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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

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Context: Heterozygous mutations in the melanocortin-4 receptor (*MC4R*) gene are the most common monogenic form of severe obesity in children. There are conflicting reports regarding the prevalence, nature, and pathogenic effects of *MC4R* mutations in adults with severe late-onset obesity.

Objective: Our objective was to determine the prevalence of *MC4R* mutations in a cohort of severely obese adults and to determine the clinical phenotype and the phenotype-genotype relationship within adult *MC4R* mutation carriers.

Design and Setting: We conducted an observational study at a referral center.

Patients or Other Participants: Participants included 769 adult patients with body mass index of at least 35 kg/m^2 and 444 nonobese control individuals.

Interventions: There were no interventions.

Main Outcome Measures: We assessed the prevalence of pathogenic *MC4R* mutations, functional characteristics of the detected mutations, phenotype, and phenotype-genotype relationship within mutation carriers.

Results: The global prevalence of obesity-specific *MC4R* mutations was 2.6%, and the 95% confidence interval (CI_{95}) was 1.5–3.7. The prevalence of *MC4R* mutations was similar in patients developing obesity in childhood (2.83%; CI_{95}, 0.9–4.8) and in patients with a later onset of the disease (2.35%; CI_{95}, 0.9–3.8). Adult obese *MC4R* mutation carriers did not present with binge eating or with any specific clinical phenotype. The severity of the functional alterations of the mutated MC4Rs and in particular the intracellular retention of the receptor correlates both with the severity and the onset of the obesity in the mutation carriers.

Conclusions: Obese adult carriers of functionally relevant *MC4R* mutations do not specifically present with binge-eating disorder or a history of early-onset obesity. The onset and severity of the obesity in the carriers is related to the functional severity of the *MC4R* mutations. **(*J Clin Endocrinol Metab* 91: 1811–1818, 2006)**

THE MELANOCORTIN-4 RECEPTOR (MC4R) is a 332–amino-acid, seven-transmembrane domain receptor that transduces its signal by coupling to the heterotrimeric Gs protein and activating adenylate cyclase (1). MC4R regulates food intake by integrating a satiety signal provided by its agonist α-MSH and an orexigenic signal provided by its antagonist agouti-related protein (AGRP). Both of these ligands are expressed in distinct neuronal populations of the arcuate nucleus of the hypothalamus and are coordinately regulated by the adipocyte-secreted hormone leptin to control food intake and maintain long-term energy homeostasis (2). Recent data suggest that MC4R exhibits a constitutive activity upon which AGRP acts as an inverse agonist (3–5). Heterozygous mutations in the *MC4R* gene have been implicated in a significant proportion of cases of severe childhood-onset obesity (6–13). These multiple studies have led to the discovery of a total of 91 mutation carriers, most of them heterozygous, in 3057 children and adolescents, representing 2.98% [95% confidence interval (CI_{95}), 2.3–3.6] of childhood-onset obesity (6–14).

The functional defects of the mutated receptor found in obese children include total loss of activity associated with

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Abbreviations: AGRP, Agouti-related protein; BMI, body mass index; CI_{95}, 95% confidence interval; GFP, green fluorescent protein; MC4R, melanocortin-4 receptor; PE, phycoerythrin; TFEQ, Three-Factor Eating Questionnaire; WT, wild-type.

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intracellular retention of the mutated protein, decrease in agonist-induced receptor activity, or decreased constitutive activity of the receptor (5, 11–13, 15). In particular, using a quantitative assay, we have demonstrated that 80% of childhood obesity-associated mutations are partially or completely intracellularly retained (11).

There have been few studies that have examined the role of *MC4R* variants in the development of severe adult-onset obesity. We had initially observed a frequency of 4% pathogenic *MC4R* mutation carriers in adult obese patients from France [209 patients with body mass index (BMI) $\geq$ 35 kg/m^2)] (16) and more recently have observed a similar frequency (3.5%) in obese adults from Northern California (166 patients with BMI $\geq$ 40 kg/m^2) (17). Such mutations segregate with obesity in the families of the probands and are absent in nonobese controls (16). In contrast, several other studies have detected a far lower frequency of pathogenic mutations in obese adults in different countries (10, 18, 19). Because the observed frequencies are further confounded by the possible early onset of the obesity observed in adult patients, these results have led to the suggestion that *MC4R* mutations cause a specific form of early-onset highly penetrant obesity and that other genes or different gene-environment interactions are implicated in later-onset severe obesity (20). In addition, given the small number of mutations previously detected in these adult patient cohorts, studies of functional alterations due to *MC4R* mutations causing severe adult obesity have been very limited, and the potential relationship between functional alterations and the phenotype, in particular the onset of obesity, have not been evaluated, especially in groups of unrelated subjects. Finally, by using a less stringent definition of pathogenic *MC4R* mutations, *i.e.* not limited to obesity-associated functional sequence variants, Branson *et al.* (18) recently suggested that severely obese adult *MC4R* mutations carriers all presented with binge-eating disorder, an observation that was not confirmed by others (21).

We here use an extended cohort of severely obese adult subjects (769 subjects with BMI $\geq$ 35 kg/m^2) to compare the prevalence and the function of *MC4R* mutations found in early- and late-onset forms of the disease, to search for specific phenotypes in adult *MC4R* mutation carriers, and to determine the presence of a genotype-phenotype relationship in adult *MC4R* mutation carriers.

