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Association of functionally significant Melanocortin-4 but not Melanocortin-3 receptor mutations with severe adult obesity in a large North American case–control study

Melissa A. Calton[1,†], Baran A. Ersoy[1,†], Sumei Zhang[1], John P. Kane[2,3,4], Mary J. Malloy[2,3], Clive R. Pullinger[2,5], Yana Bromberg[6,7], Len A. Pennacchio[8,9], Robert Dent[10], Ruth McPherson[10], Nadav Ahituv[8,9,11] and Christian Vaisse[1,*]

[1]Diabetes Center, [2]Cardiovascular Research Institute, [3]Department of Medicine, [4]Department of Biochemistry and Biophysics, [5]Department of Physiological Nursing, University of California San Francisco, San Francisco, CA 94143, USA, [6]Department of Biochemistry and Molecular Biophysics, Columbia University, New York, NY 10032, USA, [7]Columbia University Center for Computational Biology and Bioinformatics (C2B2), New York, NY 10032, USA, [8]U.S. Department of Energy, Joint Genome Institute, Walnut Creek, CA 94720, USA, [9]Genomics Division, MS 84–171, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA, [10]Division of Cardiology, University of Ottawa Heart Institute, Ottawa, Canada K1Y 4W7 and [11]Department of Biopharmaceutical Sciences and Institute for Human Genetics, University of California San Francisco, San Francisco, CA 94143, USA

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Functionally significant heterozygous mutations in the Melanocortin-4 receptor (*MC4R*) have been implicated in 2.5% of early onset obesity cases in European cohorts. The role of mutations in this gene in severely obese adults, particularly in smaller North American patient cohorts, has been less convincing. More recently, it has been proposed that mutations in a phylogenetically and physiologically related receptor, the Melanocortin-3 receptor (*MC3R*), could also be a cause of severe human obesity. The objectives of this study were to determine if mutations impairing the function of MC4R or MC3R were associated with severe obesity in North American adults. We studied MC4R and MC3R mutations detected in a total of 1821 adults (889 severely obese and 932 lean controls) from two cohorts. We systematically and comparatively evaluated the functional consequences of all mutations found in both *MC4R* and *MC3R*. The total prevalence of rare *MC4R* variants in severely obese North American adults was 2.25% ($CI_{95\%}$: 1.44–3.47) compared with 0.64% ($CI_{95\%}$: 0.26–1.43) in lean controls ($P < 0.005$). After classification of functional consequence, the prevalence of MC4R mutations with functional alterations was significantly greater when compared with controls ($P < 0.005$). In contrast, the prevalence of rare *MC3R* variants was not significantly increased in severely obese adults [0.67% ($CI_{95\%}$: 0.27–1.50) versus 0.32% ($CI_{95\%}$: 0.06–0.99)] ($P = 0.332$). Our results confirm that mutations in *MC4R* are a significant cause of severe obesity, extending this finding to North American adults. However, our data suggest that *MC3R* mutations are not associated with severe obesity in this population.

INTRODUCTION

Obesity results from the interaction between a genetic predisposition and deleterious environmental factors (1). Both common variants with small effects (2,3) and rare variants with larger individual effects have been shown to contribute to the genetic predisposition to obesity (4). In particular, rare heterozygous mutations

*To whom correspondence should be addressed at: Diabetes Center, University of California San Francisco, 513 Parnassus Avenue, HSW 1113, San Francisco, CA 94143, USA. Tel: +1 415 514 0530; Fax: +1 415 564 5813; Email: vaisse@diabetes.ucsf.edu
†The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

in the coding sequence of the Melanocortin-4 receptor (*MC4R*) gene account for a significant number of severe obesity cases (5–10). MC4R belongs to the family of seven *trans*-membrane G-protein-coupled receptors (GPCRs) and is expressed at low levels in hypothalamic nuclei involved in the regulation of food intake and in particular neurons of the para-ventricular nucleus (11). 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 (12,13). 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 (14). Mice lacking both alleles of *mc4r* (*mc4r* −/− mice) develop a maturity onset hyperphagic obesity syndrome by 10 weeks of age (13). Mice heterozygous for a *mc4r* deletion (*mc4r* +/− mice) show an average weight that is intermediate between that of wild-type (WT) and *mc4r* −/− mice (13).

