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Prevalence of mutations in *LEP*, *LEPR*, and *MC4R* genes in individuals with severe obesity

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ABSTRACT. Obesity is a major public health concern; despite evidence of high heritability, the genetic causes of obesity remain unclear. In this study, we assessed the presence of mutations in three genes involved in the hypothalamic leptin-melanocortin regulation pathway (leptin, *LEP*; leptin receptor, *LEPR*; and melanocortin-4 receptor, *MC4R*), which is important for energy homeostasis in the body, in a group of patients with severe obesity. For this study, we selected 77 patients who had undergone bariatric surgery and had a pre-operative body mass index (BMI) >35 kg/m^2, early onset and a family history of being overweight.

Candidate genes were screened by direct sequence analysis to search for rare genetic variations. The common *LEP* -2548 G/A polymorphism was also evaluated for its influence on the BMI (in obesity patients) and for obesity risk, using a case-control study involving 117 healthy individuals. Two different non-synonymous alterations in *MC4R* were found in two patients: the p.(Thr112Met), previously described in the literature as a probable gene involved in the obesity phenotype, and the novel p.(Tyr302Asp) variant, predicted to be pathogenic by *in silico* evaluations and family segregation studies. The *LEP* -2548 G/A polymorphism was not associated with the BMI or obesity risk. In conclusion, we have reported a novel mutation in *MC4R* in a family of Italian patients with severe obesity. Screening for *MC4R* could be important for directing the carriers of mutations towards therapy including partial agonists of the *MC4R* that could normalize their appetite and inhibit compulsive eating. Next-generation sequencing could be used to clarify the genetic basis of obesity in the future.

Key words: Obesity; Leptin; Leptin receptor; Melanocortin-4 receptor; Polymorphism

INTRODUCTION

Obesity is currently a major public health problem. Its incidence is increasing and it affects persons of all ages. According to the World Health Organization (WHO, 2015), worldwide obesity has more than doubled since 1980. In 2014, more than 1.9 billion adults, 18 years and older, were overweight. Of these over 600 million were obese. In 2013, 42 million children under the age of 5 were overweight or obese. Moreover, most of the world's population lives in countries where overweight and obesity kills more people than underweight. Overweight and obesity are, in fact, risk factors for type 2 diabetes, cardiovascular diseases, cancers and other health problems with substantial direct and indirect health care costs (Chan and Woo, 2010).

The etiology of obesity is multifactorial, involving genetic and environmental factors, and can be defined as a result of a prolonged imbalance between the calorie intake and energy utilization. Energy balance is regulated by many interacting neurotransmitters and neuropeptides, which create a complex network of afferent and efferent signals. The main elements of this control system are hypothalamic peptidergic neurons in the leptin-regulated melanocortin pathway (Schwartz et al., 2000). Leptin is a protein hormone consisting of 167 amino acids and with a molecular weight of 16 kDa, encoded by the *LEP* gene (also known as the obesity gene) on chromosome 7, which is produced largely by adipose cells. Its circulating concentrations are proportional to the fat content of the body (De Pergola et al., 2008).

Mobilization of the lipid energy reserves during fasting triggers a series of signals that decrease the leptin levels, increasing the sensation of hunger (Mantzoros, 1999). Leptin acts on cells through its membrane receptor LEPR B, which belongs to the first class of the cytokine receptor family (Tartaglia, 1997), and is expressed by two different sets of neurons in the arcuate nucleus of the hypothalamus: AgRP/Npy neurons that express orexigenic peptides (which increase the sensation of hunger), agouti-related proteins, and neuropeptide Y (the expression of which is inhibited by leptin), and Pomc/Cart neurons that express the

two anorexigenic neuropeptides that suppress the appetite, cocaine-and-amphetamine-related transcript (CART) and the peptide α-melanocyte-stimulating hormone (α-MSH). The latter hormone is derived from the proteolytic action of proconvertase enzymes PC1 and PC2, encoded by the proopiomelanocortin gene (*POMC*). The peptides α-MSH and CART are synthesized in the presence of leptin (Bates and Myers, 2003). AgRP and α-MSH compete in the hypothalamus to bind to melanocortin receptors, especially subtype 4 (MC4R), a 332-amino acid protein belonging to a family of seven transmembrane G protein-coupled receptors (Gantz et al., 1993). This competition is critical for maintaining energy homeostasis (Raffin-Sanson et al., 2003).

