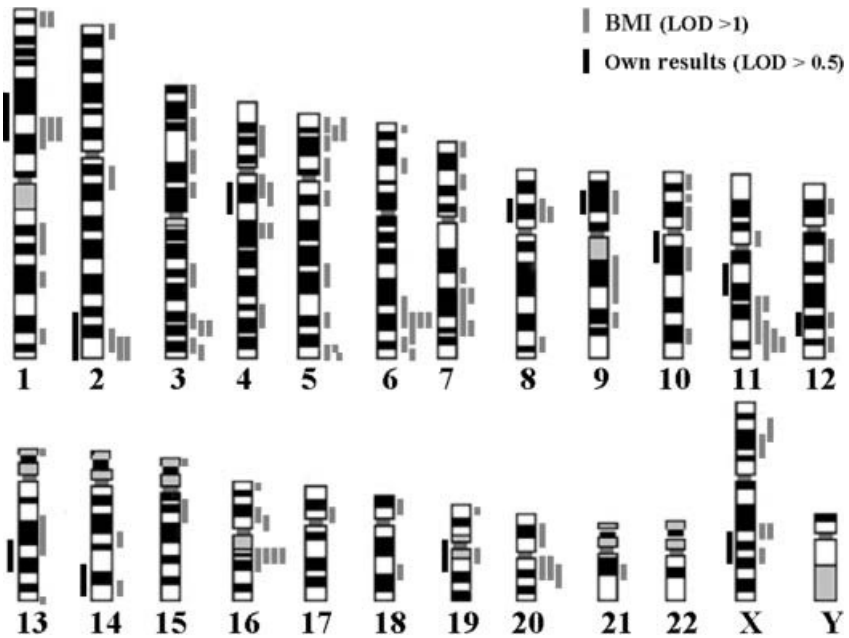

Figure 1 Chromosomal regions which were identified by linkage studies (20–40) for phenotype body mass index (BMI) (status: 12/2002). Each bar represents one positive linkage finding. LOD, Logarithm of odds.

ies has already unequivocally been shown to be involved in body weight regulation.

Ascertainment schemes

To both detect specific alleles involved in body weight regulation and to fully understand their functional role, cases, controls, trios, extreme sibships and extended pedigrees have been ascertained by different groups worldwide in ethnically diverse populations. Populations include both children and adults. In addition, several large epidemiological samples and cohorts exist which can be used to follow up on an allele of interest. In our opinion, this diversity is to be encouraged because it allows synergistic strategies. However, it should briefly be pointed out that the issue of genomic controls is important in case-control studies (41). The transmission disequilibrium test (TDT) (42) is frequently preferred if parents can readily be ascertained. The limitations of extreme sibpair approaches need to be kept in mind. Ziegler *et al*. (43) performed sample size calculations for the different designs and discussed these strategies in the context of body weight. Furthermore, infrequent major genes and minor gene effects (alleles associated with small relative risks) can only be picked up by analysing thousands of families, because allele sharing only slightly surpasses the expected rate of 50% (44).

Merging genetic studies in different species

QTL studies have been ongoing for several years in rodents, pigs and cattle. A priority for future research into the genetic mechanisms involved in body weight regulation is to bring experts together who work on different species. The occurrence of a functionally relevant single nucleotide polymorphism (SNP) in the pig melanocortin-4 receptor gene (*MC4R*) (45) is just one example illustrating the potential inherent to this approach. Some QTL peaks identified in pigs map to homologueous human chromosomal regions which have also been identified in linkage studies of human obesity. Quite possibly, the same gene(s) underlie these peaks, thus indicating conservation not only of genes and pathways but also of the mechanisms leading to genetic variability of body weight. An additional advantage pertains to the ease with which relevant phenotypes can be assessed in non-human species.

Research is required to assess the implications of studies in invertebrates. Thus, genomewide RNAi analysis of Caenorhabditis elegans revealed that out of the total of 16 757 genes 305 and 112 gene inactivations led to a decreased and increased fat storage, respectively (46). Thus, approximately 2.5% of all genes in this invertebrate are involved in fat storage. An interesting hypothesis resulting from this study is that in humans more genes might be relevant for underweight than for obesity.

Candidate gene approach

The candidate gene approach has proven to be successful for obesity. Thus, the conservation of hypothalamic pathways in rodents and humans has certainly aided in determining suitable candidate genes (47–52). This particularly applies to those candidate genes originally identified in animal models. All the spontaneously occurring obesity mutations in mice (47,53–56) either have been found to harbour functionally relevant mutations in humans too (48,57), or have led to the identification of a system/pathway in which other genes were found to be mutated [e.g. carboxypeptidase mutations in mice (58) and prohormone convertase 1 gene mutations in humans (59)].

However, it should be pointed out that most of these mutations in humans were not detected via a classical mutation screen. Instead, specific endocrinological findings such as elevated proinsulin levels (59) or hypoleptinemia (48,60) led investigators to screen specific genes with clear-cut *a priori* hypotheses. Functionally relevant mutations in the *MC4R* currently represent the only exception to this rule; this is related to the fact that obesity resulting from *MC4R* mutations is not distinguishable from 'normal' obesity. It is apparent, that mutation screens of larger samples are required to identify alleles involved in obesity not readily associated with a specific endocrinological or behavioural phenotype.

We perceive two major perspectives for the candidate gene approach both of which can certainly apply at the same time.

1. Within linkage regions the candidate gene approach is frequently resorted to in complex phenotypes when the number of putative genes has been narrowed down. Undoubtedly, we should witness the success of this approach for specific candidate chromosomal regions for obesity in the near future.
2. Some candidate genes warrant consideration independently of whether or not they are localized within chromosomal regions of interest. Such genes are considered because they are involved in relevant pathways as shown in animal models or via other evidence. This approach should not be viewed as an alternative to the identification of the genes contributing to linkage peaks. Instead such studies are complementary because as illustrated above linkage studies cannot readily lead to the detection of minor gene effects or infrequent major gene effects.

Validation of an obesity gene

Irrespective of the approach for the choice of a particular candidate gene, we need to devise how to conclude that a specific allele indeed predisposes to obesity (61). The current literature abounds with positive association studies some of which have been followed up with negative or

equivocal results (62). Many of the positive results must be viewed critically because multiple tests were performed to achieve the respective 'significant' result. It is crucial that negative findings are also published; hence, every effort should be made to encourage researchers to publish these finding. To avoid discouraging other researchers from conducting studies, high standards should be set for publication of such negative findings. Journal editors need to be aware of their responsibility. Potentially, specific journals might be required to competently deal with these issues. To allow a better interpretation of negative findings, the power of the study for a given (previously reported) effect should be stated.

We deem it important that positive findings are followed up in a systematic fashion thus enabling the scientific community to conclude whether or not the identified allele is indeed involved. For this purpose, defined population (epidemiological) samples could be referred to in addition to large trio samples to allow the TDT (42); meta-analyses should prove helpful when a sufficient number of studies are available. At some point a decision needs to be reached as to whether current evidence is sufficient to unequivocally conclude that a particular allele(s) is involved. A final decision on the epidemiological level should be based on an appropriate meta-analysis of all available studies.

This procedure should be formalized by, for instance, a committee whose task would be to rank candidate gene findings according to the empirical evidence from improbable to probable and finally to definite. Rules for determining this status need to be defined. Such a formalization would entail many benefits, the foremost of which would be the separation of solid findings from false-positive or equivocal findings. The committee would need to consider if ethnicity, gender, age and other variables have an impact on the respective association. Gene–gene interactions can reliably be assessed if the contribution of the single genes has been shown without doubt.

False-negative findings are also an issue of concern (61). Most current candidate gene studies suffer from a lack of power because of small sample sizes. Therefore, the power of these studies to detect a given effect should be stated for the negative finding. Another way of addressing this issue is to include relative risks and sample size calculations in association studies based on the premise that the observed non-significant difference in allele frequencies between cases and controls is indeed real. This will enable follow-ups on negative association studies. Estimations of allele- and/or genotype-specific relative risks and attributable risks should be presented in all positive association studies.

The thoroughness with which a particular candidate gene has been investigated requires attention. In many studies only one SNP was analysed, while in other studies two or more SNPs and haplotypes were addressed. A systematic mutation screen of the coding region represents an attempt to detect all mutations within a candidate gene. Finally, the promoter region can also be screened. Nevertheless, even this arduous approach does not totally allow the exclusion of a particular gene; theoretically a regulatory sequence can have quite a distance from the gene. The methodology also warrants consideration; thus, mutation detection rates upon use of single strand conformation analysis or other methods are not 100%.