Subjects and Methods

Experimental subjects

The 769 obese unrelated adult subjects were part of a previously described population (16) and a group of obese subjects prospectively recruited after 2001 at the Hôtel-Dieu Hospital, Paris, France. Informed consent was obtained for all subjects, and the protocol was approved by the Local Ethics Committee (Comités Consultatifs de Protection des Personnes dans la Recherche Biomedicale Hôtel-Dieu, Paris). The criterion for inclusion was a BMI of at least 35 kg/m^2. Z-scores for BMI were calculated using the median, SD and Box-Cox power for the individual's age and sex of the French age 0–87 yr BMI charts (22).

Weight histories were obtained from the childhood health records (mandatory in France) and records of height and weight measurements at conscription for men. Two hundred eighty-three patients had a BMI of at least 30 kg/m^2 at age 20 yr, and 425 had a BMI less than 30 kg/m^2. Metabolic complications of obesity were evaluated by carbohydrate, insulin, and lipid measurements. Diagnosis of type 2 diabetes was based on the 1997 American Diabetes Association criteria. Diagnosis of hypertriglyceridemia was based on hypolipidemic treatments and/or criteria defined in the National Cholesterol Education Program Adult Treatment Panel III (serum triglycerides $\geq$ 2.21), and diagnosis of hypertension was based on hypotensive treatment and/or on World Health Organization criteria (blood pressure $\geq$ 160/90 mm Hg).

Quantitative food intake was individually assessed by a dietary history taken by registered dieticians the first day of investigation (23). Qualitative food intake was evaluated using the Three-Factor Eating Questionnaire (TFEQ), a psychometric instrument developed for the study of eating behavior and as described (23, 24). It measures three dimensions of human eating behavior: restraint, disinhibition, and hunger. Restrained eating is defined as the tendency to restrict food intake to control body weight. Disinhibition is the inability to resist emotional and social eating cues. Hunger is the subjective feeling of hunger (23).

As a second approach, we retrospectively assessed binge eating based on the Diagnostical and Statistical Manual of Mental Disorders, 4th edition. Each subject completed a questionnaire using the fully validated eating disorder questionnaire of Spitzer et *al.* (24). In addition, patients were evaluated by a psychologist, a dietitian, and a physician who conducted independent semistructured interviews with the subjects. Diagnostic criteria for binge-eating disorder included at least twice-weekly binge eating over a minimum of 6 months. A binge-eating event was defined as rapid consumption of an unusually large amount of food in the absence of hunger, causing the subject to feel embarrassed, depressed, and/or guilty and out of control. There was no purging behavior in any of the recruited subjects.

Nonobese control subjects were selected from participants in a French prospective study on the efficacy of daily supplementation with antioxidant vitamins and minerals in reducing the major health problems and the cause of premature death in a large population of healthy volunteers [Supplémentation en Vitamines et Minéraux Antioxydants (SU.VI.MAX) Study]. Details on the rationale, design, and methods of the study and baseline characteristics of the subjects have been described elsewhere (25). In total, 13,017 subjects (5,141 men, 45–60 yr; 7,876 women, 35–60 yr) were included between October 1994 and June 1995. The follow-up period ended in September 2002. During the 8-yr follow-up, participants were invited to three clinical examinations (1995–1996, 1997–1998, and 2001–2002), including anthropometric measurements. Controls were randomly selected from the subgroup of subjects for which DNA was available and who had never been obese according to retrospective and prospective weight records. This control group consisted of 444 individuals, 70% female, with a mean age of 51 $\pm$ 0.31 yr and mean BMI of 22.2 $\pm$ 0.1 kg/m^2. Informed consent for the use of genomic DNA had been obtained at entry in the study.

Direct nucleotide sequencing of the MC4R gene

Genomic DNA was extracted from peripheral leukocytes for all subjects. Two primers, MC4R-AF (5′-ATCAATTCAGGGGGACACTG-3′) and MC4R-ER (5′-TGCATGTTCCTATATTGCGTG-3′), were used in a PCR to amplify the entire coding region of the *MC4R* gene as described (16). The sequencing reaction was performed with the BigDye terminator kit (Applied Biosystems, Foster City, CA) under the standard manufacturer's conditions. Each PCR product was sequenced using MC4R-AF, MC4R-ER, and two internal primers, MC4R-CF (TGTAGCTCCTTGCTTGCATC) and MC4R-CR (GGCCATCAGGAACATGTGGA). Sequencing was performed on an ABI PRISM 3700 automated DNA sequencer (Applied Biosystems).

Genomic amplification and construction of wild-type (WT) and mutant MC4R expression vectors

Because *MC4R* is a single-exon gene, we amplified the variant and WT *MC4R* genes from the available genomic DNA of the patients carrying the mutations using the primers MC4R-AF and MC4R-ER and cloned the PCR product into the pcDNA3 expression vector (Invitrogen, Carlsbad, CA). To construct the Flag-MC4R-EGFP fusion protein used in the membrane expression assay, WT and mutant *MC4R* were amplified by PCR from the pcDNA3 expression vector using a primer encoding the Flag epitope sequence (MDYKDDDDK) added 5′ of MC4R-AF and the primer MC4R-ER. PCR products were subcloned in pEGFP-N1 (Clon-

tech, Palo Alto, CA). All expression vectors were sequenced to establish the presence of the mutation and the absence of PCR-induced mutations.