In this study, we analyzed three genes of the hypothalamic leptin-melanocortin pathway that regulates the eating behavior and for which there is evidence of mutations that may alter the gene function, thereby inducing over-eating and obesity. These genes included the leptin-coding gene *LEP*, the leptin receptor-coding gene *LEPR*, and the melanocortin-4 receptor-coding gene *MC4R* (Sarzynski et al., 2011; Dougkas et al., 2013). Previous studies have indicated that this pathway may be important for energy homeostasis (Balthasar et al., 2004, 2005), while genetic studies have shown that mutations in certain genes involved in this pathway may express an obesity-causing phenotype. In particular, MC4R deficiency is known to be the most common cause of monogenic obesity by increasing the appetite and diminishing satiety, and mutations in this gene are thought to explain 2-6% of all cases of extreme obesity in children and adolescents. Alternately, mutations in *LEPR* and *LEP* are very rare (Albuquerque et al, 2014; Wabitsch et al., 2015). The aim of this study was to assess the *LEP*, *LEPR*, and *MC4R* genes for mutations in a group of gastric bypass patients with severe obesity [body mass index (BMI) > 35]. Bariatric surgery patients were chosen because they were more likely to have early onset of the disease and various comorbidities. With regard to the gene *LEP*, we also conducted a further case-control study on one of the most studied polymorphisms identified in the 5'-untranslated region of the *LEP* gene (-2548 G/A; rs7799039), reported to affect expression of the hormone, probably at the transcriptional level (Hoffstedt et al., 2002), and to be associated with obesity (Skibola et al., 2004).

Studies regarding the role of this polymorphism in obesity remains controversial; however, since its effect could be specific to an ethnicity (Zhang et al., 2014; Fujiwara et al., 2015), we evaluated this polymorphism for possible associations with obesity in Italian individuals.

MATERIAL AND METHODS

Study subjects

We chose patients who had undergone bariatric surgery because they were more likely to have had early onset of obesity and various comorbidities. The obesity in such subjects is resistant to conventional medical and dietetic therapy, which is why the subjects resort to surgery after unsuccessful attempts to diet and frequent oscillations in body weight. All patients met the international selection criteria for bariatric surgery. In this study, we employed the criteria of the Società Italiana di Chirurgia dell'Obesità e delle Malattie Metaboliche, based on those established by the Consensus Development Conference (1991) of the National Institutes of Health.

Seventy-seven patients (62 females, 15 males; Table 1) were recruited to this study. All 77 patients volunteered to participate in this genetic study, and were enrolled after genetic counseling (to explain risks and benefits of genetic testing). Each blood sample was

accompanied by written informed consent to genetic testing by the patients. The informed consent forms included a provision to consent to the use of anonymized genetic results for research. This study only reports data from those patients who consented to the use of their data (anonymized) for research.

A cohort of 117 Italian subjects without obesity (63 females, BMI = 20.9 ± 1.6; 54 males, BMI = 21.9 ± 1.3) was selected from DNA samples available in our laboratory, in order to assess the distribution of the *LEP* -2548 G/A polymorphism with respect to the genotype by case-control evaluation.

Table 1. Demographic and clinical characteristics of patients before intervention.