We are already witnessing a commercialization of molecular genetic findings in obesity. Diagnostic tests based on specific polymorphisms or mutations are available commercially (e.g. Internet). Unfortunately, in some of these cases the consumer is led to believe that genotyping of a particular polymorphism will allow detection of the 'fat gene', thus indicating that the seriousness of such providers cannot be taken for granted. In our opinion, the consumer/patient should have access to molecular genetic testing after having been informed of potential implications. Clearly, only those tests should be made available that pertain to polymorphisms or mutations whose functional relevance has been established unequivocally and which occur with a frequency large enough to warrant screening efforts. In our opinion, this is currently only the case for *MC4R* mutations, which occur in up to 2–4% of extremely obese probands. An individual should obtain a clear grasp of the implications of such a finding for herself/himself, for other family members and potentially for future offspring. The individual should also obtain a feeling of to what extent her/his obesity results from a specific mutation or polymorphism. Thus, it is evidently debatable if polymorphisms associated with a relative risk < 2 should be screened for. Such an approach might be warranted in the future if several polymorphisms involved in body weight regulation have been identified, which in a specific combination could have a larger impact.

We need to analyse potential effects of genetic testing on the obese subject; what if any are the consequences of a 'positive' test? Will such a result lead to alleviation, because the carrier now has pinpointed a major reason for the obesity and as such no longer needs to blame herself/himself? Or, might the knowledge of being a carrier of a functionally relevant mutation have negative effects in that the individual stops restraining her/his eating behaviour, because the detection of a genetic basis of the obesity is viewed as an excuse for not having to exert further control? and finally, what are the implications of genetic testing at the societal level? Will public knowledge of the genetic basis of obesity as exemplified by specific diagnosable mutations entail a reduction in stigmatization?

Genome scans

As has already been pointed out, linkage genome scans performed in obesity have come up with some consistent

regions (20–40). Fine mapping is ongoing; the TDT is of great value for detection of linkage disequilibrium (42). The next years will reveal to what extent single mutations or SNPs and haplotypes underlie these peaks. Furthermore, it seems possible that some of the peaks actually represent the combined effect of SNPs or haplotypes at more than one locus. The fact that gene and regional chromosomal duplications have occurred frequently (63) indicates that gene clusters might indeed play a role.

Genotyping

The advent of high throughput technology offers the chance to genotype more than 10 000 samples a day, thus potentially enabling genomewide association studies. Undoubtedly, scientific groups have already or will devise ways and means to obtain access to high throughput facilities. This in itself might turn out to become crucial for obtaining a competitive edge. SNPs and the extents of linkage disequilibrium blocks need to be identified. Additionally, the genotyping strategy becomes more and more important because concepts like DNA pooling and haplotype-tagging SNPs might improve efficiency. A substantial proportion of the work is currently shifting from lab work to computer work.

Study designs and statistical analysis

With the large amount of data coming up, studies have to be planned carefully and appropriate statistical methods need to be chosen. Kaplan and Morris (64) have investigated association studies for fine mapping in complex diseases and show that rare disease alleles are hard to detect. Another feature that has to be sorted out are the strengths and limitations of the analyses of haplotypes, that is, in trios the haplotype sharing analysis can be inferior to the TDT analysis at single SNPs (65). Discussing all proposed statistical approaches is beyond the scope of this article; an excellent overview of these important issues is given by Terwilliger and Göring (66). Topics include quantitative/qualitative phenotypes, admixture mapping, gene–environment interactions and gene–gene interactions. All the proposed methods are based on sound statistical considerations; future applications will show which of these methods are indeed powerful tools for identifying genes.

In light of the vast number of candidate genes, SNPs and haplotypes, false-positive results because of multiple testing will become exceedingly frequent; testing of SNPs will basically occur on a genomewide level (41). Appropriate decision rules and additional (high throughput) molecular studies in other samples are required to determine whether or not the polymorphism is to be pursued by, for instance, genotyping it in a second sample. Because 'significant' findings will additionally require functional studies, efforts must be maximized to allow identification of those genes and alleles which are indeed involved in the phenotype. Specific guidelines for a sequential procedure entailing a high probability of identifying an allele with an impact on the phenotype could prove valuable in this situation.

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4. Summary of the studies and discussion in the context of obesity genetics

Two different genome wide approaches to identify candidate genes will be discussed, focussing on differences between the analyses applied in this thesis (Saar et al., 2003, Hinney et al., 2007). Additionally, the role of the three investigated candidate genes in body weight regulation will be summarized in the context of the recent findings in obesity research (Friedel et. al., 2002, 2005, and 2007). Finally, the importance of such studies for obesity treatment interventions will be considered in the discussion of these results (Reinehr et al., 2009).

4.1 Genome-wide approaches to identify chromosomal regions/ candidate genes/genetic variants involved in body weight regulation

During the last two years, technical approaches to detect novel genes/genomic regions by genome-wide approaches changed dramatically. Genome-wide linkage studies for obesity have been conducted since 1997 (Comuzzie et al., 1997), the first two positional candidate genes were identified in 2003 (Suviolathi et al., 2003; Boutin et al., 2003), a third followed in 2005 (Meyre et al., 2005) – however, still none of these candidate genes have been robustly and consistently confirmed in a larger number of independent studies. The implementation of GWAs during the last two years, however, led to the identification of a larger number of new candidate genes for obesity.

The results of the last decades revealed that linkage studies have primarily been successful in detecting genes underlying monogenic disorders and that they have rather limited power to detect genes with modest or small effect (oligogenes, polygenes) which are more typical in complex diseases. In comparison, GWAs have the statistical power to detect such smaller gene effects. For obesity, the first GWA was published in 2006, followed by the identification of the first polygenes in 2006 and 2007 (Herbert et al., 2006; Sladek et al., 2007).

In the following, two genome wide scans, a linkage scan as well as a GWA will be presented and discussed in the context of current obesity research.

4.1.1 Identification of chromosomal regions involved in the aetiology of early onset obesity using linkage analysis in 89 families of German origin with two or more (extremely) obese children

Genome-wide linkage scans are used for the identification of chromosomal regions harbouring candidate genes. Genome scans for complex disorders are based on the assumption that the similarity in the phenotype of affected sibs is based on the same genotype (allele sharing). The genome scan of Saar et al. (2003) represented the first scan for early onset obesity. The scan was based on 89 families with 2 or more obese children. A total of 369 individuals were initially genotyped for 437 microsatellite markers, a second independent sample of 76 families was genotyped using microsatellite markers that were localized in regions for which maximum likelihood binomial logarithm of the odd (MLB LOD) scores used for the concordant sib pair approach exceeded 0.7 in the first sample. The regions with MLB LOD scores >0.7 were on chromosomes 1p32.3-p33, 2q37.1-q37.3, 4q21, 8p22, 9p21.3, 10p11.23, 11q11-q13.1, 14q24-ter, and 19p13-q12 in sample 1; MLB LOD scores on chromosomes 8p and 19q exceeded 1.5. In sample 2, MLB LOD scores of 0.68 and 0.71 were observed for chromosomes 10p11.23 and 11q13, respectively.

Similar to Saar et al., Meyre et al. (2004), also conducted a genome-wide linkage study for childhood obesity-associated traits (e.g. BMI >95th, 97th and 99th percentile (PCT95, 97, 99) and age of adiposity rebound (AAR)). A set of 431 microsatellite markers was genotyped in 506 subjects from 115 multiplex French Caucasian families, with at least one child with a BMI >PCT95. Among these 115 pedigrees, 97 had at least two sibs with a BMI >PCT95. Fine-mapping was performed for the seven most promising loci. Nonparametric multipoint analyses revealed six regions of significant or suggestive linkage on chromosomes 2q33.2-q36.3, 6q22.31-q23.2, and 17p13 for PCT95, PCT97, or PCT99 and 15q12-q15.1, 16q22.1-q24.1, and 19p13.3-p13.11 for AAR. The strongest evidence of linkage was detected on chromosome 6q22.31 for PCT97 (maximum likelihood score: 4.06) at the marker D6S287. This logarithm of odds score was significant at a genome-wide level of 0.05 when using permutations (empirical genome-wide p = 0.01 [CI: 0.0027-0.0254]).

A third scan investigated longitudinal BMI data from childhood to adulthood in 782 siblings from the Bogalusa Heart study (Chen et al., 2004). Because of the differences in study design and analysed phenotypes (i.e. the sib pairs were not ascertained for obesity), this study is hard to compare with Saar et al., (2003) and Meyre et al., (2004). Therefore, we will restrict the subsequent discussion to the first to scans.

