Novel functional variants in the *MC4R* gene among severely obese children from Iberian population

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Running title: *MC4R* and *LEPR* analysis in Iberian population.

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ABSTRACT

Background: To investigate the role of the high susceptibility mutations in the *MC4R* and *LEPR* genes in obese individuals belonging to families with evident hereditary patterns of severe and early overweight among western Iberian population. Relevant clinical aspects were described for each mutation carrier found in our cohort.

Methods: A total of 202 unrelated and severely obese patients since childhood, who referred, at least, two other cases of morbid obesity among first or second-degree relatives were enrolled. Bidirectional sequencing of the *MC4R* gene was carried out; the *LEPR* gene was analysed in 15 unrelated individuals from the 202 patients. Bioinformatic analysis was carried out to predict the possible impact of newly identified missense variants. Segregation analysis and/or genotype-phenotype description of each identified mutation or variants of unknown significance was carried out.

Results: Two *MC4R* gene mutations, p.R147G and p.G323E, were described for the first time, and p.R7H, p.G32E, p.H76R, p.S127L, p.T150I, p.I251L and p.G252S variants had been previously reported. A new dinucleotide TA insertion at -77pb pertaining to the initiation codon of the *LEPR* gene was found in the heterozygous state in one patient.

Conclusions: Previously published and the two new *MC4R* gene mutations were predicted to be deleterious and co-segregate with obesity; altered feeding behaviours were overrepresented among clinical signs and symptoms associated to severe overweigh in our individual carriers. The inefficient methods of treatment, to manage the disease among carriers, may promote objectives to optimise the increasing knowledge derived from the genetic counselling process. *LEPR* gene appeared implicated in a group our families.

INTRODUCTION

The complexity of monogenic forms of obesity has been slowly disappearing increasingly elucidated when single genetic alterations are described as deleterious causes. After discarding other signals and/or symptoms to which obesity is related as a main part of more complex genetic disease, patients affected with non-syndromic obesity who display a Mendelian pattern of inheritance, account for approximately 5% of all severe and early onset obese cases (Bell *et al*., 2005). This group represents the most extreme obese phenotype, among which individuals carrying mutations in the melanocortin-4 receptor (*MC4R*) and/or leptin receptor (*LEPR*) genes become obese at a very young age, remaining severely obese into adulthood (Ramachandrappa & Farooqi, 2011). Most of these mutations result from disruption of the leptin/melanocortin pathway in the central nervous system (CNS), which is critical in the regulation of energy balance (Coll *et al*., 2004). Until now, approximately 20 genes have been implicated in non-syndromic forms of monogenic obesity (Razquin *et al*., 2011; El-Sayed Moustafa & Froguel, 2013).

Melanocortin-4 receptor (*MC4R*) deficiency is the most common cause of monogenic obesity by increasing appetite and diminishing satiety. It is thought to explain 2-6% of extremely obese children and adolescent cases (Farooqi *et al*., 2003; Hinney *et al*., 2003, 2006; Santini *et al*., 2004, Wang & Tao, 2011). *MC4R* is a membrane-bound G-protein–coupled receptor that activates adenylate cyclase in response to the α-melanocyte–stimulating hormone (α-MSH) (Stutzmann *et al*., 2007). The expression of *MC4R* is restricted to the brain, where it is found in hypothalamic nuclei involved in food intake regulation (Mountjoy *et al*., 1994). The *MC4R* activation leads to decreased food intake and increased energy expenditure. Mutations in the single exon of the *MC4R* gene (18q21.3) lead to a reduced function. These deleterious variants

appeared normally in the heterozygous state, to which a dominantly inherited pattern have been attributed and is actually widely accepted; other family members also mutation carriers and obese have corroborated the codominant model for a group of mutations to this gene for high susceptibility to severe overweight. Different missense, nonsense, and frameshift *MC4R* mutations have been described (Human Mutation Database, HGMD) in a wide patient series with distinct ancestral origins. Currently, the clinical genotype-phenotype relationships in adult *MC4R* mutation carriers constitute an essential objective to define and corroborate the severity of each functional alteration (Hinney *et al*., 2003; 2006; Lubrano-Berthelier *et al*., 2006; Wang & Tao, 2011). To date, more than 150 different *MC4R* mutations, mostly leading to a reduced function, have been detected in obese individuals (Hinney *et al*., 2013). On the other hand, independent genome-wide association studies (GWAS) have shown obesogenic effects of several single nucleotide polymorphisms located downstream of the *MC4R* gene (Loos *et al*., 2008; Scherag *et al*., 2010). Leptin receptor deficiency is a very rare cause of severe early-onset obesity. Until now, only two studies have reported mutations in *LEPR* gene (1p31) associated with severe obesity. Farooqui *et al*. (2007) estimated the prevalence of *LEPR* mutations around 3% in a cohort of 300 subjects with severe early onset obesity. Probands were found homozygous for frameshift, nonsense and missense mutations resulting in an impaired receptor signalling, and one patient displayed compound heterozygous for a missense mutation and a frameshift (Farooqi *et al*., 2007). More recently, homozygosity for a novel *LEPR* missense mutation (p.P316T) was reported in two Egyptian cousins with severe early onset obesity (Mazen *et al*., 2011). In addition, contributions of three common *LEPR* polymorphisms (p.K109R, p.Q223R and p.K656N) have been widely investigated through association studies. The p.Q223R polymorphism has been associated with obesity and predicts a small percentage of body

weight and body composition variability, but not p.K109R or p.K656N (Yiannakouris *et al*., 2001). A recent genome wide association study revealed *LEPR* gene polymorphisms p.K109R, p.Q223R, rs2767485, rs1751492 and rs4655555 significant associated with leptin receptor levels in the circulation (Sun *et al*., 2010).