Cell surface expression of WT and mutated MC4Rs

Cell surface expression of WT and mutant MC4R was measured using a previously validated FACS-based method (11). Briefly, HEK 293 cells were transiently transfected with a chimeric receptor in which we added an N-terminal Flag epitope and C-terminal (intracellular) green fluorescent protein (GFP). Twenty-four hours after transfection, immunostaining was performed in nondetergent conditions using a primary monoclonal anti-Flag antibody (M2; Sigma Chemical Co., St. Louis, MO) and a secondary phycoerythrin (PE)-conjugated antimouse (Caltag, Burlingame, CA). Cell fluorescence was analyzed through a FACSCalibur Beckton-Dickinson flow cytometer (Beckton-Dickinson Immunocytometry Systems, San Jose, CA). Using the Cell Quest software, 10,000–15,000 cells were analyzed for each sample. For each cell, the GFP emission represents total expression of the receptor whereas PE emission represents membrane-expressed receptor. To limit artifacts caused by high receptor overexpression, we limited our analysis to cells expressing low levels of the receptor (11). The ratio PE emission/GFP emission was determined for each individual cell for the predetermined range of GFP emission level using the FlowJo software (Tree Star Inc., San Carlos, CA). ANOVA analysis of the data was performed using JMP3 software (SAS Institute Inc., Cary, NC).

Basal activity of MC4R mutants

HEK 293 cells were maintained in α-MEM supplemented with 10% calf serum (Hyclone, Logan, UT), 0.002 M L-glutamine, nonessential amino acids, and penicillin/streptomycin at 37 C and 5% CO_2. Cells were seeded 1 d before transfection at 10^5 cells per well in 12-well dishes. Cells were transiently transfected (Effectene Transfection Reagent; QIAGEN, Chatsworth, CA) with 0.25 μg WT or mutant MC4R or Flag-MC4R expression plasmid DNA (pcDNA 3.1), 50 ng of the CRE-luciferase plasmid, a cAMP-inducible luciferase reporter gene (26) used to measure the intracellular production of cAMP, and 5 ng of a Renilla luciferase expression plasmid PRL RSV to control for transfection efficiency. A pcDNA 3.1 plasmid expressing β-galactosidase was used as a negative control. Twenty-four hours after transfection, cells were split into 96-well plates (6500 cells per well) and in 48-well plates (5000 cells per well) for the Renilla luciferase assays. Cells were incubated for 6 h at 37 C in stimulation medium (11) (basal condition) or with AGRP 83–132 (200 nM), α-MSH (10^{-7} M), or cAMP (1 mM). Firefly and Renilla luciferase activities were assessed as previously described (11). Each assay was performed in triplicate. A minimum of six independent experiments was performed for each mutant (three on the MC4R backbone and three on the Flag-MC4R backbone). Basal and AGRP values are expressed as a percentage of the basal values obtained for the WT receptor in each experiment. α-MSH values are expressed as a percentage of the WT α-MSH stimulation. Each data point represents the mean ± SEM of at least six experiments.

α-MSH response of MC4R mutants

α-MSH response of MC4R WT and mutant receptors was measured as previously described (11). Data points represent the mean of at least three independent experiments performed in triplicate.

Statistical analysis

The χ^2 and *t* tests, and ANOVA analysis of the data were performed using JMP3 software (SAS Institute) or GraphPad Prism (Graphpad Software, San Diego, CA).

Results

Prevalence and nature of MC4R mutations in a large cohort of severely obese adults

We had previously demonstrated the significance of *MC4R* mutations in the predisposition to severe adult obesity in a cohort of 209 patients (16). For the purpose of the present study, and in particular to determine the presence of a phenotype/genotype relationship within *MC4R* mutation carriers, we extended this cohort by sequencing the *MC4R* gene in an additional 560 patient subjects with a BMI above 35 kg/m^2. The entire cohort was 75% female with a mean age of 44 ± 12 yr, mean BMI of 47.5 ± 7.8 kg/m^2, and a mean BMI Z-score of 3.4 ± 0.9.

A total of 43 patients carried a variation in the *MC4R* coding sequence. Of these, nineteen patients carried the Ile103Val variant (including one homozygous carrier) and three patients carried the Ile251Leu variant. These two variants have previously been found in nonobese controls (16, 27). One patient carried a silent variant (C-593-T).

TABLE 1. Characteristics of obesity-associated *MC4R* mutation carriers

Patient	*MC4R* mutation	Sex	Age (yr)	BMI (kg/m^2) at age 20 yr	Age (yr) at maximum BMI	Maximum BMI (kg/m^2)
1	Thr 11 Ser	F	43	19	43	46
2	47–48 Ins G	F	20	42	19	42
3	Arg 18 Cys	F	42	29	41	58
4	Arg 18 His	F	33	NA	33	41
5	Tyr35stop-Asp37Val	F	60	32	40	71
6	Ile 69 Met	M	53	25	53	46
7	Tyr80Stop/Ile301Thr	M	32	44	32	54
8	Val 95 Ile	M	46	26	45	48
9	Ile 102 Thr	F	56	27	56	41
10	Thr 150 Ile	M	46	32	46	50
11	Thr 150Ile	F	51	NA	51	49
12	Ala 154 Asp	F	43	29	43	43
13	Arg 165 Trp	F	45	35	45	67
14	Ile 170 Val	F	25	30	25	50
15	Gly 231 Ser	M	48	26	45	46
16	732 Ins GATT	F	34	29	34	62
17	Leu 250 Gln	F	25	31	25	59
18	Gly 252 Ser	F	53	21	53	50
19	Ser 295 Pro	F	44	28	35	44
20	Arg 305 Trp	F	20	42	20	42

Patient 5 carries two mutations on the same allele. Patient 7 carries two mutations on different alleles (compound heterozygote). F, Female; M, male.

