Genetic Analysis of Extreme Obesity

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Declaration of originality

I hereby declare that this work is a product of my own and all else is appropriately referenced. Work derived through collaboration and assistance has been acknowledged in the text, while a list of references is given in the bibliography.

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Abstract

Obesity is an increasing health problem worldwide as a result of the changing environment, with calorie-dense food and a sedentary lifestyle. However, numerous twin studies indicate that genetics plays a significant role in determining which individuals become obese or stay lean while sharing the same 'obesogenic' environment. Genetic research in obesity has two major goals: the first being to elucidate the pathophysiological basis of obesity, and the second is to provide an evidential foundation for personalised medicine.

An obesity cohort was established, consisting of over a thousand severely-obese individuals (mean BMI 48.1 kg/m^2 [± 8.67 SD]) undergoing bariatric surgery, as the basis for genetic and phenotypic analysis of the severely obese. The relatively high proportion of metabolically-healthy, but severely-obese individuals in this cohort illustrated some of the many unknown pathophysiological mechanisms, while a consistent increase of public distress among the more severely obese confirmed the ongoing stigmatisation of the obese in UK society.

Screening for the most common form of Mendelian obesity, MC4R deficiency identified a lower than anticipated prevalence (0.77%) in the bariatric cohort, but analysis of treatment outcomes indicated that bariatric surgery both (RYGB and VSG) is effective for the individuals affected. Lifestyle intervention for children with MC4R deficiency, on the other hand, appeared to have less beneficial outcomes long term.

Using whole exome sequencing on 40 super-obese bariatric participants, a higher than anticipated prevalence of putative Mendelian obesity (20.5%) was found, including several novel disruptive variants in known obesity genes. Finally, a novel Mendelian obesity and diabetes syndrome was detected, in a consanguineous family with a complex obesity phenotype, caused by a homozygous truncating mutation of the *CPE* gene (c.76_98del; p.Glu26ArgfsX68).

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CHAPTER 1

INTRODUCTION

1.1 Overview

This thesis describes the collection and phenotypic characterisation of a large cohort of obese patients, followed by molecular genetic analysis aimed, firstly, at determining the prevalence of monogenic forms of obesity and, secondly, the implications of such strong genetic effects on the outcomes of treatment.

Genetic studies have contributed immensely towards the understanding of the physiology of obesity, including elucidating appetite and other regulatory pathways. This introduction outlines how through investigation of monogenic murine models of obesity, leading to the discovery of the first human obesity genes, some major advances in obesity research were achieved. The currently active areas of genetic research in obesity will be discussed, followed by some of the practical implications of this new knowledge for clinical practice.

Genetic investigation of obesity has two major goals. First, it is important to reach a better understanding of the pathophysiological basis of obesity and related comorbidities, including type 2 diabetes mellitus (T2DM) and cardiovascular disease. Secondly, translation of such knowledge provides an opportunity to introduce personalised medicine for obese patients: by enabling proper diagnosis of the causal disease underlying obesity, optimisation of treatment can be realised.

1.1.1 Obesity as a worldwide epidemic

There has been a recent dramatic rise in the prevalence of obesity, and if current trends continue, by 2025, almost one fifth of the world population will be classified as obese, with a body mass index (BMI) >30 kg/m^2 [1]. This rising prevalence in obesity and consequent increases in T2DM, cardiovascular and respiratory disease, osteoarthritis, hypertension and certain types of cancers, represents a major threat to public health [2].

The United Kingdom currently has one of the highest prevalences of obesity (BMI >30 kg/m^2) within Europe, with around a quarter of adults in 2014 being classified as obese, while 2% of males and 4% of females were classified as morbidly obese (BMI >40kg/m^2) [3]. The general population can be categorised into five groups according to BMI: underweight (BMI < 18.5 kg/m^2), normal weight (BMI: 18.5 - 24.9 kg/m^2), overweight (BMI: 25 - 30 kg/m^2), class I obesity (BMI: 30.0-34.9 kg/m^2), class II obesity (BMI: 35.0 - 39.9 kg/m^2) and class III obesity (BMI ≥40 kg/m^2) [2]. With a special interest in this thesis in the extremes of obesity, two additional classes will be used: class IV obesity (BMI: 50-59.9 kg/m^2) and class V obesity (BMI ≥60 kg/m^2), from here onwards referred to as super obese and extreme obese, respectively. Although changes in prevalence in these extreme obesity classes are less well-studied, the data that has been collected indicates that the rise in prevalence is possibly highest among these groups [4,5].

The recent dramatic rise in the prevalence of obesity is due to environmental factors, such as an increase in easy accessibility to high-energy-dense food and a reduction in physical activity requirements in daily life; often referred to by the umbrella terminology 'obesogenic' environment. It is remarkable, however, that even though Westernised people all share this same obesogenic environment, not everybody is obese. In fact, body weight remains relatively constant over long periods of time both for individuals in the normal weight range, as well as for obese people. The variation of body weight between people sharing the same environment is strongly influenced by genetic factors. Twin studies indicate that the heritability of obesity lies between 31% and 90% [6], with many genes playing a significant role having already been identified. A large proportion of the heritability is, however, still unexplained, making this such an important area of research.

1.2 Genetics and obesity

1.2.1 Twin studies

Among the general population, and unfortunately also still among some professionals, obesity is still often considered as a consequence of personal choice to eat too much and not exercise enough, even though twin studies have been showing for decades that the situation is not that simple [7].

Twin studies are used to disentangle the relative contributions of genes and environment to human traits, by comparing the specific trait concordance rate in monozygotic twins with that in dizygotic twins. Other approaches to estimating the heritability of a trait are family segregation studies (where familial risk decays with degree of kinship), or adoption studies (either where twins are reared apart, or where adopted children's phenotype is compared with their birth families, versus their adopted families). One pioneering study [8], performed in the early 1990's, analysed a Swedish twin registry consisting of 93 pairs of identical twins reared apart, 154 pairs of identical twins reared together, 218 pairs of fraternal twins reared apart, and 208 pairs of fraternal twins reared together. The results showed that there was almost no difference in BMI between the monozygotic twins, no matter whether they were reared apart or together. In another study published in the same year, monozygotic twins were overfed with a total excess amount of 84,000 kcal over 100 days. During overfeeding, individual changes in body composition varied considerably, with about three times more variance among twin pairs than within twin pairs [9]. Numerous other twin studies followed, providing strong evidence that the regulation of body weight is highly heritable with heritability estimates of BMI between 31% and 90% (median of 73%) [6]. Noteworthy, is a recently published meta-analysis by Polderman, *et al.* of virtually all twin studies published between 1950 and 2012, on a wide range of traits and including a total of almost 14.5 million twin pairs [10]. For the trait 'weight maintenance' 223 studies were included with a total of 134,867 twin pairs, giving an overall heritability of 72.6%, probably giving the best estimate of heritability of weight in a variety of environmental circumstances.

Although some of the twin studies did show an interaction between environmental factors and genetic heritability (mainly age and time period over which the study was performed), the high heritability estimates of the numerous twin studies consistently support the importance of genetic factors [6,10].

1.2.2 From mouse models to the first human obesity genes

Leptin and the leptin receptor

The identification of the first human genes underlying monogenic obesity relied heavily on study of mouse models, and pioneered the understanding of the physiology of weight and glucose metabolism. The *obese (ob)* and *diabetic (db)* mice were first described over 50 years ago by the Jackson laboratory, with both models developing early-onset morbid obesity, hyperphagia, insulin resistance and hyperglycaemia [11,12]. Cloning and sequencing studies of these mouse models led to the identification of leptin in the *ob/ob* mouse, and the leptin receptor in the *db/db* mouse [13,14]. Leptin is produced primarily by adipocytes and plays an important role in the energy homeostasis, by acting as a signalling hormone regulating feeding behaviour and energy expenditure [15]. After the discovery of leptin, the first human cases of leptin deficiency were found by sequencing the *LEP* gene in two severely-obese cousins from a consanguineous family of Pakistani origin. The patients' serum leptin levels were very low despite their severe obesity, and a homozygous frameshift mutation in the *LEP* gene was detected in each patient [16]. Identification of additional humans with disrupting variants in the *LEP* gene showed that leptin deficiency causes a clinical phenotype of hyperphagia, severe early-onset obesity, hypogonadism, and impaired immunity [17,18]. Daily injections of recombinant human leptin reversed these symptoms and led to sustained, beneficial effects on appetite, fat mass, and hyperinsulinaemia [17].

Similarly, after the identification of a mutation in the leptin receptor in the *db/db* mouse, the first case of human leptin receptor deficiency was detected in a morbidly obese proband with a consanguineous family [19]. Subsequent cases indicated, that in addition to obesity, leptin receptor deficiency also

causes hyperphagia, alterations in immune function and delayed pubertal development, although features were less severe than seen in leptin-deficient patients [20].

Low leptin serum levels can be used to detect leptin deficiency, however, if normal leptin levels are detected it does not exclude the presence of deleterious *LEP* variation: in two recent cases of severely obese children with hyperphagia and normal circulating levels of leptin, point mutations were found in the *LEP* gene leading to an inactive hormone and therefore, while the mutant form of leptin was still expressed, it could not bind to, or activate, the leptin receptor [21,22].

Tubby, Yellow and fat mice

In addition to the obese (*ob*) and diabetes (*db*) mouse models, another three classic 'monogenic murine models of obesity' with naturally occurring mutations, contributed extensively towards the understanding of the underlying mechanisms of obesity: the yellow (e.g., A^{vy}), fat (*fat*) and tubby (*tub*) mouse models [23].

The 'yellow' or A^{vy} mice take their name from the yellow coat colour seen in this strain of obese mice, in which a dominant, constitutive ectopic *Agouti* transcription causes obesity and insulin resistance [24]. Agouti peptide is a high-affinity antagonist of the melanocyte-stimulating hormone receptor (MC1R) found in the skin, explaining the effect on mouse coat colour. The agouti peptide is also an antagonist of the hypothalamic melanocortin-4 receptor (MC4R), thereby blocking the melanocyte-stimulating hormone (MSH) derived from arcuate nucleus proopiomelanocortin (POMC) neurons [25].

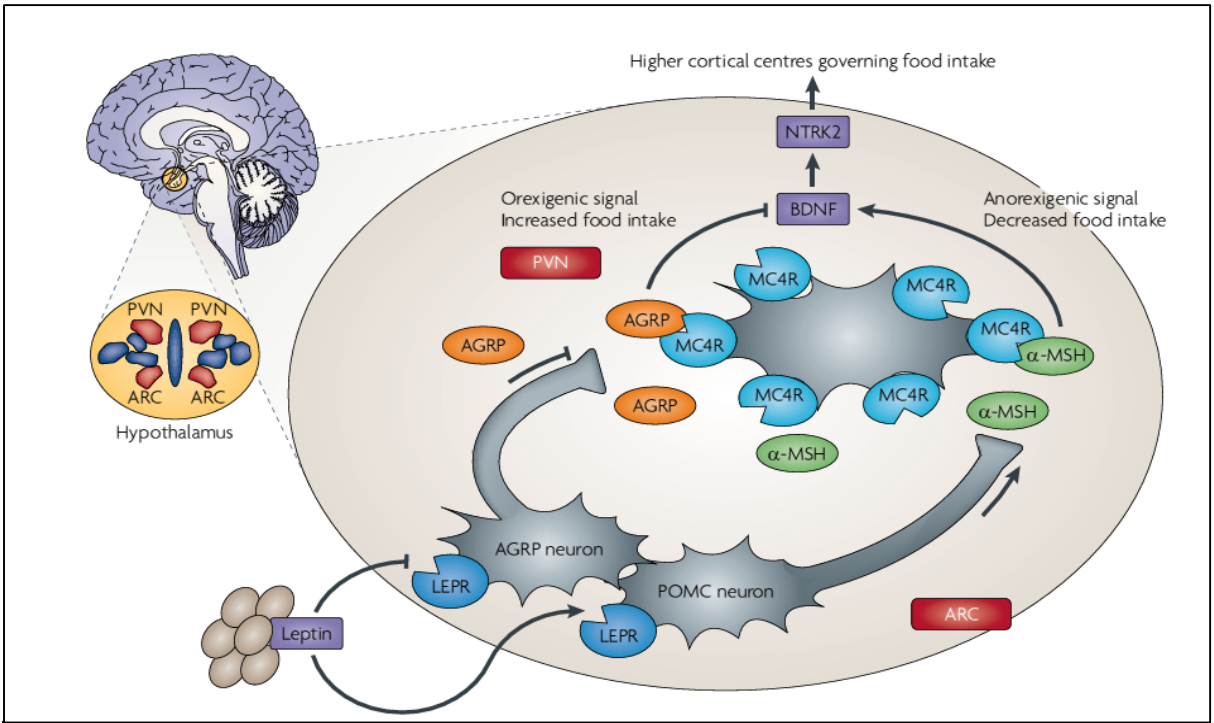


Figure 1.1: Leptin-melanocortin pathway. This figure gives an overview of the appetite regulation pathway in which the hypothalamus plays a significant central role. In the normal human physiological situation leptin binds to leptin receptors located on the agouti-related protein (AGRP)-producing neurons and POMC-producing neurons, in the arcuate nucleus of the hypothalamus. On activation of the leptin receptor, AGRP production is inhibited while the production of POMC is stimulated. POMC is cleaved into a range of peptides including adrenocorticotrophin (ACTH) and melanocyte stimulating hormones (α, β, and γ-MSH). AGRP and MSH compete to bind to the melanocortin 4 receptor (MC4R). MC4R will produce an orexigenic signal after being deactivated by binding AGRP or produce an anorexigenic signal after being activated by binding MSH (source: Walley, *et al*. [36]).

The first recessive mutations in POMC were detected in a compound heterozygous state in an individual with severe obesity, ACTH deficiency and read hair, explained by the disruption of the dual role of MSH in regulating food intake and influencing hair pigmentation [26]. Further cases of POMC dysfunction showed that the hypopigmentation is not always present in non-Caucasians, and that the actual position of the variants found are important, identifying the importance of β-MSH in human physiology (a form of MSH not present in murine models) [26-28].

The *fat* mouse elicits a more slowly developing obesity and hyperinsulinaemia, caused by an autosomal recessive mutation in the pro-hormone processing peptide, CPE [29]. Carboxypeptidase E (CPE), is involved in the biosynthesis of many neuro-peptides, including POMC, pro-insulin, and many other anorexigenic and orexigenic peptides [30]. Up until now, no disrupting variants were reported in humans in *CPE*. However, as a part of this thesis the first case of CPE deficiency was detected in a severely obese individual with hypogonadism, T2DM and intellectual disability (chapter 8 and reported in PLOS ONE [31]).

Tubby mice develop milder obesity and hyperinsulinaemia than the other obesity syndrome models. The mutated gene responsible for the tubby obesity phenotype was been identified as *TUB* [32]. Tub is a substrate of insulin receptor tyrosine kinase and leptin receptor-Janus kinase 2 (JAK2) in hypothalamic nuclei. Inhibition of Tub expression in the hypothalamus in mice led to increased food intake, fasting blood glucose, and blunted the effect of insulin or leptin on POMC processing [33]. One case of homozygous loss of *TUB* in humans has been reported so far: A homozygous truncating variant was detected in *TUB* in a boy with obesity and deteriorating vision caused by retinal dystrophy [34].

MC4R

In addition to the natural occurring mutations, *ob*, *db*, A^{vy} and *fat*, targeted disruptions of specific genes have also played a pivotal role in elucidating the leptin-melanocortin pathway.

The *Mc4r* mouse model was developed shortly after the discovery of leptin [35]. Melanocortin 4 receptor (MC4R) encodes a G-coupled receptor which is highly expressed in the hypothalamus, and produces an orexigenic effect after being deactivated by binding AGRP or produce an anorexigenic effect after being activated by binding MSH [36]. Heterozygous knockout of *Mc4r* in mice results in intermediate phenotype to that seen in wild-type and homozygous knockout littermates [35].

Within a year of the development of the mouse model, the first two cases of MC4R deficiency in humans was reported: two independent studies published in the same issue of Nature Genetics

reported heterozygous frameshift mutations in a severely obese child [37] and an adult with childhood onset of obesity respectively [38], causing dominantly inherited obesity.

Since the initial two reports, numerous cases of MC4R deficiency have been reported, with homozygous mutation carriers having a more extreme phenotype than the heterozygous mutation carriers, reflecting the situation in mouse models [39]. MC4R deficiency cause an autosomal dominant form of obesity, with clinical features of hyperphagia, severe early-onset obesity, increased lean mass, increased height and severe hyperinsulinemia in children [39]. Variants of *MC4R* leading to reduced receptor functioning are found in 0.5%-2.5% of obese adults [40-46], and up to 6% in severe early-onset obesity cases [39,42,45,47-50], making it the most common form of Mendelian obesity known to date. Given the high frequency of MC4R deficiency and its typical phenotype, assessment of *MC4R* variants is increasingly seen as an important diagnostic investigation to be included in the standardised clinical evaluation of severely-obese children.

Other Mendelian forms of obesity involved in the leptin-melanocortin pathway

The *PCSK1* gene encodes the prohormone convertase (PC1/3), which takes care of the cleavage of POMC into separate hormones. PC1/3 acts proximal to CPE in processing of prohormones and neuropeptides, and a similar phenotype as the CPE deletion case reported in this thesis and the *fat* mouse phenotype is seen in patients with molecular defects in *PCSK1* [51-53].

SIM1 (single-minded homologue 1) is essential for proper development of the paraventricular (PVN) nuclei of the hypothalamus, where MC4R signalling takes place. *Sim1* haploinsufficient mice have obesity, and haploinsufficiency of *SIM1* in humans causes dominantly-inherited obesity [54-56].

The neurotrophin, brain-derived neurotrophic factor (BDNF) inhibits food intake through activation of its receptor Trkb (encoded by the *NTRK2* gene), and murine models of with knock out of BDNF exhibit obesity with increased food intake, as well as hyperactivity. Disruptive variants both in *BDNF* and *NTRK2* have shown to cause a dominant form of obesity in humans with cognitive impairment [57-60].

1.2.3 Copy number variants and obesity

Copy number variants (CNVs), a form of structural variation including deletions or duplications in the genome, can also directly cause obesity: one large CNV of 593kb located at chromosome 16p11.2 (at 29.5–30.1 Mb), was initially discovered in obese children with developmental delay, but replication in population cohorts indicated this deletion is a significant contributor to common obesity, with 0.7% of the morbidly obese individuals carrying this deletion, while it was absent among the non-obese adults [61]. Compared to early-onset obesity seen in the Mendelian forms of obesity described above, the 16p11.2 deletion leads to a more strongly-expressed obesity phenotype in adults, while a more variable phenotype is seen in childhood [61]. Interestingly duplications of the same region have an opposite effect, and are associated with underweight instead of obesity [62].

Within the same chromosomal region of 16p11.2 another CNV of 220 kb was detected causing obesity (distal from the 593kb deletion described above, located at 28.73–28.95 Mb)[63]. This deletion was found to cause severe childhood onset of obesity, while the impact on obesity status in adult carriers appears less pronounced (although an increase in BMI can still be found) [64].

1.2.4 Syndromic obesity

In addition to the monogenic forms of obesity described above, with obesity as its main phenotype, a number of different obesity syndromes have been identified. In these disorders, obesity itself is not the predominant presenting feature, but patients are mostly referred for genetic investigation because of other clinical signs, such as developmental delay, dysmorphic features, and/or other developmental abnormalities [65]. Prader-Willi syndrome (PWS) is the most common syndromic cause of obesity, associated with mental retardation. It is an imprinted autosomal dominant disorder with typical characteristics including hyperphagia leading to severe obesity, intellectual deficiency, a range of dysmorphic features and hypogonadism. PWS results from loss of activity of the paternal copy of a region of chromosome 15, either by paternal deletions of the 15q11-13 region, by the presence of

two maternal homologues (uniparental disomy) or due to a microdeletion of the imprinting centre [66]. Several genes have been found not to be expressed in PWS patients, including the *SNRPN* gene and the HBII-85 snoRNA family. However, further investigation is still needed to fully understand how these genes affect feeding behaviour [67,68].

Albright hereditary osteodystrophy (AHO) is another imprinted autosomal dominant disorder, resulting from germline variants in *GNAS1* that decrease expression or function of Gsα protein. AHO is characterised by short stature, obesity, skeletal defects and impaired olfaction, but is not known to result in intellectual deficiency. This disease is caused by heterozygous mutations of *GNAS1*, but obesity only develops when the variant is inherited maternally [69]. Bardet Biedl Syndrome (BBS) is a heterogeneous genetic disease characterized by postaxial polydactyly, obesity, developmental delay, retinal dystrophy, anosmia and genital and renal abnormalities[70]. To date, more than 18 genes have been identified to be associated with BBS. So far, the cilium has been held responsible for the bulk of the pathology, but the precise mechanisms and pathways involved are only just being revealed [71,72].

1.2.5 Contribution of Mendelian obesity to the 'common' obese population

The discovery of monogenic, monolocus and syndromic forms of obesity has made a tremendous contribution to the understanding of the physiology underlying appetite and feeding behaviour, and made genetic counselling, and in rare cases treatment of affected individuals, possible. However, since, most of the Mendelian obesity cases have private mutations and were mostly only screened for in individual families, the contribution of Mendelian disease to the 'common' obese population is hard to estimate.

One exception to this is MC4R deficiency. With the *MC4R* gene consisting of only one exon covering a 999bp coding region (Uniprot: P32245), it is feasible to investigate for variants by PCR and Sanger

sequencing. It is, therefore, unsurprising that a number of researchers have screened their cohorts (both small and large) for variants, with almost every nonsynonymous variant found to date also having been analysed for pathogenicity through *in silico* analysis or direct assessment of localisation and function [50]. Therefore, good estimates of the prevalence of true MC4R deficiency in the obese population do exist. However, so far screening for *MC4R* variants in large un-selected population cohorts has not been performed. Examining the prevalence of known pathogenic *MC4R* variants in open databases such as NHLBI GO Exome Sequencing Project (ESP, including sequencing data of 6503 unrelated individuals), gives a prevalence of 0.5% for damaging *MC4R* variants. Although the range of BMIs included in this population cohort cannot be freely obtained, this cohort is generally considered as a valid representation of the European-American population, indicating that MC4R deficiency may be one of the most common genetic disorders within the European-American population, and is even more prevalent than more familiar heritable diseases such as cystic fibrosis [73].

Another example of a significant Mendelian contributor to common obesity, is the 593 kb deletion at chromosome 16p11.2. Originally detected in extremely obese children with intellectual disability, screening for this deletion in a larger cohort of over 16,000 individuals, indicated that the deletion also accounted for 0.7% of the obesity cases in the 'common' obese population, without any indication that cognitive deficits were present. This is an excellent example of the identification of a novel genetic cause of disease in patients with extreme phenotypes, followed by further exploration highlighting contribution of the same genetic defect to the 'common' form of the disease in the wider population.

1.2.6 Common variants-common disease hypothesis

Although the real contribution of Mendelian forms of obesity to the common obese population is currently unknown, it has generally been considered to be too rare to count for a significant proportion.

In all of the cases discussed above, gene discovery has included the investigation of severely-affected probands and their families. Other researchers have preferred to adopt a population based approach in order to identify novel genes and loci involved in the pathway to common obesity, including numerous genome-wide association studies (GWAS). These studies are based on the hypothesis that the high heritability of common diseases is caused by the combination of a number of common, mildly deleterious genetic variants (minor allele frequencies >5%) rather than highly penetrant individual mutations as seen in Mendelian disease. GWAS are based on a hypothesis free approach, in which frequencies at each variant included are tested for an association with the phenotype of interest. Even though associations can be strong, this does not indicate the variants are causal themselves, but are most likely in linkage disequilibrium with the causal defect [74].

The strongest and most well-replicated BMI-associated variants detected by GWAS to date can be found in the *FTO* (fat mass and obesity associated [75-78]) and the *MC4R* regions [76,77,79]. Numerous other loci have been identified: a recent meta-analysis of nearly 340,000 individuals (carried out by the GIANT consortium) identified a total of 97 genome-wide significant loci (meaning a p-value < $0.5x10^{\wedge-8}$) associated with BMI [80]. However, all of these obesity-related variants are characterised by very modest effect sizes, and in aggregate these 97 loci still only account for ~2.7% of the heritability of BMI [80]. Although genome-wide estimates suggest that common variation accounts for ~20% of BMI variation overall, these loci only have very limited clinical predictive value: a 3.3 kg/m^2 difference in mean BMI was seen between the people carrying the highest number of BMI-increasing alleles compared to the individuals with the smallest number of BMI-increasing alleles [80].

1.2.7 Missing heritability

The known common variants associated with BMI and obesity only explain a minor part of the heritability of BMI identified by twin studies, so it is clear that the heritability of obesity is not accounted for by common genetic variants [81]. Different hypotheses have been advanced to explain

this ‘missing heritability’ of human obesity, including unaccounted effects of CNVs and/or epigenetic events. Another possibility is that the missing heritability may reflect the presence of rare genetic variants missed by prior GWAS and linkage approaches, but possibly detectable using next generation sequencing technologies (NGS). Additionally, as most genes function in complex networks, gene-gene interactions (epistasis) as well as gene-environment interactions should also be taken into consideration.

As described by R.A. Fisher, any statistical deviation from the additive combination of two loci in their effects on a phenotype can be described as epistasis [82]. As one gene may mask the effect of another gene, or several genes may only work together, the effects of one single gene on heritability cannot not be identified without knowing the effects of the other genes [81]. Therefore, the overall effect of all variants identified so far may be of much greater or smaller significance than their summed individual effects.

Finally, with environmental factors playing such an important role in the recent obesity epidemic, specific environmental factors interacting with obesity-predisposing genes should also be considered (e.g. physical activity, diet, educational status, age, sex) [83]. Gene-environment interactions have been well studied using common variation. Interestingly, even some Mendelian forms of obesity result in a variable phenotype, which could be explained by the underlying genetic heterogeneity and gene–gene interactions, but interactions with environmental factors may also very well contribute. Investigation of the potential effects of gene-gene and gene-environmental interactions of variants will require analysis of very large-scale cohorts with careful geno- and phenotyping [84].

1.3 NGS and the discovery of new genes

The development of methods for coupling targeted capture and massively parallel DNA sequencing, commonly referred to as next generation sequencing or new generation sequencing (NGS) has led to novel opportunities for an unbiased approach of searching for disease associated variants [85]. Within this introduction-chapter, I will mainly focus on whole exome sequencing (WES; the targeted sequencing of the subset of the human genome that is protein coding or known to be regulatory), since this is the method applied within two of the chapters included in this thesis (chapter 7 and 8).

Before NGS technology became available, Sanger sequencing, considered the 'first generation sequencing' method dominated genetic research and led to numerous accomplishments, including the completion of the Human Genome Project in 2001 [86]. However, traditional Sanger sequencing approaches are limited in scalability. The major advance of NGS is the ability to produce an enormous dataset cheaply and rapidly, which has enabled cost-effective identification of nearly all of the coding sequence variation present in an individual human genome. Whole genome sequencing, however, produces very large datasets (posing considerable computational challenges) the vast majority of which are currently uninterpretable. For this reason, many researchers prefer to undertake WES, interrogating only the 1-3% of the genome known to be coding or have regulatory function (Figure 1.2). After the first commercial NGS platform was brought onto the market in 2005, the first successful application of WES to identify the causal genetic defect in Mendelian disease was reported in 2010: Ng, *et al*. [87], reported the identification of compound heterozygous disruptive variants in *DHOHD* as causative of Miller syndrome (a rare multiple malformation disorder, OMIM: #263750). Exome sequencing typically generates 20-50,000 variants per individual (depending on the exact version of reagents used), so it is necessary to define a filtering strategy to determine which are of clinical interest, and putatively causative of the disease under investigation. In the case of Miller syndrome, variants found through WES were filtered against public SNP databases to detect genes with two

previously unknown variants in each of the four affected individuals. This resulted in a single candidate gene, which was confirmed as causative by the detection (by subsequent Sanger sequencing) of disruptive *DHODH* variants in three additional families with Miller syndrome.

This initial study indicated that WES is a powerful tool to identify the genetic basis of rare monogenic disorders, and numerous discoveries have been made since, indicating the success-rate of WES application in this research area.

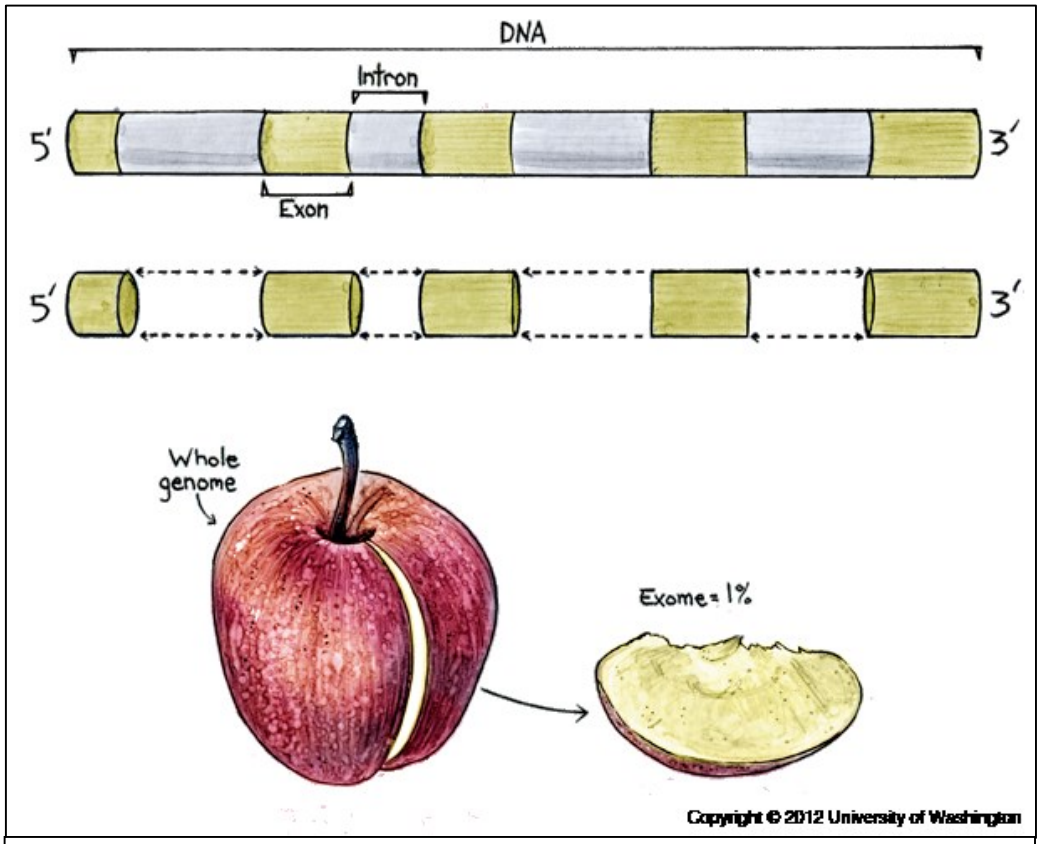


Figure 1.2: "The exome apple". The exome makes up about 1% of the genome, and consists out of exonic regions, which are the sequences that remain after introns have been removed when being transcribed into RNA.

1.3.1 Different approaches to WES

As mentioned above, the challenge of identifying the causal genetic defect in a WES dataset lies in identifying the specific clinically-relevant variant(s) among the 20,000 to 50,000 variants normally found per individual (or approximately 3 million variants in whole-genome sequencing studies). Since each individual carries, on average, 200 disrupting variants leading to an average of 20 complete knockouts of a protein, it is important to identify the correct variant causing the phenotype investigated, which for this reason cannot simply be based on finding 'a variant that disrupts a gene'. A careful study design, starting with identification of which individuals to sequence, and variant prioritisation are, therefore, crucial for a successful outcome, as well as an appropriate biological interpretation of the findings.

When looking for rare, high penetrant variants causing disease, an initial selection can be based on minor allele frequencies (MAF) of variants in populations cohorts and the functional implications of the variant: often variants with a MAF >1% within large open-access database representing the general population (such as the 1000 Genomes project (1000G), the NHLBI Exome Sequencing Project (NHLBI ESP) and Exome Aggregation Consortium (ExAC) [207-209]) are excluded, as well as variants not leading to a change in amino-acid (synonymous variant).

Different approaches can be used to detect the causal genetic defects using WES depending on the individuals investigated:

Family based approach

If the individuals investigated consist of a family with a Mendelian distribution of the targeted phenotype, detailed family history will indicate what approach to use. If a dominant inheritance pattern is seen, with multiple members affected, a *linkage-based approach* can be used: multiple affected family members can be sequenced, and a variant can be sought that is carried by all (with the

option to include non-affected members to enlarge the number of benign variants to exclude). If possible, it is best to include the most distantly-related affected individuals, to minimise the number of shared rare variants.

A *homozygosity based approach* can be used when there is a history of consanguinity in the family and a recessive pattern of inheritance is suspected. To identify the causal variant, all homozygous variants can then be prioritised. Although sequencing of only the affected proband might possibly lead to the identification of the causal variant, a high rate of consanguinity in different generations of the family (and the consequent high rate of homozygous variants) could require more affected/unaffected family members to be included. (A homozygosity-based approach was used to identify the causal variant in a consanguineous family segregating obesity with intellectual disability, as described in chapter 8 of this thesis).

When there is no history of consanguinity in the family and only the proband is affected while both parents are not, a *de novo based approach* can be applied. Here both parents as well as the proband are sequenced, and novel variants are sought within the proband. This approach is limited, however, since every individual carries around 5 novel variants not present in either parent. If none of the novel variants found are likely to explain the phenotype, variants should be screened for compound heterozygote variants (each parent carry a single variant causing recessive disease in the proband).

Unrelated individuals

With multiple unrelated individuals with a similar phenotype, different causal variants in the same gene (or genes active within one pathway) can be sought, using *parallel sequencing*. It is, however, important using this strategy that phenotypes are very well matched and are rare in the common population. When looking at more common diseases, such as obesity, a selection of individuals with an extreme form of the phenotype can be made. In investigation of common disease, however, variants present in the common population cannot as easily be excluded as when looking at rare diseases. A difference in frequency of such variants must, therefore, be sought between the extremely

affected and non-affected population. This means very large numbers are necessary to use this approach for common disease.

For the above strategy, when not enough numbers can be reached, or the phenotype is considered to be too common (or has milder forms in the reference population), a *candidate-gene approach* can be applied. This strategy requires in-depth knowledge of the pathophysiology underlying the phenotype investigated. Using this knowledge, a list of candidate genes can be produced, based on pathway analysis, known diseases with overlapping phenotypes, genes disrupted in mouse-models and previous genetic findings. To further prioritise candidate genes, variant selection could be based on prioritisation of variants likely to affect the function of the gene: Certain types of variants (frameshift, nonsense, splice site, and initiation codon variants) can often be assumed to disrupt the gene's function when the variant leads to the lack of transcription or nonsense-mediated decay of the altered transcript. For missense variants *in silico* prediction software can be used to classify the variants into variants more likely to affect the function and variants that probably do not affect function.

The approaches described above are mostly based on the assumption the disorder/phenotype being investigated is rare and caused by a monogenic causal variant. These methods can also be applied to common disease such as obesity, when it is believed that the obesity in the family or individuals investigated is caused by Mendelian disease (based on extreme phenotypes and/or family history). The difficulty is, however, that with extreme phenotypes of common disease you cannot exclude variants present in low frequencies in population cohorts (as is often the case with rare diseases such as malformation or development disorders), since milder forms of the extreme obesity investigated are likely to be included in these population cohorts as 'healthy individuals'.

'Obesity-plus' families are, therefore, a good target group for elucidating novel genes, in that the 'plus' element (mostly indicating a rare phenotype such as dysmorphic features or intellectual disability) can be approached in the same way as the rare diseases discussed above. However, it is important that the obese phenotype in that case segregates with the 'plus-phenotype'. For extreme obesity

phenotypes without the plus-phenotype, a *candidate gene* approach is most suitable. Considering that so much is already known about the different biological pathways involved in appetite regulation, an extensive candidate lists can be created in advance, and since obesity is a relatively easy phenotype to score in mouse-models (compared to cognitive dysfunction, say) biological interpretation is often possible. Using a candidate-gene approach, however, limits the un-biased approach which WES offers.

1.3.2 Standards for interpretation of sequence variants

The rapidly increasing availability of NGS and consequent existing bulk of data, has led to challenges in the clinical interpretation of sequencing variants. To improve correct interpretation and avoid confusion, standards have been created for interpretation of variants as well as on how to report them. Widely used is the Human Genome Variation Society (HGVS) nomenclature, consisting out of recommendations for the description of sequencing variants [88]. Although the term 'mutation' and 'polymorphism' are often used, they both can have several meanings and can therefore be confusing. To avoid this in this thesis I will use the term sequencing variant throughout. Similar, the term 'pathogenic mutation' can be confusing (it can mean among others 'disease causing', or 'disease causing when in a specific context'). Therefore, sequencing variants in this thesis will be classified according to the HGVS guidelines. Based on function affects these are*;* i) no functional effect, ii) probably no functional effect, iii) variant of unknown significance (VUS), iv) probably affects function, and v) affects function [88].

1.3.3 Findings so far for obesity using NGS

Whole exome sequencing has already resulted in the discovery of several highly-penetrant variants causing human monogenic obesity:

- ***HDAC8*** [89]: In a family with seven males affected by a novel syndrome of X-linked intellectual disability, hypogonadism, gynaecomastia, truncal obesity and short stature, X-chromosome

exome sequencing revealed a novel intronic variant in the histone deacetylase 8 gene (*HDAC8*) segregating with the phenotype which was absent in control datasets.

- ***MAGEL2*** [90]: Four unrelated individuals, with a Prader-Willi syndrome-like phenotype were found to carry truncating mutation on the paternal allele of *MAGEL2* (a gene within the PWS domain). The first variant was found through whole-genome sequencing. Three additional cases were identified by reviewing the results of exome sequencing of 1,248 cases in a clinical laboratory. All four subjects had autism spectrum disorder, intellectual disability and a varying degree of clinical and behavioural features of Prader-Willi syndrome, including obesity and hyperphagia.
- ***KSR2*** [91]: Sanger sequencing was used to identify the initial variants in the Kinase suppressor of Ras 2 (*KSR2*) gene in severely obese children. Whole exome sequencing datasets from another cohort of obese children were screened to identify additional variants. Although predicted-to-be-deleterious variants were also found in lean controls, this was at a significantly lower frequency than in the obese. Variant carriers exhibit hyperphagia in childhood, low heart rate, reduced basal metabolic rate and severe insulin resistance. Targeted deletion of Ksr2 also leads to obesity in mouse models.
- ***DYRK1B*** [92]: Three large unrelated families from Iran, with a Mendelian distribution of an unusual constellation of juvenile-onset central obesity (associated with early-onset coronary artery disease, severe hypertension, type 2 diabetes, and modestly elevated fasting serum triglyceride levels) were investigated using WES and linkage analysis. A single point-mutation co-segregating with the unusual phenotype was found in *DYRK1B.* Functional characterization of DYRK1B revealed the involvement of the protein with sonic hedgehog inhibition and Wnt signalling pathways and consequently adipogenesis.

As these studies show, WES is very successful in identifying novel causal genetic defects. It has, however, been suggested that this new era of NGS will decrease the need for phenotyping, with the

possibility of relatively cheaply interrogating all genes within one assay using WES [93]. Deep phenotyping, however, remains a crucial part of elucidating the consequences of variants identified. For obesity for instance, several Mendelian disorders known to cause different genetic disorders, appear after sequential further phenotyping, to also cause obesity : *PTEN* [94,95], *IGSF1* [96-98]and *POGZ [99,100]*.

All genes and genomic regions known to cause Mendelian obesity when disrupted (including *CPE*, which was discovered as part of the work described in this thesis) are summarised in Table 1.1.

Table 1.1: Obesity genes			
Gene symbol	**Gene name**	**Phenotype**	**References**
Dominant			
BDNF	Brain-derived neurotrophic factor	Childhood-onset obesity, cognitive impairment.	[58,101]
KSR2	Kinase suppressor of ras 2	Childhood-onset obesity, hyperphagia in childhood, low heart rate, reduced basal metabolic rate and severe insulin resistance.	[91]
MAGEL2	MAGE-like 2	Autism spectrum disorder, intellectual disability and a varying degree of clinical and behavioural features of PWS (including childhood-onset obesity in 75% and hyperphagia in 50% of the cases).	[90]
MC4R	Melanocortin 4 receptor	Severe obesity, increased lean mass, increased linear growth, hyperphagia, and severe hyperinsulinemia; homozygotes are more severely affected than heterozygotes.	[39,44]
NTRK2	Neurotrophic tyrosine kinase, receptor, type 2	Severe, childhood-onset obesity, hyperphagia, and cognitive impairment.	[59,60]
SH2B1	SH2B adaptor protein 1	Childhood-onset obesity, hyperphagia, disproportionate insulin resistance, spectrum of behavioural abnormalities.	[102,103]
SIM1	Single-minded (Drosophila) homologue 1	Severe obesity and a spectrum of developmental delay.	[54-56]
Recessive			
ALMS1	Alstrom syndrome 1	Alström syndrome: multisystemic, with cone–rod retinal dystrophy leading to juvenile blindness, sensorineural hearing loss, obesity, insulin resistance with hyperinsulinemia, and type 2 diabetes mellitus.	[104,105]
CEP19	Centrosomal protein 19kDa	Morbid obesity.	[106]
CPE	Carboxypeptidase E	Obesity, intellectual disability, hypogonadism and late onset type 2 diabetes mellitus.	[31]
HDAC8	Histone deacetylase 8	Intellectual disability, truncal obesity, gynaecomastia, hypogonadism and unusual face.	[89]
IGSF1	Immunoglobulin Superfamily, Member 1	Central hypothyroidism, testicular enlargement, variably low prolactin concentrations and obesity.	[96,98]
LEP	Leptin	Early-onset severe obesity, hyperphagia and hypogonadism. Some patients also have immunologic alterations.	[16,18,21,107]
LEPR	Leptin receptor	Severe obesity and hyperphagia. Some patients also have alterations in immune function, and delayed puberty due to hypogonadotropic hypogonadism.	[19,20]
PCSK1	Proprotein convertase subtilisin / kexin type 1	Severe obesity, hyperproinsulinaemia, hypogonadotropic hypogonadism. Some patients also have small intestinal dysfunction.	[51,52,108]
Recessive			
POMC	Proopiomelanocortin	Early-onset obesity, hyperphagia, isolated adrenocorticotrophin (ACTH) deficiency, and hypopigmentation of skin and hair.	[26,109]
TUB	Tubby bipartite transcription factor	Early-onset obesity and retinal dystrophy.	[34]
BBS1	Bardet-Biedl syndrome 1	Bardet Biedl syndrome: ciliopathy characterized by retinitis pigmentosa, obesity, kidney dysfunction, polydactyly, behavioural dysfunction, and hypogonadism.	[110,111]
BBS2	Bardet-Biedl syndrome 2	Bardet Biedl syndrome	[112]
ARL6 (***BBS3***)	ADP-ribosylation factor-like 6	Bardet Biedl syndrome	[113,114]
BBS4	Bardet-Biedl syndrome 4	Bardet Biedl syndrome	[115]
BBS5	Bardet-Biedl syndrome 5	Bardet Biedl syndrome	[116]

(Table continues on next page)

Gene symbol	Gene name	Phenotype	References
Recessive			
MKKS (***BBS6***)	McKusick-Kaufman syndrome	Bardet Biedl syndrome	[117,118]
BBS7	Bardet-Biedl syndrome 7	Bardet Biedl syndrome	[119]
TTC8 (***BBS8***)	Tetratricopeptide repeat domain 8	Bardet Biedl syndrome	[120]
BBS9	Bardet-Biedl syndrome 9	Bardet Biedl syndrome	[121]
BBS10	Bardet-Biedl syndrome 10	Bardet Biedl syndrome	[122]
TRIM32 (***BBS11***)	Tripartite motif containing 32	Bardet Biedl syndrome	[123]
BBS12	Bardet-Biedl syndrome 12	Bardet Biedl syndrome	[124]
MKS1 (***BBS13***)	Meckel syndrome, type 1	Bardet Biedl syndrome	[125]
CEP290 (***BBS14***)	Centrosomal protein 290kDa	Bardet Biedl syndrome	[125]
WDPCP (***BBS15***)	WD repeat containing planar cell polarity effector	Bardet Biedl syndrome	[126]
SDCCAG8 (***BBS16***)	Serologically Defined Colon Cancer Antigen 8	Bardet Biedl syndrome	[127]
LZTFL1 (***BBS17***)	Leucine zipper transcription factor-like 1	Bardet Biedl syndrome	[128]
BBIP1 (***BBS18***)	BBSome Interacting Protein 1	Bardet Biedl syndrome	[129]
IFT27 (***BBS19***)	Intraflagellar Transport 27	Bardet Biedl syndrome	[130]
CNVs			
16p11.2 deletion (593-kb; chr16: 29,500,000-30,100,000)		Obesity and high risk of autism spectrum disorder. (region includes the genes: *SPN, QPRT, C16orf54, ZG16, KIF22, MAZ, PRRT2, PAGR1, PAGR1, MVP, CDIPT, SEZ6L2, SPHD1, KCTD13, TMEM219, TAOK2, HIRIP3, INO80E, DOC2A, C16orf92, AM57B, ALDOA, RN7SKP127, AC009133.17, AC009133.21, AC009133.15, AC009133.14, AC009133.20, AC009133.12, CDIPT-AS1, CTD-2574D22.4, CTD-2574D22.2, RP11-455F5.3, RP11-455F5.4, RP11-455F5.5, SLX1A-ULT1A3, PPP4C, TBX6, YPEL3, GDPD3, MAPK3, CORO1A, BOLA2B, SLX1A, SULT1A3, RP11-347C12.3)*	[61]
16p11.2 deletion (220-kb; chr16: 28,730,000-28,950,000)		Severe childhood obesity, with a less pronounced obesity status in adults. (region includes the genes: *ATP2A1, ATXN2L, CD19, LAT, NFATC2IP, RABEP2, SH2B1, SPNS1, TUFM*)	[63]

1.4 Personalised medicine of morbid obesity

Since obesity is recognised as one of the major threats to human health worldwide, the time is ripe to use these new genetic insights into monogenic obesity, to improve clinical care. Considering the rapidly growing knowledge of genetic and molecular mechanisms of obesity, and the likelihood of further novel genes, proteins, and mechanisms being discovered, better mechanism-directed therapy combined with the more traditional lifestyle, pharmaco-therapeutic and surgical approaches, would be a logical next step.

Personalised medicine is defined as 'any clinical practice model that emphasizes the systematic use of preventive, diagnostic, and therapeutic interventions that use genome and family history information to improve health' [131]. Genetic testing has already been applied for diagnostic, prognostic and therapeutic applications in a wide variety of medical specialties, including oncology, cardiology, neurology and, most of all, paediatrics [131]. While genetic testing has even become a recommended procedure in several medical disciplines, including the use of pre-symptomatic genetic testing in oncology, the applicability of personalised medicine to common diseases, such as obesity, is still a subject of active debate.

For individuals whose obesity is caused by a Mendelian form of obesity, however, genetic analysis has a high predictive power and is necessary to enable proper genetic counselling. Additionally, it could very well contribute to choice of the right management options to treat obesity in an individualised manner. Since an extensive list of genetic defects underlying Mendelian forms of obesity is now available, with a non-trivial number of individuals potentially affected, seizing the opportunities for realising personalised medicine and, thereby, improving the quality of life for this stigmatised and under-served group of patients is crucial.

1.4.1 Genetic counselling and Lifestyle treatment

Genetic counselling is important in the management of patients with Mendelian forms of obesity, to educate them and their families about the mode of inheritance of their condition and risks for family members. This will enable screening for other individuals affected, and gives an opportunity for family planning. Careful, evidence-based advice on diets that could be maintained to limit caloric intake in patients with hyperphagia should be given, and the importance of physical activity that the patient can manage should be pointed out. The advice given should be adjusted for each patient, depending on the kind of Mendelian obesity they are diagnosed with and should ideally take an evidence-based medical approach.

The most commonly-used treatment approach for obesity is lifestyle intervention, although it is widely known that this has a poor long term effect on weight loss. Numerous clinical trials have been conducted during the last decade, and virtually all point out that an average weight loss of 5-10% can be expected [132]. Although, weight losses within this range result in beneficial clinical changes, unfortunately most people will regain their weight within a moderate amount of time, during which the clinical benefits of the weight loss will be unlikely to be sustained [132]. Surprisingly, little is known about the factors influencing the effectiveness of lifestyle intervention as a treatment option. It is very possible that different sub-forms of obesity may be differently responsive to these lifestyle intervention methods (severe monogenic forms of obesity, with hyperphagia, might make response less likely, compared to subjects with mild adult-onset obesity). If a particular lifestyle intervention approach which is most effective in specific cases of obesity (such as early-onset obesity with hyperphagia) can be detected, it would be possible to provide affected individuals and their families with targeted support.

Most studies on lifestyle intervention and hyperphagic behaviour have been performed for PWS patients. Extreme appetite behaviours are seen in PWS, including stealing and hoarding of food, consuming inedible substances, and lying about eating [133]. Various methods as well as strict dietary

monitoring have been used unsuccessfully for weight control, and it is currently generally accepted that those with PWS may be incapable of making food-related decisions. Families are, therefore, advised to constrain food-seeking behaviour (e.g. by locking kitchens cupboards and refrigerator) [134]. Strict dietary treatment, preferably starting early in life, is found most effective and can even avoid excessive weight gain in PWS [135,136].

A limited number of studies have looked at lifestyle treatment options for other Mendelian forms of obesity. One study, on children with *MC4R* variants leading to reduced receptor functioning, indicated that weight loss through lifestyle intervention is possible, but maintenance of this weight loss was much more difficult for these children compared to children with wild-type *MC4R* [48]. Hyperphagia is difficult to control, and most attempts at behaviour modification in other disorders associated with hyperphagia (such as hypothalamic obesity) have proven unsuccessful [137]. Although lessons can be learned from the more extensive experience in weight control in PWS patients, the major difference is that many of the other Mendelian forms of obesity are not associated with intellectual disability. Constraining food will, therefore, be more difficult; you cannot constrain food from a free-living adult with MC4R deficiency, by locking the fridge door. Urgent studies investigating how to best treat hyperphagia and other eating behaviour associated with these Mendelian forms of obesity are, therefore, warranted. Since there is currently no consensus regarding optimal weight management strategies, prevention might be the best option. This will, however, require an early diagnosis; preferably early in childhood before the weight gain has started, indicating the importance of pre-symptomatic genetic testing when other family members are known to be carriers.

1.4.2 Pharmacotherapy

The unsatisfactory results of lifestyle interventions make other effective methods to treat obesity an active topic of research. The non-invasive aspect of pharmacotherapy, means that it will make a good candidate for obesity treatment. Unfortunately, the history of pharmacological agents for obesity has

not been without problems and there are several examples of drugs being removed from the market due to significant side effects. One example is the relation between Pulmonary atrial hypertension and appetite suppressant drugs. Probably most infamous was the withdrawal of the combination therapy of fenfluramine and phentermine (fen/phen) by the FDA following the unexpected and potentially fatal pulmonary hypertension and valvular heart disease related to its use. Thousands of lawsuits were filed by patients treated with the drug, with over billions spend in legal costs [138,139]. Another example is two more recent drugs on the market, rimonabant and sibutramine, which provided only modest weight loss and were both associated with high attrition rates due to intolerable side effects [140]. In the case of sibutramine an increase in major adverse cardiovascular events during the Sibutramine Cardiovascular OUTcomes trial, prompted its withdrawal in Europe and the United States. Adverse psychiatric side effects of rimonabant, led to its withdrawal as well [139].

Currently five medications are approved in the USA, of which three are also available in Europe: Orlistat (a pancreatic lipase inhibitor), lorcaserin (a serotonin 2C receptor agonist), a combination of phentermine/topiramate (a sympathomimetic anticonvulsant), a combination of naltrexone/bupropion (an opioid receptor antagonist and a dopamine/noradrenaline reuptake inhibitor) and Liraglutide (a GLP-1 receptor agonist) [141]. With orlistat being the longest on the market and the safest option of these different medications, it is widely used. Recently it even became available over the counter, although its use is limited by gastrointestinal side-effects (which makes it less popular with patients). So far, no studies have been performed on the utility of these different medications in Mendelian obesity. Other pharmaco-therapeutics targeting specific forms of Mendelian obesity are currently in use, or are being developed:

Leptin agonists

As outlined earlier, leptin acts within the hypothalamic leptin–melanocortin pathway, which regulates appetite and satiety. The first steps towards personalised medicine for treatment of obesity became reality in 2002 when Farooqi, *et al.,* treated a severely-obese child who carried a homozygous

frameshift mutation in the leptin gene [17]. Leptin replacement therapy appeared to induce beneficial effects in metabolic, neuroendocrine, and immune abnormalities in patients with relative leptin deficiency. Unfortunately, no treatment is available yet for patients carrying leptin receptor disruptive variants. Individuals with 'common' obesity (without disruptive variants in the *LEP* gene) have higher serum leptin concentrations than normal-weight individuals. This observation suggests a decreased sensitivity to leptin and its effect in decreasing appetite and causing weight loss [142]. Indeed, peripheral administration of leptin in rodent models of diet-induced obesity had only marginal efficacy in induction of weight loss, and similar findings were observed in clinical trials of leptin administration to obese humans [143,144].

MC4R agonists

MC4R agonists and antagonists were not initially developed as new therapeutic analogues to treat obesity, but were designed to unravel the role of Mc4r in the energy pathway by applying them in *Mc4r* knockout mice [145]. After administration of an Mc4r agonist to wild-type mice it appeared to inhibit feeding, while administration of an antagonist resulted in enhanced feeding and obesity [146]. Regulation of body temperature, locomotor activity and metabolism contributed to these effects [147]. Not only did these findings confirm the importance of MC4R in energy balance, but also opened a new therapeutic pathway to treat obesity. It has, however, proven difficult to use MC4R as a drug target, not only because designing specific MC4R agonists without affinity for the MC3 receptor is challenging, but also because of side effects seen after MC4R agonist administration. Greenfield, *et al.* demonstrated that humans with MC4R deficiency have lower blood pressure, less hypertension, lower 24-h urinary catecholamine excretion, lower resting heart rate, and attenuated insulin-mediated, sympathetic activation compared to equally-obese humans. In overweight and obese humans without disruptive *MC4R* variants, the infusion of a highly-selective MC4R agonist led to dose-dependent increases in blood pressure and heart rate [148]. These findings might have an influence on the applicability of MC4R agonists to treat common obesity. On the other hand, treatment of patients

carrying a disruptive *MC4R* variant, might be more feasible, especially because of the lower blood pressure found in this specific group of patients. Several studies have recently analysed the *in vitro* effect of different kinds of melanocortin agonists on mutated MC4R. In this way, various synthetic ligands have been demonstrated to give distinct improvement in MC4R functional activity, depending on both agonist potency and the nature of the mutation [149-151]. These studies demonstrate the importance of functional characterization of *MC4R* variants to categorise the different classes of *MC4R* variants, so that identification of variant carriers who might benefit the most from MC4R agonist therapy can take place. Since the majority of the disruptive *MC4R* variants appear to disrupt trafficking of receptors to the cell surface, rather than affinity for the ligand, recovering cell surface expression could also be an interesting therapeutic pathway in this group of patients [152]. The first clinical trial with MC4R agonist treatment of individuals carrying disruptive MC4R variants is currently being performed [153], with initial results indicating a positive effect on weight loss without clinically important effects on heart rate or blood pressure.

Another study investigated the therapeutic effect of a MC4R agonist (setmelanotide) in two patients with POMC deficiency. Both patients showed a sustainable reduction in hunger and substantial weight loss following treatment, indicating the usability of this kind of medication in this specific patient group [154]. Several medications have been trialled to treat hyperphagia and obesity seen in PWS patients, but most were ineffective or showed severe side effects. Beloranib (a methionine aminopeptidase 2 inhibitor) showed promising results with significant weight reduction, but trials were terminated due thromboembolic disease detected in the participants [155]. Liraglutide on the other hand shows promising results in undergoing trials on weight loss as well as glucose metabolism [156,157]. Interestingly, one of the medications currently approved by the FDA (the combination of naltrexone and bupropion) has been shown to act through indirect enhancement of the POMC neurons (and therefore leads to increased MC4R activation), which makes it an interesting therapeutic option this specific group of patients with Mendelian obesity [158].

1.4.3 Bariatric surgery

Bariatric surgery is currently the most effective therapy for morbidly obese patients, with considerable reduction in weight, alongside a remarkable improvement of comorbid conditions. Roux-en-Y gastric bypass (RYGB), the most common bariatric operation in the UK, results in 20-40% weight loss initially, the majority of which is maintained over at least 15 years [159]. Adjustable gastric banding (AGB) results in slightly less weight loss, around 15–30% [160], and vertical sleeve gastrectomy (VSG) in 15-20% [161].

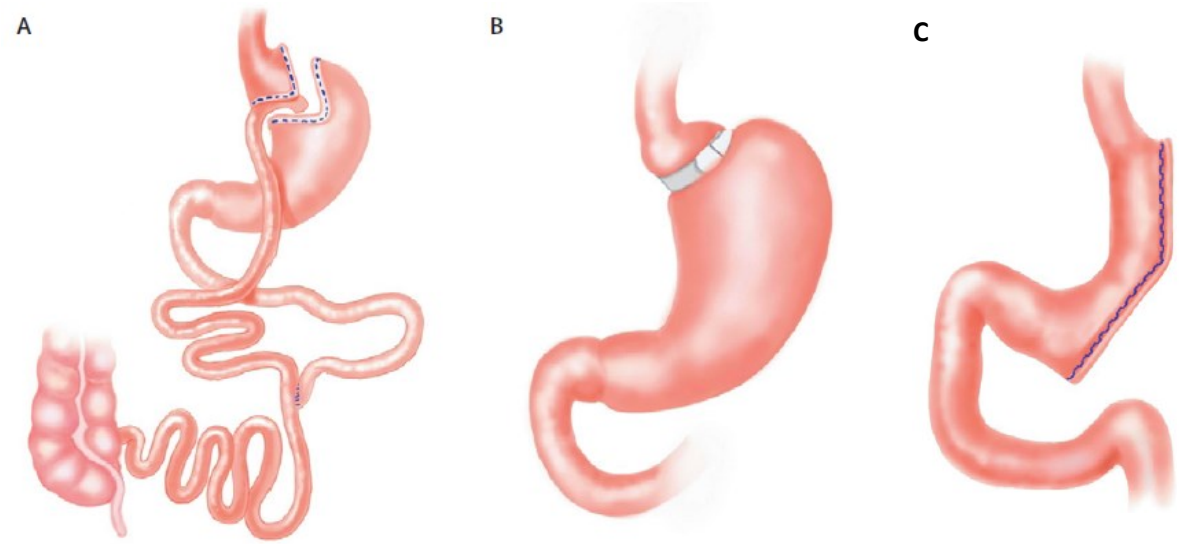


Figure 1.3: Bariatric surgery types. A) Roux-en-Y gastric bypass B) Adjustable gastric band C) Vertical sleeve gastrectomy. (source: Dixon, *et al*. [239])

Bariatric surgery also has a beneficial effect on obesity-associated comorbidities, including dyslipidaemia, non-alcoholic steatohepatitis, sleep apnoea, asthma, cardiac dysfunction, arthritis and infertility. An improvement of hypertension, remarkably enough, has not been consistently detected. Particularly noteworthy is the rapid reduction of type 2 diabetes after RYGB. Interestingly, this resolution frequently seems to be independent of weight loss, and occurs within days of surgery [159,162-166]. Although the various surgical options are generally classified into different types of procedure – restrictive, malabsorptive or hybrid- in fact, relatively little is known about the true mechanisms causing weight loss after surgery. Despite the generally favourable outcomes of bariatric

surgery, approximately 10%–40% of patients do not achieve successful long-term weight loss (BMI <36, or loss of weight >20%) [167]. Like the mechanisms of weight loss, the factors and mechanisms underlying variation in outcomes after surgery are poorly understood. Given that bariatric surgery is an invasive treatment for obesity and is often viewed as the "last resort" for patients, research focused on understanding the basis of a particular patient's obesity might be a useful prognostic indicator or be used to guide choice of surgery type.

Little research has been done on the implications of syndromic forms of obesity on the outcome after bariatric surgery. Bariatric surgery has been reported in several case reports of patients with PWS, with variable outcomes. Scheimann, *et al.,* reviewed the different bariatric techniques implemented in patients with PWS in 2008, concluding that although some smaller case series reported short-term success, there was little justification for subjecting PWS patients to the potential risks of surgical interventions [168]. However, a recent study in which 24 patients with PWS underwent VSG, showed good weight loss results, and even after 5 years there was no significant difference with a control group, which might indicate VSG is a good treatment option for patients with PWS [169]. In another case report, a patient with Bardet-Biedl syndrome treated with RYGB surgery enjoyed prominent weight loss and significant improvement of related comorbidities [170].

Reports of the outcomes of surgery in MC4R-deficient patients have, unfortunately been marred by inclusion of non-pathogenic variants, and/or short follow-up periods (see Table 1.2). To detect the influence of heterozygous disruptive *MC4R* variants on the outcome of bariatric surgery, three recent studies reported genetic screening of cohort of obese patients undergoing RYGB [169,171-173]. All showed no difference in weight loss after surgery between patients MC4R deficiency and patients without, but longer follow up give contradictory results. Hatoum, *et al.*, on the other hand, reported substantial less weight loss in mice carrying a homozygotic *MC4R* mutation, suggesting that at least one normal copy of *MC4R* is necessary for sustained effects of RYGB. One case report of a patient with compound heterozygosity and complete loss of function of both copies of *MC4R* showed no weight

loss after LAGB and truncal vagotomy, and even experienced weight gain 12 months post-op [174]. Only one study has been conducted on weight loss after LAGB in patients MC4R deficiency, reporting a significantly lower weight loss and less improvement of metabolic syndrome compared to non-mutation carriers [175]. A rat study on the influence of Mc4r on the outcome after VSG suggests that both body weight and glucose metabolism are not mediated by alterations in Mc4r activity [176].

Although the mechanisms of weight loss after RYGB, LAGB and VSG are not fully understood, it is reasonable to think that gastric restrictive operations such as gastric banding, much like conventional dieting, rely more heavily on impulse control and, therefore, are less likely to maintain long-term weight loss in patients suffering from hyperphagia. The malabsorptive and hormonal mechanisms of RYGB, on the other hand, could make it a more suitable procedure for MC4R deficient patients. However, studies with longer follow-up are needed to determine whether the positive outcomes of RYGBP in MC4R deficient patients are maintained long term.

Number of affected patients (total cohort)	**Monogenic disorder**	**Surgery type**	**Age**	**BL-BMI** (± SD)	**Weight loss metrics used (Follow up time points)**		**Difference with control group**	**Reference**
24 (na)	PWS	VSG	10.7	46.3 (±12.2)	BMI change (1 and 5 yrs)	-14.7; -10.7	Ns	Algathani, *et al.* 2016 [169]
3 (na)	PWS	VSG (n=2)	15 and 23	50 and 46	%WL (1 yr)	-27.2 and -25.4	na	Fong, *et al*. 2012 [177]
	PWS	MGBP (n=1)	18	44	%WL (1 yr)	-37.1	na	
1 (na)	BBS	RYGB	16	52.3	%WL (1 and 3 yrs)	-28,2; -33.3	na	Daskalakis, *et al*. 2010
19 (300)[A]	*MC4R* (het)	AGB	47 ± 3	45 ± 1.0	%BMI change (1 and 2 yrs)	-12; -20	↓	Potoczna, *et al*. 2004 [178]
1 (na)	*MC4R* (hom)	AGB	18	54.2	%WL (1 yr)	+4.0	↓	Aslan, *et al*. 2011[174]
4 (92) [B]	*MC4R* (het)	RYGB	45.5 ± 7.5	53.9 ± 8.3	%EBWL (1 yr)	-66	ns	Aslan, *et al*. 2011 [171]
15 (972) [B]	*MC4R* (het)	RYGB	45.9	50.1	%WL (1 and 2 yrs)	-35.4; -39.0	ns	Hatoum, *et al*. 2012 [172]
6 (1433)	*MC4R* (het)	RYGB	?	49.3 ± 6.4	BMI change [C]	-6.2 ± 6.7	↓	Moore, *et al*. 2014 [173]
4 (135) [B]	*MC4R* (het)	LAGB (n=3)	16.5 ±1.2	54.4 ± 8.6	%WL [C]	-26.2 ± 7.6	ns	Censani, *et al.* 2014 [179]
	MC4R (het)	VSG (n=1)			%WL [C]	-35.3	ns	

Table 1.2: Overview of publications on individuals with Mendelian obesity undergoing bariatric surgery. For all studies, percentage weight loss (%WL) was calculated if data was available, otherwise the metrics used in the paper are listed. A difference from a control group was noted only if a control group was included into the study: ns, no significant difference; ↓, weight loss lower than control group; na, not applicable. PWS, Prader-Willi syndrome; BBS, Bardet Biedl syndrome; *MC4R*, pathogenic variants in the *MC4R* gene in heterozygous (het) or homozygous (hom) state; VSG, vertical sleeve gastrectomy; RYGB, Roux-en-Y gastric bypass; AGB, adjustable gastric banding; %WL, percentage weight loss; %EBWL, percentage excess body weight loss. [A] included 13 non-pathogenic mutations. [B] included non-pathogenic mutations. [C] Variable follow up times.

1.5 Summary

As can be seen in this introduction, genetic research in obesity has contributed immensely towards the understanding of underlying mechanisms of the obesity pathophysiology. Not only did the identification of Mendelian forms of obesity elucidate appetite-regulatory pathways for research purposes, they also provided immediate clinical utility by enabling diagnosis (and in rare cases treatment) for the individuals involved. Building on these achievements, we can see the importance of identifying novel Mendelian forms of obesity. There is an urgent need to determine the true prevalence of these disorders among the more severely-affected obese individuals seeking treatment, and to investigate how these new insights can be translated to improve treatment of the individuals involved. Keeping this in mind, I came to the following aims for my PhD project:

1.6 Overall Aims

Hypothesis

Different pathways leading to obesity will respond differently to the different range of bariatric and lifestyle procedures. Identification of rare highly-penetrant forms of obesity will, therefore, be useful as a prognostic indicator and a guide for choosing the right surgical procedure.

1) To generate a new cohort of obese adults seeking bariatric surgery, and investigate the influence of extreme forms of obesity on phenotypic characteristics.

Patients undergoing different forms of bariatric surgery will be recruited, prospectively followed and detailed phenotypes (including health measurement and eating and psychological behaviour) investigated at baseline and following surgery.

2) To carry out screening for MC4R deficiency in the adult bariatric cohort, and in a cohort of obese Dutch children undergoing intensive lifestyle intervention.

Participants will be screened for *MC4R* variants (MC4R deficiency is the most common form of Mendelian obesity, known to date), using Sanger sequencing, and the baseline characteristics and outcomes of therapeutic intervention will be assessed in carriers of these variants compared to the rest of the cohort.

3) To identify novel Mendelian forms of obesity, and to detect the prevalence of Mendelian forms of obesity (other than MC4R deficiency) among the extreme obese individuals.

A selection of 'extreme' bariatric patients, without *MC4R* variants, will undergo further genetic analysis in the form of whole exome sequencing. This includes 40 extremely obese individuals without any other specific phenotype, and one 'obesity-plus' family.

4) To investigate the implications of such novel Mendelian forms of obesity for response to bariatric surgery.

The implications of these novel rare causal variants found will be investigated for phenotypic characteristics, including bariatric surgery outcomes.

CHAPTER 2

MATERIALS & METHODS

2.1 PMMO cohort

As a part of this PhD project a cohort of morbidly obese participants was created as a part of a larger research study entitled 'Personalised Medicine of Morbid Obesity', from here on abbreviated as PMMO [180,181]. The PMMO project is an observational research study of morbidly obese individuals pursuing bariatric surgery. The overall aim of this project is to investigate the genetic architecture of obesity and T2DM, and to identify factors that influence the outcomes of bariatric surgery. The second overall aim of the PMMO research study is to explain the mechanisms that underline T2DM remission following bariatric surgery.

This multi-centre research project is still ongoing and therefore only data available up to date have been included in this thesis. The PMMO project includes the collection of an extensive list of phenotypic data and multiple sample collection (incl. DNA, RNA, serum, urine and faeces samples, as well as tissue biopsies) collected at multiple time points throughout the participant's weight loss journeys.

For this thesis the PMMO cohort was used to analyse the clinical aspects and genetic architecture (in terms of rare highly penetrant variants) of the severely obese population. Therefore, the methods described in this chapter will focus on the recruitment, data collection and collection and processing of samples, specifically used for the analysis described in this thesis.

The PMMO research study was approved by the NRES Committee London - Riverside (REC reference 11\LO\0935), and was performed in accordance with the principles of the Declaration of Helsinki.

2.2 Research participants

Participants were recruited at three different hospital sites; The Imperial College NHS Weight Centre, Chelsea Westminster Hospital NHS healthcare centre and Derby Royal Hospital NHS healthcare centre.

Patients pursuing bariatric surgery or having undergone such a procedure were invited to participate into this study. Initial baseline data was collected at the pre-assessment clinic, with six subsequent study visits coinciding with the clinical follow up appointments, to minimise the participant's discomfort (blood sampling for research purposes could in this way be combined with clinical blood sampling), and to optimise follow-up compliance.

The PMMO study was originally set up as a prospective study, in which all participants would be recruited in a prospective manner. However, after an initial start it turned out that using this approach the number necessary for the analysis of this thesis (*n*=1000) would not be feasible to reach within time: the waiting time between recruitment and the actual surgery date (around 6 to 12 months) delayed follow up possibilities and, on top of that, there was a great proportion of recruits that did not undergo surgery at all (27% of participants recruited at baseline still to current date have not undergone surgery for multiple reasons). The protocol was therefore amended, through the application of a substantial amendment procedure, and participants could also be recruited in a retrospective manner.

All participants gave written informed consent to participate in this study. Copies of an example patient information sheet and consent form can be found in Appendix 2.1 (page 303).

Of the participants recruited at the Imperial Weight Centre I personally recruited, collected samples and data for around 800 of the participants included in this thesis.

2.2.1 Inclusion criteria

1) Adults (18-65 years old) with a BMI >35 kg/m^2, and pursuing bariatric surgery.

2) Adults (18-65 years old) having undergone bariatric surgery, and had a BMI >35 kg/m^2 before undergoing treatment.

2.2.2 Exclusion criteria

Receiving or intending to receive medication not approved by the European Medicines Agency (EMA) or current participation in other clinical research trials.

2.2.3 Data collection

Participants could be included into this study before undergoing bariatric surgery, in which case data was collected in a prospective manner, while for participants that were recruited after they had undergone bariatric surgery the data was collected in a retrospective manner through retrieving data from medical notes. A schematic overview of the study visits is given in figure 2.1, while an overview of the collected data at each time points can be found in Table 2.2 (found at the end of this section).

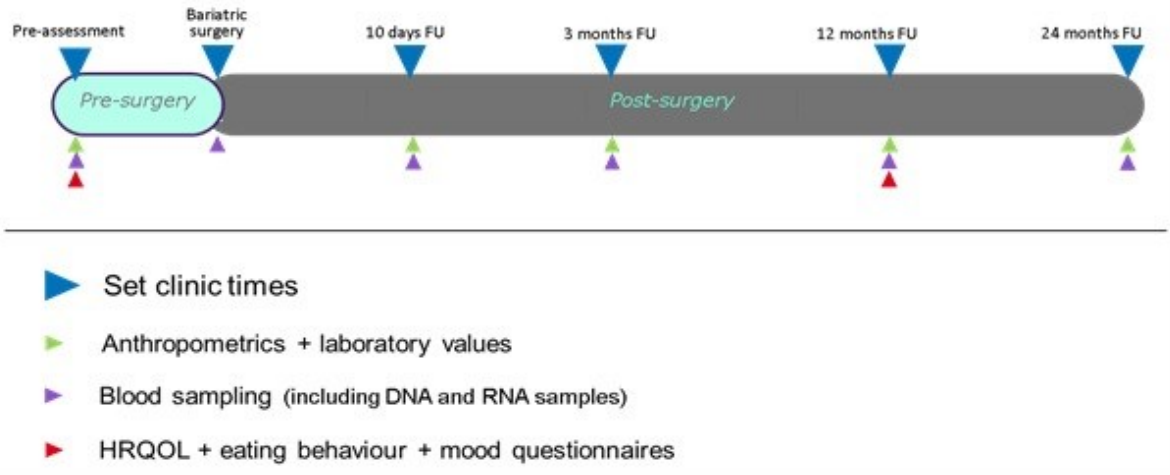


Figure 2.1: Time line of study visits of the PMMO research study. Participants could be recruited before undergoing bariatric surgery or at any time after having undergone surgery. Research sample collection would take place at the day of recruitment.