The main goal of this study was to screen for mutations in the *MC4R* gene in a sample of children with morbid obesity from the western Spanish population, who were evaluated along with their families. A group of Portuguese severely obese children was also included in the analysis. *LEPR* gene was screened in a very specific group of patients with high risk criteria. Familial co-segregation analysis of *MC4R* mutations or *LEPR* variant of unknown significance was also performed.

PATIENTS AND METHODS

Patients

A total of 202 unrelated subjects from the Iberian Peninsula were selected as candidates for genetic analysis of the *MC4R* gene. The 170 severely childhood obese since childhood patients were selected from families screened as a part of a study to detect cases of morbid obesity in Extremadura (western Spain) (González *et al*., 2012) and whose pathogenesis can be attributed primarily to genetic alterations. It was considered as essential to select patients who had reached a weight greater than + 3 SD before 14 years, and which overweight had been maintained, belonging to families with at least two other individuals who developed a similar phenotype among its first and/or second degree members. The extensive phenotypic description of each patient allowed us to identify other causes of non syndromic obesity and/or the existence of dysmorphic signs and/or intellectual disabilities which discarded the patient for this series, the enormous progress in understanding the molecular basis of syndromic obesity, discards

its approach with the strategies suggested in this article. The existence of grade 3 overweight (commonly called morbid obesity) was considered to be a body mass index (BMI) greater than or equal to 40 kg/m². In children, this grade corresponds to a BMI greater than the 95th percentile for age-matched and sex-matched control subjects from our population data. Individuals carrying mutations and/or variants of unknown significance (VUS) in the sequenced genes, gave their consent to contact at least first degree family members to carry out the segregation analysis.

The 32 Portuguese subjects aged 6-12 years old (21 boys; 11 girls) were selected from a previous study comprising 1433 children that volunteered from public schools in central region of Portugal, and classified using age and sex specific BMI cut-offs provided by the International Obesity Task Force (IOTF) (Albuquerque *et al*., 2012). All children selected for *MC4R* genetic analysis were above the 99 percentile.

The study protocol was approved by the Ethical Committee of the Infanta Cristina Hospital and by *Direção-Geral de Inovação e de Desenvolvimento Curricular,* the ethical Committee of the Portuguese Ministry of Education. The study was conducted in accordance with the Declaration of Helsinki and its subsequent revisions, as well as the institutional guidelines of the University of Coimbra. Written informed consent was previously obtained from all patients and the children´s parents prior to enrolment.

Anthropometric measurements and Clinical valuation

All subjects underwent anthropometric measurements of height, weight, waist and hip circumference using a standardized protocol during interview and clinical examination. Body weight (kg) and height (cm) were taken with participants dressed in lightweight clothing without shoes to determine the BMI. Waist circumference (WC) (cm) was measured midway between the lowest rib and the iliac crest, to the nearest 0.1

cm after inhalation and exhalation. Hip circumference (cm) was measured at the point over the buttocks yielding the maximum circumference. BMI was calculated by dividing weight (in kilograms) by height (in meters) squared (kg/m^2). Using the WC criterion, WC = 90 cm for men or WC = 80 cm for women, patients were classified as abdominal obese (Alberti *et al*., 2005). The characteristics and composition of the study sample is summarized in Table 1.

The patients of Spanish origin were exhaustively clinically evaluated in the Pediatric and Endocrinology Services of the Infanta Cristina Hospital, according to the signals and symptoms they presented, and the description of their familial phenotypes. The aim was to rule out other etiologies causing severe obesity, above all the monogenic obesity syndromes often associated with mental retardation, dysmorphic features, and/or congenital developmental abnormalities (OMIM: http://www.ncbi.nlm.nih.gov/omim/). For each patient, a personal data questionnaire and construction of a broad family tree referring to comorbidities and previous pathologies was performed. It was considered essential to obtain a quality family history, also addressing origin and possible consanguinity. Each patient was examined and treated by paediatric endocrinologists, and recruited after discarding other etiologies causing severe obesity as syndromes often associated with mental retardation, dysmorphic features, and/or congenital developmental abnormalities.

Sequence analysis of MC4R and LEPR genes

In the Spanish series, genomic DNA was extracted from peripheral blood using the QIAamp DNA Kit (Qiagen, GmbH, Hiden, Germany) following the manufacturer's instructions, whereas in the Portuguese patients, DNA was collected from buccal swabs

using the PureLink Pro 96 Genomic DNA Kit (Invitrogen Corporation, Carlsbad, CA, USA).