Numerous studies, mostly in European populations, have documented a prevalence of 2.67% ($CI_{95\%}$: 2.1–3.6) of nonsense, frameshift and missense *MC4R* mutations in early onset obesity (15). More recently, such mutations have also been implicated in the development of severe adult obesity in these same populations. For example, *MC4R* mutations were found in 2.35% ($CI_{95\%}$: 0.9–3.8) of severely obese French adults (15). Fewer studies, in relatively small number of patients, have explored the prevalence of *MC4R* mutations in severely obese North American patients (9,16,17). Results have been inconsistent leading to the suggestion that environmental differences could selectively impact the relative effects of *MC4R* mutations in these populations.

Another melanocortin receptor, the Melanocortin-3 receptor (MC3R), is closely related to MC4R and is expressed in the arcuate nucleus of the hypothalamus (11,18). MC3R has also been implicated in long-term energy homeostasis in rodents (19,20). Homozygous null *mc3r* −/− mice have an increased fat mass through increased feed efficiency (19,20).

Evidence for a causative role for MC3R in human obesity is scarce. Few heterozygous *MC3R* mutations have been detected in early onset obese patients (21,22). Large-scale studies, systematically comparing the prevalence of rare *MC3R* mutations in cases and controls to formally determine if, similarly to *MC4R*, such mutations are associated with severe obesity have not yet been performed.

In this study, we used two cohorts of North American adult subjects to compare the prevalence and the function of MC4R and MC3R mutations found in patients with severe obesity and in non-obese controls. Our results confirm that *MC4R* mutations are a significant cause of severe obesity and extend this finding to North American adults. In contrast, our data do not support a similar role for *MC3R* mutations in the predisposition to this condition.

RESULTS

Prevalence and nature of *MC4R* mutations in two North American cohorts of severe obese adults

We analyzed the prevalence and nature of *MC4R* coding region mutations detected in 1821 adults (889 severely obese and 932 controls) from our previously reported cohorts (23,24). A total of 26 subjects carried rare variants in the *MC4R* coding region (Table 1): 20 in the severely obese group (2 subjects both carried Leu211Del and Pro299His mutations) and 6 among the controls. In severely obese adults, 17 missense mutation carriers, 2 nonsense mutation carriers and 3 frameshift mutation carriers were identified (for a total of 20 different carriers). Six control subjects carried six different missense mutations. The total prevalence of rare *MC4R* variants in severely obese North American adults was 2.25% ($CI_{95\%}$: 1.44–3.47) compared with a prevalence of 0.64% ($CI_{95\%}$: 0.26–1.43) in lean controls ($P = 0.005$) (OR = 4.3).

In addition, a total of 41 subjects carried the common variant Val103Ile and 12 subjects carried the common variant Leu251Ile (Table 2). The frequency of these two variants was similar to that described in other studies (2.43–5.31% for Val103Ile and 0.96–3.23% for Leu251Ile) (9,15,17,25,26). The prevalence of these polymorphisms was not statistically different between case and controls in our studies ($P = 0.775$ and 0.774 respectively), most likely due to limited power to detect an association, and thus failed to replicate the small, but significant association with decreased body weight seen in a larger study for the Val103Ile variant (27).

Comparative functional analysis of severe adult obesity-associated *MC4R* mutations

Nonsense and frameshift mutations in GPCRs generally lead to loss of function of the protein, and we considered them, *a priori*, to be functionally severe. However, most of the detected rare *MC4R* mutations are missense mutations, which may or may not significantly affect the function of the receptor. We therefore determined whether documenting the functional consequences of all mutations would strengthen the observed association between rare *MC4R* mutations and severe obesity. We systematically evaluated the agonist activation of each of the missense MC4R mutants found in obese and lean subjects to that of the WT receptor in an *in vitro* assay. We chose this assay as it will more sensitively detect most functional alterations in MC4R including impairment in membrane expression and/or ligand binding (10). We considered a missense mutation to have functional alteration when: (1) a mutation had α-MSH dose response that did not reach a maximum, (2) a mutation had an EC_{50} that was significantly different ($P < 0.05$) compared with that of the WT receptor, and/or (3) a mutation had an E_{max} <50% of WT receptor maximum activity. Including the 2 nonsense and 2 deletion mutations, 10 mutations, found only in obese subjects (in a total of 12 obese carriers), modified the agonist activation of the receptor in our *in vitro* assay while only 1 mutation found in control subjects (Arg305Gln) had such an *in vitro* phenotype (Table 1 and Fig. 1). After classification of functional consequences, the prevalence of functionally altered *MC4R* mutations was significantly greater than compared with controls ($P = 0.001$) (OR = 12.7) (Fig. 2).