	Male No. 15	Female No. 62
Mean age, years ± SD (min-max)	42.87 ± 9.98 (31.00-62.00)	45.53 ± 8.81 (23.00-64.00)
Mean onset, years ± SD (min-max)	10.33 ± 4.13 (0.00-17.00)	8.85 ± 4.74 (2.00-17.00)
Mean height, cm ± SD (min-max)	175.53 ± 6.77 (164.00-186.00)	161.16 ± 6.21 (146.00-175.00)
Mean weight, kg ± SD (min-max)	142.95 ± 31.30 (93.00-205.00)	118.32 ± 19.31 (79.60-179.00)
Mean BMI ± SD (min-max)	46.61 ± 8.50 (33.70-59.30)	45.76 ± 7.26 (35.60-63.40)
Mean BCM ± SD (min-max)	42.93 ± 13.57 (30.00-75.20)	32.65 ± 8.51 (12.90-59.20)
Mean circ. waist, cm ± SD (min-max)	129.18 ± 17.12 (109.00-165.00)	117.98 ± 11.48 (96.00-144.00)
Mean circ. hips, cm ± SD (min-max)	130.89 ± 18.87 (104.00-163.00)	133.97 ± 13.61 (110.00-170.00)
Mean WHR ± SD (min-max)	0.99 ± 0.08 (0.87-1.13)	0.88 ± 0.09 (0.76-1.13)
Mean ECW % ± SD (min-max)	41.48 ± 6.25 (30.20-48.50)	43.25 ± 6.51 (19.00-64.90)
Mean ICW % ± SD (min-max)	58.52 ± 6.25 (51.50-69.80)	56.30 ± 5.65 (35.10-70.30)
Mean phase angle ± SD (min-max)	7.24 ± 1.86 (5.40-11.00)	6.73 ± 1.36 (3.10-11.30)

Genetic evaluations

DNA was extracted from 0.5 mL whole blood using a commercial kit (Blood DNA kit E.N.Z.A., Omega bio-tek; Norcross, GA, USA). The coding and adjacent intron regions of the genes *LEP* (NM_000230; 2 exons), *LEPR* (NM_002303; 18 exons), and *MC4R* (NM_005912; 1 exon) were analyzed by polymerase chain reaction (PCR) and direct sequencing. The PCR mixture contained 40 ng DNA, 10 pmol/µL of each primer, 2.5 µL 10X PCR buffer, 0.15 mM dNTPs, 1 mM $MgCl_2$, and 0.75 U Taq DNA polymerase, Recombinant (Thermo Fisher Scientific, Waltham, MA USA). The reaction was carried out in a thermocycler (Esco, Hamburg, Germany) using the following cycling conditions: initial denaturation at 95°C for 10 min; 35 cycles of denaturation at 95°C for 30 s, annealing at 30 s, and polymerization at 72°C for 40 s; and a final extension at 72° for 5 min. The annealing temperatures and number of cycles were specific for each primer pair (listed in **Table S1**).

Three microliters of the amplification product was electrophoresed on a 2% agarose gel stained with Gel Red (Biotium, Hayward, CA, USA) and observed under ultraviolet light. The sizes of the PCR products were estimated by comparing with a 100-bp DNA marker ladder (Fermentas, Vilnius, Lithuania).

All PCR products were purified using the Cycle Pure Kit (Omega bio-tek; Norcross, GA, USA) and sequenced with the same PCR amplification primers (separately), and the following cycling protocol: 30 cycles of denaturation at 96°C for 30 s, annealing at 55° for 20 s, and extension at 60° for 4 min. The sequencing mixture was composed of 5 µL DCTS Quick Start Master Mix (GenomeLabTM DCTS-Quick Start Kit; Beckman Coulter, Brea, CA, USA), 4 µL sequencing primer (1 pM/µL), and 4 µL amplified PCR product (final volume, 20 µL). The product was purified again, suspended in 40 µL Sample Loading Solution (GenomeLabTM DCTS-Quick Start Kit; Beckman Coulter), and analyzed in a CEQ 8000 sequencer (Beckmann Coulter).

The electropherograms of all amplified fragments were analyzed using the ChromasPro v.1.5 software (Technelysium Pty. Ltd., Queensland, Australia).

