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Research article

## Novel Mutations in Obesity-Related Genes in Turkish Children with Non-Syndromic Early Onset Severe Obesity: A Multicentre Study

**Short Title: Novel Mutations in Non-Syndromic Early Onset Severe Obesity**

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### Abstract

**Objective:** Non syndromic monogenic obesity is a rare cause of early onset severe obesity in the childhood period. This form may not be distinguishable from other forms of severe obesity without genetic analysis, particularly if patients do not exhibit any physical abnormalities or developmental delay. The aim of this study is to screen 41 different obesity-related genes in children with non-syndromic early onset severe obesity.

**Methods:** Children with severe (BMI-SDS>3) and early onset (<7 years) obesity was screened by next-generation sequencing based targeted DNA custom panel for 41 known-obesity-related genes and results were confirmed by Sanger technique.

**Results:** Six novel variants were identified in five candidate genes in seven out of 105 children with severe obesity; two in *SIMI* (p.W306C and p.Q36X), one in *POMC* (p.Y160H), one in *PCSK1* (p.W130G fs Ter8), two in *MC4R* (p.D126E) and one in *LEPR* (p.Q4H). Additionally, two previously known variations in *MC4R* were determined in four patients (p.R165W in three, and p.V166I in one).

**Conclusion:** We identified six novel and four previously described variants in six obesity-related genes in 11 out of 105 children with early onset severe obesity. The prevalence of monogenic obesity was found as %10.4 in our cohort.

**Keywords:** severe obesity, novel mutations

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## **Introduction**

Common forms of obesity are caused by a combination of environmental and behavioral factors, together with an underlying genetic predisposition to obesity. The etiology of childhood obesity is multifactorial, and non syndromic early-onset severe obesity is usually monogenic, while other forms of obesity are polygenic and occur due to the cumulative effect of multiple susceptibility genes which regulate energy intake and expenditure. It has been reported that non-syndromic monogenic obesity is very rare, not exceeding 7% of childhood obesity cases (1-3). However, this ratio varies with ethnic characteristics and the proportion of consanguineous couples within any given population. To date mutations in several genes have been described which cause the development of early-onset severe obesity in children although, with advances in genetic testing, more genetic causes of obesity are being identified. Most of these genes such as *LEP*, *LEPR*, *SIM1*, *POMC*, *PCSK1*, *MC4R* are involved in the central regulation of satiety via the leptin-melanocortin signaling pathway. Therefore variants in any of these genes cause overt changes in food intake, body weight and energy expenditure and are also associated with some forms of neuroendocrine and immun dysfunction (4-6).

Syndromic obesity is usually diagnosed clinically with features such as hyperphagia, early-onset severe obesity, developmental delay or other findings caused by defects in the responsible gene. However, it may not be possible to diagnose the underlying genetic defect solely on the basis of clinical findings in some types of monogenic obesity. For example, mutations in the *MC4R* lead to the most prevalent form of monogenic obesity, and because the clinical features resemble those found in exogenous obesity, differential diagnosis can only be confirmed by detection of genetic variants (4-7). Although, treatment options are limited in early-onset severe obesity, with the exception of leptin deficiency due to leptin gene mutations, new specific drugs will offer a novel therapeutic option for those patients with monogenic obesity due to *MC4R* or *POMC* dysfunction (8,9). Consequently, genetic testing should be offered in children with early onset severe obesity as they may be suitable candidates for current or promising new drugs such as MC4R agonists. The present study, therefore, has been conducted to assess the variants of 41 different obesity-related genes in Turkish children with non-syndromic early onset severe obesity.

## **Material and Methods:**

The study population was selected from severe obese patients referred to our center for genetic analysis from different centers in geographically diverse parts of Turkey. Inclusion criteria for children and adolescents were obesity onset less than seven years of age, and a BMI-SDS >3. Patients taking any drugs or followed up with any specific endocrine disorders such as Cushing syndrome or hypothyroidism and those with syndromic features were not included in the study. The study protocol was approved by the regional ethical committees (Malatya Clinical Research Ethics Committee, 21.01.2018, no:2018-20), and informed consent was obtained from the parents of all the children before their participation.