Prevalence and nature of *MC3R* mutations in two North American cohorts of severe obese adults

To determine whether, as is the case for *MC4R*, rare *MC3R* mutations are associated with obesity, we examined the

Table 1. Summary of unique or rare mutations identified in the coding regions of *MC4R* and *MC3R*

Gene	Mutation DNA	Protein	No. severely obese carriers ($n = 889$)	No. control carriers ($n = 932$)	*In silico* prediction PolyPhen	SNAP	*In vitro* functional study Classification	Assayed difference compared with WT
MC4R								
Cohort I								
	c.11C>T	Ser4Phe[a,b]	1	0	+	+(0; 58%)	−	
Severely	c.105C>A	Tyr35stop[a]	1	0	Nonsense	Nonsense	+	Nonsense
obese	c.606C>A	Phe202Leu[b]	1	0	−	−(0; 53%)	−	
	c.634_635delT	Leu211Del	1	0	Frameshift	Frameshift	+	Frameshift
	c.691G>A	Gly231Ser	1	0	−	−(4; 85%)	−	
	c.706C>T	Arg236Cys[b]	2	0	++	+(3; 78%)	−	
	c.806T>A	Ile269Asn[b]	2	0	+	+(4; 82%)	+	EC_{50}
	c.812G>T	Cys271Phe[b]	1	0	−	+(5; 87%)	+	E_{max}[d]
	c.895C>T	Pro299Ser[b]	1	0	+	+(4; 82%)	+	E_{max}[d]
	c.919C>T	Gln307stop[b]	1	0	Nonsense	Nonsense	+	Nonsense
Lean	c.335C>T	Thr112Met	0	1	−	+(3; 78%)	−	
	c.473A>G	His158Arg[b]	0	1	++	+(6; 93%)	−	
	c.523G>A	Ala175Thr	0	1	−	−(3; 78%)	−	
	c.719A>G	Asn240Ser[b]	0	1	−	−(3; 78%)	−	
Cohort II								
	c.89C>T	Ser30Phe[b]	1	0	+	+(4; 82%)	−	
Severely	c.95G>A	Gly32Glu	1	0	−	−(1; 82%)	−	
obese	c.182A>G	Glu61Lys	1	0	+	+(5; 87%)	+	E_{max}[d]
	c.380C>T	Ser127Leu	1	0	+	−(0; 53%)	+	No E_{max}
	c.634_635delT	Leu211Del	2	0	Frameshift	Frameshift	+	Frameshift
	c.896C>A	Pro299His	2	0	+	+(6; 93%)	+	E_{max}[d]
	c.907G>A	Ala303Thr	1	0	−	+(2; 70%)	+	EC_{50}
	c.976T>C	Cys326Arg	1	0	++	+(4; 82%)	−	
Lean	c.583A>G	Ile195Val[b]	0	1	−	−(0; 53%)	−	
	c.914G>A	Arg305Gln[b]	0	1	++	+(4; 82%)	+	E_{max}[d]
	Total		20[c]	6				
MC3R								
Cohort I								
	c.260T>C	Ile87Thr[a,b]	1	0	+	+(0; 58%)	−	
Severely	c.779C>T	Ala260Val[a,b]	1	0	−	−(3; 78%)	−	
obese	c.824T>C	Met275Thr[a,b]	1	0	++	+(4; 82%)	−	
	c.397_726delins228	c.397_726delins228[a,b]	1	0	Del/ins	Del/ins	+	Del/ins
Lean	c.205A>T	Ser69Cys[a,b]	0	1	−	−(1; 69%)	−	
	c.245T>C	Phe82Ser[a,b]	0	1	+	+(4; 82%)	+	E_{max}[d]
Cohort II								
Severely	c.839C>G	Thr280Ser[a,b]	1	0	++	+ (2; 70%)	+	E_{max}[d]
obese	c.889C>G	Leu297Val[a,b]	1	0	−	+ (3; 78%)	−	
Lean	c.245T>C	Phe82Ser[a,b]	0	1	+	+ (4; 82%)	+	E_{max}[d]
	Total		6	3				

For PolyPhen predictions: benign is denoted as −, possibly damaging is + and probably damaging is ++. For SNAP predictions: neutral is denoted as '−' and non-neutral as '+', both followed by (RI; % expected accuracy). For *in vitro* classification: no functional alteration denoted as '−' and functional alteration as '+', followed by reason for functional alteration classification. Mutations are classified as having an *in vitro* functional alteration when: response did not reach a maximum, EC_{50} was significantly different ($P < 0.05$) compared with that of the WT by ANOVA followed by Dunnett's post-test, and/or had an E_{max} <50% of WT receptor maximum activity.

[a]Mutations never previously described.

[b]Mutations for which α-MSH dose–response had never been described.

[c]Two patients from Cohort II carry both Leu211del and Pro299His mutations.

