A NOVEL MUTATION Thr162Arg OF THE MELANOCORTIN 4 RECEPTOR GENE IN A SPANISH CHILDREN AND ADOLESCENTS POPULATION

Short title: Mutations in MC4R in a Spanish children and adolescents population

MC Ochoa[1], C Azcona[2], H Biebermann[3], H Brumm[3], C Razquin[1], A-K Wermter[4], JA Martínez[1], J Hebebrand[5], A Hinney[5], MJ Moreno-Aliaga[1], A Marti[1] and GENOI members.

[1]Department of Physiology and Nutrition, University of Navarra, 31008 Pamplona (Navarra), Spain.

[2]Pediatric Endocrinology Unit, Deparment of Paediatrics, University Clinic, University of Navarra, 31008 Pamplona (Navarra), Spain.

[3]Institute for Experimental Pediatric Endocrinology, Charité Universitätsmedizin Berlin, Berlin, Germany.

[4]Clinical Research Group, Department of Child and Adolescent Psychiatry, University of Marburg, 35033 Marburg, Germany.

[5]Department of Child and Adolescent Psychiatry; University of Duisburg-Essen, Essen, Germany

Other GENOI members are: A Patiño, M Chueca, M Oyarzabal, R Pelach.

Key terms: MC4R, obesity, children

Correspondence to: Dra. A Marti, Department of Physiology & Nutrition, University of Navarra, 31008 Pamplona, Spain; Phone: 34-48-42 56 00; Fax: 34-48-42 56 49;

E-mail: amarti@unav.es

Abstract

Objective

The melanocortin 4 receptor gene (*MC4R*) is involved in body weight regulation. While many studies associated *MC4R* mutations with childhood obesity, information on *MC4R* mutations in Spanish children and adolescents is lacking. Our objective was to screen a population of children and adolescents from the north of Spain (Navarra) for *MC4R* mutations and to study the phenotypes of carriers and their families. In addition, functional assays were performed for a novel *MC4R* mutation.

Methods

The study was comprised of 451 Spanish children and adolescents (49% boys), aged 5-18 year. According to the International Obesity Task Force (IOTF) criteria, the groups included 160 obese, 132 overweight and 159 normal-weight control subjects.

Results

One novel (Thr162Arg) and three known non synonymous mutations in the *MC4R* gene (Ser30Phe, Thr150Ile, Ala244Glu) were detected heterozygously. The *MC4R* mutations were found in three male (one obese and two overweight) and two female subjects (one obese and one overweight). The novel mutation did not appear to lead to an impaired receptor function. An unequivocal relationship of *MC4R* mutations with obesity in pedigrees together with an impaired function of the encoded receptor could not be established for any of the mutations.

Conclusions

The presence of heterozygous *MC4R* mutations in obese and overweight subjects indicates that these mutations may be a susceptibility factor for obesity development, but lifestyle factors, such as exercise or sedentary activities, may modify their effect.

Introduction

The melanocortin 4 receptor (MC4R) exerts an important role in controlling food intake in humans. Since 1998, investigators have reported at least 72 mutations in the *MC4R* gene [1] and an impaired MC4R function seems to induce severe obesity soon after birth [2]. *MC4R* mutations are more frequent in northern European populations as compared to Mediterranean or Asian populations; its prevalence ranges from 0.5% to 6% [2]. Currently, a total of 91 mutation carriers have been reported in 3057 children and adolescent subjects, most of them heterozygotes, which represents 2.98% of childhood-onset obesity [3].

Although there is a consensus that *MC4R* mutations may excert a monogenic effect on extreme obesity, the direct involvement of *MC4R* mutations in causing obesity has been questioned by studies that have detected low-frequency mutations in adult control subjects. Marti et al. [4] and Jacobson et al. [5] reported two normal-weight subjects carrying mutations that encoded partially active receptors [6]. Hinney et al. [1] found six non-obese carriers of *MC4R* mutations with impaired receptor function *in vitro*. Thus, it is worth mentioning that the loss of *MC4R* function *in vitro* does not necessarily lead to obesity.

Furthermore, a number of studies have revealed discrepancies among investigators on the effect of a given mutation on obesity development, which may be partially explained by environmental influences. Lifestyle factors, especially those related to diet and physical or sedentary activities, can interact with genetic factors and may mask genotype influences [7, 8]. Our objective was to examine a children and adolescent

population (obese, overweight and normal-weight subjects) from the north of Spain (Navarra) for mutations in the *MC4R* gene and to test the relevance of family history of obesity. The sedentary and physical activities of all subjects were carefully examined. A functional characterization of a novel mutation of the *MC4R* gene was also performed.