All identified genetic variants were searched for in the professional Human Genome Mutation Database (HGMD) (http://www.biobase-international.com/product/hgmd). Polymorphisms were excluded by additional searches in the public database of single nucleotide variants (dbSNP, www.ncbi.nlm.nih.gov/SNP/) and the Exome Variant Server (EVS) database (http://evs.gs.washington.edu/EVS/); missense variants with an allelic frequency >1% were deemed to be non-pathogenic. The pathogenicity of new nucleotide alterations involving a change in amino acids was determined using the PolyPhen 2 algorithm (Polymorphism Phenotyping v2; http://genetics.bwh.harvard.edu/pph2) (Adzhubei et al., 2010), considering the HumVar-trained model and the SIFT algorithm (Sorting Intolerant From Tolerant; http://sift.bii.a-star.edu.sg/) (Kumar et al., 2009), by comparing the properties of the wild-type amino acid with those of the variants when possible (http://www.russelllab.org/aas/aas.html) (Betts and Russell, 2003). For all new synonymous and intronic variants near the splicing sites, we searched for splicing defects and any variations in exon splicing enhancers (ESEs) and exon identity elements using on-line Human Splicing Finder software v.3 (http://www.umd.be/HSF3/) (Desmet et al., 2009).

The distribution of the *LEP* -2548 G/A polymorphism was determined in this case-control study by analyzing DNA from 117 healthy controls by *Hha*I restriction enzyme analysis (Şahın et al., 2013). The HaploPainter software (http://haplopainter.sourceforge.net/index.html) was used to determine the pedigrees of the patients.

Statistical analysis

We investigated the possibility of associations between the *LEP* -2548 G/A SNP and BMI by analysis of variance (ANOVA). In particular, we performed one-way ANOVA (considering the genotype of *LEP* -2548 G/A and the quantitative BMI) and two-way ANOVA (also considering the second demographic factor "gender"). The Levene test was used to test the ANOVA assumption of "equal variances in the population" before conducting ANOVA.

In this case-control study, the χ^2 test was used to evaluate the differences in SNP distribution between the two populations and the odds ratio statistic as a measure of the risk of obesity in *LEP*-2548 G/A SNP carriers. The Hardy-Weinberg equilibrium (HWE) for *LEP* -2548 G/A SNP was tested to compare the observed genotype frequencies with the expected genotype frequencies in the control population (http://www.genes.org.uk/software/hardy-weinberg.shtml) in order to exclude sampling bias, mistyping of genotypes, or spurious gene associations caused by population stratification. All statistical analyses were conducted using the MedCalc software (MedCalc Software bvba, Ostend, Belgium).

RESULTS

Sanger sequencing analysis did not show any major nucleotide alterations in the exon regions of *LEP* or *LEPR*; however, four non-synonymous alterations were detected in *MC4R* in six (6/77) of the patients (all females) (Table 2). Two of these alterations, the p.(Val103Ile) and p.(Ile251Leu) variants, identified in three and one patients in our cohort, respectively, are common polymorphisms (annotation frequencies: 1.85 and 1.16%, respectively) in the EVS database. The search in the HGMD professional database revealed an association between both

variants and autosomal dominant obesity; in particular, the p.(Val103Ile) variant is reported as a "disease-associated polymorphism with supporting functional evidence", whereas the p.(Ile251Leu) variant has been reported as a "probable disease causing mutation". However, their very high frequencies in the normal population mark them to be common polymorphisms; therefore, these variants are excluded from any dissertation in this study.

The p.(Thr112Met) variant, identified in one patient of our cohort, associated with the "autosomal dominant obesity" phenotype by HGMD, is a very rare variant reported at a frequency of 0.07% in EVS. On the other hand, the p.(Tyr302Asp) variant (identified in one patient) is described for the first time in this study. All bioinformatic tests conducted on the new mutation p.(Tyr302Asp) in *MC4R* predicted its damaging effect on the (resultant) protein function (Table 2). Figure 1 shows the results of the family segregation study for this new mutation.

Table 2. Prediction of functional effects of genetic mutations.