[d]Decreased.

coding sequence of this gene in the same 889 cases and 932 controls.

The haplotype of two previously described common variants Thr6Lys and Val81Ile (28) were identified in 13 obese and 11 controls (not significant, $P = 0.950$; Table 2). In addition, one mutation, Arg257Ser, was found in both control and severely obese subjects and was not considered further in the analysis (Table 2). Six severely obese and three controls carried rare heterozygous mutations in *MC3R* (Table 1). The prevalence of rare *MC3R* variants in the severely obese patients was 0.67% ($CI_{95\%}$: 0.27–1.50) compared with 0.32% ($CI_{95\%}$: 0.06–0.99) in controls (not significant, $P = 0.332$).

Comparative functional analysis of severe adult obesity-associated *MC3R* mutations

As with MC4R, we systematically and comparatively evaluated the agonist activation of each of the mutant *MC3R* found in obese and lean subjects. Two *MC3R* mutations identified in two obese adults and one *MC3R* mutation identified in

Table 2. Summary of common variants identified in *MC4R* and *MC3R*

Gene	Variant	No. severely obese carriers ($n = 889$)	No. lean control carriers ($n = 932$)	*P*-value
MC4R	Val103Ile	19	22	0.755
	Leu251Ile	6	6	0.774
MC3R	Arg257Ser	1	7	0.070
	Thr6Lys/Val81Ile Haplotype			
	Wild-type	391	414	0.950
	Heterozygous for 6K/81I	93	113	
	Homozygous for 6K/81I	13	11	

The number of severely obese adult carriers (out of $n = 889$) and lean control carriers (out of $n = 932$) for each variant for both *MC4R* and *MC3R* are listed.

two control subjects were functionally altered *in vitro* (Table 1 and Fig. 3). Arg257Ser, found in both case and control subjects, was also classified as a mutation with functional consequence. The prevalence of variants with functional alterations was the same in cases and controls ($P = 0.625$; Fig. 2).

In silico analysis of severe adult obesity-associated *MC4R* and *MC3R* mutations

To determine whether *in silico* approaches could also be used to determine the association of functionally significant mutations in MC4R and MC3R with obesity, we used the computer mutation prediction programs PolyPhen (29) and screening for non-acceptable polymorphism (SNAP) (30–32). After classification of functional consequences, the prevalence of functionally altered MC4R mutations was significantly greater compared with controls for both the prediction tools, PolyPhen ($P = 0.001$) and SNAP ($P = 0.002$) (Fig. 2). However, as with our *in vitro* analysis ($P = 0.625$), the prevalence of MC3R variants with functional alterations by both *in silico* prediction methods was the same in cases and controls (PolyPhen $P = 0.442$ and SNAP $P = 0.277$) (Fig. 2). For both MC4R and MC3R, there was no significant difference between the prevalence of mutations classified as having a functional alteration in the *in vitro* assay and *in silico*. However, there were a number of discrepancies between both methods, which indicates that at an individual mutation level *in silico* approaches cannot replace functional characterization (Table 1 and Fig. 2).

DISCUSSION

This study confirms that mutations in *MC4R* are a significant cause of severe human obesity and extends this finding to severely obese North American adults. Indeed, we find the same combined prevalence of such mutations [2.25% ($CI_{95\%}$: 1.44–3.47%)] in our severely obese adult patient populations as that previously described in French adults [2.35% ($CI_{95\%}$: 0.90–3.80%)] with severe obesity (BMI > 35 kg/m^2) (15)