Materials and methods

The study population, recruited from the pediatric departments of two hospitals and three community health centres, was composed of 451 Spanish children and adolescents (49% boys), aged 5-18 years. Obesity was defined according to Cole et al. [9] . Thus, the study group included 160 obese, 132 overweight, and 159 normal weight individuals. Non-obese participants were healthy subjects coming to the community health centres for routine medical examination or to be vaccinated. Eighty-three per cent of obese subjects were recruited through the hospitals, whereas 62% of overweight participants attended the health centres. Exclusion criteria were (i) exposure to hormonal treatment or ii) development of secondary obesity due to an endocrine disorder or other illness. Ethical approval was granted by the Ethics Committee of the University of Navarra. All parents and subjects who are 12 years of age or older provided written informed consent, whereas children younger than 12 years gave verbal consent to participate in the study. The reported investigation was carried out according to the Declaration of Helsinki II principles.

Anthropometric measurements were made by standard procedures and fat mass was measured by bioelectrical impedance (TBF-300 A Body Composition Analyzer/Scale, TANITA©, Tokyo, Japan). Following a 12-h fast, venous blood samples were obtained and serum glucose, lipids, insulin, cortisol and leptin were determined for

93% of the subjects. Trained researchers conducted face-to-face interviews and helped 80% of the participants and their parents fill out questionnaires. A recently validated physical activity questionnaire – the Spanish version of the physical activity questionnaire used in the Nurses' Health Study and the Health Professionals' Follow-up Study – was used.[11] . It included 17 activities and sports and nine option responses for frequency, ranging from "never" to "11 or more hours per week". A multiple of resting metabolic rate (MET score) was assigned to each activity. With this information, we computed an activity metabolic equivalent index (METs hours/week), which represents the amount of weekly leisure physical exercise for each participant [12, 13] . The sedentary activities were computed through questions pertained to time spent watching TV, playing computer or video games, reading, and sleeping during the day. In order to assess for hyperphagia, participants were asked to rate their appetite from 1 to 5.

Blood samples were taken for extraction and characterization of genomic DNA from leucocytes. Single strand conformational polymorphism (SSCP) screening for mutations in the *MC4R* gene was performed as previously described [14] . PCR products of samples with aberrant SSCP pattern were resequenced to identify specific nucleotide changes. For the unambiguous assignment of the genotypes, allele discrimination was made independently by at least two experienced researchers. Discrepancies were solved by repeating.

Family members of pedigrees, including carriers of mis-sense mutations, were asked to participate in the study. Anthropometric measurements were obtained and face-to-face interviews were conducted with each family member. The questionnaire included 8 questions about physical activity and 11 questions about binge eating disorder (BED), based on diagnostic criteria of the DSM-IV [15] . A "BED score" was calculated

ranging from 0 to 11 based on the number of positive answers to the DSM-IV questionnaire. Blood samples from volunteer family members were drawn for genotyping.

Functional characterization of the Thr162Arg mutant MC4R

The novel mutant MC4R identified in this study (Thr162Arg) and the wild-type MC4R were cloned in a pcDps expression vector. For functional studies, mutant and wild-type receptors were transiently transfected into COS-7 cells using Metafectene (Biotex, Munich, Germany) according to the manufacturer´s protocol. To investigate signal transduction properties, cells were labelled with 2 µCi/ml of [^{3}H]adenine (Amersham, Little Chalfont, UK) 72 hours after transfection. One day later, Cyclic AMP (cAMP) accumulation assays were performed. Cells were stimulated with increasing concentrations of α-melanocyte stimulating hormone (α-MSH), its potent analog, [Nle[4], D-Phe[7]]-α-MSH (NDP-MSH), or β-MSH; cAMP-assays were performed as described elsewhere.[16]. Cyclic AMP accumulation data were analysed using GraphPad Prism (GraphPad Software, San Diego, CA, USA). All data of the functional study were obtained from at least five independent experiments.