### **Anthropometric Measurements:**

All patients were examined in the morning after an overnight fasting. Height and weight were measured by experienced nurses from the pediatric endocrinology-outpatient clinic. BMI was calculated as body weight in kilograms divided by the square of height in meters. BMI and BMI-SDS were calculated using age and gender specific percentiles of Turkish children from established reference data (10).

### **DNA Preparation:**

Genomic DNA was isolated from peripheral blood mononuclear cells (PBMCs) using the QiAmp DNA Blood Mini Kit (cat. no. 51106, Qiagen, Hilden, Germany). DNA purity and quality was confirmed by agarose gel electrophoresis. DNA concentration was measured by Qubit (Life Technologies, Singapore). Before the library preparation, appropriate dilution was made for each sample.

### **Next Generation Sequencing:**

Sequencing libraries were prepared according to the manufacturer's instructions using CDHS-1346Z-901 QIASeq™ Targeted DNA Custom Panel (ref. no. 333525, Qiagen, Hilden, Germany) that includes all exomes with 10bp exon-intron junctions of 41 target genes (*DYRK1B*, *LEP*, *LEPR*, *MC4R*, *NROB2*, *POMC*, *UCP3*, *ADRB2*, *ADRB3*, *AGRP*, *MC3R*, *NTRK2*, *PCSK1*, *SIMI*, *CARTPT*, *ENPP1*, *PPARG*, *PPARGC1B*, *PYY*, *SDC3*, *UCP1*, *ADIPOQ*, *NAMPT*, *CFD*, *RETN*, *PPARGC1A*, *CCK*, *NPY*, *SLC2A4*, *ADD1*, *SREBF1*, *PTPNI*, *IRS-1*, *GHRL*, *BDNF*, *NEGR1*, *SH2B1*, *GIPR*, *TMEM18*, *FTO*, *SLC22A1*). Briefly, the samples were enzymatically fragmented and molecularly barcoded and passed through the stages of library generation, target enrichment, sample indexing and, amplification. The concentration of each library was determined by using QIAseq Library Quant Assay Kit (ref.no. 333314, Qiagen, Hilden, Germany) according to the manufacturer's instructions. Each library was diluted into 4 nM, and pooled in equimolar ratio. The final pool was denatured with freshly prepared 0.2 N NaOH and then diluted to 20 pM and sequenced as 251x2 bp paired-end chemistry according to the sequencer manufacturer's instructions (MiSeq, Illumina, San Diego, CA) (11).

### **Sequencing Data Analysis**

Demultiplexed FASTQ files were processed individually using Qiagen Bioinformatics solutions. Secondary analysis was performed by using Qiagen, QCI Analyze Universal 1.5.0. Tertiary analysis and interpretation were performed using Qiagen Clinical Insight Interpret (All programs from Qiagen, Hilden, Germany).

### **Sanger Sequencing:**

Detected variants were also analysed and confirmed by Sanger sequencing according to the manufacturer's protocols. The amplicons were analyzed by direct sequencing with ABI 3500 (Life Technologies, Waltham, Massachusetts, USA). Analysis of sequence results was done by Mutation Surveyor Programme (SoftGenetics, USA).

### **Data Analyzing:**

Mutations and/or polymorphisms were screened for using next-generation sequencing. All the genes that were investigated have various roles in energy homeostasis such as energy intake, energy expenditure, adipose tissue functions and glucose metabolism. Genetic variant pathogenicity was examined using the following standard *in silico* analyses; MutationTaster, PolyPhen-2, CADD, Stratum and I-Mutation-2.0: prediction. novel mutations detected were verified by Sanger sequencing (12-14).