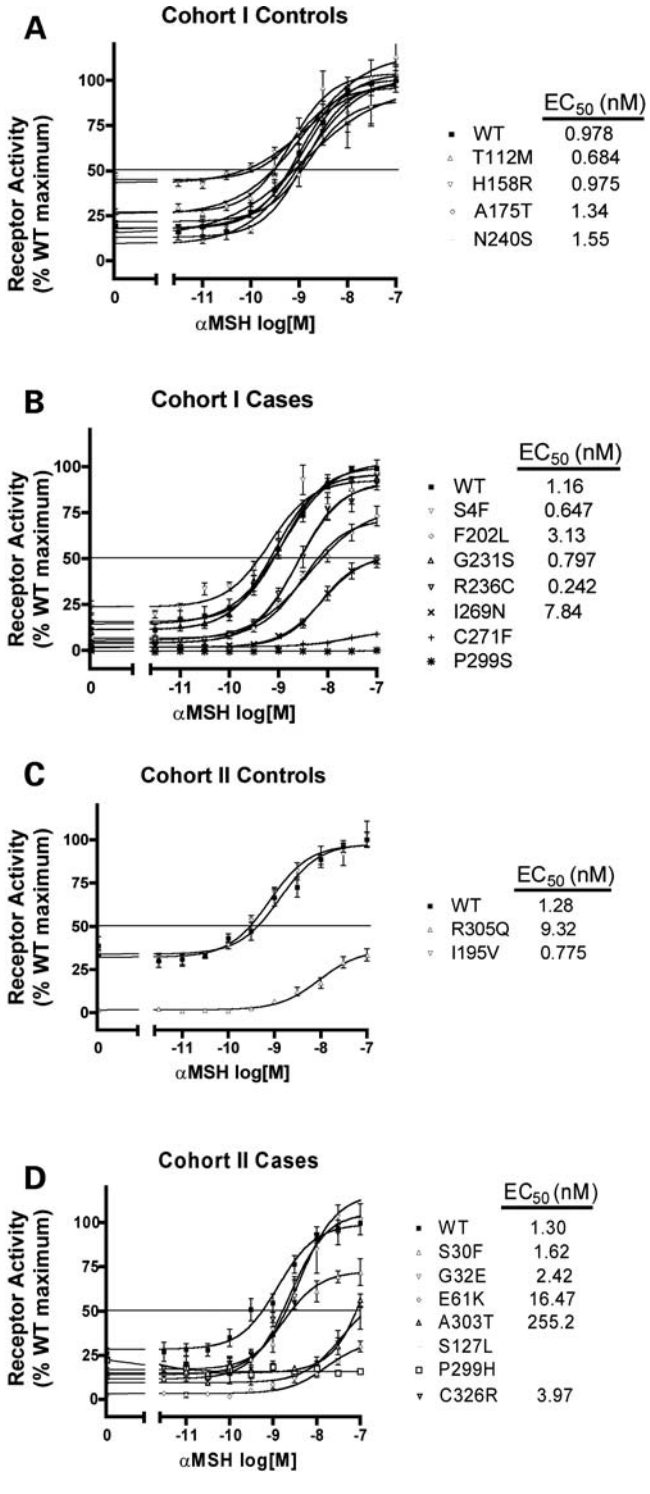

Figure 1. Functional analysis of mutant MC4Rs. α-MSH dose–response curves of mutants identified in (**A**) lean controls of Cohort I ($n = 554$), (**B**) severely obese cases of Cohort I ($n = 510$), (**C**) lean controls of Cohort II ($n = 378$) and (**D**) severely obese cases of Cohort II ($n = 379$). Data points represent mean ± SEM of at least three experiments performed in triplicate. Mean ($CI_{95\%}$) of the EC_{50} (nM) is indicated for each variant, when the variant response reached a maximum. For comparison purposes, the activities range from basal activity (0%) to the maximal activity (100%) of each receptor.