To investigate cell surface expression, MC4R wild-tupe and Thr162Arg mutant were N-terminally HA-tagged and cell surface ELISA studies were performed. In brief, 72 h after transfection, cells were washed two times with Dulbecco's phosphate buffered saline (DPBS) and fixed for 30 min in 4% formaldehyde in DPBS followed by two times washing in DPBS. Cells were then incubated in blocking buffer (10% fetal calf serum-supplemented Dulbecco`s minimal essential medium) for 1 h at 37°C followed by a washing step in DPBS and a 2-h incubation in blocking buffer with 1 µg/ml biotin labelled anti-HA monoclonal antibody (Roche) at 37°C. After three washes in

DPBS, cells were incubated in blocking buffer with 1:5000 diluted streptavidin labelled peroxidase (Dianova, Hamburg, Germany) at 37°C for 1 h followed by three times washing . The colour reaction was carried out in a buffer containing 0.1% H_2O_2 and 10 mg o-phenylendiamine in 0.1 M citric acid and 0.1 M Na_2HPO_4 at pH 5.2. The reaction was stopped after 10 min with 1M Na_2SO_3 in 1M HCl. Colorimetry was carried out using an Anthos reader 2001 (Anthos Labtech Instruments, Salzburg, Austria). The data of cell expression were calculated from 10 independent experiments.

Results

One novel and three known nonsynonymous mutations in the *MC4R* gene were detected heterozygously in two girls and three boys from a total of 451 (49 % boys) children and adolescents from a Navarra population group (Table 1). A synonymous variation has also been found in an obese child. Two children were heterozygous for the Val103Ile polymorphism (1 obese and 1 overweight) and 11 subjects carried the Ile251Leu polymorphism heterozygously (two obese, three overweight and six normal-weight subjects).

The prevalence of nonsynonymous mutations was 1.2% in the obese group and 2.3% in the overweight group. Phenotypic characteristics of participants (carriers and non-carriers of *MC4R* mutations) are summarized in Table 2. Information on physical and sedentary activities is also detailed. The obese and overweight study groups showed lower levels of physical activity as measured by metabolic equivalents (METs hours/week) than the normal weight subjects (37.3 and 27.6 respectively, *vs*. 18.2

METs hours/week). The differences were statistically significant ($p<0.001$, Kruskal-Wallis test). Moreover, normal-weight subjects spent less time engaging in sedentary activities (16.8 h/week *vs.* 17.0 h/week for the overweight group and 19.6 h/week for the obese group; $p=0.019$) than obese or overweight subjects. In Fig. 1a, a summary of the information regarding *MC4R* mutation carriers and their families is presented.

Finally, we evaluated binge eating disorder (DSM-IV [15] in *MC4R* mutation carriers. None of these individuals fulfilled the DSM-IV criteria for BED. There was no statistically significant difference for BED score between carriers and noncarriers of *MC4R* mutations (Fig. 1). Hyperphagia was also evaluated with the aid of a scale ranging from 1 to 5. As seen in Table 2, four out of six *MC4R* mutation carriers showed high levels of hyperphagia (score of 5).

Discussion

One novel (Thr162Arg) and three known (Ser30Phe, Thr150Ile, Ala244Glu) non-synonymous mutations in the *MC4R* gene were detected heterozygously by screening of 451 Spanish children and adolescents (49% boys). *MC4R* mutations were found in three male (one obese and two overweight) and two female subjects (one obese and one overweight). Differences in mutation frequency or obesity degree were not attributed to sex. However, previously, in a family–based setting, Dempfle et al. [17] found that *MC4R* mutation allele effects on BMI were stronger in females compared to males.

The prevalence of *MC4R* mutations was 1.7% in the obese and overweight groups of the Spanish children and adolescents. In the so far only study on *MC4R* mutations in

Spanish adults the frequency was 0.6% [4] . Similarly, lower values of prevalence for *MC4R* mutations in adult populations from other countries have been reported [1] .

The frequency of *MC4R* mutations observed in the present study is similar to values reported for obese children and adolescents from different European countries-Italy [18] , France [19] , Germany [20] , the UK[21] and Finland [22] - being the calculated mean mutation frequency of 2·94% (SE: 0·88).

Because discrepant results are reported in the literature concerning the presence of binge-eating disorder in *MC4R* mutation carriers, we performed face-to-face interviews with the aid of a BED questionnaire in 11 mutation carriers. None of the subjects fulfilled the diagnostic criteria for this eating disorder. Only the father of the index patient harbouring the Thr150Ile mutation, who transmitted the mutation, reported binge eating occasionally (twice a week).

Assuming an inverse relationship between obesity and levels of physical activity [23] , and that the protective effect of physical activity for obesity development may depend on the genotype [24] , the present study sought to evaluate physical activity and sedentary behavior of *MC4R* mutation carriers.

