Clinical Chemistry 51:8
1358–1364 (2005)

Molecular Diagnostics and Genetics

Six Novel Mutations in the Proopiomelanocortin and Melanocortin Receptor 4 Genes in Severely Obese Adults Living in Southern Italy

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Background: **The genetic characterization of obese individuals could clarify the molecular mechanisms underlying body weight regulation and lead to targeted therapy. Here we report variants of the proopiomelanocortin (*POMC*) and melanocortin receptor 4 (*MC4R*) genes detected in severely obese adults living in southern Italy.**

Methods: **A total of 196 unrelated nondiabetic severely obese individuals [111 females and 85 males; mean (SD) age, 32.2 (11.5) years; mean body mass index, 48.8 (8.1) kg/m^2] and 100 normal-weight healthy volunteers (34 males and 66 females) entered the study. *POMC* and *MC4R* were genotyped by sequencing analysis. Leptin, insulin, glucose, and the lipid profile were measured in fasting serum samples. We used the protein truncation test to verify the stop-codon mutation. Anthropometric measurements, sitting blood pressure, and heart rate were also recorded.**

Results: **Of the obese participants, 1.5% had mutations in *POMC* exon 3 (new mutations, P231L and E244X; known, R236G) and 2.5% had MC4R mutations (new mutations, W174C, Q43X, S19fsX51, and I317V; known, A175T). These mutations were not present in the controls. Gene polymorphisms were identified in similar percentages of severely obese and nonobese individuals, i.e., respectively, 52.5% and 51% (*POMC*) and 1% and 2% (*MC4R*).**

Conclusions: **We detected 2 new *POMC* mutations and 4 new *MC4R* mutations in a large number of severely obese adults living in southern Italy. These mutations, not present in normal-weight individuals, are further evidence that defects in the melanocortin pathway are related to severe obesity.**

Obesity is caused by a dysregulation in energy homeostasis that promotes an increase in body fat stores *(1)*. The neuroendocrine system regulates energy balance by controlling appetite, as well as food intake and utilization *(2, 3)*. Leptin, a cytokine-like peptide produced by adipose tissue in proportion to adipose mass, acts on the melanocortin system, inducing proopiomelanocortin (POMC)[6] synthesis in the hypothalamic arcuate nucleus *(4)*. The action of POMC-derived peptides, α-, β-, and γ-melanocyte–stimulating hormone (MSH), and adrenocorticotropic hormone (ACTH), is mediated by a family of guanosine triphosphate–binding protein receptors, one of which, melanocortin receptor 4 (MC4R), is produced at high concentrations within the central nervous system and plays an important role in the control of food intake and energy balance *(4)*. The first compelling evidence that the *POMC* gene is involved in the control of human appetite came from a report of 2 defects in ACTH synthesis and in *POMC* gene translation in 2 children affected by hyperphagia and uncontrolled obesity *(5)*. Investigation of other *POMC* mutations confirmed the link between this

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Received January 5, 2005; accepted May 4, 2005.
Previously published online at DOI: 10.1373/clinchem.2005.047886

[6] Nonstandard abbreviations: POMC, proopiomelanocortin; MSH, melanocyte-stimulating hormone; ACTH, adrenocorticotropic hormone; MC4R, melanocortin receptor 4; 3′-UTR, 3′ untranslated region; and BMI, body mass index.

gene and obesity in humans and mice *(4, 6–9)*. Increased food intake, obesity, and hyperinsulinemia are features of *MC4R* knock-out mice *(10, 11)*, and various *MC4R* missense and nonsense mutations have been reported in patients with severe early-onset obesity *(12–15)*. These findings prompted us to study the *POMC* and *MC4R* genes in relation to serum leptin concentrations and to anthropometric measurements in a large group of severely obese adults from southern Italy. Our aims were to identify mutations in susceptibility genes in relation to obesity and to identify subgroups in whom major genes exert greater phenotypic effects *(16)*.