These two studies are the only genome-wide linkage scans for phenotype early onset

obesity. In light of the potentially stronger genetic determination of childhood and adolescent BMI and the possibility of age-dependent genetic influences on body weight, genome-wide scans based on children and adolescents are of obvious interest. Furthermore, scans based on children and adolescents entail the advantage that the parents can be readily ascertained, thus enabling determination of the identity by descent status. It was hypothesized that a genome scan based on childhood- and adolescent-onset obesity has a greater potential to detect relevant chromosomal regions than a scan based on obese adult sib pairs. This hypothesis originates from data indicating a potentially higher genetic load in childhood and adolescent obesity (Pietiläinen et al., 1999). The ascertainment of the sample investigated by Saar et al., (2003) was based on the extremely concordant sibling pair approach (ECSP; Risch and Zhang, 1995). They included German obese index patients and their siblings via BMI >PCT95 for one sibling and >PCT90 for the other(s). In comparison, Meyre et al., (2004) included French families with at least two probands with a BMI >PCT95 before the age of 8 and two living parents and additionally 18 pedigrees with at least two sibs and only one proband with a BMI >PCT95 for analysis of age of adiposity rebound. Despite the stricter inclusion criteria of Meyre et al., the German sample has a is slightly higher BMI (mean BMI 32 vs. 29) and older than the French sample (mean age 13.6 vs.11.9). Furthermore, Meyre et al. used their whole sample of 115 families for the scan as well as for the fine mapping while Saar et al. divided their sample in an initial scanning sample and a second sample for independent fine mapping. Moreover, the strongest signal of Meyre et al. (2004) was a maximum likelihood score of 4.06 on chromosome 6q; Saar et al., observed no suggestive peak of linkage (LOD >2.2) with obesity. Beside a signal on chromosome 2q33 (Meyre et al., 2004) and 2q37 (Saar et al., 2003) was no overlap in chromosomal regions identified in both scans. Interestingly, no linkage with morbid obesity in adults was reported for this region. It might be possible that the molecular determinants of the severe forms of early onset obesity are different from those associated with morbid obesity in adults. Until today, no candidate gene contributing to these linkage peaks was identified.

Nevertheless, both genome-wide linkage studies discussed and followed up candidate genes derived from their scans. Saar et al. describe *glutamic acid decarboxylase 2* (*GAD2*; chr.10), *angiotensin receptor-like 1* (*AGTL1*), *ciliary neurotrophic factor* (*CNTF*), galanin (*GALN*), and *uncoupling proteins 2* and *3* (*UCP2, 3*) as promising candidate genes localized within the chromosome 10 and 11 peak regions. However, they found no evidence for association of *CNTF, UCP2* and *GAD2* with obesity (Münzberg et al., 1998; Schäuble et al., 2003; Swarbrick et al., 2005). In contrast, Meyre et al. (2005) performed fine mapping on chromosome 6q and identified several variants of ectonucleotide pyrophosphatase/phosphodiesterase (*ENPP1*) to be associated with obesity. This finding

was replicated in some (Böttcher et al., 2006; Bochenski et al., 2006; Meyre et al., 2007; Prudente et al., 2007; Valli-Jaakola et al., 2008; Jenkinson et al., 2008; González-Sánchez et al., 2008) but not all subsequent studies (Lyon et al., 2006; Weedon et al., 2006; Grarup et al., 2006; Seo et al., 2008).

Despite the heterogeneity of samples and obesity-related phenotypes investigated in these studies it remains an open question if *ENPP1* is a true positive finding. The recently published GWAs for obesity and diabetes do not support the role of *ENPP1* in the aetiology of obesity or NIDDM. However, one should note that even more consistently associated genes may not light-up among the top hits of a GWA as recently reviewed by Frayling et al. (2007) for NIDDM. Altogether, both linkage scans for childhood and early onset obesity were not (Saar et al., 2003) or not convincingly successful (Meyre et al., 2004) in identifying candidate genes for obesity.

Following Altmüller et al. (2001), most genome-wide scans are not able to detect “significant” linkage as defined by Lander and Kruglyak (1995). The results of Saar et al. fit within this category. Both the relatively small sample size and the fact that the scan was based on sibling pairs might mainly account for the discouraging outcome. Due to the presumably large genetic heterogeneity of the phenotype obesity, the reliance on single sibling pairs entails the disadvantage that different genetic variants may only be present in a limited number of families. Even for major genes this will reduce the chances to observe a high LOD score. The identification of major genes is more likely in large pedigrees with several affected family members. Whereas genome-wide linkage analyses were useful instruments identifying relevant genes for monogenetic disorders, they mainly failed in the search of candidate genes for complex disorders like obesity. This conclusion is supported by the study of Saunders et al. (2007), who performed a meta-analysis of 37 published genome-wide linkage scans. Despite having substantial statistical power even for smaller genetic effects, they did not identify specific loci for BMI or obesity. The *FTO* gene locus on chr.16q12.2 showed nominal evidence for linkage in the pooled analysis. As *FTO* is one of the most convincing genetic findings in obesity and as the study has sufficient statistical power, one would have estimated a larger linkage signal.

Reasons might be that microsatellite - based linkage scans are not suitable for the detection of small genetic effects expected for obesity or heterogeneity of genetic loci.

4.1.2 Identification of genetic variants involved in body weight regulation using a genome-wide association study (GWA) for extreme, early onset obesity

GWA studies offer a new conceptual framework to identify common genetic variants underlying common diseases (Thomas et al., 2006). In 2007, the detection and publication of genes/SNPs for complex disorders like NIDDM increased rapidly (.e.g. Scuteri et al., 2007; Zeggini et al., 2007; Scott et al., 2007; Frayling et al., 2007; Sladek et al., 2007).

We performed a GWA for early onset (extreme) obesity. 440,794 SNPs from the Genome-Wide Human SNP Array 5.0 (Affymetrix) were genotyped in 487 extremely obese young German individuals and 442 healthy lean German controls. The most promising markers were followed up using a family-based association approach based on 644 independent families with at least one obese offspring and both parents for confirmatory analyses. We aimed to identify and subsequently confirm the 15 SNPs with a minor allele frequency (MAF: =10 %), which provided the lowest p-values in the GWA by four genetic models: additive, recessive, dominant and allelic. Six SNPs in *FTO* (*fat mass and obesity associated gene*) within one LD block including the GWA SNP with the lowest p-value (rs1121980; nominal $p=1.13x10^{-7}$, corrected p=0.0494) belonged to the 15 SNPs showing the strongest evidence for association with obesity. For our confirmatory analyses we genotyped 11 of these 15 SNPs in 644 independent families (of the six *FTO* SNPs we chose only two representing the linkage disequilibrium block). For both of the two *FTO* SNPs the initial association was confirmed (both Bonferroni corrected p<0.01). However, none of the nine non-*FTO* SNPs revealed significant transmission disequilibrium in the family study.

Altogether, our GWA study for extreme early onset obesity substantiated that variation in *FTO* strongly contributes to the development of early onset obesity. *FTO* is one of the genes picked up in GWA studies for NIDDM (Frayling et al., 2007; Sladek et al., 2007) where an adjustment for BMI revealed that the effect was more likely due to this quantitative phenotype (Frayling et al., 2007). In a replication study investigating 13 samples with 38,759 individuals and a meta-analysis, Frayling et al. (2007) showed that the A-allele of the variant rs9939609 was associated with an increased risk to develop obesity. These results were independently supported in 8,000 individuals from different populations (Dina et al., 2007) and in a GWA study for obesity-related traits in an epidemiological cohort (Scuteri et al., 2007). According to Frayling et al. (2007), the risk for being obese is increased by 31% for carriers of a single copy of the risk allele. This risk is doubled for homozygous carriers (67%), whereas the average weight gain is approximately 3.0 kg among them. One can estimate that about one sixth of the population of European descent is homozygous for the risk allele. Therefore, the

link between *FTO* and obesity seems to be one of the strongest genotype-phenotype associations detected by GWA studies (Barabasi, 2007).

Ioannidis et al., (2007) re-analyzed the data from three GWA studies on type 2 diabetes (Scott et al., 2007; Saxena et al., 2007; Frayling et al., 2007) and found that for 5 of the 11 genetic variants that are considered as ‘‘confirmed’’ susceptibility loci for NIDDM there was still moderate to very large between-study heterogeneity across the different GWA investigations. For *FTO* rs8050136, the random effects summary odds ratio yielded a weak p= 0.015, as compared with the impressive $p = 1.3x10^{-12}$ originally reported indicating a large between-study heterogeneity (77%). Despite this heterogeneity, the Wellcome Trust investigators have indeed found that this variant is a susceptibility marker for increased body mass index and obesity (Frayling et al., 2007). Following Frayling et al. (2007), susceptibility to NIDDM might be mediated through the effect on body mass index and is not an independent effect which might explain part of the inconsistency seen for different populations. Thus, the observed heterogeneity for association to NIDDM is also explained by the study design of the three GWA investigations. For example, Saxena et al. (2007) used a tightly matched case-control sample in the discovery phase, where cases and controls had been matched for body mass index and thus it is not surprising that there was no residual effect of this *FTO* variant on the risk of NIDDM.