The Ser30Phe mutation of the *MC4R* gene was found in two obese subjects (male, aged 12 and female, aged 14). There are four known functional studies for the Ser30Phe variant itself, three *in vitro* assays established a normal receptor activity [20, 25, 26] , but one demonstrated a lower response to the agonist [19] (Table 4). Concerning the family of the proband -carrier of the Ser30Phe mutation-, his father and his brother were both carriers and obese, even though they reported to spend more than 30 METS

hours/week in physical activities. This mutation was first found in a 15-year-old German obese adolescent who also harboured another mis-sense mutation (Gly252Ser) [14] . In German populations, at least four other Ser30Phe carriers have been reported: two obese children [20], and two non-obese adults [1] . To our knowledge, this is the first time that this mutation usually associated with childhood obesity was found in a non-German population.

A novel nonsynonymous mutation of the MC4R gene consisting of the replacement of threonine for arginine at position 162 located in the 2nd intracellular loop of the MC4R was detected heterozygously in an overweight 13-year-old Spanish girl. All family members carrying the mutation were overweight or obese, while noncarriers were of normal weight. We have also conducted the first functional *in vitro* assay for the MC4R Thr162Arg mutation (Table 3). It appears that the mutated receptor had normal activity, while a slightly partial inactivation was found in maximal stimulation properties after the addition of α- or β-MSH agonist. Position 162 in the MC4R shows a high grade of conservation. Due to this condition, together with the fact that all experiments were conducted in an overexpression system using transient transfections, a significant influence of this mutation *in vivo* cannot be excluded.

The Thr150Ile variant of the MC4R was first described and characterized by Vaisse *et al.*[29]. It caused a decrease in receptor activation due to a reduction in receptor binding. Two recent studies also showed that this mutation modified not only the ability of the receptor to be activated by its ligand, but also its constitutive activity [3, 27] . In our Spanish population, two descendents of a father heterozygous for the Thr150Ile mutation (BMI=25.3 kg/m²) were also carriers of the mutation (one overweight and

one normal weight). Both carriers were physically active, spending more than 29.5 METs hours/week in physical activities and little time in sedentary activities, (Fig. 1, family B). Similarly, the Ala244Glu mutation of the *MC4R* gene also impaired the receptor function *in vitro*, as reported in three published studies [19, 20, 26] . In our study, the proband harbouring this mutation was overweight and very active (65.6 METs hours/week). It is likely that high level of physical activity of these carriers have aided in their prevention of obesity.

In regard to the two *MC4R* polymorphisms, two children (one overweight boy and one obese girl) had the Val103Ile SNP and 11 subjects (seven boys) carried the Ile251Leu SNP of the *MC4R* gene (two obese, three overweight and six normal-weight subjects). The frequency of the Ile251Leu SNP in the obese group was 1.3%, with similar results in the overweight and normal-weight groups. A comparable value (2.5%) was obtained after the screening of a Spanish adolescent population from Santander (n=118, 48% males, age mean=15.5 years, BMI mean= 22.6 kg/m^2) [28] . The observed values were similar to previous reports from other European countries: Germany (1.3%) [14,] and France (1.4%) [29] , which included subjects with severe childhood obesity and a family history of obesity.

The Val103Ile polymorphism was detected in two subjects (one obese and one overweight). Similarly, the frequency in the Spanish adolescent population (Santander) was 0.8% [28] . In a number of studies, this variant was the most prevalent polymorphism of the MC4R receptor, associated with a decreased body weight [30] . In our Spanish population, the frequency of the Ile103 SNP is too low to support any conclusion. This also occurred with adult Spanish subjects [4] .

In summary, in our Spanish children and adolescents population we found one novel and three already known nonsynonymous mutations of the *MC4R* gene in heterozygous carriers. Specifically, an overweight subject (13-year-old girl) had the novel mutation Thr162Arg, which does not appear to alter *in vitro* receptor activity. Meanwhile, two overweight physically active subjects were carriers of mutations, which led to an impairment of the receptor function *in vitro* (Ala244Glu and Thr150Ile). A third mutation, Ser30Phe, was present in two obese children (14 and 12 years old).

We conclude that the presence of heterozygous *MC4R* mutations in obese and overweight subjects indicates that there may be a susceptibility factor for obesity development. Lifestyle factors, such as exercise or sedentary activities, may also modify this effect.