Demographic and anthropometric data

Ethnic background was verbally collected from the participants, as well as weight history (age of onset of obesity and highest weight). Height and weight was measured upon recruitment and was repeated for every follow up visit at 10 days and 3, 12 and 24 months following surgery. Body mass index was calculated by dividing body mass (kg) by squared height (m). Blood pressure and pulse were collected from the clinical measurements for the similar time points as weight was collected. Medical history, age (calculated for the day of surgery, or recruitment day if the participant did not undergo surgery) and gender were collected from the medical files.

Blood phenotypes

As a part of the participant's clinical care fasting blood tests were performed at the pre-assessment clinic (baseline) and at 3, 12 and 24 months following surgery. Blood test results included into this study were: insulin (mmol/L), glucose (mmol/L), HbA1c (mmol/mol), total cholesterol (mmol/L), LDL (mmol/L), HDL (mmol/L), triglycerides (mmol/L), free T4 (pmol/L), and TSH (mU/L).

Family history

Family history was collected through drafting family pedigrees of up to three generations through direct interview with the participants. Specific detail was paid to obesity and weight history in family members (also including contradictions with lean family members) and T2DM diagnosis. Other specific features checked included learning difficulties, born defects, multiple miscarriages and psychiatric disorders, such as autism and schizophrenia.

Obesity related phenotypes

T2DM was defined as receiving treatment with glucose lowering medication or having an HbA1c level of >48 mmol/L, and was further subdivided into insulin-treated T2DM (ITT2DM) and participants on oral treatment. Participants with Type 1 diabetes mellitus were grouped into a separate category.

Hypercholesterolemia was defined as receiving statin treatment upon recruitment or having a total cholesterol level of >5.15 mmol/L. T2DM and hypercholesterolemia were assessed at baseline and re-assessed for each follow up visit by analysing medication lists and HbA1c levels for T2DM, and total cholesterol, LDL, HDL and triglyceride levels for hypercholesterolemia.

Obstructive Sleep apnoea (OSAP) was assessed at baseline as a part of the pre-assessment clinic, and patients with symptoms of snoring and day-time somnolence were referred for a sleeping test to diagnose or exclude OSAP. Mobility problems were assessed at baseline by recording the requirement of walking aids or a wheelchair (while more in depth analysis was performed through the SF36 questionnaire, described below). Hypothyroidism was defined as receiving thyroid replacement therapy or having abnormal TSH levels (>4.7 mU/L) and/or abnormal free T4 levels (<10 pmol/L).

Psychological screening

All patients undergoing bariatric surgery at the Imperial Weight Centre received psychological screening as a part of their clinical care. Psychological assessments are performed by specifically trained psychologists using standardised screening tools based on DSM V criteria. Data regarding binge eating disorder and other eating disorders, depression and any other psychiatric disorder (such as, substance abuse, autism related traits, post-traumatic stress syndrome, anxiety disorder, bipolar disorder and schizophrenia) were collected from this assessment. BED was further sub-categorised into current active BED or a history of BED if no binge episodes had occurred for at least 6 months. Depression was further sub-categorised as currently receiving treatment (with anti-depressants or therapy) or having received such treatment in the past (which was classified as a history of depression).

Questionnaires

A total of seven questionnaires were collected at baseline and at 12 months following surgery; two assessing health related quality of life (HRQOL), three assessing eating behaviour, and another two

assessing mood. Each questionnaire was select on the basis of good internal consistency and test-retest reliability, as well as having been validated in the obese population.

Health related quality of life:

- **Short Form 36 health survey** (SF36) [182]: The SF36 assesses 8 different domains of HRQOL; General health, physical functioning, limitations in daily activities as a result of physical health problems, limitations in daily activities as a result of emotional health problems, vitality, bodily pain, emotional well-being, and social functioning. The SF36 has good internal consistency (*Cronbach's alpha*: 0.79-0.92 [183]), also in the obese population [183,184].
- **Impact of weight on quality of life** (IWQOL-lite) [185]: The IWQOL assesses 5 different domains of weight related QOL; physical functioning, self-esteem, sexual life, public distress and work problems. The IWQOL was specifically designed to assess for the effect of weight on health related aspects, and has good internal consistency (*Cronbach's alpha*: 0.87) [185].

Eating behaviour:

- **Dutch eating behaviour questionnaire** (DEBQ) [186]: The DEBQ measures dietary restraint, emotional eating and external eating. This measure is internally consistent (Cronbach's alpha range: 0.80–0.95 for the different subscales) and has been validated for use in the obese population.
- **Three factor eating questionnaire** (TFEQ) [187]: The TFEQ is a 51-item questionnaire assessing dietary restraint, disinhibition (loss of control over eating), and subjective feelings of hunger. This measure has a good internal consistency (Cronbach's alpha range: 0.79 to 0.92 for the different subscales), but has variable results in the obese population [188].
- **Eating Disorder Examination Questionnaire** (EDEQ) [189]: The EDEQ assesses eating disorder psychopathology, through the domains dietary restraint and disordered eating patterns (eating concern, weight concern, shape concern). Internal consistency is good (*Cronbach's*

alpha range: 0.77 to 0.84 for the different subscales), but some scales are limited for the obese population [188,190].

Mood

- **Positive and Negative affect scale** (PANAS) [191]: PANAS measures the positive and negative affect scale, with low positive affect scores reflecting 'sadness and lethargy' whereas high positive affect scores reflect 'high energy, full concentration, and pleasurable engagement' and low negative affect scores describe 'a state of calmness and serenity' whereas high negative affect scores suggest 'subjective distress and unpleasurable engagement'. Internal consistency is good (: 0.83 -0.90 for positive affect and 0.85 - 0.93 for negative affect)[192].
- **Hospital Anxiety and Depression score** (HADS) [193]: HADS is self-rating instrument for anxiety and depression in patients with both somatic and mental problems, with good internal consistency (Cronbach's alpha range: 0.78-0.93 and 0.82-0.90, for the anxiety and depression scale respectively) [111].

"Monogenic-obesity-like" risk-score

Several obesity-related phenotypes previously reported to be associated with monogenic forms of obesity (early onset of obesity, binge eating disorder and hyperphagia [39,41,42,50,178,194]) were combined together in a so called "monogenic-obesity-like" risk-score. The scoring was done as followed:

One point was given for each phenotype present in the individual, while zero points were given when the phenotype was not present (Table 2.1):

- For early onset obesity, the cut off was set at 10 years old, so one point was given when the age of onset of an individual's obesity was at or before the age of 10.
- For BED one point was given if a current diagnosis was present or if the participants suffered from BED in the past.

- Since hyperphagia was not directly measured in the PMMO cohort, domains of eating behaviour questionnaires previously shown to correlate with hyperphagia and/or BED were used [188,195-197]: the domains 'hunger' and 'disinhibition' measured using the TFE-questionnaire, and the domains 'emotional eating' and 'external eating' measured using the DEBQ. For each domain quartiles were calculated for the overall scores found in the PMMO cohort. One point was given for each domain when an individual scored within the 4th quartile (and therefore was among the most severely affected of the total cohort).

The scoring system has been schematically summarised in Table 2.1. Although "monogenic-obesity-like" risk-score creates a score from 0 (no phenotypes) to 6 (all six phenotypes), the score was stratified into two groups: participants scoring 0-3 (low "monogenic-obesity-like" risk-scorers) and participants that scored 4-6 (high "monogenic-obesity-like" risk-scorers).

Monogenic-obesity-like phenotype		**Score:**
Onset of obesity	Before or at 10 years old, enter 1 →	
	After 10 years old, enter 0 →	
Binge eating disorder (BED)	Current diagnosis, or a history of BED, enter 1 →	
	No current diagnosis nor history of BED, enter 0 →	
EDEQ – Emotional*	Score 50 or higher, enter 1 → Score below 50, enter 0 →	
EDEQ - External*	Score 35 or higher, enter 1 → Score below 35, enter 0 →	
TFEQ - Inhibition*	Score 11 or higher, enter 1 → Score below 11, enter 0 →	
TFEQ - Hunger*	Score 11 or higher, enter 1 → Score below 11, enter 0 →	
Total "Monogenic-obesity-like" risk-score: (sum of individual components)		

Table 2.1: Instruction on how to calculate the Monogenic-obesity-like risk-score.

* scores were calculated by using the 4th quartile cut-off point of the overall scores found in the PMMO cohort.

Surgery and follow up

Operations included laparoscopic RYGB, VSG and restrictive gastric band placement. Any per-operative or long term complications were collected.

Research visits following surgery were scheduled simultaneously with the participant's clinical appointments at 10 days and 3, 12 and 24 months following surgery. At these visits anthropometric measurements (weight, blood pressure, pulse) and laboratory measurements (HbA1c (mmol/mol), total cholesterol (mmol/L), LDL (mmol/L), HDL (mmol/L), triglycerides (mmol/L)) were collected. General health was screened with each participant, and any changes with possible influence on weight

were noted (such as pregnancy, emergency/selective surgery, newly diagnosed psychiatric/somatic disorders, newly diagnosed mobility problems), and the decision of excluding participants for weight loss analysis was decided on a case to case basis.

On overview of the data collection for each visit is given in the table 2.2.

Data collected:	Study visits					
	Baseline	**Surgery day**	**0.25 mths**	**3 mths**	**12 mths**	**24 mths**
†Demographics:						
Ethnicity	✓					
Age & gender	✓					
General health	✓	✓	✓	✓	✓	✓
Anthropometrics:						
Height (m)	✓					
Weight (kg)	✓		✓	✓	✓	✓
BMI (kg/m^2)	✓		✓	✓	✓	✓
Blood pressure (mmHg)	✓		✓	✓	✓	✓
Pulse (n/min)	✓		✓	✓	✓	✓
†Family History (up to 3 generations):						
Obesity/ Overweight	✓					
T2DM	✓					
Learning difficulties	✓					
Born defects	✓					
Miss carriages	✓					
Ethnicity of parents	✓					
†Medical history:						
Weight history	✓					
General medical history	✓					
Comorbidities:						
T2DM status	✓		✓	✓	✓	✓
T2DM, medication use	✓		✓	✓	✓	✓
Sleep apnoea	✓				✓	
Hypertension, medication use	✓		✓	✓	✓	✓
Cardio vascular disease	✓				✓	
PCOS	✓					
Psychological screening:						
Bing eating disorder	✓					
Depression	✓					

(Table continues on next page)

Other psychiatric disorders	✓					
Bariatric surgery:	✓					
Surgery type		✓				
Surgery related complications		✓	✓	✓	✓	✓
Laboratory values:						
Insulin (mmol/L)	✓		✓	✓	✓	✓
Fasting glucose (mmol/L)	✓		✓	✓	✓	✓
HbA1c (mmol/mol)	✓			✓	✓	✓
Cholesterol (mmol/L)	✓			✓	✓	✓
LDL (mmol/L)	✓			✓	✓	✓
HDL (mmol/L)	✓			✓	✓	✓
Triglycerides (mmol/L)	✓			✓	✓	✓
Vitamin D (nmol/L)	✓			✓	✓	✓
Thyroid function	✓					
Kidney function	✓					
Quality of life questionnaires:						
Short Form 36 health survey	✓				✓	
Impact of Weight on Quality of Life-lite	✓				✓	
Eating behaviour questionnaires:						
Dutch Eating Behaviour Questionnaire	✓				✓	
Three Factor Eating Questionnaire	✓				✓	
Eating Disorder Examination Questionnaire	✓				✓	
Mood disorder questionnaires						
Positive and Negative Affect Scale	✓				✓	
Hospital Anxiety Depression Scale	✓				✓	

Table 2.2: Overview of data collected at the different study visits.

†These items are collected at the day of recruitment, so a participant was recruited post-surgery these items were collected post-surgery as well.

2.2.4 Sample collection

For the studies described in this thesis two research samples were collected per participant; a whole blood sample for genomic DNA extraction and another whole blood sample for RNA extraction. The samples were collected upon recruitment. If it was not possible to collect a blood sample for DNA extraction, a saliva sample was taken instead.

DNA and RNA sample collection and handling

Whole blood samples (BD Vacutainer, EDTA (k2)) were collected upon recruitment and in stored in a -20 freezer until processing for gDNA extraction. If a blood sample could not be retrieved for DNA extraction, a saliva sample was collected using the Oragene-DNA (500 OG) collection kit, Genotek Inc., Canada. Blood samples for RNA extraction were collected at the same time as the sample for DNA was collected, using PAXgene collection tubes (Preanalytix, Hombrechtikon, Switzerland).

2.3 Other cohorts used

2.3.1 Heideheuvel cohort

The Heideheuvel cohort was created by Dr Olga van der Baan and Dr Mieke van Haelst, and consisted out of 113 severely obese children (aged 10-18 years old) recruited at the Childhood Obesity Centre Heideheuvel, Paediatric Hospital Merem, Hilversum, The Netherlands. All children received lifestyle intervention, which is described into more detail in Appendix 2.2 (page 315).

Exclusion criteria to participate in this study were:

- Severe psychiatric disorder (e.g., schizophrenia, severe autism)
- Intellectual disability
- Obesity caused by endocrine disorders

- Use of medication that could cause significant weight gain or weight loss

EDTA blood samples were collected and DNA was extracted at the medical genetics department of the University Medical Centre Utrecht, The Netherlands.

2.3.2 NutriTech Cohort

NutriTech is a consortium of 23 international Partners [198] and is funded by the European Commission FP7 (2012-2015) [199]. The overall goal of the NutriTech project is to identify the effect of diet on phenotypic flexibility. As a part of this project a cohort of 74 healthy overweight to class I European Caucasian obese individuals (BMI range: 24.9-35.8 kg/m2) was created, for a human intervention study. Analysis performed on this cohort included genomics, transcriptomics, proteomics, metabolomics, laser scanning cytometry, NMR based lipoprotein profiling and advanced imaging by MRI/MRS.

This study was approved by the National Research Ethics Service Committee London (study number 12/L0/0139).

For this PhD, baseline characteristics in terms of height, weight, age, gender and ethnicity of this cohort was used. Saliva samples for DNA extraction were collected using the Oragene-DNA (500 OG, Genotek Inc.), and were processed by myself using the methods described in section 2.1.3. DNA samples were submitted for genome wide SNP analysis and WES as described in section 2.4.4 and 2.4.5 respectively. Genome wide SNP and WES data created for this cohort was used as a control dataset for WES analysis of super obese individuals described in chapter 6.

2.3.3 Obesity plus family

A final group of participants used in this thesis was a family with a Mendelian distribution of a complex obesity phenotype. This family was recruited by Dr Tony Goldstone at Hammersmith Hospital, Imperial College Healthcare NHS Trust, London UK. Clinical data from the proband and family members and

family history was collected by Dr Tony Goldstone. Whole blood samples were collected for DNA and RNA extraction and processed by myself as described in section 2.1.3.

This study was approved by the National Research Ethics Service Committee London – West London (study number 12/LO/0396) and National Research Ethics Service Committee London - Fulham (study number 07/Q0411/19). All individuals included in this study gave written informed consent.

2.4 Genetic analysis

2.4.1 Genomic DNA extraction

A proportion of the gDNA extraction was performed by LGC genomics, UK, using standard methods (*n*=224). The remaining gDNA samples were extracted from 3ml EDTA blood samples by me and another PhD student, Hanis N. Ramzi, using the Gentra-puregene Blood Kit, by Qiagen, Hilden Germany. DNA extraction was performed as per manufacturer's protocol, and included cell lysis, protein precipitation, DNA precipitation, ethanol wash and DNA hydration.

gDNA was extracted from saliva samples (collected using Oragene-DNA (500 OG) kits) following manufacturer's instruction, and included a nuclease incubation step, removing impurifications, DNA precipitation, ethanol wash and DNA hydration.

2.4.2 Sample QC

DNA was quantified using a spectrophotometer (NanoDrop Lite Spectrophotometer 120V, Thermo Fisher Scientific) before submitted to PCR. If quality or concentration was poor, a second EDTA blood sample (if available from the other study visits) was processed for DNA extraction. No further DNA preparations were necessary for PCR.

To prepare samples for the whole exome library preparation, 4µg of gDNA was diluted in a total volume of 50µl. Spectrophotometer measurements were used to ensure DNA was of good quality (with an OD 260/280 ratio between 1.8 and 2.0) and fluorometric quantitation was used to ensure the correct DNA concentration (Qubit, thermofisher scientific).

To prepare samples for genome wide SNP sequencing samples were normalised to 40ng/µl. Again spectrophotometer measurements were used to ensure DNA was of good quality (with an OD 260/280 ratio between 1.8 and 2.0) and fluorometric quantitation was used to ensure DNA concentration (Qubit, thermofisher scientific).

2.4.3 *MC4R* Sanger sequencing

To sequence the coding region of the *MC4R* gene the entire 999-pb coding region was amplified using two primer pairs. For amplicon 1 (635 bp) the following primers were used F1: TTTACTCACAGCAGGCATGG; R1: CCAACCCGCTTAACTGTCAT and for amplicon 2 (763bp) the following primers were used: F2: CATCACCCTATTAAACAGTACAG and R2: TACAATATTCAGGTAGGGTAAGA. PCR was performed on around 100ng DNA in a reaction mixture (using the GoTaq Flexi DNA polymerase kit, Promega) and run on a G-STORM GS4 thermal cycler (Somerton Biotechnology Centre, UK). Standard PCR setting were used with an annealing temperature of 53°C for amplicon 1 and an annealing temperature of 60°C for amplicon 2. All PCR products were run on a 1% agarose gel at 70V for 45 minutes, using electrophoresis, to inspect PCR products and exclude contamination.

PCR purification was performed using exoSAP-IT (USB products, Affymetrix, USA). Sequencing reactions were carried out by the MRC Core Genomics Laboratory, using an ABI 3730xl sequencer. The primers initially used in the PCR reaction were also used for sequencing.

Sequence data was analysed using the CodonCode Aligner software (CodonCode Corporation, US). Alignment was performed against the wild type sequence using an inbuilt feature of ClustalW.

Sequences suggesting nucleotide changes were verified by repeating the sequencing using the reverse primers initially used in the PCR reaction.

DNA samples of 46 participants of the PMMO cohort and two children from the Heideheuvel cohort were sequenced through collaborative work at the University Medical Centre Utrecht, The Netherlands, under their diagnostic service protocol. Conditions for this can be found in Mul, *et al*. [176] All other DNA samples were sequenced by me at Imperial College London.

For prediction of the consequences of the variants in the sequence, literature and genetic variation databases were searched for observations of the variants in normal weight controls and for loss of function in *in vitro* studies: when in the literature it was reported that the variant co-segregate with obesity in families and has been shown to influence the function of MC4R *in vitro*, mutation were considered to be pathogenic. Mutations were considered not to be pathogenic, when they were reported at a comparable frequency in lean and obese cohorts and did not appear to affect the function of MC4R in *in vitro* studies. If in the literature contradicting results were found, or if mutations were novel, *in silico* prediction programs were used (SIFT and PolyPhen [200,201]) to predict the mutations' deleteriousness. All variants were classified according to the HGVS criteria [88]: 1) no functional effect, 2) probably no functional effect, 3) variant of unknown significance (VUS), 4) probably affects function, and 5) affects function [88].

2.4.4 Whole exome sequencing

WES was performed on a total of 116 individuals for this thesis (43 super obese and 73 NutriTech individuals), in three batches all using the same protocol settings. For the first batch of 28 super obese samples I performed the sequencing library preparations with the assistance of Dr Anna Zekavati, while for the remaining samples (15 super obese and 73 NutriTech) library preparation was performed by Dr Anna Zekavati on collaborative basis. Sequencing was performed by the Genomics Laboratory, MRC Clinical Sciences Centre, Imperial College London, UK. Read quality control (QC), reference mapping and variant calling on the raw WES data was performed by Dr Michael Mueller, while CNV

calling was performed by Dr Alona Sosinsky and Dr Dalia Kasperaviciute, all as a part of collaborative work with the NIHR Imperial BRC Genomics Facility, Imperial College London, UK.

Exome capture and sequencing

Whole-exome sequencing libraries were prepared using SureSelectXT Human All Exon V4+UTRs (71Mb) (Agilent Technologies, Santa Clara, CA). To shear the genomic DNA into fragments of 150-200bp, DNA was sheared using a Covaris S220 instrument. To assess quality of the sheared DNA a Bioanalyzer DNA 1000 chip (Agilent Technologies) was used.

For each sample DNA-ends were repaired and ligated. Half of the adapter-ligated libraries volumes (remaining stored for possible future purposes) were amplified with 6 PCR cycles, after which another quality assessment step was performed using the Bioanalyzer.

Hybridisation of each amplified library individually (750 ng) took place using 120nt Biotinylated RNA baits, specific to regions of interest, in solution for 16 hours for target enrichment. Genomics DNA-bait hybrids were captured by magnetic streptavidin beads, followed by purification.

To amplify the captured libraries a 12 cycle amplification step was performed to add sample-specific index tags to each library for multiplex sequencing purposes following Agilent SureSelect instructions. Index-tagged libraries were purified, and a final quality assessment using the Bioanalyzer and the Library Quantification Kit (Kapa Biosystems) was performed. Four indexed libraries were multiplexed, loaded on a single lane of an Illumina flowcell (v3), and sequenced on a HiSeq25000 platform generating 100bp paired end reads (performed by the Genomics Laboratory, MRC Clinical Sciences Centre).

Bioinformatics

Read QC and reference mapping

The quality of sequencing data was assessed with FastQC version 0.10.0 *(http://www.bioinformatics.babraham.ac.uk/projects/fastqc)*. BWA mem version 0.7.2

(*http://arxiv.org/abs/1303.3997?context=q-bio*) was used to map sequencing reads to the GRCh37 (hg19) reference assembly of the human genome. To reduce false positive read mapping the hs37d5ss decoy sequences (ftp://ftp.1000genomes.ebi.ac.uk/vol1/ftp/technical/reference/phase2_reference_assembly_sequence/README_human_reference_20110707) obtained from the 1000 genomes project FTP server (ftp://ftp.1000genomes.ebi.ac.uk/vol1/ftp/technical/reference/phase2_reference_assembly_sequence/hs37d5.fa.gz) were included as mapping targets. After reference mapping, duplicate reads were marked with Picard tools version 1.85 (http://picard.sourceforge.net/).

SNV and short indel calling

Processing of mapped reads and calling of single nucleotide variants and short insertions/deletions was carried out with the Genome Analysis Toolkit (GATK) version 3.3: reads mapping to known indel regions were realigned using the GATK Indel Realigner to reduce false positive SNP calls resulting from mapping artefacts around indels. Base call quality scores were recalibrated using the GATK BaseRecalibrator.[202] Variants were called with GATK HaplotypeCaller algorithm and variant calling scores recalibrated with GATK VSQR. Quality filtered variants were annotated with Annovar release 2014Jul14.[203]

Copy number variation calling

Reads were filtered to retain only non-duplicate reads with a minimum mapping quality of 20 that mapped as proper pairs. Copy number variable exons were predicted from filtered reads with the Bioconductor R package ExomeDepth version 1.0.7.[204] Copy number was assessed across the Agilent SureSelect Human All Exon V4+UTRs target regions. Reference sets were selected from unrelated proband samples. Targets on the X chromosome were assessed using reference sets of same sex individuals.

2.4.5 Variant selection

Following the WES steps described above a vcf-file containing all variants (~20,000 per participant) in annotated format was created. Two separate variant selection pipelines were designed for further analysis; one to detect the contribution of variation within obesity genes to obesity in a cohort of 40 unrelated severe obese candidates (chapter 7), while a second pipeline was designed to detect the causal variant of Mendelian obesity in a family with a complex obesity phenotype (chapter 8).

Variant selection pipeline I:

A variant selection pipeline was designed for detecting the prevalence of high penetrance variants in known and candidate obesity genes (chapter 7).

Step I: Creating a list of (candidate) obesity genes:

A selection of 36 Mendelian human-obesity genes was made using OMIM and literature search. All genes and genomic regions reported to cause obesity in humans when disrupted, in a dominant or recessive mode of inheritance, were included (Appendix 2.3, page 319). A second list of candidate-obesity genes was created by enlisting all genes known to cause obesity or weight increase in mice once disrupted (*n*=165, Appendix 2.3, page 319). This list was based on a recently published review by Yazdi, *et al.* [205] enlisting genes known to affect weight in mice when disrupted or overexpressed. This list was updated for use in humans by retrieving human orthologs of the genes enlisted and subsequently excluding and including genes through a literature and mouse genome database (MGD) search [206].

Step II: Categorising variants according to their functional implications:

All non-synonymous variants found within the coding regions and UTRs of the selected (candidate) obesity genes were scaled according to PolyPhen2 (Hvar) and SIFT [200,201]. For PolyPhen the following scale was used: probably damaging (≥ 0.909), possibly damaging (0.447 ≤ pp2_hvar ≤ 0.909); benign (pp2_hvar ≤ 0.446). For SIFT the following scale was used: Deleterious (sift ≤ 0.05); tolerated (sift>0.05). For this analysis, all variants found in the obesity genes were assigned to the

following four categories (with category 1 variants expected to be least harmful and category 4 variants expected to be most harmful to concerning genes):

1) Synonymous variants: all coding variants not affecting amino-acid sequence.
2) Likely functional variants: all variants affecting amino-acid sequence (including nonsynonymous, frameshift, non-frameshift and nonsense mutations).
3) Deleterious variants: nonsynonymous variants, predicted-to-be deleterious by at least one of the two in silico prediction programs ('possibly damaging' and 'probably damaging' for PolyPhen, 'deleterious' for SIFT), and all nonsense and frameshift mutations.
4) Deleterious, rare variants: all deleterious variants that were rare (all variants with a MAF >0.01 in 1000G, ESPN and/or ExAC in any ethnicity group were excluded [207-209]).

Step III: Detecting Mendelian forms of obesity:

Group 4 variants in the 36 human-obesity genes, adjusted for mode of inheritance for the particular gene concerned, were classified as putative Mendelian. Each of these likely Mendelian variants was further explored by literature research, to assess whether they were likely to result in Mendelian obesity. All remaining variants were confirmed using Sanger sequencing (see section 2.4.6).

Step IV: Detect the contribution of variation in obesity genes to the extreme obese phenotype:

As previously reported, multiple rare variants can have a combined strong effect on phenotypes [210]. Therefore, frequencies of the different variant groups were examined, first for the human-obesity genes, then for the candidate-obesity genes, followed by a selection of genes taken from the human- and candidate-obesity lists, based on their tolerance to variation. Previous studies have shown a wide variety between genes and their tolerance to variation, with the expectation that genes with low tolerance are more likely to cause disease. Petrovski, *et al.* [211] have developed a Residual Variation Intolerance Score (RVI-Score), which can be applied to all human genes, to indicate how tolerant genes are to functional variation. This RVI-score was used to make a selection of obesity genes less tolerable to variation (en therefore possibly more likely to cause disease) by selecting all genes with a RVI-score

below 0, which left a remaining number of 94 (candidate) obesity genes less tolerable to functional variation (Appendix 2.3, page 319).

Step V: Detect novel human-obesity genes from the candidate-obesity list:

To uncover new human-obesity genes, a discrepancy was sought in variant frequency among the candidate genes in the super obese compared to the overweight controls, in an attempt to identify genes carrying significantly more damaging variants among the super obese. Since most deleterious variants among these genes were rare (and therefore unlikely to reach significant difference in frequency with the relative small number of participants included in this part of the study), a further selection was made of genes carrying highly likely to be damaging variants (frameshift or nonsense mutations) only present in super obese individuals in genes with low RVI-score, for further analysis. Further analysis included interpretation of functional effect of variants found, pathway analysis of genes involved and literature research.

Variant selection pipeline II (chapter 8)

A second variant selection pipeline was designed to identify the causal genetic defect of a complex obesity phenotype distributed in Mendelian fashion among a consanguineous family (as described in Chapter 8).

First all variants found in the family members included were screened against the human obesity-gene list described above and a similar list created for intellectual disability [31], to exclude known genetic causes of obesity and intellectual disability.

Further variant selection was based on the family history of consanguinity and the recessive pattern of the phenotype in the family. Therefore, all homozygous exonic variants present in the proband but not present or in a heterozygous state in the non-affected mother and sister were considered. Although it is most likely the mother will carry one copy of the causal variant (with the family history of consanguinity, and two affected siblings), variants not present in the mother were considered as

well. This to make sure to not miss (very rare, but not impossible) mechanisms such as uniparental disomy, or the less rare possibility of missing variants in WES data because of poorly covered regions. Although a homozygous variant is expected in the proband, because of the consanguinity of the parents compound heterozygous variants within the same gene were included as well, including CNVs covering regions mutated in the proband.

For the remaining variants all synonymous variants and variants with a minor allele frequency of 0.01 in the 1000 Genomes project phase 1 release (1000G), the NHLBI Exome Sequencing Project (NHLBI ESP) and Exome Aggregation Consortium (ExAC) were excluded [207-209]. Variants that were predicted-to-be benign by two out of three *in silico* prediction programs (SIFT, Polyphen2, PROVEAN1) were also excluded [200,201]. Only variants with a read depth of at least 4 were considered.

The remaining variants were assessed for likelihood of causing the phenotype by looking at the function of the gene the variant was found in, the predicted variant pathogenicity, the conservation of the gene and overall gene variation in 1000G, NHLBI ESP and ExAC.

2.4.6 Variant confirmation using Sanger sequencing

Sanger sequencing was used to confirm putative Mendelian forms of obesity in Chapter 7 as well as in Chapter 8. Primer pairs were designed for each variant using Primer 3 Web version 4.0.0. [212] and were checked for specificity using Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Primers used to amplify the different variant regions are given in Table 2.4.

To amplify the different regions covering the variants, a similar PCR set up was used for all:

- A master-mix (using the GoTaq Flexi DNA polymerase kit, Promega) was used to prepare PCR on three samples for each variant: The proband carrying the variant detected through WES, a positive control and a negative control.

- PCRs were run on an on a G-STORM GS4 thermal cycler (Somerton Biotechnology Centre, UK) with the following conditions listed in Table 2.3.

- The appropriate annealing temperature for each reaction was calculated by subtracting 5°C from the melting temperature (Tm) of the primer with the lowest Tm of the primer pair.

Steps	**Temperature**	**Time**	**Number of cycles**
Initial duration	95°C	2 min	1 cycle
Denaturation	95°C	1 min	30 cycles
Annealing	42°C-68°C	Calculated per reaction by multiplying 1 min per kb to be covered.	
Extension	72°C	1 min	
Final extension	72°C	5 min	1 cycle
Table 2.3: PCR thermal cycling conditions			

All PCR products were examined using gel electrophoresis, to inspect the quality of the PCR products, signs of unspecific binding and possibilities of contamination.

If the above approach did not deliver a satisfactory PCR product, annealing temperatures were adjusted accordingly. If this still did not lead to a satisfactory PCR product, PCR setting were reflected in depth for the specific region targeted and adjusted accordingly:

- To amplify the 447bp region covering the first exon of CPE (for variant confirmation for Chapter 8), PCR setting had to be adjusted for the high GC-content of the region, and therefore an increased denaturation temperature of 98°C was used with a subsequent annealing temperature of 63°C**.**

Gene (variant)	Chapter	Cohort	Primers	Product size	Annealing temperature
SH2B1 (p.Gly545Ser)	Chapter 7	severely obese	F: AGGTACCGGAGGTGTGAGTG R: AAATGGACTGGAACCACAGG	619bp	62°C
SH2B1 (p.Pro16Arg)	Chapter 7	severely obese	F: CCTCGTGTGTGCCTCTCTCT R: CGTGGGACTCACAGAACTCC	214bp	59°C
SH2B1 (p.Ser410Phe)	Chapter 7	severely obese	F: CAGCACCATCTTCCCTGTCT R: CTAAGGCTCCACCCTTACCC	220bp	61°C
IGSF1 (p.Arg1295Ter)	Chapter 7	severely obese	F: ACCACCTGGTTCACAGAAGG R: AGAGTGTGGGGCAATACCAG	252bp	63°C
LEPR (p.Ser389Asn)	Chapter 7	severely obese	F: TGGTGGATGAATTTAGCTGAGA R: GGCATTCATGTTCATTGCAG	154bp	63°C
LEPR (p.Ser1014Cys)	Chapter 7	severely obese	F: AGGACGAAAGCCAGAGACAA R: AAATGCCTGGGCCTCTATCT	185bp	59°C
NTRK2 (p.Ile741Val)	Chapter 7	severely obese	F: GTGACTGATGCCTCCCTGTT R: AGACCCATTGCACACCTCAT	211bp	62°C
NTRK2 (p.His638Leu)	Chapter 7	NutriTech	F: AAGGATGCCAGTGACAATGC R: TGAACCCTCCACTCCTGAAC	235bp	62°C
SIM (p.Ile313Val)	Chapter 7	NutriTech	F: CGTGAGGACATAGTTGACGC R: CTCCTGTCTCTCCGTCAGTG	167bp	62°C
CPE (p.Glu26ArgfsTer68)	Chapter 8	Obesity plus family	F: GGAAGGTGAGGCGAGTAGAG R: CCCTTACCAGGCTCATGGAC	447bp	63°C

Table 2.4: Primer sequences used for confirmation analysis. Primers and annealing temperatures that were used to amplify each region covering a variant (listed in the first column) are listed here. F, forward primer; R, reverse primer.

All PCR products were purified using exoSAP-IT (USB products, Affymetrix, USA) and sequenced in both directions using the similar primers used to amplify the region. Sequencing reactions were carried out by the MRC Core Genomics Laboratory, Imperial College London, using an ABI 3730xl sequencer. Sequencing products were aligned to a wild type reference using CodonCode Aligner software (CodonCode Corporation, US).

2.4.7 Family segregation analysis

To enable family segregation analysis, blood and saliva samples were collected from the family members of the proband described in chapter 7. All family members were screened for the *CPE* p.Glu26ArgfsTer68 variant using the same PCR and sequencing methods described above. Phenotypes were compared to the genotype per family individual, to confirm the homozygous state of this variant was not found in healthy family members (which would exclude causality of this variant).

2.4.8 Genome-wide SNP analysis

Forty of the super obese included for WES analysis (excluding the 3 samples used for family analysis in chapter 6) and the 74 NutriTech individuals, were submitted for genotyping. Genotyping was performed by the High Throughput Genomics, Wellcome Trust Centre for Human Genetics, University of Oxford, using Illumina HumanOmniExpress arrays.

Samples were processed in two batches using the Infinium HTS assay (Illumina Inc., San Diego, California, USA) on HumanOmniExpress-24v1.0 BeadChips. The arrays consist of >700,000 markers with genome-wide coverage.

Quality controls of genotyping, population structure analysis and CNV calling were performed by Nikman A. Nor Hashim as a part of his own PhD, and are therefore not further covered in this thesis. His population structure and CNV data were, however, essential for verifying the ethnicity of individuals included for WES in chapter 7 and to confirm the findings of a large CNV found through exome read depth analysis.

2.4.9 RNA expression analysis

RNA was extracted from whole blood samples for RNA quantitative expression analysis was used for functional analysis in chapter 8, while Sanger sequencing of cDNA was used in chapter 7 to confirm variants and deletions found through WES. RNA extraction, reverse transcription and quantitative PCR was performed under close supervision from Dr Jess Buxton.

Total RNA was isolated from whole blood samples collected with PAXgene tubes (Preanalytix, Hombrechtikon, Switzerland). The PAXgene blood RNA kit (Qiagen Ltd, Manchester, UK) was used to extract the RNA following manufacturer's instructions. Reverse transcription to obtain cDNA was carried out with 500ng total RNA using the RT^2 Easy First Strand kit (Qiagen Ltd), which includes an initial step to ensure all genomic DNA is eliminated.

Quantitative PCR to detect expression of *CPE*

Quantitative PCR was used to examine *CPE* expression in total RNA from whole blood samples of the proband and heterozygous sister, and to assess whether detected levels were comparable to matched controls. Six controls were selected from the PMMO cohort, matched for BMI, age, gender and T2DM. All were screened using the Sanger sequencing methods described above to exclude carrier status of the *CPE* deletion.

Quantitative PCR was performed on each sample in triplicate, on a CFX384 real-time PCR detection system (Bio-Rad Laboratories, Hemel Hempstead, UK), using RT² SYBR Green qPCR Mastermix with primer assays for *CPE* (NM_001873, which amplifies a 90bp product within exon 8 and 9 (Figure 2.2) and the housekeeping gene *HPRT1* (NM_000194) (Qiagen Ltd). Relative expression levels for the proband, sibling and six control samples were determined using the ΔΔCt method using a common reference sample [213].

Figure 2.2: mRNA of *CPE*. The coding region of mRNA *CPE* is notated in non-italic capitals. Start of each exonic region is underlined and marked with respective exon number. The 23bp deletion discovered in the family reported here is noted with '76-98 deletion'. The * marks the location where the stop codon is expected due to the frameshift caused by the deletion. Highlighted in grey is the area covered by the primer assay used for the mRNA expression analysis.

Sanger sequencing of cDNA for *NTRK2*-deletion confirmation

A predicted deletion of exon 19 of the *NTRK2* gene was detected through read depth analysis of WES data. Since WES only covers exonic regions (and limited untranslated regions surrounding the exons), the breakpoint locations of the deletion were not known. Therefore, the size of the deletion could be anywhere between 71.5 kb (distance between exon 18 and 20) and 235 bp (size of exon 19, Figure 2.2).

To confirm the predicted deletion, primer pairs were designed to cover exon 19 in cDNA using PCR (one forward primer located within exon 18, one reverse primer located within exon 20, and a second forward primer overlapping partly exon 18 and 20, Figure 2.2): F1: TGACCAACCTCCAG CATGAG, F2: CAAGTTCCTCAGGTCGGTGGC, R1: AAATCTCCCACAACACGACC. PCR was run on cDNA from the participant with the predicted deletion, one control cDNA and one control gDNA sample. Standard PCR settings were used as described above and the different thermal cycling settings applied are given in Table 2.5.

Breakpoint mapping for the *NTRK2*-deletion

A second attempt to cover this large region was by long-range PCR. Nine primers were designed (three forward and six reverse, to be combined into 18 different combinations) to cover this 71.5 kb region (figure 2.2 ant Table 2.6). The LongAmp Taq PCR kit (New England, Biolabs) was used to set up the reactions, with 5 µl Lonamp Taq buffer, 0.75 µl 10 mM dNTPS, 1 µl forward primer, 1 µl reverse primer, 1 µl LonAmp Taq polymerase, 6.25 µl nuclease free water and 10 µl of DNA sample per reaction. Reactions were run on a G-STORM GS4 thermal cycler (Somerton Biotechnology Centre, UK) with different annealing temperatures (Table 2.5).

Figure 2.3: *NTRK2*-deletion primer design. A schematic overview of exons 18-20 of *NTRK2*.The breakpoints of the predicted deletion of exon 19, could be located anywhere in the 6.8kb and 60kb region noted in between the exonic regions. Primer locations used for PCR on cDNA (in green) and for long-range PCR on gDNA (in blue) are schematically represented in this figure.

Steps	Temperature	Time	Number of cycles
settings PCR of cDNA			
Initial duration	95°C	2 min	1 cycle
Denaturation	95°C	1 min	30 cycles
Annealing	50°C-65°C	1 min	
Extension	72°C	1 min	
Final extension	72°C	5 min	1 cycle
settings long-range PCR of gDNA			
Initial duration	95°C	2 min	1 cycle
Denaturation	95°C	1 min	30 cycles
Annealing	50°C-65°C	1 min	
Extension	72°C	10 min	
Final extension	72°C	10 min	1 cycle

Table 2.5: PCR thermal cycling conditions

F1	AGAACTCCCCTCCCTCAGATGATGG
F2	GTGTCAGTCCTCCTCACATCAATGCC
R1	AAATCTCCCACAACACGACC
R2	TGTTGCTTCAGGTTACCTCCCACATG
R3	CTACAGGAAACAGTGGGGTGGAAGC
R4	AGCCAGAGTCCCAGCTTGTATCAAAA
R5	CAGGGAAAGGGAGAGAGATGGCAAAT
R6	CTCTCAGGAAAGTCAAGGGTCATGGT

Table 2.6: Primer sequences for long-range PCR

2.5 Statistical analysis

All continuous variables were tested for normality (Kolmogorov-Smirnov and Shapiro-Wilks tests). Visual inspection of the variables distribution was explored by histograms, Q-Q plots and box plots to verify normality. Continuous data is presented as mean with standard error of the mean or standard deviation or as median and interquartile range, where appropriate. Categorical variables are summarised with the use of frequencies.

In each chapter of this thesis, differences between groups at baseline were compared using Student's T-test (ANOVA for more than 2 groups) or Mann Whitney U test (Kruskal-Wallis test for more than 2 groups) for non-parametric data. Comparisons of categorical variables used Chi-square test.

Questionnaire analysis

Comparison analysis for each questionnaire scale between different baseline factors was performed using ANCOVA, with gender, age, BMI, T2DM status and ethnicity included as covariates.

Weight loss measurements

Differences within groups were assessed by paired Student's T-test (2 measurements only) or repeated measurements ANOVA (multiple measurements).

Differences between groups in weight loss trajectories were initially assessed using mixed measures ANOVA, with correction for baseline BMI, gender, age, ethnicity and T2DM status. However, due the relative large number of missing data (caused by the still ongoing follow-up of a selection of participants, and loss to follow up), further analysis was performed on singular measure time-points following treatment intervention:

To assess what weight loss metric was best to use to correct for the differences in BMI at baseline, the different weight loss metrics used in the literature (listed in table 2.7) were compared. ANOVA was used in order to analyse the WL metric differences between the different obesity classes for 12 months

follow up data. A linear regression model was used to find correlation between each WL metric and preoperative BMI.

Weight loss metric:	Abbreviation:	Calculation:
Body mass index	BMI	Baseline measurements were ignored and the final measured BMI at one year follow up was used.
Percentage weight loss	%WL	$\left(\frac{\text{(12 ms weight - BL weight)}}{\text{BL weight}}\right) \text{x } 100$
BMI change	△BMI	$\text{(12 ms BMI - BL BMI)}$
Percentage excess body weight loss	%EBWL	$\left(\frac{\text{((25 x height}^2\text{) - BL weight)}}{\text{(12 ms weight - BL weight)}}\right) \text{x } 100$

Table 2.7: Weight loss metrics analysed for the PMMO cohort. Measurements were calculated for 12 months follow up data. 12 mths, measurement collected at 12 months following surgery; BL, measurement collected at baseline.

For weight change following lifestyle intervention in children (Chapter 6), BMI standard deviation scores (BMI-SDS) were calculated for each time-point, to correct for differences in age, gender and height. BMI-SDS was calculated using the age- and sex-normative data from the Dutch National Growth Study of 1997 [214].

Changes beyond weight loss following bariatric surgery

Chi-square tests were used to evaluate the changes in the proportion of diabetes participants with an HbA1c value of ≤48 mmol/mol, or classified as hypercholesterolaemia (total cholesterol of >6.15 mmol/L). Mixed measures ANOVA, with correction for baseline BMI, gender, age, ethnicity was used to analyse continues laboratory measurements and comparison analysis between the surgery groups.

Changes in questionnaire data following surgery within groups were assessed by paired T-test, while differences between surgery groups were assessed using repeated measures ANOVA, with correction for baseline BMI, gender, age, ethnicity and T2DM status.

Multiple regression analysis was used to examine whether baseline characteristics had a predictive value towards %WL seen following RYGB or VSG at 12 and 24 months following surgery.

High scorers and low scorers for the "monogenic-obesity-like" risk score were compared using Ancova, corrected for BMI, gender and ethnicity, while binominal data were compared using chi-square.

Variation analysis in obesity genes

Frequencies of Mendelian disease and rare, deleterious variants were compared using Fisher's exact test, while mean number of variants per functional category were compared using student's T test.

Throughout the thesis, a p-value of <0.05 was considered significant, and multiple comparison analysis was corrected post-hoc using Bonferroni and listed with the results when applied.

The statistical analyses were carried out using IBM SPSS Statistics for Windows, Version 20.0. released 2011 (IBM Corp. Armonk, NY).

2.6 Schematic overview of the methods used in the different chapters

	Chapter 3: Creation of PMMO cohort	Chapter 4: Bariatric surgery outcomes	Chapter 5: The effect of MC4R variants on weight loss in patients undergoing bariatric surgery	Chapter 6: The effect of MC4R variants on weight loss in children undergoing intensive lifestyle treatment	Chapter 7: High penetrant variants in obesity genes	Chapter 8: Discovery of a new form of Mendelian obesity and diabetes in humans
Cohort used:						
PMMO cohort	✓	✓	✓	-	✓	-
Heideheuvel cohort	-	-	-	✓	-	-
NutriTech cohort	-	-	-	-	✓	-
Obesity plus families	-	-	-	-	-	✓
Clinical Phenotypes used:						
Anthropometric measurements	✓	✓	✓	✓	✓	✓
Medical history	✓	✓	✓	-	✓	✓
Family history	-	-	-	-	✓	✓
Weight history	✓	✓	✓	-	✓	✓
Questionnaire data	✓	✓	-	-	✓	-
Weight loss trajectories	-	✓	✓	✓	✓	-
Genetic analysis used:						
DNA preparation	-	-	✓	-	✓	✓
MC4R sequencing	-	-	✓	✓	✓	✓
Whole Exome sequencing	-	-	-	-	✓	✓
RNA expression analysis	-	-	-	-	✓	✓

CHAPTER 3

CREATION OF THE PMMO COHORT

3.1 Introduction

This chapter describes the recruitment and baseline characteristics of a large cohort of bariatric surgery patients for study.

Bariatric surgery is the most successful treatment currently available for obesity. Although the number of patients undergoing this invasive treatment is increasing, the selection criteria within the UK to undergo bariatric surgery are still stringent. Only individuals with a BMI of >40 kg/m2, or with a BMI of >35 kg/m2 and a major obesity-related comorbidity (such as T2DM, OSAP or infertility problems) are currently eligible to undergo bariatric surgery, according to up-dated NICE guidelines (2014) [215]. Besides fulfilling these basic criteria, patients are expected to have undergone stringent lifestyle adjustments in attempt to lose weight, and failed at these multiple times. Therefore, it can be expected that patients undergoing bariatric surgery within the NHS healthcare system, are among the most severely-affected obese individuals within the UK.

The general population can be categorised into five groups according to BMI: underweight (BMI < 18.5 kg/m^2), normal weight (BMI: 18.5 - 24.9 kg/m^2), overweight (BMI: 25 - 30 kg/m^2), class I obesity (BMI: 30.0-34.9 kg/m^2), class II obesity (BMI: 35.0 - 39.9 kg/m^2) and class III (BMI ≥40 kg/m^2). [2] Since the distribution of BMI among the bariatric population exceeds this BMI range, with often a mean BMI of over 45 kg/m^2, two additional classes are often used in describing the severely obese individuals in bariatric cohorts: class IV or super obesity (BMI 50.0-59.9 kg/m^2) and Class V or super-super obesity (≥60 kg/m^2). In order to create a cohort of severely obese individuals to identify the prevalence and novel genetic factors related to obesity, bariatric patients represent an excellent target population. Investigation of this patient group would not only enable identification of the most extreme obese individuals and their phenotypic characteristics, it also immediately provides directly clinical applicable outcomes on treatment options.

Although the prevalence of the severely obese is increasing [5], there is still much to learn about the phenotypes correlating with extreme obesity, including the prevalence of co-morbidities, psychological disorders, or best treatment options.

The main aim of this part of the work was to create a cohort of severely obese participants, to identify the phenotypic features related with the most severely obese, providing a baseline by which to measure treatment success.

The cohort reported here is a part of an ongoing clinical trial named Personalised Medicine of Morbid Obesity (PMMO) [180,181]. Recruitment and follow-up of participants is still ongoing. Here I have reported the results so far, with a specific focus on obesity-related phenotypes and features of the cohort relevant to the following chapters.

3.2 Aims of the study

1) To create a cohort of morbidly obese participants undergoing bariatric surgery for genotype-phenotype correlation analysis.
2) To determine the anthropomorphic, clinical and psychological characteristics of this population group, providing a baseline for assessment of treatment success (which is the subject of Chapter 4 of this thesis).
3) To determine baseline characteristics of those with the most extreme obesity (BMI >50 kg/m^2, and BMI >60 kg/m^2).

3.3 Results

3.3.1 Recruitment data

A total of 1080 participants had been recruited into this study in time for preparation of this analysis. Figure 3.1 gives an overview of the recruitment numbers and the proportion lost to follow-up at the different stages of the study. Of this overall number, 36 participants were recruited at the Chelsea Westminster Hospital NHS healthcare centre, 42 participants were recruited at the Derby Royal Hospital NHS healthcare centre, and the remaining 1002 participants were recruited at the Imperial College NHS Weight Centre. For all participants, baseline data was collected, but a small number had to be excluded from further analysis because of crucial baseline data that was missing, such as height or weight (*n*=5).

In total 466 participants were recruited before undergoing surgery and the clinical data was collected in a prospective manner, while 658 participants were recruited post-surgery, in which case the data was collected retrospectively from their clinical notes. Of all participants recruited, 46 were fitted with an adjustable gastric band (AGB), 551 had a Roux-en-Y gastric bypass (RYGB) and 352 had a vertical sleeve gastrectomy (VSG). 124 participants did not undergo surgery; some were still awaiting a surgery date (*n*=47), while the other participants will not undergo bariatric surgery treatment for multiple reasons (including change of mind: because of the risks of the surgery or personal circumstances that were not suitable to support major surgery-, not meeting NICE criteria, or they were lost to follow-up). A loss to follow-up was seen following all three surgery types, with 2.2%, 13.0% and 9.7% lost to follow up at 12 months following surgery for AGB, RYGB and VSG, respectively. At 24 months this increased to 6.5%, 31.1% and 31.7%, respectively. Since the study that these data was collected from is still ongoing, some participants were still awaiting their follow up visits at the time of writing this thesis (24 months, *n*=313; 12m months, *n*=110; and 24 months, *n*=23).

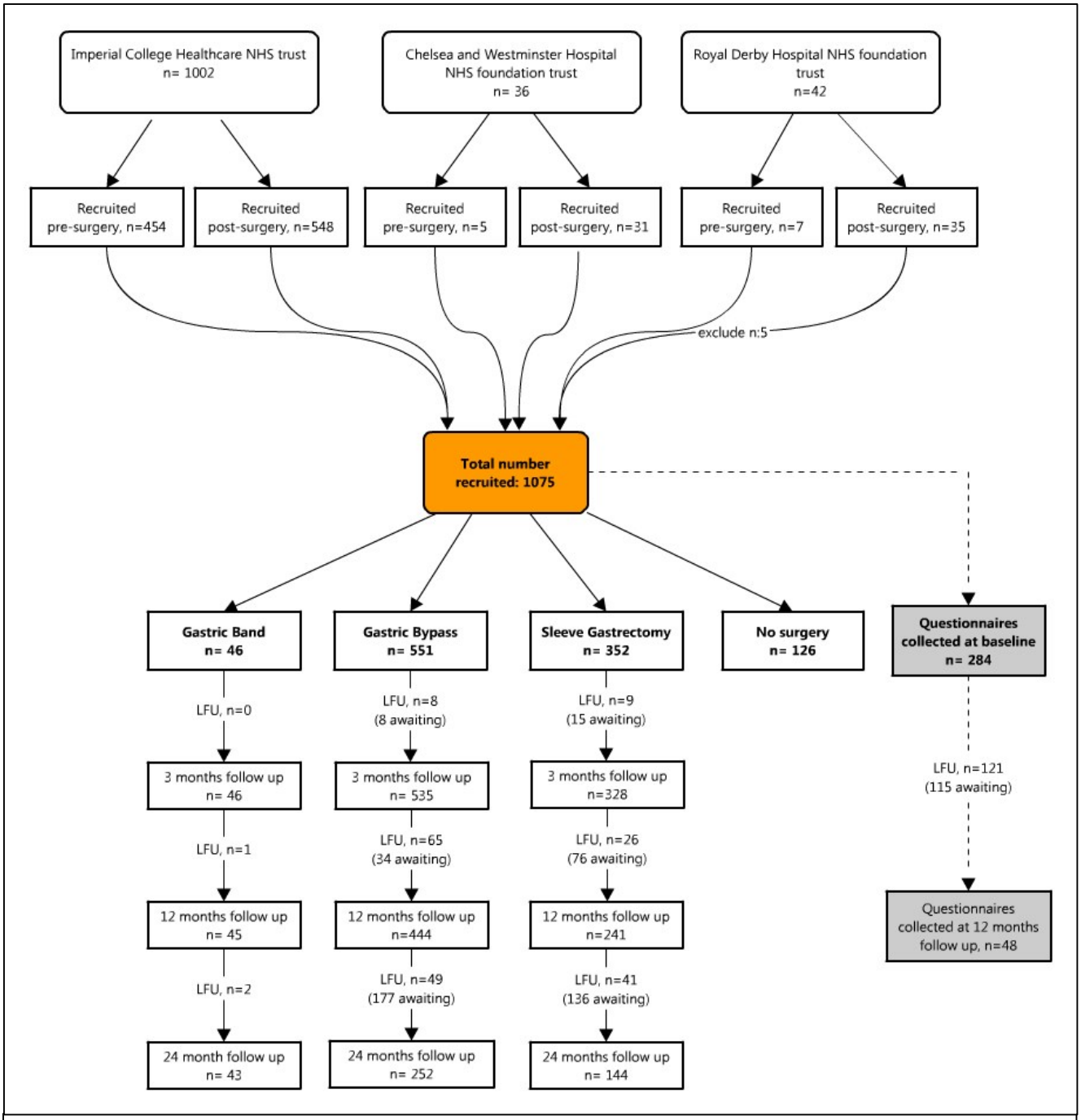


Figure 3.1: Recruitment flowchart of the PMMO study. Research participants recruited at the different study sites, before or after undergoing bariatric surgery. LFU; lost to follow up. Follow up times indicate the time after surgery date. 'Awaiting' indicates the number of participants that are still awaiting their follow-up visit in the ongoing study.

3.3.2 Clinical measurements

Baseline characteristics are summarised in Table 3.1. The participants in the cohort had a mean age of 45.6 (± 11.1 SD), a mean BMI of 48.1 kg/m^2 (± 8.67 SD), a mean weight of 134.3 kg (± 28.1 SD) and 74.0% were female.

The distribution of BMI showed skewness in positive direction (Figure 3.2). This is most likely caused by the 'floor effect' created by the eligibility criteria of undergoing bariatric surgery in the UK: a minimum BMI of 35 kg/m^2 is normally required to be eligible for surgery, while there is no upper limit of BMI. As seen in Figure 3.2, only a very few individuals with a BMI below 35 kg/m^2 at recruitment were included (all had a weight-history of BMI >35 kg/m^2).

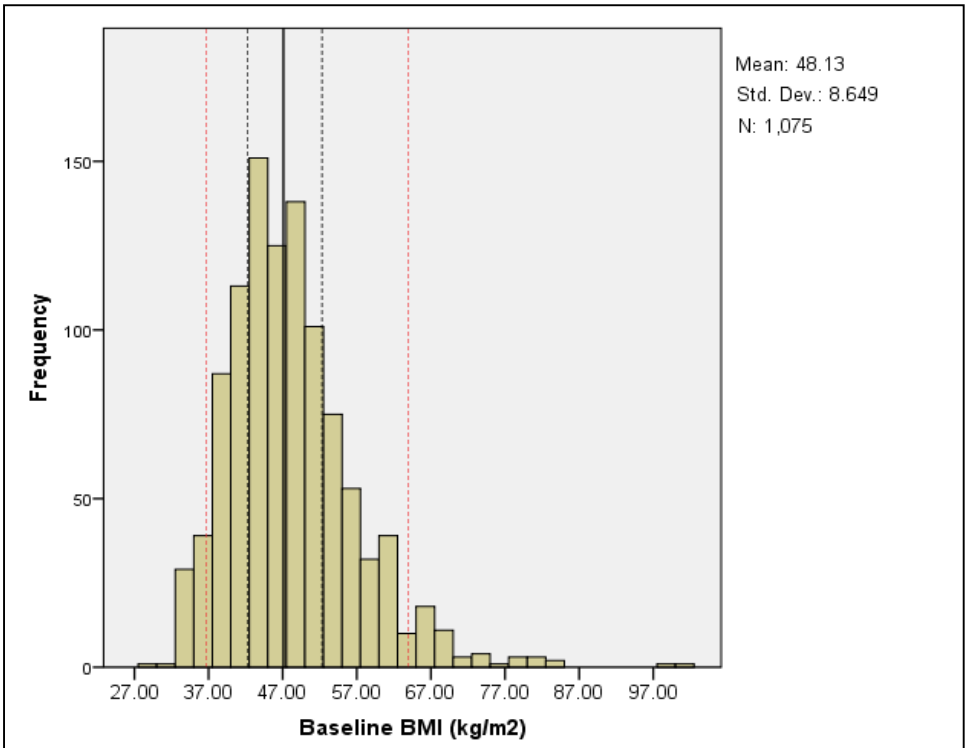


Figure 3.2: Histogram showing distribution of BMI. Vertical black lines indicate the quartiles. The red vertical lines indicate the 95th percentiles. The distribution shows a significant positive skewness (*p*: <0.05). BMI: body mass index.

BMI levels did not significantly differ among the participant groups opting for the different surgery types (Table 3.1). Off all participants included in the PMMO cohort, almost a quarter (24.3%) were classified as having class IV obesity (super obesity) with a BMI ranging between 50.0 and 59.9 kg/m^2, and 8.9% had class V obesity (extreme obesity), with a BMI of ≥ 60 kg/m^2.

An average prevalence of 37.9% was seen for T2DM in the cohort, with a significantly higher prevalence in the particpants undergoing RYGB compared to the other surgery types (reflecting the clinical efficacy of this surgery type for diabetes). As expected, the mean HbA1c levels were significantly higher in the participants with T2DM, compared to the participants without T2DM (63.22 vs. 39.39 mmol/mol, p <0.000), indicating a poor control of glucose levels in the diabetic participants. Indeed only about a quarter (24.8%) of the individuals diagnosed with T2DM had an HbA1c value of ≤ 48 mmol/mol (a level which generally can be interpreted as an acceptable glycaemic control in diabetic patients [216]). In total, six individuals with type 1 diabetes mellitus were included, of whom 4 underwent RYGB.

A high prevalence of other co-morbidities related to obesity were seen in the cohort, with a significantly higher prevalence of mobility problems, binge eating disorder (BED) and depression rates seen in the participants undergoing VSG.

Almost half of the cohort (45.8%) had a lifetime history of depression and were still under active treatment for this at the day of recruitment (in the form of counselling and/or anti-depressant medication), or received such treatment in the past. A more detailed overview of the prevalence of different psychiatric disorders seen in the cohort can be found in Appendix 3.1 (page 329).

Forty-four participants had revisional bariatric surgery after their initial surgical treatment, because of insufficient weight loss (revision from initial AGB to either VSG or RYGB: n=27; revision from initial VSG to RYGB: n=17).

There was a high selection bias in the participants that underwent adjustable gastric banding. A large proportion of this group was recruited post-surgery at a specific clinic which they attended because of complications following surgery or insufficient weight loss. Therefore, they are not representative of the wider patient group undergoing adjustable gastric banding and, therefore, were excluded in the comparison analysis for surgery outcomes. Baseline characteristics are given but should be interpreted with caution in consideration of the high selection bias.

		Complete cohort		Different surgery groups				
				Gastric band	*Gastric bypass*	*Gastric sleeve*	*No surgery*	*P-value*
Number		*1075*		*46*	*551*	*352*	*126*	---
Gender (% female)		74.0		84.8	74.0	73.3	72.6	0.289
Age		45.6 (± 11.1)		45.1 (± 11.2)	45.7 (± 10.7)	45.1 (± 12.0)	47.7 (± 12.0)	0.081
Height		1.67 (± 0.09)		1.663 (± 0.09)	1.674 (± 0.09)	1.668 (± 0.10)	1.670 (± 0.10)	0.692
Weight		134.3 (± 28.1)		123.2 (± 21.53)	134.1 (± 25.00)	137.0 (± 31.76)	131.2 (± 31.12)	0.009[a]
BMI		47.1 [42.3-52.3]		**44.4 [41.1-49.8]**	47.3 [42.9-52.3]	**47.5 [42.7-53.8]**	**45.9 [39.9-51.5]**	0.033[b]
Age of onset obesity <10 years old (%)		36.2		26.9	36.5	37.0	35.7	0.721
Type 2 diabetes - **T2DM (%)** - **ITT2DM (%)**		 37.9 9.0		 **19.6** **0**	 **41.6** **12.2**	 **23.0** **3.7**	 38.7 10.5	 0.000 0.000
Type 1 diabetes (n)		6		0	4	1	1	---
Obstructive sleep apnoea (%)		27.2		22.0	27.0	30.9	19.7	0.720
Requires walking aid or wheelchair (%)		12.5		2.2	**10.8**	**18.3**	14.5	0.004
PCOS (% of females)		20.5		18.5	20.0	23.5	14.9	0.871
Binge eating disorder (%)		16.4		18.2	**12.6**	**21.2**	20.0	0.010
Depression (%) **History of depression (%)**		23.0 45.8		25.7 51.4	**19.2** 41.9	**27.8** 56.8	26.3 39.0	0.017
History of psychiatric disease (other than depression) [¥] **(%)**		6.7		2.9	6.4	8.2	7.0	0.067
Hypothyroidism (%)		9.7		10.3	9.6	9.6	9.9	0.993
Hypercholesterolemia (%)		33.3		28.2	37.9	25.0	36.9	0.238
Blood phenotypes:								
Participants with T2DM:	**HbA1c (mmol/mol)**	63.2 (± 18.3)		52.8 (± 9.4)	**65.6 (± 19.0)**	**57.1 (± 15.6)**	63.9 (± 18.2)	0.005[c]
	HbA1c ≤ 48 mmol/mol (%)	24.8		50.0	18.9	40.0	27.3	0.000[c]
Participants without T2DM:	**HbA1C (mmol/mol)**	39.4 (± 5.1)		41.3 (± 3.1)	39.4 (± 5.4)	39.5 (± 5.0)	38.3 (± 3.8)	0.184
	Insulin	29.9 (± 44.25)		27.7 (± 20.88)	25.9 (± 27.88)	35.0 (± 58.35)	22.2 (± 20.76)	0.345
	Fasting glucose	5.1 (± 1.50)		5.0 (± 0.63)	5.1 (± 2.08)	5.1 (± 0.82)	4.9 (± 0.70)	0.885
Participants on statin treatment:	**Cholesterol (mmol/L)**	4.51 (± 1.18)		4.84 (± 1.12)	4.40 (± 1.18)	4.67 (± 1.19)	4.42 (±0.75)	1.000
	Triglycerides (mmol/L)	2.40 (± 2.19)		2.15 (± 0.67)	2.26 (± 1.71)	2.33 (± 2.38)	1.79 (±0.75)	1.000
	LDL (mmol/L)	2.49 (± 1.01)		2.53 (± 0.93)	2.42 (± 1.03)	2.65 (± 1.02)	2.55 (± 0.85)	1.000
	HDL (mmol/L)	1.13 (± 0.40)		1.32 (± 0.25)	1.12 (± 0.45)	1.11 (± 0.29)	1.25 (± 0.37)	1.000

(Table continues on next page)

		Complete cohort		Different surgery groups				
				Gastric band	*Gastric bypass*	*Gastric sleeve*	*No surgery*	*P-value*
Blood phenotypes:								
Participants not on statin treatment:	**Cholesterol (mmol/L)**	5.06 (± 0.95)		5.26 (± 1.24)	5.13 (± 0.95)	4.97 (± 0.93)	4.99 (± 0.89)	1.000
	Triglycerides (mmol/L)	1.72 (± 1.05)		1.63 (± 0.71)	1.74 (± 1.23)	1.71 (± 0.85)	1.57 (± 0.61)	1.000
	LDL (mmol/L)	3.11 (± 0.85)		3.21 (± 1.05)	3.20 (± 0.86)	3.00 (± 0.83)	3.15 (± 0.79)	1.000
	HDL (mmol/L)	1.22 (± 0.56)		1.30 (± 0.29)	1.20 (± 0.54)	1.19 (± 0.42)	1.22 (±0.38)	1.000
Ethnicity:								
- British (%)		49.2		56.5	51.5	44.0	50.4	0.190
- Irish (%)		2.1		2.2	2.2	2.6	0.8	
- Other Caucasian (%)		9.5		10.9	9.3	9.4	10.2	
- Caribbean (%)		5.8		4.3	6.0	5.4	6.3	
- African (%)		3.5		2.2	2.7	5.7	1.6	
- Any other black background (%)		0.7		2.2	0.7	0.9	0	
- Indian (%)		4.7		4.3	4.9	4.5	4.7	
- Pakistani (%)		1.2		0	0.7	2.0	1.6	
- Bangladeshi (%)		0.3		0	0.2	0.6	0	
- Any other Asian background (%)		2.3		2.2	0.9	3.4	5.5	
- White and Black Caribbean (%)		1.9		0	2.0	2.0	1.6	
- White and Black African (%)		0.6		0	0.7	0.6	0	
- White and Asian (%)		0.7		2.2	0.5	0.9	0.8	
- Any other mixed background (%)		1.0		2.2	0.4	2.0	0.8	
- Any other (%)		16.4		10.9	17.1	16.2	15.7	

Table 3.1: Baseline characteristics of PMMO cohort. Data is presented as mean (± SD) and median [IQR], unless otherwise indicated. BMI, body mass index; T2DM, type 2 diabetes mellitus (on oral glucose lowering medication and/or insulin); ITT2DM, insulin-treated type 2 diabetes mellitus.

[¥] Prevalence of different psychiatric disorders can be found in Appendix 3.1. [A] Not significant between groups after Bonferroni post-hoc corrections. [B] Significant between gastric band and gastric sleeve, and gastric band and no-surgery group. [C] Significant between gastric bypass and gastric sleeve group. Highlighted in red are the mean difference which are significant at <0.05 level, following post-Bonferroni corrections.

3.3.3 Extremes of Obesity

To identify how the most extreme obese individuals within this cohort differ phenotypically from the remaining morbidly obese cohort, a comparison analysis was performed between the different obesity classes (II – V) (Table 3.2).

The ethnic background of the class IV and V obese individuals differed from the remaining cohort, in that they were more likely to be European Caucasian (72.8% and 74.0% respectively vs 61.2% in the overall cohort, p <0.0000).

As expected the prevalence of obstructive sleep apnoea and percentage of individuals depending on walking aids or a wheelchair for their mobility was highest among the class V obese (46.7% vs 27.6% and 26.2% vs 12.5% respectively). Interestingly the prevalence of early onset obesity increased with increasing BMI, from 25.3% in class II obese to 64.1% in class V obese (*p:* <0.000, indicating early onset obesity might result in higher BMI in adult life).

The prevalence of T2DM was highest among the class II and V obese (most likely due to selection bias in the obesity class II group). More unexpected was the decrease in prevalence of hypercholesterolemia with each increasing obesity class, with the highest prevalence in the class II obese (44.9%) and the lowest prevalence in the class V obese (22.9%, *p:* 0.005). Since in previous studies it was reported this feature is seen more pronounced in males, analysis was repeated with segregation for gender. Segregation was also used to correct for the two diagnostic criteria used for hypercholesterolemia, one being the use of statin treatment upon recruitment and the other being newly diagnosed cases with a total cholesterol levels of >5.18 mmol/mol.

Segregation analysis for gender and diagnostic criteria showed that the correlation was indeed stronger in males (Figure 3.3 and Appendix 3.2 [page 330]). Although all cholesterol measurements (total cholesterol, LDL, HDL and triglycerides) were lower with increasing BMI class, the only significant difference was seen in males not on statin treatment for triglycerides (*p*: 0.009 Appendix 3.2).

Selection bias is less likely to occur on basis of hypercholesterolemia in itself compared to T2DM, since it is not considered a comorbidity that make individuals eligible for bariatric surgery according to NICE guidelines [215], and it does not have any direct clinical symptoms when not treated. A possible in-direct selection bias through T2DM could explain the higher prevalence of hypercholesterolemia in the lower obesity classes, if hypercholesterolemia had a higher occurrence among the participants also diagnosed with T2DM. Indeed, of all participants with hypercholesterolemia, about two thirds also were diagnosed with T2DM within all obesity classes (73.3-76%, *p*: <0.000 for all obesity classes, Appendix 3.2 [page 330]).

	Complete cohort	Difference in obesity level				
		Class II	Class III	Class IV	Class V	*p*-value*
Number	1075	126	592	261	96	---
BMI (kg/m²), range	29.0 - 100.4	35.0 – 39.9	40.0 – 49.9	50.0 – 59.9	60.0 – 100.4	---
BMI (kg/m²)	47.1 [42.3-52.3]	**37.8 [35.9-38.7]**	**44.9 [42.9-47.7]**	**53.3 [51.5-55.8]**	**64.6 [61.5-69.1]**	0.00000
Weight (kg)	134.7 (± 28.09)	**103.9 (± 12.94)**	**126.6 (± 16.25)**	**151.3 (± 18.87)**	**183.8 (± 28.41)**	0.00000
Weight (kg), range	74.0 - 282.0	74.0 – 140.0	84.4 – 180.0	112.6 - 220.0	135.5 - 282.0	---
Height (m)	1.67 (± 0.09)	1.67 (± 0.10)	1.67 (± 0.09)	1.67 (± 0.09)	1.66 (± 0.10)	0.505
Gender (% female)	73.5	75.8	74.6	72.4	66.7	0.363
Age	45.7 (± 11.10)	45.9 (± 11.34)	45.7 (± 11.18)	45.0 (± 10.79)	47.0 (± 11.12)	0.475
T2DM (%): **- none** **- T2DM** **- ITT2DM**	 61.9 29.0 9.0	 **54.6** **34.2** **11.2**	 60.9 28.7 10.5	 **69.8** **24.3** **5.9**	 58.5 35.1 6.4	0.028
Ethnicity (% European Caucasian)	61.2	**53.5**	**55.4**	**72.8**	**74.0**	0.000000
Hyper-cholesterolemia (%)	33.8	**44.9**	**35.2**	**27.3**	**22.9**	0.005
Hypothyroidism (%)	9.6	10.3	8.5	9.2	15.7	0.227
Depression (%)	45.7	47.6	44.0	48.3	44.7	0.815
Onset obesity < 10 years old (%)	36.3	**25.3**	**29.9**	**40.7**	**64.1**	0.000000
Binge eating disorder (%)	16.3	16.5	14.9	16.5	23.0	0.383
PCOS (% in females)	20.6	28.6	19.5	20.0	10.3	0.086
OSAP (%)	27.6	**18.8**	**24.9**	**31.5**	**46.7**	0.000007
Requirement for walking-aid or wheelchair (%)	12.5	**6.7**	**10.9**	**13.9**	**26.2**	0.000313

Table 3.2: Baseline characteristics among the different obesity classes. Data is presented as mean (± SD) and median [IQR], unless otherwise indicated. BMI, body mass index; T2DM, type 2 diabetes mellitus; ITT2DM, insulin-treated type 2 diabetes; PCOS, polycystic ovary syndrome; OSAP, obstructive sleep apnoea. Highlighted in red are the mean difference which are significant at <0.05 level, following post-Bonferroni corrections.