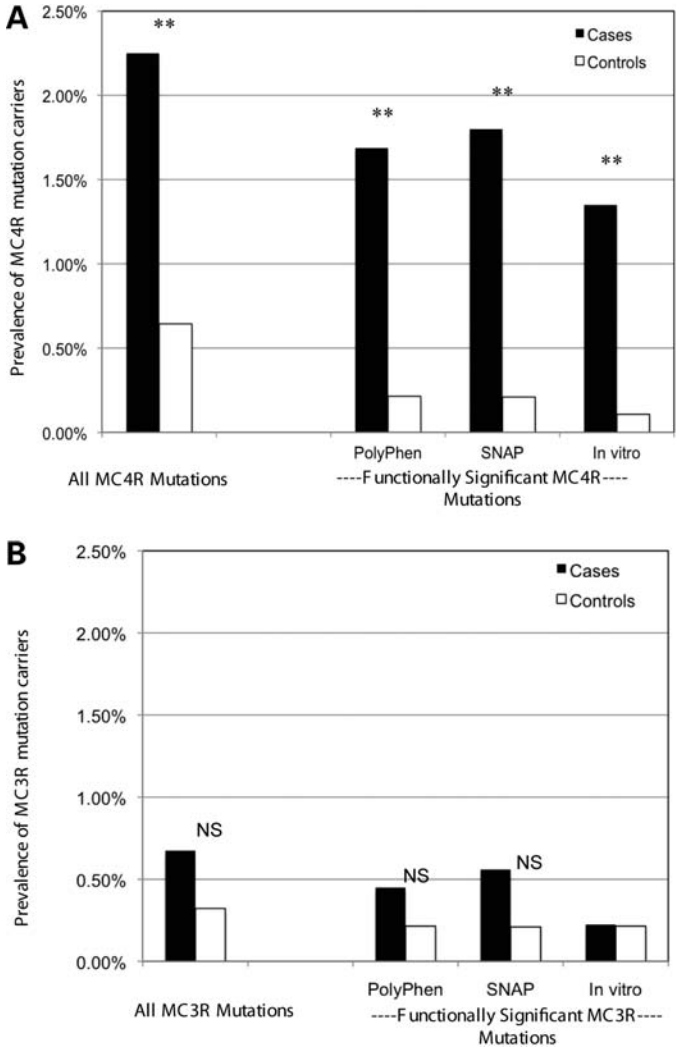

Figure 2. Prevalence of rare *MC4R* (**A**) and *MC3R* (**B**) mutation carriers in severely obese and lean subjects. Prevalence of carriers is determined by combining both Cohort I and Cohort II (a total of 889 cases and 932 controls). The prevalence of total rare mutation case carriers was compared with control carriers. These mutations were then grouped according to results of the functional studies. Comparison of carriers between cases and controls were made using two-tailed Fisher's exact test. $^{**}P < 0.005$; NS, not significant.

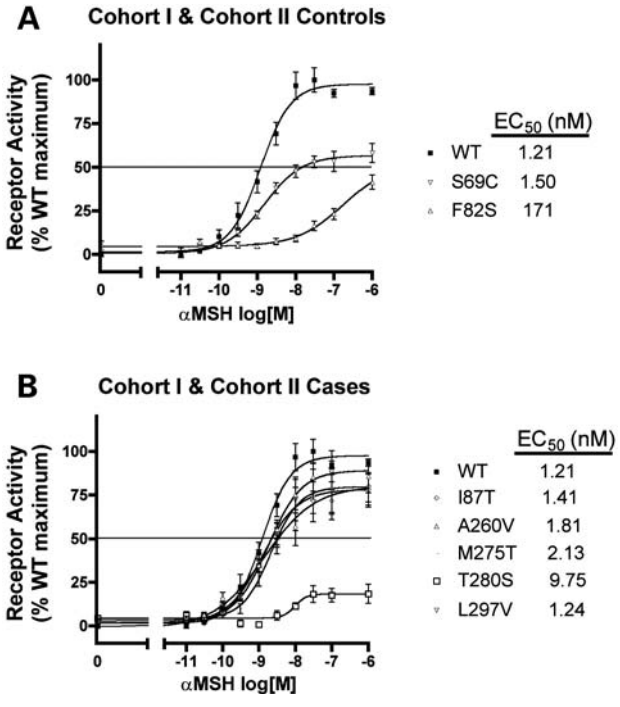

Figure 3. Functional analysis of mutant MC3Rs. α-MSH dose–response curves of mutants identified in (**A**) lean controls of Cohorts I and II ($n = 932$) and (**B**) severely obese cases of Cohorts I and II ($n = 889$). Data points represent mean ± SEM of at least three experiments performed in triplicate. Mean ($CI_{95\%}$) of the EC_{50} (nM) is indicated for each variant, when the variant response reached a maximum. For comparison purposes, the activities range from basal activity (0%) to the maximal activity (100%) of each receptor.

and in young adult Danish males (BMI ≥ 31 kg/m^2) (25). This prevalence also matches the frequency of *MC4R* mutations detected in cohorts of patients with childhood obesity of different origins (1–6%) (5–10,25,33,34). Rare *MC4R* mutations are also found in non-obese controls albeit with a significantly lower frequency. The prevalence of such mutations in our control population (0.64%) is strikingly similar to that described for a very large population-based sample of adults from Germany [0.66% ($CI_{95\%}$: 0.44–0.96%)] (35) and in most non-obese control populations. Although the significant difference in the prevalence of rare *MC4R* mutations between cases and controls supports a causative role for such mutations in the severe obesity of these patients, this observation underlines the importance of systematically evaluating the functional consequences of such mutations. Indeed, restricting the association analysis to only mutations with a significant effect on the function of MC4R significantly increases the odds ratio of association of rare MC4R mutations with severe obesity.

When compared with MC4R, the importance and role of MC3R mutations in the pathogenesis of obesity in rodents and humans has been less clear. When compared with *mc4r* −/− mice, *mc3r*−/− mice have a milder phenotype, limited to an increase in body fat, and unlike in heterozygous *mc4r* +/− mice, no energy homeostasis phenotype has been observed in heterozygous *mc3r* +/− mice (13,19,20,36).