Acknowledgements

We thank all individuals and their families for their participation. This work was supported by the Deutsche Forschungsgemeinschaft, the German Bundesministerium für Bildung und Forschung (BMBF; NGFN2; ; 01GS0482; 01GS0492), Gobierno de Navarra (Departamentos de Salud y Educación) and University of Navarra (Línea Especial Nutrición, Obesidad y Salud). M.C. Ochoa received a grant for University Exchange from the DAAD (German Academic Exchange Service).

Table 1. Frequency of *MC4R* mutations and polymorphisms in 292 Spanish obese and overweight, children and adolescents

Amino acid change	Nucleotide substitution	Obese (n=160)	Overweight (n=132)	Normal weight (n=159)
Ser30Phe	89C>T	2	0	0
Thr150Ile	449C>T	0	1	0
Thr162Arg	485C>G	0	1	0
Gly167Gly, silent	501G>A	1	0	0
Ala244Glu	731C>A	0	1	0
Val103Ile SNP	307G>A	1	1	0
Ile251Leu SNP	751A>C	2	3	6

Table 2. Phenotypic characteristics of the Spanish children and adolescents population groups and MC4R gene mutation carriers

	Obese	Overweight	Control	Ser30Phe	Ser30Phe (501G>A)	Gly167Gly	Thr150Ile	Thr162Arg	Ala244Glu
N	n=160	n=132	n=159	Obese	Obese	Obese	Overweight	Overweight	Overweight
Age (yr)	11.4 (0.23)	11.7 (0.22)	11.8 (0.22)	14	12	11	16	13	15
Sex (Female/Male)	83/77	67/65	79/81	Female	Male	Male	Male	Female	Male
Height (m)	1.50 (0.01)	1.49 (0.01)	1.50 (0.01)	1.62	1.57	1.55	1.82	1.51	1.71
Actual weight (kg)	67.4 (1.69)	53.7 (1.27)	42.5 (1.07)	79.8	66.2	62.0	86.0	52.3	77.0
BMI (kg/m^2)	28.9 (0.4)	23.5 (0.2)	18.4 (0.2)	30.4	26.9	25.8	26.0	22.6	26.3
BMI-SDS	4.35 (0.12)	1.99 (0.05)	0.04 (0.07)	3.54	3.39	3.36	1.56	2.48	1.84
WHR	0.88 (0.007)	0.84 (0.008)	0.81 (0.005)	NA	0.91	0.86	0.81	0.73	0.82
% fat mass	36.9 (0.6)	28.3 (0.7)	17.0 (0.6)	42.1	31.7	39.8	14.3	34.1	22.2
Glucose (mM)	4.95 (0.04)	4.87 (0.05)	4.64 (0.04)	4.72	4.94	5.00	5.38	5.22	NA
Insulin (pmol/L)	113.3 (5.60)	75.9 (6.89)	47.2 (3.41)	76.8	57.6	47.4	36.0	47.4	NA
TC (mM)	4.18 (0.07)	4.28 (0.07)	4.28 (0.07)	3.62	3.46	5.48	4.24	3.93	2.15
TG (mM)	1.00 (0.05)	0.83 (0.05)	0.71 (0.03)	0.23	0.48	1.46	1.40	0.85	NA
HDL (mM)	1.24 (0.03)	1.43 (0.04)	1.60 (0.03)	1.29	1.32	1.34	1.16	1.42	3.67
LDL (mM)	2.59 (0.06)	2.55 (0.07)	2.35 (0.06)	1.99	1.94	3.49	2.46	2.12	1.63
Leptin (ng/ml)	39.6 (1.66)	21.4 (1.49)	10.5 (0.94)	56.0	31.0	21.4	7.0	13.0	NA
Cortisol (nmol/L)	425.0 (15.7)	436.1 (22.1)	419.5 (22.6)	276	336.7	171.1	297.2	NA	NA
METs-hours/week	18.2 (0.45)	27.6 (1.82)	37.3 (1.76)	10.1	13.1	26.2	29.5	NA	65.6
Sedentary activities (h/week)	19.6 (0.97)	17.0 (1.03)	16.8 (0.91)	4.5	23.7	23.5	5	NA	4.5
Hyperphagia				-	+	+	+	-	+

Values are mean (SD) when indicated. WHR, waist-to-hip ratio; TC, total cholesterol; TG, triglycerides; HDL, high density lipoprotein; LDL, low density lipoprotein; NA, no avalaible. Hyperphagia (+) was considered when they rated their appetite as a 5