Materials and Methods

PARTICIPANTS

The obese group included 196 unrelated severely obese individuals [111 females (56.6%) and 85 males (43.4%); 17–70 years of age] recruited by the outpatient clinic of the Department of Clinical and Experimental Medicine, University "Federico II", Naples, Italy. Except for a 28-year-old African woman who had lived in Italy for the past 20 years, all participants were Caucasian and had lived in southern Italy for at least 3 generations. Inclusion criteria were no diabetes, no coronary heart disease, and severe obesity classified as body mass index (BMI; weight/height^2) >40.0 kg/m^2. For all participants we obtained the following measurements: BMI, waist and hip circumference (in cm), and fat and fat-free mass by impedentiometry *(17)* (STA/BIA; Akern). The general characteristics of the obese group are reported in Table 1. Of the obese participants, 91.2% had a familial history of disease: obesity, 26.3%; obesity plus hypertension plus diabetes, 2.1%; hypertension, 14%; diabetes, 5.3%; hyperlipidemia, 2.3%; and neoplasia, 1.2%. As controls, 100 (34 males and 66 females) normal-weight healthy individuals (BMI <25 kg/m^2; age range, 26–76 years) were recruited from the Laboratory Medicine Hospital and underwent genetic testing for obesity. These controls were also tested to calculate the reference values for serum leptin.

Written informed consent was obtained from all participants. The research was approved by the Ethics Committee of the School of Medicine, University of Naples "Federico II" and was in accordance with the principles of the Helsinki II Declaration.

Table 1. General and biochemical characteristics of severely obese adults, living in southern Italy, who were studied (n = 196).[a]

	Males	Females
No.	85	111
Age, years	32.3 (10.5)	32.4 (12.5)
BMI, kg/m^2	48.2 (8.3)	49.3 (8.1)
Waist-to-hip ratio	1 (0.06)[b]	0.96 (0.07)
Fat-free mass,[c] %	56.9 (5.4)[b]	48.3 (6.0)
Fat mass,[c] %	43.1 (5.5)[b]	51.7 (6.8)
Systolic blood pressure, mmHg	134.1 (16)[b]	123.2 (16)
Diastolic blood pressure, mmHg	85.9 (10)[b]	79.3 (10)
Heart rate, beats/min	79.8 (12)	78.8 (10)
Total cholesterol, mmol/L	4.5 (0.8)[d]	4.9 (1.0)
HDL-cholesterol, mmol/L	1.0 (0.2)[b]	1.2 (0.2)
LDL-cholesterol, mmol/L	2.8 (0.8)	3.0 (0.9)
Triglycerides, mmol/L	1.7 (1.1)	1.5 (0.6)
AST,[e] U/L	29.9 (13.0)[d]	24.6 (14.2)
ALT, U/L	52.6 (31.5)[b]	32.1 (22.3)
Leptin,[f] μg/L	67.6 (48.1)[b]	141.5 (54.4)
Insulin, mIU/L	26.9 (18.5)[d]	20.3 (9.5)
Glucose, mmol/L	5.4 (1.8)	5.1 (0.8)
HOMA[g]	6.5 (4.4)[b]	4.6 (2.4)
TSH, mIU/L	1.7 (0.8)	2.3 (2.5)
FT_3, ng/L	2.9 (0.5)	2.9 (0.5)
FT_4, ng/L	62 (51)	59 (53)
ACTH, ng/L	39.9 (37.7)	24.4 (11.1)
Cortisol,[h] μg/L	136.7 (44.8)	133.2 (59.8)

[a] All values are the mean (SD).
[b] $P < 0.001$.
[c] Measured in 86 patients (39 males and 47 females).
[d] $P < 0.05$.
[e] AST, aspartate aminotransferase; ALT, alanine aminotransferase; HOMA, homeostasis model assessment; TSH, thyroid-stimulating hormone; FT_3, free triiodothyronine; FT_4, free thyroxine.
[f] Reference intervals for normal-weight controls (see the section on patients in the *Materials and Methods*) were 1.1–19.7 μg/L in males and 2.3–56.1 μg/L in females.
[g] HOMA: fasting insulin (mIU/L)/[22.5 × $e^{-\ln(\text{glucose, in mmol/L})}$].
[h] Measured in plasma, whereas all other biochemical values were measured in fasting serum.