### **Results:**

A total of 105 patients meeting the inclusion criteria were included in the study. *Table 1* shows the key clinical and genetic characteristics of the children carrying the obesity-related gene variations. We described six novel mutations in five candidate genes in seven out of the 105 patients, and previously described mutations in *MC4R* were detected in four patients. The novel variations detected were two in *SIMI*, one in *POMC*, one in *PCSK1*, one in *LEPR* and two in *MC4R*, *Table 1*. Family members of these affected children were also genetically screened for the same pathogenic variants. Family pedigrees of the children carrying the novel variations are shown in *Figure 2*.

### **Genetic Results:**

*Table 1* shows the key clinical characteristics of the patients carrying novel mutations. We identified six novel mutations potentially contributing to the subject's severe obesity. In patient 1, a novel homozygous *SIMI* variant (p.W306C, c.918 G>T in exon 8) was detected. He was two years and three months of age at the time of the study, his BMIS-DS was 5.6, and obesity onset age was one and a half years. His birth weight was 2900 g. He had no any other endocrinological or developmental abnormalities. His parents were second degree relatives. His father and mother were also obese and both were heterozygous for the same variation.

The second patient (Patient 2) carrying a novel heterozygous *SIMI* variant (p.Q36X, c.106 G>T in exon 1) was five years old, her birth weight was 2300 g, her BMI-SDS was 4.7, and she had severe obesity from two years of age. Her growth velocity and developmental history were normal, and she has no additional endocrinological or developmental abnormalities. There was no consanguinity in her family, her father was obese and heterozygous for the same variants.

A novel heterozygous *POMC* variant (p.Y160H, c.478 T>C in exon 3) was detected in a male patient who was 14 years old (patient 3). He had no other abnormalities except severe obesity and hyperphagia. There was no consanguinity in his family, but obese mother was heterozygous for the same variation.

In patient 4, a novel heterozygous *PCSK1* variant (p.W130G fs Ter8, c.388delT) was detected. He was two years and four months old, and had no any endocrinological abnormalities. His parents were not obese and have no any genetic variation.

Two siblings in the same family (Patient 5 and 6) were homozygous for a novel *MC4R* variant (p.D126E, c.378C>A in exon 1). They had severe obesity, intractable hyperphagia and accelerated growth which is typical for *MC4R* deficiency. Their parents were severely obese and close relatives, and they were heterozygous for the same variation.

The last female patient (Patient 7) was 14 years old, severely obese and heterozygous for a novel *LEPR* variant (c.12A>C, p.Q4H in exon3) (Figure1). Her obese father was heterozygous for the same variant. In addition to these novel variants, previously described mutations in *MC4R* in four patients (p.R165W, c.493C>T in exon 1 in three of four, and p.V166I, c.496G>A in exon 1 in one) (15,16).