Table 3. Functional assay for a novel non-synonymous (Thr162Arg) mutation in the *MC4R* gene

		NDP-α-MSH		α-MSH		ß-MSH		Cell surface expr.
	Basal	Max	EC50	Max	EC50	Max	EC50	
Wild-type	1.00	1.00	0.33±0.24	1.00	2.0±0.58	1.00	6.0±1.7	100
Thr162Arg	0.88±0.20	1.04±0.38	0.86±0.52	0.78±0.18	1.5±0.75	0.8±0.17	6.6±3.4	97±15

For functional characterization cAMP assays were performed as described in Materials and Methods. Max and EC50 values were determined from concentration-response curves of agonists (endogenous agonists 0.1 - 1000 nM, NDP-α-MSH 0.01 - 100 nM) using GraphPad Prism. Max values are given as percentage of maximum stimulation of the wild-type receptors. Data are presented as means ± SEM of five in duplicate performed independent experiments. Cell surface expression levels were measured by indirect cellular ELISA. Specific optical density (OD) readings (OD value of HA-tagged construct minus OD value of GFP-transfected cells) are given as percentage of wild-type HA-tagged MC4R. The nonspecific OD value (GFP) was 0.14 ± 0.04, and the OD value of the wild-type HA-tagged MC4R was 1.37 ± 0.29. ELISA data are given as mean ± SD of three independent experiments, each carried out in sextuple.

Table 4. Summary of some characteristics of the *MC4R* mutations according to the literature

Mutation	Membrane expression (%wt)	Basal activity (% wt)	Alpha-MSH EC_{50} (fold over weight)	Function	Study
Ser30Phe	ND	140	0.45	Similar to wild type	Hinney et al.[20]
	>100	ND	1.98	Reduced response to agonist	Lubrano-Berthelier *et al.* [19]
	ND	ND	Similar to wild type	Similar to wild type	Santini *et al*. [25]
	Similar to wild type	ND	0.65	Similar to wild type	Xiang *et al* [26]
Thr150Ile	ND	ND	23.9	Reduced response to agonist	Vaisse *et al*. [29]
	>100	<10	9.63	Reduced basal function and response to agonist	Lubrano-Berthelier *et al.*[3]
	>100	7	19.6	Reduced basal function and response to agonist	Govaerts *et al.* [27]
	~60	ND	12.5	Reduced response to agonist	Xiang *et al* [26]
Thr162Arg	97 ± 15	88 ± 20	Similar to wild type	Similar to wild type	This study
Ala244Glu	ND	50	8.33	Reduced basal function and response to agonist	Hinney et al. [20]
	90	ND	6.30	Reduced response to agonist	Lubrano-Berthelier *et al.*[19]
	~70	ND	1.74	Reduced response to agonist	Xiang *et al*. [26]

ND, not determined

References

1 *Hinney, A., Bettecken, T., Tarnow, P., Brumm, H., Reichwald, K., Lichtner, P., Scherag, A., Nguyen, T.T., Schlumberger, P., Rief, W., Vollmert, C., Illig, T., Wichmann, H.E., Schafer, H., Platzer, M., Biebermann, H., Meitinger, T. & Hebebrand, J. (2006) Prevalence, spectrum, and functional characterization of melanocortin-4 receptor gene mutations in a representative population-based sample and obese adults from Germany. Journal of Clinical Endocrinology and Metabolism, 91, 1761–1769.*

2 *Farooqi, I.S. & O'Rahilly, S. (2005) Monogenic obesity in humans. Annual Review of Medicine, 56, 443–458.*

3 *Lubrano-Berthelier, C., Dubern, B., Lacorte, J.M., Picard, F., Shapiro, A., Zhang, S., Bertrais, S., Hercberg, S., Basdevant, A., Clement, K. & Vaisse, C. (2006) Melanocortin 4 receptor mutations in a large cohort of severely obese adults: prevalence, functional classification, genotype–phenotype relationship, and lack of association with binge eating. Journal of Clinical Endocrinology and Metabolism, 91, 1811–1818.*

4 *Marti, A., Corbalan, M.S., Forga, L., Martinez, J.A., Hinney, A. & Hebebrand, J. (2003) A novel nonsense mutation in the melanocortin-4 receptor associated with obesity in a Spanish population. International Journal of Obesity and Related Metabolic Disorders, 27, 385–388.*