As BMI is a heritable measure of obesity that can be routinely and easily measured in large cohorts, it is a readily accessible trait useful to screen for genetic variants associated with the aetiology of obesity. There have been many publications reporting association between common genetic variants and BMI, but few of them were reproducible in multiple populations (Rankinen et al., 2006). An example is SNP rs7566605, upstream of the *INSIG2* gene, which was found to be associated with obesity as measured by BMI (Herbert et al., 2006). The association between increased BMI and homozygosity for the minor allele was first observed in data from a GWA scan of 86,604 SNPs in 923 individuals from the Framingham Heart Study offspring cohort. The association was reproduced in four additional study groups, but was not seen in a fifth cohort. To further assess the general reproducibility of this association, rs7566605 was genotyped in nine large cohorts from eight populations across multiple ethnicities (total n = 16,969). The SNP was tested for association with BMI in each sample under a recessive model using family-based, population-based, and case-control designs. Significant ($p < 0.05$) association was observed in five of eight study groups. Moreover, there was even variability in the strength of association evidence across examination cycles in a longitudinal assessment of the same unrelated individuals of the Framingham Heart Study Offspring cohort. A combined analysis revealed significant independent validation of this association in both unrelated (p = 0.046) and family-based (p =

0.004) samples. The estimated risk conferred by this allele is small, and could easily be masked by small sample size, population stratification, or other confounders. The validation studies suggest that the original association is less likely to be spurious, but the failure to observe an association in every data set suggests that the effect of SNP rs7566605 on BMI may be heterogeneous across population samples.

The poor rate of reproducible findings in association studies in general and obesity in particular are likely due to stochastic variation leading to false-positive findings, underpowered attempts to reproduce associations with modest effects, systematic bias due to technical artefacts or population stratification, and perhaps true heterogeneity in effect across populations due to differences in genetic or environmental modifiers (Lohmueller et al., 2003; Clayton et al., 2005). Thus, new reports of association require rapid, well-powered studies to validate true associations or to identify false positives that could otherwise trigger costly and time-consuming investigation of spurious findings (Lyon et al., 2006). As an example, Loos et al. (2008) analyzed GWA-data from 16,876 individuals of European descent to identify common variants influencing BMI. Besides *FTO,* the second strongest association signal (rs17782313) was identified 188 kb downstream of *MC4R*. The finding was confirmed in additional 60,352 individuals. Although the functional relevance of the SNP is yet unknown, this finding is one of the best supported findings in obesity genetics.

4.2 Investigation of candidate genes for obesity

Findings from formal genetic studies and animal models have indicated that approximately 50 – 80 % of BMI variance is due to genetic factors (Maes et. al 1997), a fact which has led to a great interest in conducting candidate gene studies for body weight regulation. The identification of such candidate genes can be carried out in different ways: (a) Functional candidate genes which are known to be involved in energy metabolism (i.e. *GLUT4*, involved in glucose metabolism) or genes known from obese animal models (i.e. the obese *Bdnf*+/- mice); or (b) positional candidates that were identified in linkage peaks via genome-wide scans for BMI or related phenotypes (i.e. *DGAT2* in a linkage region for BMI on chromosome 11q13). The first milestone in the genetics of obesity was the positional cloning of the mouse obese gene and its human homologue *leptin*. Since then the genetics of obesity has rapidly grown. Many candidate genes presumed to be involved in body weight regulation have been investigated. Studies usually started with a mutation screen of the gene in obese individuals followed by case-control association studies for detected variants.

Examples of functional candidate genes presented here include two studies investigating the

genetic impact of *BDNF* (Friedel et al., 2005) and *GLUT4* (Friedel et al., 2002) on body weight regulation, whereas *DGAT2* (Friedel et al. 2007) represents both a positional and functional candidate gene.

4.2.1 Analysis of the role of brain-derived neurotrophic factor precursor gene (*BDNF*) as a candidate gene for body weight regulation and activity (Friedel et al. 2005)

Several lines of evidence indicate an involvement of BDNF in body weight regulation and activity: Heterozygous *Bdnf* knockout mice (*Bdnf*$^{+/-}$) are hyperphagic, obese, and hyperactive; furthermore, central infusion of BDNF leads to severe, dose-dependent appetite suppression and weight loss in rats. Altogether, this phenotype makes BDNF a suitable candidate gene for obesity, eating disorders, and even attention-deficit/hyperactivity disorder (ADHD).

We screened the translated main exon of *BDNF* by SSCP (single strand confirmation analysis) and dHPLC (denaturing high pressure liquid chromatography) for mutations in a total of 370 German obese or underweight individuals. Three variants were identified apart from the previously known SNP p.Val66Met (rs6265): (i) We found the previously detected non-conservative amino acid substitution p.Thr2Ile (Weese-Mayer et al., 2002) in a single extremely obese male who inherited the mutation from his obese mother. It is unknown whether the Ile2 variant affects the mode of action of the signal peptide. Functional studies need to be conducted in order to investigate the possible effect of the mutation at Ile2 and how it may be linked to the clinical obesity phenotype. (ii) The novel variant c.273G>A was detected once in an extremely obese male. We assumed that there was no major effect because this mutation is silent. (iii) The 3´UTR variant c.*137A>G was detected in one underweight control (BMI 19.7 kg/m2). For this variant an influence on the mode of action of BDNF is unlikely. (iv) We did not detect an association between obesity, anorexia nervosa (AN) or ADHD and SNP p.Val66Met or c.-46C>T in the genomic region of *BDNF*. For bulimia nervosa (BN), we found a trend towards a potential association with -46T but we were not able to follow-up on this result due to our limited number of BN cases. Furthermore, the p-value would not have been of interest if adjustments for multiple testing would have been performed. In case that our observation indicated a true positive signal two different mechanisms might explain the finding: First, the c.-46C>T variant is in linkage disequilibrium with a yet unknown variant or an unknown susceptibility gene directly involved in the aetiology of BN. Alternatively, this variant itself entails an increased risk that may result from an alteration in the translation efficacy (Shintani et al., 1992). No data with regard to the potential functional consequences of this variant are available yet. Moreover, our results were not in line with Ribases et al. (2003), who reported an association of the Met66-allele

with AN in a Spanish sample. To address some of the problems of frequently observed inconsistencies, parts of our data on patients with AN or BN and controls were included in a recent meta-analysis pertaining to the polymorphisms Val66Met and c.-46C>T. The meta-analysis revealed an association of the Met66 variant with eating disorders as well as evidence for an association of the -270C (-46T) BDNF variant and age at onset of weight loss (Ribases et al., 2004). In conclusion, our results do not suggest a large impact of genetic variation in BDNF for the phenotypes AN, BN, ADHD, or obesity.

In our publication, we assumed that larger independent samples need to be assessed to exclude moderate genetic effects of the two investigated polymorphisms. Interestingly, Thorleiffson et al., (2008) identified *BDNF* (Val66Met) in a GWAS investigating more than 30,000 individuals as one of seven loci that associated with measures of obesity. Therefore, Val66Met might be involved via a moderate or small genetic effect in the aetiology of obesity.

Another line of evidence pertaining to the role of BDNF comes from studies showing that BDNF and its receptor TrkB (tyrosine kinase B) are downstream components in the MC4R-mediated control of energy balance. Xu et al. (2003) reported that mouse mutants which express decreased amounts of the BDNF receptor TrkB are characterized by hyperphagia and maturity-onset obesity similar to MC4R mutants. This suggests a role for TrkB in energy balance. Additionally, Tsao et al. (2008) showed that peripheral administration of neurotrophin-4 (NT4), a natural ligand of TrkB, suppresses body weight and appetite in several murine models of obesity. Two additional studies, however, suggest that BDNF and TrkB are more likely related to rare monogenic obesity than to polygenic obesity:

Gray et al. (2006) report an 8-year-old girl with hyperphagia and severe obesity, impaired cognitive function, and hyperactivity who harboured a *de novo* chromosomal inversion, 46,XX,inv(11)(p13p15.3), a region encompassing the *BDNF* gene. Haploinsufficiency for *BDNF* was associated with increased *ad libitum* food intake, severe early-onset obesity, hyperactivity, and cognitive impairment. Yeo et al. (2004) described an 8-year-old boy with a complex developmental syndrome and severe obesity who was heterozygous for a missense mutation resulting in a Tyr722Cys substitution in the neurotrophin receptor TrkB. This mutation markedly impaired receptor autophosphorylation and signalling to MAP kinase (mitogen-activated protein kinase). The associated impairment in memory, learning and nociception seen in the proband reflects the crucial role of TrkB in the human nervous system.