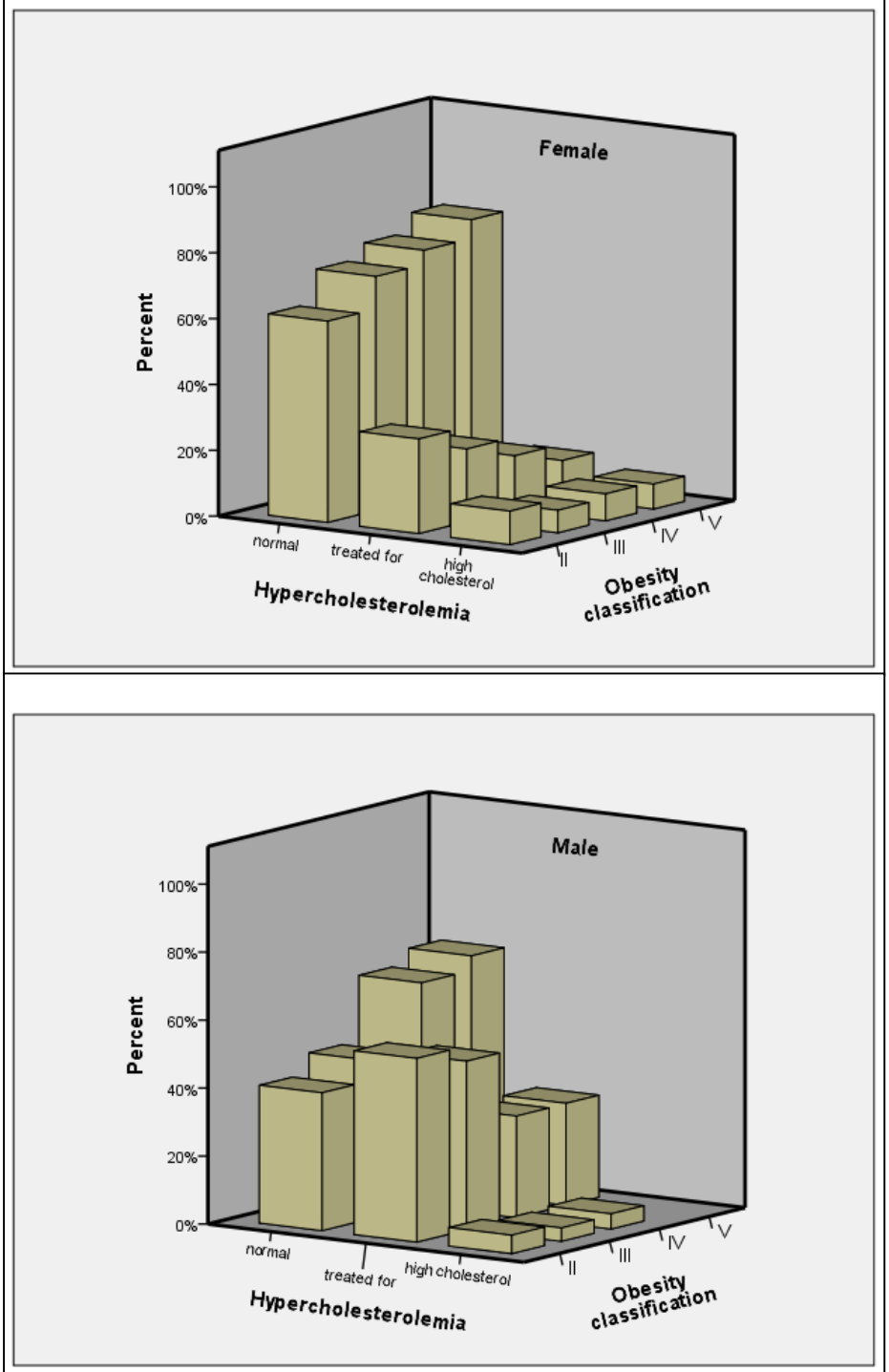


Figure 3.4: Hypercholesterolemia by obesity classification in females (top panel) and males (bottom panel). Diagnosis of hypercholesterolemia was segregated into gender (female, *n*=748; male *n*=283), diagnosis method and obesity class. Female, *r:* 0.116, *p*-value*:* 0.002. Male; *r:* 0.207, *p*-value*:* 0.001.

3.3.4 Questionnaire data

All participants recruited in a prospective manner were asked to fill in a set of questionnaires before they underwent surgery, if they were fluent in reading/writing English. Since the cohort includes both participants recruited in a prospective and retrospective manner, a comparison between the two groups was made to identify any differences in participant's characteristics or possible data collection. Table 3.3 shows an overview of the characteristics, with the only significant difference being in the prevalence of T2DM, which was more frequent in participants recruited pre-surgery.

Of all participants recruited pre-surgery, 284 participants returned the set of questionnaires, giving a return rate of 60.9%. About one third had filled in the set of questionnaires online, while the others filled in the set of questionnaires on paper and returned them by post. Both groups were asked to fill in the questionnaires at home within a week of the recruitment day. The participant baseline characteristics were compared between the participants returning the questionnaires and between the participants that did not (Table 3.3). The only significant difference was seen in the ethnic background, with participants returning the questionnaires more likely to be Caucasian, and a higher prevalence of T2DM in the participants returning the questionnaires.

There was no significant difference in baseline characteristics in participants filling in the questionnaires on paper or online (Table 3.3). A comparison was made between the questionnaire results filled in online vs the paper set of questionnaires to find out if differences in the collection methods would influence the results, but no significant differences were seen (Appendix 3.3, page 330).

	Participants recruited pre-surgery	**Participants recruited post-surgery**	*p-value*	**Participants recruited pre-surgery** [A]			*p-value*
				No questionnaires filled in	*Questionnaires filled in on paper*	*Questionnaires filled in on-line*	
Number	*457*	*612*	--	*183*	*199*	*85*	--
Gender (% female)	72.0	75.5	0.113	71.8	72.3	1.66	0.492
Ethnicity (% Caucasian)	60.7	60.8	0.519	**52.5**	**62.3**	**74.1**	0.009[*]
Age	45.3 (± 11.0)	45.4 (± 11.0)	0.384	45.6 (± 11.9)	46.2 (± 10.9)	47.2 (± 10.4)	0.554
Height	1.67 (± 0.10)	1.67 (± 0.10)	0.998	1.67 (± 0.09)	1.68 (± 0.09)	1.66 (± 0.08)	0.304
Weight	134.0 (± 27.9)	134.6 (± 27.9)	0.724	133.8 (± 28.6)	136.1 (± 28.3)	129.2 (± 26.5)	0.302
BMI	46.9 [41.9-52.4]	47.2 [42.5-52.4]	0.486	47.2 [42.5-52.3]	47.5 [41.6-53.5]	45.1 [40.5-51.5]	0.246
TD2M (%)	**42.0**	**34.5**	0.008	**35.5**	**45.4**	**41.2**	0.031[†]
Requires walking aid (%)	10.3	14.5	0.090	13.2	7.7	18.2	0.103
Obstructive sleep apnoea (%)	25.9	28.3	0.398	28.5	27.6	15.3	0.440
PCOS (% females)	17.6	22.8	0.143	20.6	22.2	26.7	0.864
Lifetime depression (%)	44.6	46.4	0.300	45.8	45.9	46.1	0.284
Lifetime BED (%)	16.9	16.1	0.486	17.8	18.1	8.4	0.598
%WL at 1 year							
- RYGB	31.2 (± 7.9)	31.1 (± 8.3)	0.900	31.1 (± 9.1)	31.0 (± 8.5)	31.9 (± 8.5)	0.932
- VSG	26.9 (± 9.3)	26.0 (± 9.7)	0.554	26.1 (± 9.6)	27.6 (± 10.8)	25.9 (± 7.3)	0.730

Table 3.3: Baseline characteristics of questionnaire return rate. Data is presented as mean (± SD) and median [IQR], unless otherwise indicated. T2DM, Type 2 diabetes mellitus; BMI, body mass index; PCOS, polycystic ovary syndrome; BED, binge eating disorder; %WL, percentage weight loss; RYGB, roux-en-y gastric bypass; VSG, vertical sleeve gastrectomy. [A] Participants that underwent Gastric banding were excluded because of low number (n=10) and bias in selection. [†]Significant between participants having filled questionnaire (on paper or on-line) and participants that did not fill in questionnaires. Highlighted in red are the mean difference which are significant at <0.05 level, following post-Bonferroni corrections.

Comparison analysis of baseline phenotypes with baseline questionnaire data was performed to find any effect on quality of life, eating behaviour or mood, with gender and age having the biggest effect (Tables 3.4, 3.5 and 3.6):

Overall females reported lower scores among all domains in quality of life compared to males, indicating greater impairments. Significantly greater impairments in females compared to males were especially seen in the domains covering mental health: more severely affected vitality, emotional well-being, bodily pain, sexual life, public distress and self-esteem (Figure 3.5 and Figure 3.6, Table 3.4).

A significantly greater dietary restraint was seen in females (2 out of the 3 subscales covering restraint), and a significant increase in disordered eating (pre-occupation with shape and weight) (Table 3.5).

Also among questionnaires, looking at mood disorders (PANAS and HADS) females showed significantly increased scores in depression and anxiety rates (Table 3.6).

As expected, a trend of decreased physical health could be seen with increasing age (Figures 3.7 and 3.8). Interestingly, however, several domains associated with mental health showed a positive correlation with increasing age: emotional well-being and self-esteem showed less impairment with increasing age (Figures 3.7 and 3.8), dietary restraint decreased with older age, and lower anxiety scores were seen with older age.

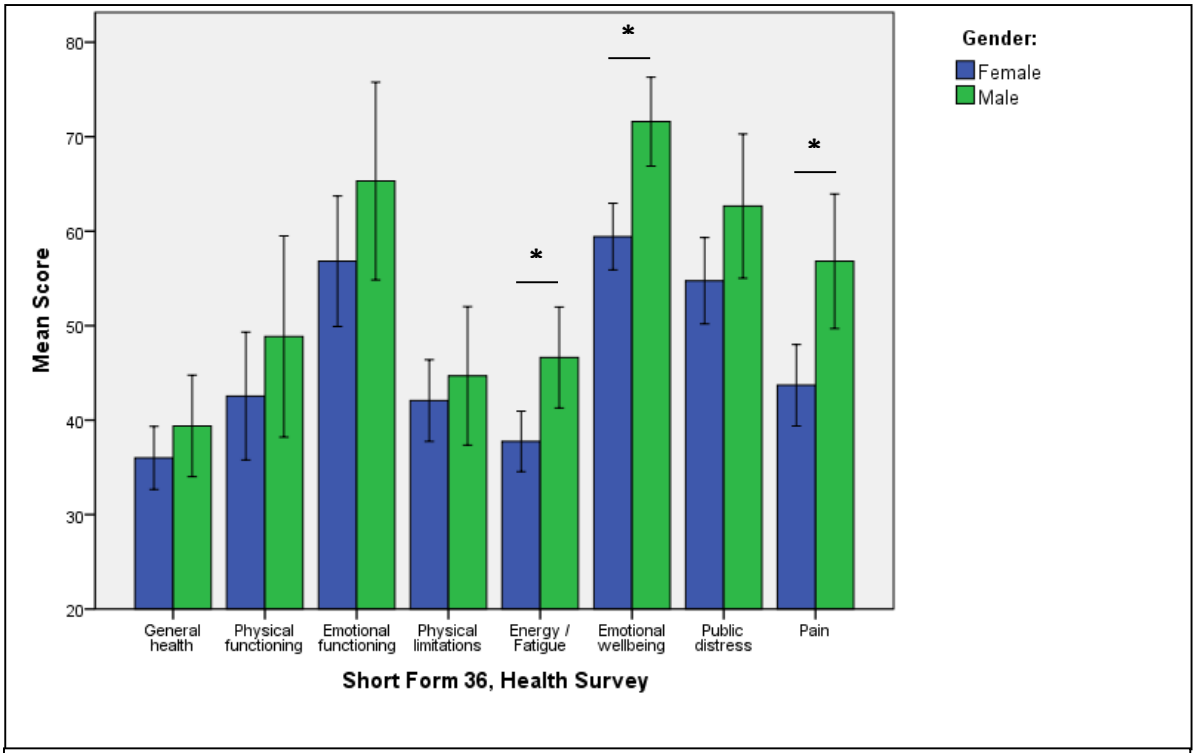


Figure 3.5: SF 36 health survey by gender. Scores were adjusted for BMI, age, T2DM and ethnicity. * indicate significant difference at <0.05 level, following post-Bonferroni corrections. (female, n= 206; male, n= 79)

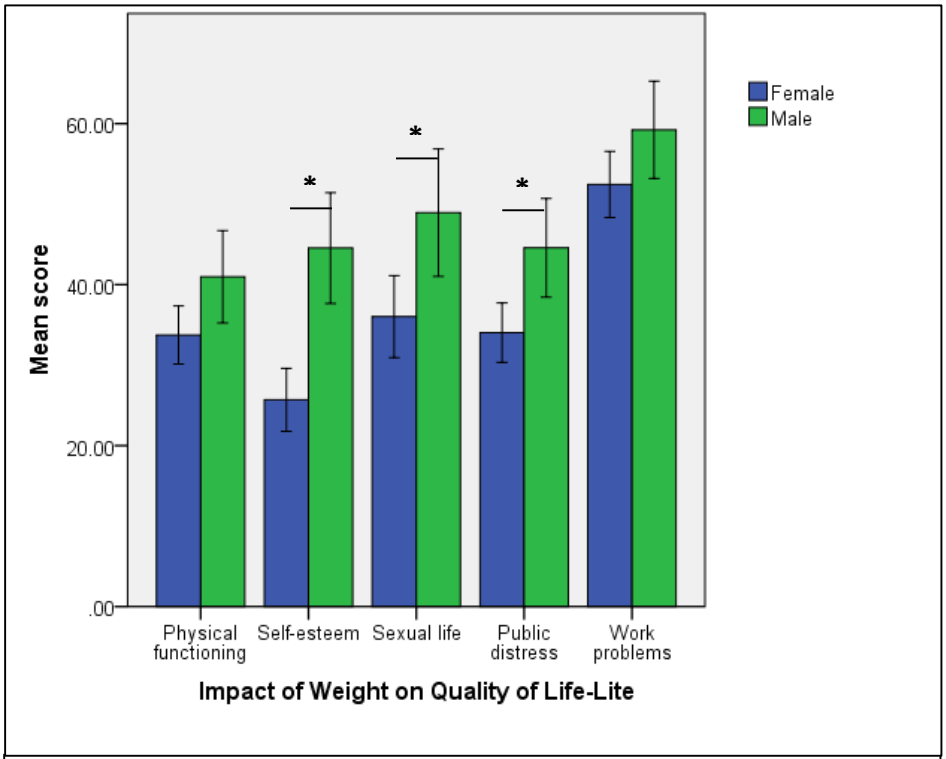


Figure 3.6: IWQOL by gender. Scores were adjusted for BMI, age, T2DM and ethnicity. Scores were adjusted for BMI, age, T2DM and ethnicity. * indicates a significant difference at <0.05 level, following post-Bonferroni corrections. (female, n= 206; male, n= 79)

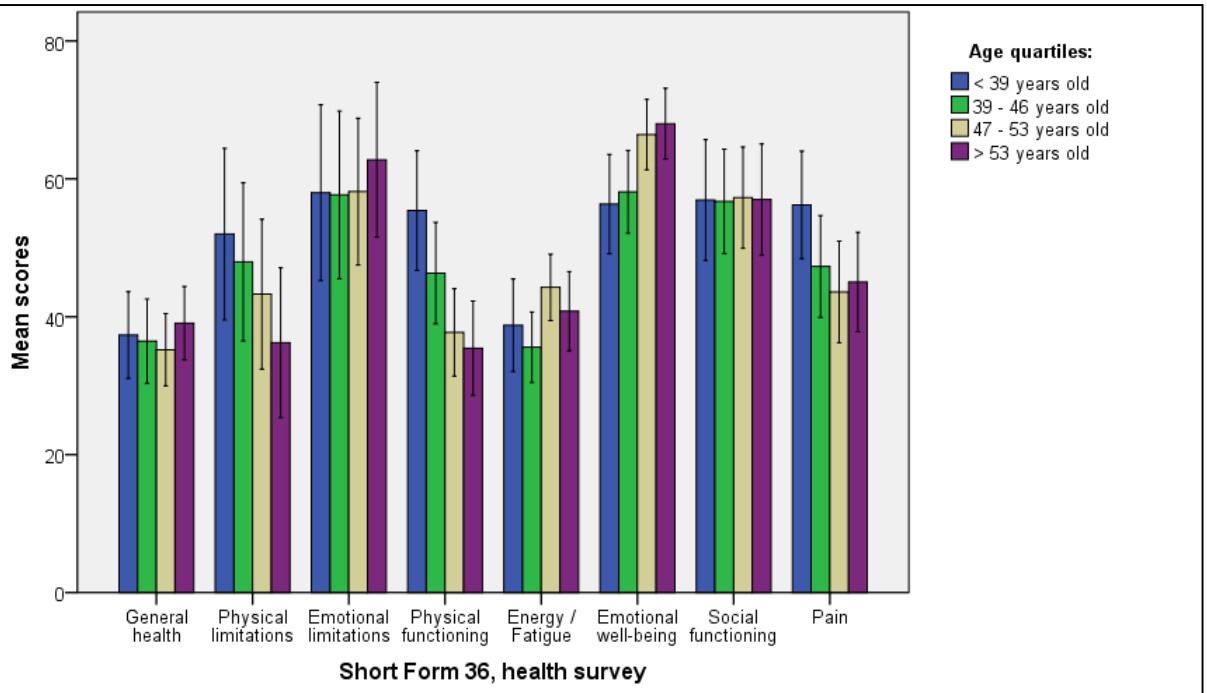


Figure 3.7: SF36 health survey by age groups. Significant differences were found in the following scales: physical limitations (p-value: 0.029), physical function (p-value: 0.0001) and emotional wellbeing (p-value: 0.003). Scores were adjusted for BMI, gender and ethnicity. Bonferroni corrections were applied. (<39 years, n=69; 39-45 years, n=68; 47-52 years, n=77; >53 years, n=70)

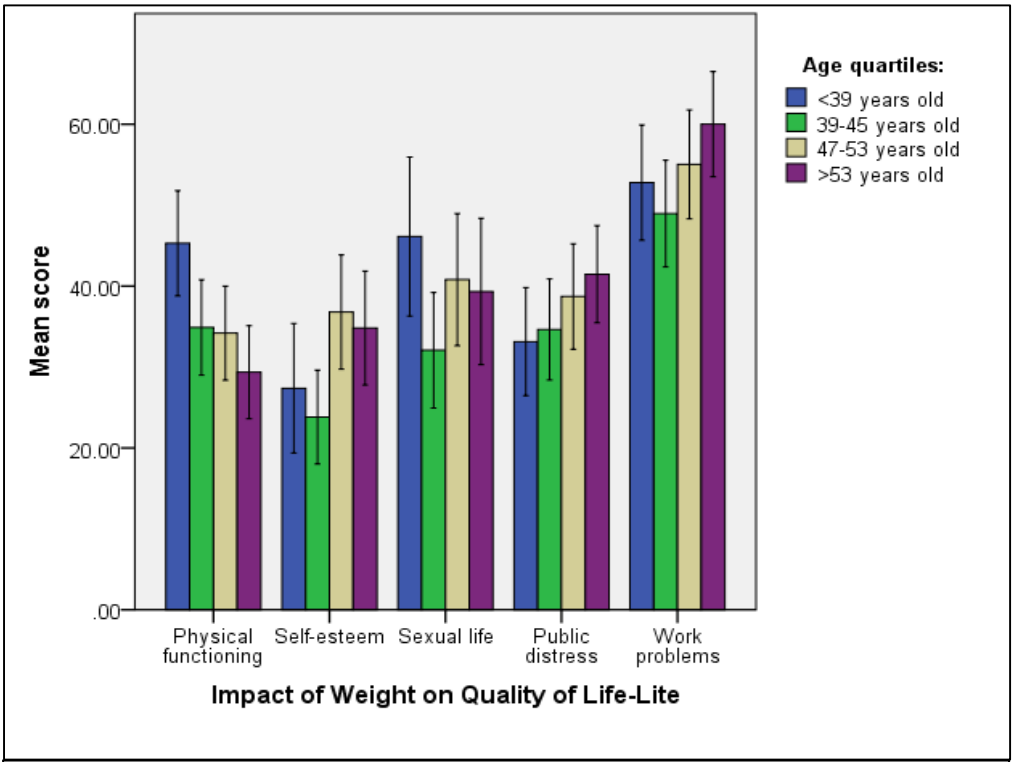


Figure 3.8: IWQOL scales by age group. Significant differences were found in the following scales: physical functioning (p-value: 0.0001) and self-esteem (p-value: 0.029). Scores were adjusted for BMI, gender and ethnicity. Bonferroni corrections were applied. (<39 years, n=69; 39-45 years, n=68; 47-52 years, n=77; >53 years, n=70)

Possibly most interesting, the only domain that was associated with BMI within the three groups of questionnaires (quality of life, eating behaviour and mood questionnaires) was the domain that covered public distress. Ideally a comparison would be made between all the four different obesity classes, but this was not undertaken due the small number of questionnaires returned: comparison between the super obese (BMI ≥50 kg/m^2) and morbidly obese (BMI <50kg/m^2) indicated that the super obese were more severely affected than the morbidly obese in the public distress domain. Also, an overall correlation between higher BMI and more severe public distress scores was seen, whilst highly-related sub-domains, such as self-esteem, were not affected by the difference in BMI (Table 3.4-3.6 and Figure 3.9).

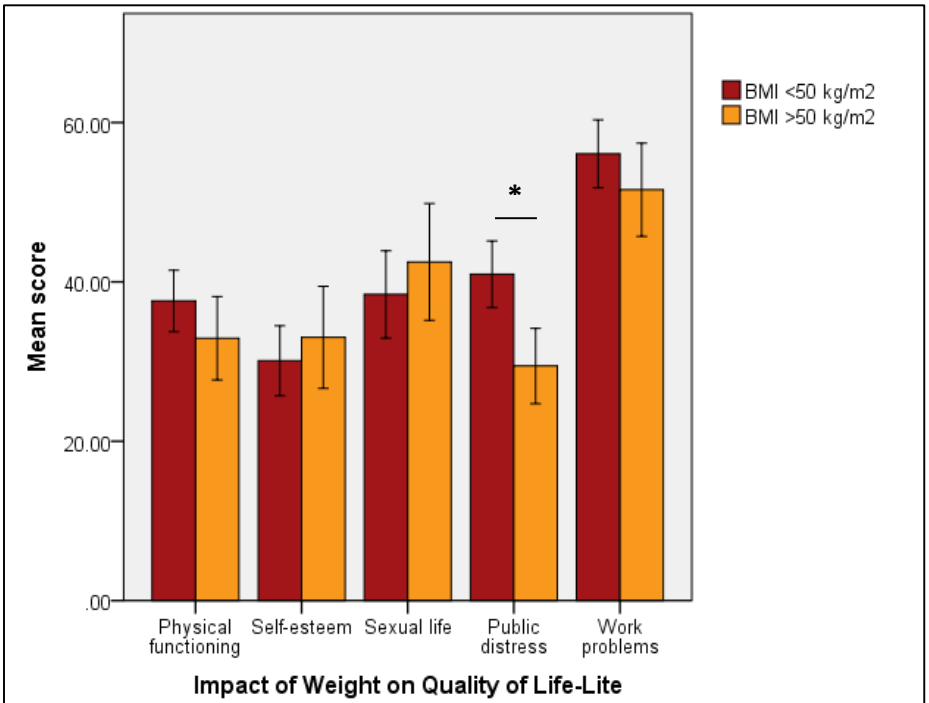


Figure 3.9: BMI and public distress. Scores were adjusted for age, T2DM, gender, BMI and ethnicity. * indicates a significant difference at <0.05 level, following post-Bonferroni corrections. (BMI <50 kg/m^2, n=183; BMI >50 kg/m^2, n=97)

Finally, differences were seen among the different ethnic backgrounds. Non-Caucasians showed significantly less limitations, due to emotional health and physical health, and scored better in four out of five domains in the IWQOL. They also had less restrained eating behaviour (three out of three domains covering restraint were significant) and scored lower in the anxiety as well as depression scores. Further analysis compared the three main different ethnic groups included in the PMMO cohort (European- Caucasian [n=143], Indian [n=11] and Caribbean [n=15]) to investigate if any group in particular differed from the other in relation to weight related quality of life. However, no differences were seen between the three main ethnic groups (Appendix 3.4, page: 335).

	Gender: Female (n=206) vs. Male (n=79)			BMI: <50 kg/m² (n=183) vs. ≥50 kg/m² (n=97)			Comorbidities: T2DM (n=157) vs. no T2DM (n=124)			Ethnicity: Caucasian (n=188) vs. no Caucasian (n=97)			BMI (kg/m2) (n=284)		Age (years) (n=284)	
	Mean difference	**SEM**	**P value**	**Mean difference**	**SEM**	**P value**	**Mean difference**	**SEM**	**P value**	**Mean difference**	**SEM**	**P value**	**r**	**P-value**	**r**	**P-value**
Physical Health:																
SF36 - General Health	-3.788	3.547	0.219	-1.562	3.399	0.646	-1.950	3.303	0.556	5.742	3.547	0.107	0.089	0.185	0.022	0.745
SF36 - Limitations due to physical health	-9.138	7.204	0.126	9.597	6.904	0.166	-7.409	6.708	0.271	1.515	7.204	0.834	0.001	0.989	**-0.635**	0.029
SF36 - Physical functioning	-5.779	4.202	0.171	6.222	4.312	0.151	-7.331	4.297	0.090	1.525	4.614	0.741	-0.125	0.063	**-0.737**	0.000
SF36 - Pain	**-13.774**	4.260	0.004	3.040	4.417	0.492	.783	4.292	0.855	8.956	4.609	0.054	-0.012	0.856	-0.119	0.074
IWQOL - Physical function	**-8.002**	3.477	0.022	-6.055	3.301	0.532	-2.729	3.377	0.420	**7.319**	3.298	0.028	-0.120	0.063	**-0.279**	0.000
Mental Health:																
SF36 - Energy/fatigue	**-7.933**	3.204	0.014	0.516	3.274	0.875	.597	3.181	0.851	-2.745	3.416	0.423	0.029	0.664	0.73	0.277
SF36 - Emotional well being	**-10.052**	3.277	0.002	-0.463	3.261	0.887	5.097	3.168	0.109	4.483	3.195	0.172	0.042	0.533	**0.437**	0.003
SF36 - Social functioning	-7.856	4.617	0.090	3.584	4.547	0.432	-2.976	4.418	0.501	5.588	4.744	0.240	0.000	0.999	0.029	0.663
SF36 - Limitations due to emotional health	-9.206	7.106	0.171	8.512	6.732	0.208	-4.080	6.542	0.534	**13.976**	6.665	0.037	0.017	0.801	0.046	0.488
IWQOL - Self-esteem	**-16.170**	4.110	0.000	-2.433	3.886	0.532	-4.232	3.992	0.290	7.499	3.903	0.056	0.077	0.237	0.115	0.015
Other:																
IWQOL - Sexual life	**-12.211**	5.084	0.017	-0.045	4.802	0.992	-3.769	4.938	0.446	**10.165**	4.828	0.036	0.109	0.096	-0.095	0.144
IWQOL - Public distress	**-12.181**	3.716	0.001	**11.586**	3.564	0.001	-3.375	3.609	0.351	**8.483**	3.520	0.017	**-0.309**	0.000	0.186	0.190
IWQOL - Work problems	-5.209	3.872	0.180	6.256	3.642	0.087	-0.210	3.761	0.956	**11.490**	3.660	0.002	-0.072	0.284	0.105	0.118

Table 3.4: Quality of life questionnaires and baseline phenotypes. SF36, short form 36 health survey; IWQOL, Impact of weight on quality of life-lite; SEM, standard error of the mean; BMI, body mass index; T2DM, type 2 diabetes mellitus. Covariates included in the analysis were: age, T2DM, Gender, BMI and ethnicity. For SF36 the lower the score the more disability, while for the IWQOL the higher the score the more disability. Highlighted in red are the mean difference which are significant at <0.05 level, following post-Bonferroni corrections.

	Gender: Female (n=206) vs. Male (n=79)			BMI: <50 kg/m² (n=183) vs. ≥50 kg/m² (n=97)			Comorbidities: T2DM (n=157) vs. no T2DM (n=124)			Ethnicity: Caucasian (n=188) vs. no Caucasian (n=97)			BMI (kg/m2) (n=284)		Age (years) (n=284)	
	Mean difference	**SEM**	**P value**	**Mean difference**	**SEM**	**P value**	**Mean difference**	**SEM**	**P value**	**Mean difference**	**SEM**	**P value**	**r**	**P-value**	**r**	**P-value**
Dietary restraint																
DEBQ-Restraint	**3.267**	1.171	0.006	0.532	1.109	0.632	1.945	1.084	0.263	**-2.269**	1.126	0.050	-.065	.280	**0.104**	0.028
TFEQ-Restraint	**2.147**	0.634	0.001	0.176	0.601	0.770	0.733	0.587	0.263	**-1.392**	.603	0.027	-.074	.238	0.078	0.207
EDEQ-Restraint	0.116	0.227	0.611	-0.270	0.215	0.211	**0.441**	0.221	0.047	**-0.521**	.218	0.020	.012	.852	**0.019**	0.048
Disinhibited eating																
DEBQ-Emotional	3.929	2.105	0.063	-2.029	1.994	0.310	-1.541	1.949	0.430	3.639	2.039	0.076	.063	.078	-0.117	0.50
DEBQ-External	-1.244	1.091	0.255	0.715	1.034	0.490	-1.813	1.066	0.090	0.312	1.057	0.768	.061	.309	-0.099	0.098
TFEQ-Disinhibition	0.011	0.561	0.984	0.100	0.531	0.851	-0.604	0.519	0.246	0.699	0.543	0.199	.058	.354	-0.052	0.406
TFEQ-Hunger	-0.630	0.600	0.295	0.380	0.569	0.505	-0.848	0.556	0.128	-.273	0.581	0.639	-.036	.569	-0.119	0.056
Disordered eating (preoccupation with):																
EDEQ-Weight	**0.572**	0.186	0.002	0.006	0.177	0.972	0.032	0.173	0.852	-0.199	0.181	0.271	-.058	.362	**-0.023**	0.002
EDEQ-Eating	0.310	0.242	0.202	-0.026	0.229	0.911	-0.108	0.224	0.631	-0.289	0.234	0.218	-.020	.750	-0.105	0.098
EDEQ-Shape	**0.529**	0.208	0.009	0.012	0.197	0.760	0.135	0.193	0.483	-0.133	0.202	0.511	-.102	.108	-0.120	0.057

Table 3.5: Eating questionnaires and baseline characteristics. DEBQ, Dutch Eating Behaviour Questionnaire; TFEQ, Three factor eating questionnaire; EDEQ, Eating disorder examination questionnaire; SEM, standard error of the mean; BMI, body mass index; T2DM, type 2 diabetes mellitus. Covariates included in the analysis were: age, T2DM, Gender, BMI and ethnicity. Highlighted in red are the mean difference which are significant at <0.05 level, following post-Bonferroni corrections.

	Gender: Female (n=205) vs. Male (n=79)			BMI: <50 kg/m² (n=183) vs. ≥50 kg/m² (n=97)			Comorbidities: T2DM (n=157) vs. no T2DM (n=124)			Ethnicity: Caucasian (n=181) vs. no Caucasian (n=97)			BMI (kg/m2) (n=284)		Age (years) (n=284)	
	Mean difference	**SEM**	**P value**	**Mean difference**	**SEM**	**P value**	**Mean difference**	**SEM**	**P value**	**Mean difference**	**SEM**	**P value**	**r**	**P-value**	**r**	**P-value**
PANAS – Positive scale	-1.348	1.362	0.324	0.585	1.288	0.650	0.225	1.239	0.856	-1.111	1.397	0.393	-0.008	0.903	0.110	0.064
PANAS – Negative scale	0.129	1.336	0.923	-1.017	1.271	0.425	-2.235	1.222	0.069	-1.806	1.279	0.159	0.077	0.237	**-0.137**	0.016
HADS – Anxiety	**1.745**	0.716	0.016	-0.481	0.687	0.484	-0.533	0.660	0.420	**-1.792**	0.681	0.009	-0.011	0.866	**-0.088**	0.004
HADS – Depression	**2.175**	0.723	0.003	-0.020	0.683	0.977	0.391	0.657	0.552	**-2.014**	0.688	0.004	-0.040	0.523	-0.107	0.083

Table 3.6: Mood disorder questionnaires and baseline characteristics. PANAS, positive and negative affect schedule; HADS, Hospital anxiety and depression score; SEM, standard error of the mean; BMI, body mass index; T2DM, type 2 diabetes mellitus. Covariates included in the analysis were: age, T2DM, Gender, BMI and ethnicity. Highlighted in red are the mean difference which are significant at <0.05 level, following post-Bonferroni corrections.

Questionnaire data and different types of surgery at baseline:

To investigate whether any differences existed between participants undergoing RYGB or VSG, in terms of eating behaviour, quality of life or mood, a baseline comparison was made between the two particpant groups.

There was no difference in clinical baseline characteristics among the participants that filled in the set of questionnaires and opted for the different types of surgery, apart from the prevalence of T2DM which was higher in participants undergoing RYGB (Table 3.7).

Participants undergoing VSG had more severely impaired weight-related quality-of-life, and scored worse in all five domains of the IWQOL, while non-weight-related quality-of-life scores were not significantly different (Table 3.8).

A significantly higher score in emotional eating, hunger and a pre-occupation with eating (all associated with binge-eating disorder) were seen in participants undergoing VSG, compared to participants undergoing RYGB (Table 3.9). This is in line with the higher prevalence of BED seen in the VSG group in the overall cohort (Table 3.1).

Also, the HADS scores for depression and anxiety were higher in participants undergoing VSG (Table 3.10).

	All participants that filled in questionnaires [A]			
	Gastric bypass	*Gastric sleeve*	*No surgery*	*P-value*
Number	*130*	*63*	*81*	
Gender (% female)	71.8	73.0	71.6	0.979
Ethnicity (% Caucasian)	62.7	57.0	60.0	0.543
Age	46.52 (± 11.0)	44.68 (± 9.9)	47.21 (± 10.9)	0.370
Height	1.675 (± 0.08)	1.677 (± 0.09)	1.672 (± 0.10)	0.948
Weight	133.6 (± 23.9)	139.8 (± 34.4)	131.0 (± 29.7)	0.171
BMI	47.6 (± 7.19)	49.3 (± 9.36)	46.7 (± 9.28)	0.171
TD2M (%)	**50.4**	**33.3**	43.8	0.018*

Table 3.7: Baseline characteristics of participants that filled in questionnaires. Data is presented as mean (± SD), unless otherwise indicated. [A] Participants that underwent Gastric banding were excluded because of low number (n=10) and bias in selection. * Significant between the Gastric bypass and Gastric sleeve group. Highlighted in red are the mean difference which are significant at <0.05 level.

	RYGB vs. VSG		
	Mean difference	SEM	P value
SF36 - General Health	7.209	4.406	0.311
SF36 -limitations due to physical health	5.395	8.932	1.000
SF36 -limitations due to emotional health	6.545	8.758	1.000
SF36 - Physical functioning	8.523	5.744	0.419
SF36 - Energy/fatigue	3.842	4.182	1.000
SF36 - Emotional well being	5.755	4.162	0.506
SF36 - Social functioning	11.236	5.905	0.176
SF36 - Pain	6.194	5.802	0.862
IWQOL - Physical function	**12.031**	4.165	0.004
IWQOL - Self-esteem	**9.951**	4.745	0.038
IWQOL - Sexual life	**10.034**	5.842	0.088
IWQOL - Public distress	**11.395**	4.285	0.009
IWQOL - Work problems	**19.083**	4.154	0.000

Table 3.8: Quality of life questionnaires and surgery type. Highlighted in red are the mean difference which are significant at <0.05 level, following post-Bonferroni corrections.

	RYGB vs. VSG		
	Mean difference	SEM	P value
DEBQ- Restraint	-1.097	1.380	1.000
DEBQ- Emotional	**-8.057**	2.420	0.003
DEBQ- External	-3.001	1.267	0.056
TFEQ- Restraint	-0.108	0.751	1.000
TFEQ- Disinhibition	-1.326	0.645	0.124
TFEQ- Hunger	**-1.799**	0.699	0.032
EDEQ- Restraint	-0.444	0.267	0.294
EDEQ- Weight	-0.194	0.221	1.000
EDEQ- Eating	**-0.788**	0.238	0.017
EDEQ- Shape	-0.278	0.248	0.790

Table 3.9: Eating behaviour questionnaires and surgery type. Highlighted in red are the mean difference which are significant at <0.05 level, following post-Bonferroni corrections.

	RYGB vs. VSG		
	Mean difference	SEM	P value
PANAS – Positive scale	1.631	1.598	0.926
PANAS – Negative scale	-0.893	1.579	1.000
HADS – Anxiety	**-0.716**	0.859	1.000
HADS – Depression	**-0.466**	0.853	1.000

Table 3.10: Mood disorder questionnaires and surgery type. Highlighted in red are the mean difference which are significant at <0.05 level, following post-Bonferroni corrections.

3.4 Discussion

In this chapter I describe the creation of a cohort of over a thousand morbidly obese individuals seeking bariatric surgery, with deep phenotyping in terms of anthropometrics, psychological behaviour and clinical measurements. Within this cohort, a wide range of extreme phenotypes were included, with a main focus for this thesis on severe obesity, with the expected and several novel unexpected co-morbidities.

Creation of a cohort of severely obese participants undergoing bariatric surgery

A cohort of 1075 morbidly obese individuals seeking bariatric surgery was created. The mean BMI of 48.1 kg/m2 (range: 29.0 - 100.4 kg/m^2), and with over one-third of the cohort being classified as class IV obesity (super obesity; BMI >50 kg/m^2), it can be concluded this cohort exists out of extreme phenotypes within the BMI range.

A combination of prospective and retrospective study approach was used, but comparison analysis showed this created no difference in participant characteristics. Therefore, the data collected here could generally be interpreted as representative of the wider cohort.

As expected, a higher prevalence of T2DM was seen among the participants opting for RYGB compared to VSG surgery. This most likely due to RYGB being advised as the best treatment option for patients with T2DM [5,215]. A higher prevalence of binge-eating disorder and mobility problems (the requirement for walking aids or wheelchair) was higher among the VSG participants. Although the BMI was not significantly higher in the VSG group, it is possible patients with a higher BMI in combination with severe health problems, such as reduced mobility, will opt for this potentially surgically simpler and less risky surgery type. It is also note-worthy that the participants opting for the different surgery types had significant difference in quality of life, eating behaviour and mood scores. Overall,

participants undergoing VSG had a bigger impairment of their weight-related quality-of-life, more disordered eating and scored higher on the depression and anxiety scales. This is in line with the higher rates of depression, binge eating and mobility impairments seen among the VSG participants. These baseline differences compared to the participants undergoing RYGB have so far not been reported before [217-219], and are important to take in consideration when evaluation comparative outcome data on these two surgeries, when not randomised into a clinical trial.

The severely obese: Class IV and V obesity

To further examine the co-morbidities and other health-related phenotypes in the extremely obese, comparison analysis was done between the different obesity classes II-V. To be able to separate the more severely obese within this cohort two additional obesity classes were used, class IV and class V obesity, to describe the population with a BMI of 50.0-59.9 kg/m^2 and ≥60 kg/m^2 respectively. Although the general population can be categorised into five groups according to BMI, with the highest obesity class III covering a BMI of ≥40 kg/m^2, the distribution of BMI among this bariatric population exceeded this BMI range, with a mean BMI of over 48.1 kg/m^2.

As expected, co-morbidities such as OSAP and severe mobility limitations (which require walking-aid or wheelchair usage) were more frequent among those who were most obese: these co-morbidities are highly dependent on the direct weight burden.

In comparison, two other co-morbidities, highly correlated with obesity, showed more counter-intuitive results: the highest prevalence of T2DM (45.4%) was not detected in the highest obesity category within the cohort (class V obesity), but in the lowest BMI range (Class II obesity) instead. The lowest prevalence of T2DM (30.2%) was seen in the Class IV obesity group. A likely explanation for this could be selection bias. Patients with a lower BMI are more likely to opt for bariatric surgery if they suffer from severe comorbidities such as T2DM, while patients with higher BMI levels might also consider undertaking this invasive treatment option without suffering from severe comorbidities, but

just to treat obesity in itself. On top of that, patients with a BMI <40 kg/m^2 will only qualify for bariatric surgery when diagnosed with an obesity-related comorbidity, such as T2DM, according to the NICE guidelines. [220] This possibly could explain the high prevalence of T2DM seen in the participants at the lower BMI range within this cohort.

The other obesity-related comorbidity which gave an unexpected result was the significant decrease in prevalence of hypercholesterolemia with each increasing obesity class: 44.9%, 35.2%, 27.3% and 22.9% for Class II, Class III, Class IV and Class V obesity respectively (p-value 0.005). To see if this could also be caused by selection bias, the hypercholesterolemia cases were divided into 'treated with statin' and 'newly diagnosed' cases. In some cases, statin treatment might have started before entering into the study, on more conservative criteria than others (which might be especially the case in patients with T2DM), which might explain the higher prevalence of hypercholesterolemia cases in the lower BMI groups: this possibility was explored. Indeed, the biggest proportion of the hypercholesterolemia cases consisted out of individuals already treated with statins upon recruitment. However, the percentage of newly diagnosed cases of the overall hypercholesterolemia cases was similar for each obesity class (so the absolute number of newly-diagnosed cases was significantly higher among the lower obesity classes compared to the higher BMI groups). The possible 'increased likelihood of treatment' of hypercholesterolemia in the lower BMI classes is, therefore, less likely to explain the higher prevalence of hypercholesterolemia. However, further analysis did show a high correlation of hypercholesterolemia with the diagnosis of T2DM within all obesity classes (73.3-76.0% of the cases with hypercholesterolemia were also diagnosed with T2DM). Therefore, the selection bias of T2DM cases into the lower BMI classes could indirectly also lead to the higher prevalence of hypercholesterolemia in these groups. This explanation holds up looking at the matching prevalence seen in the different obesity classes for T2DM and hypercholesterolemia (Class II: 45.4% and 44.9%, Class III: 39.4 and 35.2%, Class IV: 30.2% and 27.3% for T2DM and hypercholesterolemia respectively). However, the discrepancy of T2DM and hypercholesterolemia in

obesity class V, with a BMI >60 kg/m^2: 41.5% vs. 22.9% indicates this did not hold up for the most extreme BMI cases.

A decrease in the prevalence of T2DM and hypercholesterolemia for class IV and V obese compared to class III and II obese, has been reported several times before. Contradictory results have been reported regarding the prevalence of T2DM in populations with a BMI above 50kg/m^2: several studies reported the expected increase while others reported a stagnation of the prevalence once a BMI of 50 kg/m^2 was reached [221-224]. Reports on the prevalence of dyslipidaemia in the severely obese, on the other hand, seem to be more consistent: the super obese (BMI >50 kg/m^2) showed significant better lipid profiles compared to the morbidly obese [221-225]. Unfortunately for most of the studies that do cover BMI ranges above 50 kg/m^2, the cohort investigated existed out of patients recruited from weight loss clinics (bariatric surgery clinics as well as lifestyle treatment clinics), and, therefore, selection bias cannot be excluded. Only two studies based on population cohorts showed similar findings; an increase in the prevalence of dyslipidaemia that peaked for individuals with obesity class II and hit a plateau or decreased for obesity class III individuals [226,227]. For T2DM both studies reported a gradual increase among all obesity classes included. Unfortunately, both studies did not cover the extreme ranges of BMI (class III was the highest BMI group).

A final observation made among the more severely obese is possibly less unexpected, but still important to take into consideration: The proportion of individuals in whom the onset of obesity was at or before the age of ten, increased with BMI class; starting with 25.3% in the Class II obese, followed by a gradual increase up to 64.1% in the Class V obese. This could indicate that, at least among the obese bariatric surgery population, an early onset of obesity leads to a higher mean BMI than late onset obesity. The increased risk of developing (morbid) obesity in adult life following childhood obesity is well defined [228,229]. However, to my knowledge, no studies have been published so far describing the increased history of childhood onset of obesity in the more severely obese compared to the morbidly obese. This finding could be important, especially for studies trying to identify patients

at risk for genetic causes of obesity, since this could indicate that more severely affected obesity cases have a higher genetic contribution, as has been found for childhood obesity [39,45,49,50,65]. The results reported here are, however, on self-reported childhood onset of obesity. There is a possibility of recall bias, which could have led to an intensification of the weight history of the severely obese compared to the less severely affected individuals within this cohort.

Quality of life, eating behaviour and mood phenotypes

Within the cohort, participants were asked to fill in a set of questionnaires (if recruited in a prospective manner, n=466), to assess their quality of life, eating behaviour and mood. Although an incomplete response rate was seen (60.8%), no differences could be detected between the participants that had filled in the questionnaire set and the participants that did not, apart from their ethnicity (which could be easily explained by the fact only participants fluent in English were asked to fill in the questionnaires) and T2DM status. Possibly participants with a diagnosis of T2DM were more concerned about their health, and, therefore, more keen to help out with research.

Health related quality of life

Here, we used two assessment tools to screen for health related quality of life (HRQOL). The first (SF36) is a generic HRQOL assessment tool, while the second assessment tool (IWQOL) was specifically designed to measure HRQOL in the obese population [185,230]. While several factors are known besides obesity itself to affect HRQOL in the obese population, relatively little is known about HRQOL in the severely-obese population.

Although it has repeatedly been shown that obesity and an increase in BMI have a negative effect on the different aspects of HRQOL measurements [184,185,230-234], we could not find such correlations in our cohort. The severely obese individuals were not more severely affected than the morbidly obese in any domain, besides public distress. What makes this finding even more interesting is that highly related domains, such as self-esteem, did not correlate with BMI. While self-esteem mostly

involves someone's evaluation of oneself, public distress relates mostly to how others react to the obese person (questions include experience of discrimination, ridicule and worry about fitting in chairs, fitting through isles) [185,234]. Therefore, it can be plausible that within this cohort the individuals visually suffering from one of the 'more extreme forms of obesity', might be more effected by public distress, while other features more based on actual health do not score worse in the super obese. Although multiple studies have looked at the impact of severe obesity on quality of life compared to normal and over-weight individuals, only one study looked at the impact of BMI differences within a morbid obese cohort. White, *et al.* [235], found the relationship between BMI and quality of life to be attenuated when reaching more extreme BMI levels, confirming our findings here. Interestingly the strongest correlation with BMI they found within the obese cohort was also within the domain covering public distress.

Although the general correlations with BMI could not be repeated, previously reported correlations with gender, ethnicity and age could be repeated here:

Overall worse scores were seen in females compared to males in different domains of quality of life, suggesting that, for females, severe obesity was more likely to result in diminished quality of life. In particular, significantly greater impairments were seen in the domains covering mental health. This is consistent with previous studies, reporting poorer quality of life in female obese individuals compared to male obese individuals within similar BMI range [184,185,231,234].

As reported by others, we also found non-Caucasians to feel less limited due to emotional health, compared to Caucasians [184,231,235]. Although only one domain was affected from the general HRQOL questionnaire (SF36), all domains of the weight specific HRQOL questionnaires (IWQOL) were significantly more impaired among the Caucasians. It is not surprising that in several comparison analysis the IWQOL gave stronger results than the SF36, since disease-specific HRQOL measures are specifically designed to assess limitations and characteristics associated with the disease (in this case obesity), and are generally considered more sensitive in disease-specific populations [184]. The

differences here could be explained by cultural differences. Although a difference in interpretation of language, cannot be excluded. Participants who were not fluent in English were excluded for this analysis, but not all participants had English as their first language. Sequential analysis between the different ethnic groups included in the cohort reported here, could not clarify which groups were more significantly different from the others. It is therefore important to remember to include ethnic background as a potential confounder in analysis of this kind of data.

Another interesting finding within the quality of life measurements within this severe obesity cohort was the affect that age had on the different domains of quality of life. As expected, and reported before, domains involving physical health were more severely impaired with increasing age [234,236,237]. However, other domains involving mental health (emotional well-being and self-esteem) actually improved with age. Although an improvement of self-esteem with increasing age has been shown in general population cohorts [238], only one study has shown this positive effect of age on mental health within an obese populations before [234]. This study was limited to the use of the IWQOL assessment tool only, so this is the first time an improvement of self-esteem and mental well-being with increasing age has been seen in an obese population using two independently health related quality of life assessment tools.

Eating behaviour

The results obtained from the eating behaviour questionnaires were less consistent than the results from the quality of life questionnaires. One consistent finding however was the lower scores in restrained eating in the non-Caucasian participants compared to the Caucasians. Three out of the three domains covering restraint (from three different questionnaires, DEBQ, TFEQ and EDEQ) showed significantly less severe dietary restraint in non-Caucasians compared to Caucasians. Previous contradicting results indicate further research in this area is warranted [239-241]

Both gender and age appeared to affect dietary restraint as well (with females having higher restraint, and restraint getting more severe with older age), but both analysis only showed significant differences in two out of the three scales that covered dietary restraint, which makes the interpretation less reliable.

Again, no differences were seen in eating behaviour scores between the morbidly obese and the super obese, nor were any correlations found with BMI. This contrasts with previous reports, which consistently found relations with obesity and increasing BMI [239,240,242-244].

Mood

Mood (in the form of depressive and anxious disability) were assessed through two questionnaires, PANAS and HADS. Both females and Caucasians scored worse in anxiety and depression levels using HADS, compared to males and non-Caucasians respectively. These results could not be repeated using PANAS, and, therefore, should be interpreted with caution. Previous studies, however, do show similar results with high rates of depression and anxiety in bariatric cohorts as well as increased severity in females and differences between individuals of different ethnic background [245-248]

The most surprising result overall from the questionnaire data was that (besides the domain covering 'public distress') none of the domains covering quality of life, eating behaviour or mood revealed a correlation with BMI within this cohort. This contrasts with previous studies showing strong correlations with BMI, but could be explained by the overall extreme BMI range within the cohort described here, and possibly could question the usability of these different questionnaires within a bariatric cohort. Indeed, all of the questionnaires used here were selected based on their validation within the obese population, but none of them, besides the IWQOL, were validated specifically within a bariatric population. Surprising is however that other previous reported correlations such as gender, age and ethnicity could be repeated here, possibly indicating the effect of BMI stabilises once a

'threshold of highest BMI' is reached, while other features are still measurable in the extreme population.

Further validation analysis is therefore warranted to indicate the utility of these questionnaires for research and clinical settings in the bariatric population. A further detailed research plan regarding validation and reliability testing in the severe obese bariatric cohort of these different questionnaires can be found in the future research plan section of the last chapter of this thesis (page 275).

3.5 Conclusion

In this chapter I described the creation of the PMMO cohort, consisting of severely obese individuals undergoing bariatric surgery, to be further investigated by genetic analysis. The in depth phenotyping of this cohort showed some interesting findings; unexpectedly, the more severely obese individuals turned out to be metabolically healthier than the less severe obese included. If this holds up in larger population cohorts, and is not only due to selection bias, it needs to be further investigated. Surprisingly, no differences were seen between the super obese and morbid obese individuals in quality of life, eating behaviour or depression scales, besides experiencing greater public distress (covering discrimination and ridicule). This could, however, be explained by the general negative attitudes towards obese individuals in current society, and points out the necessity of creating a better understanding of obesity and its causes.

CHAPTER 4

BARIATRIC SURGERY OUTCOMES

4.1 Introduction

This chapter describes the analysis of outcomes after bariatric surgery, contextualised to the baseline participant characteristics discussed in Chapter 3.

Bariatric surgery is currently the most effective treatment available for morbid obesity, and has been applied at an increasing rate within the UK. Between 2011 to 2013, 32,073 bariatric surgery operations were performed in the United Kingdom & Ireland [5]. The different types of surgery included in this study for outcome analysis, RYGB and VSG, have previously shown good outcomes in terms of weight loss and improvements of other health factors, such as T2DM, lipid levels and mobility [249]. RYGB is still the most common bariatric operation performed within the UK and results in ~20-40% weight loss, while for VSG a slightly lower range of weight loss is seen of ~15-20% [160,161,249]. Following RYGB, a positive change in quality of life and depression rates have been reported [232,245], while no such data for VSG exists.

Although an overall positive outcome in health improvements is seen following either surgery type, exemptions are found, with individuals losing insufficient weight (<20% from start weight), or showing no improvement of obesity-related comorbidities, such as T2DM [167,249]. Some have even reported an increase in depression and suicidal rates and addictive behaviour [250]. It is therefore important to identify (bio)markers useful for prioritising the scarce NHS resources to those most likely to benefit, avoiding surgical risk in those less likely to respond well, and to make sure extra care is given to the individuals most at risk of serious complications such as psychological deterioration.

4.2 Aims of this study

1) Analyse weight loss and health outcomes beyond weight loss following RYGB as well as VSG.

2) Identify factors influencing weight loss, quality of life and other health factors in the bariatric population.

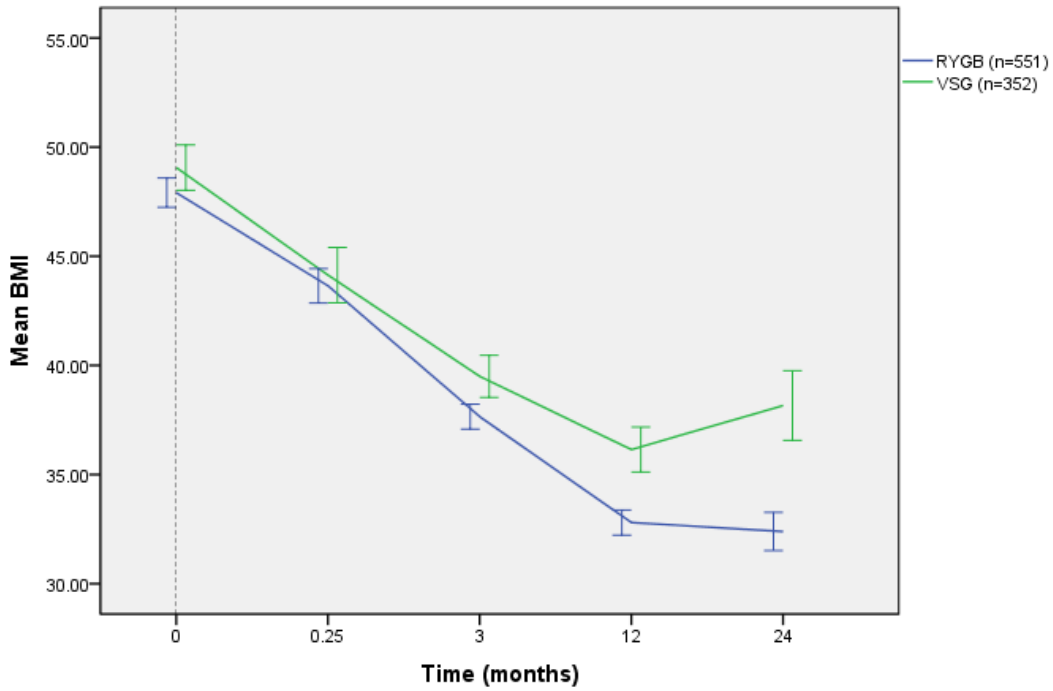
3) Investigate factors associated with monogenic obesity and their influence on health factors and weight loss following bariatric surgery.

4.3 Results

4.3.1 Weight loss trajectories

As expected, study participants showed a significant weight loss and change in BMI following both RYGB and VSG (Figure 4.1 and Table 4.1). A significant change in BMI could already be detected at the first follow up measurement taken at 10 days following the surgery day (although this may be largely attributable to pre-surgery weight loss through pre-surgery diets), and continued to significantly change until 12 months after the surgery day. Following the initial 12 months an overall stabilisation in weight change was seen in participants that underwent RYGB and a small increase in weight in participants that underwent VSG.

Mixed linear regression modelling, adjusted for age, baseline-BMI, gender and ethnicity revealed a significantly steeper weight loss trajectory in the participants who had undergone RYGB compared to participants who had undergone VSG (*p*: <0.000, Figure 4.1). A significant difference in BMI change was detectable between participants undergoing RYGB compared to VSG, from 3 months onwards following the surgery (Table 4.1). This, even though both participant groups are advised by the bariatric surgery clinic to be on a similar diet of soft foods until 3 months after the surgery. It is only after these initial three months they are advised to go back to 'normal' foods.



Time:	0	0.25	3	12	24
RYGB:	47.91	43.65	37.75	32.80	32.39
	(n=551)	*(n=548)*	*(n=535)*	*(n=444)*	*(n=226)*
VSG:	49.06	44.14	39.50	36.14	38.16
	(n=352)	*(n=349)*	*(n=328)*	*(n=241)*	*(n=114)*

Figure 4.1: Weight loss trajectories in RYGB and VSG group. Mean BMI (± SEM) at the different time points, for RYGB (Roux-en-Y gastric bypass) and the VSG (vertical sleeve gastrectomy) group. Linear mixed modelling showed a significant difference between the two trajectories (*p*: <0.0000). BMI values kg/m^2 and number of participants per time point are given below the graph.

Time (mths)	**RYGB (n=551)**			**VSG (n=352)**			**Difference between groups**	
	Mean BMI (SD)	**Δ-BMI from baseline**	**p-value***	**Mean BMI (SD)**	**Δ-BMI from baseline**	**p-value***	**Mean (SE) [95% CI]**	**p-value****
0	47.91 (± 7.81)	--	--	49.06 (± 9.66)	--	--	-1.15 (0.62) [-2.36 to 0.06]	0.064
0.25	43.65 (± 6.75)	-4.35	0.000	44.14 (± 8.54)	-4.16	0.000	-0.49 (0.75) [-1.96 to 0.98]	0.705
3	37.75 (± 5.83)	-10.23	0.000	39.50 (± 7.77)	-8.69	0.000	-1.85 (0.53) [-2.95 to -0.74]	0.004
12	32.80 (± 5.90)	-15.60	0.000	36.14 (± 7.80)	-11.93	0.000	-3.34 (0.50) [-4.50 to -2.18]	0.000
24	32.39 (± 6.45)	-16.18	0.000	38.16 (± 9.06)	-10.94	0.000	-5.77 (0.91) [-7.56 to -3.98]	0.000

Table 4.1: Change in BMI following surgery. The BMI-SDS change over time for the RYGB (Roux-en-Y gastric bypass) and VSG (vertical sleeve gastrectomy) group are listed. Data is presented as mean (± SD), unless otherwise indicated.

*compared to baseline BMI **change BMI in RYGB group compared to VSG group. Bonferroni corrections were applied.

In an attempt to more effectively correct for differences in baseline-BMI and weight for further analysis, regression analysis was performed between the different weight loss metrics most commonly used in the literature for bariatric surgery studies and baseline BMI measuremnts. Metrics included were: percentage weight loss (%WL), total BMI, change in BMI (△BMI) and percentage excess body weight loss (%EBWL). Regression analysis between the different weight loss metrics used and baseline BMI was significant for each metric, however, baseline BMI could least be accounted for the variability in %WL following both RYGB as VSG compared to the other metrics tested (Table 4.2). This is confirmed by visualising the distributions for the different weight loss metrics by two obesity classes in Figure 4.2.

Roux-en-Y gastric bypass						
Models *	**12 mths FU****	***r***	***r²***	**95.0% Confidence Interval**		**P-value**
				Lower bound	**Upper bound**	
%WL	-31.09 (±8.15)	0.194	0.038	-0.314	-0.107	0.000073
BMI	32.79 (±5.90)	0.732	0.535	0.523	0.626	0.000000
△BMI	-14.99 (±5.09)	0.627	0.393	-0.476	-0.374	0.000000
%EBWL	-68.69 (±20.28)	0.418	0.175	0.891	1.367	0.000000
Vertical sleeve gastrectomy						
Models *	**12 mths FU****	***r***	***r²***	**95.0% Confidence Interval**		**P-value**
				Lower bound	**Upper bound**	
%WL	-26.28 (±9.64)	0.154	0.024	-0.284	-0.025	0.020
BMI	36.14 (±7.80)	0.770	0.593	-0.557	-0.692	0.000000
△BMI	-12.92 (±6.15)	0.587	0.345	-0.443	-0.308	0.000000
%EBWL	-56.23 (±24.32)	0.279	0.078	0.387	1.021	0.000019

Table 4.2: Linear regression between weight loss metrics applied at 12 months following surgery and baseline BMI. %WL, percentage weight loss; BMI, body mass index at 1 year follow up; △BMI, delta BMI; %EBWL, percentage excess body weight loss. *r*, coefficient of correlation; r^2, coefficient of determination. * Models adjusted for gender, age and ethnicity. ** Mean values (± standard deviation) as measured at 12 months following surgery date.

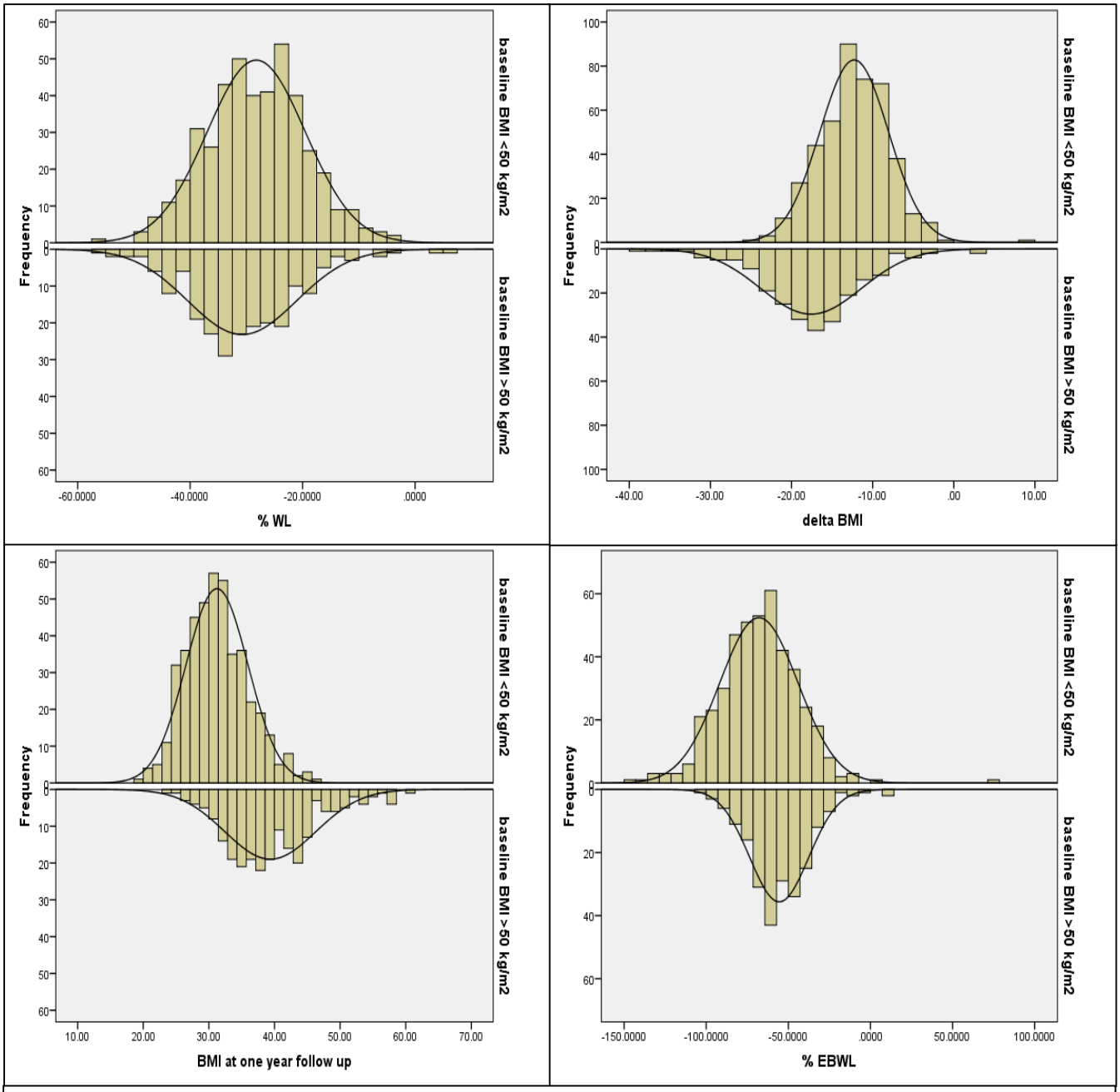
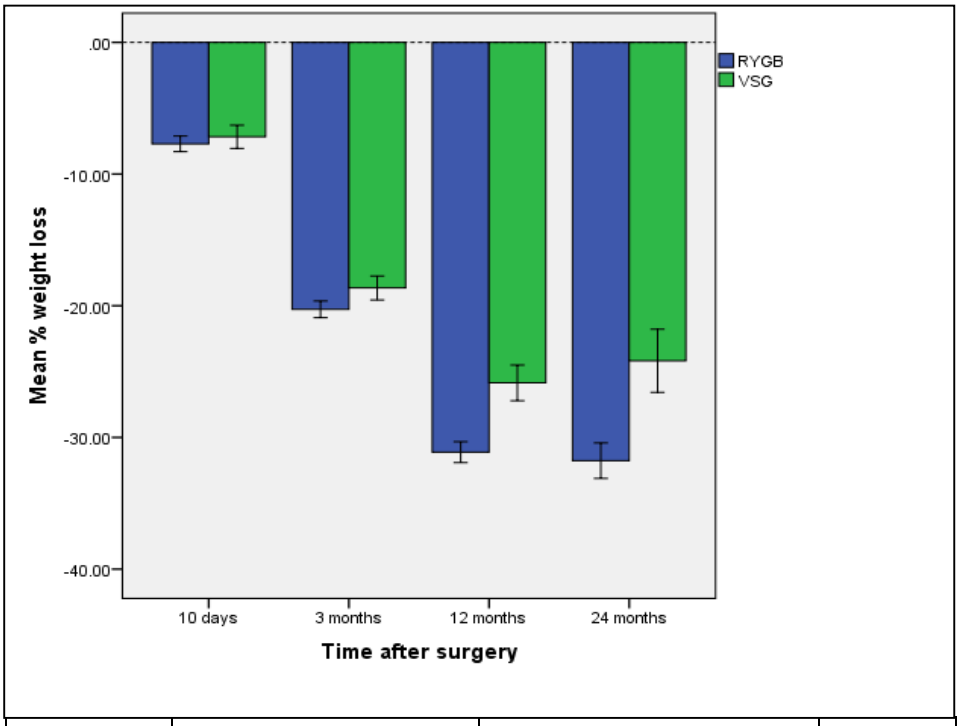


Figure 4.2: Weight loss metrics by morbid obese vs super obese population. Distributions of different weight loss metrics are given for 12 months following surgery. %WL, percentage weight loss; BMI, body mass index; △BMI, delta body mass index; %EBWL, percentage excess body weight loss.

Following these results %WL was used for further analysis and was calculated for the different follow up time points for both RYGB and VSG. Again a significant difference was seen in %WL between the RYGB and VSG participants at 3, 12 and 24 months following surgery, but not at 10 days following surgery (Figure 4.3).



Time	RYGB		VSG		P-value
	n	% weight loss	n	% weight loss	
0.25 mths	548	-7.71 (± 5.14)	349	-7.17 (± 5.98)	0.301
3 mths	535	-20.28 (± 6.50)	328	-18.64 (± 7.34)	0.003
12 mths	444	-31.11 (± 8.13)	241	-26.25 (± 9.63)	<0.0000
24 mths	226	-31.76 (± 9.98)	114	-24.55 (± 12.96)	<0.0000

Figure 4.3: Percentage weight loss. Significant differences were seen at 3 months (*p*-value: 0.021), 12 months (*p*-value: <0.0000) and 24 months (*p*-value: <0.0000), but not at 10 days (*p*-value: 0.510).

4.3.2 Health changes beyond weight loss

Type 2 diabetes mellitus:

In participants that underwent either RYGB or VSG a significant change was seen in HbA1c values, in both participants with and without T2DM (Table 4.4). There was also a significant increase in the percentage of participants with T2DM that had an HbA1c value of ≤48 mmol/mol (including both those participants on glucose lowering medication and participants that were taken off medication): 18.9% at baseline vs. 74.1% at 12 months following surgery in the RYGB group, and 40.0% at baseline vs. 84.0% 12 months following surgery in the VSG group (inter group difference, p-value: 0.221). Participants treated for T2DM with oral or insulin medication were in most cases kept on a low dose of Metformin, even when their HbA1c levels reached normal values (this according to local clinical protocols). Therefore, it was unfortunately not possible to look at the proportion of T2DM cases that resolved following surgery, since current criteria for T2DM remission include normal glucose and HbA1C values for at least 1 year's duration in the absence of active pharmacologic therapy [251]. Further analysis of T2DM improvements and remission in this cohort is outside the scope of this thesis, and will be covered by other PhD students in ongoing projects.

Lipid profiles:

Of the participants not on statin treatment, a change of hypercholesterolaemia prevalence from 12.6% to 2.1% and 8.0% to 6.0% was seen for the RYGB and VSG groups respectively (inter group difference, p-value: 0.054). Since treatment with statin is normally not altered following bariatric surgery according to clinical standards, participants treated with statins were excluded for this analysis. In the group of participants that underwent RYGB, a significant positive change was seen in lipid levels (in total cholesterol, triglycerides, HDL and LDL). In the group of participants that underwent VSG this was only seen in triglycerides and HDL, while no significant change was seen in the clinically most important LDL levels and total cholesterol (significantly different from the RYGB group, Table 4.4).

	RYGB							VSG							RYGB vs. VSG
	Baseline	**3 mths (n=530)**	**p-value***	**12 mths (n=440)**	**p-value***	**24 mths (n=215)**	**p-value***	**Baseline**	**3 mths (n=320)**	**p-value***	**12 mths (n=240)**	**p-value***	**24 mths (n=105)**	**p-value***	**p-value ****
T2DM (at baseline):															
- HbA1c	65.6 (± 19.0)	45.79 (±10.45)	0.000	43.56 (± 10.68)	0.000	43.74 (±11.07)	0.000	57.1 (± 15.6)	41.67 (± 7.09)	0.000	41.87 (± 10.42)	0.000	40.20 (± 8.64)	0.000	0.000
- HbA1c ≤48 mmol/mol (%)	18.9	66.0	0.0001	75.7	0.0001	74.1	0.0001	40.0	83.6	0.0001	86.0	0.0001	84.0	0.0001	0.221
No T2DM (at baseline):															
- HbA1c	39.4 (± 5.4)	35.76 (± 3.63)	0.000	34.07 (± 3.64)	0.000	34.39 (± 3.89)	0.000	39.5 (± 5.0)	35.16 (± 3.89)	0.000	35.09 (± 3.75)	0.000	34.30 (± 3.05)	0.000	1.000
Cholesterol:															
- Total	4.90 (± 1.07)	4.08 (± 0.93)	0.000	4.21 (± 0.89)	0.000	4.42 (± 1.00)	0.000	4.91 (± 1.00)	4.72 (± 0.99)	0.190	4.78 (± 0.95)	0.636	4.88 (± 0.88)	1.000	0.000
- Triglycerides	1.90 (± 1.42)	1.50 (± 0.63)	0.018	1.23 (± 0.51)	0.000	1.26 (± 0.64)	0.000	1.83 (± 1.30)	1.56 (± 0.65)	0.267	1.45 (± 1.05)	0.021	1.40 (± 0.67)	0.031	0.232
- HDL	1.18 (± 0.51)	1.08 (± 0.40)	1.000	1.30 (± 0.30)	0.000	1.46 (± 0.37)	0.000	1.18 (± 0.40)	1.14 (±0.28)	1.000	1.31 (± 0.33)	0.000	1.36 (± 0.32)	0.000	0.752
- LDL	2.96 (± 0.98)	2.37 (± 0.82)	0.000	2.36 (± 0.78)	0.000	2.39 (± 0.84)	0.000	2.94 (± 0.88)	2.91 (± 0.85)	1.000	2.83 (±0.85)	1.000	2.92 (± 0.85)	1.000	0.001

Table 4.4: Changes in HbA1c and cholesterol. Participants were divided and kept into the 'T2DM' (type 2 diabetes mellitus) or 'no T2DM' group according to the diagnosis made at baseline. Data is presented as mean (± SD), unless otherwise indicated. * compared to baseline value. ** difference between RYGB (roux-en-Y gastric bypass) and VSG (vertical sleeve gastrectomy) group. Bonferroni corrections were applied.

4.3.3 Changes in questionnaire data following surgery

Twelve months following surgery, participants who returned the set of questionnaires at baseline were asked to fill in a second set of questionnaires, similar to the one filled in at baseline. 48 (RYGB, n=31; VSG, n=17) of the total 169 participants that filled in a questionnaire at baseline and reached their twelve months follow up appointment, returned the set of questionnaires, leading to a response-rate of 28.4%. Since numbers are small, particularly for VSG, some aspects of this work should be interpreted with caution.