GENOMIC DNA AMPLIFICATION AND SEQUENCING

Genomic DNA from obese and nonobese individuals was extracted from whole blood (Nucleon BACC-2; Amersham Biosciences Europe). The coding sequences of both *POMC* and *MC4R* were amplified with primers chosen by the PRIMER 3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The primers were selected to generate fragments encompassing *POMC* exons 1 and 2 and to obtain overlapping fragments of exon 3 of *POMC* and of *MC4R* gene transcripts. *POMC* was amplified (GeneAmp PCR System 9700; Applied Biosystems) in a final volume of 25 μL containing a PCR mixture [10 μM each primer, 1× PCR buffer, 200 μM each deoxynucleotide triphosphate, and 0.5 U of *Taq* DNA polymerase (Perkin-Elmer Cetus)] and 100 ng of genomic DNA. The *MC4R* PCRs were performed with a final volume of 50 μL containing 50 ng of genomic DNA; 1 U of *Taq* DNA polymerase (Invitrogen Life Technologies); 200 μM each deoxynucleotide triphosphate, 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 2.5 mM $MgCl_2$, 0.2 μg/mL bovine serum albumin, and 10 μM of the corresponding primers (see the Appendix in the Data Supplement at that accompanies the online version of this article at http://www.clinchem.org/content/vol51/issue8). The PCR fragments were separated by electrophoresis on a 1.5% agarose gel and purified. Both strands were then sequenced in both directions (BigDye Terminator v3.1 cycle sequencing method on an ABI-Prism 3100 Genetic Analyzer; Applied Biosystems). The *POMC* and *MC4R*

nucleotide and amino acid sequences were numbered according to nucleotide 1 in the reference sequence (GenBank accession nos. V01510 and NP_000930 for *POMC*; S77415 and P32245 for *MC4R*). Mutations were named according to standard guidelines *(18)*. We confirmed the *MC4R* mutations by restriction fragment length polymorphism mapping (Q43X, W174C, and S19fsX51) and with the amplification refractory mutation system (I317V). For further details, please contact the corresponding author.

HPLC analysis

To analyze the 9- and 18-bp insertion (codons 93–99) polymorphisms in exon 3 of the *POMC* gene, we designed amplification primers using the PRIMER 3 program (forward, 5′-GTCTTCCCCCAGGAGTGC-3′; reverse, 5′-AGACGTCCTCGCGCTTCT-3′). The analysis was performed according to standard procedures with the WAVE DNA Fragment Analysis System (3500 HT; Transgenomic).

Protein truncation test

We verified the presence of the E244X mutation in *POMC* with the in vitro protein truncation test by adding 5 μL of PCR product and 1 μL of [^{3}H]leucine to a reticulocyte lysate (Promega) under the manufacturer's recommended reaction conditions. The upstream primer contained eukaryotic signals for efficient transcription and translation (the T7 promoter sequence and a translation initiation site). The oligonucleotide sequences were 5′-T7-GAGTGCATCCGGGCCTGCAAG-3′ for the amplification of fragment B and 5′-CGATCCATGCTGCTGTTATTT-3′ for the 3′ untranslated region (3′-UTR). The PCR was performed as follows: 1 cycle at 94 °C (5 min); 35 cycles at 94 °C (30 s), 60 °C (30 s), 72 °C (45 s); and 10 min of final extension at 72 °C. The molecular masses of the PCR products (wild-type and mutant) were assessed by electrophoresis on a 1.5% agarose gel. A 9-μL aliquot of the reaction products was denatured with 3 μL of loading buffer and loaded on a 15% sodium dodecyl sulfate–polyacrylamide gel. The molecular masses of the mutated and wild-type peptides were assigned based on comparison with molecular mass calibrators (range, 6.9–205 kDa; Kaleidoscope Prestained Standard; Bio-Rad Laboratories) analyzed under the same experimental conditions. The gel was then fixed, dried, and autoradiographed.