To date only two common variants (28) and four rare mutations in heterozygous carriers (21,37) have been described in the coding region of the *MC3R*. The two common *MC3R* variants Thr6Lys and Val81Ile, which were also detected in the current study, were not associated with any obesity-related phenotypes, therefore likely representing benign polymorphisms (28,38–41).

With respect to rare mutations in *MC3R*, one mutation, Ile183Asn, has been detected in one severely obese girl and her obese father, which was absent in control subjects and was characterized to be functionally inactive (21,22). More recently, three MC3R mutations were identified in a study of 290 severely obese Italian adults, mean BMI of 44.2 ± 5.9 kg/m^2. These three mutations were absent in 215 non-obese controls (37). In that study, however, controls were only screened for mutations found in cases, but the *MC3R* gene was not systematically sequenced in these controls. Two of these mutations were studied in the two families and

segregated with obesity in the family members (total of four relatives). Only one of the mutations, Ile335Ser, demonstrated *in vitro* functional abnormalities (37).

Our study, the largest yet to evaluate the prevalence of *MC3R* mutations in severe human obesity, does not support a significant role for mutations in this gene in this condition. The prevalence of such mutations in cases is 0.67% ($CI_{95\%}$: 0.27–1.50) and is the same in controls. Systematic *in vitro* study of these mutations also demonstrates that functionally significant mutations are as frequent in both cohorts. Interestingly, the prevalence of rare MC3R variants found in both groups is also the same as the prevalence of rare MC4R variants (whether considering all or only restricted to functionally significant) found in controls or in the general population, further suggesting that it corresponds to the 'background' frequency of rare mutations in these genes.

Our data do not eliminate a possible role for some of the individual *MC3R* mutations in the obesity of the carriers, in particular if some of these mutations have a pleiotropic or dominant negative effect. However, formal demonstration of the phenotypical effect of such specific mutations would require the study of very large multigenerational pedigrees to reach statistical significance. Such pedigrees are rarely available.

The recent completion of large genome scans has demonstrated that a certain number of common variants are associated with severe obesity but has also strongly suggested that, in aggregate, such common variants will only account for a small portion of the overall genetic predisposition to this condition (2,3,42). This observation has led to the re-visiting of the hypothesis that rare variants could account for the majority of one individual's predisposition to severe obesity and has led to the suggestion that large-scale systematic sequencing of patients will be required to detect genes in which rare mutations predispose to the disease. Our results underline some of the possible limitations in the outcomes and interpretations of this approach. First, it is clear that the mere presence of rare heterozygous variants in cases is not sufficient to implicate a gene in the condition as such variant can be present at the same prevalence in controls, reflecting the background level of benign mutations in the gene. Second, differentiating functionally relevant mutations from functionally neutral mutations through specific *in vitro*/*in vivo* assays might be required to strengthen the association of rare mutations in a particular gene with a common phenotype. Finally, at an individual level, demonstration of the role of a specific mutation in a common condition might be limited.

MATERIALS AND METHODS

Subjects

US case–control study (Cohort I) (23,43). Patients and controls were recruited through an ongoing study on the genetic determinants and clinical implications of severe obesity as well as from the Cardiovascular Research Institute Genomic Resource, a population-based investigation of cardiovascular disease both established at the University of California, San Francisco (UCSF). Severely obese adult patients ($n = 510$) were selected for a BMI ≥ 40 kg/m^2. BMI was 47.9 $\pm$ 8.3 kg/m^2, age 48.3 $\pm$ 12.1 years, 73% female and 85% Caucasian. Controls ($n = 554$) were chosen on the basis of a BMI ≤ 25 (BMI 22.9 $\pm$ 1.4 kg/m^2) and were matched for sex (68% female), age (51.3 $\pm$ 4.5y) and ethnicity (82% Caucasian).

The UCSF Committee on Human Research approved the protocols, and informed written consent was obtained from all patients. Genomic DNA was extracted from white blood cells by standard methods.

Canadian case–control study (Cohort II) (24,43). Patients were recruited from the Ottawa Hospital Weight Management Clinic and controls were recruited from the Ottawa region. Severely obese Caucasian adult patients ($n = 379$) were selected for a BMI ≥ 40 kg/m^2. BMI was 49.0 $\pm$ 8.8 kg/m^2, age 49.5 $\pm$ 10.7 years and 63% female. Caucasian controls ($n = 378$) were chosen on the basis of a BMI below the 10th percentile for age and sex (BMI 19.4 $\pm$ 1.6 kg/m^2) and were matched for sex (64% female) and age (45.5 $\pm$ 13.0 years).