Gene	Nucleotide change	Amino acid change	Accession No.[1]	MAF%[2]	Mutation taster	Polyphen score[3]	Sift score
MC4R	c.335C>T	p.(Thr112Met)	rs13447329	0.069	P	B	D
	c.904T>G	p.(Tyr302Asp)	new	-	DC	PrD	D
LEP	IVS2: c.145-50C>T		rs17151914	0.860		-	
LEPR	IVS3: c.-20-15A>T		rs116571599	0.698	-	-	-
	IVS7: c.703+57T>G		new	-	-	-	-
	IVS12: c.1604-74A>C		new	-	-	-	-
	IVS20: c.2673+52C>T		rs80343559	0.715	-	-	-
	c.637T>C	p.(Leu213Leu)	new	-	-	-	-

[1]dbSNP accession No. [2]MAF from Exome Variant Server, European Allele Frequencies. [3]Score from HumVar-trained model; Mutation Taster score system: polymorphism (P), disease-causing (DC); Polyphen 2 score system: benign (B), possibly damaging (PoD, less confident prediction), probably damaging (PrD, more confident prediction); SIFT score system: Tolerated (T), damaging (D).

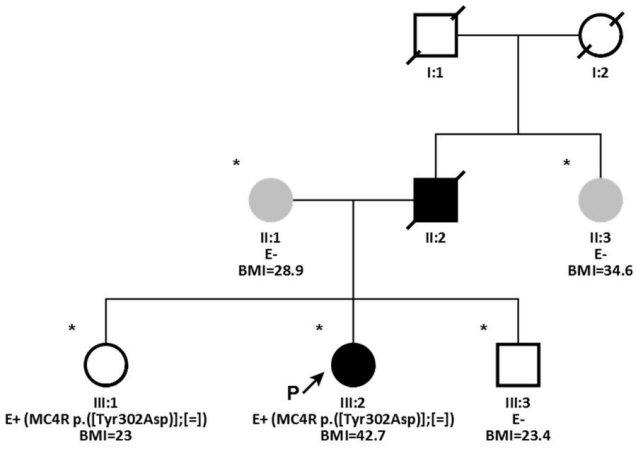

Figure 1. Pedigree and genotype of an Italian family with the novel p.(Tyr302Asp) *MC4R* mutation, showing an autosomal-dominant pattern of inheritance, with incomplete penetrance. BMI, body mass index; *documented clinical evaluation; E+ and E-, positive and negative to genetic test, respectively. Obesity classes (BMI): normal (18.5-24.9), overweight (25.0-29.9). Obesity class I (30.0-34.9), obesity class II (35.0-39.9), obesity class III (> 40). Color code: white, normal phenotype; gray, mild phenotype; dark, severe phenotype.

We also identified a broken ESE motif in the synonymous c.637T>C *LEPR* p.(Leu213Leu) variant, whereas none of the *LEP* and *LEPR* intronic variants affected the splicing function in any way.

In this case-control study, the HWE test confirmed that the *LEP* -2548 G/A SNP was in equilibrium (data not shown) in the control population; however, we could not find any significant differences in its distribution between the cases and controls (Table 3). One-way ANOVA identified no significant associations between this SNP and the BMI (P = 0.18) (Figure 2); similarly, two-way ANOVA (considering "genotype" and "gender" as factors and BMI as the dependent variable) also showed no significant associations (P = 0.71) (Figure 3).

Table 3. Genotype distribution in cases and controls.

LEP -2548 G<A distribution in cases and controls										
	Cases			Controls			AA *vs* GG		AA *vs* AG	
Genotype	A/A [N (%)]	A/G [N (%)]	G/G [N (%)]	A/A [N (%)]	A/G [N (%)]	G/G [N (%)]	OR	P < 0.05	OR	P < 0.05
Male	4 (25%)	9 (56%)	3 (19%)	12 (22%)	35 (65%)	7 (13%)	0.77	0.78	1.29	0.71
Female	15 (25%)	34 (56%)	12 (20%)	13 (21%)	28 (44%)	22 (35%)	2.11	0.15	0.92	0.86
Total	19 (25%)	43 (56%)	15 (19%)	25 (21%)	63 (54%)	29 (25%)	1.46	0.38	1.08	0.81

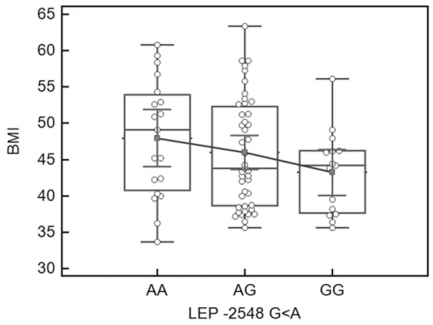

Figure 2. Distribution of the *LEP* -2548 G/A genotype in relation to the body mass index (BMI) of the patients. The box plot shows median (internal horizontal lines) BMI values by genotype, the interquartile range (IQR, central box, 25-75th percentile) and error bars (1.5 x IQR); points represent single samples by genotype (A/A, A/G, and G/G, x-axis) and BMI (y-axis). Bars indicate 95% confidence interval of the mean. Gene *LEP* -2548: genotypes A/A, A/G, and G/G; BMI, body mass index expressed in kg/m^2.