Bidirectional sequence analysis of genomic DNA to cover the entire single coding exon sequence of the *MC4R* gene RefSeqmRNA NM_005912.2 (chromosome 18: 58,038,564-58,040,001), was initially performed on the 202 subjects from the non syndromic and severe childhood obesity series. After discarding mutations and/or variants of unknown significance in the *MC4R* gene, the 18 coding exons and flanking intronic regions of the *LEPR* gene RefSeqmRNA NM_002303.5 (chromosome 1: 65,886,248-66,107,242) were also subjected to bidirectional sequencing in 15 unrelated individuals from the 202 patients, because they exhibited delayed puberty due to hypogonadotropic hypogonadism and short stature, in addition to hyperphagia and severe obesity. Primer sequences used for amplification of both *MC4R* and *LEPR* genes were designed with Primer 3 software (Rozen & Skaletsky, 2000) and are available upon request. Sequencing was performed on an ABI 3130 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) using Big Dye Terminator v3.1 Cycle Sequencing Chemistry (Applied Biosystems, Foster City, CA, USA), according to protocols recommended by the manufacturer. Base calling was performed with Sequencing Analysis v5.2 (Applied Biosystems, Foster City, CA, USA). The sequences were analyzed with the Staden package software (Staden, 1996) and the Applied Biosystems SeqScape Software v2.5. The obtained sequences were compared with the standard sequence (ENSG00000166603 for *MC4R* and ENSG00000116678 for *LEPR*).

Bioinformatics analysis

To predict the possible impact of newly identified and not previously published missense variants, Alamut (Interactive Biosoftware, Rouen, France) and PolyPhen2

(Polymorphism Phenotyping v2) software (Adzhubei *et al*., 2010), was used to predict the possible phenotypic effect of aminoacid changes. For potential transcription factor binding site analysis the TFSearch software (http://www.cbrc.jp/research/db/TFSEARCH.html) was used based on the TRANSFAC database (Heinemeyer *et al*., 1998). DNA sequences encompassing approximately 20bp upstream and downstream the polymorphic site were provided as input sequence data. Taxonomy matrix was entered as "vertebrate" and the threshold score was set at 85.0 for the analysis.

RESULTS

MC4R gene analysis

In the total cohort of 202 unrelated subjects with severe early-onset obesity, *MC4R* gene sequencing enabled the identification of ten different missense variants: p.R7H, p.G32E, p.H76R, p.V103I, p.S127L, p.R147G, p.T150I, p.I251L, p.G252S, and p.G323E (Table 2). All the patients were found heterozygous for a single genetic change, except a compound heterozygous patient carrying both of the p.S127L and p.G252S mutations. Mutations p.R147G and p.G323E are described for the first time, whilst p.R7H, p.G32E, p.H76R, p.S127L, p.T150I, p.I251L and p.G252S variants had previously been reported (Table 2). The p.V103I substitution was previously described as a common polymorphism, reaching in this study sample an allele frequency of 1.24% (Table 2).

Regarding the Portuguese children, only the p.V103I polymorphism was found at the heterozygous state in a 6 years-old boy with a BMI Z-score of 2.60 (Height: 134.4 cm; weight: 42.7 kg), an allele frequency of 1.6% (1/32).

Bioinformatic analysis using Alamut software, which integrates protein structure and interaction predictions with conservation information, shows that the possible effect of the firstly identified p.R147G and p.G323E substitutions was predicted to be deleterious, having a high score of 1 meaning "pathological effect". The 323Gly amino acid is located in the intracytoplasmic final β turn of the protein and showed full conservation among mammals but not with non-mammals (Fig 1).

LEPR **gene analysis**

The screening of the entire coding and flanking regions of the *LEPR* gene in 15 unrelated individuals with morbid obesity, selected on the basis of additional clinical signals, allowed the identification of 14 different previously described polymorphisms: six were found in the coding region (p.K109R, p.Q223R, p.S343S, p.R612H, p.K656N and p.P1019P) and 8 were intronic SNPs (Table 3). A new variant in the promoter region that consists in a dinucleotide insertion TA, located at the 77 base pair upstream the initiation codon was found in one patient at the heterozygous state (Table 3). No other *LEPR* variants were found in our patient series.

Segregation analysis

We analysed the available DNA sample from first degree family members of the 8 individuals carrying the *MC4R* mutations, and from relatives of the patients carrying the variant of unknown significance of *LEPR*. It was observed that in all the pedigrees the mutation co-segregated with severe obesity at early onset, and with high BMI was maintained along the whole period of life or substituted by pathologic eating behaviour and/or addictions. Relevant clinical aspects are described below for each mutation

found in our cohort. Figure 2 showed segregation analysis in five families carrying different mutations, including the two new *MC4R* mutations.

Pedigree A, *MC4R* p.R7H mutation

The *MC4R* p.R7H mutation was found in a 67 year-old female. She had been morbidly obese since childhood and presented a mild mental retardation. At the age of 52, she entered haemodialysis due to a focal segmental glomerulosclerosis and she has been losing weight since then. Her son inherited the *MC4R* p.R7H mutation and presented morbid obesity and mild mental retardation as well; the genetic base of familial mental deficiency has not been identified to date. The patient was not discarded for *MC4R* sequencing because other relatives brother and daughter also affected with mental retardation never revealed overweight or obesity phenotype.