5 *Jacobson, P., Ukkola, O., Rankinen, T., Snyder, E.E., Leon, A.S., Rao, D.C., Skinner, J.S., Wilmore, J.H., Lonn, L., Cowan, G.S. Jr, Sjostrom, L. & Bouchard, C. (2002) Melanocortin 4 receptor sequence variations are seldom a cause of human obesity: the Swedish Obese Subjects, the HERITAGE Family Study, and a Memphis cohort. Journal of Clinical Endocrinology and Metabolism, 87, 4442–4446.*

6 *Tao, Y.X. & Segaloff, D.L. (2005) Functional analyses of melanocortin-4 receptor mutations identified from patients with binge eating disorder and nonobese or obese subjects. Journal of Clinical Endocrinology and Metabolism, 90, 5632–5638.*

7 *Ochoa, M.C., Moreno-Aliaga, M.J., Martínez-González, M.A., Martínez, J.A., Marti, A. & Members, G. (2006) TV watching modifies obesity risk linked to the 27Glu polymorphism of the ADRB2 gene in girls. International Journal of Pediatric Obesity, 1, 83–88.*

8 *Marti, A., Moreno-Aliaga, M.J., Zulet, A. & Martinez, J.A. (2005) Advances in molecular nutrition: nutrigenomics and/or nutrigenetics. Nutrición Hospitalaria, 20, 157–164.*

9 *Cole, T.J., Bellizzi, M.C., Flegal, K.M. & Dietz, W.H. (2000) Establishing a standard definition for child overweight and obesity worldwide: international survey. British Medical Journal, 320, 1240–1243.*

10 *Moreno, L.A., Mesana, M.I., Gonzalez-Gross, M., Gil, C.M., Fleta, J., Warnberg, J., Ruiz, J.R., Sarria, A., Marcos, A. & Bueno, M. (2006) Anthropometric body fat composition reference values in Spanish adolescents. The AVENA Study. European Journal of Clinical Nutrition, 60, 191–196.*

11 *Martinez-Gonzalez, M.A., Lopez-Fontana, C., Varo, J.J., Sanchez-Villegas, A. & Martinez, J.A. (2005) Validation of the Spanish version of the physical activity questionnaire used in the Nurses' Health Study and the Health Professionals' Follow-up Study. Public Health and Nutrition, 8, 920–927.*

12 Ochoa, M.C., Marti, A., Azcona, C., Chueca, M., Oyarzabal, M., Pelach, R., Patino, A., Moreno-Aliaga, M.J., Martinez-Gonzalez, M.A. & Martinez, J.A. (2004) Gene–gene interaction between PPARγ2 and ADRβ3 increases obesity risk in children and adolescents. International Journal of Obesity and Related Metabolic Disorders, 28 (Suppl. 3), S37–S41.

13 *Martinez-Gonzalez, M.A., Varo, J.J., Santos, J.L., De Irala, J., Gibney, M., Kearney, J. & Martinez, J.A. (2001) Prevalence of physical activity during leisure time in the European Union. Medicine and Science in Sports and Exercise, 33, 1142–1146.*

14 Hinney, A., Schmidt, A., Nottebom, K., Heibult, O., Becker, I., Ziegler, A., Gerber, G., Sina, M., Gorg, T., Mayer, H., Siegfried, W., Fichter, M., Remschmidt, H. & Hebebrand, J. (1999) Several mutations in the melanocortin-4 receptor gene including a nonsense and a frameshift mutation associated with dominantly inherited obesity in humans. Journal of Clinical Endocrinology and Metabolism, 84, 1483–1486.

15 *American Psychiatric Association. (1994) Diagnostic and Statistical Manual of Mental Disorders, 4th edn. American Psychiatric Association, Washington, DC.*

16 *Biebermann, H., Schoneberg, T., Krude, H., Schultz, G., Gudermann, T. & Gruters, A. (1997) Mutations of the human thyrotropin receptor gene causing thyroid hypoplasia and persistent congenital hypothyroidism. Journal of Clinical Endocrinology and Metabolism, 82, 3471–3480.*

17 *Dempfle, A., Hinney, A., Heinzel-Gutenbrunner, M., Raab, M., Geller, F., Gudermann, T., Schafer, H. & Hebebrand, J. (2004) Large quantitative effect of melanocortin-4 receptor gene mutations on body mass index. Journal of Medical Genetics, 41, 795–800.*

18 *Miraglia Del Giudice, E., Cirillo, G., Nigro, V., Santoro, N., D'Urso, L., Raimondo, P., Cozzolino, D., Scafato, D. & Perrone, L. (2002) Low frequency of melanocortin-4 receptor (MC4R) mutations in a Mediterranean population with early-onset obesity. International Journal of Obesity and Related Metabolic Disorders, 26, 647–651.*