The remaining 20 patients (2.6%) carried nonsense, frameshift, or other missense mutations in the *MC4R* gene (Table 1). None of these mutations has been found in any nonobese control populations screened by our group or by other investigators (13, 14, 16), including 366 nonobese controls screened in our initial study (16). In addition, none of these mutations was found in an additional 444 nonobese French subjects (70% female; mean age, 51 ± 0.31 yr; mean BMI, 22.2 ± 0.1 kg/m^2) screened for the purpose of this study.

Eight of these mutations, Arg18His (G53A), Ile69Met (A207G), Tyr80stop (C240A), Ala154Asp (C461A), Gly231Ser (G691A), Ile102Thr (T305C), Ser295Pro (T883C), and Arg305Trp (C913T), have not been detected and/or extensively functionally characterized previously. Two unrelated patients were carriers of the Thr150Ile mutation (patients 10 and 11). Patient 5 carried two mutations (Tyr^{35}Stop and Asp37Val) on the same allele. Patient 7 carried two mutations (Tyr80Stop and Ile301Thr) on different alleles (compound heterozygote). Gly252Ser had been previously found on the same allele as Ser30Phe (28) (Table 1 and Fig. 1).

Comparative functional analysis of severe adult obesity-associated MC4R mutations

Functional defects associated with obesity-causing *MC4R* mutations include intracellular retention and alteration of the response to the endogenous agonist α-MSH. In addition, we have recently demonstrated that the MC4R displays a constitutive basal activity in the absence of ligand and that this constitutive activity is essential for body weight regulation in humans (5). We systematically and comparatively evaluated all three of these functional characteristics (membrane expression, activation by agonist, and basal activity) for each of the mutant MC4R found in obese adult subjects. Membrane expression of missense mutations was assessed using a sensitive quantitative FACS-based method used previously to study childhood obesity-associated mutations (Fig. 2A and supplemental data, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org) (11). Both basal (Fig. 2B) and agonist-induced (Fig. 2C) receptor activity were determined in HEK 293 cells using a cAMP-responsive luciferase reporter gene. All 19 mutations found only in obese subjects modified the function of the MC4R in at least one of the assays (Table 2). Decreased basal activity was the most common detected defect (79% of mutants). EC_{50} for α-MSH activation was altered in 64% of the studied mutants. Membrane expression was decreased significantly in 50% of the mutants.

Clinical characteristics of obese MC4R mutation carriers vs. noncarriers

Early- vs. late-onset obesity. The prevalence of carriers of functionally relevant *MC4R* mutation in this large cohort of severely obese adults was 2.6%, with CI_{95} of 1.48–3.73%, and did not differ significantly from the mean prevalence of *MC4R* mutations described in all severely obese children (6–13), including a cohort of 750 Scandinavian men with