Pedigree B, *MC4R* p.H76R

The *MC4R* p.H76R mutation was found in a 28 year-old female with morbid obesity (128 Kg, BMI 50.6 kg/m^2). She had been diagnosed with type 2 diabetes and treated with metformin. Her grandmother on her mother´s side weighted 200 kg and her father, sisters and aunts in both sides suffered from morbid obesity too. The patient died due to a surgical complication during bariatric surgery.

Pedigree C, *MC4R* p.S127L and p.G252S

The patient carrying both the mutations p.S127L and p.G252S in the *MC4R* gene was a 27 year-old female. Her history included severe smoking habit and a long condition of morbid obesity since early infancy. She had attempted suicide when she was 25 years-old and was diagnosed with personality disorder. At that moment, she

weighted 115 kg (BMI: 43 kg/m^2). Clinical evaluations performed two years after that episode revealed a 60 kg weight loss, to a current BMI of 24 kg/m^2. The patient reported that she had suddenly begun to lose weight due to an important increase in daily physical activity at work as well as to a strong aversion to food. She followed erratic eating patterns. On laboratory tests, vitamin D and folic acid deficiencies were found. One of her brothers was obese and died at the age of 13 year-old. Her 8 year-old son weighted 45 kg (3.8 SD) and her 5 year-old daughter 36 kg (5.5 SD). *MC4R* molecular analysis was performed in the patient daughter and the p.S127L mutation was found.

Pedigree D, *MC4R* mutation p.R147G

The new *MC4R* mutation, p.R147G, was found in a 29 year-old female patient and his 11 year-old son. Both of them were severely hyperphagic and presented with extreme obesity (180 kg, BMI: 74 kg/m^2 and 75 kg, BMI: 34.2 kg/m^2, +3.2 DS, respectively) since early infancy, as well as primary hypothyroidism. The mother history also included smoking habit, type 2 diabetes and polycystic ovary syndrome. She had been diagnosed with binge-eating disorder with poor response to pharmacotherapy. She severely restricted intake and lost 75 kilograms in one year, in order to improve ovulation and became pregnant, and quickly regained 26 kg in three months after delivery.

Pedigree E, *MC4R* p.T150I mutation

The p.T150I mutation was associated with BMIs higher than 40 kg/m^2, and was found as unique causal variant and in a heterozygous state. The more progressed phenotype was a morbidly obese 56 year-old woman who weighed 123 kg (BMI: 45.9 kg/m^2). She had been overweight since childhood and she had gained weight

progressively; in the last ten years, she had gained 25 kg after having stopped smoking. She reported extreme hyperphagia. She had been diagnosed with binge-eating disorder and anxiety and had been treated with topiramate, which she was intolerant to, and fluoxetine, which had shown ineffective in controlling hyperphagia. The patient had a history of arterial hypertension and hypercholesterolemia and she had undergone surgery for a non-functioning pituitary macroadenoma.

Pedigree F, *MC4R* mutation p.I251L

A 10 year-old girl and her 47 year-old father carried the *MC4R* mutation p.I251L. The girl presented severe obesity and she had been referred when she was 7 years-old (55.4 kg, IMC: 30.4 kg/m^2, +5.3 DS). The father presented impaired basal glucose and several members of the family had been diagnosed with type 2 diabetes. Her 38 year-old mother presented morbid obesity since childhood, reaching a maximum weight when she was 23 years-old (200 kg, BMI 75 kg/m^2). She had undergone bariatric surgery. Nevertheless, the complete gene sequencing failed to show the same *MC4R* mutation.

Pedigree G, *MC4R* mutation p.G323E

The *MC4R* mutation p.G323E was found in three brothers. The index patient was referred when he was 8 years-old weighing 38.5 kg (BMI 26.7 kg/m^2, +3.3 DS). At the age of 17, he developed anorexia nervosa and he received psychiatric treatment. His two brothers, also carriers, presented obesity (51.9 kg, BMI: 23,3 kg/m^2, + 0.7 DS, at the age of 13 and 46.6 kg, BMI 26.7 kg/m^2, + 2.28 DS, at the age of 8, respectively) and one of them had been diagnosed with attention deficit disorder. The father carried the mutation. Another brother and the mother were negative for the mutation.

Pedigree of the p.G32E mutation in the *MC4R* gene was not achieved because the family declined to participate in the study.

Pedigree H*, LEPR* variant 5´UTR -77insTA

The family, in which the promoter variant of unknown significance in the *LEPR* gene had been transmitted, exhibited a difficult establishment of a genotype-phenotype correlation. The index patient was a 62 year-old woman with severe early-onset hyperphagia and obesity (125 kg, BMI: 47.7 kg/m^2). She had been diagnosed with depression, anxiety and panic attacks and treated with fluoxetine and topiramate, with partial improvement of hyperphagia. Her history included high blood pressure, type 2 diabetes and a recent diagnosis of acromegaly with empty sella and pathologic pituitary octreotide uptake. She had an obese son, who did not carry the mutation in the *LEPR* gene, another obese son (BMI: 35.5 kg/m^2) who had inherited the mother mutation and a daughter who also had inherited the mutation and who had developed anorexia nervosa in adolescence and currently maintained a healthy weight.