19 *Lubrano-Berthelier, C., Durand, E., Dubern, B., Shapiro, A., Dazin, P., Weill, J., Ferron, C., Froguel, P. & Vaisse, C. (2003) Intracellular retention is a common characteristic of childhood obesity-associated MC4R mutations. Human Molecular Genetics, 12, 145–153.*

20 *Hinney, A., Hohmann, S., Geller, F., Vogel, C., Hess, C., Wermter, A.K., Brokamp, B., Goldschmidt, H., Siegfried, W., Remschmidt, H., Schafer, H., Gudermann, T. & Hebebrand, J. (2003) Melanocortin-4 receptor gene: case–control study and transmission disequilibrium test confirm that functionally relevant mutations are compatible with a major gene effect for extreme obesity. Journal of Clinical Endocrinology and Metabolism, 88, 4258–4267.*

21 *Farooqi, I.S., Yeo, G.S. & O'Rahilly, S. (2003) Binge eating as a phenotype of melanocortin 4 receptor gene mutations. New England Journal of Medicine, 349, 606–609.*

22 *Valli-Jaakola, K., Lipsanen-Nyman, M., Oksanen, L., Hollenberg, A.N., Kontula, K., Bjorbaek, C. & Schalin-Jantti, C. (2004) Identification and characterization of melanocortin-4 receptor gene mutations in morbidly obese Finnish children and adults. Journal of Clinical Endocrinology and Metabolism, 89, 940–945.*

23 *Yang, X., Telama, R., Viikari, J. & Raitakari, O.T. (2006) Risk of obesity in relation to physical activity tracking from youth to adulthood. Medicine and Science in Sports and Exercise, 38, 919–925.*

24 *Phares, D.A., Halverstadt, A.A., Shuldiner, A.R., Ferrell, R.E., Douglass, L.W., Ryan, A.S., Goldberg, A.P. & Hagberg, J.M. (2004) Association between body fat response to exercise training and multilocus ADR genotypes. Obesity Research, 12, 807–815.*

25 *Santini, F., Maffei, M., Ceccarini, G., Pelosini, C., Scartabelli, G., Rosellini, V., Chiellini, C., Marsili, A., Lisi, S., Tonacchera, M., Agretti, P., Chiovato, L., Mammoli, C., Vitti, P. & Pinchera, A. (2004) Genetic screening for melanocortin-4 receptor mutations in a cohort of Italian obese patients: description and functional characterization of a novel mutation. Journal of Clinical Endocrinology and Metabolism, 89, 904–908.*

26 *Xiang, Z., Litherland, S.A., Sorensen, N.B., Proneth, B., Wood, M.S., Shaw, A.M., Millard, W.J. & Haskell-Luevano, C. (2006) Pharmacological characterization of 40 human melanocortin-4 receptor polymorphisms with the endogenous proopiomelanocortin-derived agonists and the agouti-related protein (AGRP) antagonist. Biochemistry, 45, 7277–7288.*

27 *Govaerts, C., Srinivasan, S., Shapiro, A., Zhang, S., Picard, F., Clement, K., Lubrano-Berthelier, C. & Vaisse, C. (2005) Obesity-associated mutations in the melanocortin 4 receptor provide novel insights into its function. Peptides, 26, 1909–1919.*

28 *Ochoa, M.C., Razquin, C., Azcona, C., García-Fuentes, M. & Martinez, J.A. (2005) [Val103Ile and Ile251Leu polymorphisms in MC4R gene in Spanish children and adolescents]. Revista Española de Obesidad, 3, 250–272.*

29 *Vaisse, C., Clement, K., Durand, E., Hercberg, S., Guy-Grand, B. & Froguel, P. (2000) Melanocortin-4 receptor mutations are a frequent and heterogeneous cause of morbid obesity. Journal of Clinical Investigation, 106, 253–262.*

30 *Geller, F., Reichwald, K., Dempfle, A., Illig, T., Vollmert, C., Herpertz, S., Siffert, W., Platzer, M., Hess, C., Gudermann, T., Biebermann, H., Wichmann, H.E., Schafer, H., Hinney, A. & Hebebrand, J. (2004) Melanocortin-4 receptor gene variant I103 is negatively associated with obesity. American Journal of Human Genetics, 74, 572–581.*