#### **Discussion:**

In this study, we searched for variants in 41 genes which are known to be involved in causing obesity in patients with non-syndromic early onset severe obesity. Two novel *SIMI* variants in two unrelated patients, a novel *POMC* variant, a novel *PCSK 1* variant, two siblings with the same *MC4R* variant and a novel *LEPR* variant were identified in our cohort. Single-minded-1 gene (*SIMI*) is located on chromosome 6q16.3-q21 and consists of 11 exons spanning 75kb. *SIMI* encodes a hypothalamic transcription factor in the bHLH-PAS (basic helix loop helix/ Per Arnt Sim) family. Its main function has been described as the formation of the paraventricular nucleus of the hypothalamus which is critical for food intake regulation. *SIMI* also plays an important role in the regulation of energy homeostasis by interacting with the melanocortin signalling pathway and loss-of-function variants in this gene are one of the few known causes of monogenic obesity in both humans and mice (17,18). Recently, it has been reported that chromosomal abnormalities such as translocation between chromosome 1p22.1 and 6q16.2, deletion of the 6q16.2 region and heterozygous point mutations in *SIMI* region are responsible for early-onset severe obesity in humans (19-21). In these reports, patients had increased fat mass with increased body fat percentage in addition to hyperphagia, increased linear growth, learning disabilities, and Prader-Willi like phenotype. Experimentally, it has been observed that homozygous *Sim1* knockout mice (*sim1*  $-/-$ ) do not survive due to lack of the hypothalamic neurons which produce multiple neuropeptides including oxytocin, vasopressin, corticotropin-releasing hormone, thyrotropin-releasing hormone, and somatostatin. However, heterozygous mice (*Sim1*  $+/-$ ) develop partial failure of hypothalamic neurons resulting in hyperphagia and obesity similar to *mc4r*-mutant mice (22). In our study group, we described one patient with a homozygous missense *SIMI* variant (p.W306C, c.918 G>T in exon 8) and another patient with heterozygous nonsense *SIMI* variant (p.Q36X, c.106 G>T in exon 1). The homozygous patient had severe obesity due to hyperphagia from eighteen months of age, and his obese parents were also heterozygous for the same *SIMI* variant. This p.W306C variant is located in the PAS domain, which has a critical role in *SIMI* activity (23). Stratum and I-Mutant 2.0 prediction analysis suggest that the Gibbs free energy ( $\Delta\Delta G$ , DDG) value of this mutant protein would be -1.7 and CADD score was 35 indicating a decrease in the stability of the mutant protein structure. Therefore, this variant is likely to be pathogenic because of changes in the protein structure and redox status leading to reduced *SIMI* activity. Previously, pathogenic variants have been described in this region (23-25), and it appears that this new variant located in the same region is also pathogenic. In addition, and contrary to what might be expected, identification of accelerated growth on his physical examination and the resemblance of phenotype to the *MC4R* variants led us to hypothesise that this *SIMI* variant might induce considerable functional loss in *MC4R* activation, but functional studies would be required to confirm this.

The mother of the Patient 2 in whom a novel heterozygous nonsense *SIMI* variants (p.Q36X, c.106 G>T in exon 1, CADD score: 37) was identified did not carry the same variant, whereas his obese father was haploinsufficient for p.Q36X. This new variant located in the bHLH domain of the *SIMI* is predicted to play a significant role in DNA dimerization and binding, so it is likely to be pathogenic according to Polyphen-2 and CADD analysis. In addition to the critical location of this variant, its pathogenicity is enhanced because it also produces a premature stop codon resulting in a truncated protein. Previously, a loss-of-function, heterozygous *SIMI* variant (T46R) was described in the same region (24,25). Although, most of the heterozygous *SIMI* variants that cause obesity have been described as causing growth retardation and Prader-Willi-like syndrome in addition to the accompanying obesity (24-25), developmental and intellectual capacity was normal in our patient.

Proopiomelanocortin (POMC) is produced by the POMC/CART (cocaine and amphetamine-related transcript) neurons in the hypothalamus, and is the precursor of adrenocorticotrophic hormone (ACTH), beta-endorphin, beta-lipotropin (beta-LPH), corticotropin-like intermediate peptide (CLIP) and  $\alpha$ -,  $\beta$ -, and  $\gamma$ -melanocyte-stimulating hormones (MSH) some of which regulate melanin synthesis, adrenal functions and inhibits food intakes through interaction with the *MC4R* signalling pathway (26-28). Homozygous loss-of-function mutations in *POMC* have been reported to be very rare and a cause of severe obesity, ACTH deficiency and hypopigmentation in mice and humans (29,30). It is suggested that the *MC4R* signalling pathway is affected secondarily to the impairment of interaction with *MC4R* and  $\alpha$ -MSH in heterozygous missense *POMC* variants without complete *POMC* deficiency, and subsequently severe obesity develops in humans (29-31). In this study, a novel heterozygous *POMC* variant (p.Y160H) was described in a patient with early onset severe obesity whose obese mother was also affected by the same variant. This variant was located in the CLIP region of the ACTH domain of *POMC*. The DDG value of this mutant protein was -1.62 kJ/mol, predicted by Stratum and I-mutant 2.0 analysis, and CADD score was 25.8 leading

to a decrease in the stability of the mutant protein. PolyPhen-2 analysis predicted that this novel variant is likely to be pathogenic. Although the function of CLIP is not fully understood in humans, it is considered that variants affecting this region may confer the phenotype through an altered MC4R signalling pathway.