Biochemical assays

Serum leptin concentrations were measured in duplicate in obese and control individuals with a human leptin enzyme immunoassay (Linco Research). Total cholesterol and triglyceride concentrations were measured by standard enzymatic methods *(19, 20)*. HDL was measured after precipitation of LDL-cholesterol, and LDL-cholesterol was calculated according to the Friedewald formula. Thyroid-stimulating hormone, free triiodothyronine, and free thyroxine concentrations were measured by immunoassay (Abbott Laboratories). Serum insulin, cortisol, and ACTH were measured by chemiluminescence methods (Immulight 2000; Medical System). Glucose was measured by the hexokinase method. In each participant, the degree of insulin resistance was estimated at baseline by homeostasis model assessment as reported elsewhere *(21)*. Transaminase (aspartate transaminase and alanine transaminase) activities were measured with the IFCC enzymatic colorimetric method.

Statistical analysis

Variables that were not gaussian distributed were logarithmically transformed before statistical analyses (SPSS for Windows, Ver. 11.5; SPSS). Continuous variables are reported as the mean (SD), and categorical variables are reported as percentages. Comparisons among variables measured in females and males were performed with the unpaired *t*-test or χ^2 test. Differences among groups were considered statistically significant at $P < 0.05$.

Prediction study of protein structure

We performed a prediction study of the secondary structure of the mutated β-MSH peptide and MC4R protein with the GOR4 program (ABS-NIH) and used the PSI-BLAST multiple sequence program *(22)* for alignment of the protein sequences.

Results

Fasting serum hormone concentrations and biochemical indices evaluated in the severely obese adults examined in this study are reported in Table 1. Serum thyroid-stimulating hormone, free triiodothyronine, free thyroxine, ACTH, and cortisol values were within the reference intervals for healthy individuals in all obese patients. Serum leptin reference intervals obtained in our nonobese participants were 1.1–19.7 μg/L in males and 2.3–56.1 μg/L in females. In obese males and females, serum leptin concentrations were, respectively, 3.4 and 2.5 times higher than the upper reference limits obtained in our nonobese participants. The prevalence of the metabolic syndrome *(23)* was 61% in males and 67% in females and was diagnosed when at least 3 of the following 5 factors were outside the reference values: waist circumference, blood pressure, triglyceride and HDL-cholesterol concentrations, and glycemia.

As shown in Table 2, *POMC* or *MC4R* polymorphisms were found in 53.5% of obese and in 53.0% of nonobese participants. For *POMC* exon 3, we detected a 9-bp insertion and an 18-bp insertion between codons 93 and 99, respectively: AGC AGC CGC (S-S-G) and AGC AGC CGC AGC AGC CGC (S-S-G-S-S-G); the 8246C>T polymorphism within the 3′-UTR; and the silent polymorphism 4512C>T (C6C) in exon 2. Obese females with the 8246C>T or T>T polymorphism had lower triglyceride concentrations and higher HDL-cholesterol concentrations than obese females with the 8246C>C polymorphism ($P < 0.003$; data not shown). We detected the T112M and I103V polymorphisms in the *MC4R* gene

Table 2. Mutations and polymorphisms detected in the *POMC* and *MC4R* genes in severely obese adults (n = 196) and nonobese controls (n = 100) living in southern Italy.

	Amino acid change	Nucleotide change	Obese, n (%)	Lean, n (%)
POMC (exon 3)				
Mutations	P231L[a]	8072C>T	1 (0.5)	0
	R236G[b]	8086C>G	1 (0.5)	0
	E244X[a,c]	8110G>T	1 (0.5)	0
Polymorphisms	93–99	7658–7677 (ins AGC AGC CGC)[d]	20 (10.2)	6 (6)
	93–99	7658–7677 [ins $(AGC\ AGC\ CGC)_2$]	1 (0.5)	0
	3′-UTR	8246C>T	65 (33.1)	33 (33)
	3′-UTR	8246T>T	9 (4.6)	10 (10)
	93–99/3′-UTR	7658–7677 (ins AGC AGC CGC)/8246C>T	7 (3.6)	2 (2)
	C6C/3′-UTR	4512[e]C>T/8246C>T	1 (0.5)	0
MC4R				
Mutations	W174C[a,f]	915G>C	1 (0.5)	0
	Q43X[a]	520C>T	1 (0.5)	0
	A175T[b,c]	916G>A	1 (0.5)	0
	S19fsX51[a]	448delA	1 (0.5)	0
	I317V[a,b]	1342A>G	1 (0.5)	0
Polymorphisms	T112M	728 C>T	1 (0.5)	0
	I103V	700 A>G	1 (0.5)	2 (2)