DISCUSSION

Mutations in the leptin-melanocortin pathway are widely accepted as the most prevalent form of monogenic obesity. The *MC4R* gene, encoding for the 332-amino acid seven-transmembrane G-protein-linked receptor, is critically involved in regulating energy balance (Grantz *et al*., 1993). More than 150 variants of this gene have been described as decreasing *MC4R* activity associated with human obesity, and are usually classified into different classes depending of their molecular characteristics (Hinney *et al*., 2013). The prevalence of severe obesity associated with mutations in this pathway is

estimated around 2.5% (Beckers *et al*., 2006).

Hence, among children with severe and early-onset obesity, screening for mutations in the *MC4R* and *LEPR* genes could be the first step to determine inherited susceptibility as the main and genetically conditioned causes of the overweight. One of the most important factors that could influence the outcomes and cost/benefit ratio of the indicated genetic studies, is the criteria and selection of the candidate patients. Patients carrying *MC4R* or *LEPR* mutations show severe obesity since childhood and extreme hyperphagia, which is unresponsive to pharmacotherapy. An interesting aspect is the sudden and fast lost of important amounts of weight, sometimes with immediate rebounding, along with the development of a pathological aversion to food or anorexia nervosa in some patients. It has been reported that in *MC4R* deficiency the severity of hyperphagia appears to partially ameliorate with time and that obese adult mutation carriers report less intense feelings of appetite than children with the same mutation. Other authors defended that an age-related penetrance for *MC4R* deficiency and demonstrated a generational effect on penetrance, related to the development of an "obesogenic" environment. It may contribute to coexistence of obese non carriers individuals among relatives of those strongly genetically predisposed (Stutzmann, 2008).

The main aim in this study was to screen the entire coding region of *MC4R* and *LEPR* gene to investigate possible genetic causes in a series of severely obese Iberian population. To improve the efficiency of genetic analysis, only obese individuals with high inheritance risk criteria were included,

In our study, the frequency of heterozygous individuals with *MC4R* gene sequence variations was 3.9% (8/202), excluding the two most common polymorphism p.V103I and p.I251L. This frequency is consistent with previous studies (2-6%) in individuals

with a similar severely obese phenotype. A total of 10 different *MC4R* sequence variations were found in our series; two of them, p.R147G and p.G323E, had not been previously described in the literature or mutation databases:

The novel p.R147G mutation is the result of an A to G nucleotide transition at position c.439 resulting in the drastic non-conservative amino-acid substitution of the charged polar basic arginine (Arg) to the non-polar and most simple amino-acid glycine (Gly) at codon 147 (p.R147G). This mutation was found in a 29 year-old female patient and his 11 year-old son, both presenting extreme obesity, which suggests the partial penetrance of the p.R147G mutation in the heterozygous state. The Arg147 amino acid is located in the intracytoplasmatic H3 helix strand. In concordance, conservation analysis between different species showed that the 147Arg residue is fully conserved between mammals and non-mammals (Fig. 1) reflecting important structural and/or functional features.

The new p.G323E mutation resulting from a G to A nucleotide transition at position c.968, produces the non-conservative amino-acid substitution of a glycine (Gly) to the charged, polar acidic glutamic acid (Glu) at codon 323 (G323E) . All patients carrying a mutation in the *MC4R* gene, including the new mutations found, suffered eating disorders from hyperphagia to bulimia and/or anorexia nervosa. The adults carrying *MC4R* mutations developed phenotypes with BMIs from 43.7 to 58.3 kg/m^2 (~140 kg; ~1.55 m). The maximum weight was achieved in the second decade of life and the severe overweight was maintained in spite of clinical management and diverse treatments.

It is known that *MC4R* decreases food intake in response to α-MSH activation (Lubrano-Berthelier *et al*., 2003a, 2003b). Functional studies have previously reported four known *MC4R* mutations p.R7H, p.S127L, p.T150I and p.G252S, which are now

also found among Spanish patients. These variants demonstrated an impaired response to α-MSH, suggesting that this could be the mechanism underlying the obese phenotype (Lubrano-Berthelier *et al*., 2003a, 2003b). The p.G32E mutation described by Ahituv *et al*. (2007) and the p.H76R mutation previously described by Stutzman *et al*. (2008) did not show any effect on the cAMP response leading to increasing concentrations of α-MSH. The index case in the *MC4R* mutation p.R7H presented a focal segmental glomerulosclerosis which ended in terminal renal failure. An obesity-related glomerulopathy has been described as a secondary form of focal segmental glomerulosclerosis occurring in obese patients with a body-mass index higher than 30 kg/m^2 (Darouich *et al*., 2011). It is typically manifested by nephrotic-range proteinuria without full nephrotic syndrome, and progressive renal insufficiency. In some patients with chronic kidney disease, gastric bypass results in stabilization or improvement of their kidney disease (Darouich *et al*., 2011; Amann *et al*., 2013).

Regarding the two common *MC4R* polymorphisms, p.V103I and p.I251L, all subjects with these two mutations were found heterozygous. Its functional implication in obesity is controversial since several case-control studies found similar 103Ile frequencies between extreme obese and non-obese individuals (Meyre *et al*., 2009). Regarding the p.Ile251Leu variant, a meta-analysis conducted by Stutzmann *et al.,* (2007) confirmed the protective effect of the 251 Leucine allele. Individuals carrying this allele have a reduced risk of obesity, nearly 50%, when compared with carriers of the wild type allele (251 Ile). Nevertheless, more studies are necessary to confirm if this polymorphism is or not associated with BMI.