The proprotein convertase subtilisin/kexin type 1 gene (*PCSK1*) encodes the prohormone convertase enzyme (PC1/3) and is abundantly expressed in hypothalamus (32). PC1/3 deficiency is described as an autosomal recessive disorder. Although heterozygous PC1/3 deficiency is associated with obesity, homozygote loss-of-function mutations usually present with early onset severe obesity and hyperphagia in addition to malabsorptive diarrhea in neonatal period, central diabetes insipidus, reactive hypoglycemia and hypoadrenalism (33-35).

However, the described phenotype may be variable depending on which parts of the *PCSK1* gene structure have been affected. In Patient 4, a novel heterozygous frameshift *PCSK1* variant (p.W130G fsTer8, C388delT) was found, however the same variant was not present in his parents. This novel variant is located in a catalytic domain of *PCSK1* and leads to a frameshift mutation and deletion followed by stop-codon that is predicted to produce non-functional truncated protein. Its CADD score was 36. It has been described that pathogenic variants within the same domain reduce the PCSK1 activity (34,35). Therefore, it seems highly likely that this novel variant would be pathogenic.

MC4R is the receptor for  $\alpha$ -MSH and plays a key role in controlling energy homeostasis, food intake and satiety. *MC4R* mutations are the most common genetic cause of monogenic obesity and also contribute in polygenic forms. Loss-of-function *MC4R* mutations are associated with early onset severe obesity due to hyperphagia, hyperinsulinemia and increased linear growth. Currently more than 150 variants have been identified, and the prevalence of pathogenic *MC4R* variants reported in various obese populations is highly variable, ranging from 0.5% to 6%. (1, 36-38). We found a novel homozygous *MC4R* variant, D126E, in exon 1 in two siblings. This mutation is located on the helical transmembrane domain/putative ligand binding site (NCBI-search tool). Its DDG value was -1.33 kJ/mol predicted by Stratus and I-Mutation 2 prediction, and CADD score was 24.5 suggesting a possible decrease in the function of the mutant protein. This variant may lead to a decrease in binding capacity of MC4R to  $\alpha$ -MSH, as previously described in the pathogenic variants, I137T, R165W and G98R located in the same region of *MC4R* (39-42). Thus this novel variant, D126E, is likely to be pathogenic. Our affected siblings were extremely obese, and they had increased height velocity for age. Their parents were heterozygous for the same variant and, they were also severely obese. Additionally, we found two different previously described mutations in *MC4R* in four patients (p.R165W, c.493C>T in exon 1 in three, and p.V166I, c.496G>A in exon 1 in one), *Table 1*.

Leptin and *LEPR* mutations are associated with early onset severe obesity, severe hyperphagia, and some neuroendocrine abnormalities such as hypogonadotropic hypogonadism, impaired growth hormone secretion and hypothalamic hypothyroidism (43,44). Patient 7 had a novel heterozygous *LEPR* mutation (p.Q4H, c.12A>C in exon 3, Figure 1). She was severe obese and had no any endocrinopathy. The heterozygous *LEPR* variant detected in this patient is located in the signal peptide and may destroy protein synthesis and/or processing (sorting and location). Its DDG was -1.13 kJ/mol, signifying a decrease in protein stability and CADD score was 10. Previously, deletions causing dysfunction in the signal peptide located in the extracellular domain of *LEPR* have been reported (44). Although it is hard to speculate about this variant, without performing analysis to confirm abnormal protein processing, the patient's phenotype and heterozygosity of the obese father for the same variant led us to suppose that this novel variant is most likely pathogenic. However, definitive functional analysis should be performed to confirm pathogenicity.