[a] New mutation.
[b] 8246C>T.
[c] *POMC* polymorphism [7658–7677 bp (ins 9 bp)] also present in the mutated DNA.
[d] This variant (ins AGC AGC CGC) was detected in the homozygous state only in 1 of 196 obese persons.
[e] *POMC* exon 2 polymorphism.
[f] 8246T>T.

product. The former variant was present in only 1 obese patient, whereas the latter variant was present in 1 obese individual and in 2 nonobese individuals. We found no association between the metabolic syndrome and *POMC*/*MC4R* polymorphisms.

POMC and *MC4R* mutations were present in 8 of the 196 obese individuals: 3 had *POMC* mutations (2 novel and 1 known mutation), and 5 had *MC4R* mutations (4 novel and 1 known mutation). No mutations were found in our normal-weight adults. The phenotypic characteristics of mutation-carrying patients are reported in Table 3.

In case 1, we detected the heterozygous missense mutation R236G, which disrupts a dibasic processing site between β-MSH and β-endorphin and so produces a mutant β-MSH/β-endorphin fusion protein *(7, 8)*. The individual was wild type for the *MC4R* gene. Case 2 had the novel *POMC* heterozygous mutation P231L in β-MSH. The prediction study of the secondary structure of the P231L peptide indicated an increase in the extended strand structure (55.56% vs 38.89%) and a decrease in the random coil structure (44.44% vs 61.11%) for the mutated vs the wild-type peptide. Protein sequence alignment

Table 3. Phenotypic characteristics of mutation-carrying obese patients in southern Italy.

	BMI, kg/m²	Family history of disease	Age at onset of obesity, years	HOMA[a]	Leptin, μg/L	*POMC* variant	*MC4R* variant
Case 1[b]	52.0	Obesity (f + m); diabetes (f)	21	4.2	82.2	R236G; 8246C>T (3′-UTR)	WT
Case 2[b]	60.0	Obesity (f + m)	18	4.3	117.0	P231L	WT
Case 3[c]	48.2	Not reported	20	6.8	101.2	E244X; 7658–7677 (ins AGC AGC CGC)[d]	WT
Case 4[b]	59.0	Overweight (f + m); hyperlipidemia and diabetes (f)	8	3.9	36.1	8246T>T (3′-UTR)	W174C
Case 5	45.7	Obesity (f + m)	10	1.3	99.1	WT	Q43X
Case 6	47.7	Diabetes (m)	10	7.7	52.9	8246C>T (3′-UTR); 7658–7677 (ins AGC AGC CGC)[d]	A175T
Case 7	44.0	Obesity (f + m)	18	3.9	43.3	WT	S19fsX51
Case 8	39.2	Hypertension (m)	10	1.9	47.9	8246C>T (3′-UTR)	I317V

[a] HOMA, homeostasis model assessment; f, father; m, mother; WT, wild type.
[b] Correlated diseases: case 1, hypertension plus cholelithiasis, bilateral coxarthrosis, and spondyloarthrosis; case 2, hypertension; case 4, impaired fasting glucose.
[c] Obese African female living in Italy for the last 20 years.
[d] This variant (ins AGC AGC CGC) was detected in the homozygous state in case 3 and in the heterozygous state in case 6.

indicated that, in addition to the H-F-R-W core sequence, proline at residue 231 was also highly conserved *(22)* among different mammalian and nonmammalian species (human, pig, sheep, cow, horse, rat, mouse, pigtailed macaque, American mink, African elephant, frog, African clawed frog, chicken, and ostrich). The individual was wild type for the *MC4R* gene.

Case 3 carried a novel *POMC* heterozygous nonsense mutation that introduces a stop codon in the β-endorphin peptide (E244X) and the above-described 9-bp insertion (AGC AGC CGC, S-S-G) between codons 93 and 99 in homozygosity. After retrotranscription and translation of this mutated DNA, we verified by the protein truncation test that this variant caused the synthesis of a truncated peptide shorter (~22.0 kDa) than the wild-type peptide (~24.4 kDa; Fig. 1). The participant was wild type for the *MC4R* gene and was the only black non-Italian individual attending our outpatient clinic.