The *LEPR* gene has been suggested as responsible for energy restriction (Mars *et*

al., 2004). Mutations in *LEPR* gene are very uncommon and to date only 11 mutations have been described in association with obesity (Farooqui *et al.*, 2007; Mazen *et al.*, 2011). Results concerning the frequency of *LEPR* variants are probably due to the limited screening.

In our study we described for the first time a new *LEPR* variation corresponding to an insertion of a TA dinucleotide in the -77 bp position of the promoter region. This dinucleotide insertion was found in a 62 year-old woman with severe early-onset hyperphagia and obesity (BMI: 47.7 kg/m^2) at the heterozygous state. However, the genotype-phenotype correlation in family members seems to be more complex, as an obese son did not carry the mutation. This fact, combined with a transcription factor (TF) search analysis which revealed that the -77 TA insertion would not modify any brain tissue-specific consensus binding-site sequence, suggests that this insertion likely do not to contribute to the patient´s phenotype.

A total of 14 previously described polymorphic variations were also found in coding and intronic *LEPR* gene regions. Allele frequencies for each variant were similar to other European populations (available in Ensembl database http://www.ensembl.org/Homo_sapiens/Variation), except for the c.370+16G>T and c.2213-75A>T changes which showed allele frequencies of 40% and 53%, respectively. In our series, their prevalence were much higher as compared with the Ensembl reported data (<1%).

Concerning the non-synonymous *LEPR* polymorphisms, namely p.K109R, p.Q223R, p.K656N and p.R612H, several studies have shown inconclusive results (Gotoda *et al.*, 1997; Rosmond *et al.*, 2001; Yiannakouris *et al.*, 2001; Furusawa *et al.*, 2010). Indeed, a systematic review and meta-analysis on the association between these three *LEPR* polymorphisms and obesity-related outcomes found no relevant

associations (Bender *et al*., 2011).

However, other studies did found positive correlations, for instance, a study conducted by (Bender *et al*., 2011) showed the p.Q223R polymorphism associated with overweight, considering a BMI cut-off of 25 kg/m^2. In the same manner, a study conducted by Roman *et al*. (2006) in a Spanish sample revealed an association of the p.K656N polymorphism with decreased response to leptin and lower weight loss secondary to changes in lifestyle behaviour (Mars *et al*., 2004). In our study, the obtained prevalence for the p.Q223R and p.K656N polymorphisms was similar to that previously described in the literature for obese patients (Gotoda *et al*., 1997; Rosmond *et al*., 2001; Yiannakouris *et al*., 2001). For the p.K109R polymorphism, we found a lower frequency (10%) than that previously described results for Iberian populations (29%, data from Ensembl database).

With regard to the last polymorphism, p.R612H, Branson *et al*., (2003) described for first time this mutation with a frequency of 0.1%. One individual was heterozygous for the polymorphism (frequency = 6.7%) in our series.

CONCLUSIONS

In conclusion, to our knowledge, this is the first study to screen for *MC4R* gene mutations in a sample of Iberian population with a history of severe obesity from childhood. The two new mutations found in the *MC4R* gene, p.R147G and p.G323E, are predicted to be highly deleterious and co-segregate with obesity and/or altered feeding behaviours among carrier family members. Only a non previously reported variation was found in the promoter of the *LEPR* gene whose possible functional capability needs combined and exhaustive approaches to be elucidated.

The intronic variants rs2186248 and rs190485648 of the *LEPR* gene, were pointed out as risk genetic marker of severe overweight in our series. Different and combined strategies for genetic analysis in this group of families could identify new causal susceptibility variants to early and severe overweight.

The exhaustive description of clinical phenotype at different ages among individual carriers may be considered as essential in the investigation for functional implication of the new and previously described mutations. *MC4R* genetic variation could be assumed as a likely cause of severe obesity among the Iberian population, and therefore genetic counseling associated to high inherited risk to severe overweight might be helpful in routine clinical management. Finally, an exhaustive clinical description was provided by a multidisciplinary research team evidencing the importance of coordinating disciplines for obesity investigation.

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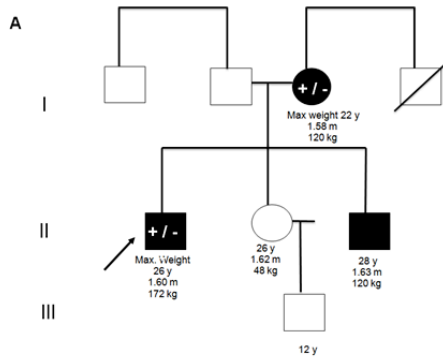
Figures Legends:

Figure 1. Three-dimensional structure of melanocortin-4 receptor (*MC4R*) gene. This figure was elaborated with the RasMol software (Sayle & Milner-white, 1995) and the PDB database.
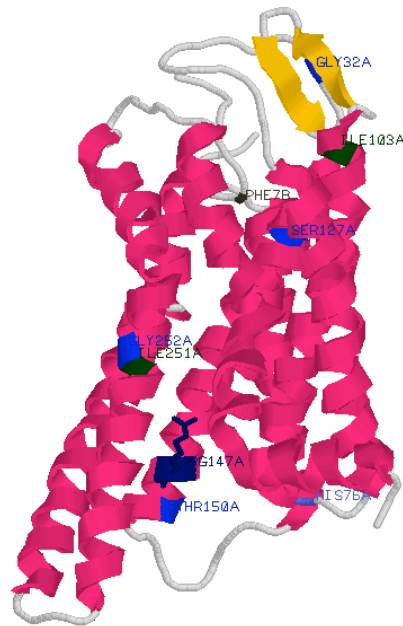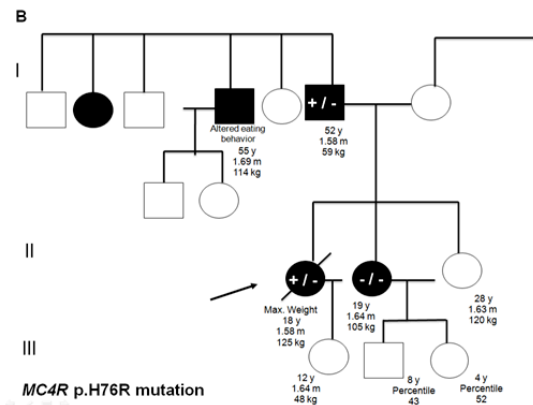

Figure 2. Pedigrees of families of *MC4R* and/or *LEPR* variant carriers. Squares and
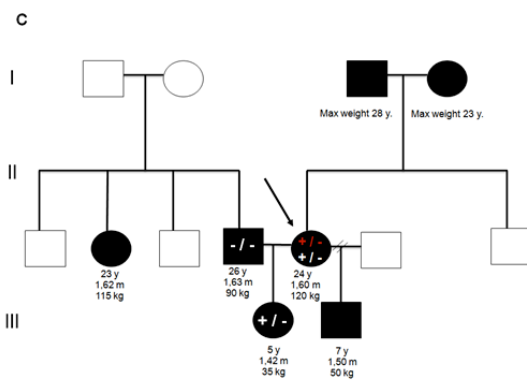
circles represent male and female family members, respectively. Black symbols denote family members with obesity, and grey symbols denote family members with overweight. Arrows indicate first individual where mutation was found. Below each individual, the age, height and weight is given along with genotype, where - represents a normal allele and + represents a mutant allele.

A

I

+/-
Max weight 22 y
1.58 m
120 kg

II

+/-
Max. Weight
26 y
1.60 m
172 kg

26 y
1.62 m
48 kg

28 y
1.63 m
120 kg

III

12 y

MC4R p.R7H mutation

B

I

Altered eating
behavior
55 y
1.69 m
114 kg

+/-
52 y
1.58 m
59 kg

II

+/-
Max. Weight
18 y
1.58 m
125 kg

-/-
19 y
1.64 m
105 kg

28 y
1.63 m
120 kg

III

12 y
1.64 m
48 kg

8 y
Percentile
43

4 y
Percentile
52

MC4R p.H76R mutation

C

I

Max weight 28 y.

Max weight 23 y.

II

23 y
1.62 m
115 kg

-/-
+/-
26 y
1.63 m
90 kg

+/-
+/-
24 y
1.60 m
120 kg

III

+/-
5 y
1.42 m
35 kg

7 y
1.50 m
50 kg

MC4R p.S127L/p.G252S mutations

D

I

52 y
1.54 m
107 kg

II

-/-

+/-
25 y
1.55 m
140 kg

28 y
1.75 m
97 kg

23 y
1.62 m
76 kg

III

+/-
11 y
1.50 m
75 kg

1 month

MC4R p.R147G mutation

E

I

II
-/-   +/-
56 y
1.65 m
125 kg

III
32 y
1.72 m
100 kg

30 y
1.78 m
95 kg

BMI>40
Before 25-
30 years

BMI>40
Before 25
years

MC4R p.T150I mutation

F

I

II
+/-   -/-
39y,
1,68 m
90 kg

Max weight 200 kg ,
17years
37y.
1,64 m
89 kg.
MC4R NORMAL

III
+/-
9 y
>3.5 SD

MC4R p.I251L mutation

G

I

II
40 y
1.59 m
90 kg
-/-

BMI>40
Before 25
years

BMI>40
Before 25
years

III
+/-
Max weight 13 y
1.56 m
59 kg

-/-
15 y

+/-
13 y
1.52 m
62 kg

MC4R p.G323E mutation

H

I

II
+/-
63 y
1.55 m
170 kg

-/-
59 y
1.60 m
75 kg

III
+/-
27 y
1.76 m
110 kg

-/-
30 y
1.93 m
135 kg

+/-
32 y
1.70 m
67 kg
Anorexia nervosa

5ÚTR -77pb insTA *LEPR*

Table 1. Characteristics of the sampled subjects screened for mutations.

Individuals	SPANISH		PORTUGUESE	
	n=170		*n*=32	
Age, years at inclusion	16.0	10	8.4	1.6
Age, years with >3 SD	6	4	-	-
Females	61%	-	35%	-
Height, cm	165.0	165.0	135.2	9.9
Weight, kg	NR	-	47.3	11.2
Percentil	>97%	-	>99%	-
Waist circumference, cm	-	-	78.3	6.6
Hip circumference, cm	-	-	89.0	8.8
WHR	-	-	0.87	0.0
Affected family members	4	-	-	-

Data are presented as mean ± standard deviation.
Abbreviations: *n,* number of subjects; SD, Standard Deviation; BMI, body mass index; BMI Z-score, body mass index standard deviation score; WHR, waist-to-hip ratio; NR, Not Representative; -, not provided data.