In literature, there are few similar studies detecting too many obesity-related genes with a targeted DNA custom panel. In a recent one by Foucan L et al, 59 obesity-related genes were screened by next-generation sequencing in 25 obese children in Guadeloupe Island, and they found 5 mutations in *MC4R*, *SIM1*, *SH2B1* and *NTRK2* genes (45). The prevalence of monogenic obesity in this cohort was 10% which is similar to the present study.

In conclusion, we identified six different novel variants within five obesity-related genes (*SIM1*, *POMC*, *PCSK1*, *MC4R* and *LEPR*) in seven out of 105 children with early onset severe obesity in Turkish population. Additionally, we found previously known mutations in *MC4R* gene in four patients, thus monogenic obesity prevalence is determined as %10.4 in our cohort. In order to understand whether these novel variants are specific to Turkish community in which consanguineous marriages are common, more broad-based genetic screening is needed.

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**Conflicts of interest:** The authors state that there is no conflict of interest.

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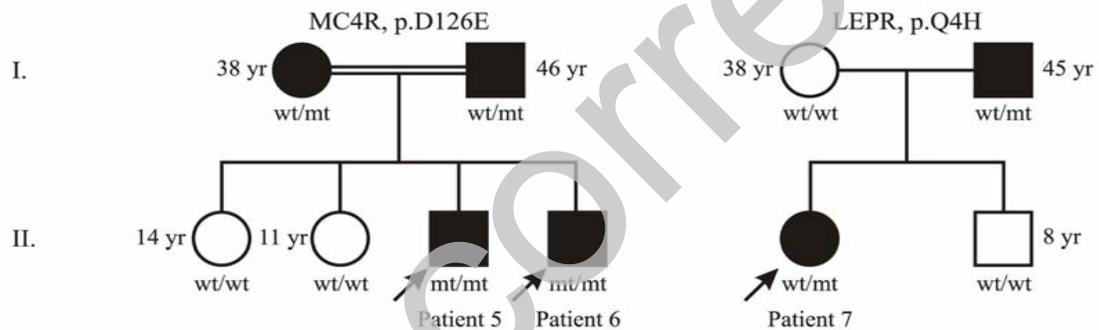
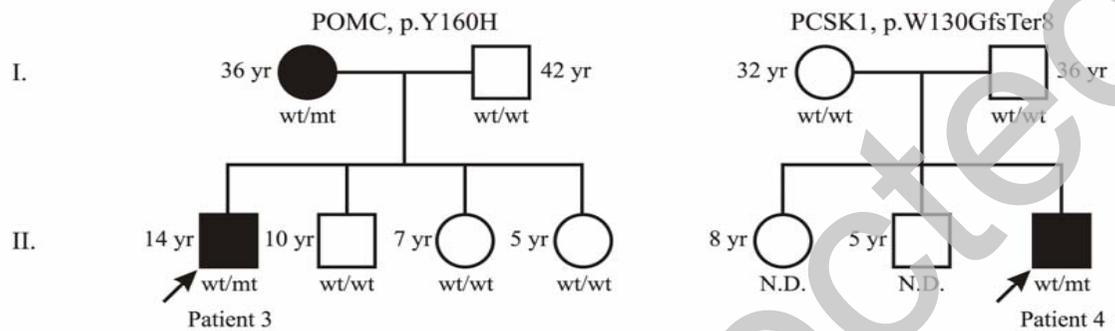
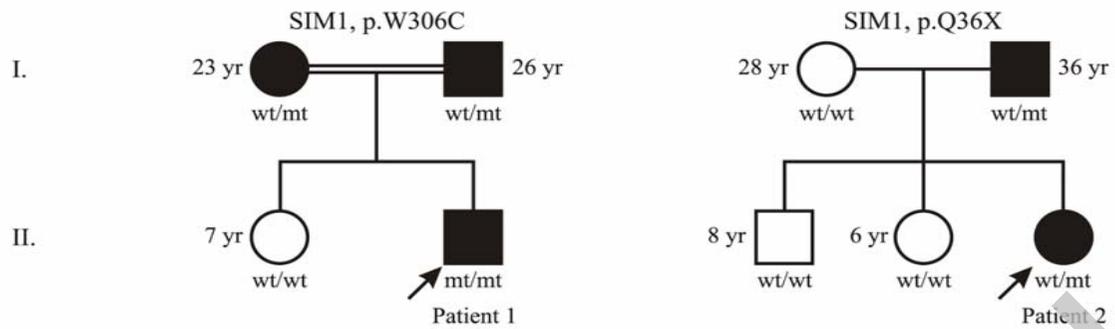
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**Figure 2.** Pedigrees of the families bearing novel variants in obesity related genes. Arrows indicate probands in each families. Genotypes were defined as wild type (wt) or mutant (mt) for corresponding variations.