Case 4 carried the *MC4R* heterozygous missense mutation W174C. This mutation was not found in our nonobese participants, nor has it been reported in other Mediterranean obese and nonobese individuals *(12, 13)*. The prediction study of the secondary structure of the W174C peptide indicated a slight decrease in the extended strand structure of the mutated fragment vs the wild-type peptide. Case 5 was wild type for the *POMC* gene and had a novel *MC4R* heterozygous nonsense mutation that introduces a stop codon (Q43X) and produces a truncated N-terminal peptide. In case 6, we identified the previously reported *MC4R* heterozygous missense mutation A175T, which has been associated with partial activity of the protein *(24, 25)*.

Case 7 was wild type for the *POMC* gene and had a 1-bp deletion (448delA) in the *MC4R* gene that causes a frameshift after codon 18 and introduces a premature stop codon at codon 51 (S19fsX51). Only the first 18 of 51 amino acids of the truncated peptide belong to the wild-type protein. For the *MC4R* gene, case 8 carried a novel heterozygous missense mutation, I317V, located in the C-terminal region of the protein. Protein sequence alignment indicated that the isoleucine residue was highly conserved among different mammalian species *(22)*.

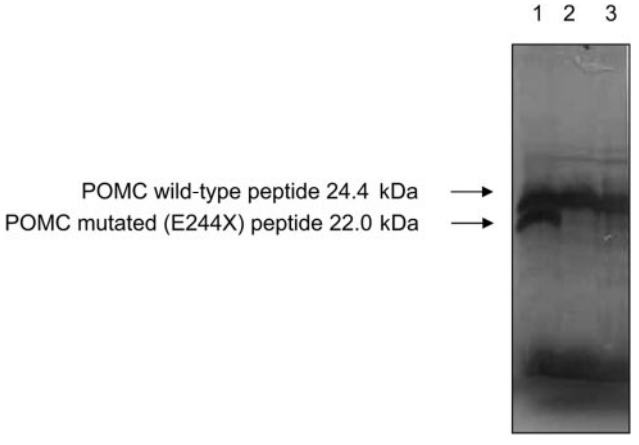

Fig. 1. Screening by protein truncation test of *POMC* exon 3 transcripts from the patients with mutations (*lane 1*, case 3) and 2 nonobese controls (*lanes 2* and *3*).

The variant introduced a stop codon at position E244 in the protein. In the heterozygous patient (*lane 1*; case 3) we observed 2 different peptides: the wild-type peptide (~24.4 kDa), which was also present in the 2 controls (*lanes 2* and *3*), and the truncated peptide (~22.0 kDa). A prestained molecular mass calibration mixture (6.9–205 kDa) was used to estimate the molecular masses of the peptides.

Discussion

We identified *POMC* mutations in 3 of our severely obese adults. Mutation R236G, in exon 3, is a known mutation that comprises part of the conserved dibasic amino acid cleavage site, K235-R236, which is recognized by prohormone convertase 2. It is associated with altered *POMC* processing and produces a β-MSH/β-endorphin fusion protein, which is less able than the wild type to activate the *MC4R* gene *(8)*. R236G was reported to cosegregate with early-onset obesity in 0.76% (2 of 262) of British obese individuals (BMI standard deviation score >2.5). In the latter study, the mutation was not present in 412 unrelated nonobese individuals; moreover, in 1 of the 2 patients with mutations, the variant cosegregated with obesity in the family over a period of 3 generations *(8)*. In a French study, R236G was present in 1.65% (3 of 182) of individuals (mean BMI >31.8 kg/m^2) in association with early-onset obesity and in 1.04% of controls *(8)*. R236G was also detected in 1.15% (1 of 87) of obese Italian children (BMI standard deviation score = 3.43) *(9)*. Interestingly, it was also identified in 0.6% (1 of 156) of individuals in a Danish study of juvenile-onset obesity (BMI = 35.8 kg/m^2) diagnosed when the individual was ~20 years of age *(7)*, which coincides with our heterozygous carrier of this variant, a female diagnosed as obese at 21 years of age.