Table 2. *MC4R* mutations found in the total (*n*=202) study sample.

Gene	Pedigree	Nucleotide change	Codon change	AA change	SNP ID	HGMD reference	N° of Subjects[1]	Allele Freq.[2]	Reference	European MAF
MC4R	A	c.20G>A	CGT>CAT	p.R7H	rs142837166	CM035774	1	0.25%	Lubrano-Berthelier (2003)	NFD
MC4R	-	c.95G>A	GGA>GAA	p.G32E	NA	CM070989	1	0.25%	Ahituv (2007)	NFD
MC4R	B	c.227A>G	CAT>CGT	p.H76R	rs199558727	CM085524	1	0.25%	Stutzmann (2008)	NFD
MC4R	-	c.307G>A	GTC>ATC	p.V103I*	rs2229616	CM030481	5	1.24%	Branson (2006)	2%
MC4R	C	c.380C>T	TCG>TTG	p.S127L	rs13447331	CM030234	1	0.25%	Lubrano-Berthelier (2003)	NFD
MC4R	D	c.439A>G	AGG>GGG	p.R147G	NA	NA	1	0.25%	*This study*	NFD
MC4R	E	c.449C>T	ACT>ATT	p.T150I	NA	CM003759	1	0.25%	Vaisse (2000)	NFD
MC4R	F	c.751A>C	ATT>CTT	p.I251L	rs52820871	CM030483	1	0.25%	Branson (2003)	<1%
MC4R	G	c.754G>A	GGC>AGC	p.G252S	rs13447336	CM990835	1	0.25%	Hinney (1999)	NFD
MC4R	H	c.968G>A	GGA>GAA	p.G323E	NA	NA	1	0.25%	*This study*	NFD

* *MC4R* mutation found in a Portuguese child.
[1] Mutations were found at the heterozygous state.
[2] Allele frequency in all study sample (*n*=202).

Abbreviations: AA, Amino acid; NA, Not available; MAF, Minor allele frequency in Ensembl database among European population; NFD, No frequency data.

Table 3. *LEPR* mutations in 15 patients with morbid obesity.

Gene	Pedigree	Nucleotide change	Codon change	AA change	Local	Phenotypic effect	SNP ID	HGMD reference	N° of Subjects[1]	Allele Freq.[2]	Reference	European MAF
LEPR	I	-77pb delA	-	-	promoter	Unp.	NA	NA	1	3.3%	*This study*	NFD
LEPR	-	c.370+16G>T	-	-	Intron	NPD	rs2186248	NA	12	40.0%	Ensembl	<1%
LEPR	-	c.326A>G	AAG>AGG	p.K109R	Exon 4	NPD	rs1137100	CM032948	3	10.0%	Souren *et al.*, 2008	39%
LEPR	-	c.668A>G	CAG>CGG	p.Q223R	Exon 6	NPD	rs1137101	CM010905	9	30.0%	Quinton *et al.*, 2001	41%
LEPR	-	c.849+49A>G	-	-	Intron	NPD	rs3828034	NA	3	10.0%	Ensembl	11%
LEPR	-	c.850-50A>C	-	-	Intron	NPD	rs3762273	NA	15	50.0%	Ensembl	50%
LEPR	-	c.1029T>C	AGT>AGC	p.S343S	Exon 9	NPD	rs1805134	CM030473	8	26.6%	Mammes *et al.*, 2001	19%
LEPR	-	c.1835G>A	CGC>CAC	p.R612H	Exon 13	NPD	rs144159890	CM030476	1*	6.7%	Branson *et al.*, 2003	<1%
LEPR	-	c.1968G>C	AAG>AAC	p.K656N	Exon 14	NPD	rs8179183	CM025915	3	10.0%	Wauters *et al.*, 2002	15%
LEPR	-	c.2213-75A>T	-	-	Intron	NPD	rs190485648	NA	15	50.0%	Ensembl	<1%
LEPR	-	c.2396-37T>A	-	-	Intron	NPD	rs3790439	NA	5	16.7%	Ensembl	39%

LEPR	-	c.2597+15G>C	-	-	Intron	NPD	rs6693573	NA	1	3.3%	Ensembl	2%
LEPR	-	c.2673+37C>A	-	-	Intron	NPD	rs12067936	NA	10	33.3%	Ensembl	41%
LEPR	-	c.2673+287A>G	-	-	Intron	NPD	rs4655566	NA	3	10.0%	Ensembl	21%
LEPR	-	c.3057G>A	CCG-CCA	p.P1019P	Exon 20	NPD	rs1805096	NA	8	26.6%	Ensembl	40%

[1] Mutations were found at the heterozygous state except *
[2] Allele frequency in the study sample (*n*=15).

Abbreviations: AA, Amino acid; NA, Not available; MAF, Minor allele frequency in Ensembl database among European population; NFD, No frequency data; NPD, No Phenotype data associated to overweight in Ensembl database; Unp, Unpredictable.