Uncorrected proof



**Table 1.** Clinical and genetic characteristics of the patients with obesity-related gene variations

Patient no,	Birth weight (gr)	Obesity onset (yrs)	Current age (yrs), gender, ethnicity	BMI SDS	Height SDS	Mutant gene	Zygoty /Variation/ protein change	Functional prediction	Clinical findings	Consanguinity	Parents' BMI (Father/mother)	Parents' zygosity (Father/mother)
1	2900	1.5	2.5, M, Kurdish	5.6	1.8	SIM1 Exon 8	Homozygous c.918G>T p.W306C	Disease causing	hyperphagia	yes	36/29	Heterozygous/Heterozygous
2	2300	2	5, F, Turkish	4.7	0.3	SIM1 Exon 1	Heterozygous c.106G>T PQ36X	Disease causing	hyperphagia	no	35/28	Heterozygous/wild
3	4000	2	14, M, Turkish	3.2	0.4	POMC Exon 3	Heterozygous c.478T>C p.Y160H	Disease causing	hyperphagia, hyperlipidemia	no	27/47	wild/heterozygous
4	2600	1	2.4, M, Turkish	3.6	-0.8	PCSK1 Exon 3	Heterozygous c.388delT p.W130G fsTer8	Disease causing	hyperphagia, IR	no	21/32	Wild/ wild
5	3250	1	6, M, Turkish	6.5	2.4	MC4R Exon 1	Homozygous c.378C>A p.D126E	Disease causing	hyperphagia, IR, hyperlipidemia, hepatosteatosis	yes	38/37	Heterozygous/Heterozygous
6	2250	1	9, M <i>Brother of patient 5</i>	3.9	3.2	MC4R Exon 1	Homozygous c.378C>A p.D126E	Disease causing	hyperphagia, IR, hyperlipidemia, hepatosteatosis	-	-	-
7	3200	2	16, F, Turkish	3.4	1.8	LEPR Exon 3	Heterozygous c.12 A>C p.Q4H	Disease causing	hyperphagia, IR, hyperlipidemia	no	33/26	Heterozygous/wild
8	3100	2.5	11,F, Turkish	3.1	2	MC4R Exon 1	Heterozygous c.493 C>T p.R165W	Previously described (R)	Hyperphagia ,hyperlipidemia,hepatosteatosis	no	35/26	Heterozygous/wild

9	3200	1	4, M, Turkish	3.2	2.2	MC4R Exon 1	Heterozygous c.496 G>A p.V166I	Previousl y described (R)	Hyperphagia , Hyperlipide mia, hepatosteato sis	no	28/33	Wild/heterozy gous
10	3000	3,5	7.5, F, Syrian	3.5	1.9	MC4R Exon 1	Heterozygous c.493 C>T p.R165W	Previousl y described (R)	hyperphagia, IR, hyperlipide mia, hepatosteato sis	yes	32/ 25	Heterozygous/ wild
11	4100	2	9, M, Turkish	3.6	2.1	MC4R Exon 1	Heterozygous c.493 C>T p.R165W	Previousl y described (r)	hyperphagia, IR, hyperlipide mia	yes	43/ 26	Heterozygous/ wild

IR; insulin resistancy ( defined as HOMA-IR >3.5; HOMA-IR= *fasting glucose (mmol/L) x fasting insulin mIU/ml/22.5*)