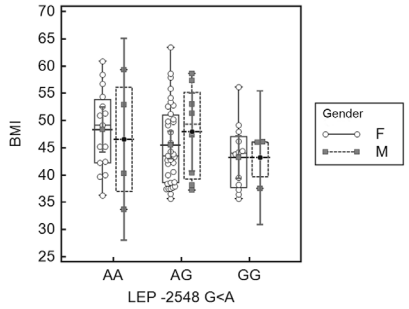

Figure 3. Two-way analysis of variance (ANOVA) of genotype, gender (factors), and BMI (dependent variable). The box plot shows median (internal horizontal lines) BMI values by genotype and gender, the interquartile range (IQR, central box, 25-75th percentile), and error bars (1.5 x IQR); points represent single samples by genotype and gender (A/A, A/G and G/G; F and M, x-axis) and BMI (y-axis). Bars indicate 95% confidence interval of the mean. *LEP* -2548 genotypes A/A, A/G and G/G; BMI, body mass index expressed in kg/m^2; gender: female (F) and male (M).

DISCUSSION

The main aim of this study was to assess the *LEP*, *LEPR*, and *MC4R* genes in gastric bypass patients with severe obesity (BMI > 35) for mutations. The results of this study revealed alterations in all three genes; however, the mutations identified in *LEP* and *LEPR* were heterozygous, and therefore did not influence the incidence of obesity in our population (as mutations in these genes normally display autosomal recessive transmission). Moreover, a majority of these mutations were in the non-coding intron portion of the gene, displaying no significant splicing motif alteration; therefore, these mutations did not exert a significant impact on splicing. An online search for splicing variations using HSF revealed an alteration in the exonic ESE site in *LEPR*, with a potential splicing alteration in the new synonymous mutation p.(Leu213Leu). ESEs are nucleotide hexamers that direct or enhance accurate splicing. Loss of ESEs could lead to exon skipping and aberrant gene regulation (Sterne-Weiler et al., 2011), but the functional significance of the variation reported in this study needs to be clarified.

Heterozygous mutations in *MC4R*, however, have autosomal dominant transmission. A majority of the functional studies into the variant p.(Thr112Met) of MC4R, first described by Hinney et al. (1999), measured the cAMP levels directly or indirectly to indicate receptor activation; therefore, these studies failed to demonstrate any association between this mutation and the receptor function (Hinney et al., 2003; Tao and Segaloff, 2005).

Based on the observation that MC4R activates MAPKs, especially ERK1/2 (Tao, 2010), in addition to the conventional Gs-stimulated adenylyl cyclase pathway, He and Tao (2014) demonstrated that this variant impaired ligand-stimulated ERK1/2 activation. They proposed that defective ERK1/2 signaling, which mediates the melanotan II-induced inhibition of food intake (Daniels et al., 2003; Tao, 2010), might cause obesity in patients harboring the p.(Thr112Met) variant of MC4R. Activation of the ERK1/2 pathway is a cellular mechanism that may underlie the regulation of energy homeostasis; mediation of this pathway by MC4R or genetic mutations could result in decreased basal or ligand-stimulated ERK1/2 signaling, which could might contribute to the pathogenesis of obesity.

The predicted pathogenicity of the new variant p.(Tyr302Asp) should be confirmed in future functional studies. However, mutations in the same codon causing changes in amino acids have already been reported, and are known to affect receptor function (Tao, 2006; Roth et al., 2009). Amino acid Tyr302 lies in the highly conserved DPLIY motif of transmembrane helix VII in human MC4R (Chapman et al., 2010); the p.(Tyr302Ala) mutation has been shown to reduce receptor signaling by leaving the receptor in a "locked-off" state (Tao, 2006).