Health related quality-of-life

There were significant improvements in all mental and physical domains of the SF36 in the RYGB group, similarly all domains of the IWQOL significantly improved in the RYGB group (Figures 4.4 and 4.5, Table 4.5). For the VSG group changes were mainly seen within the physical domains: four of the total eight domains significantly improved of the SF36 survey, and one of the five domains of the IWQOL survey significantly changed (Figures 4.4 and 4.5).

No significant differences between the RYGB and VSG group were seen in the changes within the different domains (Table 4.5). In both surgery groups, greater improvements were seen in the physical domains compared to the mental domains.

Figure 4.4: Change in SF36 health survey following surgery. All domains in had a significant change in the RYGB (Roux-en-Y gastric bypass, n=31) group. The following domains had a significant change in the VSG (vertical sleeve gastrectomy group, n=17): General health, Physical functioning, pain and Energy/Fatigue.

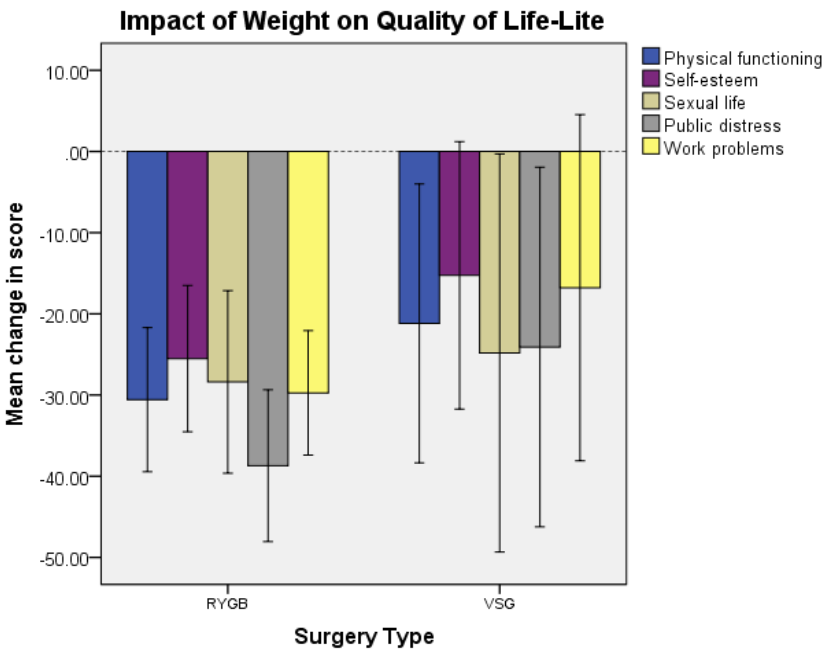


Figure 4.5: Change in IWQOL following surgery. All domains in had a significant change in the RYGB (roux-en-Y gastric bypass, n=31) group. Physical functioning had a significant change in the VSG (vertical sleeve gastrectomy, n=17) group.

	Paired Differences, RYGB group (n=31)					Paired Differences, VSG group (n=17)					Differences between groups p-value*
	Mean	SEM	95% Confidence Interval of the Difference		P-value*	Mean	SEM	95% Confidence Interval of the Difference		P-value*	
			Lower	Upper				Lower	Upper		
Physical Health											
SF36 - General Health	**-32.72**	5.32	-43.64	-21.80	0.000001	**-23.00**	5.60	-35.02	-10.99	0.001	0.294
SF36 -limitations due to physical health	**-49.11**	8.18	-65.89	-32.32	0.000002	-21.67	15.97	-55.93	12.59	0.196	0.086
SF36 - Physical functioning	**-35.14**	4.75	-44.89	-25.39	0.000000	**-28.33**	8.43	-46.42	-10.25	0.005	0.461
SF36 - Pain	**-24.46**	4.86	-34.43	-14.50	0.000028	**-21.67**	7.75	-38.29	-5.05	0.014	0.750
IWQOL - Physical function	**-31.83**	4.46	-40.46	-22.69	0.000000	**-21.18**	8.58	-40.31	-2.04	0.033	0.239
Mental Health											
SF36 -limitations due to emotional health	**-28.57**	8.35	-45.71	-11.44	0.001995	-15.56	9.69	-36.33	5.22	0.131	0.212
SF36 - Energy/fatigue	**-26.61**	4.42	-35.67	-17.54	0.000002	**-22.44**	7.59	-38.65	-6.24	0.010	0.653
SF36 - Emotional well being	**-14.00**	4.36	-22.94	-5.06	0.003386	-4.57	6.12	-17.79	8.65	0.468	0.218
SF36 - Social functioning	**-25.63**	4.39	-34.63	-16.63	0.000003	-15.00	7.83	-31.79	1.79	0.076	0.206
IWQOL - Self-esteem	**-26.67**	4.49	-35.87	-17.44	0.000000	-15.26	8.23	-33.60	3.09	0.094	0.204
Other											
IWQOL - Sexual life	**-31.18**	6.10	-43.68	-18.67	0.000022	-24.81	12.26	-52.13	2.50	0.101	0.610
IWQOL - Public distress	**-39.46**	4.57	-48.83	-30.09	0.000000	-24.09	2.22	-48.77	0.58	0.055	0.133
IWQOL - Work problems	**-29.74**	3.83	-37.62	-21.86	0.000000	-16.78	1.63	-40.54	6.98	0.228	0.159

Table 4.5: Change in quality of life questionnaires following surgery. SF36, short form 36 health survey; IWQOL, Impact of weight on quality of life-lite; SEM, standard error of the mean difference. Highlighted in red are the mean difference which are significant at <0.05 level, following post-Bonferroni corrections.

Eating behaviours

Mixed results were seen among the dietary restraint domains (Figure 4.6): the dietary restraint domain of TFEQ showed a significant increase (indicating more severe restraint) in the VSG group, while this was not significant in the RYGB group. Both the DEBQ and the EDEQ showed no significant changes in either surgery group within the dietary restraint domain. The contradictory results could indicate that (not all) dietary restraint measurements are reliable in the bariatric population.

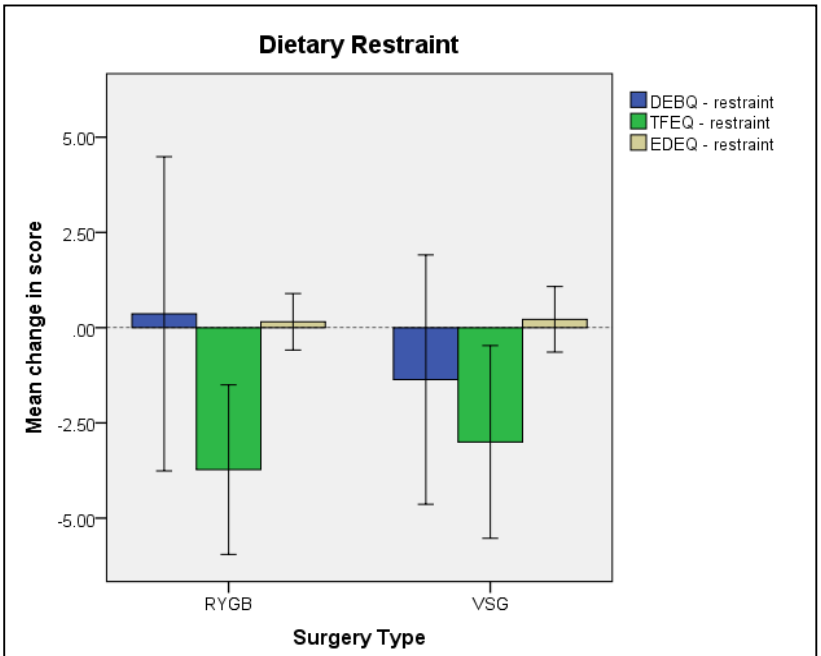


Figure 4.6: Dietary restraint change following surgery. TFEQ-restraint scores significantly changed following VSG surgery. DEBQ, Dutch eating behaviour questionnaire; TFEQ, Three factor eating questionnaire; EDEQ, eating disorder evaluation questionnaire; RYGB, Roux-en-y gastric bypass (n=31); VSG, vertical sleeve gastrectomy (n=17).

A significant improvement was seen in all domains of disinhibited eating in the RYGB group (DEBQ: emotional and external, TFEQ: disinhibition and hunger), while only the emotional domain of the DEBQ showed a significant change in the VSG group (Figure 4.7).

Disordered eating only significantly improved in the RYGB group, and no significant changes in any of the disordered eating subdomains were seen in the VSG group (Figure 4.8). The only significant difference between the RYGB and VSG group was seen in the disordered eating; a significantly greater improvement was seen in pre-occupation with weight in participants that had RYGB surgery compared to participants that VSG surgery, which was still significant after correcting for weight loss (*p:* 0.001, Table 4.6).

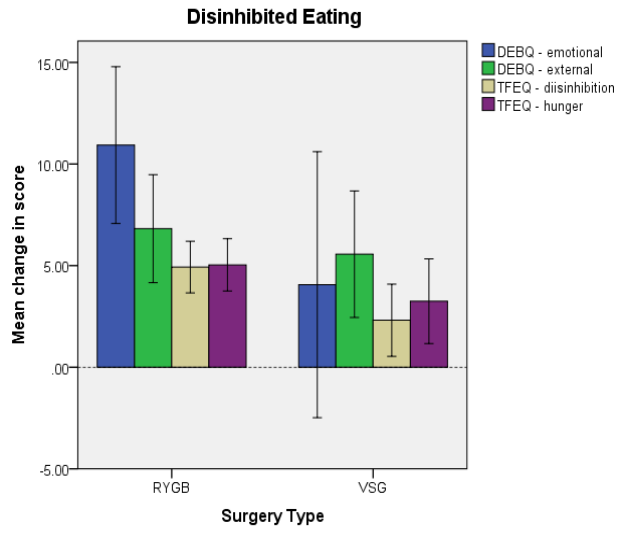


Figure 4.7: Change in disinhibited eating following surgery. All domains had a significant change in the RYGB (Roux-en-Y gastric bypass, n=31) group. External eating changed significantly in the VSG (vertical sleeve gastrectomy, n=17) group. DEBQ, Dutch eating behaviour questionnaire; TFEQ, Three factor eating questionnaire; EDEQ, eating disorder

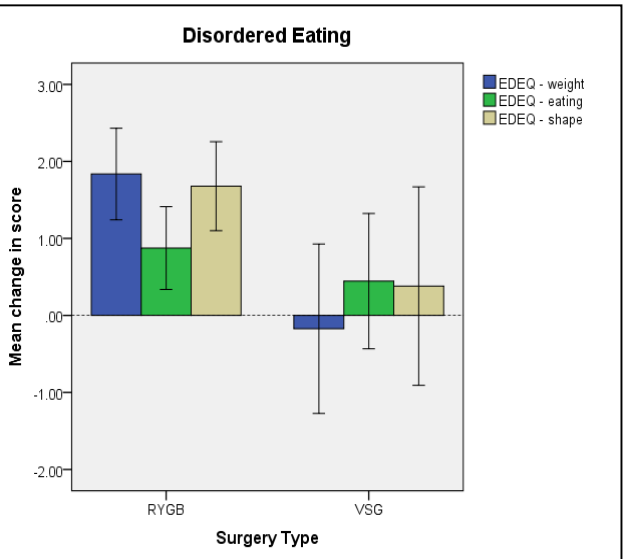


Figure 4.8: Change in disordered eating following surgery. All domains had a significant change in the RYGB (Roux-en-Y gastric bypass, n=31) group. External eating changed significantly in the VSG (vertical sleeve gastrectomy, n=17) group. DEBQ, Dutch eating behaviour questionnaire; TFEQ, Three factor eating questionnaire; EDEQ, eating disorder

	Paired Differences, RYGB group (n=31)					Paired Differences, VSG group (n=17)					Differences between groups p-value*
	Mean	SEM	95% Confidence Interval of the Difference		P-value*	Mean	SEM	95% Confidence Interval of the Difference		P-value*	
			Lower	Upper				Lower	Upper		
Dietary restraint:											
DEBQ-restraint	2.10	1.76	-1.51	5.705	0.243	-3.63	1.54	-6.92	-0.34	0.330	0.380
TFEQ-restraint	-2.90	1.01	-4.96	-.832	0.077	**-4.19**	1.10	-6.52	-1.85	0.020	0.420
EDEQ-restraint	0.25	0.37	-0.51	1.02	0.501	0.22	0.43	-0.74	1.18	0.624	0.956
Disinhibited eating:											
DEBQ-emotional	**10.77**	1.83	7.03	14.51	0.00002	4.06	3.27	-2.91	11.04	0.234	0.590
DEBQ-external	**6.40**	1.45	3.43	9.366	0.001	**5.56**	1.56	2.24	8.88	0.030	0.717
TFEQ-disinhibition	**4.69**	0.66	3.34	6.039	0.00000	2.31	0.89	0.42	4.21	0.201	0.370
TFEQ-hunger	**5.14**	0.63	3.85	6.43	0.00000	3.25	1.04	1.02	5.47	0.070	0.107
Disordered eating (pre-occupation with):											
EDEQ- Weight	**1.88**	0.29	1.28	2.47	0.00001	-0.17	0.55	-1.40	1.05	0.760	**0.021**
EDEQ- Eating	**0.88**	0.27	0.32	1.43	0.041	0.45	0.44	-0.53	1.42	0.335	0.388
EDEQ- Shape	**1.70**	0.28	1.13	2.28	0.00004	0.38	0.64	-1.05	1.82	0.567	0.340

Table 4.6: Change in eating behaviour following surgery. DEBQ, Dutch Eating Behaviour Questionnaire; TFEQ, Three factor eating questionnaire; EDEQ, Eating disorder examination questionnaire; SEM, standard error of the mean difference. Highlighted in red are the mean difference which are significant at <0.05 level, following post-Bonferroni corrections.

Mood

Again significant improvement was seen in all domains (anxiety and depression scores) in the RYGB group, while no significant changes were seen in the VSG group (Figure 4.9 and Table 4.7). However, no significant differences in this change were seen between the RYGB and VSG group.

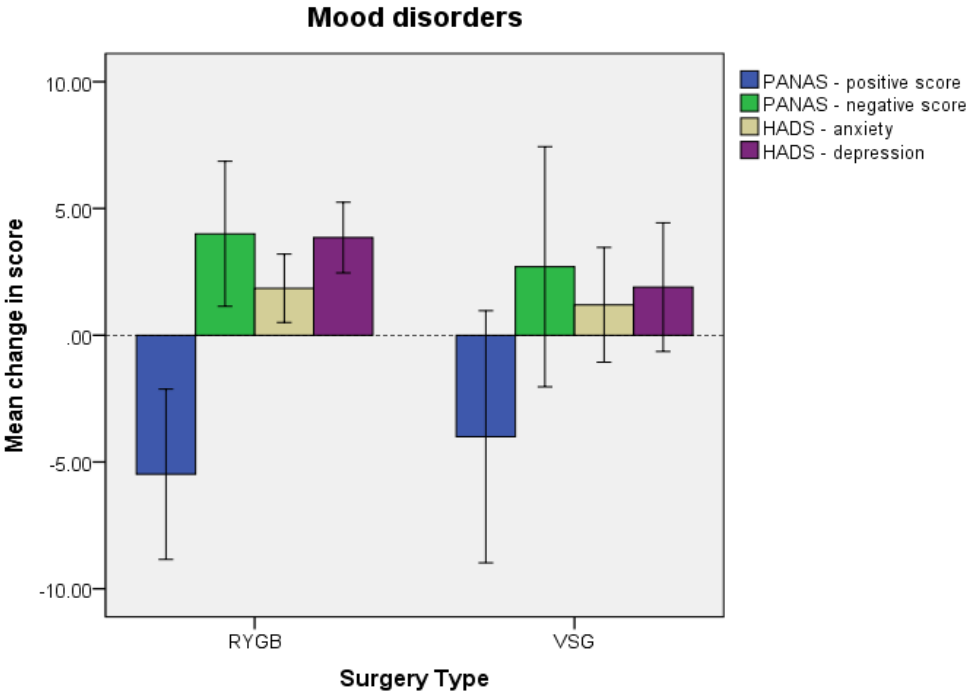


Figure 4.9: Change in mood following surgery. All domains had a significant change in the RYGB (Roux-en-Y gastric bypass, n=31) group. No significant changes were seen in the VSG (vertical sleeve gastrectomy, n=15) group. PANAS, positive and negative affect scale; HADS, hospital anxiety and depression scale.

	Paired Differences, RYGB group (n=31)					Paired Differences, VSG group (n=15)					Differences between groups p-value
	Mean	SEM	95% Confidence Interval of the Difference		p-value	Mean	SEM	95% Confidence Interval of the Difference		p-value	
			Lower	Upper				Lower	Upper		
PANAS-positive	**-5.48**	1.68	-8.93	-2.03	0.012	-4.00	2.49	-9.62	1.62	0.142	0.641
PANAS-negative	**4.00**	1.43	1.06	6.94	0.038	1.64	2.39	-3.69	6.96	0.509	0.389
HADS-anxiety	**2.03**	0.68	0.65	3.42	0.022	-0.82	1.57	-4.15	2.50	0.607	0.061
HADS-depression	**4.07**	0.68	2.67	5.47	0.000008	1.71	1.08	-0.58	3.99	0.133	0.058

Table 4.7: Change in mood following surgery. PANAS, positive and negative affect schedule; HADS, Hospital anxiety and depression score; SEM, standard error of the mean difference. Highlighted in red are the mean difference which are significant at <0.05 level, following post-Bonferroni corrections.

4.3.4 Factors influencing weight loss following surgery

Possible 'non-genetic' factors influencing weight loss:

Using multiple regression analysis, baseline characteristics were examined to see whether they had a predictive value towards %WL following RYGB or VSG. Analysis was performed in two stages with one analysis performed on the complete cohort (*n*=685) without the questionnaire data, and a second analysis was performed only including the participants that filled in the questionnaire set (*n*=169) before undergoing surgery.

The independent variables (excl questionnaire data) significantly predicted weight loss following surgery, $F(11, 119):3.011$, p-value: 0.001, r^2: 0.145. The variables adding significantly towards the prediction were: surgery type, baseline BMI and diagnosis of T2DM.

Questionnaire data:

None of the domains of the quality of life, eating behaviour or mood disorder questionnaires were predictive for weight loss.

Possible 'monogenic-obesity-like' predictive behavioural factors for weight loss

Several obesity-related phenotypes have previously been reported to be associated with monogenic forms of obesity: early onset of obesity, binge eating disorder and hyperphagia. Although in this cohort hyperphagia was not directly measured, the domains 'hunger' and 'disinhibition' measured using the TFE-questionnaire, and the domains 'emotional eating' and 'external eating' measured using the DEBQ have been associated before with hyperphagia, overeating and/or binge eating disorder [42,252].

Early onset obesity (onset before 10 years of age, compared to onset after 10 years of age) did correlate with baseline BMI and weight at baseline (Figure 4.10). The other 'monogenic-obesity-like phenotypes' did not correlate with any baseline features (Table 4.8). None of the phenotypes correlated with weight loss following surgery (Table 4.9).

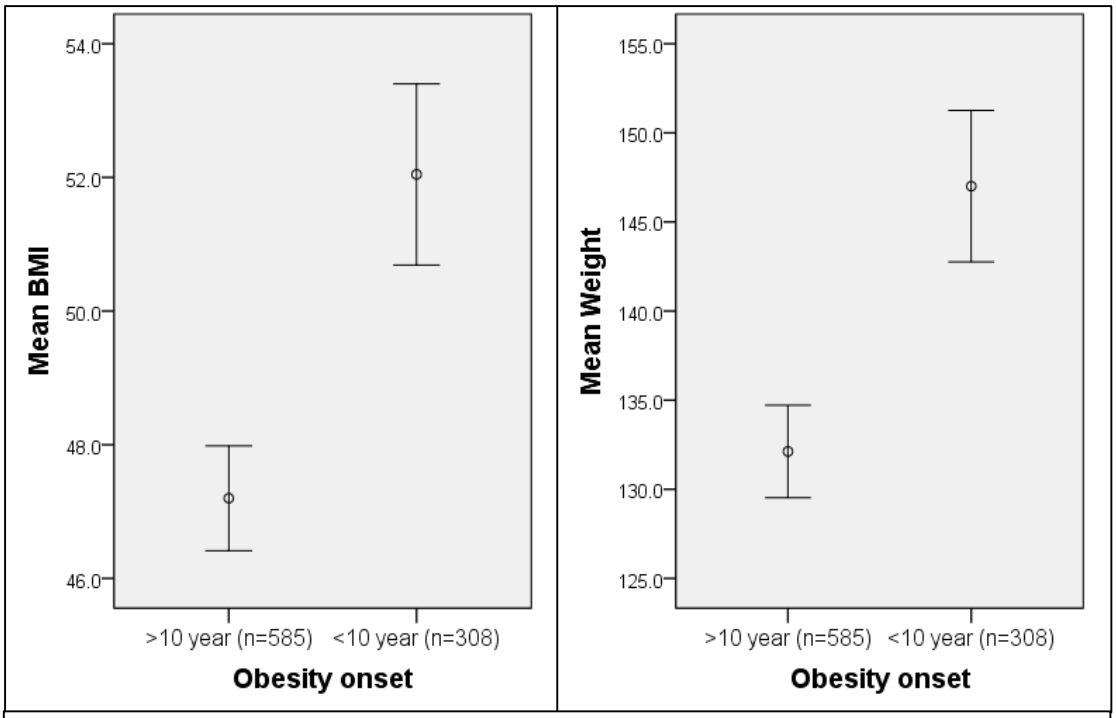


Figure 4.10: Onset of obesity and baseline BMI (left panel) and weight (right). Error bars depict standard error of the mean.

Phenotypes	**Height (m)**		**Weight (kg)**		**BMI (kg/m2)**	
	r	*P-value*	*r*	*P-value*	*r*	*P-value*
Obesity onset (*n*=903)	0.029	0.470	**0.225**	0.00000	**0.254**	0.00000
Binge eating disorder (*n*=980)	-0.017	0.627	-0.067	0.056	-0.057	0.107
TFEQ - disinhibition (*n*= 262)	0.024	0.698	0.073	0.239	0.058	0.354
TFEQ - hunger (*n*= 262)	0.036	0.572	-0.011	0.864	-0.036	0.569
DEBQ - emotional (*n*= 262)	-0.056	0.355	0.089	0.140	0.120	0.141
DEBQ - external (*n*= 262)	0.072	0.235	0.103	0.087	0.061	0.309

Table 4.8: Correlation analysis with "monogenic-obesity-like" phenotypes. TFEQ, Three factor eating questionnaire; DEBQ, Dutch Eating Behaviour Questionnaire. Highlighted in red are the mean differences which are significant at <0.05 level.

	% Weight loss at 12 months		% Weight loss at 24 months	
Phenotypes	*r*	*P-value*	*r*	*P-value*
Obesity onset	-0.063	0.216	0.050	0.495
Binge eating disorder	-0.031	0.478	-0.070	0.244
TFEQ - disinhibition	-0.037	0.677	-0.052	0.628
TFEQ - hunger	0.048	0.590	0.139	0.195
DEBQ - emotional	0.155	0.066	0.110	0.282
DEBQ - external	0.008	0.923	0.012	0.909

Table 4.9: Correlation analysis with monogenic obesity-phenotypes and weight loss. TFEQ, Three factor eating questionnaire; DEBQ, Dutch Eating Behaviour Questionnaire.

4.3.5 "Monogenic-obesity-like" risk-score

Although the individual phenotypes associated with monogenic forms of obesity were not associated with weight loss following surgery, a "monogenic-obesity-like" risk-score was developed to see whether these phenotypes would, in combination, correlate with any specific obesity-related baseline phenotype or with weight loss following surgery. This score was based on the phenotypes previously associated with monogenic obesity: diagnosis of binge eating disorder, early onset of obesity (before the age of 10) and extreme scores within hyperphagic-related eating behaviour.

Although this phenotypic risk-score creates a score from 0 (no phenotypes) to 6 (all six phenotypes), the score was subdivided into two groups: participants scoring 0-3 (*n*=242) and participants that scored 4-6 (*n*=31). This, because there was a limited number of participants with high scores. For instance, only one participant scored the maximum 6 points (meaning, being affected by all six phenotypes at the same time).

	"Monogenic-obesity-like" risk-score		
	Low score (0-3)	**High score (4-6)**	***p*-value**
N	245	31	--
Gender (% female)	72.5	74.2	0.514
Age (years)	**46.8** (±10.84)	**41.7** (±10.46)	0.013
BMI (kg/m^2)	47.66 (±8.56)	47.36 (±6.51)	0.853
Weight (kg)	134.00 (±28.54)	134.7 (±27.71)	0.894
IWQOL - Physical function	**37.05** (±24.39)	**26.96** (±20.96)	0.018
IWQOL - Self-esteem	**33.09** (±28.10)	**14.53** (±17.75)	0.003
IWQOL - Sexual life	**41.39** (±33.77)	**27.16** (±29.28)	0.009
IWQOL - Public distress	**38.01** (±24.47)	**27.41** (±25.16)	0.004
IWQOL - Work problems	**56.73** (±25.09)	**36.16** (±23.03)	0.001
Surgery type: - RYGB (%) - VSG (%)	 69.6 30.4	 50.0 50.0	0.080
%WL, 12 months	**-30.53** (±8.99)	-23.63 (±7.71)	0.000
%WL, 24 months	**-28.46** (±11.09)	-20.24 (±18.21)	0.030

Table 4.10: "Monogenic-obesity-like" risk-score. Data is presented as mean (± SD), unless otherwise indicated. Highlighted in red are the mean difference which are significant at <0.05 level, following post-Bonferroni corrections.

Interestingly, although no baseline differences in BMI or weight could be detected, participants with a higher risk-score appeared to have a worse weight-related quality of life, scoring significantly lower on all domains of the IWQOL. %WL at 12 and 24 months was also significantly different, with the participants having a high risk-score having a lower mean weight loss (Figure 4.11). To control for the difference in weight loss resulting from the two different surgery types, %WL was compared within each surgery type: again for both RYGB and VSG the participants with a higher risk-score lost less weight (Figure 4.12). Keeping the score range from 0 to 6 (without dividing them into two groups) a significant effect on weight loss was seen in the RYGB surgery group, although numbers in the higher scoring groups were small (*p*: 0.009, Figure 4.13 and 4.14).

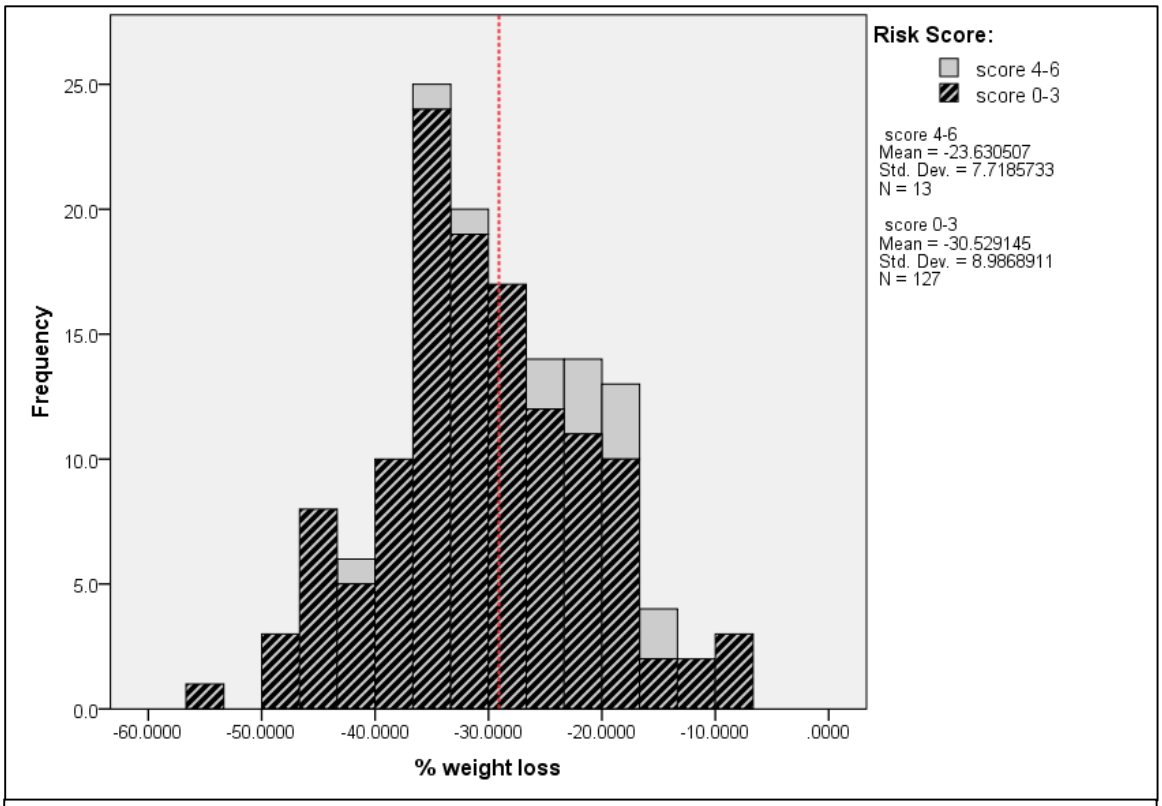


Figure 4.11: Percent weight change distribution at 12 months following surgery, with phenotypic-risk score.

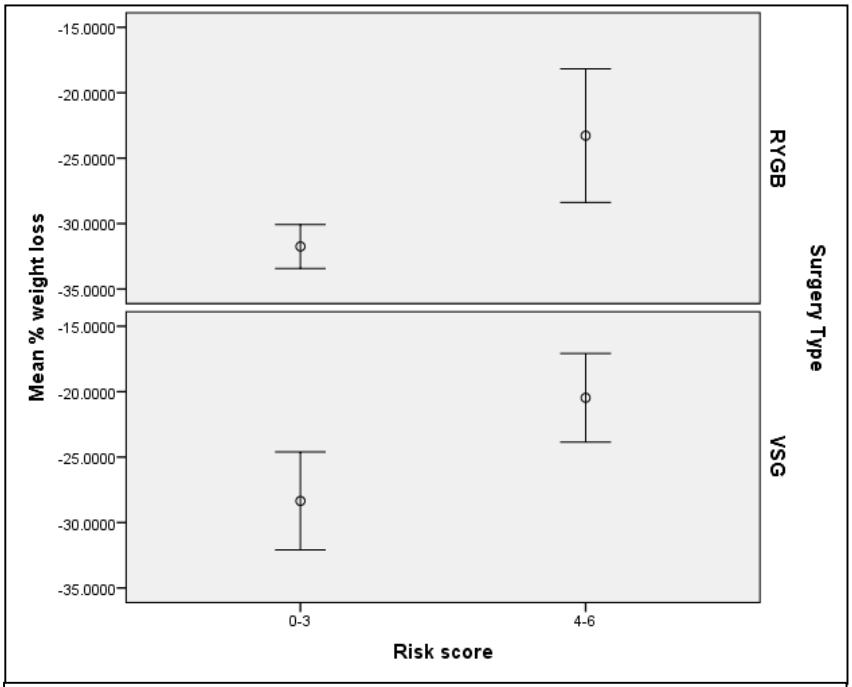
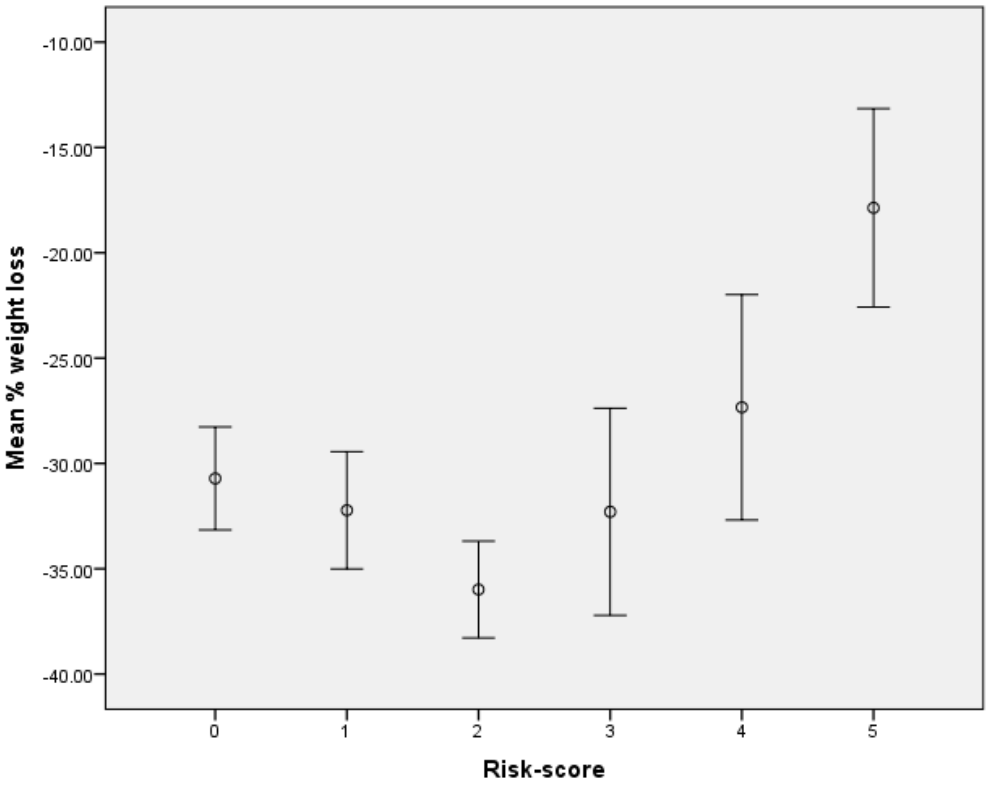
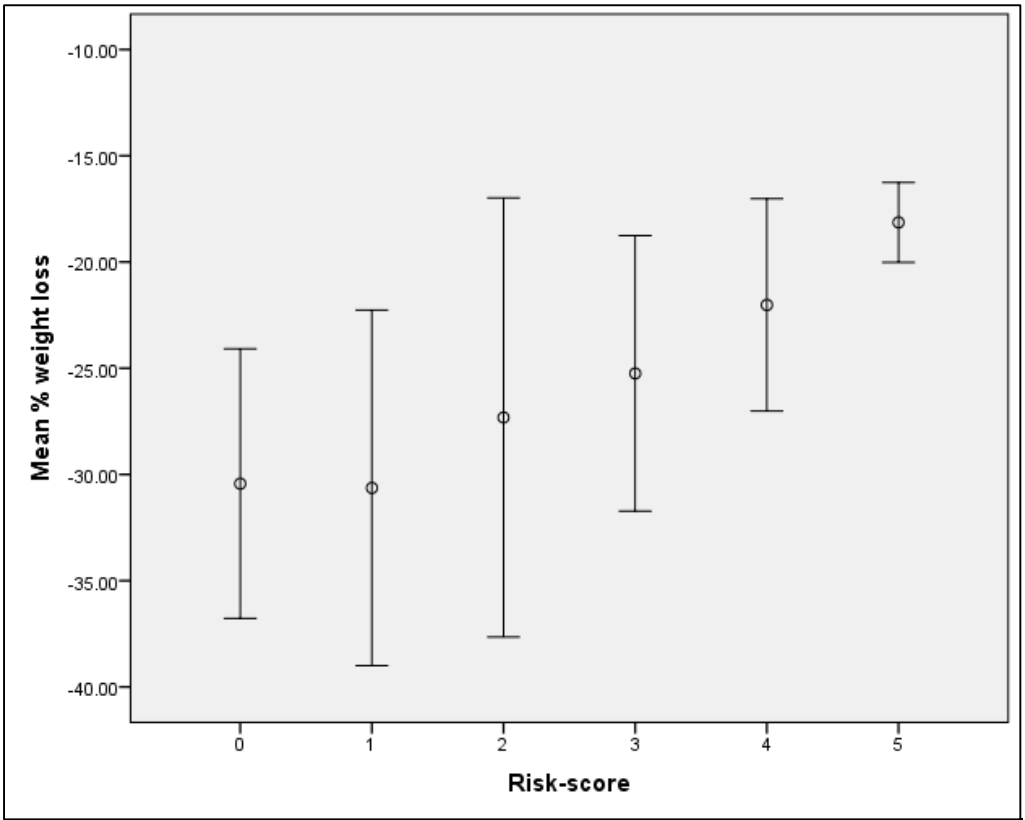


Figure 4.12: "Monogenic-obesity-like" risk-score and % weight loss 12 months following RYGB (top panel) and VSG (bottom panel). Error bars depict standard error of the means.



Risk-score	n	Mean	SEM	95% Confidence Interval	
				Lower Bound	Upper Bound
0	50	-31.117[a]	1.075	-33.253	-28.981
1	25	-31.475[b]	1.537	-34.528	-28.422
2	12	-36.474[c]	2.167	-40.777	-32.170
3	7	-32.166	2.842	-37.809	-26.522
4	4	-26.466	3.812	-34.036	-18.895
5	3	-16.894	4.402	-25.636	-8.151

Figure 4.13: "Monogenic-obesity-like" risk-score and percentage weight loss, 12 months following RYGB. [a] significantly different from risk score 5, p: 0.036. [b] significantly different from risk score 5, p: 0.033. [c] significantly different from risk score 5, p: 0.002. Bonferroni corrections were applied.



Risk-score	n	Mean	SEM	95% Confidence Interval	
				Lower Bound	Upper Bound
0	12	-32.025	2.944	-38.065	-25.984
1	4	-29.663	5.154	-40.238	-19.088
2	6	-24.340	4.514	-33.603	-15.077
3	9	-24.747	3.335	-31.590	-17.903
4	3	-23.454	5.767	-35.288	-11.621
5	2	-19.559	7.112	-34.152	-4.966

Figure 4.14: "Monogenic-obesity-like" risk-score and percentage weight loss, 12 months following VSG. No significant difference between the groups.

4.4 Discussion

Weight loss following surgery

As expected, both participants that underwent RYGB and those having a VSG showed a significant change in BMI following surgery. Multiple logistic regression modelling showed a significantly bigger change in BMI in the RYGB group compared to the VSG group.

For further analysis, weight loss at specific time points was used. Currently, some controversy exists on what weight loss tool is best used to measure weight loss following bariatric surgery and to effectively correct for differences in baseline BMI. Most commonly used metrics currently are BMI measurements at each time point (which is only valid if no differences in baseline BMI exists, such as in RTCs), △BMI, %WL or %EBWL [159,161,253]. Linear regression analysis for the different metrics calculated for 12 months follow up data, showed that of these different weight loss metrics, %WL was least affected by baseline BMI. This is in line with previous studies, and therefore this metric was used in the following analysis [254-256].

The maximum percentage weight loss of 31.76% seen in the RYGB group, and 26.25% in the VSG group is comparable to previously reported weight loss seen in cohorts with similar baseline BMI: 25% to 35% for RYGB and 20% to 30% for VSG [159,161,164-167,253,257-260]. Although several studies have been published on the effect of different weight loss surgery options, only a few of them were randomised clinical trials, mostly comparing or gastric banding or RYGB with intensive medical treatment [164,166,258-261]. The cohort reported here was also created through an observational study and was not set up as a randomised control trial. We can therefore not exclude the possibility that the difference seen in surgery outcome were due to differences in the two participant groups at baseline (described in chapter 3). So far, there is only one randomised control trial that compared RYGB with VSG, and this also showed a greater reduction in body weight after RYGB than after VSG at one and three years following surgery (27.5±7.3% vs 24.7±6.6% and 24.5±9.1% vs 21.1±8.9%

respectively) [164]. Although these weight loss percentages are lower than we have seen in our cohort, this can be explained by the lower BMI at baseline of the participants included in the RCT (36.0±3.5 in the RCT vs. 48.4± 8.67 reported here).

Health changes beyond weight loss

Analysis of health changes other than weight loss also showed significant improvements. Although T2DM remission *per se* was not examined in this analysis, HbA1C levels improved significantly for both the RYGB and VSG groups. In both groups, the percentage of diabetic participants with an HbA1c level <48 mmol/mol increased significantly, from 18.9% to 74.1% and from 40.0% to 84.0% in the RYGB and VSG group respectively. Lipid levels also significantly changed, with changes in total cholesterol, triglycerides, HDL and LDL for the RYGB group and changes in triglycerides and HDL in the VSG group. Although no significant difference was seen for the change in HbA1C between the two surgery groups, improvements in total cholesterol and LDL in the RYGB were significantly different from the VSG group, which did not show any improvements in these parameters.

Since the cohort presented here is not a RCT, it is difficult to correct for known and unknown baseline differences. However, previous studies show similar results to those reported here. A RCT comparing RYGB and VSG did not find any significant differences in improvements in T2DM remission, glycaemic control, or lipid levels [164,253]. Interestingly, however, one of the larger observational studies looking at the outcomes following VSG, reported similar results to ours, with positive changes in HDL and triglycerides, but no significant changes in total cholesterol and LDL levels [161].

Change in questionnaire data following surgery

Although the poor response rate (28.4%) of repeated questionnaires collected at 12 months following surgery led to only a small overall number of repeated questionnaire datasets (RYGB, n=31; VSG n=17), significant improvements were still found in all physical and mental health domains of the quality of life assessment tools in the participants that did return the questionnaires in the RYGB group (for both

general HRQOL, SF36, and the weight specific HRQOL, IWQOL). For the VSG group, however, changes were only seen in the physical domains. It is, therefore, not surprising that the biggest change in the RYGB group was also found in the physical domains compared to the mental domains. Although a tendency towards bigger changes in the RYGB was seen, there were no statistically-significant differences between the two groups. I am not aware of any previous studies investigating quality of life changes after VSG, but for RYGB there are several studies which have showed a significant positive influence on quality of life, with greater positive changes in physical health compared to mental health [232,262,263].

Comparing data from the three different eating behaviour questionnaires, there was no consistency in dietary restraint at baseline or following surgery. This contradiction is also visible in the limited number of studies that looked at dietary restraint following RYGB, with two previous studies reporting significant increase in cognitive restraint using the TFEQ [243,244], while one other study using the DEBQ did not see a significant change [264].

Significant improvement was seen in all domains covering disinhibited eating (emotional eating, external eating, disinhibition and hunger) in the RYGB group. This is similar to data reported in studies using TFEQ [243,244] and DEBQ [264]. Here we report for the first time, the significant improvements following RYGB in disordered eating measured by the EDEQ, covering preoccupation with weight, eating and shape. Apart from an improvement in external eating behaviour, and an increase in dietary restraint (measured using TFEQ), no significant changes were seen in the VSG group.

The mood disorder questionnaire results improved again only in the RYGB group (lower scores in depression and anxiety rates), but not in the VSG group. Several large studies have reported the decrease in prevalence of depression and the severity of depressive symptoms following RYGB or gastric banding [246,248,265,266], while no studies so far has reported changes in depression following VSG.

Although often in the questionnaire data a significant change was seen in the RYGB group, but not in the VSG group, only one domain showed significantly better improvement in the RYGB group compared to the VSG group: disordered eating with preoccupation with weight. This is most likely due the small numbers in both groups, and especially the VSG group. Longer follow up will hopefully increase the numbers and possibly will show if indeed no significant changes can be found within the VSG participants, and if this is significantly different from the RYGB group. This is especially important because of the limited data so far following the VSG procedure. Larger numbers will also enable analysis of possible predictive factors affecting quality of life, eating behaviour and mood disorder change, such as weight loss [243,245].

Predictive factors for weight loss

Multiple regression analysis indicated the only predictive factor for weight loss were type of surgery the patient underwent, baseline BMI and having a diagnosis of T2DM. This is in line with previous studies, and especially the type of surgery and baseline BMI measurements are well-known contributing factors. Although most studies correct for surgery type by segregating for this during the analysis, baseline BMI is less well accounted for, and it is thought that contradictory results of bariatric surgery comparisons in different studies may be explained by the application of different weight loss metrics used [254,255,267].

Some of the previously reported predictive factors for weight loss could not be repeated in this cohort, such as: binge eating disorder, age, gender, ethnicity and a having a history of depression [247,252,262,268,269].

"Monogenic-obesity-like" risk-score

A risk-score was designed from phenotypic characteristics that have previously been observed as major features of monogenic forms of obesity: early onset of obesity, binge eating disorder and

hyperphagia [74,84]. Although hyperphagia was not directly measured in this cohort, a selection of four domains was made from the eating behaviour questionnaires that were most related and all covered a form of overeating [187,270]. The 'hunger' scale measured using the TFEQ, measured the tendency to eat more than usual due to a loss of control over intake accompanied by subjective feelings of hunger, while the 'disinhibition' scale measures the tendency to overeat in response to different stimuli, with the feeling of losing control over dietary intake. The 'external eating' of the DEBQ explores the tendency to overeat in response to external cues (such as smell and sight), while the 'emotional eating' covers eating in response to emotional arousal states such as fear, anger or anxiety.

Since the questionnaire data was used to create this score, and only for about a quarter of the cohort the questionnaires were available, this score could only be applied to a small proportion of the complete cohort. Interestingly, of the group scored (n=276) only 31 had a core of 4 or more, indicating they had 4 of the 6 phenotypes included in this risk-score. Combining these 31 individuals as one group of 'high risk-scorers' and comparing them to the 'low risk-scorers' it appeared that although no differences could be found in gender, baseline BMI or weight, the overall weight related quality of life was worse for this group. Interestingly, weight loss at 12 months and 24 months was also significantly lower in the 'high risk-scorers' compared to the 'low risk-scorers', for both RYGB and VSG.

Since a "monogenic-obesity-like" risk-score like this has not been applied before to my knowledge, and it appears to have an effect on the participant's quality of life and treatment success, it would be interesting to see whether the individuals with the highest scores indeed have monogenic forms of obesity. Especially since it appears in this cohort, but also in previous studies, that the phenotypes included in this score on their own, do not correlate with post-surgery quality of life or weight loss outcome [243,244,252,264,269].

4.5 Conclusion

This chapter described, following on to the previous chapter, the results following bariatric surgery (RYGB and VSG) of the PMMO cohort. Previously reported outcomes in terms of weight loss could be repeated here (-31.1% for RYGB and -26.3% for VSG). For both surgery types a significant improvement was seen in T2DM status and cholesterol levels, while for the latter results following VSG were less promising. Although the questionnaire follow-up data was limited due the low return rate, significant improvements were seen overall following RYGB in quality of life, eating behaviour and mood. Although no or minor significant improvements were seen following VSG, continued follow up will need to indicate if this is due the lack of numbers or if there is really a lack of improvement following this VSG compared to RYGB.

As a part of this study a 'monogenic-obesity-like' risk score was developed, by collating phenotypes previously associated with monogenic obesity. Interestingly, a decrease in weight loss was seen with an increasing risk-score. Further analysis is required to indicate if this score will indeed predict monogenic obesity, or if it affects weight loss through other means.

CHAPTER 5

THE EFFECT OF *MC4R* VARIANTS ON WEIGHT LOSS IN PATIENTS UNDERGOING BARIATRIC SURGERY

5.1 Introduction

In the previous chapters the creation and characterisation of a bariatric patient cohort, named Personalised Medicine of Morbid Obesity (PMMO), was described. This cohort was created to enable analysis of genetic and non-genetic factors influencing body weight, obesity-related comorbidities and response to bariatric surgery. This chapter describes screening of the cohort for the most common cause of monogenic obesity, MC4R deficiency, and assessment of the implications of this condition on baseline phenotypes and outcomes after surgery.

MC4R deficiency is currently the most common known form of Mendelian obesity, with a prevalence of 2 to 6% reported in morbidly-obese adults and children respectively [39-50,271]. MC4R deficiency has often been described as a monogenic 'non-syndromic' form of obesity, with only obesity as its phenotype, while others prefer to refer to the 'MC4R deficiency syndrome' [39,41,42,272]. The latter including besides obesity, increased lean mass, increased linear growth, hyperphagia, and severe hyperinsulinemia [39].

The complex phenotype of severe obesity and hyperphagia might indicate that patients suffering from this disease might not respond to all weight loss surgery types as well as others. Although several studies have recently been published on the effect of MC4R deficiency on weight loss outcome following bariatric surgery, some contradictions exist. Limited studies report on contradictory results following gastric banding with an increased risk of re-operation [178,179], but having little or no effect in patients having RYGB [171-173]. So far, no reports are available on the effect of MC4R deficiency on surgery outcome following VSG (besides a single case report with short-term follow up [173]). Although RYGB has been the most applied bariatric surgery type within Europe, VSG is gaining more popularity within and outside Europe [5]. An urgent update regarding the feasibility of the different surgery types in MC4R deficient patients is therefore warranted.

In this chapter I describe the results of screening for *MC4R* variants in the PMMO cohort, aiming to identify baseline characteristics specific for individuals carrying such variants, which might be useful for stratification purposes. Secondly, by identifying individuals with MC4R deficiency and following their weight loss trajectories, surgery types might be identified as being more suitable for this group of patients.

5.2 Aims of the study

1) To detect the prevalence and disease-related phenotypes of MC4R deficiency in a cohort of morbidly-obese patients seeking bariatric surgery.
2) To investigate the influence of *MC4R* variants on the outcomes of bariatric surgery.

5.3 Results

5.3.1 Baseline characteristics

Of the 1,075 participants included in the PMMO cohort, 28 participants (2.6%) had to be excluded for this part of the study, because of poor quality of DNA. This was due to multiple reasons, but mostly due the improper collection of an EDTA blood or saliva sample, leading to a too small sample amount to be processed. For a small number of samples, errors were made during the DNA extraction steps, leading to poor quality of DNA samples.

For the 1,049 remaining participants, baseline characteristics are listed in Table 5.1. A similar mean BMI of 48.17 (± 8.69 SD) and age of 45.68 (± 11.11 SD) was seen as for the complete cohort described in chapter 3. As with the overall cohort, participants undergoing RYGB had a significantly higher prevalence of T2DM, whilst BED was more common among participants undergoing VSG. The participants with higher BMI ranges also tended to go more often for the VSG option.

	Total	AGB	RYGB	VSG	none	P-value
n	*1044*	*42*	*537*	*340*	*125*	--
Age	45.68 (± 11.11)	44.76 (± 11.12)	45.73 (± 10.69)	44.98 (± 11.40)	47.74 (± 11.93)	0.115
Gender (% Female)	74.0	84.1	74.2	73.0	72.2	0.439
Weight	134.50 (± 31.25)	**123.75** (± 21.75)	134.13 (± 25.03)	**137.60** (± 31.68)	131.22 (± 31.25)	0.007
Height	1.67 (± 0.09)	1.66 (± 0.09)	1.67 (± 0.09)	1.67 (± 0.10)	1.67 (± 0.09)	0.823
BMI	47.1 [42.3-52.3]	44.4 [41.0-48.8]	47.3 [42.9-52.3]	47.5 [42.7-53.8]	45.9 [39.9-51.4]	0.056
BMI classification (%) - **Class II** - **Class III** - **Class IV** - **Class V**	 15.2 50.2 25.2 9.3	 16.2 62.2 21.6 0.0	 13.5 52.0 **27.3** **7.2**	 14.3 48.2 **23.8** **13.7**	 25.2 44.5 21.0 9.2	0.002
T2DM (%)	38.0	23.1	**45.2**	**26.2**	44.3	<0.0000
BED (%)	16.5	18.2	**12.6**	**21.5**	20.0	0.015
Onset obesity <10 years old (%)	36.5	24.0	37.1	37.3	35.7	0.611

Table 5.1: Baseline characteristics of participants screened for *MC4R* variants. Data is presented as mean (± SD), unless otherwise indicated. AGB, adjustable gastric banding; RYGB, Roux-en-Y gastric bypass; VSG, vertical sleeve gastrectomy; BMI, body mass index; T2DM, type 2 diabetes mellitus (treated with oral glucose lowering medication and/or insulin); BED, binge eating disorder. Highlighted in red are the mean difference which are significant at <0.05 level, following post-Bonferroni corrections.

5.3.2 *MC4R* variants detected

A total of 71 instances of variants in *MC4R* were found, of whom 16 were considered to be rare (MAF <0.001). These 16 variants were found in 15 individuals, as two rare variants were found within one individual. Nine of these variants had previously been reported to be affecting the protein function (class 5 variants), while for two variants contradictory results were reported (class 3 variants of unknown significance). The remaining five variants were consistently reported as not to affect the receptor's function (class 2 variants).

A total of eight individuals carrying a class 5 variant affecting the function of the protein gave a prevalence of 0.77% of MC4R deficiency in the overall morbidly obese PMMO cohort, which was lower than anticipated. Sanger sequencing chromatograms of the variants identified can be found in Appendix 5.1 (page 333). For all rare variants, location, functional characterisation, and phenotypes of the participants they were found in, are summarised in Table 5.2

Of the nine class 5 variants, three were reported to cause a complete loss of function of the MC4R protein: c.631_634delCTCT (p.(Leu212fs)) [39], c.896C>A (p.(Pro299His)) [39,150], and c.812G>A (p.(Cis271Tyr)) [39,150,273]. Two of these variants (p.Leu212fs and p.Pro299His) were found in the same individual (participant 1 in Table 5.2), so further analysis was warranted to identify whether a complete knockout of the *MC4R* gene was apparent in this individual through compound heterozygosity (in which case, there would be a complete loss of MC4R function), or alternatively whether both variants were present on the same chromosome, so that the other copy of the gene remained functional.

Analysis of the sequence overlay seen following the frameshift caused by the c.631_634delCTCT variant visualised in the chromatogram and compared to the sequence in the reverse direction, it was discovered that the c.896C>A variant was located on the same allele (on the sequence that was frameshifted, as can be seen in Figure 5.1), rather than on the other chromosome.

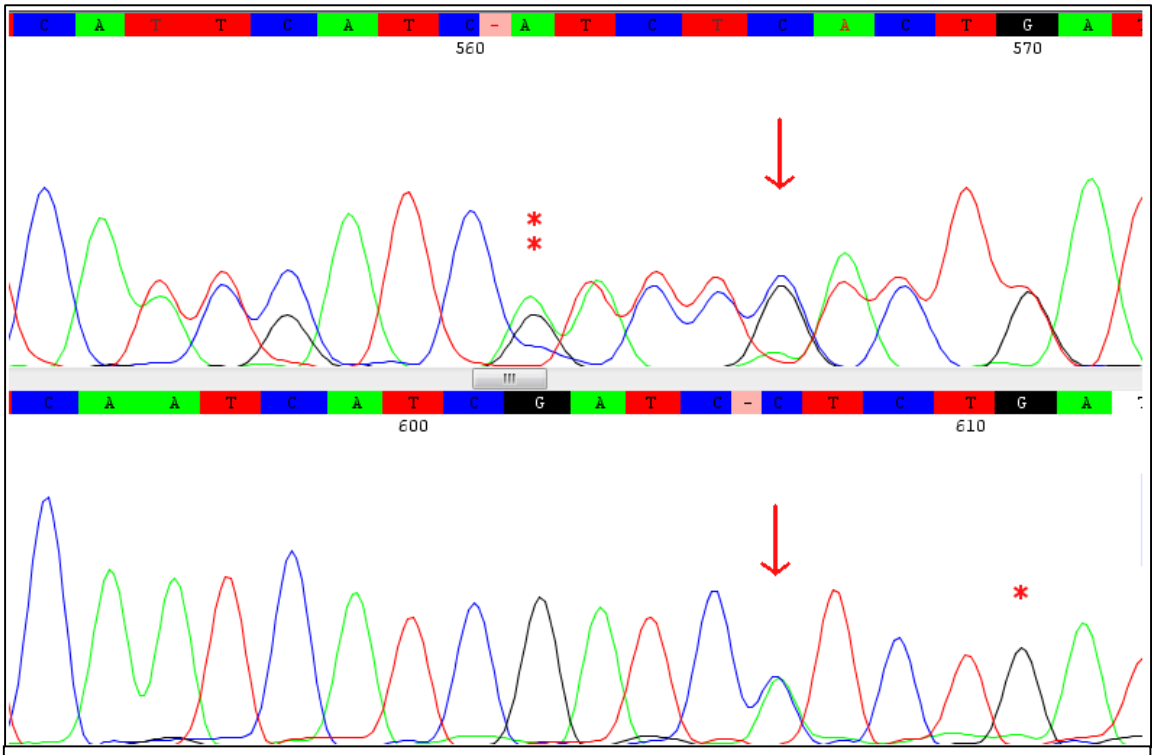


Figure 5.1: Chromatogram of c.631_634delCTCT and c.896C>A variants. The frameshift caused by the CTCT deletion (top sequence) was analysed to detect if the C>A substitution (indicated by the arrow in the bottom sequence) was on the same allele as the deletion (and should therefore have shifted). The arrows indicate the location of the c.896C>A variant at both sequences. The single asterisk indicates the nucleotide (G) that has shifted to this location in the upper sequence because of the 4bp frameshift. The double asterisk indicated the location the base located at the point-mutation has shifted to. As at the location of the mutation in the top panel a C nucleotide (not mutated allele) and G nucleotide can be seen, while at the frame-shifted point-mutation location (double asterisk) a G nucleotide (normal reference) and an A nucleotide can be seen, it can be concluded the c.896C>A variant has shifted with the frameshift caused by the c.631_634delCTCT and therefore is located on the same allele.

Of the six remaining class 5 variants, previously performed in silico studies showed for one variant a reduced cell surface expression and signalling: c.815C.T (p.(Pro272Leu) [274]; for three variants a decreased response to the agonist: c.182A>G (p.(Glu61Lys)), c.494G>A (p.(Arg165Gln)) and c.124G>A (p.(Glu42Lys)) [39,41,47,151]; for one variant a decreased constitutive activity: c.53G>T (p.(Arg18Leu)) [151]; and for one variant a decreased response to the agonist as well as a decreased constitutive activity: c.913C>T (p.(Arg305Trp)) [42]. For two variants, c.20G>A (p.(Arg7His)) and c.706C>T

(p.(Arg236Cis)), there were contradictory results in the literature regarding the effect on function of the protein, with some studies reporting them as affecting the function, while other studies reported them as not affecting the receptor [151,274-277].

A further 52 instances of more common variants were identified: 21 (2.0%) individuals carried the c.307G>A (p.(Val103Ile)) variant, and 15 (1.4%) individuals carried the c.751A>C (p.(Ile251Leu)) variant, which is similar to the frequencies that have been reported in population-based studies (MAF in ExAC are 0.017 and 0.007 respectively [208]). *In vitro* studies indicate that neither variant decreases the function of the MC4R protein, but instead, each might enhance its function: through a decrease of hAGRP potency of the p.Val103Ile variant, while an increased basal activity of the MC4R was seen in the p.Ile251Leu variant [150,151]. This is consistent with the negative association of each of these two variants with obesity in large meta-analyses [271].

Another 14 (1.3%) individuals carried the c.594C>T (p.(Ile198Ile)) synonymous variant, which has a MAF in ExAC of 0.034 in individuals with African descent, while two individuals carried the infrequent c.468G>A (p.(Gln156Gln)) synonymous variant (for which the MAF in ExAC is 0.002) [208].

In five unrelated individuals, a combination between the c.606C>A (p.(Phe202Leu)) variant and the synonymous variant p.Ile198Ile was seen, while p.Phe202Leu was not found in any participant on its own. The p.Phe202Leu variant has, like the p.Ile198Ile, a higher prevalence among the population of African descent (MAF in ExAC of 0.009 in African vs. MAF of 0.00001 in European Caucasians) [208].

Within the PMMO cohort, all of these five p.Phe202Leu; p.Ile198Ile variant combinations were found in individuals of African descent. In total there were 100 individuals with African descent (African, African-American, Caribbean or mixed background) included in the PMMO cohort and screened for *MC4R* variants, which would give a prevalence of 5% in that population group. Although the p.Phe202Leu variant has been reported in a few lean individuals on its own [40,44], and has been reported to not affect the MC4 receptor function [151,277,278], one study does report a small

reduction in cell surface expression [279]. No functional analysis has been reported on the combination of these two variants within *MC4R*.

ID	Variant characteristics						Participant phenotypes				
	Variant	**Amino acid change**	**Reported MAF**	***in silico* analysis**	***in vitro* analysis**	**Ref.**	**Age (yr)**	**BMI (kg/m^2)**	**Age of onset**	**Gender**	**Ethnicity**
1	c.631_634 delCTCT	p.Leu212fs	<0.0000	*na*	complete loss of function	[39]	57	49.4	<10	M	British
	c.896C>A	p.Pro299His	<0.0000	PD/D	complete loss of function	[150]					
2	c.913 C>T	p.Arg305Trp	<0.0000	PD/D	decreased constitutive activity and decreased response to agonist	[42]	43	49.0	<10	F	Jamaican
3	c.815 C>T	p.Pro272Leu	NR	PD/D	reduced cell surface expression and signalling	[274]	25	48.4	<10	F	Brazil
4	c.20G>A	p.Arg7His	<0.0000	B/T	contradictory studies	[150,172,272,275]	52	41.8	>10	F	Caribbean
5	c.706C>T	p.Arg236Cis	<0.0000	B/T	contradictory studies	[46,272,277]	35	39.8	<10	F	British
6	c.53G>T	p.Arg18Leu	<0.0000	B/T	decreased constitutive activity	[151,275]	58	42.9	?	F	British
	c.751A>C	p.Ile251Leu	0.007	B/T	no functional alteration						
7	c.812G>A	p.Cis271Tyr	NR	/D	complete loss of function	[39,150,273]	41	43.3	<10	F	British
8	c.124G>A	p.Glu42Lys	<0.0000	B/T	decreased response to agonist	[47]	38	53.2	>10	F	British
9	c.182A>G	p.Glu61Lys	<0.0000	PD/D	decreased response to agonist	[151,210,277]	63	46.1	<10	F	British
10	c.494G>A	p.Arg165Gln	I: <0.0000	PD/D	decreased response to agonist	[39,41,150]	47	38.4	<10	F	Caribbean
	c.594C>T	p.Ile198Ile	0.004	*na*	no known functional alteration	[278]					

(Table continues on next page)

11	c.606C>A	p.Phe202Leu	0.007	B/T	no known functional alteration	[151]	39	44.3	<10	F	African
	c.594C>T	p.Ile198Ile	0.004	*na*	no known functional alteration	[278]					
12	c.606C>A	p.Phe202Leu	0.007	B/T	no known functional alteration	[151]	47	48.2	<10	F	Caribbean
	c.594C>T	p.Ile198Ile	0.004	*na*	no known functional alteration	[278]					
13	c.606C>A	p.Phe202Leu	0.007	B/T	no known functional alteration	[151]	38	39.6	>10	F	Caribbean
	c.594C>T	p.Ile198Ile	0.004	*na*	no known functional alteration	[278]					
14	c.606C>A	p.Phe202Leu	0.007	B/T	no known functional alteration	[151]	29	39.6	>10	F	Caribbean
	c.594C>T	p.Ile198Ile	0.004	*na*	no known functional alteration	[278]					
15	c.606C>A	p.Phe202Leu	0.007	B/T	no known functional alteration	[151]	28	49.6	>10	F	African
	c.594C>T	p.Ile198Ile	0.004	*na*	no known functional alteration	[278]					

Table 5.2: Overview of variants found in the PMMO cohort and variant carrier characteristics. In silico predictions (Polyphen/SIFT): B: benign; PD: probably damaging; T: tolerated; D: damaging; na: not applicable. MAF (minor allele frequency) as noted in ExAC [208]. NP; not reported in the ExAC database.

5.3.3 Baseline characteristics of *MC4R* variant carriers

Baseline characteristics are summarised in Table 5.2. One of the eight class 5 *MC4R* variant carriers was found in a male participant, while the remaining class V variant carriers were female. There was a mixture of ethnicities, with five individuals being British. Interestingly only one class V variant carrier had a BMI >50 kg/m^2 upon recruitment and was therefore classified as class IV obesity, while the others' BMI ranged from 38.4 - 49.4 kg/m^2.

However, while analysing the weight histories collected, it emerged a further three class V variant carriers (participant 7, 9 and 10 in Table 5.2) had significantly higher weight in the past and would have classified as class IV obesity (with a BMI >50kg/m^2), but managed to lose weight through life-style adjustments before being recruited into this study and undergoing bariatric surgery. None of the nearly a hundred class V obese (with a BMI >60kg/m^2) included in the PMMO cohort carried a *MC4R* variant affecting the receptors function.

A further comparison analysis was performed between the MC4R deficient participants (including the two carriers of the p.Arg7His and p.Arg236Cys variants of unknown significance) and the remaining cohort was performed (Table 5.3). Interestingly a significantly higher proportion of these MC4R deficient participants, compared to the remaining cohort had early onset obesity, consistent with previous findings that MC4R deficiency leads to early onset obesity. No other differences in clinical phenotypes were seen between the MC4R deficient participants, the common variants carriers and the remaining cohort, specifically not in previously reported associations with height or BED [39,42,178]

Although it would be interesting to look at the quality of life, eating behaviour and mood in the MC4R deficient participants using the questionnaire data described in chapter 3, unfortunately only three of the participants of the MC4R deficient participants had filled in these questionnaires. The small number made comparison analysis impossible.

	Remaining cohort	MC4R deficient participants	*p*-value*	V103I	I251L	F202L:I198I	*p*-value**
Number	993	10		21	15	5	
Gender (female %)	73.8	90.0	0.245	75.0	64.3	100	0.433
Age	45.74 (± 11.19)	45.30 (± 11.75)	0.902	46.40 (± 7.56)	44.36 (± 9.60)	36.20 (± 7.86)	0.412
Weight	134.84 (± 28.52)	129.07 (± 20.96)	0.951	128.45 (± 17.37)	127.24 (± 16.71)	123.46 (± 14.37)	1.000
Height	1.67 (± 0.09)	1.67 (± 0.08)	0.524	1.67 (± 0.10)	1.67 (± 0.11)	1.67 (± 0.05)	0.535
BMI	48.29 (± 8.78)	45.25 (± 4.55)	0.301	46.35 (± 7.93)	45.53 (± 5.73	44.25 (± 4.66)	0.364
Highest BMI	48.38 (± 8.91)	49.81 (± 6.57)	0.632	46.35 (± 7.93)	45.53 (± 5.73)	44.25 (± 4.66)	0.450
T2DM (%)	37.8	40.0	0.884	60.0	28.6	20.0	0.252
Insulin	30.0 (± 44.21)	24.7 (±6.72)	0.896	16.68 (± 9.28)	18.33 (± 9.16)	20.90 (± 3.82)	1.000
Glucose	5.11 (± 1.55)	4.78 (± 0.29)	1.000	4.85 (± 0.33)	4.86 (± 0.54)	4.78 (± 0.29)	1.000
HbA1c	39.38 (± 5.05)	40.0 (± 4.83)	1.000	39.54 (± 4.94)	40.36 (±7.99)	39.50 (± 4.36)	1.000
BED (%)	16.5	12.5	0.760	15.8	30.0	0.0	0.662
Onset obesity <10 years old (%)	**37.0**	**83.3**	0.020	7.1	20.0	33.3	0.298

Table 5.3: Differences between MC4R deficient participants and non-variant carriers between MC4R deficient participants and non-variant carriers. Data is presented as mean (± SD), unless otherwise indicated. BMI, body mass index; T2DM, type 2 diabetes mellitus (on oral glucose lowering medication and/or insulin); BED, binge eating disorder. Highlighted in red are the mean difference which are significant at <0.05 level, following post-Bonferroni corrections. * Difference with between MC4R deficient participants and non-variant carriers ** multiple comparison analysis between common variant carriers and non-variant carriers.

5.3.4 Weight loss following bariatric surgery in *MC4R* variant carriers

Of the 10 MC4R deficient participants (including the two carriers of the p.Arg7His and p.Arg236Cys variants of unknown significance) eight had bariatric surgery treatment: five underwent RYGB, while three underwent VSG. Two individuals had not received surgery at the time of writing, and are currently on the waiting list.

With the data being analysed here, being part of a still ongoing study, some participants had not reached all follow up appointments at the time of writing: Of the five that underwent RYGB surgery, one individual was lost to follow-up 3 months following surgery and for the VSG group one individual had their surgery only recently, so no follow-up data was available yet at the time of writing this thesis. For three individuals, all time-points have been collected up to 24 months (2x RYGB, 1x VSG). For the remaining three, data was collected up to 12 months following surgery.

The following analysis was, therefore, based on a total of 5 individuals that underwent RYGB (with for one individual only limited date) and for 2 individuals that underwent VSG. In consideration of this small number, for initial analysis all MC4R deficient participants were grouped into one, no matter what surgery type they had. For sequential analysis variant carriers were divided by surgery, although caution is advised in interpreting the results because of the small numbers available to date.

MC4R deficient participants had a significant change in BMI following bariatric surgery, just like the individuals without any *MC4R* variants. A significant change was already seen 10 days following surgery and a significant change in BMI was seen up until 12 months following surgery, after which BMI change stabilised (Figure 5.2 and Table 5.4).

No significant differences were seen comparing the BMI change for the MC4R deficient participants and the non-variant carriers at each time-point (Table 5.4), nor was there a difference in weight loss trajectories (Figure 5.3).

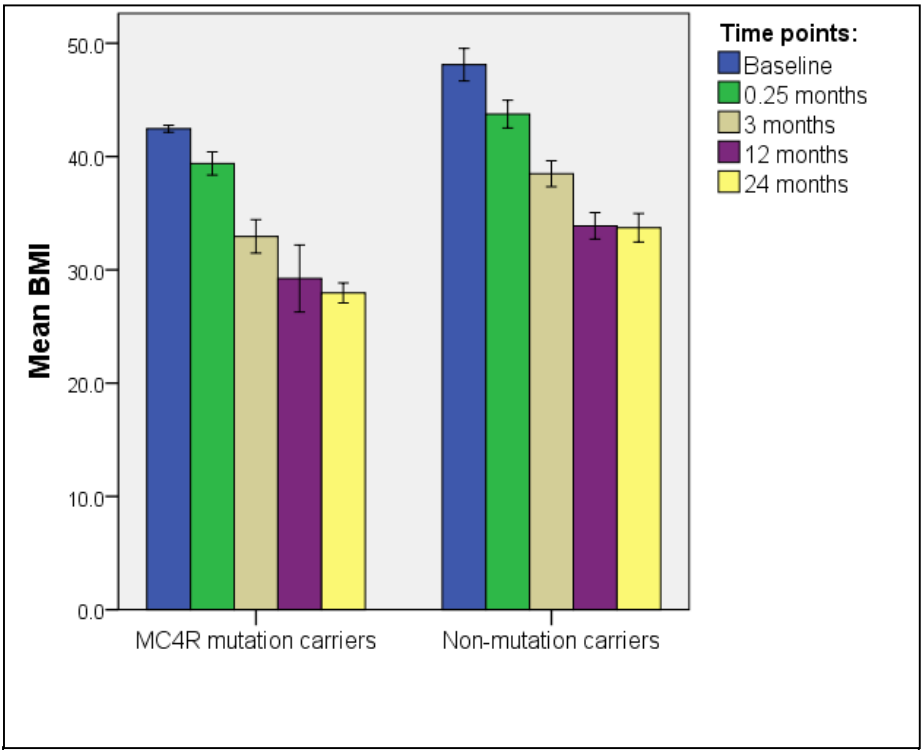


Figure 5.2: Change in BMI. Change in BMI in A) MC4R deficient participants (n=7) and in B) non-variant carriers (n=633). Error bars depict SEM.