Altogether, BDNF and its receptor seemingly play a role in energy balance. Nicholson et al. (2007) showed in rats that activation of MC4R leads to an acute release of BDNF in the hypothalamus. This release could be a precondition for MC4R-induced effects on appetite

and body temperature etc., revealing that BDNF is an important downstream mediator of the MC4R pathway. In light of the importance of BDNF for neuronal development, it is unlikely that common genetic variation which results in impaired function would only affect body weight regulation. As demonstrated by Yeo et al. (2004) and Gray (2006), impaired function of the *BDNF* and its receptor TrkB led to both extreme obesity and serious impairments in memory, cognitive function and behaviour. From an evolutionary point of view, it is unlikely that such mutations or haplotypes will become frequent in populations. As a matter of fact, the authors themselves also assume that the observed mutations in *BDNF* and *NTRK2* (encoding TrkB) more likely result in syndromal, monogenic forms of human obesity.

4.2.2 Involvement of two single nucleotide polymorphisms (SNPs) of the insulin-responsive glucose transporter 4 gene (*GLUT4*) in individuals from different weight extremes (Friedel et al. 2002)

The insulin-sensitive glucose transporter GLUT4 is the most common glucose transporter in muscle and adipose tissue (Katz et al., 1995). As GLUT4 has been shown to be dysregulated in diabetes and obesity, it was expected that the knockout of *Glut4* would result in abnormal glucose homeostasis. In contrast, homozygous *Glut4* knockout (*Glut4*$^{-/-}$) mice exhibit nearly normal glycaemia, but postprandial hyperinsulinaemia. It was shown that *Glut4*$^{-/-}$ mice clear glucose as efficiently as controls, but are less sensitive to insulin action. Besides different malfunctions they also reveal growth retardation and severely reduced adipose tissue deposits. According to the observed phenotype, it can be assumed that the Glut4 protein is not required for maintaining normal glycaemia but is essential for growth, cellular glucose and fat metabolism (Katz et al., 1995). Altogether, this makes *GLUT4* a suitable candidate gene for obesity. Our study analyzed a possible association of body weight with two SNPs in *GLUT4* in a German Caucasian population. We investigated a total of 388 probands: 212 extremely obese children and adolescents, 94 underweight students, and 82 normal-weight students. We did not detect evidence for an association of any of the analyzed SNP alleles in the vicinity of *GLUT4* to different weight categories. Hence, there is no evidence suggesting that the analyzed polymorphisms are related to body weight regulation in our study groups. Besides common variation, our results do not exclude the potential body-weight related role of rare variants or mutations in the *GLUT4*.

We re-analyzed all SNPs located in *GLUT4* genomic region in our GWA case-control data set for which a more dense set of genotypes based on the Genome-Wide Human SNP Array 6.0 (Affymetrix) is also available (Hinney et al., 2007; *described above*). None of the 16 analyzed SNPs revealed a significant p-value or a trend towards significance using a nominal alpha of 5% as a significance level. As this study has moderate power to detect the expected

effect sizes for complex traits (see Hinney et al., 2007; supplementary material) the contribution of common genetic *GLUT4* variants to early onset extreme obesity seems unlikely. In light of today's knowledge, some points might explain the negative findings of our initial study:

i) The investigated variants are not functionally relevant and they are not representative for the *GLUT4* genomic region. According to Hapmap (data release 21), there is no LD between the investigated SNPs and moreover, with exception of a small block encompassing the first two exons, there are no haplotype blocks described in *GLUT4* genomic region. So, if there are functionally relevant mutations, the investigated variants might not have been appropriate to pick these up.

ii) Because of the small sample size, our study has sufficient statistical power to detect large genetic effects only. In light of the small effects now expected to be the rule for complex diseases like obesity, statistical power/sample size are of particular importance for the interpretation of "negative" results. Of note, the subsequent analyses of our larger GWA data sets confirmed our initial negative findings.

iii) According to its crucial role in insulin signalling (McCarthy et al., 2007) *GLUT4* might not be directly involved in the aetiology of obesity and its involvement in the aetiology of NIDDM is more likely. As our sample consists of extremely obese children and adolescents with no diagnosis of NIDDM, the detection of an association to NIDDM cannot be demonstrated. Given the many upcoming GWA studies that include BMI and NIDDM associated phenotypes and which are well powered even for moderate or small genetic effects, it should be possible to elucidate the role of *GLUT4* soon. Until today, there is no hint for an association of *GLUT4* and obesity or NIDDM in published studies.

iv) Two recent findings reveal that GLUT4 might be involved in body weight regulation via the TBC1D1 gene. The potential importance of TBC1D1 in linking insulin, exercise and energy status signalling with GLUT4 membrane traffic is heightened by the discovery of Stone et al., (2006) who reported variant Arg125Trp to be involved in severe female obesity. In contrast, a mutation in the same gene was recently shown to be protective for high fat diet (HFD) induced obesity. As Chadt et al. (2008) describe, a loss-of-function mutation occurring in the lean Swiss Jim Lambert-mice (SJL) leads to a truncated protein and, therefore, markedly reduced blood glucose levels as compared to carriers of a control allele. This result might point to Tbc1d1 as the missing link between Glut4 translocation and glucose uptake, further studies are warranted to show whether Glut4 translocation is affected by this mutation.

4.2.3. Investigation of Diacylglycerol O-acyltransferase homolog 2 gene (*DGAT2*) as a positional and functional candidate gene for early onset obesity (Friedel et al. 2007)

DGAT2 is a promising candidate gene for obesity because of its function as a key enzyme in triglyceride metabolism and because of its localization on chromosome 11q13, a linkage region for extreme early onset obesity detected in our sample (Saar et al., 2003). *Dgat2* knockout mice (*Dgat2*-/-) are lipopenic, their total carcass triglyceride content is reduced by 93% (Stone et al., 2004). In contrast to *Dgat1*-/- mice, where *Dgat2* is able to compensate the role of Dgat1 in triglyceride synthesis (Smith et al., 2000), *Dgat1* was unable to compensate for the absence of Dgat2 in *Dgat2*-/- mice. These results indicate that Dgat2 might be the major enzyme of triglyceride synthesis in mice (Stone et al., 2004). Based on both positional as well as on functional arguments, we hypothesized that genetic variations in *DGAT2* also alter triglyceride synthesizing activity of the protein in humans. Genetic variations leading to a gain of function of DGAT2 may thus be associated with obesity, whereas variations entailing a reduced function might result in underweight.

Accordingly, we performed a mutation screen in 93 extremely obese children and adolescents and 94 healthy underweight controls. Association studies were performed subsequently in samples of up to 361 extremely obese children and adolescents and 445 healthy underweight and normal weight controls. Additionally, we tested for linkage and association using nuclear families at four common variants in the 165 families of our initial genome scan. The mutation screen revealed 15 DNA variants, four of which code for non-synonymous exchanges: p.Val82Ala, p.Arg297Gln, p.Gly318Ser and p.Leu385Val; ten variants were synonymous. Additionally, the small biallelic trinucleotide repeat rs3841596 was identified. None of the case control and family-based association studies showed evidence for an association of investigated variants or their haplotypes in the genomic region of *DGAT2* to obesity. In conclusion, our results do not support the hypothesis of an important role of common genetic variation in *DGAT2* for the development of obesity in our sample.

Thus, in case of an influence of genetic variation in DGAT2 on body weight regulation, it might either be conferred by the less common variants (MAF < 0.1) or the detected, rare non-synonymous variants. In contrast, Choi et al. (2007) presented data demonstrating that suppression of hepatic Dgat2 in rats with antisense oligonucleotides decreased plasma triglycerides (TG) and protected against fat-induced insulin resistance. Additionally, treatment of *ob/ob* mice with the DGAT2 antisense oligonucleotide resulted in a significant decrease in weight gain, adipose weight and hepatic TG content (Liu et al., 2008). These findings indicate that the majority of TG destined for secretion by liver is synthesized by DGAT2 and

suggests that DGAT2 may be a therapeutic target for treatment of hypertriglyceridemia, hepatic steatosis and obesity.