The second novel mutation we identified in an obese participant is a heterozygous variant in *POMC* exon 3 that produces the P231L change in the β-MSH peptide. Given the predicted secondary structure of this mutated 18-amino acid peptide, the proline→leucine exchange could modify the secondary structure of the core melanocortin sequence H-F-R-W *(26)*. The β-MSH peptide binds to MC4R, via its core sequence, with a significantly higher affinity than α-MSH and is reported to be the key ligand for MC4R that regulates feeding *(27)*. Because both the core sequence and proline residue 231 are highly conserved among species, we suggest that the leucine substitution alters the ligand affinity to MC4R and so contributed to obesity in our patient. A functional study is required to verify this hypothesis.

The third novel mutation we identified is a heterozygous nonsense mutation in *POMC* exon 3 that encodes a truncated β-endorphin peptide at codon 244. It is well known that most nonsense transcripts are recognized and efficiently degraded by nonsense-mediated mRNA decay *(28)*. This pathway may cause disease either by inducing haploinsufficiency and/or a via dominant-negative effect

of the mutated protein *(28)*. Our patient may be affected by a similar disease-associated mechanism. Interestingly, β-endorphin peptide knockout mice develop an increased fat mass late in life *(29)*, which coincides with the late-onset obesity of our patient carrying the E244X mutation in heterozygosity.

Mutations in *MC4R* cosegregate with severe early-onset obesity and are the most common monogenic cause of human obesity, with mutations spanning from causative, inactive, to partially active *(12–15, 24, 25, 30–32)*. We identified the A175T variant in the *MC4R* gene in a severely obese young male (BMI = 47.7 kg/m^2) with early-onset obesity (case 6). This variant was described in a British individual with early-onset obesity; a family history of obesity, insulin resistance, and euglycemia associated with reduced *MC4R* activity *(24)*; and a reduced ability to generate cAMP in response to ligand but a normal or near-normal ligand binding affinity for α-MSH analogs *(25)*.

The novel W174C mutation in *MC4R*, identified in a severely obese male (case 4), lies in the fourth transmembrane segment of the protein, where it produces a change of tryptophan (a highly conserved amino acid among species) to cysteine. On the basis of the predicted secondary structure, W174C could alter the protein's conformation.

Mutation S19fsX51 alters the N-terminal peptide of MC4R. In fact, only the first 18 amino acids of the peptide are conserved. Recent evidence indicates that, by acting as an intramolecular ligand for the receptor, an intact NH_2 terminus is required for receptor activity *(33)*. Loss of constitutive receptor has been associated with obesity in humans *(15, 32, 33)*.

The mutation Q43X encodes an MC4R protein truncated from the first transmembrane domain onward. Other stop-codon mutations have been described recently *(15, 32, 33)*; in particular, protein with the Y35X mutation in the N-terminal region did not bind α-MSH and showed a relatively high allele frequency (0.6%) in a Danish obese population *(34)*. Presumably, our stop-codon mutation could also be associated with no or partial MC4R activity.

The novel heterozygous missense mutation I317V is located in the C-terminal region of *MC4R*. Despite the structural similarity between isoleucine and valine, we did not find I317V in nonobese participants. Highly conserved among species, isoleucine-317 may exert an important functional role. A functional study is required to test whether this variant is associated with reduced *MC4R* membrane expression and α-MSH response as shown for the I317T variant *(14, 15, 31, 32, 35, 36)*.

In conclusion, we identified *POMC* mutations in 1.5% of our severely obese adults, which coincides with the low frequency of mutations reported previously for this gene (~1% for the most frequent R236G variant) *(8)*. These mutations do not appear to be associated with early-onset obesity in our population. We found *MC4R* mutations in 2.5% of our patients, a frequency similar to that reported for other Caucasian populations affected by both early- and adult-onset obesity, i.e., from 2% to 5.8% *(4, 24, 25, 32, 34, 37)*, but higher than frequencies recently reported for 2 other Mediterranean obese populations, 0.5% and 1.7% *(12, 13)*.

This work was supported by grants from CEINGE-Regione Campania (Convenzione-Del. G.R. 27/12/2002 No. 6276) and MIUR (PRIN 2002). We are grateful to Jean Gilder for editorial assistance and for revising the English text.

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