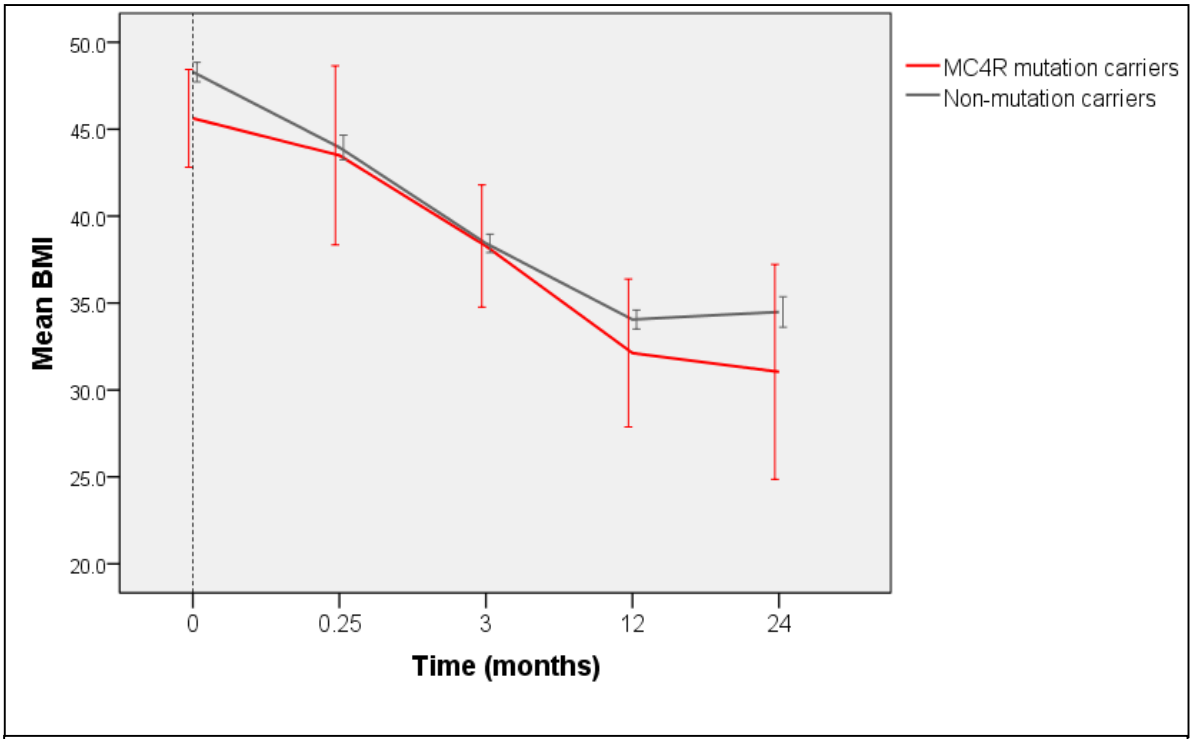


Figure 5.3: Weight loss trajectories for MC4R deficient participants. Linear mixed modelling showed no significant differences between the MC4R deficient participants (n=7) trajectories and the non-variant carriers (n=633) trajectories (p-value: 0.654). Error bars depict SEM.

Time (mths)	MC4R deficient participants (n=7)			Non-variant carriers (n=633)			Difference between groups	
	Mean BMI (SD)	Δ-BMI from baseline	p-value*	Mean BMI (SD)	Δ-BMI from baseline	p-value*	Mean (SE) [95% CI]	p-value**
0	47.49 (± 4.58)	--	--	47.90 (± 8.37)	--	--	--	---
0.25	43.49 (± 5.76)	-4.00	0.004	43.95 (± 7.56)	-3.95	0.000	-1.30 (3.44) [-8.06 to 5.46]	0.706
3	38.28 (± 4.31)	-9.21	0.000	38.43 (± 6.67)	-9.56	0.000	0.18 (2.93) [-5.57 to 5.94]	0.950
12	32.12 (± 5.21)	-14.43	0.004	34.04 (± 6.83)	-13.86	0.000	-1.86 (o,52) [-7.50 to 3.78]	0.517
24	31.04 (± 5.35)	-16.45	0.027	34.43 (± 8.01)	-13.47	0.000	-2.93 (4.38) [-11.54 to 5.67]	0.503

Table 5.4: Change in BMI in MC4R deficient participants following surgery. The BMI change over time for MC4R deficient participants and non-variant carriers are listed. *compared to baseline BMI **change BMI in RYGB group compared to VSG group. Bonferroni corrections were applied.

To best correct for baseline differences in BMI and to be able to segregate for the two different surgery types, weight loss percentage was calculated for each time point, and compared between the MC4R deficient and non-variant carriers. Again no differences were seen between the two groups at any time-point (Figure 5.4).

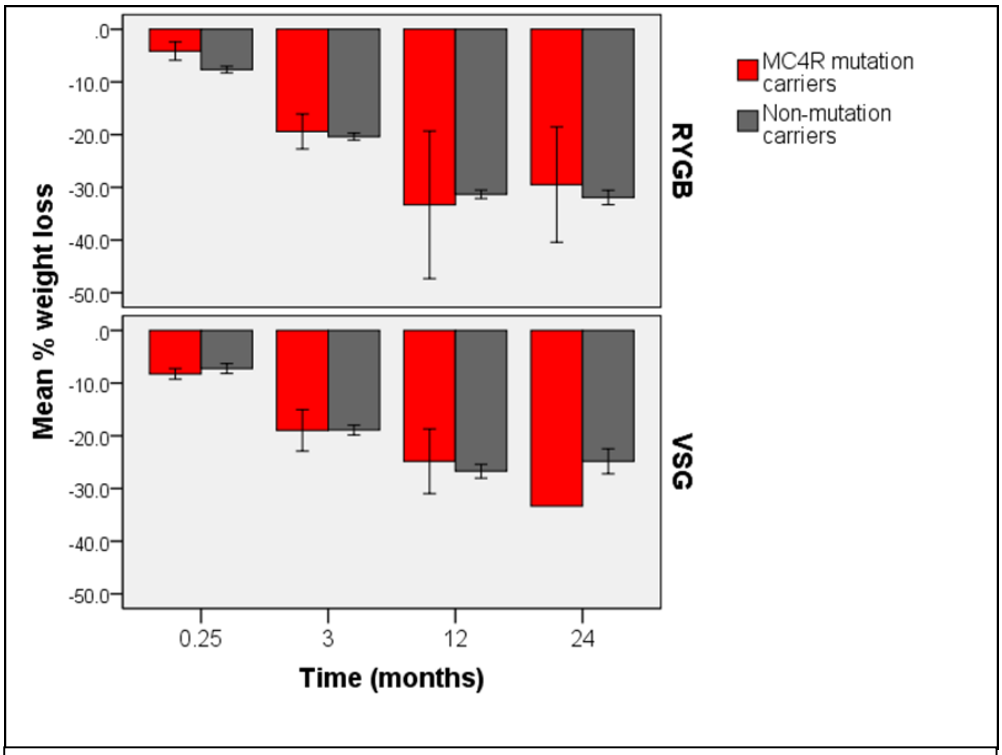


Figure 5.4: Percentage weight loss in MC4R deficient participants. RYGB, Roux-en-Y gastric bypass; VSG, vertical sleeve gastrectomy. Error bars depict SEM.

To visualise the diversity in weight loss seen among the MC4R deficient participants, the weight loss trajectories of each individual were plotted in Figure 5.5. Participant 3 was excluded, since she only attended the 3 months follow up visit and was then lost to follow up. This figure indicates that all MC4R deficient participants showed a positive change in weight following surgery and none regained weight, for as far as data was available.

Importantly all MC4R deficient participants, both the ones that had RYGB or VSG, reached a clinically significant weight loss of >20%, at 12 or 24 months following surgery (range %WL for MC4R deficient participants: 21.7 - 52.7).

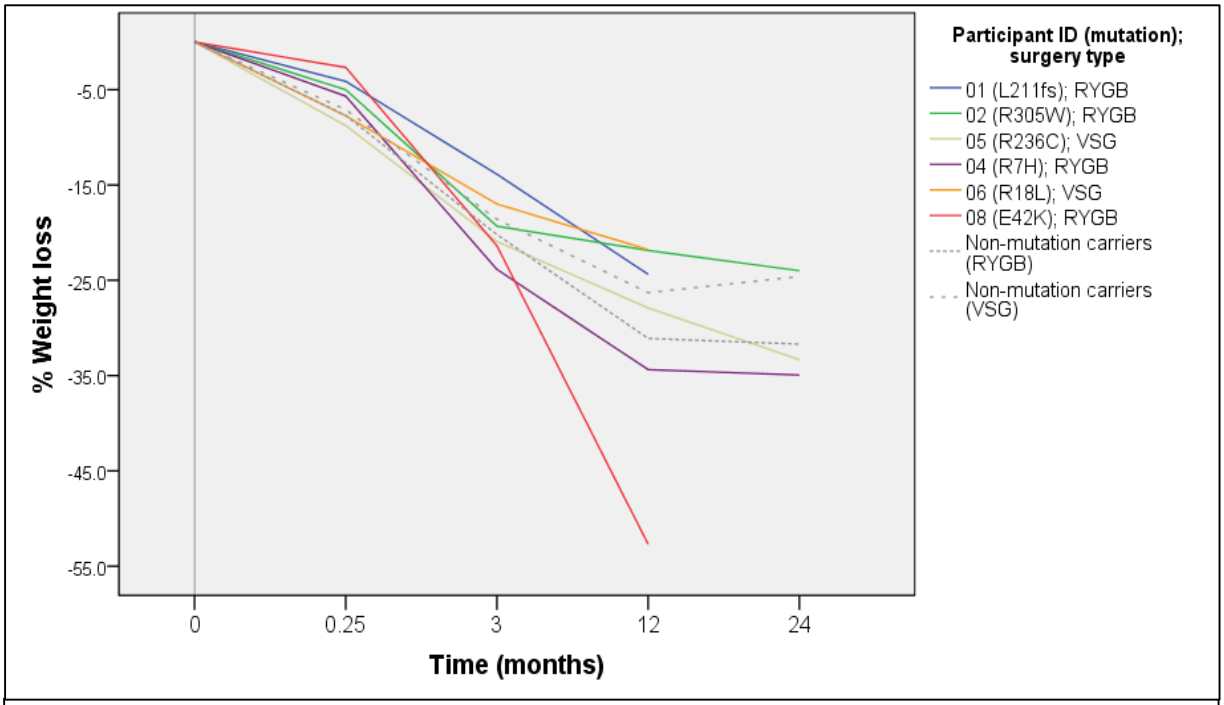


Figure 5.5: Individual weight loss trajectories of MC4R deficient participants. Participant IDs are as noted in table 5.3. A reference of the mean change in BMI seen following RYGB (Roux-en-Y gastric bypass) and VSG (vertical sleeve gastrectomy) for non-variant carriers are given in grey dotted lines.

5.3.5 Common *MC4R* variants and weight loss following surgery

Since a previously reported study showed an increase in weight loss in individuals carrying the p.Ile251Leu variant [280], we compared %WL at 3, 12 and 24 months following RYGB and VSG for the two common variants found in this cohort; p.Ile251Leu and p.Val103Ile. However, no differences in weight loss could be found (Figure 5.6).

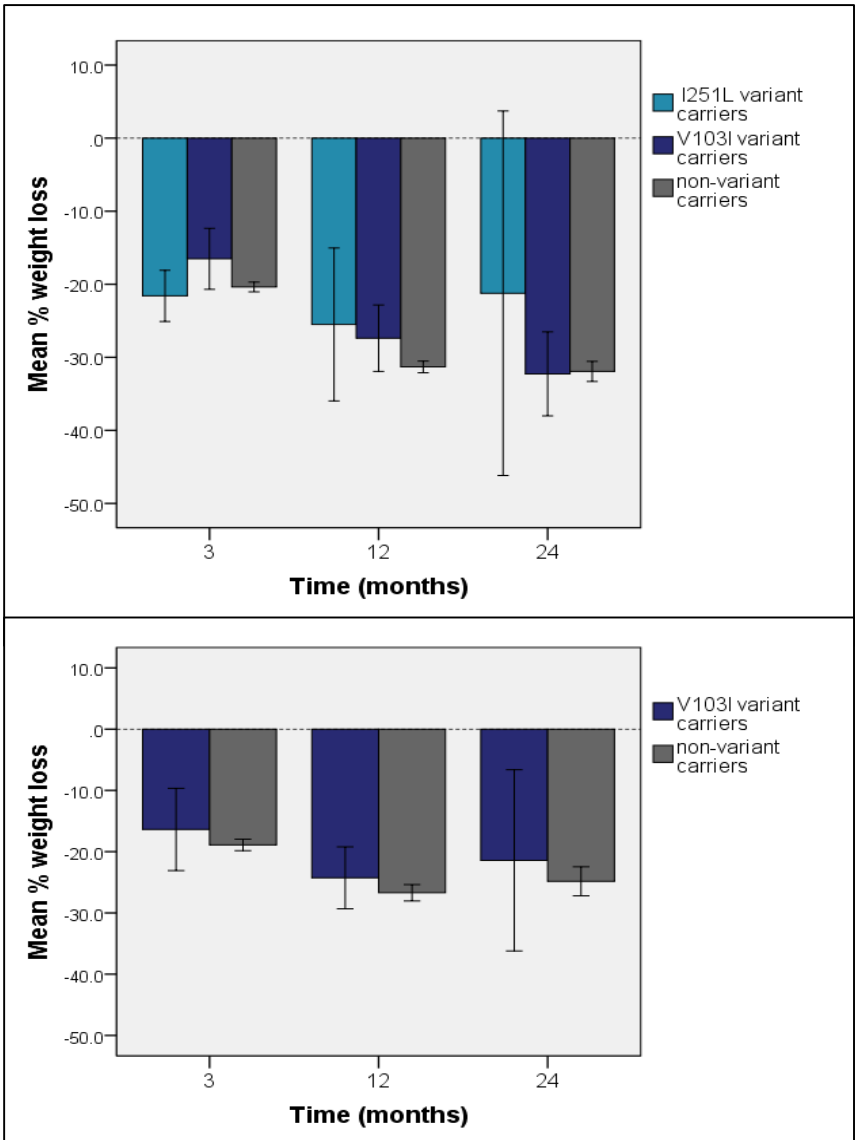


Figure 5.6: p.Val103Ile and p.Ile251Leu *MC4R* variants and percentage weight loss following RYGB (top graph) and VSG (bottom graph). RYGB, Roux-en-Y gastric bypass (p.Ile251Leu variant carriers, *n*=10; p.Val103Ile variant carriers, *n*=12); VSG, vertical sleeve gastrectomy (p.Ile251Leu variant carriers, *n*=2 [therefore not included]; p.Val103Ile variant carriers, *n*=7).

5.4 Discussion

In this chapter I have described the results of *MC4R* sequencing in the PMMO cohort, the phenotypes related to the variant carriers and weight loss seen following bariatric surgery.

_MC4R_ variant prevalence

The frequency of rare *MC4R* variants in 15 individuals (15/1044, or 1.44%) and only eight individuals carrying a variant consistently demonstrated to affect the function of the protein (carrier rate of class 5 variants, 0.77%) was lower than expected. Even including the two dubious functional variants leads to an overall lower than anticipated prevalence of 0.96%. Previous reported prevalence vary widely, with lower frequencies found in less severely obese adult populations, ranging from 0.8% to 2.6% [42,44,46,172,271], while higher frequencies are found in cohorts of obese children [39,42,47,50,281].

The two studies most comparable to the PMMO cohort investigated here are an adult obese population with juvenile onset of obesity [41] and another bariatric surgery cohort [172]. Larsen, *et al.* [41], reported a 2.5% functional *MC4R* variant carrier rate in an adult obese population with reported juvenile onset of obesity (*n*=750). The main difference from the cohort reported here is that the juvenile (age 20) onset of obesity was actually measured as a part of a clinical trial, and was a selection criterion to be included into the study, which meant all 750 participants had a definite juvenile onset of obesity. In contrast, in the cohort used in this thesis the childhood onset was verbally collected with cut-off at the age of 10, and, most importantly, was only present in 36.2% of the individuals. If we would recalculate the prevalence by only including the participants with a verbally collected childhood onset of obesity, a more similar carrier frequency of 1.85% would be reached.

Hatoum, *et al*. [172] reported in a bariatric cohort (*n*=972) a total of 16 individuals with functional *MC4R* variants, giving a prevalence of MC4R deficiency of 1.5%, which is almost double of the

prevalence found in the cohort reported here. However, they included the p.Phe202Leu and p.Arg7His variants as 'pathogenic', which could explain the discrepancy; If the p,Phe202Leu and the p.Arg7His were included as pathogenic in the cohort reported here the prevalence of 'pathogenic' variant carrier rate would go up to a more similar 1.34%.

Still, with the severe obesity, high prevalence of early onset obesity and a history of failed weight loss in this cohort, it was anticipated a higher frequency of MC4R deficiency would be found. Several possible explanations could be given to explain this discrepancy. First of all, our cohort includes several different ethnicities, which might contain ethnic backgrounds in which MC4R deficiency is less common. There are several studies, among different ethnic populations (in mostly western European countries), that have reported a significantly smaller MC4R deficiency prevalence than expected [40,43,282,283]. Secondly, the possibility of ascertainment bias must be considered. It may be that MC4R deficient individuals are not being included in our study population because of systematic factors, such as possible differences in psychology and/or other comorbidities from subjects without MC4R deficiency.

***MC4R* variant carriers**

Since it has been reported consistently that the monogenic forms of obesity are expected within the more extreme spectrum of obesity [74,84], it was anticipated the functional *MC4R* variants would be found in the more severely obese individuals within this cohort. It is, therefore, surprising that the class 5 *MC4R* variants were almost exclusively found in individuals with a BMI <50kg/m^2. Although three MC4R deficient participants did have a weight history which would classify them as Class IV obese in the past, all three had managed to lose sufficient weight to reach a lower BMI class before recruitment into this study.

We hypothesised that the low prevalence of MC4R deficiency in the most severely obese participants (if not simply stochastic) could be influenced by the reported increase in height caused by MC4R

deficiency, which would lead to relatively lower BMIs. This is reflected in previously reported BMIs of individuals with MC4R deficiency, which are generally below 50kg/m^2 [39-46,172,271].

However, it is surprising that in the PMMO cohort no increase in height in the MC4R deficient participants was found. This increase in height caused by MC4R deficiency has been questioned before in mainly adult cohorts [42,271], so it could be a feature of MC4R deficiency seen in children only (for instance they might undergo an earlier growth spurt). However, because of the multi ethnic background of the PMMO cohort, it was difficult to estimate if height really was not affected in the MC4R deficient participants. Alternatively, there might be other unknown factors affecting height within this cohort. The other main feature of the so-called MC4R deficiency syndrome, was hyperinsulinemia in children, although no increased risk of T2DM or glucose intolerance is seen in adults carrying functional *MC4R* variants compared to controls with similar degree of overweight [39]. Indeed, no higher prevalence of T2DM or an increase in fasting glucose, insulin or HbA1c was seen among the MC4R deficient participants here.

Weight loss following bariatric surgery in *MC4R* variant carriers

Of the 10 MC4R deficient participants (two variants of unknown significance included: p.Arg7His and p.Arg236Cys), eight individuals had bariatric surgery; 5x RYGB and 3x VSG. Significant change in BMI was seen in the MC4R deficient participants, and a clinically significant weight loss (>20%) was seen in all the individuals, for which sufficient data was available. No difference was seen in change in BMI or %WL in the MC4R deficient participants when compared to the remaining cohort, even not when segregated for surgery type.

Looking at previous studies on MC4R deficiency in bariatric surgery cohorts, it is reported that MC4R deficiency differentially affect response to individual bariatric procedures, raising risk of re-operation in patients undergoing gastric banding [175,178], but having little or no effect in patients having RYGB [171-173]. It should be noted, however, that in these studies a distinction was not always made

between carriers of variants affecting the protein's function or not: for instance, Potoczna, *et al*. did not separate functional from non-functional variants, and included the non-functional common variants (p.Val130Ile and p.Ile251Leu) in their study on gastric banding [151,178]. Other studies on RYGB did separate functional from non-functional variants in their analysis. However, the two studies with larger numbers included variants as 'pathogenic' while sequential *in vitro* studies indicated that these variants do not affect the function of the gene or protein: In the study by Aslan *et al*. two of the four patients supposedly carrying a 'pathogenic' variant, carried the similar p.Arg236Cys variant which was found in one of the participants here, of which pathogenicity is dubious [171,277], while Hatoum, *et al.* included a patient carrying a p.Phe202Leu variant (reported 5 times in our cohort) and one patient carrying a p.Arg7His variant in the 'pathogenic' group, although for both variants it is questionable if the affect the function of MC4R [151,172]. It is important to make a clear distinction between variants of *MC4R* that influence the receptor's function and those which do not. Including non-functional variants in the analysis of MC4R deficiency affecting the outcome following bariatric surgery, may create confusion and lead to inappropriate clinical decisions.

Findings on the effect of MC4R deficiency on weight loss following RYGB are, however, pretty consistent (including the results presented here) [171-173], indicating it is an effective treatment for MC4R deficiency. However, a few unanswered queries remain. Because of the rarity of MC4R deficiency, only small numbers were discovered in the cohorts reported up until now. Therefore, no segregation of the severity of the functional alterations of the variants has been able to be applied. Preferably, if enough functional variants are found, a distinction between the different functional effects must be made. It is reported that participants carrying a more severe functional alteration have a higher BMI and an earlier onset of obesity, while a complete knockout of *MC4R* (through homozygosity or compound heterozygosity) leads again to a more severe phenotype than heterozygote variant carriers [39,42]. Individuals carrying a variant that causes a major loss of MC4R signalling, or individuals with a complete loss of MC4R, may respond differently to surgery than individuals carrying a variant with only a mild effect on MC4R function. Although no reports are

currently available of homozygous or compound heterozygous variant carriers that underwent RYGB, a study on mice showed that mice heterozygous for *Mc4r* remain fully responsive to gastric bypass, while a complete knockout of *Mc4r* led to substantially less weight loss, and weight regain [172].

Although a substantial number of studies have covered the results of MC4R deficient patients undergoing RYGB, to my knowledge, no studies are available on VSG, beside a single case report with limited follow up [173] . Working in collaboration with Mul, *et al.* (at the start of this PhD project), we did not find any functional variants in the small cohort of participants undergoing VSG [176]. The finding that the three individuals with MC4R deficiency described here had good results following VSG is, therefore, a novel and clinically-relevant finding. Further follow up of these individuals will clarify whether weight loss is also maintained long-term: ideally, a larger number of variant carriers would be found in a larger VSG cohort, to enable proper comparison analysis with the remaining cohort.

Finally, we could not repeat the finding that the pIle251Leu *MC4R* variant led to a significant increase in weight loss following RYGB [280]. This is in line with other studies which following the initial published association also could not repeat this finding [172].

5.5 Summary

Here I report a prevalence of 0.77% of definite pathogenic variants in a morbidly obese bariatric cohort, which was lower than anticipated. Apart from the higher prevalence of early onset obesity, no other phenotypes that differed from the remaining cohort could be found. MC4R deficient participants had good weight loss following bariatric surgery, which was not significantly different from the remaining cohort. This was the case for the small number of MC4R deficient participants undergoing RYGB, which is in line with previous studies. Also for the small number of MC4R deficient participants undergoing VSG surgery clinically significant weight loss trajectories were seen, not different from the remaining cohort, which is a novel finding with possible clinical implications; RYGB is currently being

advised as best treatment option for patients with MC4R deficiency, but if it can be shown that VSG also gives good, durable results for this group this surgery type could be offered as a second feasible option.

CHAPTER 6

THE EFFECT OF *MC4R* VARIANTS ON WEIGHT LOSS IN CHILDREN UNDERGOING INTENSIVE LIFESTYLE TREATMENT

6.1 Introduction

The prevalence of *MC4R* deficiency was lower than expected in the adult morbidly obese patients undergoing bariatric surgery, with a prevalence of 0.77% as reported in the previous chapter.

In this chapter a cohort of severely obese children will be screened for *MC4R* variants to detect the prevalence of MC4R deficiency in obese children with a history of failed weight loss. As shown in the previous chapter, certain types of bariatric surgery seem affective in adults with heterozygous loss of MC4R activity. However, surgery is an invasive treatment option, and although effective can have major complications. Malabsorption is one of the main complications not entirely explored yet, and of which the long term implications for children and young adults, in which the body is still developing, are not known [284].

Therefore, it is important to investigate less invasive approaches of weight loss, especially for children. Only little is known about lifestyle treatment approaches of obesity in individuals with MC4R deficiency. So far only one study has looked at children with MC4R deficiency undergoing lifestyle treatment, and showed that children with MC4R deficiency did lose weight during the intensive treatment period, comparable to other children, but regained weight after a following period without the treatment [48].

Here I investigated the prevalence of MC4R deficiency in a cohort of 113 children with severe obesity and a history of failed weight loss treatment. All children underwent in-house lifestyle treatment of which an overview can be found in appendix 2.2, page 315. Weight loss trajectories were analysed for MC4R deficient participants.

6.2 Aims of the study

1) Detect the prevalence and characteristics of MC4R deficiency in a cohort of morbidly obese children with a history of failed weight loss management.
2) Detect if a lifestyle treatment approach to weight loss is an effective treatment option for children with MC4R deficiency.

6.3 Results

6.3.1 Baseline characteristics

113 severely obese children (aged 10-18 years old) recruited at the Childhood Obesity Centre Heideheuvel, Paediatric Hospital Merem, Hilversum, The Netherlands, were included for this study (more details on recruitment can be found in chapter 2, section 2.3.1, page 68). Of the 113 participants one was excluded for low quality of DNA which made Sanger sequencing of *MC4R* not possible. The remaining 112 individuals had a mean BMI of 40.55 (± SEM: 0.59). For further analysis, the BMI-standard deviation scores (BMI-SDS) were used to correct for difference in age, height and change in height. Children, while on weight loss treatment, still grow. So even though children might not change in weight (kg) during weight loss interventions, their BMI-SDS could still significantly change during the treatment. Baseline characteristics of this cohort are listed in Table 6.1.

Complete cohort	
Number	112
Gender (% female)	53.6
Age (year)	15 [13-16]
Weight (kg)	114.3 (±2.22)
Height (m)	1.673 (±0.01)
BMI (kg/m^2)	40.55 (±0.59)
BMI-SDS	3.67 (±0.038)
Treatment intervention (n)	
- 8 weeks in-patient treatment	21
- 12 weeks in-patient treatment	64
- 36 out-patient treatment	27

Table 6.1: Baseline characteristics. Data are given as mean (± SEM) or median [interquartile range].

6.3.2 *MC4R* variants

A total of eight variants in *MC4R* were found in seven individuals, of whom five were previously shown to affect the receptor's function (class 5 variants), giving a prevalence of 4.4% of MC4R deficiency in this cohort of obese children. All variant characteristics and participant phenotypes are listed in Table 6.2. Chromatograms of the variants can be found in Appendix 6.1 (page 338).

One variant, c.110A>T (p.(Asp37Val)), was found in an individual that also carried an c.105C>A (p.(Tyr35Ter)) variant. Although it is known that p.Tyr35Ter leads to an inactive MC4R [277] , *in silico* studies describing the effect of p.Asp37Val on the receptors function reported contradicting results [41,150]. Therefore, it was important to find out if these variants were located on the same allele in this individual or not. In order to establish this, both parents were screened for these variants. Both *MC4R* variants (p.Tyr35Ter and p.Asp37Val) were found in the mother, indicating that both variants were located on the same allele and both inherited from the mother, excluding compound heterozygosity.

A more common variant, c.307G>A (p.(Val103Ile)), which was found in two individuals, has been reported before in obese as well as lean individuals with a relatively high MAF (0.017 in ExAC), and has been shown to not affect the function of MC4R. In the contrary some studies reported for this variant an increased basal activity rate of the receptor, and an association with a lower BMI in population studies [151,271].

Comparing baseline characteristics between the complete cohort and the carriers of *MC4R* variants affecting the receptors function (from here on forward referred to as MC4R deficient participants) did not show any significant differences, besides a small but significant difference in BMI-SDS (Table 6.3).

ID	Age (yr)	Gender	Weight (kg)	Height (m)	BMI (kg/m^2)	BMI-SDS	*MC4R* variants detected	Literature			
								Reported MAF	*in silico* analysis	*in vitro* analysis	Ref.
1	16	F	127.9	1.687	44.9	3.96	c.896 C>A; p.Pro299His	<0.0000	PD/D	CE, LB, BA	[150,210, 272,277]
2	16	M	178.1	1.992	44.9	3.89	c.105 C>A; p.Tyr35Ter	<0.0000	*na*	complete loss of function	[41,150,273,277]
							c.110 A>T; p.Asp37Val	<0.0000	B/T	Contradicting results	
3	11	M	96.8	1.630	36.4	3.66	c.380 C>T; p.Ser127Leu	0.0002	PD/T	complete loss of function (Contradicting results)	[150,277, 285]
4[b]	10	M	98.3	1.620	37.6	3.88	c.105 C>A; p.Tyr35Ter	<0.0000	*na*	complete loss of function	[150,277]
5[b]	13	F	110.6	1.618	42.2	3.91	c.105 C>A; p.Tyr35Ter	<0.0000	*na*	complete loss of function	[150,277]
6	18	M	161.0	1.818	48.7	4.12	c.307 G>A; p.Val103Ile	0.017	B/T	Increased basal activity	[151]
7	13	M	112.2	1.773	35.7	4.42	c.307 G>A; p.Val103Ile	0.017	B/T	Increased basal activity	[151]

Table 6.2: Participants and *MC4R* variant characteristics. MAF (minor allele frequency) as noted in ExAC [208]. In silico predictions (Polyphen/SIFT): B: benign; PD: probably damaging; T: tolerated; D: damaging; na: not applicable. CE, Alteration cell surface expression of MC4R; LB, decreased endogenous ligand binding; BA, decreased basal activity. [b] These two individuals are siblings

A: *Complete cohort*			
	Pariticpants with no *MC4R* variants	***MC4R* deficient participants**	**p-value**
Number	107	5	--
Gender (% female)	54.2	40	0.662
Age (year)	15 [13-16]	13 [10.5-16]	0.191
Weight (kg)	113.9 (±2.23)	122.3 (±15.01)	0.434
Height (m)	1.671 (±0.010)	1.709 (±0.072)	0.451
BMI (kg/m^2)	40.52 (±0.62)	41.19 (±1.81)	0.817
BMI-SDS	**+3.66 (±0.039)**	**+3.86 (±0.052)**	0.013
Treatment intervention (n)			
- 8 weeks in-patient treatment	21	0	--
- 12 weeks in-patient treatment	59	5	--
- 36 out-patient treatment	27	0	--
B: *12 week inpatients treatment group*			
	Matched controls	***MC4R* deficient participants**	**p-value**
Number [A]	55	5	--
Gender (% female)	61.8	40	0.380
Age (year)	15 [13-17]	13 [10.5-16]	0.119
Weight (kg)	115.9 (±2.86)	122.3 (±15.01)	0.541
Height (m)	1.680 (±0.013)	1.709 (±0.072)	0.538
BMI (kg/m^2)	41.01 (±0.85)	41.19 (±1.81)	0.950
BMI-SDS	**+3.67 (±0.056)**	**+3.86 (±0.052)**	0.026

Table 6.3: Baseline characteristics. Anthropometric data of MC4R deficient participants and non-variant carriers in A) the complete cohort and B) the 12-week in-patient treatment group. Data are given as mean (± SEM) or median [interquartile range]. [A] Only participants that completed the in-patient period were included. Highlighted in red are the differences that are significant at <0.05 level.

6.3.3 Weight loss in *MC4R* variant carriers

All five individuals with MC4R deficiency underwent the same treatment intervention; 12 weeks in-patients treatment, with monthly follow up appointments during the next 9 months (Appendix 2.2, page 315).

Of the 60 individuals treated in this same treatment group, five individuals stopped their treatment within the in-patient period for various reasons (home-sickness, family problems and behavioural/motivational problems), and were excluded for further analysis. For 9 individuals the

LOCF method was applied, since the one year follow up appointment was missed (last appoint range 5-9 months). All of the MC4R deficient participants completed all visits.

Comparing the baseline characteristics between the MC4R deficient participants and the participants receiving similar treatment (matched controls) no significant differences were seen, besides in the BMI-SDS (3.86 vs. 3.67, p-value: 0.049) (Table 6.3).

Although an initial significant mean change in overweight was seen in the MC4R deficient participants, after one year follow up no significant changes in BMI-SDS survived, this in comparison with the matched controls that still showed a significant change in overweight at one year compared to baseline (Figure 6.1). Comparison analysis between the two groups for each time point, did however not show any significant differences (Table 6.3).

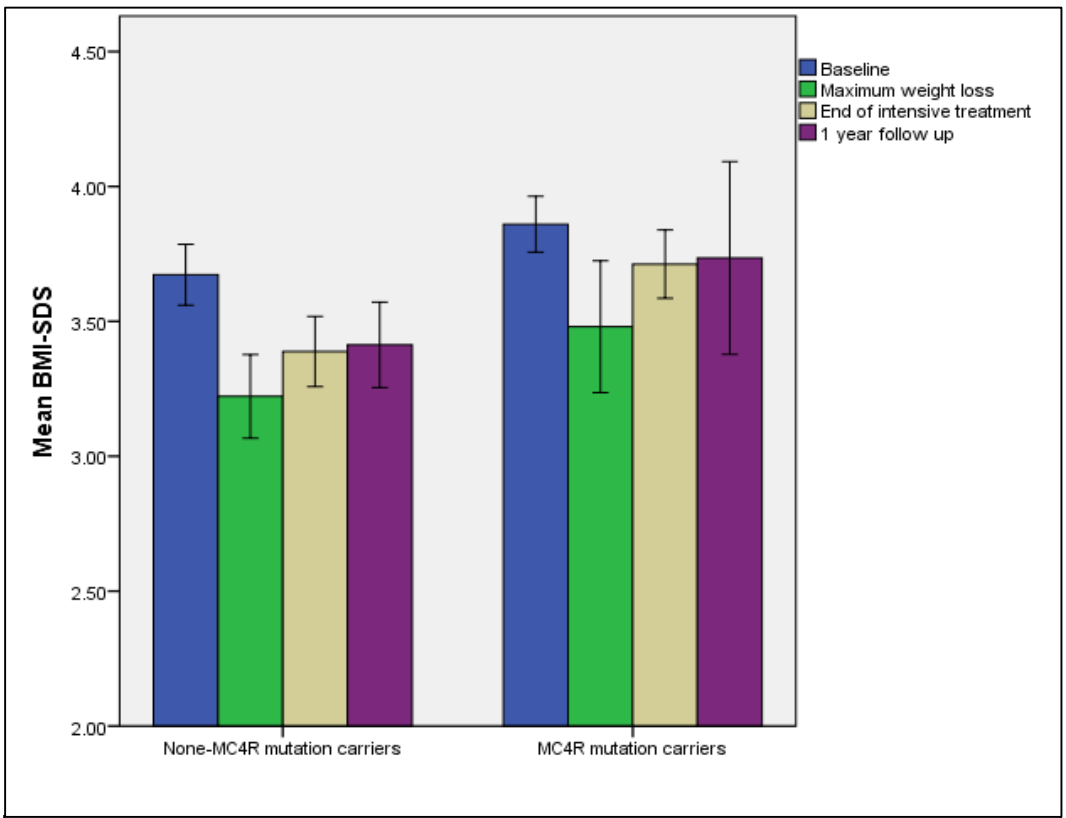


Figure 6.1: Change in BMI-SDS. Change in BMI-SDS in A) MC4R deficient participants (n=5) and in B) matched controls (n=55). Error bars depict SEM.

	Matched controls (n=55)			*MC4R deficient participants (n=5)*			*Difference between groups*
	Mean (SD) BMI-SDS	**Change in BMI-SDS from baseline**	**p-value**	**Mean (SD) BMI-SDS**	**Change in BMI-SDS from baseline**	**p-value**	**p-value***
Baseline	3.67 (±0.42)	na	na	3.86 (±0.12)	na	na	0.049
Maximum weight loss	3.22 (±0.57)	-0.45	0.000	3.48 (±0.27)	-0.38	0.027	0.201
End of intensive treatment	3.39 (±0.48)	-0.29	0.000	3.71 (±0.14)	-0.15	0.032	0.055
1 year follow up	3.42 (±0.59)	-0.26	0.000	3.71 (±0.32)	-0.15	0.366	0.205

Table 6.4: Change in BMI-SDS following treatment. The BMI-SDS change over time for the MC4R deficient and matched controls are listed. Na: not applicable * p-values listed as after post hoc Bonferroni correction.

No significant difference was seen comparing the weight loss trajectories between the MC4R deficient participants and the matched controls, using linear mixed modelling, corrected for baseline BMI-SDS (P-value: 0.45).

Also after adjusting the change in BMI-SDS for baseline BMI-SDS, by calculating the percentage change, no significant differences were seen between the two groups at the maximum reduction in overweight seen during the one-year lifestyle intervention, or during any of the other follow up points (Figure 6.2).

Looking at the maximum weight loss reached by individuals throughout the 1-year treatment, two of the five MC4R deficient participants (40%) reached a medically significant change in their BMI-SDS (i.e. >0.5), compared to 17 of the 55 matched controls (30.9%, p-value: 0.648).

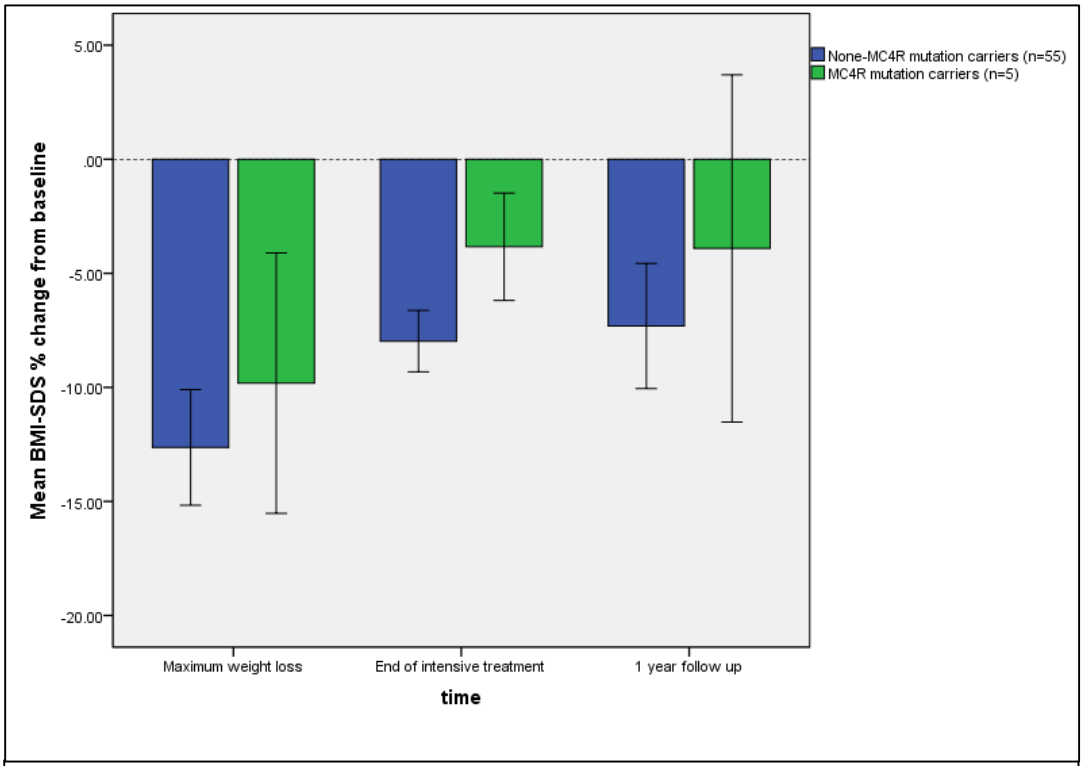
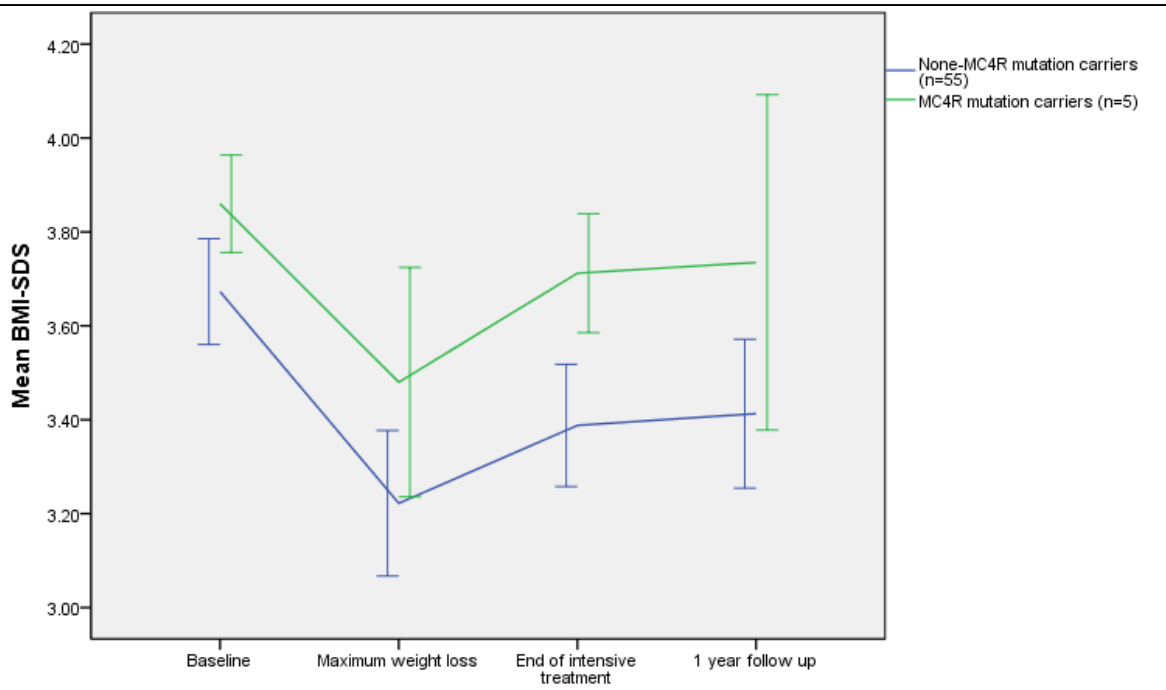


Figure 6.2: Percentage change in BMI-SDS. No significant differences were seen between the two groups in BMI-SDS percentage change from baseline.

Since such diversity in weight loss was seen among the MC4R deficient participants, the weight loss trajectories of each individual were plotted in Figure 6.3, bottom graph. This figure indicates that although the overall change in BMI-SDS was not significant, some of the individuals did seem to loose significant weight through the lifestyle intervention, while others did not.



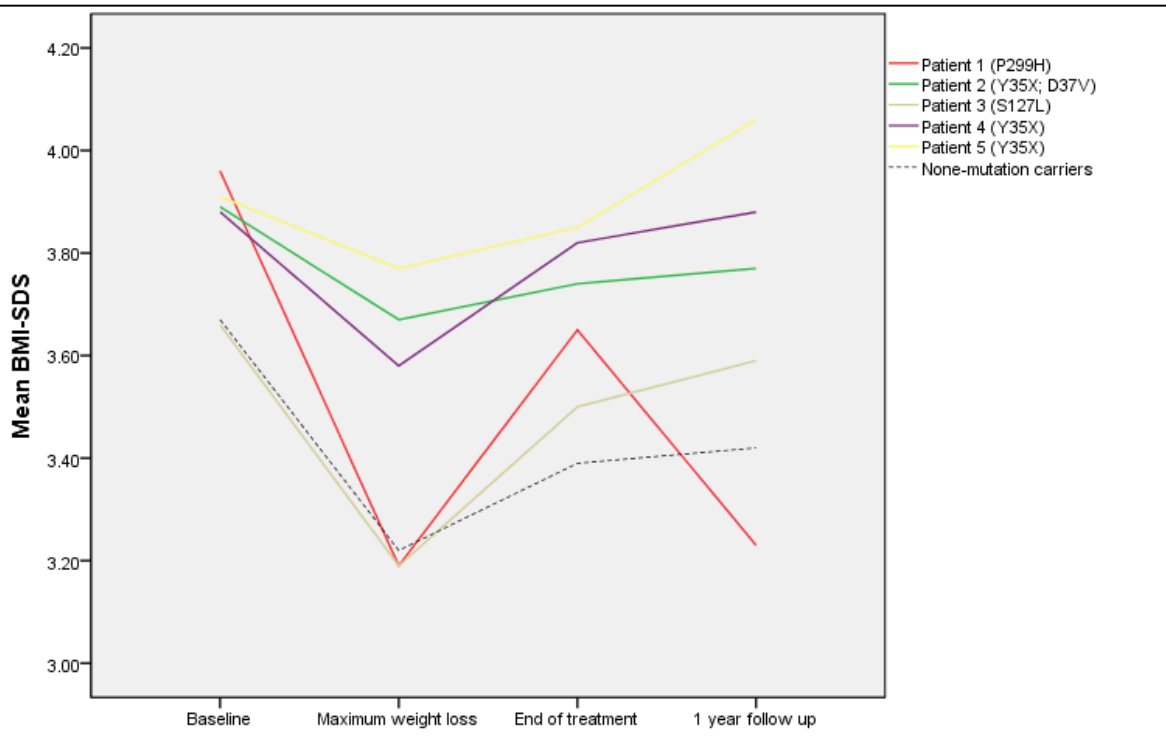


Figure 6.3: Weight loss trajectories. Top chart shows the mean BMI-SDS (±SEM) at the different time points, for the MC4R deficient participants and the matched controls. Linear mixed modelling showed no significant differences between the two trajectories (p-value: 0.45). The bottom chart shows the individual BMI-SDS measurements for the MC4R deficient participants, compared to the mean BMI-SDS measurements for the matched controls (dotted line).

6.4 Discussion

Here I screened a cohort of 112 obese children for MC4R deficiency. In five individuals a variant affecting the function of MC4R (class 5 variant) was found, resulting in an expected prevalence of MC4R deficiency of 4.4%. Comparing weight loss trajectories after lifestyle intervention in the MC4R deficient participants to matched controls did not show a significant difference.

***MC4R* variant carriers**

The prevalence of MC4R deficiency of 4.4% that was found in this cohort of obese children, with a history of unsuccessful weight loss treatment, was similar as reported by others. Several studies conducted in children cohorts of different ethnic back grounds showed a prevalence of 2% to 6% [39,41,42,45,286,287].

The lack of differences in baseline characteristics, confirms the difficulty of pre-selecting children from an obesity cohort that are more likely to have MC4R deficiency. Some studies indicate that MC4R deficiency is associated with increased linear growth rate, hyperphagia and severe hyper-insulinemia, although some discrepancies on this MC4R deficiency syndrome exist [39,41,42,50]. In the adult PMMO cohort, such associations could not be found. In this cohort of obese children, again no signs of increased linear growth were seen, which possibly might be affected by the already increased height normally seen in the Dutch population. Interestingly previous Dutch studies reporting on MC4R deficiency did not find an increase in linear growth either [50]. Unfortunately, I was not able to check for the other symptoms associated with MC4R deficiency, since the necessary data was not available.

In this study three individuals carried the same stop codon creating variant, p.Tyr35Ter. Two were siblings, and one unrelated individual also carried the p.Aps37Val variant on the same allele. Interestingly the p.Tyr35Ter and p.Asp37Val haplotype has previously been reported in Dutch, German, Norwegian and Danish obese individuals in relative high prevalence among the early onset

obese (MAF: 0.005 to 0.006), which could indicate an ancestral founder shared with the German, Scandinavian and Dutch population [41,46,50,288,289]. The p.Aps37Val has not been reported before on its own, while the p.Tyr35Ter has only been reported before in several individuals [48,277]. Similarly only the combination of these two variants can be found in ExAC in 6 non-finish European Caucasians, while neither were reported on its own [208].

Weight loss in deficient participants

In this study we showed that on average children with MC4R deficiency can lose weight through lifestyle treatment, but regain weight within one year. All 5 participants with MC4R deficiency were able to decrease their overweight, but there was a wide variety in the maximum reduction achieved between the individuals and the maintenance of this reduction.

There was no significant difference in the overall weight loss trajectories or the maximum reduction in overweight seen between the MC4R deficient participants and the matched controls (BMI-SDS: 0.38 (± 0.31) vs 0.45 (± 0.25), p-value: 0.210). One year after the initial treatment started an increase in overweight was seen in the MC4R deficient participants leading to an overweight status similar to baseline, while this was not seen in the matched controls. Intra group comparison, however, did not show a significant difference between the two groups.

Only two previous studies have looked at lifestyle interventions in children with MC4R deficiency (n=9 and n=4 for each study respectively) [48,281]. They showed similar results in children with MC4R deficiency, as in a change in BMI-SDS was seen, similar to matched controls without *MC4R* deficiency. Both studies, however, did not report on significance in BMI-SDS change within the group itself, but just mentioned there was no significant difference compared to non-variant carriers. We can therefore not conclude if the lifestyle treatments were successful in the MC4R deficient children themselves or not.

Only one of these two studies followed the patients up after initial treatment, and showed similar to here, a regain in overweigh in the MC4R deficient patients. The difference is, however, that in their study during the follow up the controls did not regain any weight, and therefore there was a significant difference between the MC4R deficient participants and the controls [48].

This discrepancy is more likely to be explained by the success of long term weight change in the controls in the study of Reinehr, *et al*.[48] compared to the non-variant carrying matched controls here. It does, however, show a similar pattern for the MC4R deficient participants as reported here: a positive change in overweight is possible in children with MC4R deficiency, but it is unlikely to be maintained over a longer period.

Interestingly in the three participants reported here with the nonsense mutation p.Tyr35Ter, a smaller reduction in BMI-SDS was seen during the intervention than in the other two participants with a less damaging *MC4R* variant (Figure 6.4). In the latter two participants a BMI-SDS change more similar to the controls was seen. This is in accordance with previous findings, that there is a strong relationship between the severity of functional alterations caused by *MC4R* variants and the age of onset and severity of obesity, with variants leading to a decreased membrane expression leading to the most severe phenotype [42].

It would therefore be interesting to see if carriers of variants with a complete loss of the receptor's function respond differently to lifestyle treatment than carriers of variants with only a reduced function. Unfortunately, the only two previous studies looking at the effect of lifestyle intervention, do not mention which variants were found in the individuals undergoing the intervention. Although baseline characteristics, including variant details, were given for the complete cohort screened, they were not given for the final group of individuals undergoing lifestyle intervention (9 individuals of the 16 variant carriers initially reported at baseline). Therefore, the severity of the functional alteration of the *MC4R* variants of the individuals that underwent lifestyle treatment cannot be assessed [48,281].

It is important to notice that two of the 5 individuals with MC4R deficiency (with the less severe disrupting *MC4R* variants) did manage to have a clinical significant decrease in their overweight (change in BMI-SDS >0.5), with one individual maintaining this over a longer period. This indicates that it is indeed possible for some individuals with MC4R deficiency to change their BMI-SDS significantly, and that interpersonal differences might be accountable for the differences seen in BMI-SDS change, in which possibly also the severity of the functional alterations of the MC4 receptor plays a role.

Limitations of this study were the small numbers MC4R deficiency cases, and that two of them consisted out of siblings. Thereby, the control/non-variant carrier group consisted out of children that also suffered from severely early onset obesity and all had a history of unsuccessful weight loss treatment. It is therefore very likely that a significant proportion of these individuals probably suffer from Mendelian obesity as well. Although MC4R deficiency was excluded, the children were not screened for any other known Mendelian forms of obesity.

Studies to date on weight loss treatment in children or adults with MC4R deficiency are limited. Therefore, this study, even with a small number, still has a clinical value in that it shows weight loss is possible for patients suffering from MC4R deficiency through lifestyle intervention, although weight regain is very likely.

The lifestyle treatment approach could possibly be adjusted to better suit the needs of patients suffering from MC4R deficiency. Longer follow up of the participants described here, could therefore be valuable. This study had a retrospective setup, and therefore all interventions and measurements took place before the diagnoses of MC4R deficiency was fed back to participants and their parents (after validation in a medically qualified lab) by a clinical geneticist in the Netherlands. Receiving the diagnosis could therefore not have had an influence on the outcomes reported here.

It would, however, be important to investigate how receiving the diagnosis influenced further weight loss/gain. We therefore aim to apply further follow up of the participants described here in

collaboration with Heideheuvel centre, and analyse if any positive or negative effects of receiving a MC4R deficiency diagnosis can be seen on lifestyle and psychological behaviour.

6.5 Conclusion

For the study described in this chapter a cohort of 112 severely obese children were screened for *MC4R* variants. In five children a functional variant (class 5 variant) was found, giving a prevalence of 4.4% of MC4R deficiency. Change in BMI-SDS after lifestyle treatment was significant in the MC4R deficient participants, but weight regain was seen in the following year. A difference between the individual participants was seen in their ability to change their overweight, indicating other factors (such as the severity of the implication of the variant) could play an important role. Further investigation is necessary to see how lifestyle interventions can be optimised for children suffering from MC4R deficiency.

CHAPTER 7

HIGH PENETRANCE VARIANTS IN OBESITY GENES

7.1 Introduction

This chapter describes the exome sequencing of 40 White Caucasian bariatric patients with BMI>50 and family history of obesity. This study was undertaken to provide an initial estimate of the prevalence of monogenic (or Mendelian) obesity in this group, and also to seek novel human obesity genes.

A number of Mendelian forms of obesity are currently known (as summarised in Table 1.1 page 36), but are often dismissed as too rare to contribute to adult "common obesity" in the general population. Additionally, many such disorders are reported to cause an "obesity plus" phenotype, with the "plus" element including features such as intellectual disability, maladaptive behaviour or hypothalamic hypogonadism, and so have been considered unlikely to be present in common obesity.

MC4R deficiency has been reported to be the most common form of monogenic obesity, with a prevalence of 2.0-6.0% in childhood obesity and 0.8-2.7% in the adult obese population [39-50,271]. Other, rarer forms of Mendelian obesity have been detected in small cohorts, but the prevalence of these disorders in adult obese individuals is unknown.

We postulated that super-obese adults with childhood onset of their obesity might be enriched for Mendelian forms of disease. As described in the chapter 5, MC4R deficiency was rare in our PMMO bariatric cohort: only 0.77% of the morbidly obese individuals carried a functional *MC4R* variant, with only one of these having a BMI >50 kg/m^2 on recruitment. Looking at the participants' weight history, it emerged that a further three would have classified as Class IV obesity if highest weight through-out lifetime had been used. Still this would only lead to a prevalence of 1.1% for MC4R deficiency in our ≥ class IV obese, which was lower than anticipated.

To evaluate whether, in the super obese (≥ class IV obese) proportion of the PMMO cohort other forms of Mendelian obesity might be more prevalent, a subgroup of 40 individuals was investigated

using WES. Screening of the WES data for all known forms of Mendelian obesity was performed, followed by the investigation of novel variants within known obesity genes and regions. A second cohort of 73 overweight to obese individuals (BMI range 25-35 kg/m^2) from the NutriTech project were analysed using WES in a similar fashion, and were used to compare the findings in the super-obese group (as described in Chapter 2, section 2.3.2, page 69). Finally, the WES data was screened for novel obesity candidate genes, focussing on genes which, when disrupted, cause obesity in mice.

7.2 Aims of this study

1) To identify novel high-penetrance variants in known and candidate obesity genes.

2) To identify the prevalence of such high penetrance variants in obesity genes in the super obese, and to compare this to prevalence in the NutriTech cohort.

3) To investigate the implications of such highly-penetrant forms of obesity for response to bariatric surgery.

7.3 Results

7.3.1 Participant characteristics

Forty super obese European Caucasian participants were selected from the PMMO cohort based on their severity of obesity (with a minimum BMI of 50 kg/m^2) and having a Mendelian pattern of obesity in their family history. Of the 40 individuals initially included, one participant had to be excluded, because population structure analysis of the genome wide SNP data revealed that person to be an ethnic outlier. For the remaining 39 super obese participants the mean BMI was 62.5 kg/m^2 (range: 50.0-87.53 kg/m^2), with 62.5% being female.

Seventy-three overweight to class I obese individuals from the NutriTech project were included as a comparison dataset [199]. All were classified as European Caucasian by population structure analysis and, as expected, the mean BMI of 29.7 kg/m2 (range: 24.9-35.8 kg/m^2) was significantly lower than in the super obese individuals from the PMMO cohort (Table 7.1). Our hypothesis was that deleterious, rare variants in obesity genes would be less frequent in this group than in the super obese.

As can be seen in Table 7.1, the super obese sub-group also significantly differed in terms of BMI, weight and onset of obesity from the other PMMO participants, indicating that they were the extremes within this cohort.

	PMMO cohort	Participants included for WES			
		Super obese cohort	P-value*	NutriTech cohort	P-value**
N	1036	39	---	73	---
Age (years)	45.56 (± 0.35)	**47.45** (± 1.74)	0.293	**58.32** (± 0.96)	0.000001
Female (%)	74.5	62.5	0.069	52.1	0.192
BMI (kg/m^2)	**47.66** (± 0.26)	**62.5** (± 1.23)	0.000000	**29.7** (± 0.33)	0.000000
Weight (kg)	**132.87** (± 0.84)	**180.0** (± 5.44)	0.000000	**84.07** (± 1.95)	0.000000
Height (m)	1.67 (± 0.09)	1.69 (± 0.11)	0.123	1.67 (± 0.22)	0.485
BMI >60 kg/m^2 (%)	**7.2**	**71.8**	0.000000	0	---
Class I obese (%)	2.9	0	---	45.2	---
Onset of obesity before 10 years old (%)	**32.2**	**100**	0.000000	unknown	---
- T2DM (%) **- ITT2DM (%)**	29.0 8.7	27.5 17.5	0.157	0 0	---
Binge eating disorder (%)	16.2	22.9	0.203	0	---

Table 7.1: Baseline characteristics of WES participants. Data is presented as mean (± SD), unless otherwise indicated. BMI, body mass index; T2DM, Type 2 diabetes mellitus; ITTDM, insulin-treated type 2 diabetes mellitus. * Super obese compared to PMMO cohort ** NutriTech compared to the super obese. Highlighted in red are the differences that are significant at <0.05 level.

7.3.2 Mendelian forms of obesity

The overall quality of the WES data was high, and for all samples >99% of reads mapped to the reference sequence. An overall number of 179,250 different variants were found in the super obese and NutriTech group together.

In total 36 genes were included as 'human-obesity genes', and are listed in Appendix 2.3 (page 319). Coverage of these 36 human-obesity genes was good for all individuals included. A total of 7,772 variants were found in the obesity genes in the super obese and NutriTech group together; a mean number of 72.08 (± 10.68 SD) variants per participant in the super obese, and a mean number of 67.96 (± 7.70 SD) variants in the NutriTech group (*p*: 0.250).

CNV interpretation through read-depth analysis predicted 10,097 CNVs in total, with 12 CNVs covering the human-obesity genes (4 duplications and 8 deletions).

Of these overall variants in the obesity genes 7,390 (95.1%) were common (MAF >1%); 3,704 (47.7%) were synonymous; 1,150 (14.8%) were non-coding; and 2,918 (37.5%) were non-synonymous.

An increase in non-synonymous and predicted-to-be-deleterious variants (by *in silico* prediction software) was seen with decreasing MAF (Figure 7.1): this is expected as, in general, harmful variants are rarer in the population than benign ones. Figure 7.1 also shows that, in themselves, predicted-to-be deleterious variants in human monogenic obesity genes are not rare: again this is expected [290]. It is, therefore, important to carefully distinguish potentially disease-causing variants from the 'normal' variants.

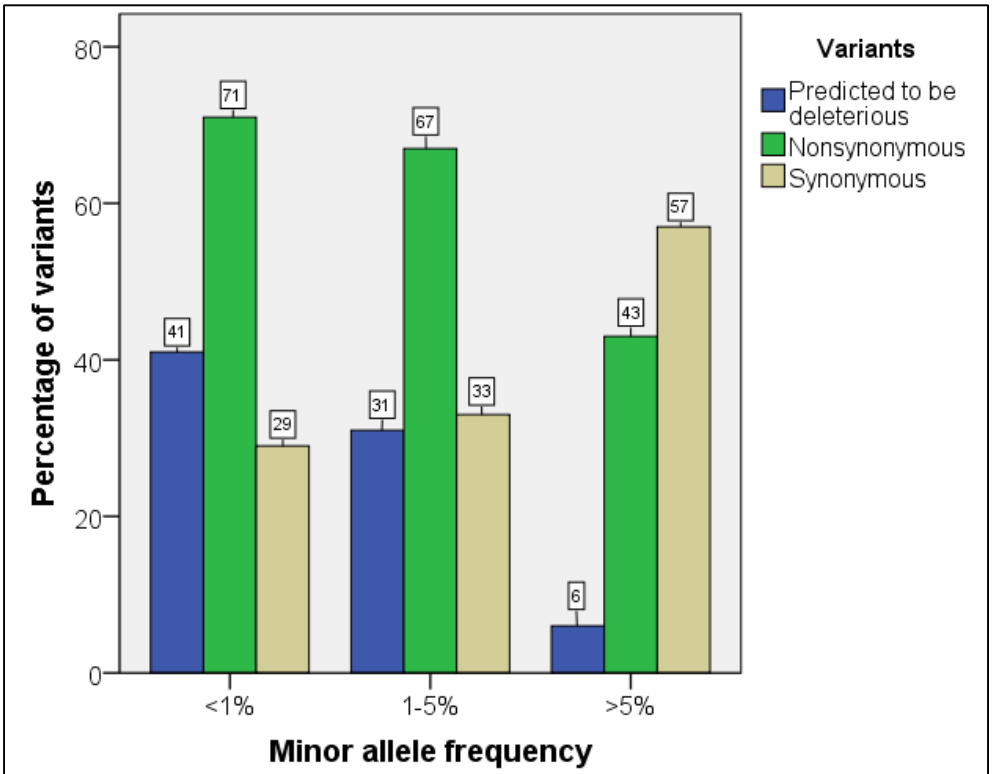


Figure 7.1: Percentage of different variants in obesity genes.

The percentage of nonsynonymous, synonymous and predicted-to-deleterious variants of the overall coding-variants within each minor allele frequency range. Since all predicted-to-be deleterious variants were also nonsynonymous, these percentages are overlapping. Percentages are given above each bar.

Putatively causative variants in known human obesity genes

To assess variants for potential to be causative of Mendelian obesity, the rare, predicted-to-be-deleterious variants in the human obesity genes were considered in the light of the mode of inheritance appropriate to the gene concerned. In this analysis, two variants were, therefore, required to be considered potentially causative of known recessive conditions and only one variant was needed for dominant conditions. The variants considered for further analysis and reasons for exclusion of other variants are summarised in Table 7.2.

Overall 56 rare, predicted-to-be-deleterious variants were found (24 in the super obese and 32 in the NutriTech group, Table 7.2). Of these variants, 12 were potentially causative of monogenic obesity after adjusting for mode of inheritance (eight in the super obese and four in the control group). Two of these variants were excluded after literature research revealed that they were also reported in lean controls, although non-penetrance in the previous cases cannot be excluded [56,91].

The remaining ten variants were confirmed using Sanger sequencing (methods section 2.4.6, page 78). One variant, *SH2B1* c.47C>G (p.(Pro16Arg)), could not be confirmed and was, therefore, excluded. In hindsight, this variant had been called in two sequencing reads from a total of only six, indicating the importance of confirming variants found through high throughput sequencing with conventional methods such as Sanger sequencing – particularly in regions with lower sequence depth.

The remaining number of putative causative variants was seven in six super obese individuals (15.4%) compared to two variants in two individuals from the NutriTech group (2.7%, *p*: 0.0081).

Putatively causative CNVs

WES data was screened for CNVs known to cause obesity and CNVs covering obesity genes; 12 such CNVs were found. Further selection took place by applying mode of inheritance for the genomic regions and genes concerned. The CNVs are summarised in Table 7.3, including details on inclusion or exclusion for further analysis. A final two CNVs were selected as highly likely to be causative of

Mendelian forms of obesity; a deletion covering exon 19 of *NTRK2* and a deletion of the proximal 16p11.2 region [61]. Both were found in super obese individuals, while no potentially-causative CNVs were found in the NutriTech participants.

This gives an overall diagnosis of putative Mendelian obesity in eight out of 39 (20.5%) super obese individuals, compared to two putative Mendelian forms of obesity in 73 (2.7%) NutriTech participants (*p*: 0.0031, Figure 7.2).

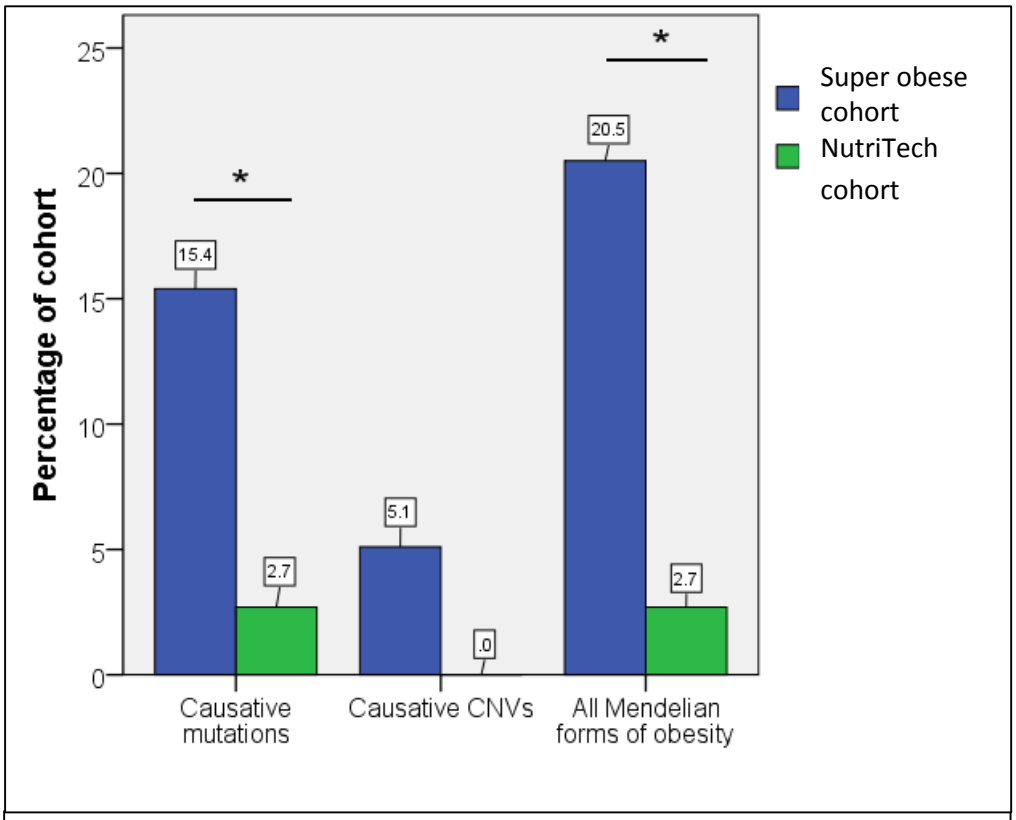


Figure 7.2: Frequencies of Mendelian forms of obesity. Mendelian causes of obesity are given for causative variants (left), causative CNVs (middle) and both causative mutations and CNVs combines (right). Percentages per cohort are indicated above the bars. * indicates a significant difference with p <0.05.

Super obese cohort (n)		NutriTech cohort (n)		Gene	Variant exonic function	Variant details	Reason for inclusion/exclusion
Het	Hom	Het	Hom				
1	0	1	0	*ALMS1*	nonsynonymous SNV	NM_015120:exon8:c.A2033G:p.Y678C	Excluded: Alström syndrome is a recessive disease
1	0	0	0	*ALMS1*	nonsynonymous SNV	NM_015120:exon16:c.G10889A:p.R3630H	Excluded: Alström syndrome is a recessive disease
1	0	0	0	*ALMS1*	nonsynonymous SNV	NM_015120:exon19:c.G11991C:p.R3997S	Excluded: Alström syndrome is a recessive disease
1	0	0	0	*ALMS1*	nonsynonymous SNV	NM_015120:exon8:c.C3376T:p.P1126S	Excluded: Alström syndrome is a recessive disease
0	0	1	0	*ALMS1*	nonsynonymous SNV	NM_015120:exon8:c.G3252C:p.Q1084H	Excluded: Alström syndrome is a recessive disease
0	0	1	0	*ALMS1*	nonsynonymous SNV	NM_015120:exon16:c.A11350G:p.I3784V	Excluded: Alström syndrome is a recessive disease
0	0	1	0	*ALMS1*	nonsynonymous SNV	NM_015120:exon10:c.C8165A:p.S2722Y	Excluded: Alström syndrome is a recessive disease
0	0	1	0	*ALMS1*	nonsynonymous SNV	NM_015120:exon8:c.C2266T:p.P756S	Excluded: Alström syndrome is a recessive disease
0	0	1	0	*ARL6*	nonsynonymous SNV	NM_001278293:exon6:c.C361T:p.R121C	Excluded: BBS is a recessive disease
0	0	1	0	*BBS1*	nonsynonymous SNV	NM_024649:exon4:c.G235A:p.E79K	Excluded: BBS is a recessive disease
1	0	0	0	*BBS12*	nonsynonymous SNV	NM_152618:exon2:c.T116C:p.I39T	Excluded: BBS is a recessive disease
0	0	1	0	*BBS2*	nonsynonymous SNV	NM_031885:exon7:c.A725G:p.N242S	Excluded: BBS is a recessive disease
1	0	0	0	*BBS4*	nonsynonymous SNV	NM_033028:exon6:c.C337T:p.L113F	Excluded: BBS is a recessive disease
1	0	0	0	*BBS4*	nonsynonymous SNV	NM_001252678:exon9:c.T190C:p.Y64H	Excluded: BBS is a recessive disease
1	0	0	0	*BBS5*	nonsynonymous SNV	NM_152384:exon7:c.A551G:p.N184S	Excluded: BBS is a recessive disease
1	0	0	0	*BBS5*	nonsynonymous SNV	NM_152384:exon1:c.G32A:p.R11Q	Excluded: BBS is a recessive disease
1	0	0	0	*BBS7*	nonsynonymous SNV	NM_018190:exon10:c.A955G:p.T319A	Excluded: BBS is a recessive disease
0	0	1	0	*BBS9*	nonsynonymous SNV	NM_014451:exon19:c.G2351A:p.R784H	Excluded: BBS is a recessive disease
0	0	1	0	*BBS9*	nonsynonymous SNV	NM_014451:exon19:c.G2357A:p.C786Y	Excluded: BBS is a recessive disease
0	0	1	0	*CEP19*	nonsynonymous SNV	NM_032898:exon3:c.G182A:p.R61Q	Excluded: CEP19 deficiency has been reported as a recessive disease
0	0	1	0	*CEP290*	nonsynonymous SNV	NM_025114:exon33:c.G4237C:p.D1413H	Excluded: BBS is a recessive disease
0	0	1	0	*CEP290*	nonsynonymous SNV	NM_025114:exon4:c.G226A:p.A76T	Excluded: BBS is a recessive disease
0	0	1	0	*CEP290*	nonsynonymous SNV	NM_025114:exon29:c.G3343A:p.E1115K	Excluded: BBS is a recessive disease
0	0	1	0	*CPE*	nonsynonymous SNV	NM_001873:exon1:c.C215T:p.A72V	Excluded: CPE deficiency has been reported as a recessive disease.