Even though we observed no evidence relating common genetic variation within the *DGAT2* gene with obesity, the lessons from DGAT-deficient mice (Smith et al., 2000; Stone et al., 2004) and suppression experiments (Choi et al., 2007) still suggest that DGAT-inhibition may be a good strategy for the treatment of obesity. Both DGAT2 and its functional homologue DGAT1 would be excellent targets for small molecule inhibitors; Matsuda and Tomada (2007) refer to more than 30 selected inhibitors of fungal and plant origin. As an example, Lee et al (2006) report the inhibition of DGAT1 by alkamides isolated from the fruits of *Piper longum* and *Piper nigrum*; whereas Chung et al. (2005) reported first *in vitro* inhibition experiments with betulinic acid from *Alnus hirsuta*. Altogether, DGAT inhibitors of natural and synthetic origin have been identified, but their selectivity toward DGAT1 and DGAT2 remains to be clarified.

Nonetheless, enthusiasm for the potential benefits of DGAT inhibition must be tempered by the reality that newly identified therapeutic targets rarely enter the stages of clinical trials. As a first step towards a potential application in humans, studies on the degree and the additional consequences/ side effects of DGAT inhibition are required.

4.3 Investigation of the influence of functional relevant MC4R-variants on weight loss during a lifestyle intervention program (Reinehr et al., 2009)

Approximately two percent of extremely obese children and adolescents are carriers of functionally relevant mutations in the *MC4R* (Hinney et al. 1999). By now more than 80 functionally relevant missense mutations in the *MC4R* are known. Most of them lead to a partial or complete loss of function of the receptor (Ho et al. 1999; Vaisse et al. 2000; Farooqi et al. 2000, 2003; Kobayashi et al. 2002; Hinney et al. 2003) and therefore, to weight gain and obesity. The aim of this study was to investigate impact and extent of weight changes after lifestyle intervention in children with *MC4R* mutations. Additionally, weight changes after the lifestyle intervention program "Obeldicks" (Reinehr et al., 2007) between children with and without *MC4R* variations were compared in a two-year longitudinal study.

Among 514 obese German children and adolescents 18 (4%) carriers with 10 functionally relevant mutations were identified, 1 (0.2%) had a mutation with unknown relevance and 14 (2.7%) children carried variations not leading to reduced receptor function. The children with and without *MC4R* mutations did not significantly differ at baseline with respect to any anthropometrical marker, cardiovascular risk factor, or hormone profile. Both children with and without *MC4R* mutations reduced their degree of overweight at the end of the intervention and they did not differ in their overweight reduction at the end of the intervention. However, the maintenance of weight loss after intervention among children with *MC4R* mutations leading to reduced receptor function was less stable in comparison to children without such mutations. In contrast, Hainerova et al. (2007) reported on four *MC4R* mutation carriers who underwent a weight reduction program and were able to maintain their weight loss ten months after the program. In either case, studies with larger sample sizes are warranted to specifically test this hypothesis. In conclusion, children with *MC4R* mutations leading to reduced receptor function are able to lose weight in a lifestyle intervention but might have greater difficulties to maintain this weight loss potentially supporting the impact of these mutations on weight status.

With regard to other genetic variants, there is also no clear evidence to indicate if specific allelic variations may influence the outcome of weight loss programs. At present, three studies have addressed this question. Soerensen et al. (2006) investigated 42 SNPs in 26 candidate genes in 648 adults on a hypocaloric diet with high or low fat content for ten weeks in eight clinical centres in Europe and did not identify any genetic polymorphisms as predictors of weight loss. In a second study, Arkadianos et al. (2007) modified the diet of half

of the participants (43 of 86) of a weight management program according to their genetic profile (24 SNPs in 19 genes, nutrigenetic subgroup). In contrast to Soerensen et al. (2006) they detected improved weight management and weight loss after 300 days follow-up for the nutrigenetic group. However, as the sample size is very small, the outcome of this study needs to be discussed critically and requires independent validation. Reinehr et al. (2009) genotyped 293 obese children attending a weight reduction programme and found that CC-homozygotes at SNP rs7566605 in the vicinity of *INSIG2* lost less weight in a lifestyle intervention.

In principle, the identification of genetic variants associated with lower or higher success rates after intervention could enable clinicians to adapt interventions to specific patient groups and to study the impact of different genetic variants on weight loss management in children and adolescents. Altogether, the available evidence to support this idea is very sparse. Soerensen et al. (2006) and Arkadianos et al. (2007) investigated polygenic effects of 42 and 24 SNPs on weight loss and its maintenance in 648 and 43 individuals and reported contrary findings. Moreover, the functional relevance of most of the polymorphisms genotyped in both studies is unknown. As a consequence, this might have contributed to the negative findings of the first study. For future studies it is demanded that only polymorphisms with robust evidence for an involvement in body weight regulation (e.g. SNPs in or near *FTO, MC4R* and maybe *INSIG2*) should be investigated. Due to the small estimated effect sizes for polygenes, these studies should be conducted in sufficiently large samples.

In contrast to the studies cited above, Reinehr et al. (2009) and Hainerova et al. (2007) investigated *MC4R* which is also a well supported major gene for obesity. Because functionally relevant *MC4R*-mutations occur in only 2-5% of extremely obese children and adolescents it is difficult to sample larger numbers of mutation carriers. As a consequence, the sample sizes in both studies were small – the resulting power problem, however, might be in part compensated by the larger effect size of MC4R mutations. As both studies reported contrary results, larger samples and meta-analyses are needed to elucidate the effect of functionally relevant *MC4R* mutations on weight loss and its maintenance. As a primary hypothesis, such studies should follow-up the results of Reinehr et al. (2009), suggesting that carriers of functionally relevant *MC4R* mutations seem to lose body weight similar to non-carries but might have larger difficulties to maintain this weight loss.

Weight loss via dieting and physical activity is one of the widely used strategies recommended for obese individuals, e.g. carriers of functionally relevant *MC4R* mutations, but so far, little is known about the medium and long-term implications and side effects of an intentional weight loss. Although weight loss is known to improve metabolic and

cardiovascular risk factors, specific risks associated with acute and specifically rapid weight loss and its long-term implications have rarely been studied. The same is true for obese children for whom short-term success of weight loss interventions is well documented while data on long-term outcomes after intervention is lacking. One frequent adverse effect of intentional weight loss is a regain of a significant amount of weight. The repeated loss and regain of body weight is called “weight cycling”. The molecular mechanisms underlying weight regain following cycles of dietary deprivation and refeeding are still poorly understood (Kochan et al., 2006).

In adults, Soerensen et al. (2005) report results of an observational study where those subjects who intended to lose weight and actually lost weight over a 6 year period had an 86% increased risk of death over a 25 year follow up period compared to those who did not intend to loose weight or intended to loose weight but did not proceeded to indeed lose weight. Thus, data on the long-term effects of intentional weight loss especially in integrated programs clearly need to be collected and analysed.

In an attempt to summarize the available evidence, Simonsen et al. (2008) conducted a review comprising studies on intentional weight loss and mortality. Of the studies evaluated, two found decreased mortality with intentional weight loss, three found increased mortality, and four found no significant associations between intentional weight loss and total mortality. Thus, the long term effects of intentional weight loss remain unclear.

With regard to the implications of dietary weight loss, its regain and the impact of genetic factors on success in a lifestyle intervention program (as described by Reinehr et al. 2009), the long term consequences should be thoroughly assessed especially in the group of children and adolescents carrying functionally relevant *MC4R* mutations.

4.4 Perspectives of molecular genetic research in human obesity (Hebebrand et al., 2003)

Since the identification of leptin in 1994 (Zhang et al., 1994) obesity research is a fast growing research field. From the very beginning until today, research approaches and dogmas changed. Following the research era of monogenes detected in large pedigrees we now search for polygenes in huge samples of independent subjects. Discussions on how to define obesity, which phenotypes to investigate and how many individuals to ascertain are recurring themes of genetic research in human obesity. Hebebrand et al. (2003) critically reflected approaches to detect mutations and polymorphisms predisposing to the development of obesity and related phenotypes and provided outlines for the future. Topics considered were a) the impact of the investigated phenotype, b) the question whether to invest in quantity or quality (i.e. the more precise phenotyping, of the assessed individuals), c) differences in ascertainment schemes, d) potential benefits of conducting studies in different species as well as e) a discussion of the candidate gene approach in comparison to genome scans and f) finally issues of genotyping.

With regard to phenotypes, we pleaded in 2003 for investigating not only the undoubtedly relevant anthropometric and endocrinological variables but to include also behavioural and sensory phenotypes like eating behaviour or differences in the perception of taste.