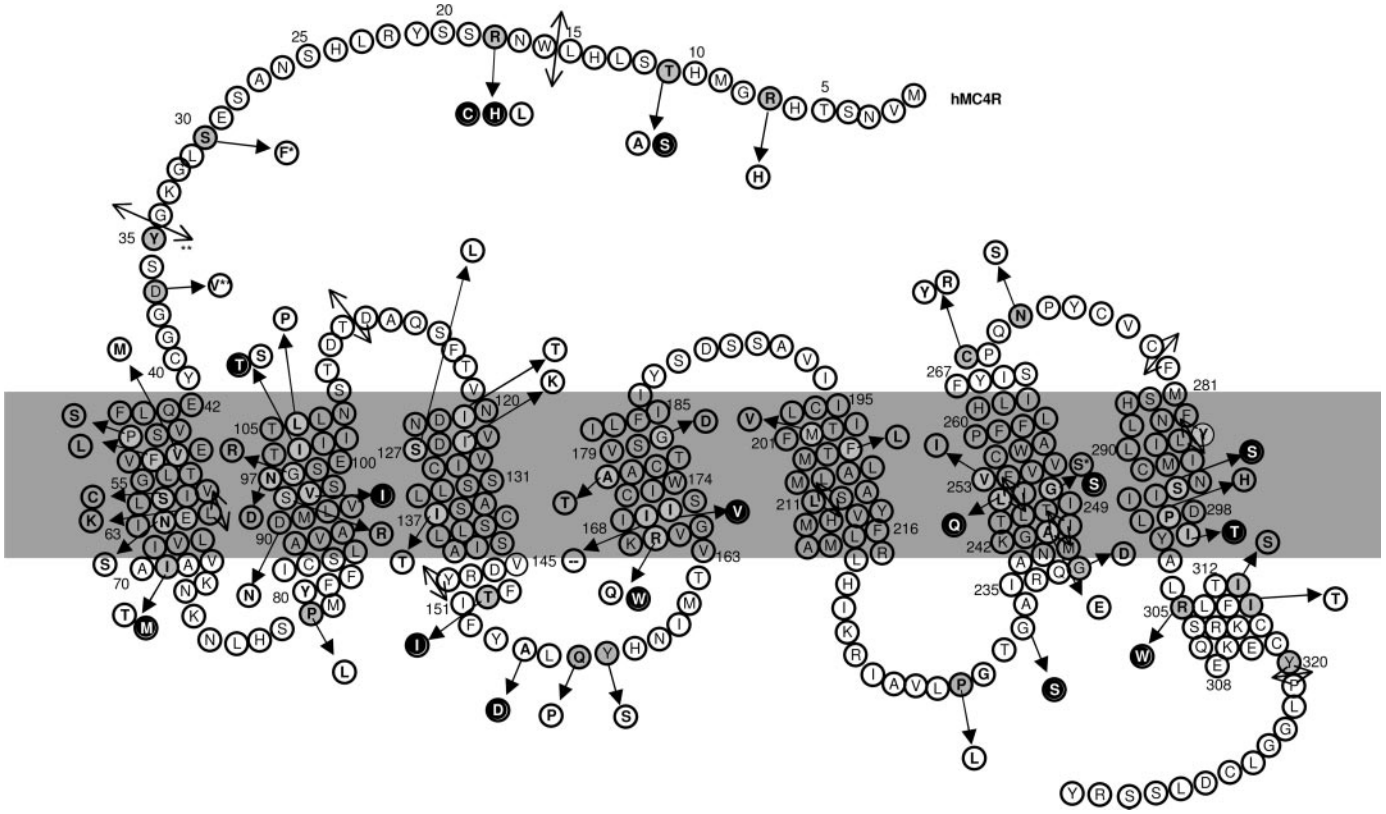

FIG. 1. Schematic representation of MC4R and the sequence variants detected in human obesity. The mutations detected in the present study are indicated in *black circles*; mutations detected in other studies are shown in *white circles*. *Double-headed arrows* indicate nonsense or frameshift mutations.