(Table continues on next page)

Super obese cohort (n)		NutriTech cohort (n)		Gene	Variant exonic function	Variant details	Reason for inclusion/exclusion
Het	Hom	Het	Hom				
0	0	1	0	*CPE*	nonsynonymous SNV	NM_001873:exon3:c.A578C:p.D193A	Excluded: CPE deficiency has been reported as a recessive disease.
0	1*	0	0	***IGSF1***	**stopgain**	**NM_001170961:exon19:c.C3883T:p.R1295X**	**Selected for further analysis.**
0	0	1	0	*KSR2*	nonsynonymous SNV	NM_173598:exon18:c.C2624T:p.S904L	Excluded: as variant has been reported in lean controls [91]
1	0	0	0	***LEPR***	**nonsynonymous SNV**	**NM_002303:exon9:c.G1166A:p.S389N**	**Selected for further analysis.**
1	0	0	0	***LEPR***	**nonsynonymous SNV**	**NM_002303:exon20:c.C3041G:p.S1014C**	**Selected for further analysis.**
0	0	**1**	0	***MAGEL2***	**nonsynonymous SNV**	**NM_019066:exon1:c.C2426A:p.A809D**	**Selected for further analysis.**
1	0	0	0	***MAGEL2***	**nonsynonymous SNV**	**NM_019066:exon1:c.C2408A:p.A803D**	**Selected for further analysis.**
0	0	**1**	0	***MAGEL2***	**nonsynonymous SNV**	**NM_019066:exon1:c.G2290A:p.A764T**	**Selected for further analysis.**
0	0	1	0	*MKKS*	nonsynonymous SNV	NM_018848:exon6:c.G1363A:p.E455K	Excluded: BBS is a recessive disease
0	0	1	0	*MKKS*	nonsynonymous SNV	NM_018848:exon3:c.T890C:p.I297T	Excluded: BBS is a recessive disease
0	0	1	0	*MKS1*	nonsynonymous SNV	NM_001165927:exon1:c.C10T:p.P4S	Excluded: BBS is a recessive disease
0	0	1	0	*MKS1*	nonsynonymous SNV	NM_001165927:exon3:c.C169T:p.R57C	Excluded: BBS is a recessive disease
1	0	0	0	***NTRK2***	**nonsynonymous SNV**	**NM_006180:exon20:c.A2221G:p.I741V**	**Selected for further analysis.**
0	0	**1**	0	***NTRK2***	**nonsynonymous SNV**	**NM_006180:exon18:c.A1913T:p.H638L**	**Selected for further analysis.**
1	0	0	0	*POMC*	nonsynonymous SNV	NM_000939:exon3:c.A641G:p.E214G	Excluded: POMC deficiency has been reported as a recessive disease.
0	0	1	0	*POMC*	nonsynonymous SNV	NM_000939:exon3:c.C429G:p.H143Q	Excluded: POMC deficiency has been reported as a recessive disease.
1	0	0	0	***PTEN***	**nonsynonymous SNV**	**NM_000314:exon5:c.A278G:p.H93R**	**Selected for further analysis.**
1	0	0	0	***SH2B1***	**nonsynonymous SNV**	**NM_015503:exon6:c.G1633A:p.G545S**	**Selected for further analysis.**
1	0	0	0	***SH2B1***	**nonsynonymous SNV**	**NM_015503: exon4:c.C1229T:p.S410F**	**Selected for further analysis.**
1	0	0	0	***SH2B1***	**nonsynonymous SNV**	**NM_015503: exon1:c.C47G:p.P16R**	**Selected for further analysis.**
0	0	1	0	*SIM1*	nonsynonymous SNV	NM_005068:exon11:c.G2119C:p.D707H	Excluded: as variant has been reported in lean controls [56]
0	0	**1**	0	***SIM1***	**nonsynonymous SNV**	**NM_005068:exon8:c.A937G:p.I313V**	**Selected for further analysis.**
2	0	0	0	*TTC8*	nonsynonymous SNV	NM_001288782:exon12:c.A659G:p.Q220R	Excluded: BBS is a recessive disease

(Table continues on next page)

Super obese cohort (n)		NutriTech cohort (n)		Gene	Variant exonic function	Variant details	Reason for inclusion/exclusion
Het	Hom	Het	Hom				
1	0	0	0	*TTC8*	nonsynonymous SNV	NM_001288782:exon13:c.C788T:p.A263V	Excluded: BBS is a recessive disease
0	0	1	0	*TUB*	nonsynonymous SNV	NM_177972:exon3:c.A121C:p.K41Q	Excluded: TUB deficiency has been reported as a recessive disease.
0	0	1	0	*WDPCP*	nonsynonymous SNV	NM_015910:exon3:c.T176A:p.I59N	Excluded: BBS is a recessive disease
0	0	1	0	*WDPCP*	nonsynonymous SNV	NM_001042692:exon4:c.G508A:p.V170M	Excluded: BBS is a recessive disease
0	0	1	0	*WDPCP*	nonsynonymous SNV	NM_015910:exon2:c.G160A:p.D54N	Excluded: BBS is a recessive disease

Table 7.2: Predicted-to-be deleterious, rare variants in obesity genes. All variants predicted-to-be deleterious and with a MAF <1% are listed here. Reasons for inclusion (highlighted in red) or exclusion for further analysis are given in the last column. All heterozygous variants found in genes involved in recessive disease were checked for compound heterozygosity (and for tri- and tetra-allelic inheritance for the *BBS* genes); one such combination was found for the two variants found in *LEPR* within one individual. Het, heterozygous; Hom, homozygous; BBS, Bardet Biedl Syndrome. * Variant was found on the X-chromosome in a male participant, so the variant was in a hemizygous state.

Super obese cohort (n)	NutriTech cohort (n)	Type of CNV	Location	BF	Reads ratio	Genes	Reason for inclusion/exclusion
1	0	deletion	chr7:33195197-33195366	3.63	0.4	*BBS9*	Excluded: BBS is a recessive disease
1	0	deletion	chr7:33195197-33195366	6.92	0.265	*BBS9*	Excluded: BBS is a recessive disease
1	0	deletion	chr7:33195197-33195366	4.58	0.37	*BBS9*	Excluded: BBS is a recessive disease
1	0	deletion	chr7:33195197-33195366	4.53	0.357	*BBS9*	Excluded: BBS is a recessive disease
1	0	deletion	chr7:33195197-33195366	3.94	0.1	*BBS9*	Excluded: BBS is a recessive disease
1	0	**deletion**	**chr9:87570170-87570529**	**3.59**	**0.5**	***NTRK2***	**Included for further analysis**
1	0	**deletion**	**chr16:29675061-30215702**	**1270**	**0.509**	*****	**Included for further analysis**
1	0	deletion	chr1:66094862-66096206	4.06	0.579	*LEPR*	Excluded: not covering the transcript associated with obesity
0	1	duplication	chr1:66094862-66096206	4.79	1.59	*LEPR*	Excluded: duplication less likely to disrupt gene function
0	1	duplication	chr2:25383662-25384584	5.4	1.54	*POMC*	Excluded: duplication less likely to disrupt gene function
0	1	duplication	chr16:29675061-30215702	763	1.47	*	Excluded: duplication of 16p11.2 proximal region leads to lean phenotype [62]
0	1	duplication	chr16:29994135-29995054	8.79	1.72	*TAOK2*	Excluded: duplication of 16p11.2 proximal region leads to lean phenotype [62]

Figure 7.3: CNVs covering obesity genes. All predicted CNVs covering human obesity genes are listed here. Reasons for inclusion (highlighted in red) or exclusion for further analysis are listed in the last column. All CNVs covering genes involved in recessive disease were checked for compound heterozygosity with other CNVs and variants listed in table 6.2 (and for tri- and tetra-allelic inheritance for the *BBS* genes). BBS, Bardet Biedl Syndrome.

* Genes included in the 16p11.2 region*: SPN,QPRT,C16orf54,ZG16,KIF22,MAZ,PRRT2,PAGR1,PAGR1,MVP,CDIPT,SEZ6L2, SPHD1, KCTD13, TMEM219, TAOK2, HIRIP3,INO80E,DOC2A,C16orf92, FAM57B,ALDOA,RN7SKP127,AC009133.17,AC009133.21,AC009133.15,AC009133.14,AC009133.20,AC009133.12,CDIPT-AS1,CTD-2574D22.4,CTD-2574D22.2,RP11-455F5.3,RP11-455F5.4,RP11-455F5.5,SLX1A-ULT1A3, PPP4C, TBX6, YPEL3, GDPD3, MAPK3, CORO1A, BOLA2B, SLX1A, SULT1A3, RP11-347C12.3*

The final 10 putative cases of Mendelian obesity are described below, with further detailed interpretation of the genes involved, prediction of the effect of the variants, phenotype of the participants involved and confirmation analysis. Details of the final selection of putative Mendelian forms of obesity are summarised in Table 7.4.

***NTRK2* deletion: p.R646R-fsX700:**

A deletion of exon 19 of Neurotrophic Tyrosine Kinase, Receptor, Type 2 (*NTRK2*, transcript NM_006180), encoding the TrkB protein, was found in one participant. This deletion was discovered by read-depth analysis from the WES data: a deletion of chr9:87570170-87570529 within reference genome CH37/hg19 (Figure 7.3). The expected number of sequencing reads for this region was 66, but only 33 were observed, giving a reads-ratio of 0.5 and a Bayes Factor: 3.59 (Table 7.3).

The deletion was found in a 46-year-old male, with a BMI of 40.9kg/m^2 (weight: 144kg, height: 1.85m) on recruitment (participant SO_01 in Table 7.4). He had been treated for obesity and dyslexia during childhood and in his mid-thirties managed to lose a significant amount of weight through lifestyle adjustment. His maximum recorded weight was 222 kg, giving him a BMI of 64.9kg/m^2. After recruitment he underwent RYGB and reached a weight loss of 12.8% of his pre-surgical weight (144kg) two years following surgery.

Disruptive point mutations in *NTRK2*, encoding the Trkb receptor, cause severe early-onset obesity in children [59,60]. In our participant, a complete deletion of exon 19 leads to a loss of 79 amino acids within the intracellular tyrosine kinase domain of the NTRK2 protein, and a subsequent frameshift of the remaining coding region, culminating in a premature stop codon at amino acid location 700 (Figure 6.5). This deletion includes the position of a previously-reported disease-causing variant, and is likely to damage the signalling capacity of the receptor.

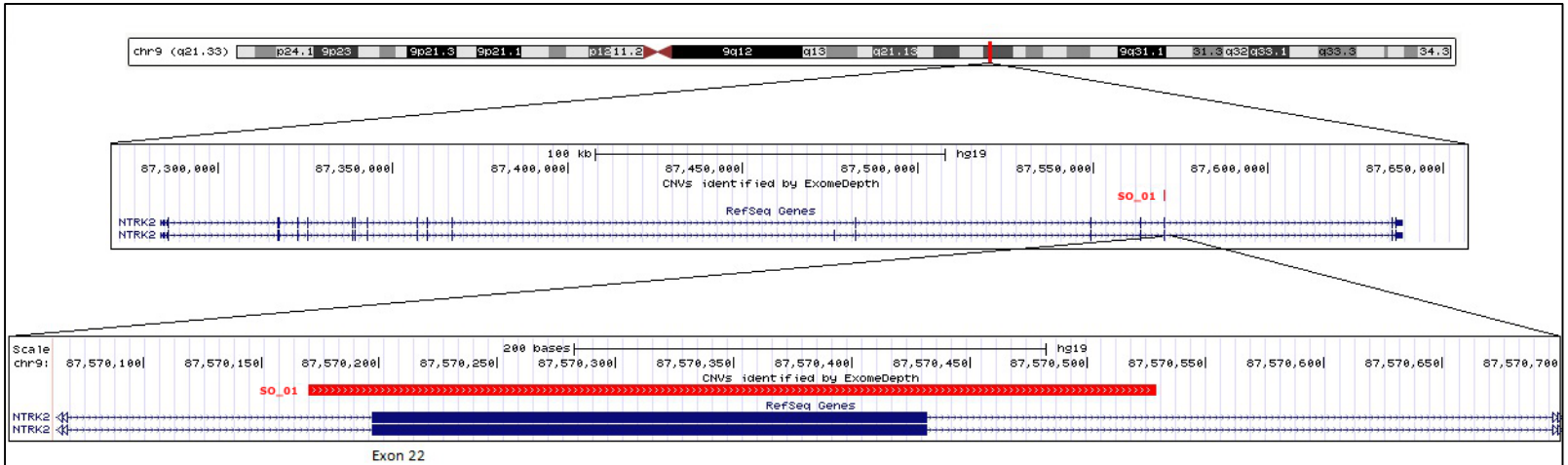


Figure 7.3: Overview of the location of the deletion of exon 22 of *NTRK2.* This image was created by uploading a customised annotation track into the UCSC Genome browser (reference genome GRCh37/Hg19) http://genome.ucsc.edu

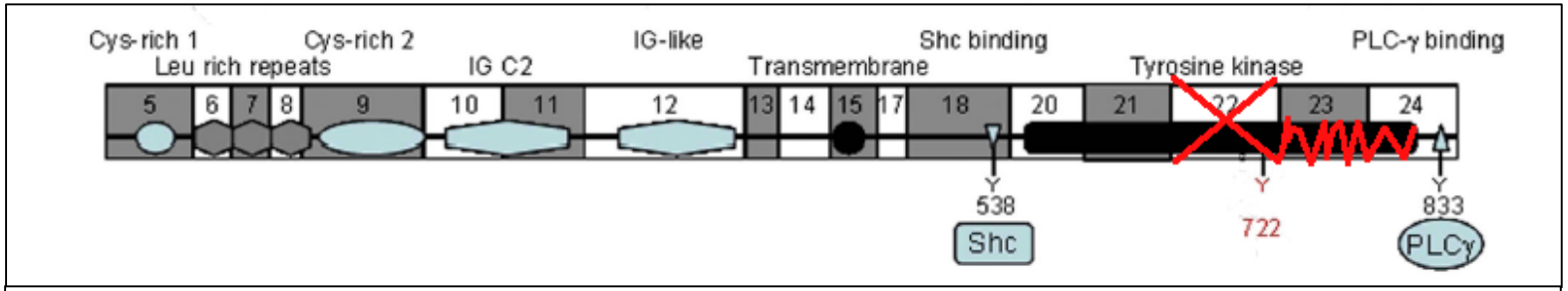


Figure 7.4: Exonic structure of *NTRK2* and protein structure of TrkB. Three tyrosines are highlighted, with Tyr538 binding to Shc and Tyr833 binding to PLCγ. A previous variant found at Tyr722 was found to impair the receptor function causing a complex developmental syndrome and severe obesity. The red indicates the exon deleted in the patient described here (exon 19 in transcript NM_006180), causing a truncation of the remaining protein. (Current figure was adjusted from the original figure as published in Yeo GS, *et al.* [60])

Genome-wide SNP analysis, Sanger sequencing of cDNA and long range PCR of gDNA was used in an attempt to confirm this deletion, but we have not yet been able to provide confirmation or to exclude the existence of this deletion:

1) *Genome wide SNP analysis* was impossible because of poor coverage of the region: Although a larger CNV covering several exons of NTRK2 could be excluded, the SNP coverage was not sufficient in the specific region of exon 19 to confirm or exclude a deletion of this exon (analysis performed by Nikman A. Nor Hashim, Figure 7.5).

2) *Sanger sequencing of cDNA* was impossible because the gene was not expressed in blood cells: A primer-pair was designed to cover exon 19 in cDNA retrieved from RNA extracted from whole blood (forward primer located within exon 18 and reverse primer located within exon 20). Unfortunately, no product could be retrieved in participant 01, or any of the 5 controls. To identify whether this was due the PCR reaction being sub-optimal, or due the lack of expression of *NTRK2* in the whole blood sample, PCR was performed using the primer pair used for confirmation of the *NTRK2* p.Ile741Val variant in participant SO_08, for which both primers were located within the exonic region of *NTRK2*. A PCR product was retrieved in gDNA of participant SO_01 and the gDNA control samples, while no product was received in cDNA of participant SO_01 or any of the control cDNAs. Therefore, it appears that there is not enough expression of *NTRK2* in the whole blood sample to be detected by RT-PCR. Although the cDNA sample of this individual was not used for any other analysis in this thesis, other analysis performed on this sample by others did give positive results, confirming that reverse transcription had been successful.

2) *Long range PCR of gDNA* was also unsuccessful: In another attempt to confirm the deletion, long range PCR was used to cover the extensive region between the possible breakpoint locations. Since the *NTRK2* deletion was predicted using read depth analysis of WES data, the location of the breakpoints of the deletion were not known. Therefore, the size of the deletion could be anywhere between 71.5 kb (distance between exon 18 and 20) and 235 bp (size of exon 19). Several primer pairs

were designed to cover this 71.5 kb region using long-range PCR (chapter 2, section 2.4.9, page 81), in an attempt to amplify the allele containing the deleted region for Sanger sequencing. Unfortunately, no PCR products were obtained, not even using primer pairs that were designed within a distance from each other so they should be able to cover the non-deleted allele. This might indicate the long-range PCR set-up needs to better optimisation for this region. Time constraints meant that this could not be carried out for inclusion in this thesis.

Although the three attempts at confirming the deletion did not work, the deletion could also not be excluded using the methods. Looking at the actual WES coverage of the region (Figure 7.6) and the deletion Bayes Factor of 3.59 (which is significant regarding the small size of the predicted deletion), the prediction of the deletion seems to be reliable.

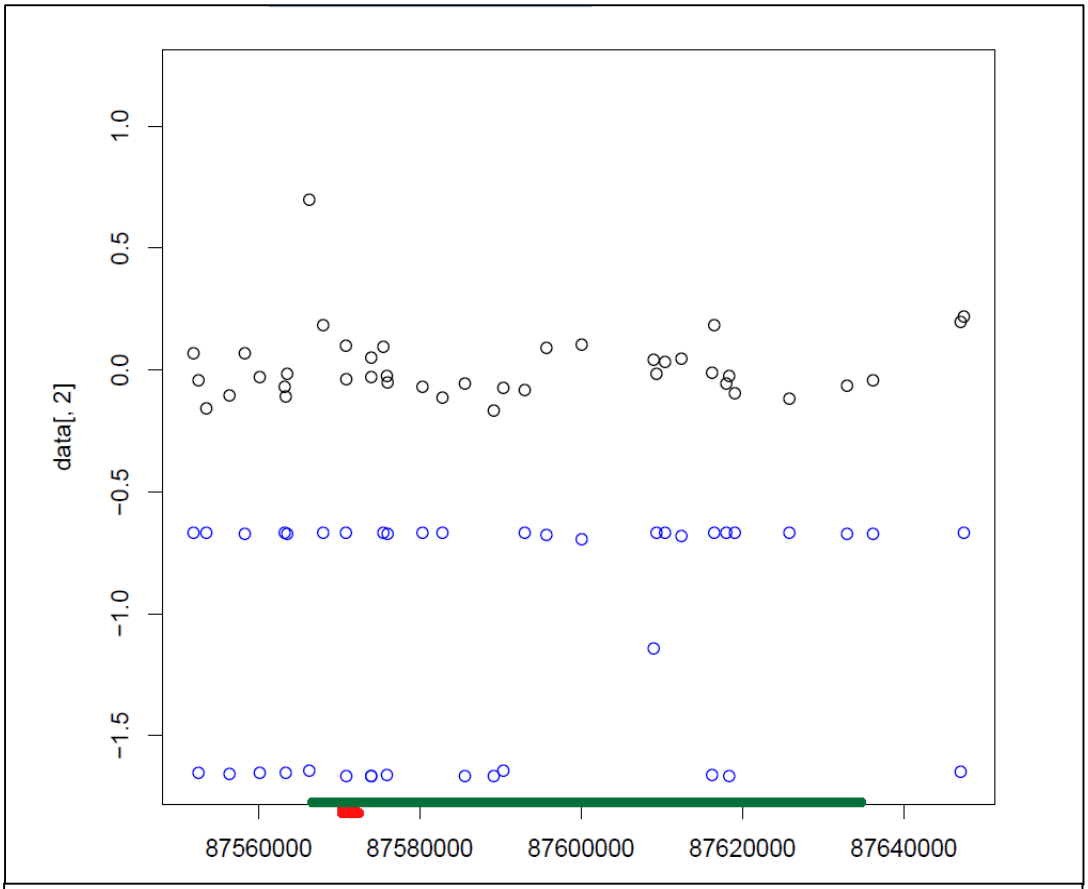


Figure 7.5: SNPs surrounding predicted deleted region. The location of the predicted deleted exon 19 is indicated in red, while the intronic region within the possible breakpoints can be located is highlighted in green. As can be seen, there was no difference in signal intensity of this region (markers in black), and there were no heterozygous SNPs in this region beside one imputated SNP (markers in blue).

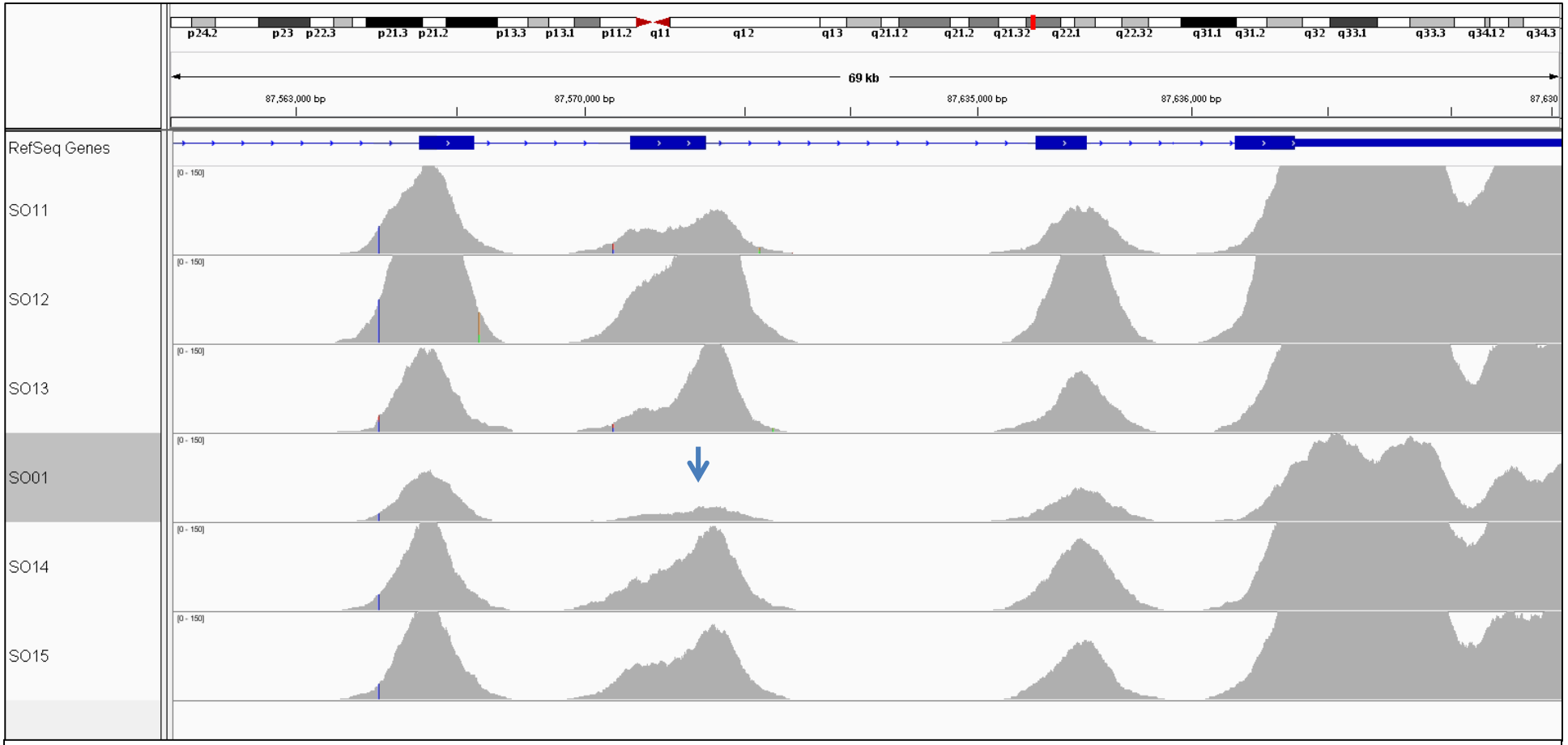


Figure 7.6: Aligned sequencing reads covering the last four exons of *NTRK2*. Sequencing reads of six different participants from the Class V severely obese cohort are visualised here (created using Integrative Genomics Viewer, IGV). Intronic regions have been shortened to visualise multiple exonic regions of exon 18-21 of *NTRK2* (transcript NM_006180) within one image. The real distance between the exonic regions can be interpreted by the location identifiers above the RefSeq gene in blue. The arrow indicates the sequencing reads of exon 19 in participant SO_01, referring to the predicted deletion

16p11.2 deletion:

In this 56-year-old woman (SO_02 in Table 7.4) with a BMI of 61.2 kg/m2 (weight: 160.5 kg, height: 1.62 m), CNV analysis of the WES data revealed a 16p11.2 deletion, covering ~30 genes (Figure 7.7). This is a known causative CNV of obesity and also confers increased risk of autism spectrum disorder [61]. The participant had insulin-dependent type 2 diabetes mellitus and sleep apnoea, for which she used a CPAP machine. There were no reported autism spectrum disorder features, but the participant had been diagnosed with dyslexia. She had undergone RYGB with a successful weight loss of 35.3%, at two years following surgery. The deletion was confirmed using genome wide SNP analysis performed by Nikman A. Nor Hashim, as described in Chapter 2.4.8, page 81. (Figure 7.8).

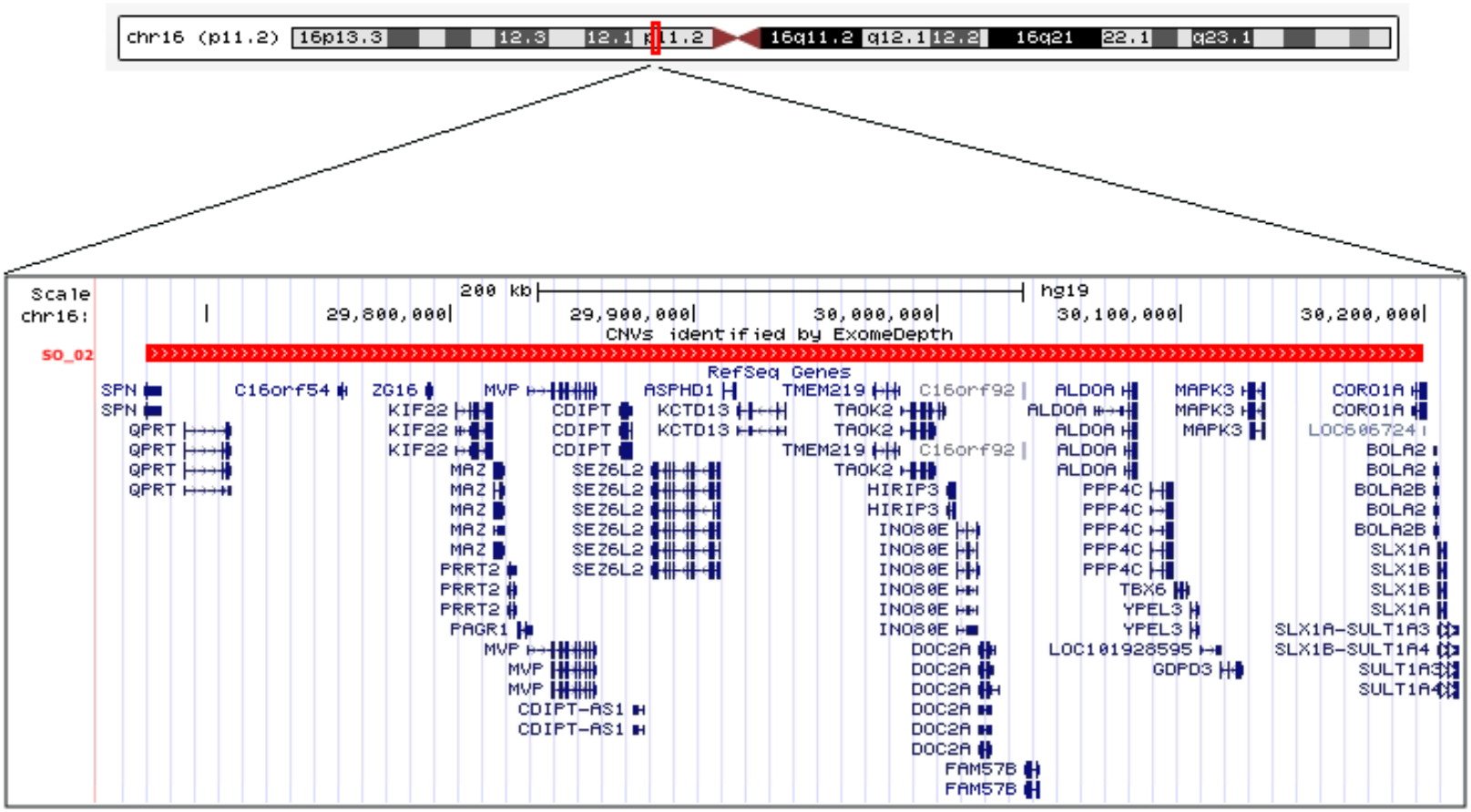


Figure 7.7: Overview of the location of the predicted deletion within the 16p11.2 region. This image was created by uploading a customised annotation track into the UCSC Genome browser (reference genome GRCh37/Hg19). http://genome.ucsc.edu

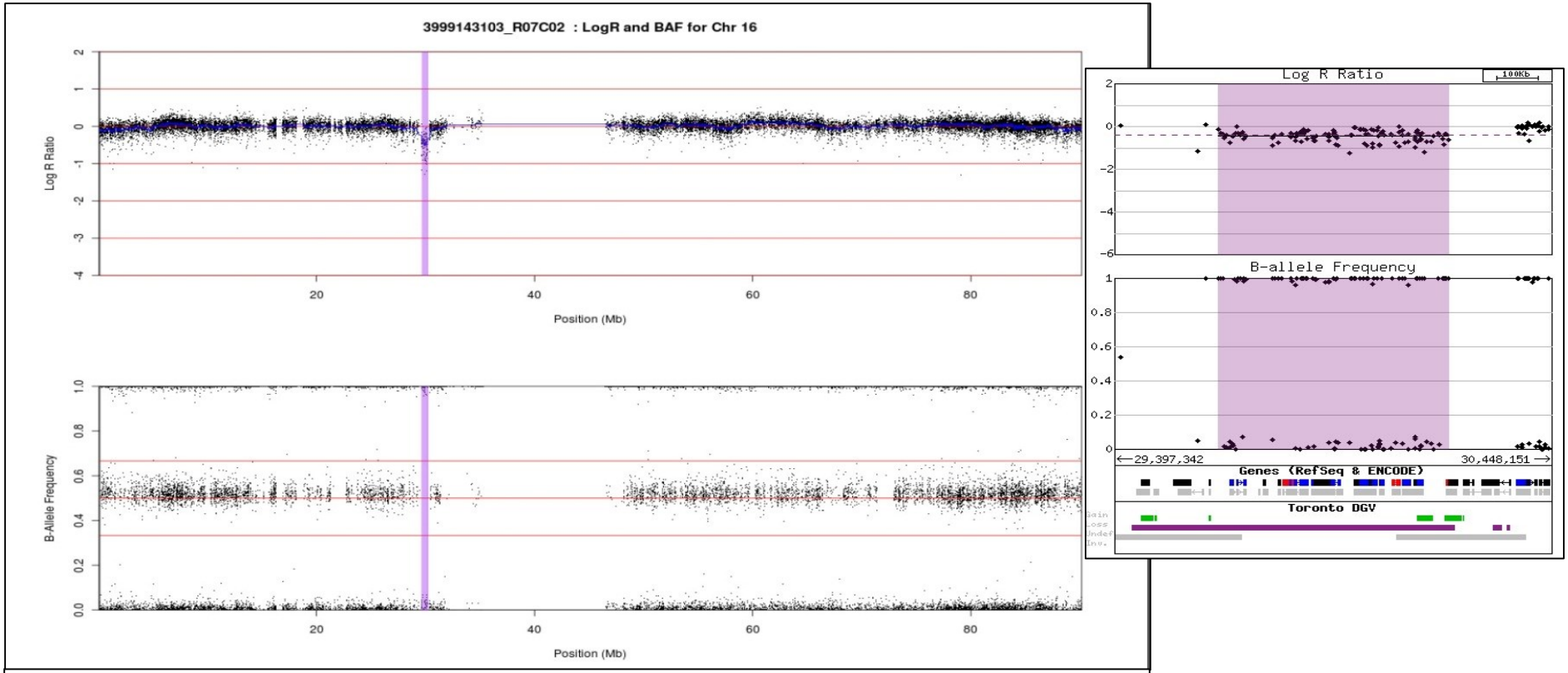


Figure 7.8: Confirmation of 16p11.2 deletion through genome-wide SNP analysis. The Log R Ratio (LRR) and B allele frequency (BAF) information were extracted from GenomeStudio version 2011.1 (Illumina Inc., San Diego, California, USA). The exported data were used to generate autosomal CNV calls using the PennCNV software (2011 version). PennCNV applies a hidden Markov model-based (HMM) model to predict CNVs on a sample-by-sample approach. LRR and BAF plots in chromosome 16 for 16p11.2 deletion carrier (SO_02) are visualised here. The purple shaded region shows signal intensity aberration in the 16p11.2 region. (Right) A zoomed-in view of the LRR and BAF plots.

***IGSF1*; p.Arg1295Ter:**

A hemizygous nonsense mutation, c.578A>C (p.(Arg1295Ter)), in Ig superfamily member 1 (*IGSF1*) was found in male participant (SO_03 in Table 7.4). Disruptive variants in *IGSF1* cause an X-linked disorder with central hypothyroidism, macroorchidism, obesity and a variable prolactin and growth hormone deficiency [96-98]. The variant p.Arg1295Ter, leads to a truncation of the final 41 amino acids which form a part of the coding region for the cytoplasmic C-tail of the protein. Participant SO_03 had no signs of hypothyroid disease (baseline measures included TSH: 1.02 mμ/L, free-T4: 12.7 pmol/L). No data was available for the other typical phenotypes of IGSF1 deficiency at the time of preparation of this thesis.

***LEPR*; p.Ser389Met and p.Ser1014Cys:**

Two variants in the leptin receptor gene (*LEPR*), c.1166G>A (p.(Ser389Met)) and c.3041C>G (p.(Ser1014Cys)), were found in a male participant (SO_04 in Table 7.4). Homozygous and compound heterozygous mutations in *LEPR* cause early onset obesity and hyperphagia, alterations in the immune system and delayed puberty due hypogonadotropic hypogonadism, although patients are less severely affected than patients with leptin deficiency [19,20]. Both variants reported here are predicted-to-be deleterious and are located within the immunoglobulin (p.Ser389Met) and intracellular domains (p.Ser1014Cys) of the receptor (Uniprot: P48357). These variants have not been reported in homozygous or compound heterozygote state before in open databases (1000 genomes, NHLBI Esp or ExAC [207-209]). The participant in whom both variants were found had a BMI of 66.7 kg/m^2 and T2DM, but no signs of an altered immune function, or hypogonadotropic hypogonadism.

***PTEN*; p.His93Arg:**

In one super obese male (SO_05 in Table 7.4), with a BMI of 63.4 kg/m^2, a predicted-to-be deleterious variant in the Phosphatase and tensin homolog gene, *PTEN*; c.278A>G (p.(His93Arg)), was found. Loss-of-function variants in *PTEN* cause Cowden syndrome, a rare complex cancer-predisposition syndrome (with features including microcephaly and obesity) [95]. However, less disrupting point mutations in

PTEN cause a milder phenotype of macrocephaly and autistic features. The variant described here was previously described as a novel germline mutation in a severely overweight four-year-old male (27.4kg, + 4 SD), with macrocephaly, speech delay, and autistic behaviour [94]. The p.His93Arg variant in *PTEN* has subsequently been shown to affect the protein's function, inhibiting phosphatase activation [291,292]. Other cases with point mutations have a similar phenotype of macrocephaly, autistic behaviour and obesity [94]. In the participant described here no autistic behaviour could be detected. No head-size measurements had been taken.

***SH2B1* variants:**

In two super obese female participants (SO_06 and SO_07 in Table 7.4), rare and predicted-to-be deleterious variants in Src homology 2 (SH2) B adaptor protein 1 (*SH2B1*) were found: c.1633G>A (p.(Gly545Ser)) and c.1229C>T (p.(Ser410Phe)). Both variants were located in the N-terminal region, which is common to all four differently spliced isoforms of *SH2B1*, and in which previous point mutations have been reported in obese individuals with maladaptive behaviour [102,103]. The first participant, carrying the p.Gly545Ser variant, had a BMI of 57.3 kg/m^2, but no other co-morbidities, while the participant with the p.Ser410Phe variant had a BMI of 76.2 kg/m^2 and suffered from hypertension and obstructive sleep apnoea. Neither participant had any sign of abnormal behavioural characteristics.

***NTRK2* variants:**

Predicted-to-be deleterious variants in *NTRK2,* c.2221A>G (p.(Ile741Val)) and c.1913A>T (p.(His638Leu)), were found in one super obese (72.0 kg/m^2) and one mildly obese male (32.1 kg/m^2) respectively (SO_08 and NT_01 in Table 7.4). Both variants were located in the tyrosine kinase domain of the receptor, in which previous point mutations were reported in obese individuals with learning disability [59,60]. Both participants had a normal intellect, and there were no signs of abnormal cognitive function.

***SIM1;* p.Ile131Val:**

In a final overweight male participant (NT_02), with a BMI of 27.9 kg/m^2, a predicted-to-be deleterious variant in single-minded 1, *SIM1* c.937A>G (p.(Ile131Val)) was found. The variant was located in the domain that acts as a secondary dimerisation interface, in which previous point mutations were found affecting the protein, leading to severe obesity [56].

Excluded variants through literature research:

Two further rare, predicted-to-be-deleterious variants were found; one variant in *KSR2*, c.2624C>T (p.(Ser904Leu), MAF: 0.001), and one variant in *SIM1*, c.2119G>C (p.(Asp707His), MAF: 0.0005), in an obese (BMI 30kg/m^2) and overweight (BMI 29kg/m^2) individual respectively. However, both variants have been reported before in lean controls as well as obese subjects, and were, therefore, considered less likely to be causative of Mendelian obesity, although variable penetrance cannot be excluded [56,91].

Other variants, reported as deleterious by multiple prediction tools, were found in *MAGEL2* (one in the super obese as well as two in NutriTech individuals). However, a phenotypic effect is only expected when the mutation is on the paternal allele, so the effect on BMI in these individuals could not be predicted without parent-of-origin analysis which could not be carried out in time for this thesis [90]. PCR of cDNA (retrieved from RNA extracted from whole blood) was used in an attempt to amplify the regions of the variants for Sanger sequencing, in order to confirm whether the allele with or without the variant is being expressed. Unfortunately, PCR produced no product on cDNA, while PCR on gDNA ran at the same time did. This could indicate the lack of expression of *MAGEL2* in the whole blood samples collected.

Participant ID	Cohort	affected gene/region	Ethnicity	BMI	T2DM	Onset obesity	BED	Obesity related comorbidities	Other	Surgery type (%WL at 12, 24 mths)
SO_01	Super obese	*NTRK2* del	British	68.5	ITT2DM	<10	no	hypertension, hypercholesterolemia, sleep apnoea	Dyslexia	RYGB (35.7; 14.3)
SO_02	Super obese	16p11.2 del	British	62.9	ITT2DM	<10	yes	hypertension, hypercholesterolemia, sleep apnoea	Dyslexia	RYGB (36.3; 35.2)
SO_03	Super obese	*IGSF1* (p.Arg1295Ter)	British	61.2	T2DM	<10	no	hypertension, sleep apnoea	none	VSG (15.3; 12.6)
SO_04	Super obese	*LEPR* (p.Ser389Asn; p.Ser1014Cys)	British	66.7	T2DM	<10	no	hypertension, hypercholesterolemia	none	VSG (32.0; --)
SO_05	Super obese	*PTEN* (p.His93Arg)	British	63.4	no	<10	no	sleep apnoea	none	VSG (19.8; --)
SO_06	Super obese	*SH2B1* (p.Gly545Ser)	British	57.3	no	<10	no	none	none	RYGB (27.8; 32.6)
SO_07	Super obese	*SH2B1* (p.Ser410Phe)	British	76.2	no	<10		hypertension, sleep apnoea, requires walking aid	none	VSG (22.0; --)
SO_08	Super obese	*NTRK2* (p.Ile741Val)	British	72.0	no	<10	no	hypertension, hypercholesterolemia, sleep apnoea, hypothyroidism	none	--
NT_01	NutriTech	*NTRK2* (p.His638Leu)	British	32.1	no	?	?	none	--	--
NT_02	NutriTech	*SIM* (p.Ile313Val)	British	27.9	no	?	?	none	--	--

Table 7.4: Participant characteristics. An overview of the clinical phenotypes of the individuals in which a putative Mendelian form of obesity was found. '?' indicates data is not known. '--' indicates item is not applicable for the participant. RYGB, Roux-en-Y gastric bypass; VSG, vertical sleeve gastrectomy.

7.3.3 Weight loss in Mendelian obesity following bariatric surgery

To enable the analysis of weight loss seen in individuals with Mendelian obesity, all super obese with putative Mendelian obesity discovered through WES and the individuals with MC4R deficiency described in chapter 4, were grouped into one 'Mendelian obesity' group, and compared to the remaining cohort. A total of 18 individuals with Mendelian obesity in total were discovered, with eight who underwent RYGB and seven who underwent VSG. Three individuals did not (yet) have surgery.

A mean weight loss of 36.6% (± 10.41 SD) and 23.1% (± 5.94 SD) was seen one year following RYGB and VSG respectively in the Mendelian obesity group (Table 6.5). There was no significant difference in %WL for the individuals with Mendelian obesity compared to the rest of the cohort at 3, 12 or 24 months following either surgery type (Table 7.5 and Figure 7.9).

Of all 15 individuals with Mendelian obesity that underwent bariatric surgery, three did not reach a clinically significant weight loss of 20%:

1) Participant SO_03 with a variant in *IGSF1* (p.Arg1295Ter) did not lose significant weight following VSG and had a revision to RYGB.
2) Participant SO_01 with a deletion of exon 19 of the *NTRK2* gene regained 30kg two years following RYGB, which lowered his %WL below 20% compared to his starting weight.
3) Participant SO_05 with a variant in *PTEN* (p.His93Arg) only reached 11.21 %WL at one year following surgery.

However, for both SO-01 and SO-05 if weight loss was calculated from their highest weight recorded before surgery, both reached a >20 %WL.

	RYGB			VSG		
%WL	**Mendelian obesity**	**Complete cohort**	**p-value**	**Mendelian obesity**	**Complete cohort**	**p-value**
5 months	-21.78 (± 4.81)	-20.28 (± 6.61)	0.778	-18.21 (± 3.02)	-18.73 (± 7.40)	0.582
12 months	-36.55 (± 10.41)	-31.21 (± 7.91)	0.166	-23.12 (± 5.94)	-26.56 (± 9.72)	0.215
24 months	-31.65 (± 5.44)	-31.88 (± 9.97)	0.772	-22.98 (± 14.65)	-24.86 (± 13.03)	0.771

Table 7.5: Percentage weight loss following surgery. Percentage weight loss (%WL) was adjusted for age, baseline-BMI, gender, ethnicity and T2DM status. The Mendelian obesity results were based on the data available for each time-point (RYGB: 3ms, n=8; 12ms, n=6; 24ms, n=4. VSG: 3ms, n=6; 12ms, n=6; 24ms, n=2). Data is presented as mean (± SD).

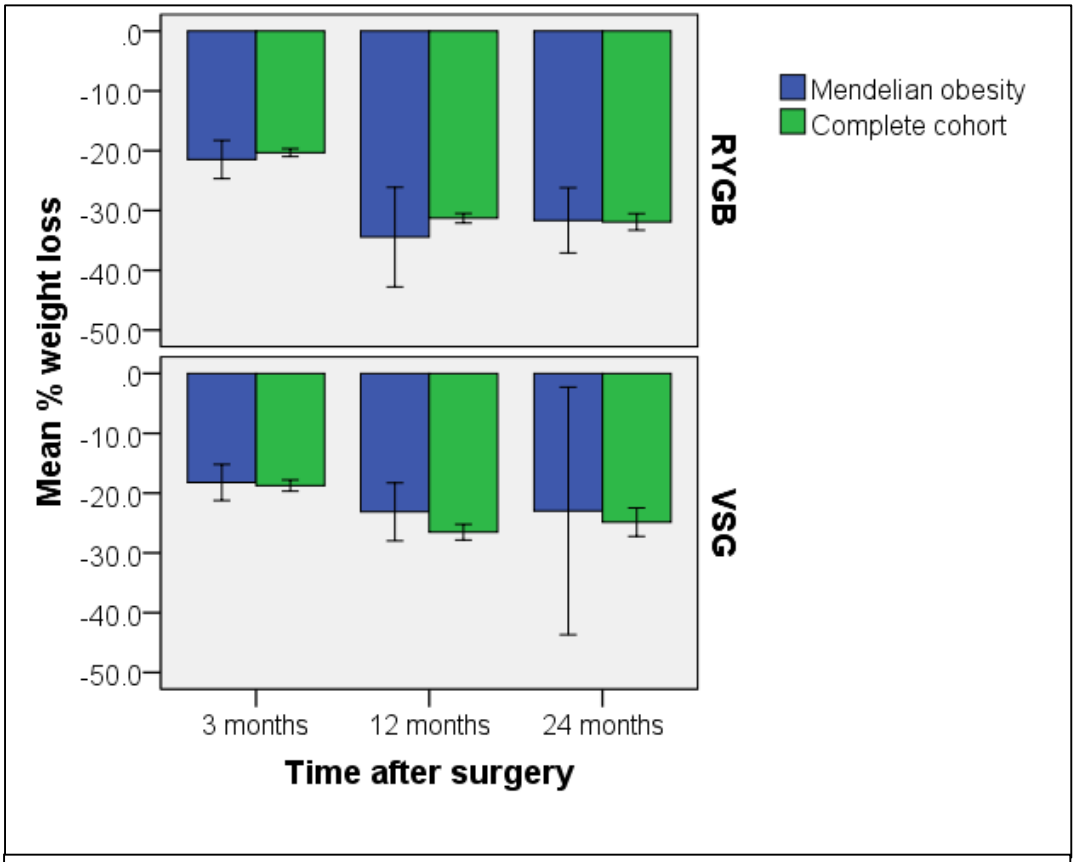


Figure 7.9: Percentage weight loss following RYGB (top panel) and VSG (bottom panel) in Mendelian obesity. Percentage weight loss was corrected for age, baseline-BMI, gender, ethnicity and T2DM status. No significant differences were seen at any time-point following RYGB or VSG between the Mendelian obesity group and the complete cohort. Error bars depict standard error of the mean.

7.3.4 Variation in human-obesity genes

In addition to cases of Mendelian obesity described above, it was hypothesised that carriage of other rare deleterious variants in obesity genes might also contribute to obesity (i.e. heterozygosity for recessive alleles, perhaps in different genes). It has previously been reported that multiple rare variants, each with moderate but significant effect, can in aggregate have a strong effect on phenotypes, especially in individuals in the extremes of the phenotype [84,210]. Therefore, we looked at the frequencies of different variant groups in the human obesity genes in the super obese and the NutriTech participants.

The mean overall number of variants per participant in the obesity genes was not different in the super obese group compared to the NutriTech group: 72.08 (± 10.7 SD) vs 68.00 (±7.70 SD) (Table 6.5).

No higher burden of deleterious variants (common and rare combined) in the human obesity genes could be found in the super obese compared to the NutriTech participants, which indicates that although having a combination of common and rare deleterious variants in this selection of genes could have an effect on BMI, it is not likely to be the only cause of the most extreme forms of obesity. Also the analysis of only rare, deleterious variants revealed no significant difference (Figure 7.11) and comparison between the two groups only became significant after adjustment for mode of inheritance of the genes concerned (as described in the previous section of this chapter).

Interestingly, looking at the proportion of non-synonymous and predicted-to-be deleterious variants found within each group, stratified into three MAF groups (MAF: <1%, MAF: 1-5%, and MAF: >5%), a tendency towards an increased proportion of non-synonymous and deleterious variants could be seen in the super obese group among the rare variants (MAF <1%), that was not present in the common variants (MAF 1-5% and MAF >5%). The difference however did not reach statistical significance (Figure 7.10).

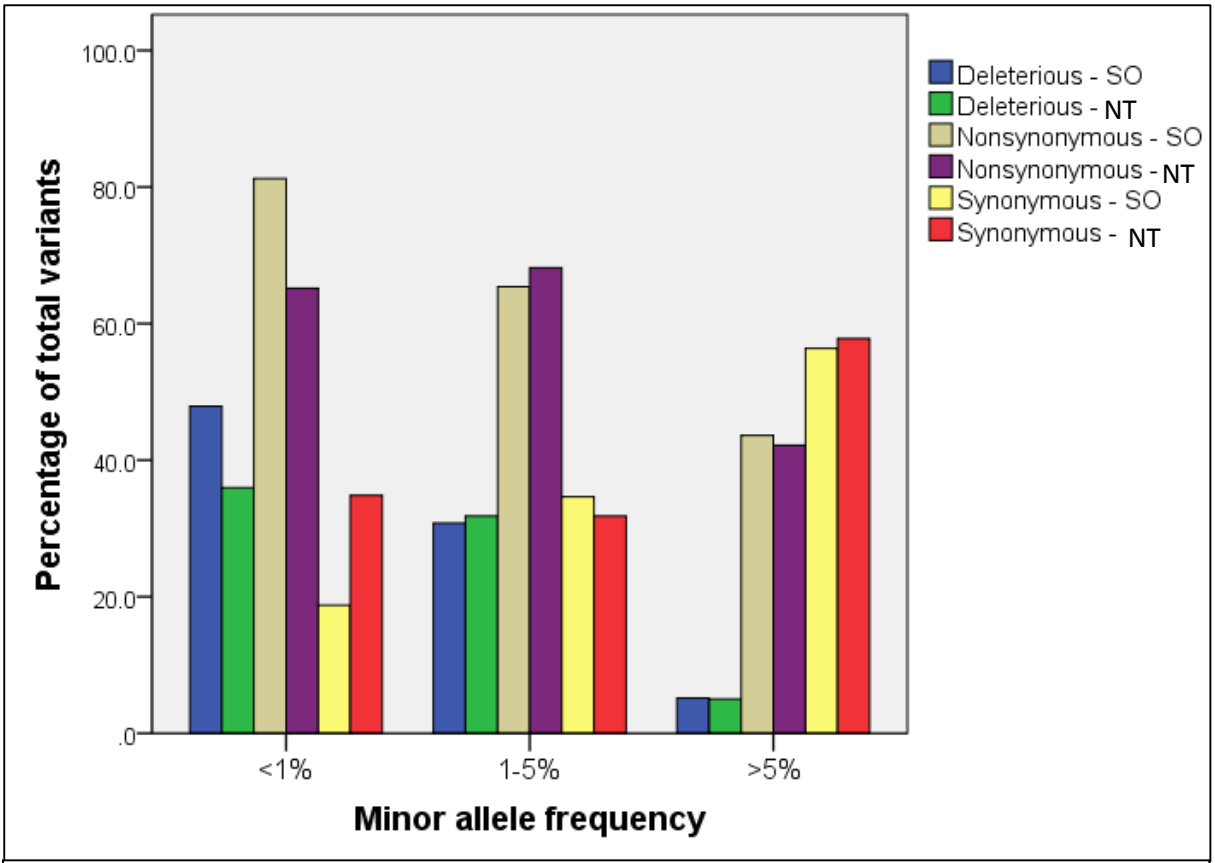


Figure 7.10: Percentages of deleterious, nonsynonymous and synonymous variants for different minor allele frequencies. All predicted-to-be deleterious variants were also nonsynonymous, and therefore these percentages are overlapping. SO, Super Obese cohort; NT, NutriTech cohort.

7.3.5 Variation in mouse-obesity genes

One aim of this study was to seek evidence for new genes causing monogenic obesity in humans. Accordingly, I next investigated a selection of genes known to cause obesity or weight increase when disrupted in mice (some overlapping with the human-obesity genes, and listed in Appendix 2.3 (page 319)). The total number of variants found in these 165 genes, was 29,873; a mean number of 269.95 (± 16.7 SD) variants per participant in the super obese, and a mean number of 265 (± 15.8 SD) variants per participant in the NutriTech participants (*p*: 0.531).

Of these overall variants in the mouse-obesity genes 28,595 (95.7%) were common (MAF >1%); 13,447 (45.0%) were synonymous; 4,251 (14.2 %) were non-coding; and 12,174 (40.8%) were non-synonymous.

Comparing the mean number of variants in the mouse-obesity genes between the super obese and NutriTech groups, there was no difference in the mean total number of variants per participant, of nonsynonymous variants or of deleterious variants (Table 7.5), nor was any difference seen between frequencies of the rare, deleterious variants (Figure 7.11).

For a final analysis, the human-obesity genes and mouse-obesity genes were combined, but only the genes known to be 'less tolerant' to variation were included (Appendix 2.3, page 319): within the human and mouse obesity genes included in this chapter, variation exists in the frequency of functional variants, with some genes carrying more variants than other genes. Petrovski, *et al*. have developed a Residual Variation Intolerance Score (RVI-Score), that assesses whether genes have relatively more or less functional genetic variation than expected, based on the apparently neutral variation found in the gene [211]. With the expectation that genes with a low intolerance score are more likely to cause disease, a selection was made among the obesity genes that had a negative score (so in which a less than expected functional variation was seen by Petrovski, *et al.* [211])

Comparison analysis was repeated with this newly-selected group of 94 genes less tolerant to variation. Again, no difference was found among the mean overall number of variants in the super obese compared to the NutriTech participants, however, a small but significantly higher mean number of non-synonymous and predicted-to-be deleterious variants was found in the super obese compared to the NutriTech participants (Table 7.6).

Our aim was to seek preliminary evidence for potential new causes of monogenic obesity in humans. Accordingly, although there was no difference in the number of rare, predicted-to-be deleterious variants in the overall number of genes less tolerant to variation (Figure 7.11) a selection was made of genes that carried severely damaging variants (such as frameshift or nonsense mutations) in the super obese, (while no predicted-to-be deleterious variants were seen in NutriTech participants) for further investigation (as described in the next section).

	Super obese cohort (n=40)	NutriTech cohort (n=73)	p-value[¥]
Human obesity genes (n=38)			
all variants	72.08 (± 10.68)	67.96 (± 7.70)	0.250
non-synonymous variants	27 [24-32]	25 [22-28]	0.060
predicted-to-be deleterious variants	5 [4-7]	5 [4-5.5]	0.081
Mouse-obesity genes (n=165)			
all variants	269.95 (± 16.7)	265 (± 15.8)	0.531
non-synonymous variants	110 [104-116]	105 [100.5-113.5]	0.063
predicted-to-be deleterious variants	21 [19-26]	20 [17-22.5]	0.156
Obesity genes intolerant to variation (n=94)			
All variants	142.15 (± 14.7)	137.15 (± 9.36)	0.059
non-synonymous variants	**47 [43-52]**	**43.7 [40.5-47.5]**	0.003
predicted-to-be deleterious variants	**5.95 [4-8]**	**4.8 [3.5-6.0]**	0.030

Table 7.6: Mean number of variants per participant in the different obesity-gene groups.

Data presented as mean (**±** SD) or median [interquartile range]. Highlighted in red are significant differences with p. <0.05. [¥] Bonferroni post-hoc corrections applied.

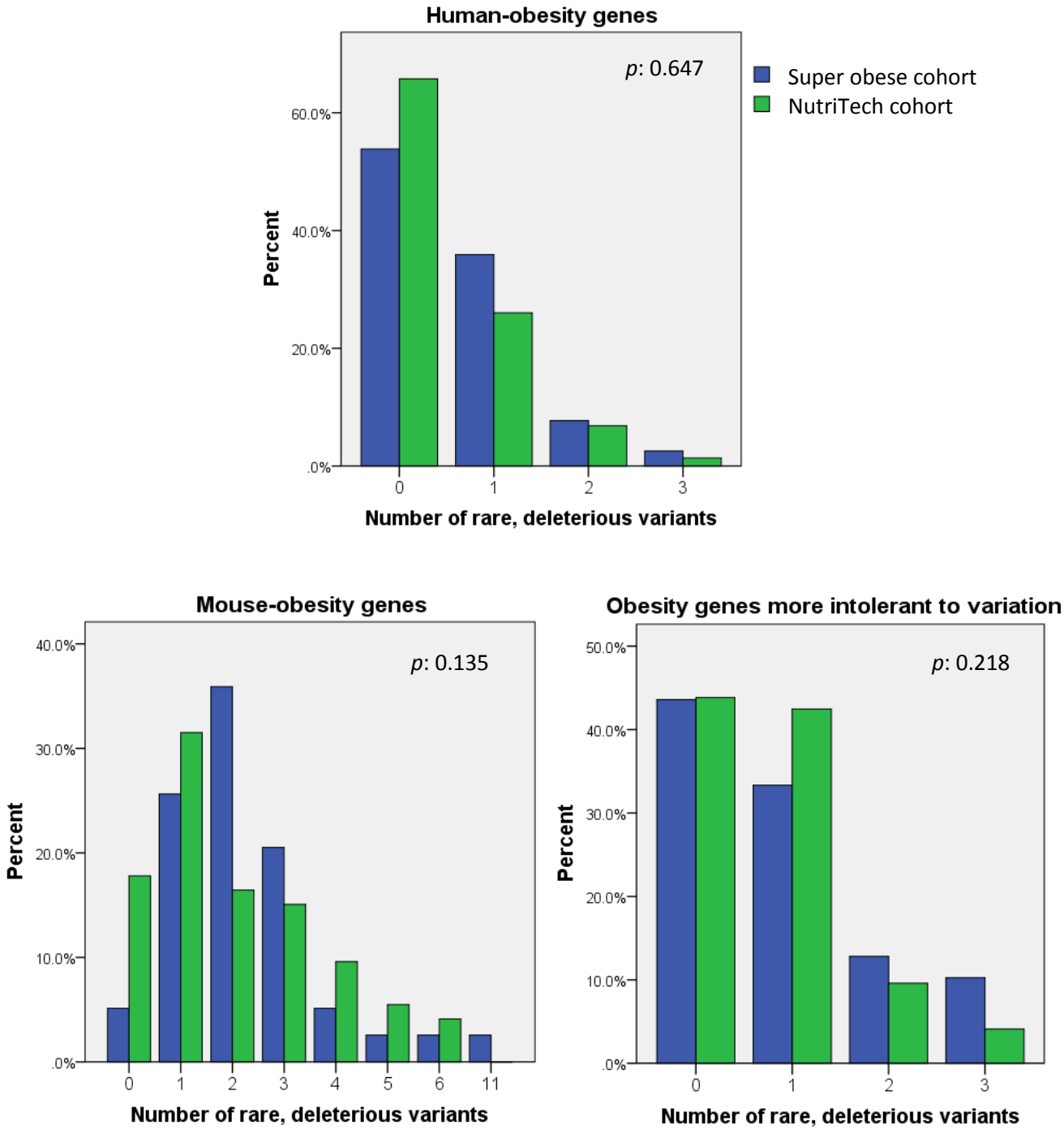


Figure 7.11: Rare, predicted-to-be deleterious variants before adjustment for mode of inheritance. Percentage of the number of individuals per cohort with 0-11 variants are given, for the human-obesity genes (top), mouse-obesity genes (bottom left) and genes more intolerant to variation (bottom right). There was no significant difference in frequencies between the two cohorts for any of the gene groups.

7.3.6 Candidate obesity-genes for further exploration

Although no human- or mouse-obesity genes were detected that carried significantly more rare and/or predicted-to-be deleterious variants in the super obese compared to the NutriTech cohort, a few variants were identified in genes that warrant further investigation:

A total of five genes, with low RVI-scores, were detected with nonsense, frameshift or homozygous/compound heterozygous predicted-to-be deleterious variants, only present in the super obese, while no deleterious variants were found in NutriTech cohort. Unsurprisingly two of these genes were known human-obesity genes namely, *LEPR* and *IGSF1*.

The other three genes were: *CORIN, MME* and *GRM8*.

CORIN

Two super obese individuals carried a highly likely to be pathogenic heterozygous variants in *CORIN,* c.971dupT (p.(Val324fs)) and c.2021G>A (p.(Trp674Ter)), while no predicted-to-be deleterious mutations were found in the NutriTech cohort. Both variants will lead to a premature truncation of the main transcript of the *CORIN* gene, and have not been reported before. Although small number of other nonsense and frameshift variants are listed in ExAC [208], none were enlisted in a homozygous state, confirming the low tolerance to functional variation in this gene.

CORIN encodes the 1,042 amino-acid protein, corin. This serine-type endopeptidase is a cardiac transmembrane serine protease that has been shown to process pro-ANP in vitro, with atrial natriuretic peptide (ANP) being a cardiac peptide that regulates blood pressure [293]. Although the known function of corin does not directly implicate its role in obesity, interactions with well-established adipogenesis-genes (*WNT* genes [294]) could indicate an (in)direct role of corin in obesity homeostasis (Figure 7.12). Indeed, Corin knock-out mice exhibited increased body weight when compared to wild-type mice, beginning at 15 weeks in both males and females, but the underlying mechanism is not clear [293]. Caution should be taken into interpreting the weight gain in these mice

knock-out models, since the weight gain could also be caused by an increase in extracellular fluid volume through sodium homeostasis.

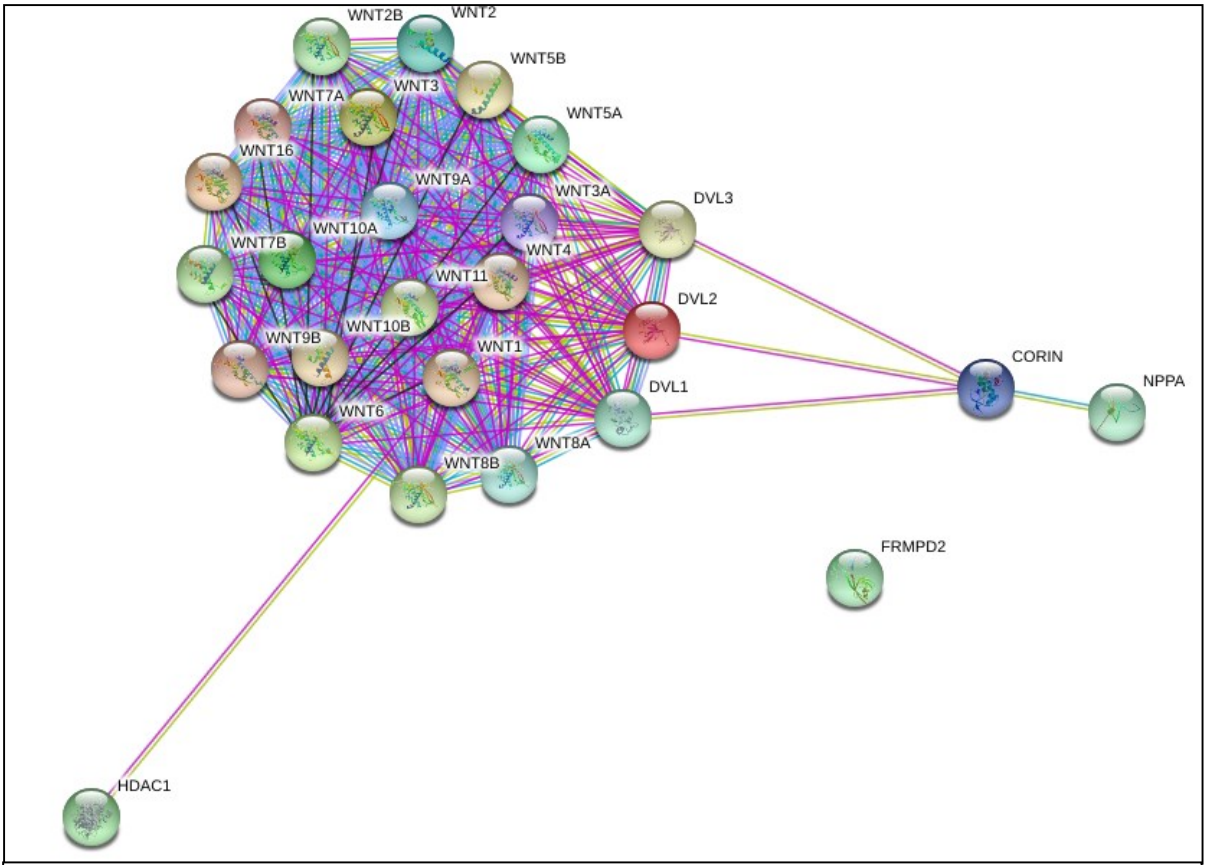


Figure 7.12: Interaction proteins for *CORIN* gene. *CORIN* has been shown to interact indirectly with multiple *WNT* genes. Source: Image generated using Retrieval of Interacting Genes (STRING) at http://string-db.org

MME

In our super obese cohort we found two novel variants in *MME* in two super obese individuals; one variant, c.1342T>C (p.(Arg448Ter)), and one predicted-to-be deleterious variant, c.674G>C (p.(Gly225Ala)). As with *CORIN*, several nonsense and frameshift variants are listed in ExAC, but none in homozygous state.

MME encodes for the 750 amino acid protein, neprilysin. Neprilysin processes several bio-active peptides, including several important orexigenic and anorexigenic compounds: NPY, galanin, and CNP [295-297]. Interestingly neprilysin is known for its role in catabolizing natriuretic peptides, including

NPA. Therefore, it could play a role in obesity homeostasis, not only through processing orexigenic and anorexigenic peptides directly, but could also play a role through the pathway described above.

Mme knockout mice have a late-onset excessive gain in body weight, through an accumulation of fat tissue, while pharmacological inhibition of neprilysin in wild-type mice increased body weight due to increased food intake [296].

GRM8

One super obese individual carried a variant, c.580C>T (p.(Arg194Ter)), in the *GRM8* gene. Again, as with the two genes described above, several nonsense and frameshift variants are listed in ExAC, but none in homozygous state.

GRM8 encodes metabotropic glutamate receptor 8, which is linked to the inhibition of the cyclic AMP cascade with high expression in human fetal and adult brains [298]. GRM8 has been reported to be involved in the regulation of neuropeptide Y and melanocortin pathways and might influence food intake and metabolism [299], and has been associated with several neurodevelopmental disorders such as Autism [300], Attention deficit hyperactivity disorder [301], and schizophrenia [302]. So far no reports have been published on the association with obesity.

Mice lacking *Grm8* exhibit an increased adiposity compared to their wild-type littermates. How this weight is caused is not clear, but it was not caused by an altered food intake [303].

7.4 Discussion

In this chapter I described how WES was used to detect the prevalence of Mendelian obesity in a super obese cohort. The frequency of putative forms of Mendelian obesity was higher in the super-obese than in overweight to mildly obese individuals from the NutriTech cohort. The results suggest that the proportion of severely-obese adult bariatric surgery patients with suspected Mendelian obesity may be non-trivial and has been underestimated in this group.

The number of overall predicted-to-be deleterious variants in (candidate) obesity genes was not different between the super obese and the NutriTech cohort. However, when only the genes known to be less tolerant of variation were included, a slight increase was seen in the number of predicted-to-be deleterious variants among the super obese. Although no novel human-obesity genes could be detected, three genes warrant further investigation were highlighted.

Mendelian Obesity

To my knowledge, this is the first time a super obese adult cohort has been screened for the combination of all known causes of Mendelian obesity. Although no WES studies looking for the prevalence of Mendelian obesity in adult bariatric patients have been published to date, previous reports indicate that about one in 20 severe childhood obesity cases are caused by disruptive mutations in the leptin-melanocortin pathway [84,304]. A frequency of 20.5% putative Mendelian obesity in a super obese cohort with self-reported childhood onset of obesity, and a history of unsuccessful weight loss through lifestyle adjustments, is therefore not unlikely. The lower frequency of 2.7% in the NutriTech cohort in comparison, indicates that it is not likely to be an accidental finding of 'normal variation'.

NTRK2 deletion:

Among the putative Mendelian causes of obesity, we report a novel CNV covering an entire exon of *NTRK2*, leading to a truncated protein and loss of the binding site of the receptor to BDNF. The TRKB receptor, which *NTRK2* encodes, and its agonist BDNF, play an important role in the control of food intake and body weight. Although a complete knockout of either gene in mice models leads to a lethal phenotype, heterozygous knock-out of *Bdnf* and partial knock-out of *Trkb* leads to hyperphagic and obese mice [305,306]. Heterozygous pathogenic point mutations in *NTRK2* have been reported in children with a complex obesity phenotype including severe hyperphagia and intellectual disability, in the form of impaired learning and memory and impaired nociception [60]. However, the participants described here had, apart from struggling with dyslexia as a child, a normal intellectual ability in adult life. This could indicate that, at least in adulthood, NTRK2 deficiency does not necessarily lead to severe intellectual disability and, therefore, could also be found in obese individuals without any other cognitive phenotypes.

16p11.2 deletion:

A second Mendelian form of obesity, a 16p11.2 deletion, was found in a severely obese female participant. That this rare deletion of over 593 kilobases at chromosome 16p11.2 causes obesity was first discovered by Walters, *et al.* in morbidly obese patients with cognitive deficits, but was subsequently found in obese individuals in the common population, without cognitive dysfunction [61]. Multiple studies since have reported on cases with 16p11.2 deletions and its related, but this is, to my knowledge, the first report of a 16p11.2 deletion carrier undergoing bariatric surgery.

Point mutations:

The multiple variants found in *SH2B1*, *LEPR*, *NTRK2*, *IGSF1, PTEN* and *SIM1*, have not been reported before, or were only reported with a rare frequency in open databases. The effect on function of the respective genes was therefore difficult to assess, especially since obese individuals are often included as 'healthy individuals' in the open databases such as NHLBI Esp, ExAC and 1000genomes. In the NHLBI

Esp database [209] for instance, 19 *MC4R* variants previously shown to be effect the receptors function by multiple *in vitro* studies can be found (9x p.Ala175Thr [150]; 2x p.Ile137Thr [150]; 2x p.Tyr35Ter [272]; p.Ser295Pro [151]; p.Ser127Leu [277]; p.Glu61Lys [151]; p.Leu54Pro [286]; p.Phe51Leu [151]; p.Cys326fs [307]), which indicates that individuals with Mendelian forms of obesity are included in this database. Variety in penetrance of disruptive variants, epistasis, gene-environment interactions should also be taken into account to possible affect the results presented here. Importantly, all sequencing analysis was performed in DNA extracted from whole blood samples, so the expression of mutations in relevant tissues could not be determined.

Interestingly, although, all individuals reported here suffered from a severe form of obesity, none showed any symptoms of the other typical features originally reported in the carriers of disease causing variants of the different genes. Specifically, no intellectual deficiency or behavioral problems were seen in the individuals described here. This indicates that, although these obesity-genes might initially be discovered in individuals with more complex phenotypes, functional variants might also be detected in individuals with non-syndromic “common” obesity.

In one severely obese individual, a previously-reported disease causing variant in *PTEN* (p.His93Arg) was found [94]. Disruptive variants in *PTEN* are also associated with in increased risk of autism and macrocephaly, and an increased risk of developing certain types of cancer, indicating the importance of genetic counseling [94,95,292]. Screening for *PTEN* variants should therefore be considered in patients with severe early onset obesity in combination with or macrocephaly, autism or cancer within a family member at young age.

Similarly, another individual carried a highly likely to be pathogenic nonsense mutation in *IGSF1*, which is associated with central hypothyroidism (with a variability of age of onset), and should be offered thyroid screening on a regular basis [96-98].

Mendelian obesity and bariatric surgery

The limited data presently available on weight-loss at 12 and 24 months following either RYGB and VSG for the individuals with Mendelian obesity in this thesis, indicates that bariatric surgery may be an effective treatment option for these patients, as has previously been shown in individuals with MC4R deficiency [171,172]. Ideally, to determine whether each different form of Mendelian obesity has similar outcomes, each form should be assessed separately. Interestingly the participant with a 16p11.2 deletion showed a successful long term weight loss after undergoing RYGB, while the individual with NTKR2 deficiency started regaining his weight 1.5 years after his surgery. Since both are the first cases of bariatric surgery reported in either 16p11.2 deletion carriers or NTRK2 deficiency, a larger number of cases for each form of Mendelian obesity and a longer follow-up period are warranted.

Variation in obesity genes

As reported before, predicted-to-be deleterious variants in obesity genes are not uncommon in general populations [210,308]. Therefore, it is not surprising that a similar number of predicted-to-be-deleterious variants were found in the super obese as well as in the NutriTech participants. It is, however, interesting that by only including the rare, predicted-to-be deleterious variants of known human-obesity genes, the number of variants was still not significantly different between the two groups. A significant difference only occurred after adjusting for mode of inheritance for the genes involved. This does not lend support to the hypothesis that multiple rare variants in known obesity genes, that individually are not pathogenic, could, in combination cause obesity. In contrast, the results presented here underline the importance of consideration of mode of inheritance in this type of analysis.

Interestingly once a selection was made among the obesity genes based on their tolerance of functional alterations, using the RVI-Score, a significant higher overall number of (common and rare) predicted-to-be deleterious variants was found in the super obese compared to the NutriTech

participants. This could indicate that more attention should be paid to genes less tolerant of variation: our data indicate that application of measures like the RVI-score should be considered while exploring causal variation of disease.

Although no novel human-obesity genes could be confirmed among the genes known to cause obesity in mice, three genes were identified that warrant further investigation: *CORIN*, *MME* and *GRM8.* These genes were selected based on the knowledge they cause obesity in mice once disrupted, their functional pathways could explain their role in obesity and because highly disruptive variants were found in the super obese cohort, while no predicted-to-be deleterious variants were found in the NutriTech participants.