It is commonly assumed that the better specific subgroups are delineated at the phenotypical level, the more homogeneous the underlying genetic factors will turn out to be. Precise phenotyping might be a prerequisite for identifying genes in the first place. On the other hand, not all conceivable phenotypes with a potential relevance in body weight regulation can be assessed in every individual who is willing to participate in a molecular genetic study. Both the endurance of the proband and the costs for phenotyping represent limiting factors. Thus, it might currently make most sense to first identify genes relevant in weight regulation in a very large phenotypically homogeneous but not too specifically characterized study group. Afterwards the effect of specific alleles should be tested in samples well characterized for specific behavioural, physiological and endocrinological phenotypes.

There are several recent publications that support this consideration: As an example, the first GWA study for NIDDM, conducted on 1924 cases and 2938 controls of a UK consortium (WTCCC, Frayling et al., 2007) revealed the currently most interesting candidate gene for obesity: *FTO*. The finding was replicated in 13 cohorts with 38,759 participants most of which stemmed from population based studies analysing BMI as a continuous trait. Since then, the association of first intron variants of *FTO* and BMI has been confirmed in many studies (e.g.

Sladek et al., 2007; Hinney et al., 2007; Kring et al., 2008; Qui et al., 2008). As the functional impact of *FTO* on body weight regulation is still unclear, the latest studies focus on more specialised phenotypes in smaller samples to figure out the possible function: Andreasen et al. (2008) showed that low physical activity might accentuate the effect of *FTO* rs9939609 on body fat accumulation, Tschritter et al. (2007) found that *FTO*-SNPs seem to be associated with cerebrocortical insulin resistance in humans. Wahlen et al. (2008) detected an association of rs9939609 to fat cell lipolysis. Klöting et al. (2008) revealed a potential inverse relationship between obesity and *FTO* gene expression in visceral adipose tissue in humans. The investigation of these more specialized phenotypes might help to narrow down the functional implications of *FTO* on obesity by stimulating ideas for functional *in vitro* and *in vivo* studies.

In 2003, we also pointed out the importance of expert cooperations to delineate the genetic mechanisms involved in body weight regulation of different species. Especially the findings of Ashrafi et al. (2003) who used genome-wide RNAi analysis of *Caenorhabditis (C.) elegans* to identify 305 and 112 gene leading to decreased and increased fat storage were promisinng. It was shown that pathways of energy homeostasis are highly conserved between human, worm (*C. elegans*), fruit fly (*Drosophila melanogaster*) and zebrafish (*Danio rerio*; Schlegel and Stainier, 2007). Because of the redundancy of the complex processes regulating the uptake, transport, catabolism and synthesis of nutrients, these species are useful to support exploration, identification and investigation of new pharmaceutical targets for metabolic diseases like obesity or NIDDM. During the last years, research in these organisms made a valuable contribution to basic research of metabolic processes (i.e. characterization of the central insulin/insulin-like growth factor signalling pathway *in Drosophila* and *C. elegans*), but it did not drive the identification of candidate genes for human obesity (Schlegel and Stainier, 2007).

For the candidate gene approach, we envisioned two strategies in 2003: first, to analyse linkage regions when the number of putative genes has been narrowed down; second, to investigate genes that are involved in relevant pathways, e.g. derived from animal models. Ideally, both approaches should be combined, because - as illustrated above - linkage studies cannot readily lead to the detection of minor gene effects or infrequent major gene effects. The candidate gene approach has led to the identification of the most monogenes for obesity, but when research switched to elucidate the role of oligo- and polygenes, it became less effective. This might also be due to the lack of good validation or confirmation studies for many of these genes. The current literature abounds in association studies claiming a new finding for which follow-up evidence frequently is either negative or equivocal (Rankinen et al., 2006). Many of the positive results must be viewed critically because multiple tests were

performed to achieve the respective 'significant' result. It is crucial that negative findings are also published; hence, every effort should be made to encourage researchers to publish their findings. Open access journals like BMC and PLoS that publish peer reviewed articles independent of the outcome if otherwise scientifically sound are a first step towards avoiding publication bias.

As suggested in 2003, high throughput technology now offers the chance to genotype thousands of samples every day. Genome-wide association studies became reality and the HapMap project contributed to databases that facilitated the selection of SNPs to tag haplotypes in order to improve the efficiency of association studies. As already foreseen five years ago, a substantial proportion of the work is currently shifting from lab to computer work. Therefore, enhancements in biostatistics and computational sciences are a still developing field in the genetics of complex diseases.

In light of the vast number of SNPs and haplotypes, the possibility of false-positive results due to multiple testing becomes an even greater challenge. Therefore, new methods to correct for genome-wide testing of markers have been proposed (e.g. Lange et al., 2004; Rakovski et al., 2008). The validation and confirmation of initial, positive findings in additional, independent samples becomes more and more a basic requirement for publication in peer reviewed journals. As a promising alternative to traditional designs, adaptive procedures that flexibly allow for design changes in order to achieve a stabilized power characteristic while controlling the overall type I error and using the information already collected are one option to address such challenges (e.g. Scherag et al., 2003; 2009*)*.

Now and then, we announced the commercialization of molecular genetic findings in obesity. And indeed, diagnostic tests based on specific polymorphisms or mutations became commercially available (e.g. deCODE, IntegraGen). Unfortunately, however, in some of these cases the consumer is misguided to believe that genotyping of a particular polymorphism will allow for the detection of the 'fat gene' indicating questionable business strategies of the provider. In our opinion, the consumer/patient should have access to molecular genetic testing after having been informed of potential implications. Clearly, only those tests should be made available that pertain to variants whose functional relevance has been established unequivocally and which occur with a frequency large enough to warrant the application of such tests. In our opinion, this is still only the case for *MC4R* mutations, which occur in up to 2–4% of extremely obese patients – *FTO* might be another candidate. An individual should obtain a clear grasp of the implications of such a finding for herself/himself, for other family members and potentially for future offspring. The individual

should also obtain a feeling of to what extent her/his obesity results from a specific mutation or polymorphism. Conducting a randomized clinical trial, Rief et al. (2007) assessed the positive and negative outcomes of informing obese individuals about the genetic aetiology of being overweight. In the case of participants with a family history of obesity the consultation which included genetic information resulted in long-term improvements of negative mood.

With regard to predictive genetic testing Cauchi et al. (2008) assessed the combined effect of SNPs identified in GWAs for NIDDM in a large French sample comparing NIDDM and normal glucose tolerant individuals. After adjustments for age, BMI and gender, subjects with at least 18 risk alleles (14.5% of French NIDDM subjects) had approximately 9-fold higher risk of developing NIDDM compared to the reference group. The estimated AUC (area under the curve) under the ROC (receiver operating characteristic) curve of a diagnostic test which also included genetic and non-genetic factors was 0.86.

Janssens et al. (2006) critically discussed such inferences with regard to public health implications and applications. They conclude that predictive genetic testing would be useful when the value it adds to existing interventions outweighs the additional personal and social costs. This requires a complete evaluation of the test's performance characteristics, including sensitivity and specificity; it's positive and negative predictive value in the population to be tested; the likelihood ratio of positive and negative test results; and the rates of false positive and false negative test results. These data are only part of the evidence base needed to recommend a test, which also includes information about effectiveness relative to existing alternatives, side effects, and costs. A risk ratio, odds ratio or population attributable risk alone which is currently most widely reported in genetic association studies cannot predict the potential usefulness for genetic testing. Ultimately, genetic discoveries may lead to better understanding of the disease process and to better therapeutic and preventive interventions. In the meantime, scientists and the media are responsible for accurately and carefully interpreting the implications of studies of genetic associations for the benefit of the general public.

5. Summary and Conclusion

The overall objective of this thesis was to study genetic mechanisms of body weight regulation. Two genome-wide approaches to identify chromosomal regions/candidate genes/genetic variants involved in body weight regulation were applied. Saar et al. (2003) presented the first genome-wide linkage scan for early onset obesity and detected suggestive evidence for linkage. Similar to other genome wide linkage scans for obesity, however, we did not identify a candidate gene for obesity. One reason might be that microsatellite-based linkage scans are not suitable for the detection of small genetic effects expected for obesity. In contrast, the first genome-wide association study for early onset obesity presented in Hinney et al. (2007) led to the re-identification of *FTO,* the currently best supported candidate gene for obesity. Thus, our investigation underlines two things: first, GWAs are in principle suitable to detect genes with small to modest genetic effect sizes, and second, with our relatively small but well defined sample of cases (early onset obese children and adolescents) and controls (adults with normal weight or underweight) it is possible to detect the same effects which required genotyping of several thousand population based unselected probands for body weight.