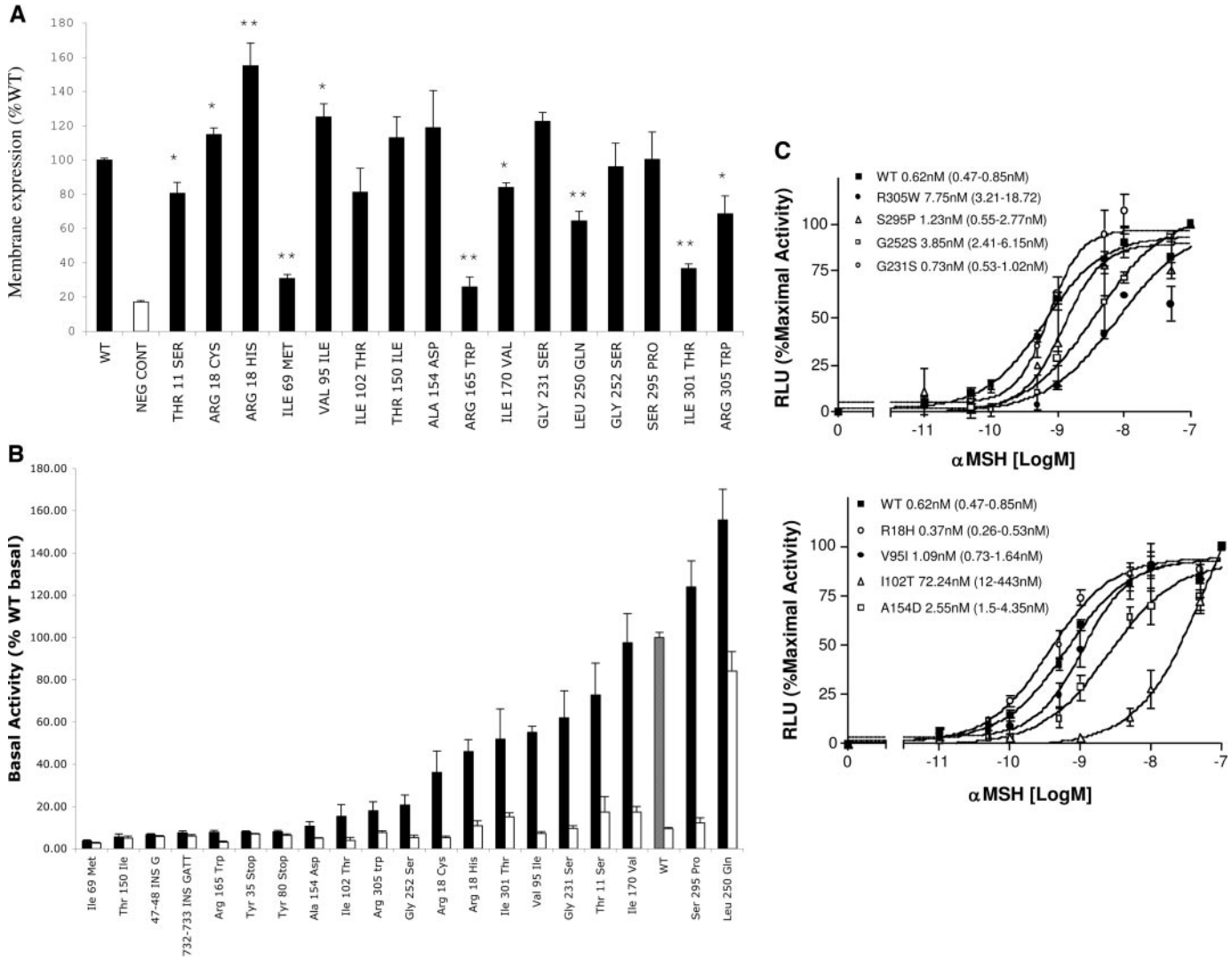

FIG. 2. A, Quantitative analysis of the membrane expression of adult obesity-associated *MC4R* missense mutants. The ratio PE emission/GFP emission, representing MC4R membrane expression relative to MC4R total expression, was calculated in each individual cell belonging to the predetermined GFP window using FlowJo software (Tree Star Inc., San Carlos, CA). Results are expressed as a percentage of the value obtained in the same experiment for the WT MC4R and are shown for WT and all *MC4R* mutations except I170V, R165W, and L250Q, which were published in a previous study (11). The numbers represent mean ± SEM of three independent experiments. The negative control is the ratio PE/GFP calculated in the absence of primary anti-Flag antibody. *, $P < 0.05$; **, $P < 0.01$. B, Basal activity of adult obesity-associated *MC4R* mutants. For each mutant, the *black bar* represents the basal activity and the *white bar* the activity in the presence of the inverse agonist AGRP (200 nM). Results represent the mean ± SEM of at least six experiments. C, Dose-response curves of α-MSH activation of obesity-associated *MC4R* mutants. Data points represent mean ± SEM of at least three independent experiments performed in triplicate. Mean (CI_{95}) of the EC_{50} (nM) is indicated for each variant. For comparison purposes, the activities range from basal activity (0%) to the maximal activity (100%) of each receptor.

juvenile onset of obesity, *i.e.* BMI above 30 kg/m^2 at age 20 yr (14). The frequency of *MC4R* mutations in patients developing obesity in their childhood (as defined by BMI > 30 kg/m^2 at age 20 yr) was 2.83%, with CI_{95} of 0.9–4.8, and was not significantly different from the frequency of *MC4R* variants in subjects with a later onset of the disease (2.35%; CI_{95}, 0.9–3.8). This result establishes that the early onset of obesity is not a specific clinical characteristic of functionally relevant heterozygous *MC4R* mutation carriers.

MC4R mutations and eating behavior. Binge eating has been reported as a specific phenotype of adult *MC4R* mutation carriers (18), but others did not confirm this finding (21). We used two approaches to test the hypothesis that obesity-associated *MC4R* mutations predispose to binge eating in the patients under study. We first used prospectively collected data from the TFEQ (23, 24). This questionnaire, validated in obese patients, evaluates dietary restraint, disinhibition, and hunger. In particular, disinhibition defines the loss of food intake control under a variety of situations and has been shown to correlate with binge eating (24). The TFEQ was administered to all patients at the time of their entry in the study (23). When compared with nonmutated severely obese patients, carriers of *MC4R* mutations and carriers of the I103V polymorphism did not differ with respect to the disinhibition, restraint, or hunger scores (Table 3).

As a second approach, we retrospectively assessed binge-

TABLE 2. Summary of functional effects of obesity-associated *MC4R* mutations detected in severely obese adults

MC4R allele	Membrane expression (% WT)	Basal activity (% WT)	α-MSH EC_{50} (nM) (WT = 0.62 nM)	Maximum α-MSH (%WT)	Classification of mutation (36)
47–48 Ins G	**IF = 0**	**≤10**	**ND**	**≤10**	Class I
Tyr35stop/Asp37Val	**IF = 0**	**≤10**	**ND**	**≤10**	
Ile 69 Met	**31 ± 2**	**≤10**	**ND**	**≤10**	
Tyr80Stop	**IF = 0**	**≤10**	**ND**	**≤10**	
Arg 165 Trp	**26 ± 6**	**≤10**	**4.97**	**≤10**	
732 Ins GATT	**IF = 0**	**≤10**	**ND**	**≤10**	
Ile301Thr	**37 ± 3**	**52 ± 14**	1.1	80 ± 16	
Thr 11 Ser	≥100	**73 ± 15**	1.73	97 ± 9	Class 2A
Arg 18 Cys	≥100	**36 ± 10**	1.02	87 ± 5	
Arg 18 His	≥100	**46 ± 6**	0.37	106 ± 22	
Val 95 Ile	≥100	**55 ± 3**	1.09	94 ± 7	
Gly 231 Ser	≥100	**62 ± 13**	0.73	100 ± 18	
Ile 170 Val	84 ± 3	98 ± 14	**7.26**	110 ± 25	Class 2B
Ile 102 Thr	81 ± 14	**16 ± 7**	**72.24**	68 ± 13	Class 2C
Thr 150 Ile	≥100	**≤10**	**5.97**	56 ± 8	
Ala 154 Asp	≥100	**11 ± 2**	**2.55**	90 ± 6	
Gly 252 Ser	96 ± 14	**21 ± 5**	**3.85**	107 ± 19	
Arg 305 Trp	69 ± 10	**18 ± 4**	**142**	74 ± 12	
Leu 250 Gln	**65 ± 6**	**156 ± 15**	0.43	79 ± 4	Class 3
Ser 295 Pro	≥100	**124 ± 12**	1.23	90 ± 7	