With all variants reported here in *CORIN*, *MME* and *GRM8* being frameshift or nonsense mutations, they are very likely to disrupt the protein function. However, they were all in heterozygous state, while so far only the effect of a complete knock-out of these genes in mouse models has been described. It is therefore, difficult to estimate what the effect of heterozygous knock-out in mice as well as in humans will be, and if this will lead to obesity.

Therefore, screening for variants in these three genes in a larger cohort of obese and non-obese individuals is necessary to confirm that these genes really cause obesity once disrupted.

7.5 Conclusion

The higher than anticipated prevalence of putative Mendelian obesity in the super obese individuals, may indicate the necessity for an expansion of genetic services available for severely obese adults. This is particularly important because some variants carry additional risks for individuals involved and their relatives (e.g. autism in Chr16p11.2 deletion carriers [61], or increased risk for certain forms of cancer in *PTEN* mutation carriers [94,95]) and to optimise personalised support of an individual's life-

long management of their obesity. Bariatric surgery in the form of RYGB and VSG seems a valid treatment option for Mendelian obesity, although further studies with stratification of the different forms of Mendelian obesity (which would require a larger number of cases) and longer term follow-up is warranted. Finally, although the combination of several predicted-to-be deleterious variants in obesity (candidate) genes is unlikely to cause severe obesity, further studies should focus on the obesity (candidate) genes with more intolerance to functional variation for identification of novel Mendelian forms of obesity.

CHAPTER 8

DISCOVERY OF A NEW FORM OF MENDELIAN OBESITY AND DIABETES IN HUMANS

8.1 Introduction

This chapter describes exome and subsequent Sanger sequencing of a consanguineous family with the proband exhibiting a complex phenotype including obesity, reproductive difficulties, diabetes and intellectual impairment, revealing for the first time a case of homozygous carboxypeptidase-E deficiency in humans.

Although multiple genes are currently known to be associated with obesity, or to cause it directly when disrupted, only a small proportion of the heritability of obesity seen in twin studies can be explained by our genetic knowledge so far (< 10% vs. 72.6% as reported in a recently published meta-analysis of twin studies [6]). Although other factors may play a role in explaining this disproportion (such as epigenetics, or multiple gene/environment interactions), it is very likely that there is still a number of Mendelian forms of obesity that have yet not been discovered.

A family-based approach to discover novel Mendelian gene defects have been shown to be a fruitful approach so far. In these studies, complex families or multiple unrelated probands with a similar phenotype of complex obesity were screened, using either traditional approaches, such as linkage analysis, or a more advanced approach, such as NGS [34,89,91,102,106]. Although individually rare, as discussed in the previous chapter (Chapter 7, page 241) the overall contribution of these different Mendelian diseases combined might turn out to be more significant in the very obese population than originally thought. Thus, targeting the more complex familial cases is a fruitful avenue for the discovery of new forms of Mendelian obesity.

The importance of finding novel forms of genetic obesity is not only to improve our understanding of the physiology of obesity pathways, but also to improve the clinical approach of the disease. Finding the genetic cause of someone's obesity will be of immediate clinical use enabling genetic screening opportunities combined with genetic counselling.

Here, I have screened a consanguineous family using WES, aiming to find the genetic defect causing the complex obesity phenotype seen segregating in this family. The proband and family screened here, were originally referred to an adult genetic obesity clinic for investigation of her morbid obesity and intellectual disability, since the phenotype was thought to resemble that of Prader-Willi syndrome. Since standard genetic investigations failed to reveal the cause of her phenotype, the patient and her family member were recruited into this study.

8.2 Aims of this study

1) To identify the genetic defect causing the complex obesity phenotype within this family, using WES.

2) To investigate the implications of the genetic defect on phenotype beyond obesity.

8.3 Results

8.3.1 Participants' characteristics

The proband was a 20-year-old Sudanese female (Figure 8.1, 1B II.6) with intellectual disability (unable to read or to write words despite adequate educational opportunity). She had a history of early onset morbid obesity with hyperphagia, and on the day of recruitment her weight was 130.2kg, with a height of 1.59m, giving her a BMI of 51.5kg/m^2. She was diagnosed with type 2 diabetes mellitus on the day of recruitment (fasting glucose 21.1mmol/L; HbA1c 114 mmol/mol). She also had hypogonadotrophic hypogonadism (primary amenorrhea; serum estradiol 78pmol/L [which is within the post-menopausal range <100pmol/L], 21.2pg/mL; LH 2.7 IU/L, FSH 2.0 IU/L). Serum hormone analysis excluded other causes of amenorrhoea, including polycystic ovary syndrome and hyperprolactinemia (testosterone 1.2nmol/L (normal <2.7), 0.35ng/mL (<0.78); normal androstenedione, 17-hydroxyprogesterone, DHEAS, prolactin).

As part of clinical care potential genetic causes of obesity and intellectual disability, Prader-Willi syndrome and Fragile X syndrome, were excluded by DNA methylation testing at the *SNRPN* locus on chromosome 15q which showed the normal methylation pattern with both parental alleles present, and demonstration of a normal number of CGG repeats in 5' UTR of *FMR1* gene at Xq27.3. No abnormality was detected by clinical array comparative genomic hybridisation (Agilent 8x60K 60mer oligo, ISCA design 024612), excluding disease causing CNVs.

The complex phenotype seen in the proband appeared to have a recessive pattern in the family: There was a history of an older brother who died of unknown cause at the age of 21 years with a similar phenotype, including childhood-onset severe obesity, intellectual disability and hypogenitalism (Figure 8.1, II.3). While other siblings (II.2, II.4, II.1 and II.3) and both parents (I.1 and I.2) were, apart from being mildly obese, unaffected. The history of consanguinity in her family (parents were first

cousins), made the likelihood of a homozygous defect causing the phenotype more likely than a compound heterozygous defect. To increase the chances of finding the causal variant, the, DNA of the mother (Figure 8.1, I.2) and one unaffected sister (II.5) were also included for WES.

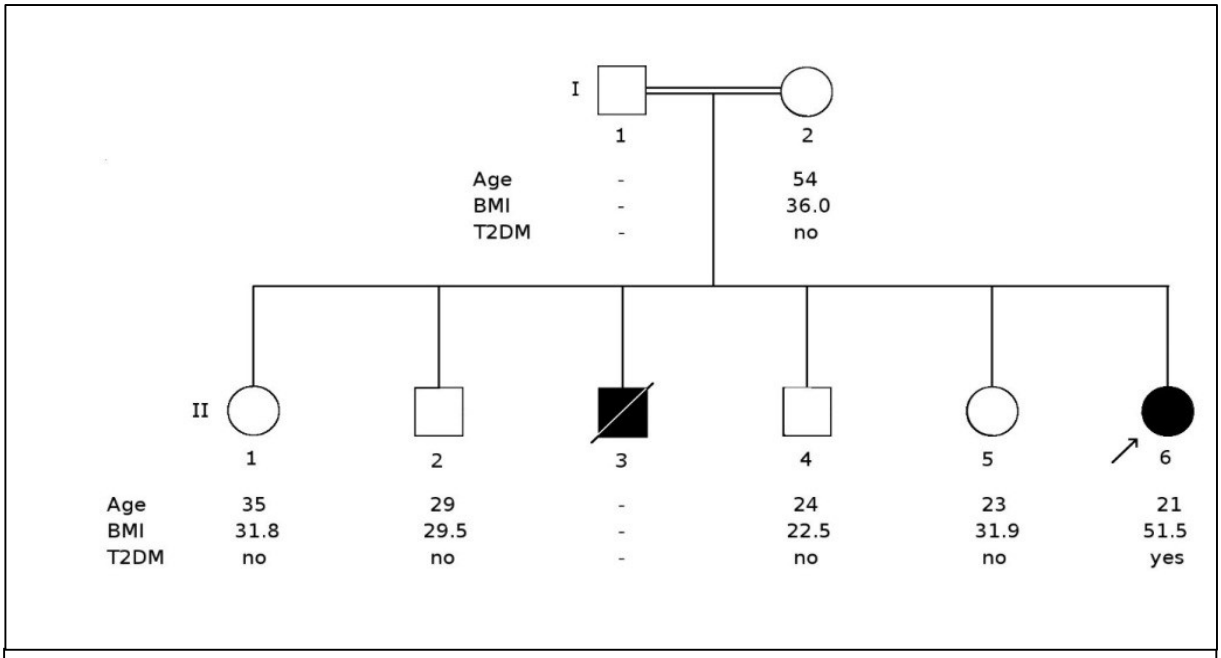


Figure 8.1: Pedigree of the affected family. Circles represent females and squares represent males. The proband is indicated by an arrow. Solid symbols represent the presence of the complete phenotype of childhood onset of obesity, hypogonadism and intellectual deficiency. Age for all individuals is given in years for the day of recruitment. BMI (body mass index) is given in kg/m^2. T2DM; Type 2 diabetes mellitus.

8.3.2 Whole exome sequencing results

The overall quality of the WES data was high: For all samples >99% of reads mapped to the reference sequence (proband: 99.95%, sister: 99.90% mother: 99.89%). An overall number of 44,120 variants were found in the family: 29,514 variants in the proband, and 30,342 and 39,439 variants in the mother and sister respectively. Table 8.1 gives an overview of the characteristics of the variants found in the proband. The high number of homozygous variants indicates that consanguinity most likely has taken place over several generations.

	Proband	
Type of variant	**Heterozygous**	**Homozygous**
Synonymous	7,432	4,086
Non-synonymous	6,362	3,508
Frameshift	100	47
Stop gain	52	11
Stop loss	7	4
Non-frameshift	182	89
Splicing	926	362
Non-coding	3,955	2,249
All variants	**19,016**	**10,356**

Table 8.1: Variant overview. Number of heterozygous and homozygous variants in the proband, mother and sister in different variant categories.

Variant selection

All variants found in the proband, mother and sister (Figure 8.1; II.6, I.2 and II.5) by WES and the predicted CNVs were screened for known obesity and/or intellectual disability causing variants. No variants were found that provided an explanation for the phenotypes. Neither were any novel, predicted-to-be deleterious variants found in any of the obesity listed in Appendix 2.3 (page 319).

In order to determine whether the phenotype in the proband could be caused by a variant in a previously-undiscovered 'obesity gene', a variant filtration strategy was designed, adjusted to the family history. Figure 8.2 gives an overview of the number of variants in the proband remaining after each step of the filtration strategy, while Table 8.2 gives an overview of the number of variants included or excluded during the family segregation step. Interestingly, as shown in Table 8.3, 205 variants that were found in a homozygous state in the proband, were not present in the mother, showing the limitations of coverage of WES.

A

Proband	Hom	Hom	Hom	Hom
Mother	Het	Het	np	np
Sister	Het	np	het	np
Total number	2,025	137	2	203

B

Proband	Hom	Hom	Hom	Hom	Hom
Mother	Hom	Het	np	Hom	Hom
Sister	Hom	Hom	Hom	np	np
Total number	5,086	2,624	0	7	2

Table 8.2: Family segregation analysis. Overview of the number of variants that were included (A) or excluded (B) of the total number of homozygous variants found in the proband during the family segregation step of the variant filtration strategy summarised in Figure 8.3. Hom, homozygous; Het, heterozygous; np, not present.

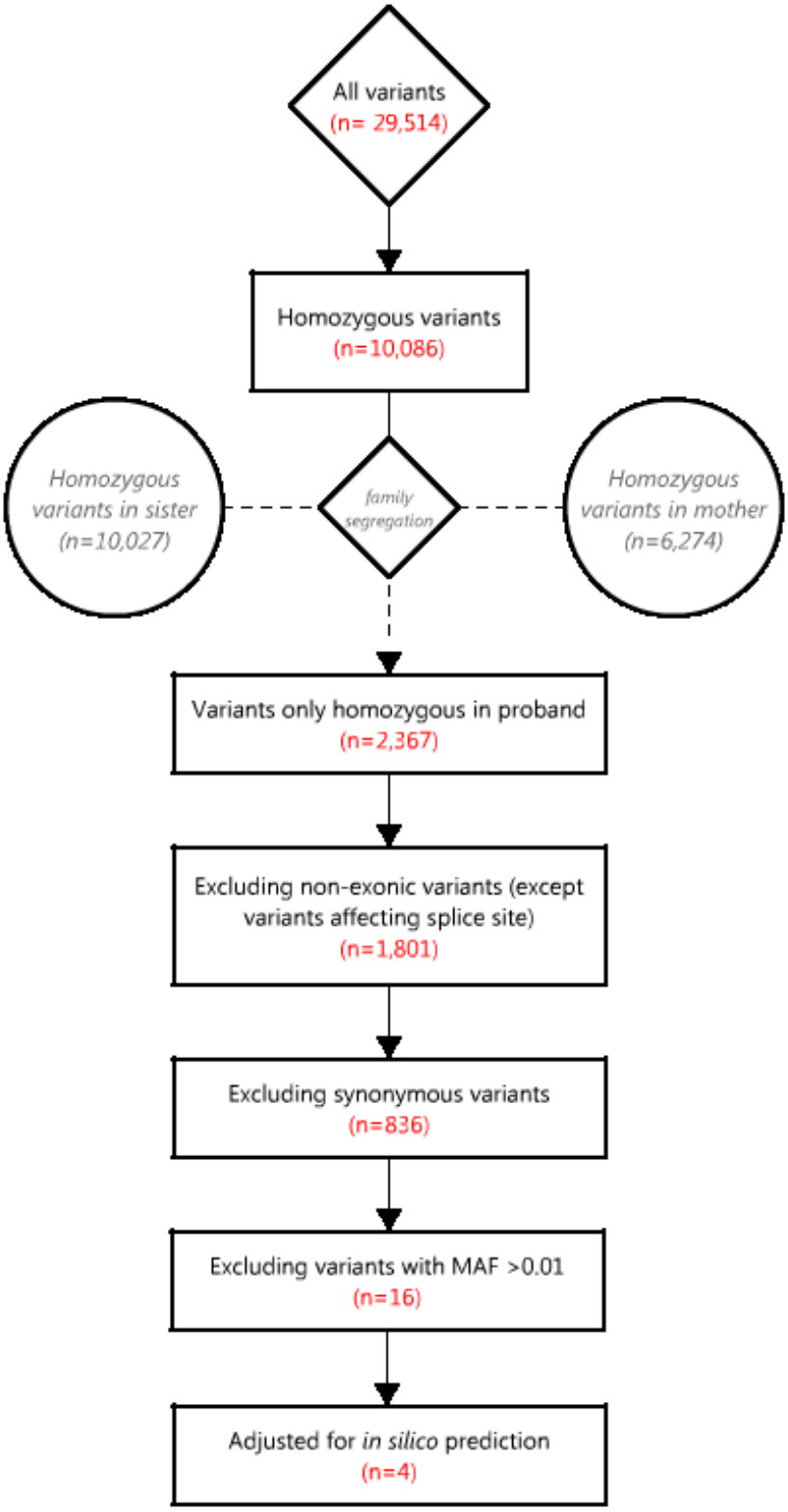


Figure 8.2: Variant filtration strategy. Overview of the number of variants in the proband remaining after each filtration step. Only variants with a sequencing depth of at least 4 were included.

8.3.3 Variant interpretation

Four homozygous, rare and predicted-to-be-deleterious variants remained after applying the filtration strategy and were found in a homozygous state in the proband, and were in a heterozygous state or absent in the mother and sister (Table 8.3). The remaining variants were further analysed by interpreting the function of the gene the mutation occurred in, the effect of the specific variants found on the gene function, and known variation in the gene through literature research and open databases.

CPE; c.76_98del (p.(Glu26ArgfsX68)):

Only one of the four variants that remained after applying the filtration strategy was within a candidate gene for obesity: a 23bp frameshift deletion in the *CPE* gene. *CPE* encodes the enzyme carboxypeptidase E, which is involved in the processing of neuropeptides and peptide hormones active in appetite and glucose metabolism pathways. The frameshift deletion found here, c.76_98del, in exon 1 of the *CPE* gene, results in a p.Glu26ArgfsX68 truncation of the protein. An exact 7 nucleotide repeat (GGGCGCC) at the breakpoints, might indicate a microhomology-mediated deletion mechanism (Figure 8.3) [309]. The start of the frameshift within the pre-protein region and the rapid truncation of the protein at amino acid location 68 (compared to the full sizes protein of 476 amino acids), indicates it will most likely lead to a non-functional or entirely absent protein.

CPE is a highly conserved gene (Table 8.4) and is widely expressed in human tissues, with high levels seen in the hypothalamus, pituitary gland and pancreatic islets. This is in line with the hormone/peptide-processing function of CPE in endocrine tissues and the central nervous system. No *CPE* null mutations have been reported in humans so far, nor has the p.Glu26ArgfsX68 variant been reported in publicly-available datasets from the 1000 Genomes project and the NHLBI Exome Sequencing Project. The deletion, however, is reported in two Caucasians in heterozygous state in the ExAC dataset [208]. No phenotypes are available for these two unrelated individuals.

Gene symbol	Variant	Exonic function	*in silico* prediction			1000 genome/ ESP650	SNP 138	OMIM
			PolyPhen-2	SIFT	PROVEAN			
CPE	p.Glu26Argfs X68	Frameshift deletion	-	-	-	np	np	Less active protein leads to pre-disposition of early onset of T2DM
MYL1	p.Met1fs	Frameshift insertion	-	-	-	np	np	-
XDH	p.Leu287Val	Missense mutation	Damaging	Deleterious	Neutral	0.0002	rs138674014	Xanthinuria Type 1
PABPC4L	p.Arg263Thr	Missense mutation	-	-	-	np	np	-

Table 8.3: Homozygous variants identified in the proband. Details of the homozygous, rare and predicted-to-be deleterious variants found in the proband, which were either absent or in heterozygous state in the mother and sister (II.5), and remained after the variant selection. np, not present in database; *CPE*, Carboxypeptidase E; *MYL1*, myosin, light chain 1; *XDH*, xanthine dehydrogenase; *PABPC4L*, poly(A) binding protein, cytoplasmic 4-like.

Genome	Assembly	Chr	CPE Peptide		CPE Exon 1
			AA length	% identity	AA sequence (GRch37/hg19, chr4:166,300,434-166,300,503)
Proband		4	86	18	…WLLGA=======RRGGHEAAPA…
Homo sapiens (human)	hg19/GrCh37	4	476	100	…WLLGAEAQEPGAPAAGMRRRRRL…
Pan troglodytes (chimpanzee)	panTro2	4	476	99	…WLLGAEAQEPGAPAAGMRRRRRL…
Gorilla gorilla (gorilla)	gorGor1	4	476	99	…WLLGAEAQEPGAPAAGMRRRRRL…
Macaca mulatta (Rhesus Macaque)	rheMac2	5	476	99	…WLLGAEAQEPGAPAAGMRRRRRL…
Mus musculus (mouse)	mm9	8	476	97	…WLLTAEAQEPGAPAAGMRRRRRL…
Monodeplhins demostica (opossum)	monDom5	5	475	92	…WLLGAAAG==AAGMRRRRRL…
Gallus gallus (chicken)	galGal3	4	426	92	…======================…
Xenopus tropicalis (frog)	xenTro2	?	475	83	…==================RRLS…

Table 8.4: *CPE* gene conservation. Overview of the conservation of *CPE* across different species. In the top row the *CPE* sequence is given as found in the proband. AA (amino acid) length and % identity are given for the complete *CPE* gene. AA sequence is given in the last column surrounding the area the p.Glu26ArgfsX68 deletion was found in (underlined is the AA where the frameshift caused by this deletion would start).

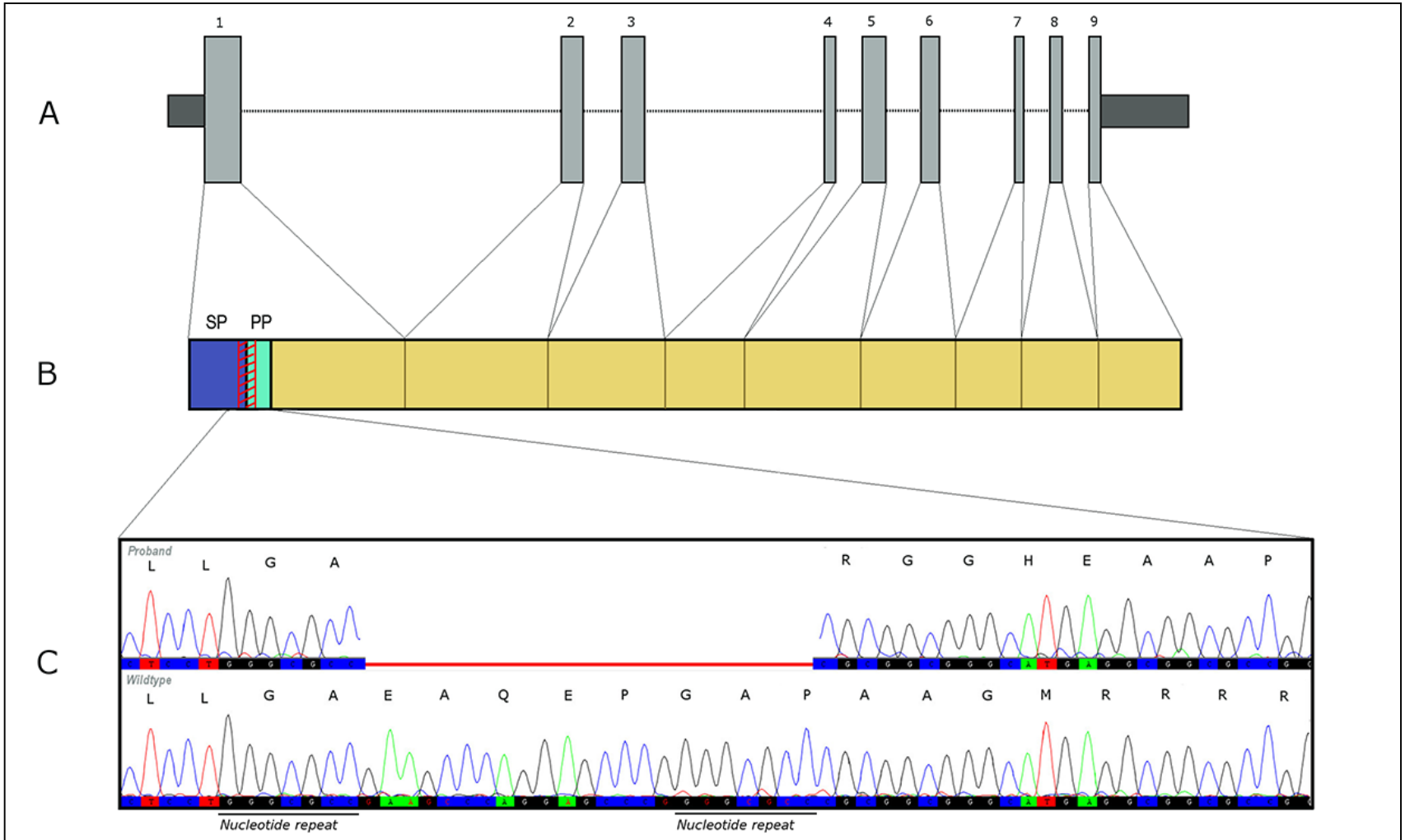


Figure 8.3: Location of c c.76_98del (p.Glu26ArgfsX68) *CPE* variant. A: Schematic overview of the exons of *CPE* (Refseq: NM_001873). Dark shaded areas are UTRs and light grey areas are coding regions. **B**: Human CPE protein (UniprotKB: P16870). Location of the Glu26ArgfsX68 variant is shown by the red diagonally striped region. SP, signalling peptide; PP, pro-peptide. **C**: Indicative chromatogram of the deletion in the proband and the normal wild-type sequence. The deletion is indicated in red. Amino acid changes caused by the frameshift are shown above the chromatogram. (Figure is as published in Alsters S., *et al*. PLOS One. 2015;10(6):e0131417

Besides the homozygous frameshift mutation in *CPE*, three other rare homozygous, predicted-to-be-pathogenic variants were found in the proband, but not or only in heterozygous state in the mother or sister (Table 8.4). However, all are less likely to contribute to the phenotype of obesity, T2DM, hypogonadotrophic hypogonadism or intellectual disability seen in the proband.

XDH: c.859C>G (p.(Leu287Val)):

Homozygous disrupting variants in *XDH,* encoding for Xanthine dehydrogenase, are known to cause Xanthinuria type I (OMIM #278300). Xanthinuria type 1 is characterised by the formation of xanthine renal stones, leading to the possibility of renal failure. Around 150 cases have been reported so far, and none have been associated with obesity or any of the other phenotypes reported in the proband [310]. The proband had no history of kidney stones or renal failure and the variant found (rs138674014) has so far not been linked to Xanthinuria type 1. The variant p.Leu287Val was predicted-to-be deleterious by both SIFT and Polyphen Hvar, but definite pathogenicity cannot be shown. rs138674014 is reported in both the NHLBI Esp and ExAC databases, with a MAF of 0.002 in the south Asian population. However, all instances were in heterozygous state.

PABPC4L: c.788G>C (p.(Arg263Thr)):

Not much is known about the function of *PABPC4L*, besides that it is expressed in the brain and multiple other tissues. A recent study on rare CNVs found an association, although not at genome-wide significance levels, between a deletion covering PABPC4L and treatment resistant depression [311]. The proband, however, does not have a history of depression. The variant p.Arg236Thr has not been reported before in the literature or in any of the open databases, nor are there any homozygous nonsense of frameshift mutations listed.

MYL1: c.1dupA (p.(Met1fs)):

MYL1 encodes a myosin alkali light chain active in embryonic, foetal and adult fast-twitch skeletal muscle [312]. The variant found in the proband, a deletion of the first nucleotide of the coding region of *MYL1*, might appear to cause a frameshift starting from the first amino acid sequence, but the

repeat of 10 similar nucleotides preceding the deletion in the non-coding region makes it less likely that an actual frameshift will occur (Figure 8.4). Examination of this specific nucleotide repeat, preceding the coding region of MYL1, in the ExAC dataset, shows that such a variation in this region is not particularly rare (minor allele frequency up to 0.03041 across different populations), and is in multiple individuals reported in homozygous state.

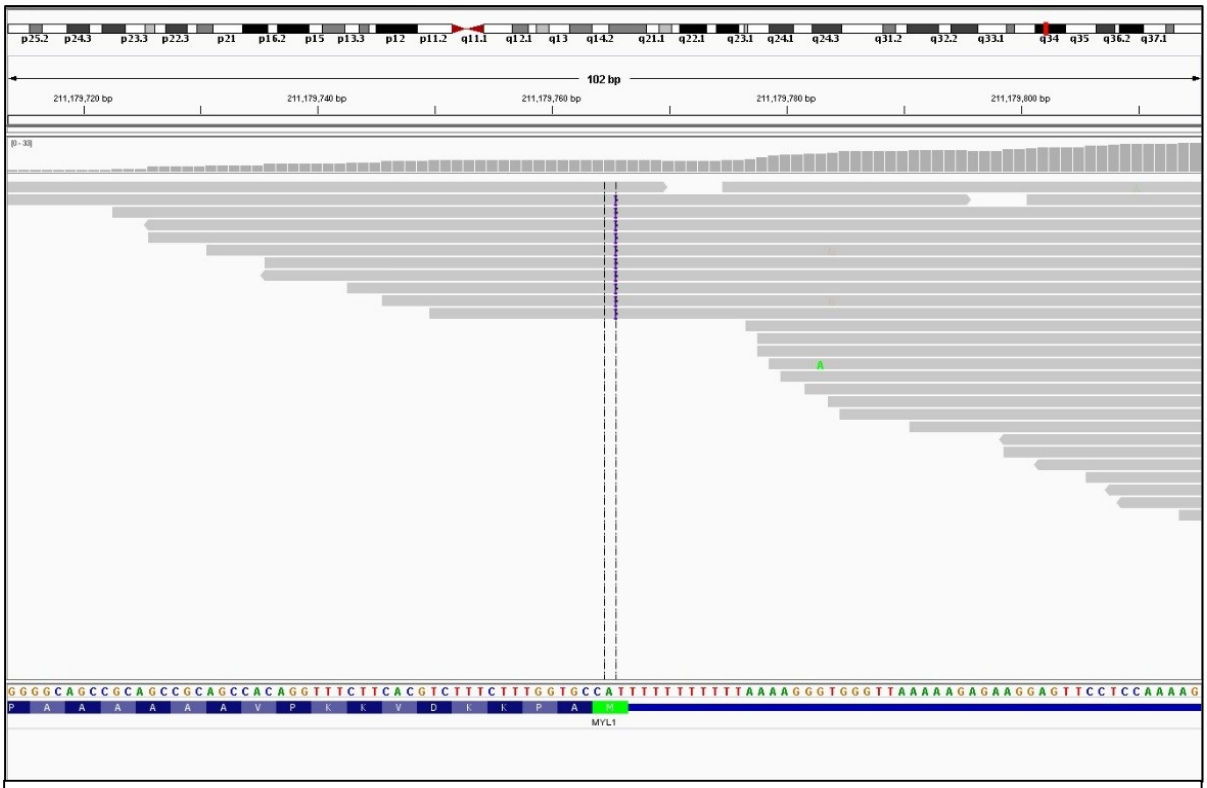
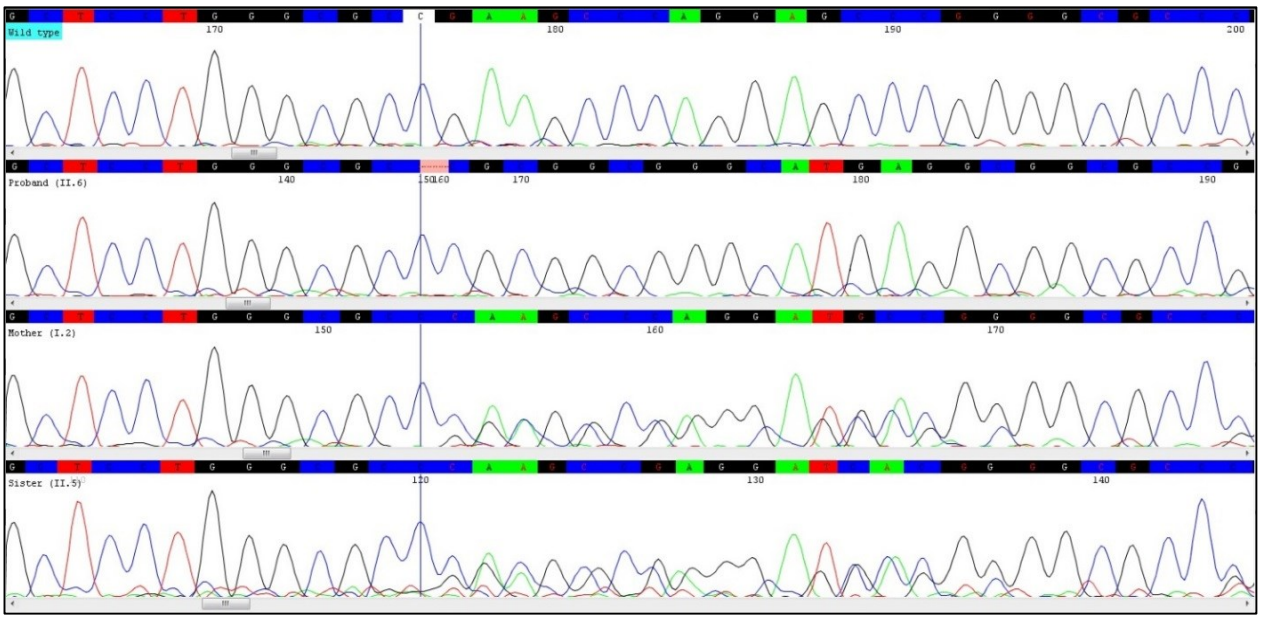


Figure 8.4: p.Met1fs variant in *MYL1*. IGV screenshot of the area surrounding the start codon of the coding region of *MYL1* (antisense strand). A deca-thymine nucleotide repeat can be seen preceding the start codon.

8.3.4 Variant confirmation and family segregation

Sanger sequencing was used to confirm the most likely variant to cause the phenotype in the proband, namely the deletion in CPE. Homozygosity for the p.Glu26ArgfsX68 variant was confirmed in the proband (Figure 8.1; II.6) and heterozygosity in her mother (I.2) and sister (II.5): Sequencing results were aligned to a wild type reference sequence and showed the typical frameshift caused by a deletion (Figure 8.5A). Segregation analysis of the variant in the other family members, for whom DNA was available, increased the likelihood of a homozygous knockout of this gene causing the proband's phenotype: None of the unaffected siblings carried the deletion in a homozygous state. In the two brothers (II.2 and II.4) the deletion was found in a heterozygous state, while the oldest sister (II.1) did not carry the deletion (Figure 8.5B). Since no DNA was available for the diseased brother with the same phenotype as the proband, or for the unaffected father, they could not be tested for carrier status. Family segregation details are summarised in Figure 8.6.

A



B

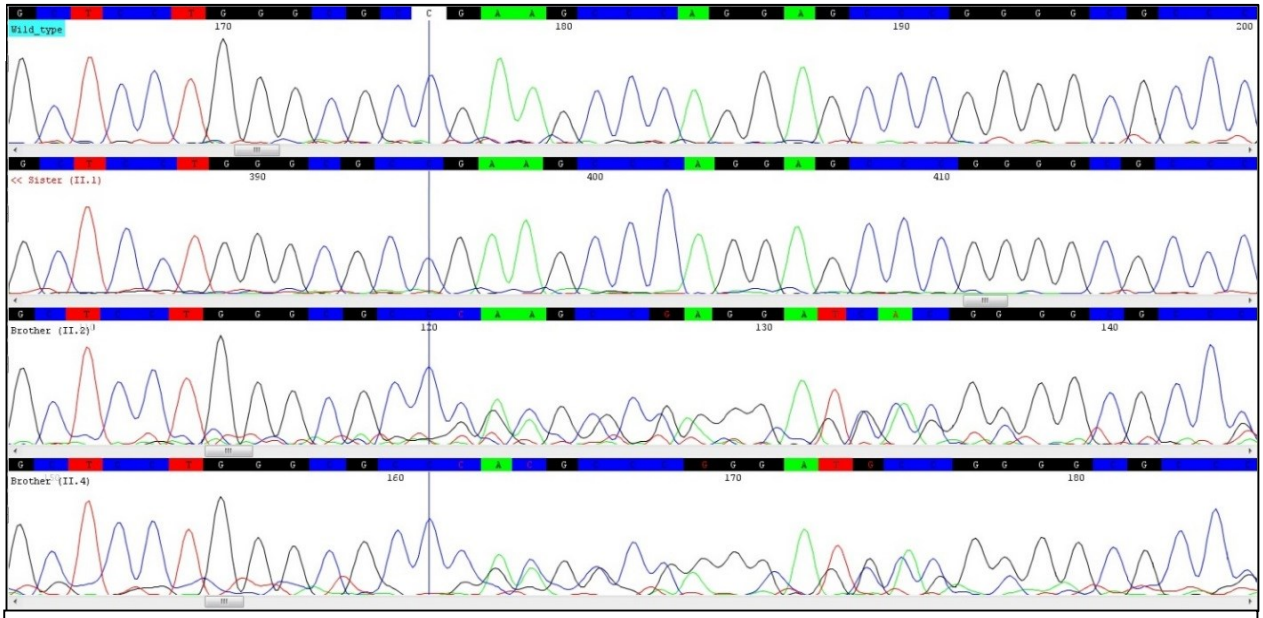


Figure 8.5: Chromatogram of the p.Glu26ArgfsX68 region. A) The p.Glu26ArgfsX68 variant found through WES was confirmed using Sanger sequencing in the proband (Fig 6.7, II.6), mother (I.2) and sister (II.5). B) Further family segregation showed the heterozygous state of the variant in the two brothers (II.2 and II.4) and a non-carrier status in the oldest sisters (II.1).

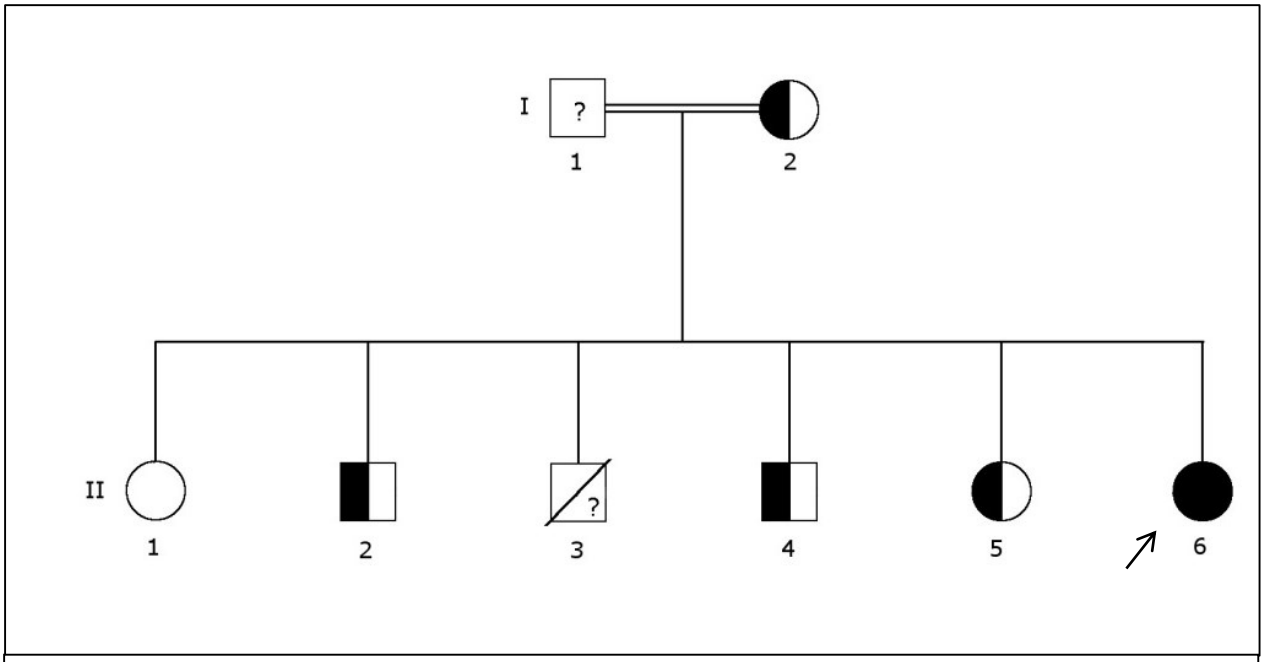


Figure 8.6: Family segregation of the p.Glu26ArgfsX68 variant. Circles represent females and squares represent males. The proband is indicated by an arrow. Solid symbols indicate homozygosity for p.Glu26ArgfsX68, while half solid symbols indicate heterozygosity and open symbols non-carriers. The question mark in I.1 and II.3 indicate that genotype is not known.

8.3.5 CPE mRNA expression analysis

Since the variant found in *CPE* causes a frameshift and premature truncation of the protein, it is likely to be deleterious and to be silenced by nonsense-mediated decay. To confirm this, mRNA analysis was performed using real time PCR on the proband (Figure 8.6, II.6), a heterozygous sister (II.5) and 6 matched female controls.

The six controls had an age range of 32-59 years, their BMI range was 47.8-53.3 kg/m^2, and three were diagnosed with T2DM, while the other three did not have T2DM. The overall mean coefficient of variation (CV) for Ct (threshold cycle) values of replicate samples (for which amplification products were obtained) was 2% for *CPE* and 1% for *HPRT1*, with a mean CT of 34.43 (SD = 1.21) for the *CPE*

assay and a mean Ct of 29.15 (SD = 0.11) for the *HPRT1* assay. Ct values for the controls, proband, sister and reference samples obtained for the *CPE* and *HPRT* assays and ΔΔCt values for all test samples are listed in Table 8.6.

No *CPE* expression was detected in blood RNA from the proband after 40 cycles of amplification, while low but detectable levels were present in the sister and six control samples and the reference sample. The value for normalised *CPE* expression in the heterozygous sibling was at the lower end of the range seen in the controls (Figure 8.7). Expression of the housekeeping gene *HPRT1* was detected in the proband, sister and all control samples, demonstrating that lack of detectable *CPE* expression in the proband was not due to insufficient or poor quality cDNA template.

Sample	**Ct for *HPRT* product**	**Ct for *CPE* product**	**ΔΔCt**
Ctrl 1	27.52	35.28	2.12
Ctrl 2	28.98	34.48	-0.14
Ctrl 3	29.80	33.61	-1.83
Ctrl 4	30.83	36.31	-0.16
Ctrl 5	28.67	34.02	-0.29
Ctrl 6	29.02	35.54	0.88
Proband	30.17	No amplification	N/A
Sibling	28.66	34.81	0.51
Reference	29.00	34.64	N/A

Table 8.6: Threshold cycle and ΔΔCt values for the *CPE* and *HPRT* assays. Ct, threshold cycle; higher values indicate lower transcript levels. Mean Ct values of triplicates are given, except for Ctrl 1, in which the Ct of duplicates is given (one failed to amplify).

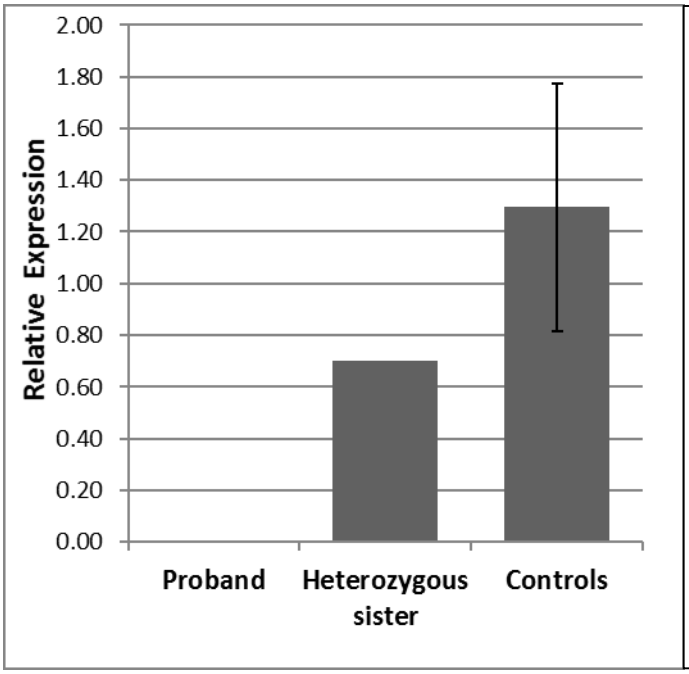


Figure 8.7: CPE mRNA expression levels. Real time PCR analysis of *CPE* mRNA expression in blood samples from the proband (II.6), heterozygous sibling (II.5) and six controls. For controls mean ± SEM (standard error of the mean) is depicted. All analyses were conducted in triplicate. (Figure is as published in Alsters S., *et al*. Plos One. 2015;10(6):e0131417)

8.4 Discussion

In this chapter I describe the discovery of a novel Mendelian form of obesity and diabetes; a homozygous frameshift mutation in *CPE* leading to a lack of its expression. The phenotype of the proband included severe, early onset obesity, hyperphagia, intellectual disability, T2DM and hypogonadotrophic hypogonadism.

Carboxypeptidase E (CPE) is an enzyme involved in the processing of majority wide range of neuropeptides and peptide hormones, removing C-terminal basic residues following initial cleavage by an endopeptidase, and therefore is active in several physiological pathways [313]. Although many studies been directed at determination of how Cpe deficiency affects mouse models, no homozygous pathogenic *CPE* mutations have ever been reported in humans previously. Several researchers have screened human populations for such variants. Utsunomiya, *et al.*, screened 269 Japanese subjects with T2DM, but did not find any variants affecting the coding region. [314] Chen, *et al.*, described a heterozygous missense mutation (p.Arg283Trp) resulting in a less active enzyme, which was reported to affect age of onset of T2DM in individuals already susceptible for T2DM, in specific Ashkenazi families [315]. There are no previous reports of any homozygous pathogenic variants in the *CPE* gene.

That CPE deficiency should result in monogenic obesity is not unexpected: pathogenic variants in the *PCSK1* gene leading to a deficiency of proprotein convertase 1/3 (PC1/3), a protein pre-processing the same peptides as CPE, are known to cause Mendelian obesity [51]. Also pathogenic variants in POMC, which is directly processed by PC1/3 and CPE, lead to Mendelian obesity [51,52]. Patients with reduced PC1/3 activity also develop hypogonadotrophic hypogonadism, similar to the phenotypes seen in the proband investigated here and her deceased brother. [52].

Much of our understanding of CPE function comes from two mouse models: *fat/fat* mice (with a naturally-occurring point mutation (Ser202Pro) inactivating *Cpe)* and *Cpe* knockout mice, generated

by deletion of exons 4 and 5 from the *Cpe* gene [29,316]. *fat/fat* mice and *Cpe* knockout mice have similar phenotypes, including slowly developing, adult-onset obesity with hyperproinsulinaemia, infertility, anxiety, depression, memory deficits and neurodegeneration of hippocampal neurons [29].

The obesity seen in these mouse models is due to an imbalance of the orexigenic and anorexigenic peptides. Since a large number of the appetite-regulatory peptides are directly processed by CPE, the mature active levels of anorexigenic peptides are significantly lower in the *fat/fat* mice and knockout mice than seen in wildtype littermates (including α-melanocyte-stimulating hormone, Cocaine- and Amphetamine-Regulated Transcript, prothyrotropin releasing hormone, oxytocin and neurotensin [316-318]). Although some of the orexigenic peptides are processed by CPE as well (such as neuropeptide-Y), a number of orexigenic peptides are not processed by CPE, which lead to normal levels of these peptides in CPE-deficient mice, leading to the ultimate phenotype of obesity and hyperphagia [30,317,319]. Interestingly, when *fat/fat* mice are limited to amount of food comparable to that consumed by wild type mice, the *fat/fat* mice still gain significantly more weight (although less then when given access to unlimited food), indicating the weight gain is due to overeating as well as an imbalance in the energy homeostasis [30].

The similar phenotype of obesity and hyperphagia seen in the mice models lacking active Cpe, and the proband and her diseased brother reported here provides strong support to the hypothesis that the homozygous null mutation in the *CPE* gene is a plausible explanation for the complex phenotype of the proband.

Besides being obese, both mouse models also show slowly increasing glucose concentrations leading to hyperglycaemia. This is considered to be most likely to reflect obesity-associated insulin resistance, but a lack of fully processed insulin and insulinotropic GLP-1possibly might also play a role [29,316]. A similar form of T2DM seen in the proband, could indicate that indeed hyperglycaemia is another symptom seen in CPE deficient humans (although the hyperproinsulinaemia seen in the patients with PC1/3 deficiency leads to hypoglycaemia).

The proband presented here exhibits hypogonadotrophic hypogonadism and intellectual disability, which may be diagnostic features of CPE deficiency and so genetic investigation of CPE is warranted in similar cases where other known genetic causes have been excluded, especially with co-existent obesity. The chances of finding similar cases might be low, however, as we might expect complete CPE deficiency to be rare. This is confirmed by the rare occurrence of heterozygous predicted-to-be deleterious variants and the absence of homozygous frameshift or stop-codon generating variants in publicly available datasets (the 1000 Genomes project, the NHLBI Esp and ExAC). However, the finding of two Caucasians in the ExAC database heterozygous carrying exactly the same deletion as found in the Sudanese family described here, could point towards the existence of hotspot for breakpoints leading to this variant. The finding that for this the deletion the breakpoints are aligned with a nucleotide repeat (pointing towards a microhomology-mediated deletion mechanism, Fig. 7.4) could indicate that this deletion may not be a unique occurrence. The high denaturation temperature that was needed to amplify the region for PCR, due to high local CG content, could explain why this variant has not been seen before in large scale next generation sequencing cohorts. This is supported by the relatively low average coverage of this region in the open databases available (Fig. 7.9). It is, therefore, important to screen larger groups of morbidly obese individuals to find the real prevalence of this variant and predict the effect of a heterozygous knock out of the CPE gene.

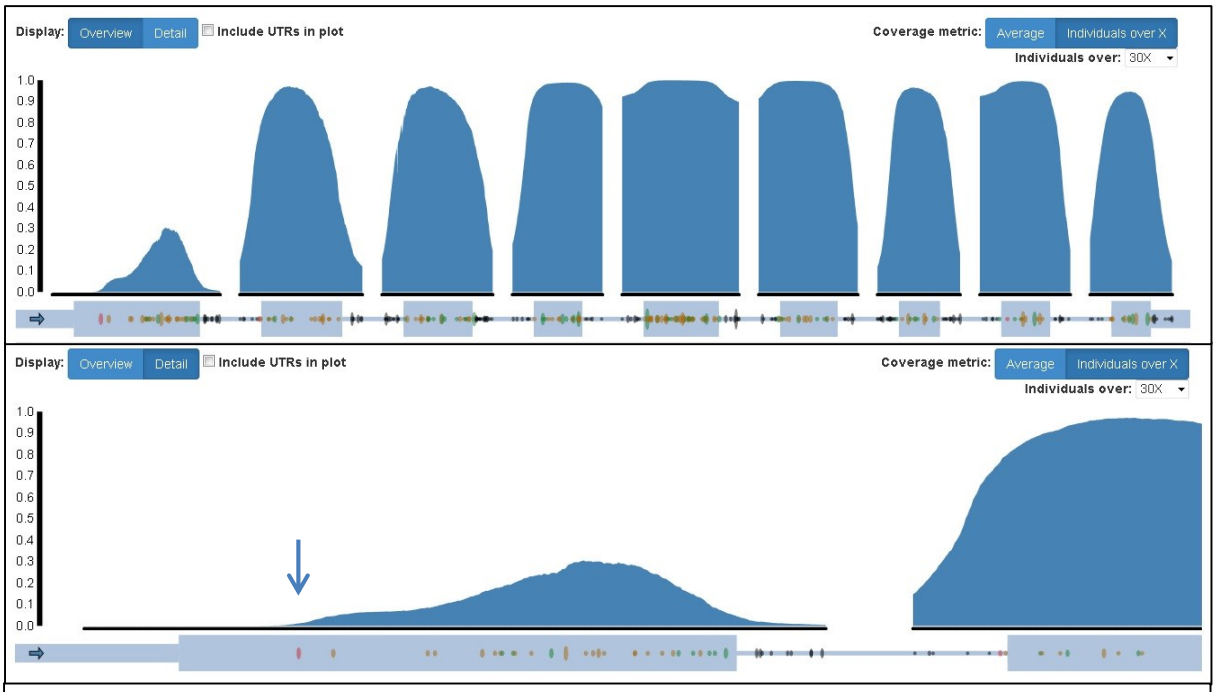


Figure 8.9: Sequencing depth of *CPE* in Exac. An overview of the percentage of individuals with a minimum sequencing depth of 30x for A) the complete *CPE* gene and B) exon 1 of *CPE*. The arrow indicates the location of the p.Glu26ArgfsX68 mutation. Data is as downloaded from the Exome Aggregation Consortium (ExAC), Cambridge, MA (URL: http://exac.broadinstitute.org) [Nov 2015].

Ongoing research, in collaboration with Dr T.A. Goldstone, will involve peptide analysis. In both mouse models lacking Cpe and patients with PC1/3 deficiency it has been shown that for numerous neuro- and hormone-peptides the processing is affected, leading to abnormal levels of the mature peptides. Further analysis will need to confirm whether the same is true for the proband lacking CPE and, if so, which peptides are affected. It is expected that the levels of similar peptides will be increased or decreased as seen in the mouse models, but since the physiology of humans is different from mice, difference might be seen. Although not expected, the chance of finding no peptides affected at all is there as well. This could indicate the function of CPE is not affected in the proband after all, which is very unlikely since no expression of mRNA of the *CPE* gene could be found in the proband. A better explanation, in that case, would be that other enzymes can partially compensate for the lack of CPE. It has already been shown in *fat/fat* mice that the low levels of (instead of a complete lack of) certain mature peptides normally processed by CPE, can be explained by carboxypeptidase D (CPD) processing

[320]. It is therefore important to investigate whether CPD is upregulated in the proband described here.

Assessment of circulating levels of hormones in heterozygote family members might also shed some light on the effect of carrying a heterozygote knockout mutation of the *CPE* gene. Although a heterozygous putatively-pathogenic mutation has been described in humans before, no analysis of peptide levels and phenotype has been reported. Although different studies on peptide levels in heterozygous *Cpe* knockout mice give contradictory results [108,319], mice heterozygous for the Ser202Pro mutation (*fat/+* mice) appear to not show any phenotypic differences from wild-type littermates [319]. Further analysis in humans will indicate if this is also true for heterozygous CPE knockout in humans.

Cpe was identified as the causative gene in the *fat/fat* mouse two decades ago, around the same time leptin was identified as the missing hormone in the *ob/ob* mouse, and mutations in its receptor *Lepr* caused the *db/db* mouse phenotype [29]. However, unlike the subsequent identification of human mutations in *LEP* and *LEPR*, a causative mutation in *CPE* has to our knowledge not been described in humans. This case of *CPE* knockout in humans now confirms that a similar phenotype is seen in humans as in the *fat/fat* and *Cpe* knockout mice. This identifies that indeed CPE is a key player in body weight regulation and glucose haemostasis in humans. This is only the third example in which congenital deficiency of a pro-hormone/peptide processing enzyme has been shown to cause human disease, in addition to PC1/3 in human obesity, and PCSK9 in autosomal dominant hypercholesterolemia [321]. Ongoing detailed phenotyping of the homozygote proband and heterozygote family members, including assessment of circulating levels of hormones regulating glycaemia and appetite regulation, will further clarify the role of the CPE pro-hormone/peptide processing enzyme in human physiology.

8.5 Conclusion

In this chapter I described how WES was used to detect a novel Mendelian form of obesity and T2DM, caused by a homozygous frameshift mutation in *CPE* (p.Glu26ArgfsX68). The deletion was found in a proband from a consanguineous family, presenting with severe early onset obesity, hyperphagia, T2DM, hypogonadism and intellectual disability. A similar phenotype was seen in the *Cpe* knock-out mice, as well as in the obese *fat* mouse model (which have been shown to carry a natural occurring functional mutation in *Cpe*). These data add to the growing list of monogenic obesity genes in humans, which will help provide diagnostic and therapeutic opportunities for this group of patients.

CHAPTER 9

CONCLUSIONS AND FUTURE WORK

9.1 Conclusions

Genetic research in obesity has seen major advances throughout the last decades, with the discovery of important appetite-regulatory pathways through obese murine models, to the discovery of genetic defects causing obesity in humans, to genome-wide association studies. The genetic research area has profited for several years from the exciting era of high throughput sequencing, by which the whole genome (or exome) can be analysed using only one assay, and this has already produced several important findings for monogenic obesity [89-92,322]. The work presented in this thesis contributes towards this area of research in several ways:

As a part of this research project, a cohort was created of over a thousand severely-obese individuals pursuing bariatric surgery: I managed the recruitment of this cohort for two years and have personally recruited and taken clinical histories from over 800 patients. The creation of the PMMO cohort not only enabled the genetic analysis performed in this thesis, but is also a basis for many other projects to follow (see future work, page 275). The deep phenotyping of the individuals included provides an excellent foundation to examine phenotypes beyond obesity for genetic studies, but has also provided a good cohort for analysing features associated with extreme forms of obesity in itself.

Although the most severely obese individuals in the PMMO cohort did show an increase in weight-related limitations, such as reduced mobility and OSAP, other obesity-related morbidities were not more prevalent among the most severely obese PMMO participants. Indeed, the more severely obese within the PMMO cohort were actually metabolically healthier than the less severely obese participants. If this is a feature that holds up in larger general population cohorts (and not just an effect of selection bias in bariatric referrals) needs to be further investigated: the metabolically healthy super obese individuals form a very interesting subgroup for further analysis. Surprisingly, the super obese did not show greater impairment in their quality of life, nor were any differences seen in eating behaviour or depression scales. The only psychological/emotional dimension that was

experienced by the super obese more profoundly was public distress. With public distress mainly being influenced by the experience of discrimination and ridicule [185], it is distressing, but perhaps unsurprising, that this is the most pronounced dimension associated with the most severely obese. Although the disassociation of other health, mental and eating related factors with BMI could be explained by the fact that the super obese individuals were more metabolically healthy then the less severely obese, the persistent increase in public distress with higher BMIs points to an ongoing problem in society: the general negative attitudes towards obese individuals. That obese individuals experience discrimination, stigmatisation and bullying, have been shown repeatedly in research studies [7,323-325]. It is, therefore, very possible that the more visually super obese individuals within our cohort are more affected. Stigmatisation of obesity should not be taken lightly, as it has been shown to not only correlate with an increase in depression, general psychiatric symptoms, and body image disturbance [326], it has also been shown to negatively influence weight loss [324]. Although no correlation between weight loss and public distress could be found in our cohort, it would be of interest to investigate whether any associations exist with other co-morbidities.

This apparently higher prevalence of obesity stigmatisation shows that the speculation about obese lacking willpower, eating too much and not exercising enough, is still very common in the UK population, even though numerous studies throughout the last decades have shown that the situation (particularly for the most severely affected) is more complex than that, and this is supported by the data presented in this thesis.

It is known that Mendelian disease can cause severe and disabling obesity, and here we have shown these Mendelian forms of obesity are not uncommon in the severely obese undergoing bariatric surgery, as well as in obese children. Although the frequency of MC4R deficiency was lower than expected in the PMMO bariatric cohort, almost one in 20 of the severely obese children screened carried a variant affecting the function of *MC4R*. Subsequently, we discovered that among 39 super obese individuals with early onset obesity over one in five suffered from a putative Mendelian disorder

causing obesity. Among these eight cases of Mendelian obesity, two were previously reported (a deletion in 16p11.2 [61] and a point-mutation in *PTEN* (p.His93Arg) [94]), while the remaining six were novel genetic aberrations in known obesity genes (*NTRK2*, *SH2B1*, *IGSF1* and *LEPR*). Among these novel findings we reported here for the first time a deletion covering an entire exon (exon 19 in transcript NM_006180) of the *NTRK2* gene. Technical difficulties have impeded direct validation of this deletion, but WES read depth analysis looked very confirmative, with a Bayes factor of 3.59. The remaining variants were predicted-to-be deleterious variants (in *NTRK2*, *SH2B1* and *LEPR*) and a hemizygous nonsense mutation in *IGSF1*. In comparison, only two of such putative Mendelian forms of obesity were found in a cohort of 73 overweight and mildly obese individuals (NutriTech); giving a putative diagnostic yield of Mendelian obesity of 20.5% in the super obese individuals, compared to 2.7% in the overweight to obese participants (*p*: 0.0031).

The higher than anticipated prevalence of putative Mendelian obesity in the super-obese individuals with early onset, and the high prevalence of MC4R deficiency in the obese children, indicate the necessity of genetic services being made available for the severely obese, even when there are no other dysmorphic or cognitive phenotypes. Interestingly the child cohort reported here, was recruited in the Netherlands, where screening of severely obese individuals (especially with childhood onset) is already in practise within their national healthcare system [327]. It is time to consider for the NHS within the UK, instituting a similar service, which should, at the very least, cover the proven to be more common obesity genetic disorders; MC4R deficiency and 16p11.2 deletion.

The two previously-described genetic disorders (16p11.2 deletion and a variant in *PTEN* (p.His93Arg)) that were found here, have both been shown to also cause an increased risk of autism, while functional variants in *PTEN* have also been associated with increased risk of certain cancers [61,94]. These two instances indicate the importance of having such tests available, to enable proper genetic counselling and provide further treatment/screening when necessary.

As a part of the work presented in this thesis, treatment success of the individuals with Mendelian obesity was investigated. It was shown that bariatric surgery, in the form of RYGB or VSG, both seem to be effective (at least for initial weight loss) in these individuals. Every individual lost weight following surgery, although 3 individuals (with the *NTRK2* deletion*, IGSF1* variant, or *PTEN* variant) did not reach the clinically significant weight loss target of 20%. Longer follow-up of these individuals will indicate whether maintenance of weight loss similar to the remaining cohort will also be achieved. Currently some contradictory results on long term weight loss following RYGB exist [172,173], while no long term data exists at all for patients with Mendelian obesity undergoing VSG.

Our results of lifestyle intervention in children with MC4R deficiency are in accord with findings of a previous study, indicating that these individuals are able to lose weight through lifestyle adjustment, but have much greater difficulties maintaining this weight loss[48]. There is very limited evidence on how the obesity and eating behaviour disturbances associated with MC4R deficiency should best be treated. Our results, combined with previous studies indicate that bariatric surgery may be a good option for weight loss, although long term data is limited. For children and young adults, however, bariatric surgery is often not an option and clinical trials to investigate the feasibility of such treatments in children and adolescents have only just begun. Urgent studies are, therefore, warranted to assess how best to treat people with Mendelian obesity, especially when bariatric surgery or lifelong restriction of the food environment (as for PWS patients in specialist therapeutic communities) is not an option.

In the final chapter of this thesis, I described how WES was used to identify a novel Mendelian form of obesity and diabetes: CPE deficiency. Although *Cpe* was identified as the causative gene in the *fat/fat* mouse two decades ago, no complete disruption of this gene was ever described in humans before now. The phenotype of the proband included severe, early onset obesity, hyperphagia, intellectual disability, T2DM and hypogonadotrophic hypogonadism. The comparable phenotype of obesity, hyperphagia and T2DM seen in the *fat/fat* mice models and the patients reported with PC1/3

deficiency (a protein that functions proximally of CPE, and which also causes hypogonadotrophic hypogonadism once disrupted), indicates that these features are probably all a part of the CPE-deficiency syndrome.

This novel finding of a complete disruption of *CPE* in humans indicates the importance of the CPE protein in weight regulation and glucose metabolism, and is only the third ever reported pro-hormone processing enzyme deficiency disorder. How CPE deficiency contributes to the wider obesity population will need to be further investigated by screening a bigger proportion of obese individuals. Although the frameshift deletion reported here is very rare, it has been reported in heterozygote form in two unrelated individuals in ExAC [208]. This could indicate (together with the finding that for this the deletion the breakpoints were aligned with a nucleotide repeat, pointing towards a microhomology-mediated deletion mechanism) that this deletion was not a unique occurrence. With the family members of the proband reported here, being heterozygote carriers of the deletion and obese, it is important to find out whether heterozygous knock out of *CPE* indeed causes an intermediate phenotype of the severe complex obesity seen in the proband.

The work described in this thesis shows the importance and power of WES in detecting novel genetic disorders causing Mendelian disease, as well as a screening tool to detect the prevalence of deleterious variants in a selected group of disease-causing (or candidate) genes. Although we cannot really still describe high throughput sequencing as a 'new or novel area of genetic research', with the first commercial kit being on the market for more than 10 years now, the costs of WES (and whole genome sequencing) has dropped so dramatically, that the approach has now become more accessible for a wider research area. With the very fruitful examples WES has brought to us so far within obesity research, including the results presented here, and so many unsolved genetic mysteries out there, it can be expected many more exciting findings will follow.

9.2 Future work

The body of work presented in this thesis delivered several interesting findings that need further investigation in order to validate, further explain and/or extend the current results reported here.

Continuation of the PMMO project

Although the data reported in this thesis provide an extensive overview of the phenotypic variation seen among the severely obese and give some insight into the effect of bariatric surgery beyond weight loss, a continuation of the follow-up of the participants is warranted. This is to supply currently missing data and increase participant numbers, to give the most accurate results on improvements in obesity-related comorbidities and important related health issues such as quality of life, mood disorders and eating behaviour. There should be specific emphasis on individuals with Mendelian obesity, to ensure that longer-term follow-up data on such patients undergoing bariatric surgery can be provided.

The self-reported quality of life, eating behaviour and mood questionnaires should be assessed to evaluate the reliability and validity of these research instruments in a bariatric cohort. Although some interesting findings regarding public distress features were revealed in the PMMO dataset, the lack of other correlations previously reported, raises questions on the reliability of the questionnaires in such an extreme obesity cohort. Internal consistency, reliability between the different questionnaire results and actual disorders present in the individuals should be investigated, followed by extensive predictive analysis, in an effort to collate the different questionnaire results into one predictive score.

The finding, that among the more severely obese individuals in the PMMO cohort there was relatively lower prevalence of T2DM and hypercholesterolaemia needs to be validated in larger general population cohorts, to ensure that this is not merely caused by selection bias in bariatric referrals.

Through collaborative work with PhD students in our research group, screening of two large birth cohorts (North Finnish Birth Cohorts 1986 (*n*= 6,800) and 1966 (*n*= 6,000)), the UK biobank data (*n*=500,000) and the China Kadoorie Biobank (*n*= 500,000) will be investigated to see whether this finding can be repeated [328-332]. Although class IV and V obesity is rare in the general population (with the current prevalence of >class III obesity in the UK being estimated to be within 0.001-0.003 [1]), the large numbers in these population cohorts will hopefully ensure enough cases to perform replication of our findings.

Continuation of the study performed at Heideheuvel centre

Further follow-up of the MC4R deficient children who received an intensive lifestyle intervention at Heideheuvel will also need to be performed, to detect response to receiving a diagnosis of Mendelian obesity. It is important to see how services in genetic facilities can be improved for the patients involved, and therefore the results of giving genetic counselling need to be carefully monitored in terms of psychological impact, effect on health behaviours and any other unexpected complications. Ideally a similar approach will also be applied to the individuals diagnosed in the PMMO cohort.

Functional analysis of variants detected

A limitation in the work presented in this thesis is the lack of further functional analysis of the discovered novel variants in the known obesity genes, to identify the level of pathogenicity of the variants on the protein function. Although such analysis is currently not performed by myself or members in our lab, collaborative work is currently being initiated to perform cell biological analysis on a selection of the novel variants found in the super obese through WES in the known obesity genes. Specifically, CRISPR-cas9 gene editing approaches will be used to generate the mutants on a standard background (an induced pluripotent cell line) for functional analysis.

Direct screening for known obesity-causing variants in the remaining PMMO cohort

The results presented here indicating that the prevalence of Mendelian obesity is higher in this patient population than initially anticipated, warrants further analysis of the remaining cohort, which will indicate whether this holds up for a larger number, and especially if this is also true the less severely-affected obese individuals within the cohort. Ideally you would like to perform WES (or WGS) on the entire cohort, which would produce an enormous amount of unbiased data that could be used to not only identify the prevalence of Mendelian obesity, but could also be used to identify novel genes involved in obesity pathogenesis and obesity related morbidities. The application of WES in such large numbers has proven challenging to manage and analyse [333,334], taking a lot of man hours for the resultant dataset to be interpreted. However, the main reason to opt for other methods is the costs of sequencing such a large number, which in the ideal situation would be performed, but in the real world is not always possible.

Another approach to identify the prevalence of currently known Mendelian forms of obesity is to directly screen for the known obesity causing variants, instead of sequencing the whole exome. Although this will not enable the identification of novel variants, the prevalence of known variants can be determined in a more cost-effective way. This is especially true considering the amount of work that is involved in the interpretation of novel variants, which ideally should be characterised by functional analyses to confirm the effect on the protein (even if located in known obesity genes): a direct analysis of known disease causing variants is much more straight forward to interpret.

In addition to known disease causing variants, a selection of predicted-to-be-deleterious variants found through WES in monogenic obesity genes, as well as in a selection of candidate obesity genes, can be included on such a genotyping screening tool as well. Ideally, rare predicted-to-be deleterious variants in these genes reported in open databases (keeping in mind that individuals with monogenic forms of obesity are included in these cohorts, as was seen for MC4R deficiency) can be included as well. In this way variants can be sought with a significantly higher frequency among the obese cohort

compared to a control set, and associations can be sought with the extensive set of phenotypes collected for this cohort (including T2DM diagnosis and remission, eating behaviour and weight loss following intervention).

Currently, this approach is being explored and set up by a PhD student, under supervision of Prof. A. Blakemore, by designing a customised genotyping chip (Affymetryx Axiom), which will include all known disease causing variants of obesity and diabetes, GWAS-identified SNPs for obesity and diabetes, and additional variants identified from our exome sequencing datasets reported here. This specifically-designed chip will be used to screen the PMMO cohort in a project I will be directly involved in through a post-doctoral position.

This planned work builds on the data presented in this thesis, but detection of monogenic obesity-causing variants in a larger number of PMMO participants will provide additional power to carry out a more robust investigation into the implications of these highly-penetrant genetic factors on response to surgical intervention.

Ultimately, I hope that my work (as presented in this thesis and planned continuation of the research as described above) will provide a firm basis for the development of a more personalised approach to obesity care.

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Appendix

Appendix 2.1 Participants information sheet and consent form

In this appendix a copy of the participant information sheets (PIS) and consent forms (CF) used for this study can be found. A separate PIS and CF was used for patients recruited pre-surgery and for patients recruited post-surgery.

Imperial College London

Ref: 11\LO\0935, Version 8, 16th July 2014

Prof Alexandra I F Blakemore
Commonwealth Building 5.S5a
Hammersmith Hospital
Hammersmith Campus
Du Cane Road, London W12 0NN

THIS INFORMATION SHEET IS VALID FOR USE UNTIL 30 November 2017

INFORMATION SHEET FOR RESEARCH PARTICIPANTS AT NHS HOSPITAL TRUSTS - PRE SURGERY

- **You will be given a copy of this Information Sheet and a signed copy of your consent form to keep, should you decide to participate in the study**.

STUDY TITLE: PERSONALISED MEDICINE FOR MORBID OBESITY

You are being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

If you do decide to take part, please let us know beforehand if you have been involved in any other study during the last year. You are free to withdraw at any time without explanation. Thank you for reading this.

WHAT IS THE PURPOSE OF THE STUDY?

This study aims to investigate the genetic cause of obesity and diabetes, and also its implication on the outcomes of bariatric surgery (obesity surgery) in terms of weight loss and diabetes resolution. We will further investigate the mechanisms that underline diabetes remission following surgery. We will also look at how other factors, such as mood, your way of handling emotions, and your exercise levels affect the outcomes of surgery.

The management of obesity is challenging and obesity surgery is by far the most effective treatment currently available. Obesity surgery carries different risks and benefits and it is important to balance these by choosing the right procedure for each patient. In particular, some patients fail to achieve the expected weight loss or experience complications and re-operations. It was previously shown that certain genetic differences account for 5-6% of morbid obesity cases and patients with different genetic variants (different forms of the same gene) may respond differently to surgical procedures.

Bariatric surgery has further been shown to result in rapid type 2 diabetes mellitus remission in some patients. Currently, researchers are still unable to predict remission of diabetes (where clinically blood sugar level and insulin response returns to normal levels) following bariatric surgery which is crucial for assessing the risks and benefits of bariatric surgery for obese diabetic patients.

WHY HAVE I BEEN CHOSEN?

We are recruiting obese adults with BMI > 35 kg/m^2 and/ or patients pre- and post-bariatric (obesity) surgery.

You should **not** take part in this study if you (1) have donated blood in the last three months and/ or (2) are currently receiving or intend to receive treatment with a new drug that has not yet been approved by the European Medicines Agency (EMA) within the next 2 months.

DO I HAVE TO TAKE PART?

-
- It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason and without affecting your future treatment.

WHAT WILL HAPPEN TO ME IF I TAKE PART?

Visit 1: Screening visit

If you meet the criteria, a member of our research team will telephone or write to you to obtain consent to proceed onto the first study visit (screening visit). During the screening visit the study will be explained to you in person, confirmation of your understanding of the study will be sought and the consent form signed. After consent has been obtained, you will undergo the following procedure at the screening visit:

- You will have your family history recorded as well as height, weight, head circumference, foot- and hand size taken. Sometimes, a photographic documentation will also be obtained from you to be studied by a clinical geneticist for the purpose of this research.

- We will ask you to complete a number of questionnaires online after obtaining informed consent at your screening visit. For this, you will be given a link and login details to access and complete all questionnaires at home. These questionnaires ask you about your eating habits, personality and mood, and smoking behaviour, and will be used to help us examine behavioural changes. In total the questionnaires will be answered in two separate occasions, which are after the initial screening, and after study visit 6.

- We will also use the data collected from your blood results upon your initial referral to screen for clinical markers such as levels of glucose, insulin and C-peptide.

- With your permission, we will also take a sample of DNA from your blood or saliva to look for genetic variations that may affect the outcomes of the surgery.

- If you have a suitable smartphone, you will also be invited to consider using a free commercially-produced app called MOVES (http://www.moves-app.com/) to record your day-to-day walking, together with a new app called MyWICompanion which has been specially designed at Imperial College London for this study. These two apps work together to record your mood and exercise activities throughout your weight loss journey.

- If you are a bariatric surgery patient, you will be asked to bring an early morning urine sample as well as a faeces sample for later clinical and microbiological analyses. You need to store your faeces sample in your domestic freezer before you come arrive at the screening visit.