Moreover, this thesis comprised the examination of three candidate genes for obesity. Mutation screens and association studies investigating *BDNF* as a candidate gene for body weight regulation and activity (Friedel et al., 2005) revealed that there is no association between genetic variation in *BDNF* and obesity, eating disorders or ADHD. Similarly, in a study of SNPs in *GLUT4* in samples from different weight extremes (Friedel et al., 2002), we did not detect association with obesity. In light of today's knowledge, the study had some methodological problems; but we were able to support our negative conclusions by reassessing our GWA data set for the *GLUT4* genomic region. Finally, mutation screening and association studies for *DGAT2* as a positional and functional candidate gene for early onset obesity on chromosome 11q13 (Friedel et al., 2007) also revealed no association with obesity. Of note, this study led to the detection of four rare non-synonymous changes that might be relevant for underweight and warrant further research.

Performing a *MC4R*-mutation screen in 514 obese children and adolescents we detected 18 (3.5%) carriers of 10 functionally relevant mutations. These individuals underwent a weight reduction program and we observed that carriers of functionally relevant *MC4R* mutations are able to reduce their body weight, but that they seem to have difficulties to sustain this weight loss over time. Of course, this finding needs to be validated in larger study groups – and as long as the implications of dietary weight loss and weight regain as well as the long time consequences are unknown, it should be critically discussed if obese children and

adolescents, especially carriers of functionally relevant mutations, can benefit from dietary weight loss.

Common obesity is caused by a complex interplay of genetic background and environmental factors. While monogenic forms of obesity are well understood, GWAs now seem to offer the option to detect oligo- and polygenes. As these genes are typically characterized by small to modest genetic effect sizes but are more common they might be more important than monogenes with regard to clinical implications.

Personalized medicine with predictive genomic profiling to identify risk factors and to allow for personalized nutrition and to come up with lifestyle health recommendations is one application of genetic research. That this goal, based on today's knowledge, is still fantasy was recently shown by Janssens et al. (2008) who performed a meta-analysis of data from the literature on purchasable genetic tests. The authors show that there is insufficient scientific evidence to conclude that genomic profiles are useful in measuring genetic risk for common diseases or in developing personalized diet and lifestyle recommendations for disease prevention.

In sum, this work is part of a puzzle that might lead to evidence-based, personalized medicine which will be based on a solid scientific base by investigating the molecular genetic mechanisms of body weight regulation with regard to confirmed findings in independent large samples and by more carefully addressing methodological flaws.

Zusammenfassung und Schlussfolgerungen

Das übergeordnete Ziel dieser Arbeit war es, die genetischen Grundlagen der Körpergewichtsregulation zu untersuchen. Es wurden zwei unterschiedliche genomweite Ansätze zur Identifizierung von in die Gewichtsregulation involvierten chromosomalen Regionen/Kandidatengenen/Genvarianten angewendet. Mit Saar et al. (2003) wurde die erste genomweite Kopplungsanalyse für frühmanifeste Adipostas vorgestellt, in der wir „suggestive evidence“ für Kopplung detektieren konnten. Wie in vielen anderen Kopplungsstudien konnten wir kein validiertes Gen für Adipositas identifizieren. Grund hierfür könnte sein, dass Mikrosatelliten-basierte Kopplungsstudien nicht geeignet sind, die kleinen genetischen Effekte, die wir bei Adipositas erwarten, zu detektieren. Im Gegensatz dazu führte die erste genomweite Assoziationsstudie für frühmanifeste Adipositas (Hinney et al., 2007) zur Re-Identifizierung von *FTO,* dem derzeit am besten validierten Kandidatengen für Adipositas.

Damit bestätigen unsere Studien zwei Punkte: Erstens, GWAs sind prinzipiell geeignet, Gene mit kleinen bzw. moderaten Effekten zu detektieren und zweitens: es ist möglich, mit unserem relativ kleinem, aber gut definierten Kollektiv aus Fällen (Kinder und Jugendliche mit frühmanifester Adipositas) und Kontrollen (normal- und untergewichtige gesunde Erwachsene) die gleichen Effekte zu detektieren, für die in bevölkerungsbasierten (für Körpergewicht unselektierten) Ansätzen mehrere tausend Individuen genotypisiert werden müssen.

Weiterhin wurden in dieser Arbeit die Analysen dreier Kandidatengene für Adipositas zusammengefasst. Die Untersuchung des *BDNF* als Kandidatengen für Adipositas und Aktivität mittels Mutationsanalyse und nachfolgende Assoziationsstudien (Friedel et al., 2005) ergab keinen Hinweis auf die Assoziation genetischer Varianten im *BDNF* mit Adipositas, Essstörungen oder ADHS. Im Rahmen der Analyse von SNPs im *GLUT4*-Gen in Kollektiven unterschiedlicher Gewichtsklassifikation (Friedel et al., 2002) konnte ebenfalls keine Assoziation zu Adipositas detektiert werden. Im Lichte des heutigen Wissens hatte diese Studie einige methodische Probleme; allerdings konnten wir die Negativergebnisse durch die re-Analyse unserer GWA-Daten für die genomische Region des *GLUT4* bestätigen. Schlussendlich konnte mittels Mutationsanalyse und Assoziationsstudien am *DGAT2*-Gen, einem positionellen and funktionellen Kandidatengen für frühmanifeste Adipositas auf Chromosom 11q13 (Friedel et al., 2007) ebenfalls keine Assoziation zu Adipositas gezeigt werden. Allerdings wurden im Rahmen dieser Studie vier seltene nicht-synonyme Varianten identifiziert, die für Untergewicht relevant sein könnten und weitere Analysen erfordern.

Mittels Mutationsanalyse des MC4R-Gens bei 514 adipösen Kindern und Jugendlichen konnten wir 18 (3.5%) Träger von 10 funktionell relevanten Mutationen identifizieren. Die untersuchten Individuen nahmen an einem Gewichtsinterventionsprogramm teil und wir konnten feststellen, dass die Träger funktionell relevanter Varianten zwar in der Lage waren, Ihr Körpergewicht zu reduzieren, allerdings im Vergleich zu den anderen Teilnehmern größere Probleme hatten, ihren Gewichtsverlust längerfristig zu halten. Dieser Befund sollte vorerst in einer größeren Studiengruppe bestätigt werden. Solange die Auswirkungen von diätinduziertem Gewichtsverlust und erneuter Gewichtszunahme sowie deren Langzeitwirkungen unbekannt sind, sollte kritisch diskutiert werden, ob adipöse Kinder und Jugendliche, besonders die Träger funktionsrelevanter Mutationen, wirklich von diätinduziertem Gewichtsverlust profitieren.

Adipositas entsteht durch das komplexe Zusammenspiel der genetischen Ausstattung mit Umweltfaktoren. Während die monogenen Formen der Adipositas gut erforscht sind, bieten GWAs nun die Möglichkeit, oligo- und polygene Effekte zu identifizieren. Da diese Gene typischerweise durch kleine bis moderate genetische Effektstärken charakterisiert dafür aber wesentlich häufiger sind, kann man annehmen, dass sie im Hinblick auf die klinischen Implikationen wichtiger als die Monogene sind.

Personalisierte Medizin, prädiktives genetisches Profiling zur Identifizierung von Risikofaktoren und Empfehlungen für eine personalisierte Ernährung und einen gesunden Lebensstil ist eine Anwendungsmöglichkeit der Genforschung. Das diese Ziel auf der Basis heutigen Wissens noch immer Phantasie ist, zeigten Janssens et al., (2008). Sie führten eine Metaanalyse der Literatur zu käuflich erhältlichen Tests durch. Die Autoren konnten zeigen, das derzeit noch ungenügend wissenschaftliche Hinweise existieren, die genomisches Profiling als nützliches Werkzeug zur Bestimmung des genetischen Risikos allgemeiner Erkrankungen bzw. personalisierte Diäten und Hinweise zum Lebensstil zur Krankheitsprävention rechtfertigen würden.

Zusammenfassend lässt sich sagen, dass diese Arbeit Teil eines Puzzles ist, das vielleicht zu evidenzbasierte, personalisierter Medizin und Ernährung führt, die auf einer soliden wissenschaftlichen Basis aus der Analyse der genetischen Mechanismen der Adipositas unter Berücksichtigung bestätigter Ergebnisse in unabhängigen großen Studiengruppen und dem sorgfältigen Umgang mit methodischen Schwierigkeiten ruht.

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Essen, den

Susann Friedel

8. Wissenschaftliche Publikationen und Vorträge

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