For missense mutants, membrane expression represents the ratio of PE emission over GFP emission in the FACS assay described in Fig. 2A. Results are expressed as a percentage of the value obtained in the same experiment for the WT *MC4R*. The *numbers* represent mean ± SEM of three independent experiments. For frameshift and nonsense mutants, absence of membrane expression was demonstrated by immunofluorescence (IF = 0). The basal activity values are from Fig. 2B. EC_{50} values were calculated as described in Fig. 2C. Maximum α-MSH is the response to 10^{-7} M α-MSH obtained in the basal activity assay. Results are expressed as a percentage of the value obtained in the same experiment for the WT *MC4R*. The *numbers* represent mean ± SEM of at least three independent experiments. Values that significantly differ from WT ($P < 0.05$) are indicated in *bold*. The mutation classification used in the last column has been recently described (36). Briefly, class 1 mutations are those that are largely intracellularly retained, resulting in a major loss of MC4R signaling. MC4Rs with class 2 mutations are expressed at the cell membrane but display a decreased constitutive activity (class 2A), a decreased response to the agonist (class 2B), or both (class 2C). The pathogenicity of these mutations can be linked to the decreased anorexigenic activity of the receptor. Class 3 mutants have an increased basal activity, and their pathogenic effects are unclear. Leu250Gln is intracellularly retained and displays a decrease in maximal α-MSH activation, whereas Ser295Pro has a slightly increased EC_{50}. ND, Not determined.

eating disorder using the DSM IV criteria (29) in a subsample of the patients. Of the five *MC4R* mutant carriers who were examined (patients 3, 6, 9, 14, and 19), none reported binge-eating disorder, whereas one of 19 (5.3%) nonmutated obese subjects presented with binge-eating disorder. Therefore, binge-eating disorder is not a specific characteristic of obese adult patients carrying *MC4R* variants.

Other phenotypes in adult MC4R mutation carriers. We did not observe any differences in anthropometric measures, including the degree of obesity, and in the severity of obesity complications between obese adult *MC4R* mutation carriers and noncarriers (Table 3). Fasting hyperinsulinemia was recently described as a specific feature of children carrying *MC4R* mutations (12), although we did not observe it in a 7-yr-old carrier

TABLE 3. Obesity, metabolic phenotype, and eating behavior of *MC4R* mutation carriers as compared with the patient cohort

	Cohort (n = 731)	*MC4R* mutation carriers (n = 19)	*MC4R* Val103Ile carriers (n = 19)
Sex ratio (% female)	75	75	79
Age (yr)	44 ± 12	41 ± 12	45 ± 11
BMI (kg/m^2) (Z-score)	47 ± 8 (3.6 ± 0.7)	48 ± 7 (3.7 ± 0.7)	47 ± 6 (3.4 ± 0.5)
BMI max (kg/m^2) (Z-score)	50 ± 7 (3.8 ± 0.9)	50 ± 9 (3.8 ± 0.7)	50 ± 6.3 (3.4 ± 0.6)
BMI age 20 yr (kg/m^2) (Z-score)	29 ± 7 (2.0 ± 1.5)	30 ± 7 (1.6 ± 1.6)	28 ± 4 (1.9 ± 1.7)
Leptin (ng/ml)	51.2 ± 27.5	51.9 ± 7	59.1 ± 25.2
Height (cm) (Z-score)	165.5 ± 9.1 (0.20 ± 1.69)	164.7 ± 8.6 (0.17 ± 1.11)	163.4 ± 10.6 (−0.14 ± 2.08)
Waist to hip ratio	0.93 ± 0.11	0.97 ± 0.17	0.96 ± 0.13
Fasting insulinemia[a] (μU/ml)	17.2 ± 11.8	18.6 ± 9	20.2 ± 11.6
Fasting glycemia[a] (mM)	5.5 ± 0.7	5.7 ± 0.7	5.7 ± 0.7
HOMA[a]	4.3 ± 3.1	4.4 ± 1.8	5.2 ± 3.0
Diabetes (%) (CI_{95})	34 (30–39)	20 (3–37)	36 (15–57)
Hypertension (%) (CI_{95})	49 (45–53)	37 (16–58)	42 (21–63)
Hypertriglyceridemia (%) (CI_{95})	46 (42–50)	53 (29–77)	61 (38–83)
Total calorie intake (kcal)	2361 ± 1067	2412 ± 392	2200 ± 690
TFEQ			
Restriction	9.5 ± 4.7	8.4 ± 1.4	8.5 ± 4.3
Disinhibition	8.6 ± 3.5	9.13 ± 0.82	8.5 ± 2.5
Hunger	5.8 ± 3.5	6 ± 1	5.5 ± 3.9

Numbers are mean ± SEM or percentage. HOMA, Homeostasis model assessment.
[a] After exclusion of patients with diabetes.

of a homozygous null *MC4R* mutation (30). In this cohort of adult patients, there was no difference in the prevalence of type 2 diabetes between carriers and noncarriers of *MC4R* mutations. In addition, in the nondiabetic patients, there were no differences in fasting insulinemia or in the parameter of insulin sensitivity as evaluated by the homeostasis model assessment index between carriers and noncarriers of *MC4R* mutations.