Number of visits

If you are a bariatric surgery patient, all the medical checks are satisfactory, and you are happy to participate in the study, you will be asked to be involved in six subsequent study visits at your hospital following your first screening visit: at the time of surgery (visit 2), two days after the surgery when still being in the hospital (visit 3), then again at 10 days after surgery and 6, 12 and 18 months after surgery (visits 4 to 7 respectively). Except for study visit 7, all efforts will be made to ensure participation in the study will not require any additional hospital visits, above and beyond the standard follow-up procedure.

Your second study visit will happen during bariatric surgery. During your operation, we will take a very small sample of muscle tissue from your abdominal wall, liver tissue from your left liver lobe, fat from under the skin at one of your incisions and fat inside your abdomen.

Your third study visit will be 2 days after the surgery while you are still in the hospital and involves one blood sample and body weight measurements.

On your 4th study visit (10 days after surgery), we will take your body weight, and blood and urine sample. This will be during your routine follow-up appointment.

The same applies for your 5th visit; we will take again your body weight and blood and urine sample.

Your 6th study visit will be when you come for your 12 months follow-up appointment. We will take your body weight and blood sample from you. Additionally you have to fill in psychological questionnaires.

Your last study visit (study visit 7) at 18 months is not part of your clinical follow-up appointments. We will ask you to come back to the hospital where we will take your blood and body weight and you need to provide us with an early urine and faeces sample. As for visit 1, you will be given appropriate container to store your urine and faeces samples. You need to store your faeces sample in your domestic freezer before you come to visit 7.

Each study visit can last up to one hour. You will be asked to abstain from alcohol and strenuous exercise for 24 hours before the visit.

DNA and RNA will be prepared from blood and tissue samples for the purpose of the study. We will use the most up-to-date and efficient methods of DNA analysis available to us at the time. Your sample will be stored for up to 15 years. If new methods arise during the time of the study, we will apply these if feasible. Sometimes this may mean that samples are sent outside Imperial College London, to other research institutes (nationally or internationally) that we work with, or as a part of a commercial DNA processing service. We would only send your sample to partners working with us on this research providing a confidential service. You will only be contacted by our research team in the event that a genetic cause for your obesity has been identified.

Blood samples will be used to screen for clinical markers such as levels of glucose, insulin and C-peptide. No more than 100 mls of blood will be taken from each participant during the entire study which roughly amounts to a volume of 20 teaspoons (no more than 15 mls on each visit).

WHAT ARE THE SIDE EFFECTS OR RISKS OF TAKING PART?

We do not anticipate any significant side effects from taking part in the study. You may experience pain or mild discomfort from giving a blood sample due to venepuncture which involves of inserting a needle into your arm to withdraw venous blood. The complications of tissue collection during surgery, especially liver biopsy can be that of bleeding, leakage causing abdominal inflammation or requiring further interventions (second operation), or abscess infection. The risk of developing serious complications such as clinically significant blood loss in a liver biopsy and tissue collection such as adipose tissue is 0.5% and 0.1% respectively. This risk will be further reduced as the liver biopsy will be done with direct vision. To ensure your comfort and to minimise risks, study visits will be conducted by experienced researchers and surgeons.

Please report any unusual or unpleasant experiences immediately to the senior clinician (Dr Le Roux on: 079 7071 9453). Although we do not anticipate any adverse effects, you will be provided with contact numbers and clear instructions that, if you feel unwell, you should call us.

WHAT ARE THE POSSIBLE DISADVANTAGES OF TAKING PART?

Some inconvenience may result from the completion of the psychological questionnaires which can be time-consuming, but this will be explained to you in detail before you decide whether or not to participate. There are minor risks of placing an intravenous catheter to draw blood from a vein, and rarely the risk of infection, but we do not anticipate any problems arising from participation in this study. The risks of tissue collection during surgery can be bleeding, leakage causing peritonitis or requiring further interventions (second operation), or may progress to an abscess infection. To ensure your comfort and to minimise risks, experienced researchers and surgeons will conduct study visits.

WHAT ARE THE POSSIBLE BENEFITS OF TAKING PART?

1) Direct benefits: The GPs of the patients identified with genetic causes for their obesity will be informed, the patients and in addition to their family members can be referred for formal genetic counselling by their GPs. This referral is optional.
2) We hope the future benefits will be:
    a) Be able to predict which patients benefit most from bariatric surgery optimising NHS services by assessing risk versus benefit balance for each patient.
    b) Identification of new therapeutic targets

c) For monogenic (caused by single gene disorder) obesity and diabetes, this study will provide the basis of personalised medicine i.e. choice of surgery type and management protocols.

-

WHAT IF NEW INFORMATION BECOMES AVAILABLE?

Sometimes during the course of a research project, new information becomes available about the project that is being studied. If this happens, your research doctor will tell you about it and discuss with you whether you want to continue in the study. If you decide to continue in the study you will be asked to sign an updated consent form. Also, on receiving new information your research doctor might consider it to be in your best interests to withdraw you from the study. If relevant new information that may medically benefit you emerges, this will be communicated to your GP.

WHAT HAPPENS WHEN THE RESEARCH STUDY STOPS?

Once the study has finished, you or your GP can be informed of the study results if you or your GP wishes to be informed. If you have any problems immediately following the study, then you should contact one of the research doctors on the numbers provided below.

WHAT IF SOMETHING GOES WRONG?

Imperial College London holds insurance policies which apply to this study. If you experience serious and enduring harm or injury as a result of taking part in this study, you may be eligible to claim compensation without having to prove that Imperial College is at fault. This does not affect your legal rights to seek compensation.

If you are harmed due to someone's negligence, then you may have grounds for a legal action. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been treated during the course of this study then you should immediately inform the senior clinician Dr Le Roux, Office: XXX/ XXX and the researcher Dr Alsters XXX.

WILL MY TAKING PART IN THIS STUDY BE KEPT CONFIDENTIAL?

All information and samples which are collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital will have your name and address removed so that you cannot be recognised from it. The data collected with the phone-apps, will be stored anonymously on our servers. At no time, would your personal details, such as your name and address be shared with other partners. At the start of the study it is a requirement that your GP is informed, with your consent, of your participation in this study.

IMPLICATIONS OF GENETIC TESTING FOR INSURANCE

Association of British Insurers (ABI) has extended its moratorium of not using predictive genetic testing results in their decision making for insurance applications until 2017. At least until 2017 there are no anticipated implications of genetic testing for obesity in terms of insurance. However there may be implications in terms of insurance policies in future if the current moratorium is not extended.

WHAT WILL HAPPEN TO THE RESULTS OF THE RESEARCH STUDY?

The results are likely to be published in the year following the study. Your confidentiality will be ensured
at all times and you will not be identified in any publication.

WHO IS ORGANISING AND FUNDING THE RESEARCH?

The study is funded by the Biomedical Research Centre and is organised by the Department of Medicine, Imperial College London. The lead scientist (called the Principal Investigator) of this study is Professor Alex Blakemore (http://bit.ly/1mrbZE8).

PAYMENT

We do not have funding to provide payment for participation in the study. Participation in the study is completely voluntary.

WHO HAS REVIEWED THE STUDY?

This study has been reviewed by the London–Riverside Research Ethics Committee or your Local Ethics Committee.

Contact for further information

If you experience any problems during the study, you may withdraw at any stage and this will not affect your future treatment. The medical doctors involved in the study at Imperial College Healthcare NHS Trust are Dr Le Roux, the surgeon Mr Olbers, and Dr Alsters. For further information and enquiries, Dr Le Roux and Dr Alsters will be available by telephone during working hours (Dr Le Roux and Dr Alsters XXX/ XXX), or you can contact your local Trust. The hospital switchboard at Imperial College Healthcare NHS Trust (XXX) has home and mobile phone numbers for the doctor involved in the study and can contact them at any time outside normal working hours.

Thank you for taking the time to read the information about our study and for considering taking part.

Prof Alexandra I F Blakemore
Commonwealth Building 5.S5a
Hammersmith Hospital
Hammersmith Campus
Du Cane Road, London W12 0NN

Imperial College London

Participant Consent Form for NHS HOSPITAL TRUSTS - PRE SURGERY

Title of project: Personalised Medicine for Morbid Obesity

Name of Principal Investigator: Prof A. Blakemore. Please initial each statement:

1. I confirm that I have read and understand the Participant Information Sheet Protocol Version 8 dated 16th July 2014 for the above study. ☐

2. I have had the opportunity to ask questions and discuss this study. All my questions have been answered fully and I have received enough information about the study. ☐

3. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, and without my medical care or legal rights being affected. ☐

4. I agree that my medical notes and data collected from the study may be accessed by individuals involved in the study; Imperial College London, Imperial College Healthcare NHS Trust or other Trust, or by regulatory authorities where it is relevant to my taking part in this research. ☐

5. I give permission for my data to be used for research by individuals involved in the study and Imperial College Healthcare NHS Trust so long as they do not contain identifying personal information. ☐

6. I give permission for the data collected in the questionnaires to be used for the purposes of the study. ☐

7. I give permission for the blood test results collected upon my initial referral to the service to be used for the purposes of the study. ☐

8. I give permission for my General Practitioner to be informed of my participation in this study and the results of any medical tests from my visits i.e. blood tests. ☐

9. I give permission for anonymised data on my exercise and mood, recorded through the smartphone apps MOVES and MyWICompanion, to be used in this study ☐

10. I agree for a DNA sample to be taken and stored to look for changes that may be involved in obesity and the control of appetite. This may include sending my anonymised sample to other research centres in or outside the UK and may include commercial companies. ☐

11. I am happy for my photographic images to be stored and studied by a clinical geneticist for the purpose of this research. ☐

12. I agree to my samples being collected as detailed in the patient information sheet and/ or my tissue samples collected during my surgery. ☐

13. The indemnity arrangements have been discussed with me. ☐

14. I agree to take part in the above study. ☐

15. I am happy to be contacted for possible participation in future research studies. ☐

_____	_____	_____
Name of Subject (block capitals)	Signature	Date
_____	_____	_____
Principal Investigator	Signature	Date

Imperial College London

Ref: 11\LO\0935, Version 8, 16th July 2014
Prof Alexandra I F Blakemore
Commonwealth Building 5.S5a
Hammersmith Hospital
Hammersmith Campus
Du Cane Road, London W12 0NN

THIS INFORMATION SHEET IS VALID FOR USE UNTIL 30 NOVEMBER 2017

INFORMATION SHEET FOR RESEARCH PARTICIPANTS AT NHS HOSPITAL TRUSTS - POST SURGERY

- **You will be given a copy of this Information Sheet and a signed copy of your consent form to keep, should you decide to participate in the study**.

STUDY TITLE: PERSONALISED MEDICINE FOR MORBID OBESITY

You are being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

If you do decide to take part, please let us know beforehand if you have been involved in any other study during the last year. You are free to withdraw at any time without explanation. Thank you for reading this.

WHAT IS THE PURPOSE OF THE STUDY?

This study aims to investigate the genetic cause of obesity and diabetes, and also its implications for the outcomes of bariatric surgery (obesity surgery) in terms of weight loss and diabetes resolution. We will alsolook at how other factors, such as mood, your way of handling emotions, and your exercise levels affect the outcomes of surgery.

The management of obesity is challenging and obesity surgery is by far the most effective treatment currently available. Obesity surgery carries different risks and benefits and it is important to balance these by choosing the right procedure for each patient. In particular, some patients fail to achieve the expected weight loss or experience complications and re-operations.It was previously shown that certain genetic differences account for 5-6% of morbid obesity cases and patients with different genetic variants (different forms of the same gene) may respond differently to surgical procedures.

The management of obesity is challenging and better understanding of the cause of obesity will help find more effective treatments for patients.

WHY HAVE I BEEN CHOSEN?

We are recruiting adults who had bariatric surgery (obesity surgery) in the past and (used to) have a BMI of > 35 kg/m^2.

You should **not** take part in this study if you (1) have donated blood in the last three months and/ or (2) are currently receiving or intend to receive treatment with a new drug that has not yet been approved by the European Medicines Agency (EMA) within the next 2 months.

DO I HAVE TO TAKE PART?

- It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason and without affecting your future treatment.

WHAT WILL HAPPEN TO ME IF I TAKE PART?

If you are interested to take part in this study and meet the above criteria, you can contact our research team. During your visit the study will be explained to you in person, confirmation of your understanding of the study will be sought and the consent form signed. After consent has been obtained, you will undergo the following procedure:

- You will have your family history recorded as well as height, weight, head circumference, foot- and hand size taken.
- We will use the data collected from your previous visits to the bariatric clinic to look at weight changes before and after surgery, and blood results to screen for clinical markers such as levels of glucose, insulin and C-peptide.
- With your permission, we will also take a sample of DNA from your blood or saliva to look for genetic variations that may affect the outcomes of the surgery.
- We will ask you to complete a number of questionnaires online after obtaining informed consent at your screening visit. For this, you will be given a link and login details to access and complete all questionnaires at home. These questionnaires ask you about your eating habits, personality and mood, and smoking behaviour, and will be used to help us examine behavioural changes.
- If you have a suitable smartphone, you will also be invited to consider using a free commercially-produced app called MOVES (http://www.moves-app.com/) to record your day-to-day walking, together with a new app called MyWICompanion which has been specially designed at Imperial College London for this study. These two apps work together to record your mood and exercise activities throughout your weight loss journey.

- Sometimes we ask participants to bring an early morning urine sample and/or a faeces sample for later clinical and microbiological analyses. If you were asked to provide a faeces sample, you would need to store the sample in your domestic freezer before you come to the screening visit. This is only applicable if this has previously been discussed with you in person.

Your study visit can last up to one hour. You will be asked to abstain from alcohol and strenuous exercise for 24 hours before the visit.

DNA and RNA will be prepared from saliva or blood samples for the purpose of the study. We will use the most up-to-date and efficient methods of DNA analysis available to us at the time. Your sample will be stored for up to 15 years. If new methods arise during the time of the study, we will apply these if feasible. Sometimes this may mean that samples are sent outside Imperial College London, to other research institutes (nationally or internationally) that we work with, or as a part of a commercial DNA processing service. We would only send your sample in an anonymous form to partners working with us on this research providing a confidential service. You will only be contacted by our research team in the event that a genetic cause for your obesity has been identified.

WHAT ARE THE SIDE EFFECTS OR RISKS OF TAKING PART?

We do not anticipate any significant side effects from taking part in the study. You may experience pain or mild discomfort from giving a blood sample due to venepuncture which involves of inserting a needle into your arm to withdraw venous blood.

WHAT ARE THE POSSIBLE DISADVANTAGES OF TAKING PART?

Some inconvenience may result from the completion of the psychological questionnaires which can be time-consuming, but this will be explained to you in detail before you decide whether or not to participate. There are minor risks of placing an intravenous catheter to draw blood from a vein, and rarely the risk of infection, but we do not anticipate any problems arising from participation in this study.

WHAT ARE THE POSSIBLE BENEFITS OF TAKING PART?

3) Direct benefits: The GPs of the patients identified with genetic causes for their obesity will be informed, the patients and in addition their family members can be referred for formal genetic counselling by their GPs. This referral is optional.
4) We hope the future benefits will be:
    d) Be able to predict which patients benefit most from bariatric surgery optimizing NHS services by assessing risk versus benefit balance for each patient.
    e) Identification of new therapeutic targets
    f) For monogenic (caused by single gene disorder) obesity and diabetes, this study will provide the basis of personalised medicine i.e. choice of surgery type and management protocols.

WHAT IF NEW INFORMATION BECOMES AVAILABLE?

Sometimes during the course of a research project, new information becomes available about the project that is being studied. If this happens, your research doctor will tell you about it and discuss with you whether you want to continue in the study. If you decide to continue in the study you will be asked to sign an updated consent form.

WHAT HAPPENS WHEN THE RESEARCH STUDY STOPS?

Once the study has finished, you or your GP can be informed of the study results if you or your GP wishes to be informed. If you have any problems immediately following the study, then you should contact one of the research doctors on the numbers provided below.

WHAT IF SOMETHING GOES WRONG?

Imperial College London holds insurance policies which apply to this study. If you experience serious and enduring harm or injury as a result of taking part in this study, you may be eligible to claim compensation without having to prove that Imperial College is at fault. This does not affect your legal rights to seek compensation.

If you are harmed due to someone's negligence, then you may have grounds for a legal action. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been treated during the course of this study then you should immediately inform the senior clinician Dr Le Roux, Office: XXX/ XXX and the researcher Dr Alsters XXX/ XXX.

WILL MY TAKING PART IN THIS STUDY BE KEPT CONFIDENTIAL?

All information and samples which are collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital will have your name and address removed so that you cannot be recognised from it. The data collected

with the phone-apps, will be stored anonymously on our servers. At no time, would your personal details, such as your name and address be shared with other partners.

At the start of the study it is a requirement that your GP is informed, with your consent, of your participation in this study.

IMPLICATIONS OF GENETIC TESTING FOR INSURANCE

Association of British Insurers (ABI) has extended its moratorium of not using predictive genetic testing results in their decision making for insurance applications until 2017. At least until 2017 there are no anticipated implications of genetic testing for obesity in terms of insurance. However there may be implications in terms of insurance policies in future if the current moratorium is not extended.

WHAT WILL HAPPEN TO THE RESULTS OF THE RESEARCH STUDY?

The results are likely to be published in the year following the study. This information can be received by you or your GP Practice upon request by contacting one of the doctors in the study (Dr Le Roux and Dr Alsters XXX/ XXX). Your confidentiality will be ensured at all times and you will not be identified in any publication.

WHO IS ORGANISING AND FUNDING THE RESEARCH?

The study is funded by the Biomedical Research Centre and is organised by the Department of Medicine, Imperial College London. The lead scientist (called the Principal Investigator) of this study is Professor Alex Blakemore (http://bit.ly/1mrbZE8).

PAYMENT

We do not have funding to provide payment for participation in the study. Participation in the study is completely voluntary.

WHO HAS REVIEWED THE STUDY?

This study has been reviewed by the London–Riverside Research Ethics Committee or your Local Ethics Committee.

Contact for further information

If you experience any problems during the study, you may withdraw at any stage and this will not affect your future treatment. The medical doctors involved in the study are Dr Le Roux and Dr Alsters. For further information and enquiries, Dr Le Roux and Dr Alsters will be available by telephone during working hours (Dr Le Roux and Dr Alsters XXX/XXX, or you can contact your local trust. The hospital switchboard at Imperial College Healthcare NHS Trust (XXX) has home and mobile phone numbers for the doctor involved in the study and can contact them at any time outside normal working hours.

Thank you for taking the time to read the information about our study and for considering taking part.

Prof Alexandra I F Blakemore
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Imperial College London

Participant Consent Form for NHS HOSPITAL TRUSTS - POST SURGERY

Title of project: Personalised Medicine for Morbid Obesity

Name of Principal Investigator: Prof A. Blakemore. Please initial each statement:

1. I confirm that I have read and understand the Participant Information Sheet Protocol Version 8 dated 16th July 2014 for the above study.

2. I have had the opportunity to ask questions and discuss this study. All my questions have been answered fully and I have received enough information about the study.

3. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, and without my medical care or legal rights being affected.

4. I agree that my medical notes and data collected from the study may be accessed by responsible individuals involved in the study; Imperial College London, Imperial College Healthcare NHS Trust or other Trusts, , or by regulatory authorities where it is relevant to my taking part in this research.

5. I give permission for my data to be used for research by individuals involved in the study and Imperial
College Healthcare NHS Trust so long as they do not contain identifying personal information.

6. I give permission for the blood test results collected upon my initial referral to the service to be used for the purposes of the study.

7. I give permission for my GP to be informed of my participation in this study and the results of any medical tests from my visits.

8. I agree for a DNA sample to be taken and stored to look for changes that may be involved in obesity and the control of appetite. This may include sending my anonymised sample to other research centres in or outside the UK and may include commercial companies.

9. I give permission for anonymised data on my exercise and mood, recorded through the smartphone apps MOVES and MyWICompanion, to be used in this study

10. I give permission for the data collected in the questionnaires to be used for the purposes of the study.

11. I agree to my samples being collected as detailed in the patient information sheet.

12. The indemnity arrangements have been discussed with me.

13. I agree to take part in the above study.

14. I am happy to be contacted for possible participation in future research studies.

_____	_____	_____
Name of Subject (block capitals)	Signature	Date
_____	_____	_____
Principal Investigator	Signature	Date
_____	_____	_____
Name of Person taking consent	Signature	Date

Appendix 2.2: Lifestyle intervention in Heideheuvel cohort.

113 severely obese children (aged 10-18 years old) recruited at the Childhood Obesity Centre Heideheuvel, Paediatric Hospital Merem, Hilversum, The Netherlands, were included for this study. All children received lifestyle treatment at the Heideheuvel centre. Depending on family preference, and external factors such as healthcare funding, patients received one of the below treatment options.

Comparing these different treatment interventions were not within the scope of this study, and randomised clinical trials published by the Heideheuvel centre have compared these different treatments extensively [335]. A short summary will be given here, to enable further analysis on this cohort.

Inpatient treatment program of 12 weeks (Figure appendix 2.2, treatment 1):

A program during which the patients were hospitalised for 12 weeks. A program within the hospital was being followed during the week days, while weekends were spend at home. Children slept and went to school at or near the treatment centre. The program included exercise, nutrition and behaviour advice and additional therapy when needed:

- Exercise: There was a 30-60 minutes exercise program 4 days per week and existed out of group session ($n \leq 10$) guided by an exercise therapist and encouragement of participation in daily outside activities. Although the group sessions were mandatory, the outside activities were not.
- Nutrition and behaviour therapy: Nutrition and behaviour education took place once per week and used a non-diet approach. Focus was on improving the quality of the dietary intake and on trying to establish controlled eating behaviour (by creating more awareness of their feelings of hunger and satisfaction).

- Behavioural therapy: Every child received private sessions with a child psychologist for behaviour modification. Sessions were adjusted per needs of the child and included topics such as self-regulation, self-awareness and goal setting.
- Weekly exercise, behaviour and nutritional education for the parents/caregivers of the children included above-mentioned components but with more in-depth knowledge.
- Individual meetings with dieticians, psychologists, and/or social workers were available for child and/or caregivers and organised when needed.

Inpatient treatment program of 8 weeks (Figure appendix 2.2, treatment 2):

- Similar setup as the 12 weeks inpatient treatment, but instead there was an 8 week hospitalised program.

Outpatient treatment (Figure appendix 2.2, treatment 3):

- Received 12 visits at increasing time intervals for a 6-month period. Children and parents received the same sessions regarding nutrition and behaviour therapy as were given to the patient undergoing the inpatient treatment set up. Exercise took place once a week at the treatment centre, but the other three sessions were expected to be undertaken at home.

For all three different treatment groups the individuals received follow up appointments once a month, during which also measurements were taken, up until one year after the initial treatment started.

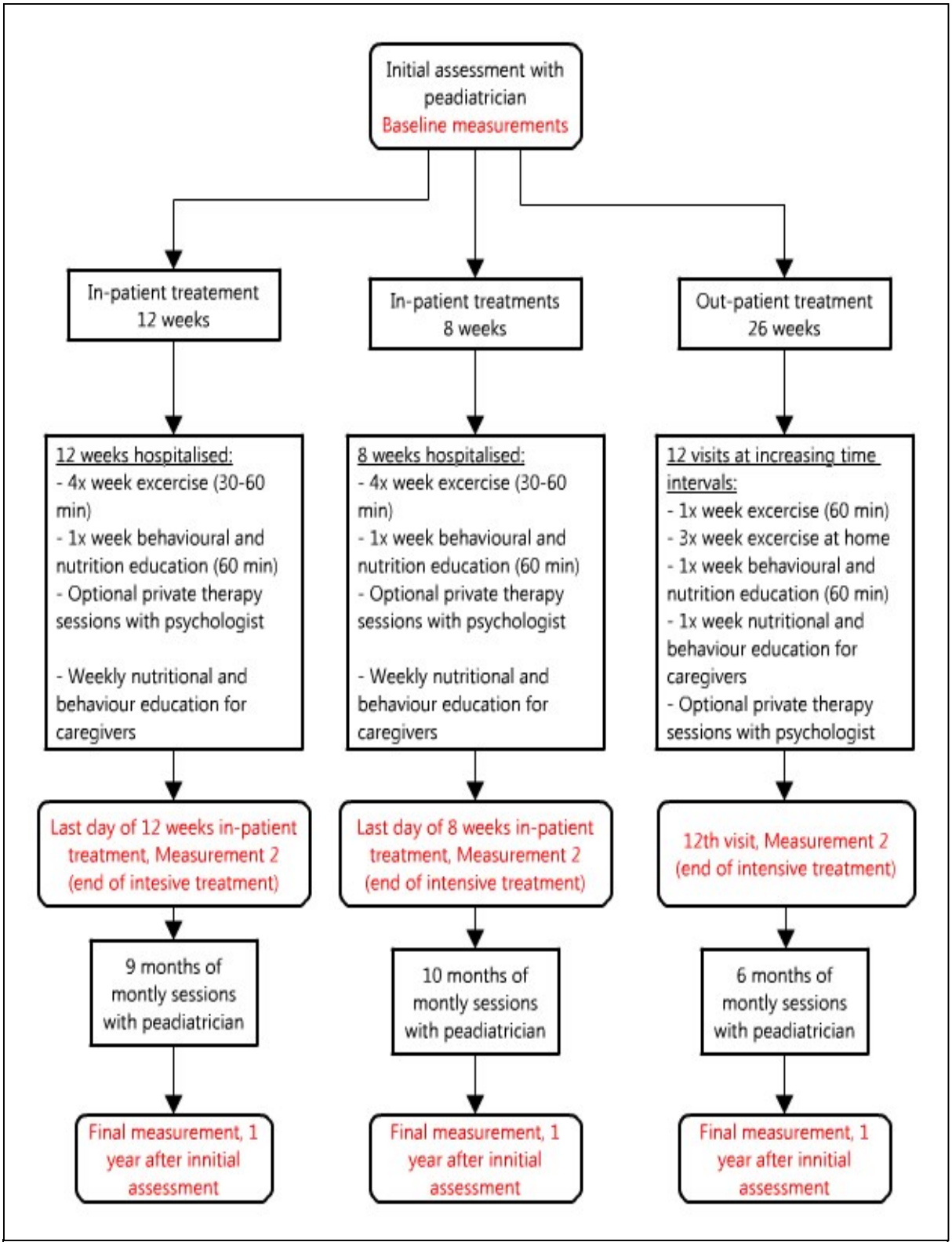


Figure appendix 2.2: Lifestyle interventions. This flowchart gives an overview of the three different lifestyle interventions patients were given at the Heideheuvel centre. Highlighted in red are the different time points measurements were collected for the patients. A fourth measurement collected was the maximum weight loss seen per individual during the one-year treatment (and could therefore be during the intensive treatment period, or after).

Appendix 2.3 Human and mouse obesity genes

In this appendix all known genes causing obesity in humans and/or mice when disrupted are listed. The first 36 genes in the list cover the human-obesity genes, while highlighted in grey are the genes with a high RVI-score, indicating an above average tolerance to functional variation. These genes were excluded for the last step of variant analysis performed in chapter 7 (7.3.5 Variation in mouse-obesity genes, page 230).

no	Gene symbol mice	Gene symbol humans	phenotype in mice	phenotype in humans	Phenotype in mice	RVIS	RVIS %	reference
1	ALMS1	ALMS1	Obesity	Obesity	Knock-out	2.29	98.28	Humans: Marshall, JD. 2007. EJHG Mice: Collin, GB. 2005. Hum Mol Genet
2	ARL6	ARL6 (BBS3)	Not known	Obesity	xx	-0.10	46.20	Humans: Chiang, AP. 2004. Am J Hum Genet
3	BBIP1	BBIP1 (BBS18)	Not known	Obesity	xx	x	x	Humans: Scheidecker, S. 2014. J med genet
4	BBS1	BBS1	Adult-onset obesity in 10% of mutants	Obesity	Knock-out	-0.26	34.88	Humans: Mykytyn, K. 2002. Nat Genet Mice: Kulaga, HM. 2004. Nat Genet
5	BBS10	BBS10	Not known	Obesity	xx	-0.35	29.43	Humans: Stoetzel C. 2006. Nat Genet
6	BBS12	BBS12	Not known	Obesity	xx	1.38	94.60	Humans: Stoetzel C. 2007. Am J Hum Genet
7	BBS2	BBS2	Adult-onset fat mass gain	Obesity	Knock-out	-0.84	11.36	Humans & Mice: Nishimura, DY. 2004. Proc Natl Acad Sci USA
8	BBS4	BBS4	Adult-onset obesity	Obesity	Knock-out	0.11	62.10	Humans: Mykytyn, K. 2001. Nat Genet Mice: Mykytyn, K. 2004. Proc Natl Acad Sci USA
9	BBS5	BBS5	Not known	Obesity	xx	0.04	56.92	Humans: Li, JB. 2004. Cell
10	BBS7	BBS7	Obesity	Obesity	Knock-out	-0.40	26.73	Humans: Badano JL. 2003. Am J Hum Genet Mice: Zhang, Q. 2013. J Cell Sci
11	BBS9	BBS9	Not known	Obesity	xx	1.05	91.37	Humans: Nishimura DY. 2005. Am J Hum Genet
12	BDNF	BDNF	Adult-onset obesity in heterozygotes	Obesity	Knock-out	0.44	77.57	Humans: Han JC. 2008. N Engl J Med Mice: Coppola, V. 2004. Neuroreport
13	CEP19	CEP19	Obesity	Obesity	Knock-out	0.01	54.95	Mice and humans: Shalata, A. 2013. Am J Hum Genet
14	CEP290	CEP290 (BBS14)	Not known	Obesity	xx	0.37	75.31	Leitch, C. 2008. Nat Genet
15	CPE	CPE	Obesity	Obesity	Knock-out	-0.05	50.22	Humans: Alsters, S. 2015. PLOS One Mice: Cawley, NX. 2004. Endocrinology
16	HDAC8	HDAC8	Not known	Obesity	xx	-0.27	33.97	Harakalova, M. 2012. J Med Genet

17	IFT27	IFT27 (BBS19)	Not known	**Obesity**	xx	0.39	76.05	Aldahmesh MA. 2014. Hum Mol Genet
18	IGSF1	IGSF1	Not known	**Obesity**	xx	-0.62	17.47	Joustra, S. 2013. J Clin Endocrinol Metab
19	KSR2	KSR2	Obesity	**Obesity**	Knock-out	x	x	Humans: Pearce, L. 2013. Cell Mice: Revelli, JP. 2011. Obesity
20	LEP	LEP	Obesity	**Obesity**	Knock-out	0.06	58.26	Humans: Montague, C.T. 1998. Nature Mice: D'Souza, AM. 2014. Endocrinol
21	LEPR	LEPR	Obesity	**Obesity**	Knock-in	-0.22	37.70	Humans: Clement, K. 1997. Nature Mice: Bates, SH. 2003. Nature
22	LZTFL1	LZTFL1 (BBS17)	Not known	**Obesity**	xx	79.89		Humans: Marion, V.2012. J Med Genet
23	MAGEL2	MAGEL2	Increased BW & adiposity	**Obesity**	Knock-out	x	x	Humans: Schaaf, C.P. 2013. Nat Genet Mice: Bischof, JM. 2007. Hum Mol Genet
24	MC4R	MC4R	Obesity	**Obesity**	Knock-out	-0.18	40.36	Humans: Farooqi, I.S 2003. N Engl J Med Mice: Huszar, D. 1997. Cell
25	MKKS	MKKS (BBS6)	Obesity	**Obesity**	Knock-out	0.35	74.58	Human: Slavotinek AM. 2000. Nat Genet Mice: Fath, MA. 2005. Hum Mol Genet
26	MKS1	MKS1 (BBS13)	Not known	**Obesity**	xx	50.45		Leitch, C.C. 2008. Nat Gen
27	NTRK2	NTRK2	Obesity	**Obesity**	knock out	-0.49	22.65	Yeo, G.S. 2004 Nat Neurosci
28	PC1/3	PCSK1	Increased adiposity in heterozygotes	**Obesity**	Knock-out	-0.42	25.73	Humans: Jackson, R.S.1997. Nat Genet Mice: Zhu, X. 2002. Proc Natl Acad Sci USA
29	POMC	POMC	Obesity under HFD	**Obesity**	Knock-out	x	x	Humans: Krude, H. 1998 Nat Genet Mice: Challis, BG. 2004. Proc Natl Acad Sci USA
30	SDCCAG8	SDCCAG8 (BBS16)	Not known	**Obesity**	xx	42.23		Otto, E.A. 2010. Nat Genet
31	SH2B	SH2B1	Obesity	**Obesity**	Knock-out	-0.24	36.17	Humans: Douche. 2012. J CLin Invest Mice: Ren, D. 2005. Cell Metab
32	SIM1	SIM1	Obesity in heterozygotes	**Obesity**	Knock-out	-0.82	11.88	Humans: Holder, JL. 2000. Hum Mol Genet Mice: Michaud, JL. 2001. Hum Mol Genet

33	TRIM32	TRIM32 (BBS11)	Not known	**Obesity**	xx	-0.60	18.06	Stoetzel, C. 2007. Hum Genet
34	TTC8	TTC8 (BBS8)	Not known	**Obesity**	xx	0.33	73.54	Ansley, S.J. 2003. Nature
35	TUB	TUB	Adult-onset obesity	**Obesity**	Knock-out	-0.91	10.12	Humans: Borman, A.D. 2014. Hum Mutat Mice: Voros, G. 2004. J Thromb Haemost
36	WDPCP	WDPCP (BBS15)	Not known	**Obesity**	xx	0.47	78.74	Kim, S.K. 2010. Science
37	ACADVL	ACADVL	Adult-onset fat mass gain	Not known	Knock-out	0.03	55.79	Exil, VJ. 2003. Circ Res
38	ADRA1B	ADRA1B	Accelerated weight gain on HFD	Not known	Knock-out	-0.60	17.75	Burcelin, R. 2004. J Biol Chem
39	ADRB1	ADRB1	Obesity	Not known	Knock-out	x	x	Bachman, ES. 2002. Science
40	ADRB2	ADRB2	Obesity	Not known	Knock-out	0.42	77.06	Soloveva, V. 1997. Mol Endocrinol
41	ADRB3	ADRB3	Obesity on HFD	Not known	Knock-out	0.08	60.09	Susulic, VS. 1995. J Biol Chem
42	RAGE	AGER	Increased BW	Not known	Knock-out	0.60	82.74	Leuner, B. 2012. Z Gerentol Geriatr
43	AT2R	AGTR2	Increase in BW in females only	Not known	Knock-out	0.31	72.23	Samuel, P. 2013. PLoS One
44	ANGPTL6	ANGPTL6	Obesity and insulin resistance	Not known	Knock-out	x	x	Oike, Y. 2005. Nat Med
45	APOB	APOB	Increased BW*	Not known	Knock-out	1.42	94.85	Siri, P. 2009. J Biol Chem
46	APOE	APOE	Obesity	Not known	Knock-out	x	x	Zhang, T. 2013. Reproduction
47	AQP7	AQP7	Adult-onset obesity	Not known	Knock-out	0.11	61.91	Hibuse, T. 2005. Proc Natl Acad Sci USA
48	AR	AR	Obesity, decreased energy expenditure	Not known	Cre/LoxP	-0.62	17.31	Fan, W. 2005. Diabetes
49	ALP1 (ASZ1)	ASZ1	Accelerated weight gain on HFD	Not known	Knock-out	-0.34	30.56	Narisawa, S. 2003. Mol Cell Biol
50	ATXN2	ATXN2	Obesity under HFD	Not known	Knock-out	-1.00	8.54	Kiehl, T. 2006. Biochem Biophys Res Commun
51	BRD2	BRD2	Obesity	Not known	Knock-out	-0.02	52.25	Wang, F. 2009. Biochem J
52	BRS3	BRS3	Obesity	Not known	Knock-out	-0.36	28.63	Ohki-Hamazaki, H. 1997. Nature

53	CTRP9	C1QTNF9	Increased bodyweight & adiposity	Not known	Knock-out	0.24	69.37	Wei, Z. 2014. Am J Physiol Endocrinol Metab
54	CAPN10	CAPN10	Increase in body weight	Not known	Knock-out	-0.33	30.92	Cheverud, JM. 2010. J Lipid Res
55	CART (CARTPT)	CARTPT	Adult-onset obesity	Obesity	Knock-out	0.33	73.11	Wierup, N. 2005. Regul Pept
56	CAV3	CAV3	Increased adiposity	Not known	Knock-out	0.44	77.70	Capozza, F. 2005. Am J Physiol Cell Physiol
57	CCKBR	CCKBR	Obesity	Not known	Knock-out	-0.27	34.60	Lavine, J. 2010. Endocrinol
58	CDH2	CDH2	Increased adiposity	Not known	Transgenic	-1.06	7.52	Castro, CH. 2004. J Cell Sci
59	CDKN1A	CDKN1A	Increased adiposity	Not known	Knock-out	0.46	78.46	Naaz, A. 2004. FASEB J
60	CHOP	CEBPB	Obesity under HFD	Not known	Knock-out	x	x	Grant, RW. 2014. J Biol Chem
61	CHGA	CHGA	Increased adiposity	Not known	Knock-out	2.11	97.88	Bandyopadhyay, G. 2012. J Biol Chem
62	CLOCK	CLOCK	Obesity	Not known	Knock-out	-0.53	20.78	Turek, F. 2005. Science
63	CHEMR23	CMKLR1	Adult-onset obesity	Not known	Knock-out	0.44	77.80	Rouger, L. 2013. J Endocrinol
64	CB2R	CNR2	Increase in body weight and hyperphagia	Not known	Knock-out	0.09	60.47	Agudo, J. 2010. Diabetologia
65	CORIN	CORIN	Increased bodyweight	Not known	Knock-out	-0.15	42.34	Chan, JC. 2005. Proc Natl Acad Sci USA
66	CPT1	CPT1c	Obesity under HFD	Not known	Knock-out	-0.71	14.78	Gao, FX. 2009. Diabetologia
67	CRH	CRH	Excess fat accumulation & muscle atrophy	Not known	Transgenic	x	x	Stenzel-Poore, MP. 1992. Endocrinology
68	CRY1	CRY1	Obesity under HFD	Not known	Knock-out	-0.89	10.30	Barclay, JL. 2013. Am J Physiol Endorinol Metab
69	CSF2	CSF2	Adult-onset obesity	Not known	Knock-out	0.41	76.67	Reed, JA. 2005. J Clin Invest
70	CYP19A1	CYP19A1	Elevated gonadal fat pad weight	Not known	Knock-out	-0.56	19.73	Misso, ML. 2005. Horm Metab Res
71	P62 (DCTN4)	DCTN4	Adult-onset obesity and hyperphagia	Not known	Knock-out	-0.51	21.56	Harada, H. 2013. J Neurosci
72	D2	DIO2	Increased BW & adiposity	Not known	Knock-out	0.04	56.64	Marsili, A. 2011. PLoS One
73	PREF1	DLK1	Obesity	Not known	Knock-out	0.55	81.60	Moon, YS. 2002. Mol Cell Biol
74	DPT	DPT	Increased subcutaneous fat	Not known	Knock-out	0.68	85.04	Takeda, U. 2002. J Invest Dermatol

75	DRD3	DRD3	Increased adiposity and obesity	Not known	Knock-out	-0.36	29.16	McQuade, JA. 2004. Behav Brain Res
76	ATX	ENPP2	Increase in adiposity in fat-specific knockout under HFD	Not known	Cre/LoxP	0.16	64.96	Dusaulcy, R. 2011. J Lipid Res
77	ESR1	ESR1	Obesity	Not known	Knock-out	0.24	69.46	Heine, PA. 2000. Proc Natl Acad Sci USA
78	FABP4	FABP4	Obesity in homozygotes under HFD	Not known	Knock-out	0.17	65.33	Hotamisligil, GS. 1996. Science
79	GPR120	FFAR4	Obesity under HFD	Not known	Knock-out	x	x	Hirasawa, A. 2005. Nat Med
80	FKBP51	FKBP51	Increase in body weight under HFD	Not known	Transgenic	x	x	Yang, L. 2012. Am J Physiol Endocrinol Metab
81	FOXA2	FOXA2	Heterozygotes develop obesity under HFD	Not known	Knock-out	0.04	56.92	Wolfrum, C. 2003. J Clin Invest
82	FOXO3A	FOXO3	Obesity	Not known	Knock-out	-0.49	22.36	Fang, C. 2008. Am J Physiol
83	FSHR	FSHR	Obesity	Not known	Knock-out	-0.11	45.49	Danilovich, N. 2000. Endocrinology
84	GAST	GAST	Obesity	Not known	Knock-out	0.39	75.87	Cowey, SL. 2005. Cancer
85	GHRH	GHRH	Increased adiposity	Not known	Transgenic	0.17	65.33	Cai, A. 1999. Endocrinology
86	GNAS	GNAS	Maternal inheritance of mutant allele leads to obesity	Not known	Knock-out	-0.02	52.32	Germain-Lee, EL. 2005. Endocrinology
87	GPD2	GPD2	Increased BW & adiposity in females	Not known	Knock-out	0.22	68.49	Alfadda, A. 2004. Am J Physiol Regul Integr Comp Physiol
88	GPR26	GPR26	Obesity	Not known	Knock-out	x	x	Chen, D. 2012. PLoS One
89	GPR39	GPR39	Obesity	Not known	Knock-out	-0.09	47.06	Moechars, D. 2006. Gastroenterology
90	GRM8	GRM8	Increased adiposity	Not known	Knock-out	-0.57	19.04	Duvoisin, RM. 2005. Eur J Neurosci
91	HDC	HDC	Increased BW & adiposity	Not known	Knock-out	-0.24	36.17	Hara, J. 2001. Neuron
92	PGDS	HPGDS	Obesity	Not known	Knock-out	0.19	67.03	Tanaka, R. 2009. Biochem Biophys Res Commun
93	HRH1	HRH1	Late onset obesity	Not known	Knock-out	0.37	75.43	Masaki, T. 2004. Diabetes
94	HRH3	HRH3	Increased BW & adiposity	Not known	Knock-out	-0.38	27.69	Takahashi, K. 2002. J Clin Invest

95	HSD11β2	HSD11B2	Increased adiposity	Not known	Transgenic	-0.47	23.25	Masuzaki, H. 2001. Science
96	HTR2C	HTR2C	Late onset obesity	Not known	Knock-out	-0.27	34.32	Nonogaki, K. 2003. Diabetes
97	ICAM1	ICAM1	Late onset obesity/accelerated under HFD	Not known	Knock-out	0.25	69.66	Gregiore, FM. 2002. AM J Physiol Endocrinol Metab
98	IFRD1	IFRD1	Increased adiposity	Not known	Transgenic	-0.38	27.69	Wang, Y. 2005. J Biol Chem
99	IL18	IL18	Increased BW	Not known	Knock-out	-0.01	52.85	Netea, M. 2006. Nature Medicine
100	IL-1RI	IL1R1	Adult-onset obesity	Not known	Knock-out	0.09	60.47	McGillicuddy, FC. 2013. Am J Physiol Endocrinol Metab
101	IL6	IL6	Increased BW & adiposity	Not known	Knock-out	0.73	85.98	Wallenius, V. 2002. Nat Med
102	INSR	INSR	Increased adiposity & obesity	Not known	Cre/LoxP	-2.14	1.49	Cariou, B. 2004. Endocrinol
103	IRS1	IRS1	Increase weight gain	Not known	Knock-out	-1.30	4.97	Shirakami, A. 2002. J Endocrinol
104	IRS2	IRS2	Increased adiposity	Not known	Cre/LoxP	x	x	Lin, X. 2004. J Clin Invest
105	JAK2	JAK2	Increased adiposity	Not known	Cre/LoxP (Adipose)	-0.37	28.22	Sy, S. 2014. Diabetalogia
106	KCNJ11	KCNJ11	Increased BW & adiposity	Not known	Knock-out	0.24	69.46	Kanezaki, Y. 2004. Endocr J
107	GIRK4	KCNJ5	Increased BW & adiposity	Not known	Knock-out	-0.53	20.70	Perry, CA. 2008. Proc Natl Acad Sci USA
108	KDM3A	KDM3A	Obesity	Not known	Knock-out	-0.73	14.24	Okada, Y. 2010. J Androl
109	GAL-3	LGALS3	Late-onset obesity	Not known	Knock-out	0.93	89.70	Pang, J. 2013. PLoS One
110	LIPC	LIPC	Increased adiposity	Not known	Knock-out	-0.71	14.78	Farahani, P. 2004. Obes Res
111	MC3R	MC3R	Obesity	Not known	Knock-out	-0.40	26.73	Butler, AA. 2000. Endocrinolopgy
112	MED13	MED13	Obesity	Not known	Cre/LoxP	-1.43	4.05	Grueter, C. 2012. Cell
113	MEST	MEST	Increased adiposity	Not known	Transgenic	-0.03	51.40	Takahashi, M. 2005. Am J Physiol Endocrinol Metab
114	MAGP-1	MFAP2	Increased BW & adiposity	Not known	Knock-out	-0.19	39.68	Weinbaum, JS. 2008. J Biol Chem
115	NEP (MME)	MME	Adult-onset obesity	Not known	Knock-out	-0.69	15.32	Becker, M. 2010. PLoS One
116	MMP11	MMP11	Obesity	Not known	Knock-out	-0.16	42.16	Andarawewa, KL. 2005. Cancer Res

117	MMP19	MMP19	Accelerated weight gain on HFD	Not known	Knock-out	-0.13	44.03	Pendas, AM. 2004. Mol Cell Biol
118	MRAP2	MRAP2	Obesity	Not known	Knock-out	-0.23	36.86	Asai, M. 2013. Science
119	MT1A	MT1A	Adult-onset obesity	Not known	Knock-out	0.57	81.78	Beattie, JH. 1998. Proc Natl Acad Sci USA
120	NBEA	NBEA	Increased BW & adiposity in heterozygotes	Not known	Knock-out	-1.89	1.98	Olszewski, P. 2012. PLoS Genet
121	SRC-1	NCOA1	Obesity	Not known	Knock-out	-2.19	1.39	Picard, F. 2002. Cell
122	NEIL1	NEIL1	Obesity	Not known	Knock-out	0.80	87.54	Sampath, H. 2011. Am J Physiol Endocrinol Metab
123	NGN3	NEUROG3	Obesity	Not known	Knock-out	0.24	68.98	Anthwal, N. 2013. Dis Model Mech
124	NHLH2	NHLH2	Adult-onset obesity	Not known	Knock-out	0.12	62.38	Jing, E. 2004. Endocrinology
125	NMU	NMU	Increased BW & adiposity	Not known	Knock-out	0.48	79.04	Handa, R. 2004. Nat Med
126	NPB	NPB	Mild obesity	Not known	Knock-out	x	x	Kelly, MA. 2005. Proc Natl Acad Sci USA
127	GPR7 (NPBWR1)	NPBWR1	Adult-onset obesity	Not known	Knock-out	0.46	78.46	Gu, W. 2004. J Mol Neurosci
128	NPC1	NPC1	Dose-dependent weight gain under HFD	Not known	Knock-out	-0.05	50.02	Jelinek, D. 2010. Obesity
129	NPY1R	NPY1R	Obesity	Not known	Knock-out	-0.18	39.95	Kushi, A. 1998. Proc Natl Acad Sci USA
130	NPY2R	NPY2R	Obesity	Not known	Knock-out	-0.58	18.59	Lin, D. 2006. Endocrinol
131	NPY5R	NPY5R	Increased adiposity	Not known	Knock-out	-0.14	43.29	Marsh, DJ. 1998. Nat Med
132	NR5A1	NR5A1	Adult-onset obesity	Not known	Knock-out	-0.05	50.22	Majdic, G. 2002. Endocrinol
133	LRH-1	NR5A2	Mild obesity	Not known	Knock-out	-0.29	33.20	Hattori, T. 2014. Endocr J
134	NTSR1	NTSR1	Adult-onset obesity	Not known	Knock-out	0.67	84.70	Remaury, A. 2002. Brain Res
135	OGG1	OGG1	Increased adiposity in HFD	Not known	Knock-out	0.71	85.73	Sampath, H. 2012. PLoS One
136	OMA1	OMA1	Obesity	Not known	Knock-out	1.24	93.42	Quiros, PM. 2012. EMBO
137	OSMRβ	OSMR	Increase in BW and hyperphagia	Not known	Knock-out	1.59	95.81	Gotardo, EM. 2013. J Nutr Sci Vitaminol
138	OXT	OXT	Obesity	Not known	Knock-out	0.64	83.98	Nishimori, K. 2008. Prog Brain Res

139	PARP1	PARP1	Adult-onset obesity	Not known	Knock-out	-0.97	8.98	Devalaraja-Narashimha, K. 2010. J Endocrinol
140	PCYT2	PCYT2	Obesity	Not known	Knock-out	-0.45	24.19	Fullerton, MD. 2009. J Biol Chem
141	PEG3	PEG3	Obesity	Not known	Knock-out	1.51	95.45	Curley, JP. 2005. FASEB J
142	PGP	PGP	Increased BW & adiposity	Not known	Knock-out	x	x	Foucaud-Vignault, M. 2011. PLoS One
143	PLAC 8	PLAC8	Increase in adiposity	Not known	Knock-out	-0.03	51.04	Jimenez-Preitner, M. 2011. Cell Metab
144	PLSCR1	PLSCR1	Increased adiposity	Not known	Knock-out	0.39	76.05	Zhou, Q. 2002. Blood
145	PLSCR3	PLSCR3	Increased BW & adiposity	Not known	gene-trap	x	x	Wiedmer, T. 2004. Proc Natl Acad Sci USA
146	PPARA	PPARA	Increase in adiposity	Not known	Knock-out	-0.36	29.31	Miyazaki, M. 2004. J Biol Chem
147	PPARδ	PPARG	Obesity under HFD	Not known	Knock-out	-0.31	31.93	Kocalis, H. 2012. PLoS One
148	PGC-1α	PPARGC1A	Obesity	Not known	Knock-out	-0.11	45.57	Leone, TC. 2005. PLoS Biol
149	PPIF	PPIF	Late-onset obesity	Not known	Knock-out	x	x	Luvisetto, S. 2008. Neurosci
150	PPIR3A	PPP1R3A	Increased BW & adiposity	Not known	Knock-out	2.67	98.86	Delibegovic, M. 2003. Diabetes
151	PPKAA2	PRKAA2	Increased adiposity	Not known	Knock-out	-0.67	15.62	Villena, JA. 2004. Diabetes
152	PRL	PRL	Increased BW	Not known	Knock-out	-0.16	41.64	Perez-Villamil, B. 1992. J Endocrinol
153	PRRP	PRLH	Increased BW	Not known	Knock-out	-0.30	32.62	Lawrence, C. 2002. Endocrinol
154	GPR10	PRLHR	Adult-onset obesity	Not known	Knock-out	0.46	78.46	Ishii, M. 2003. Proc Natl Acad Sci USA
155	PROX1	PROX1	Obesity in heterozygotes	Not known	Knock-out	-0.67	15.62	Harvey, NL. 2005. Nat Genet
156	PTPN11	PTPN11	Obesity	Not known	Knock-out	-0.43	25.15	Zhang, EE. 2004. Proc Natl Acad Sci USA
157	PYY	PYY	Obesity	Not known	Knock-out	0.52	80.46	Batterham, R. 2002. Nature
158	RAI1	RAI1	Obesity in heterozygotes	Not known	Knock-out	-3.68	0.25	Bi, W. 2005. Hum Mol Genet
159	RPGRIP1L	RPGRIP1L	Obesity	Not known	Knock-out	1.08	91.76	Vadnais, C. 2013. BMC Genomics
160	RSC1A1	RSC1A1	Obesity	Not known	Knock-out	1.07	91.67	Osswald, C. 2005. Mol Cell Biol
161	SFRP1	SFRP1	Increase in BW and adiposity under HFD	Not known	Knock-out	-0.47	23.04	Gauger, KJ. 2013. PLoS One
162	FATP4	SLC27A4	Obesity in homozygotes under HFD	Not known	Knock-out	-1.08	7.20	Lenz, LS. 2011. J Biol Chem
163	ZNT7	SLC30A7	Obesity in males only	Not known	Knock-out	-0.69	14.97	Huang, L. 2012. J Biol Chem

164	SOCS1	SOCS1	Liver degeneration, obesity	Not known	Knock-out	x	x	Starr, R. 1998. Proc Natl Acad Sci USA
165	SOCS3	SOCS3	Obesity under HFD	Not known	Knock-out	-0.03	51.04	Sachithanandan, N. 2010. Hepatology
166	SPARC	SPARC	Increased adiposity	Not known	Knock-out	0.31	72.38	Bradshaw, AD. 2003. Proc Natl Acad Sci USA
167	SPONDIN 2	SPON2	Obesity	Not known	Knock-out	-0.02	52.09	Zhu, LH. 2014. J Hepatol
168	STAT3	STAT3	Obesity	Not known	Cre/LoxP	-0.49	22.36	Cui, Y. 2004. Mol Cell Biol
169	STAT5B	STAT5B	Increased adiposity	Not known	Knock-out	-0.89	10.30	Gao, Q. 2004. Proc Natl Acad Sci USA
170	T-BET	TBX21	Obesity	Not known	Knock-out	-0.74	13.94	Kim, K. 2013. J Nutr Biochem
171	THRA	THRA	Increased BW & adiposity	Not known	Knock-out	-0.56	19.31	Udy, GB. 1997. Proc Natl Acad Sci USA
172	TIMP-2	TIMP2	Obesity and hyperphagia	Not known	Knock-out	-0.32	31.46	Stradecki, HM. 2011. J Neuroendocrinol
173	TNF-α	TNF	Increased BW & adiposity	Not known	Knock-out	-0.03	51.40	Salles, J. 2012. J Nutr Biochem
174	TAp63	TP63	Obesity	Not known	Knock-out	-0.91	9.96	Su, X. 2012. Cell Metab
175	TRPV4	TRPV4	Increased BW & adiposity	Not known	Knock-out	-1.30	4.95	O'Conor, J. 2013. Ann Rheum Dis
176	TXNIP	TXNIP	Increased fat to muscle ratio	Not known	Knock-out	0.08	60.31	Stubdal, H. 2000. Mol Cell Biol
177	UCP1	UCP1	Late-onset obesity with HFD	Not known	Knock-out	0.20	67.19	Kontani, Y. 2005. Aging Cell
178	WDTC1	WDTC1	Obesity in heterozygotes	Not known	Knock-out	-0.44	24.46	Hader, T. 2003. EMBO
179	ZEB1	ZEB1	Obesity	Not known	Knock-out	0.56	81.67	Saykally, JN. 2009. PLoS One
180	CDKN1B	CDKN1B	Increased adiposity	Not known	Knock-out	-0.01	53.51	Naaz, A. 2004. FASEB J
181	CRY2	CRY2	Obesity under HFD	Not known	Knock-out	-0.14	43.77	Barclay, JL. 2013. Am J Physiol Endorinol Metab
182	MT1B	MT1B	Adult-onset obesity	Not known	Knock-out	0.41	76.67	Beattie, JH. 1998. Proc Natl Acad Sci USA

Appendix 3.1: Overview psychiatric disorders

The table in this appendix gives an overview of the prevalence of different psychiatric disorders in the PMMO cohort. All participants included for this analysis were screened by a psychologist using the DSM-V criteria. Participants recruited at Royal Derby Hospital, NHS Foundation Trust and Chelsea Westminster Hospital, NHS Foundation Trust did not receive such screening and were therefore excluded (n=72), as well as other participants for which data was not available (n=12).

	Number	**Percentage of complete cohort screened (n=991)**
(history of) Psychosis	6	0.61%
Bipolar disorder	12	1.21%
Schizophrenia	4	0.40%
Autism spectrum	3	0.30%
(history of) alcohol and/or drug abuse disorder	7	0.71%
Anxiety disorder	19	1.92%
Post-traumatic stress disorder	5	0.50%
(history of) Self harm	4	0.40%
Borderline personality disorder	2	0.20%
Childhood behavioural problems	2	0.20%
Obsessive Compulsive Disorder	2	0.20%
Total	66	6.66%

Table appendix 3.1: Overview of psychiatric disorders.

Appendix 3.2 Hypercholesterolemia

The tables in this appendix summarise the hypercholesterolemia for gender, obesity classes and diagnostic criteria differences. Table 3.3.1 and 3.3.2 show the proportion of newly diagnosed cases are similar for each obesity class group. Table 3.3.3 give the mean value of total cholesterol, LDL, HDL and triglycerides separated for gender and participants receiving on not receiving statin treatment. The final table gives the proportion of cases with hypercholesterolemia that were also diagnosed with T2DM.

		Obesity classes				Total
		II	III	IV	V	
treated for	Count	20	63	20	9	112
	% within Obesity_class_4	54.1%	49.6%	29.9%	30.0%	42.9%
high cholesterol	Count	2	5	3	0	10
	% within Obesity_class_4	5.4%	3.9%	4.5%	0.0%	3.8%
none	Count	15	59	44	21	139
	% within Obesity_class_4	40.5%	46.5%	65.7%	70.0%	53.3%
	Count	37	127	67	30	261

Table appendix 3.2.1: Hypercholesterolemia stratified for diagnostic criteria in males

		Obesity classes				Total
		II	III	IV	V	
treated for	Count	31	81	28	6	146
	% within Obesity Class	28.7%	22.1%	16.4%	11.3%	20.9%
high cholesterol	Count	11	25	14	4	54
	% within Obesity Class	10.2%	6.8%	8.2%	7.5%	7.7%
none	Count	66	26	129	43	499
	% within Obesity Class	61.1%	71.1%	75.4%	81.1%	71.4%
	Count	108	367	171	53	699

Table appendix 3.2.2: Hypercholesterolemia stratified for diagnostic criteria in males

	Females, on statin treatment							Males, on statin treatment						
	Class III (n=112)		Class IV (n=28)		Class V (n=6)			Class III (n=83)		Class IV (n=20)		Class V (n=9)		
	Mean	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*P-value*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*P-value*
Cholesterol	4.6412	.12981	4.7043	.27957	4.3833	.43314	0.845	4.4733	.14224	4.0667	.28413	3.9286	.49122	0.267
LDL	2.6088	.12254	2.5557	.26817	1.7167	.14993	0.363	2.4451	.13168	2.4062	.27871	2.2386	.38351	0.861
HDL	1.2916	.05834	1.0977	.05303	1.0500	.19435	0.154	.9940	.02751	.9143	.03890	.9943	.12649	0.434
Triglycerides	2.0507	.18361	2.6486	.52051	1.7525	.36298	0.351	2.9759	.38664	2.1507	.27267	1.5157	.25799	0.251

	Females, no treatment							Males, no treatment						
	Class III (n=363)		Class IV (n=143)		Class V (n=47)			Class III (n=81)		Class IV (n=47)		Class V (n=21)		
	Mean	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*P-value*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*P-value*
Cholesterol	5.1225	.05537	5.0834	.08566	5.1735	.14548	0.508	4.9481	.15420	4.7917	.16288	4.4667	.26809	0.695
LDL	3.1801	.05196	3.0785	.08037	3.2437	.19828	0.864	2.9898	.15208	2.9283	.13616	2.7479	.23328	0.300
HDL	1.2126	.01874	1.2270	.03814	1.3318	.11140	0.212	1.3252	.18691	.9871	.03281	1.0729	.05733	0.292
Triglycerides	1.7093	.07169	1.6563	.07998	1.5894	.13726	0.784	**2.1550**	.15759	1.8262	.14876	**1.2727**	.12321	0.009

Table appendix 3.2.3: Lipid levels, segregated for gender, statin treatment and obesity class.

BMI class	Type 2 diabetes	Hypercholesterolemia		
		Yes	No	P-value
II	No	24.0%	67.0%	0.027
	Yes	76.0%	33.0%	
III	No	24.8%	75.0%	0.000
	Yes	75.2%	25.0%	
IV	No	29.8%	78.3%	0.000
	Yes	70.2%	21.7%	
V	No	26.7%	66.7%	0.000
	Yes	73.3%	33.3%	

Table appendix 3.2.4: Percentage of cases with hypercholesterolemia that are also diagnosed with T2DM.

Appendix 3.3: Questionnaires results online vs. on paper

The tables in this appendix show the differences in questionnaire results between the questionnaires filled in on paper and the questionnaires filled in online. No significant differences were seen in any of the questionnaires.

	Questionnaire response: Paper vs. Online		
	Mean difference	SEM	P value*
SF36 - General Health	-8.39	-8.39	0.052
SF36 -limitations due to physical health	-5.66	5.91	0.339
SF36 -limitations due to emotional health	-8.62	5.97	0.151
SF36 - Physical functioning	-5.63	3.85	0.144
SF36 - Energy/fatigue	-4.70	2.88	0.105
SF36 - Emotional well being	-1.09	3.05	0.722
SF36 - Social functioning	-2.95	4.10	0.473
SF36 - Pain	-4.49	3.91	0.252
IWQOL - Physical function	4.45	1.50	0.051
IWQOL - Self-esteem	2.89	1.14	1.00
IWQOL - Sexual life	1.87	0.77	0.212
IWQOL - Public distress	3.94	1.21	0.074
IWQOL - Work problems	3.19	1.53	0.505
*Adjustment for multiple comparisons: Bonferroni. Corrected for gender, BMI, ethnicity and age			

	Questionnaire response: Paper vs. Online		
	Mean difference	SEM	P value*
DEBQ-Restraint	-1.04	1.06	0.327
DEBQ-Emotional	-0.53	1.90	0.780
DEBQ-External	1.65	1.00	0.100
TFEQ-Restraint	-0.74	0.58	0.204
TFEQ-Disinhibition	0.89	0.48	0.067
TFEQ-Hunger	0.91	0.52	0.082
EDEQ-Restraint	0.01	0.22	0.956
EDEQ-Weight	0.48	0.18	0.068
EDEQ-Eating	0.27	0.26	0.291
EDEQ-Shape	0.64	0.20	0.059
*Adjustment for multiple comparisons: Bonferroni. Corrected for gender, BMI, ethnicity and age.			

	Questionnaire response: Paper vs. Online		
	Mean Difference	SEM	P value*
PANAS – positive	1.74	1.36	0.201
PANAS – negative	0.90	1.35	0.507
HADS – anxiety	0.40	0.73	0.587
HADS - depression	1.28	0.73	0.083
*Adjustment for multiple comparisons: Bonferroni. Corrected for gender, BMI, ethnicity and age.			

Appendix 3.4: Main ethnic groups and IWQOL

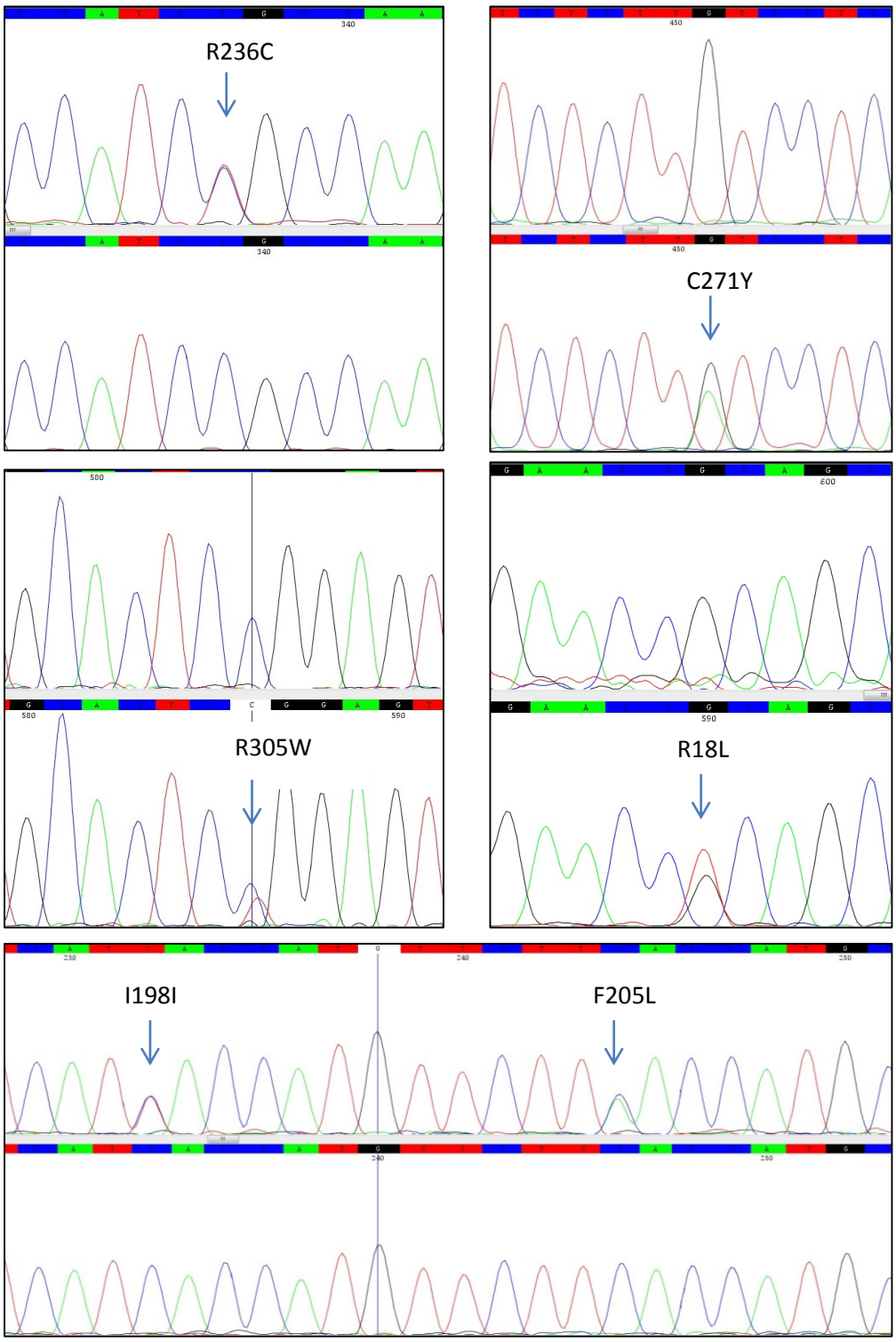
In the table in this appendix the three main ethnic groups included in the PMMO cohort (European Caucasian [n=143], Indian [n=11] and Caribbean [n=15]) are compared regarding weight related quality of life using the IWQOL questionnaire. No significant differences were seen between the three main groups.

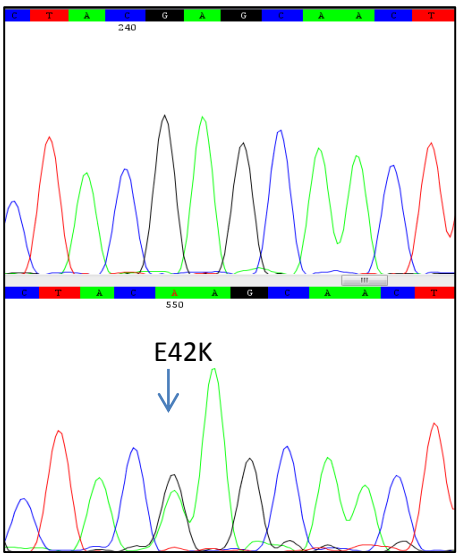
Dependent Variable	Main ethnicity groups		Mean Difference	SEM	p-value*
Physical functioning	European Caucasian	Indian	-10.22598	7.42418	0.510
		Caribbean	4.81534	6.43161	1.000
	Indian	European Caucasian	10.22598	7.42418	0.510
		Caribbean	15.04132	9.45478	0.340
Self-esteem	European Caucasian	Indian	-7.02646	9.01762	1.000
		Caribbean	11.57993	8.34365	0.501
	Indian	European Caucasian	7.02646	9.01762	1.000
		Caribbean	18.60639	11.84949	0.354
Sexual life	European Caucasian	Indian	-7.06378	10.67006	1.000
		Caribbean	4.48925	9.24672	1.000
	Indian	European Caucasian	7.06378	10.67006	1.000
		Caribbean	11.55303	13.57436	1.000
Public distress	European Caucasian	Indian	-9.64902	7.75804	0.646
		Caribbean	7.55877	6.93754	0.832
	Indian	European Caucasian	9.64902	7.75804	0.646
		Caribbean	17.20779	10.02405	0.263
Work problems	European Caucasian	Indian	-2.84628	8.09334	1.000
		Caribbean	11.52872	6.92616	0.294
	Indian	European Caucasian	2.84628	8.09334	1.000
		Caribbean	14.37500	10.25585	0.489

Table Appendix 3.6: IWQOL and the main different ethnic groups included in the cohort.

* Corrected for gender, BMI, Type 2 diabetes mellitus diagnosis and age.

Appendix 5.1: Chromatograms of *MC4R* mutations (Chapter 5)

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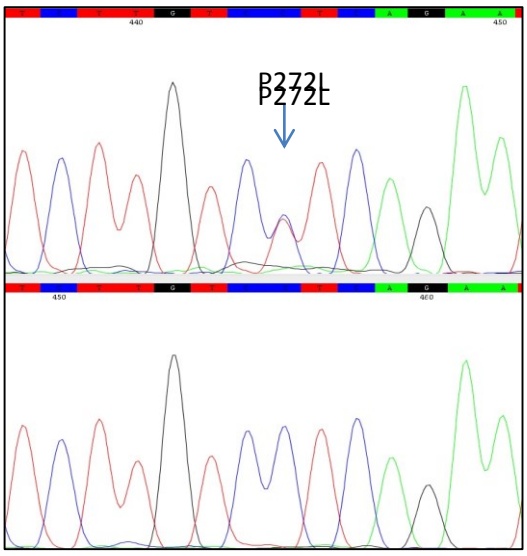
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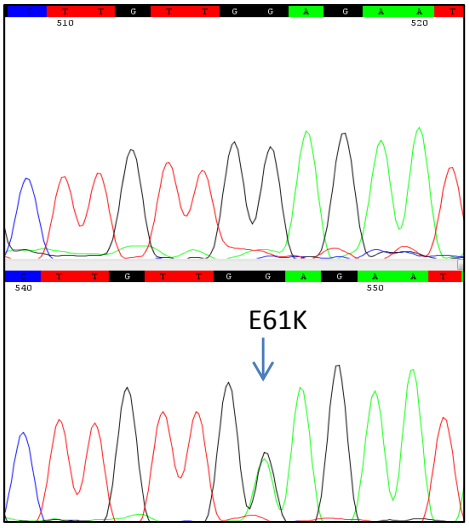
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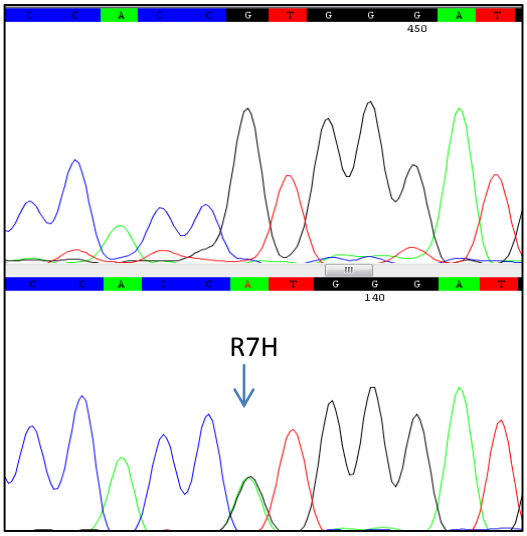
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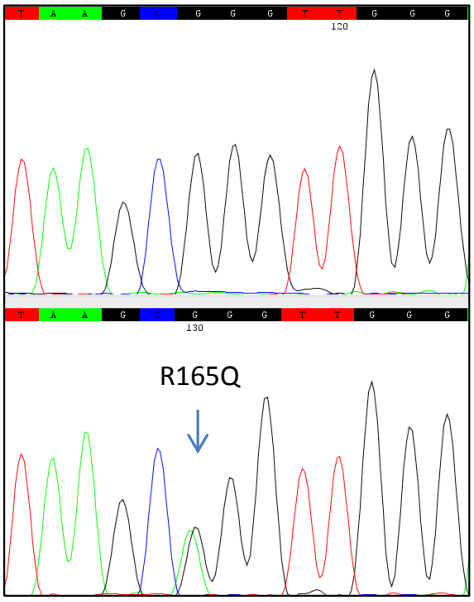
R165Q

Appendix 6.1: Chromatograms of *MC4R* variants (Chapter 6)