Another functional study conducted by the same group reported an association between the genetic mutation resulting in a p.(Tyr302Phe) substitution in the resultant protein and obesity; the authors also claimed that this mutation induced reduced cell surface expression but conserved signaling; therefore, it was considered to be less severe than p.(Tyr302Ala) (Roth et al., 2009). Moreover, the amino acid change (tyrosine to phenylalanine) was predicted to be less deleterious as the two amino acids differed by only one hydroxyl group on the benzene ring. In our patient, we identified a p.(Tyr302Asp) mutation, causing a substitution of an aromatic, partially hydrophobic tyrosine with a negatively charged polar aspartate, which could presumably be a deleterious change (Betts and Russell, 2003).

When the family members of the proband were segregated, we observed the p.(Tyr302Asp) variant in her sister (BMI = 23.0) and a milder phenotype in her mother (overweight BMI = 25.0-29.9) and paternal aunt (class 1 obesity BMI = 30.0-34.9), both of

whom expressed the commonly occurring codon (Figure 1). The father (who suffered from diabetes and died of respiratory failure) was not available for genetic evaluation; however, he was an obligate carrier of the mutation and was described as having severe obesity. While the mild phenotype in the two wild-type relatives can be explained based on their age and lifestyle, the phenotype in the proband's sister can be explained by the variable penetrance of *MC4R* mutations (Dubern et al., 2001). Taken together, these observations seem to indicate that the new p.(Tyr302Asp) *MC4R* variant plays a functional role in receptor activity, thereby affecting the receptor cell surface expression and/or decreasing the receptor signaling, in response to anorexigenic α-MSH peptides.

The association study showed no statistically significant differences in the distribution of the *LEP* -2548 G/A SNP among cases and controls; as this could be attributed to the small sample sizes of the case and control groups, the conclusion could be biased or misleading. Therefore, these results should be validated in a larger case-control study.

Although significance was not attained, the BMI appeared to be influenced by the genotype in patients with obesity, with higher values in A/A carriers, which was in stark contrast with the results of other clinical studies that reported a highly significant relationship between the *LEP* -2548 G/G alleles and obesity (Wang et al., 2006; Riestra et al., 2010), but in line with the results of certain other studies (Hoffstedt et al., 2002; Nieters et al., 2002). This trend was clearer when the population was divided according to the genotype and gender: we observed a greater difference in mean BMI in females (AA = 48.33, A/G = 45.52, and G/G = 43.22) and a weaker difference in males (AA = 46.55, A/G = 47.91, and G/G = 43.20); however, this result failed to attain statistical significance probably because of the small number of individuals expressing the *LEP* -2548 A/A homozygous variant in our population.

In conclusion, verification of the presence/absence of the familial mutation in other family members may help elucidate the causative nature of the genetic alterations responsible for a phenotype. In any case, preliminary analyses of our data suggested that 2/77 (2.5%) of the included patients, representing the Italian population with non-syndromic obesity, have a mutation in the *MC4R* gene, which could influence the phenotype of the individual(s). These results suggest that genetic screening for *MC4R* could be important for directing the carriers of mutations in this gene towards therapies with partial agonists of the MC4R that are currently under development, which could function as valid therapeutic substances against obesity, normalizing the appetite and inhibiting compulsive eating due to genetic mutations affecting the receptor protein expression.

This study has several limitations, such as its small sample size and retrospective design. Moreover, variants in three genes are unlikely to explain a complex phenotype such as obesity and nutrition, or complex diseases such as obesity.

We are therefore developing a genetic test based on next-generation sequencing for syndromic and non-syndromic obesity to simultaneously analyze 25 genes (the list of syndromes and genes is available on request). Despite the multifactorial nature of this disease, this approach makes us confident that we can improve our understanding of the genetic causes of obesity in the near future.

Conflicts of interest

The authors declare no conflict of interest.

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Supplementary material

Table S1. Polymerase chain reaction primer pairs and annealing temperatures.