Phenotype/genotype relationship within adult obese MC4R mutation carriers

We evaluated the relationship between the type and extent of the functional alterations caused by mutant *MC4R* and the precocity or severity of the obesity in the mutation carriers. The precocity of obesity was assessed by the Z-score for BMI at age 20 yr, and the severity of obesity was assessed using the maximal lifetime BMI Z-score.

When considered independently, we found a positive relationship between the presence of any of the functional defects and both the onset and the severity of the obesity in the carriers (Table 4). This relationship was strongest and most significant for the intracellular retention, making this the best predictor of both the onset and the severity of the obesity in *MC4R* mutation carriers.

Discussion

The genetic predisposition to obesity may result from the additive and interactive effects of common genetic variants each with a small effect on the phenotype, from multiple rare mutations with different environment-dependent penetrance in a large set of genes, or from a combination of both. Although theoretical considerations underlying both theories are driving the debate on the genetic architecture of the predisposition to obesity in humans, mutations in *MC4R* offer a paradigm allowing the exploration of the implications of the second hypothesis.

Our study confirms that mutations in *MC4R* are a significant cause of severe human obesity and extends this finding to patients with a later onset of the condition. Although it had been suggested that obesity caused by *MC4R* mutations should be isolated from common obesity on the basis of its early onset and its high penetrance, the frequency of 2.6% (CI_{95}, 1.48–3.73) of pathogenic *MC4R* mutations in adult subjects with severe obesity reported here matches the frequency of *MC4R* mutations detected in cohorts of patients presenting with childhood obesity of different origins (6–14). In addition, our finding of a similar frequency of *MC4R* mutations in patients with a later onset of the disease clearly confirms that childhood onset of obesity is not a specific clinical phenotype differentiating adult *MC4R* mutation carriers from other severely obese patients.

The prevalence of *MC4R* mutations reported here does not include sequence variants for which there is no functional evidence of a deleterious effect and/or that are present at a similar frequency in nonobese individuals, such as Ile103Val or Ile251Leu. The frequencies of rare pathogenic variants observed here is in agreement with previous studies performed in adult populations. For example, in the study performed in obese adult Swiss patients, the frequency of *MC4R* mutations is 1.7% when polymorphisms are excluded (14, 18). We support the argument made by others in reference to this report that including common polymorphisms in the study of obesity caused by *MC4R* mutations may create confusion and lead to inappropriate clinical decisions (31–33).

We did not detect any phenotype differences between adult *MC4R* mutation carriers and noncarriers of equivalent BMI. Our study confirms in particular that binge eating is not a specific phenotype of adult *MC4R* mutation carriers and that glucose metabolism is not specifically altered in these patients. Together, these data suggest that, in a clinical context, no specific phenotype can be used to predict the presence of an *MC4R* mutation in severely obese adult patients. It is possible, however, that other phenotypes not studied here might permit this differentiation. For example, in children, patients with *MC4R* mutations seem to present with an increased bone density as measured by dual-energy x-ray absorptiometry. Although we have not found any evidence of a relationship between *MC4R* mutations and body composition evaluated by dual-energy x-ray absorptiometry in our group of severely obese adult subjects (data not shown), it should be noted that the use of this technique is notoriously unreliable in severely obese subjects (34, 35). It should also be noted that effects of *MC4R* mutations on bone density, stature, or food intake could be more salient during periods of somatic growth.

We previously demonstrated that intracellular retention was a frequent feature of *MC4R* mutations associated with childhood obesity being observed for 80% of studied mutations (11). The results obtained here confirm this finding, because 70% of the mutations found in patients with early onset of obesity (BMI $\geq$ 30 kg/m^2 at age 20 yr) led to intracellular retention of the receptor, whereas only 23% of the *MC4R* mutations detected in patients with late-onset obesity were intracellularly retained. This result also further supports the use of intracellular retention as a relevant criteria for the functional classification of *MC4R* mutations (36).

In summary, although we did not find a difference in the prevalence of *MC4R* mutations in severely obese patients with late-onset *vs.* early-onset obesity, we found an association between the severity of the functional alterations in the MC4R and the onset of obesity in the carriers. These findings extend previous observations of a phenotype-genotype relationship within *MC4R* mutation carriers and suggest that it is allelic

TABLE 4. Relationship between the functional effects of *MC4R* mutations and the obesity in the carriers

MC4R function	Intracellular retention		Constitutive activity		EC_{50} for α-MSH		Maximum α-MSH response	
	No	Yes	Normal	Decreased	Normal	Increased	Normal	Decreased
Maximal lifetime BMI Z-score	3.53 ± 0.77	4.35 ± 0.57[a]	3.73 ± 0.81	3.94 ± 0.81	3.73 ± 0.77	3.96 ± 0.82	3.57 ± 0.73	4.11 ± 0.82
Z-score at age 20 yr	1.66 ± 1.21	3.26 ± 1.18[a]	1.74 ± 1.65	2.66 ± 1.34	1.54 ± 1.58	2.72 ± 1.32	1.42 ± 1.24	3.01 ± 1.13[a]

For every function studied, the mutations are classified as affected if the results presented in Table 2 significantly differ from the WT MC4R. The Z-scores were calculated using the LMS parameters [power in the Box-Cox transformation (L), median (M), and generalized coefficient of variation (S)] for the French BMI curves (22).

[a] $P < 0.01$ (*t* test).

heterogeneity rather than genetic heterogeneity that determines the onset of obesity in humans.

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