The institutional review boards of the University of Ottawa Heart Institute and the Ottawa Hospital approved this study and informed consent was obtained from all participants. Genomic DNA was extracted from white blood cells by standard methods. This cohort has previously been used to sequence for variants implicated in obesity (24).

Sequencing

Two primers, MC4R-AF (5′-ATCAATTCAGGGGGACACT G-3′) and MC4R-ER (5′-TGCATGTTCCTATATTGCGT G-3′), were used in PCR to amplify the entire coding region of the *MC4R* gene as described (44). Two primers, MC3R-F (5′-AAGTTCTCCCTATGTCTCCAAGC-3′) and MC3R-R (5′-CAAACGACAAGTACAATCATGGC-3′), were used in PCR to amplify the entire coding region of the *MC3R* gene. The sequencing reaction was performed with the BigDye terminator kit (Applied Biosystems, Foster City, CA, USA) under the standard manufacturer's conditions. Each PCR product was sequenced using MC4R-AF, MC4R-ER, MC3R-F, MC3R-R and two internal primers, MC4R-CF (5′-TGTAGCTCCTT GCTTGCATC-3′) and MC4R-CR (5′-GGCCATCAGGAA-CATGTGGA-3′). Sequencing was performed on an ABIPRISM 3700 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA).

In vitro analysis of missense MC4R and MC3R mutations

MC4R and MC3R expression vectors. WT and mutant alleles of *MC4R* and *MC3R* gene were amplified and cloned directly from the genomic DNA of the patient. Both one exon genes were cloned in the vector pcDNA 3.1 (Invitrogen, San Diego, CA, USA). All expression vectors were sequenced to establish the presence of the mutation and the absence of any induced mutations.

Assay of MC4R and MC3R activity. HEK293 cells stably expressing or transiently transfected to express the luciferase reporter under the control of a cAMP responsive promoter (10,23) were maintained in α-MEM supplemented with 10%

calf serum (Invitrogen, San Diego, CA, USA), L-glutamine, non-essential amino acids and penicillin/streptomycin. Transfection and dose response assays were performed as previously described (10,23). Briefly, different concentrations of α-MSH (Sigma, St Louis, MO, USA) were added to the medium at the desired concentration. Luciferase activity, representing the cAMP production through MC4R or MC3R activation, was assessed using the Steady-Glo Luciferase Assay System (Promega, Madison, WI, USA) and a microplate luminescence counter (Packard Instrument, Downers Grove, IL, USA). Luciferase activity upon MC4R or MC3R activation was normalized over the transfection efficiency, and the results were finally normalized as a percentage of the maximum stimulation observed for the WT receptors.

In silico prediction of missense mutation effects

PolyPhen Prediction: Missense variants identified by sequencing were classified based on their potential impact on protein function or structure (benign, possibly damaging or probably damaging) using a new version of the PolyPhen method (29). These predictions are based on the analysis of multiple sequence alignments of homologous proteins, functional annotation and structural information if available (29). The new version of PolyPhen constructs multiple sequence alignment using a pipeline of several existing programs for aligning sequences, alignment quality control and clustering of sequences. PolyPhen defines the predictions of the mutations as follows: (1) probably damaging: it is with high confidence that it is suppose to affect protein function or structure, (2) possibly damaging: it is suppose to affect protein function or structure and (3) benign: it is most likely that it lacks any phenotypic effect (http://genetics.bwh.harvard.edu/pph).

SNAP prediction. SNAPs is a neural-network-based method (30) that uses, among other things, information about sequence conservation, per residue predictions of secondary structure, solvent accessibility and flexibility, and, if available, experimental functional annotations. The server output is a binary classification of the mutation's functional effect (neutral/non-neutral) in combination with the reliability index (RI) of prediction (integer score ranging from zero to nine) (32), which correlates well with the expected accuracy of the prediction (31).

Statistical analysis

Prevalence and confidence intervals were calculated using GraphPad Statistics Software. Common variants and haplotype associations with obesity were analyzed by performing a χ^2 analysis-of-contingency table with Yates' correction. The prevalence of rare mutation carriers and the prevalence of functionally significant rare mutation carriers (as defined by *in silico* and *in vitro* experiments) in severely obese subjects compared with those prevalences in lean subjects were analyzed for a significant difference ($P < 0.05$) by two-tailed Fisher's exact test. Best-fit estimates of the EC_{50}s (the concentration of ligand needed to achieve 50% of maximum effect) and the 95% confidence intervals were obtained by non-linear regression fitting of the sigmoidal dose–response curves using Prism 4. Mutant receptor EC_{50}s were compared with WT receptor EC_{50} and significance ($P < 0.05$) was determined by ANOVA followed by Dunnett's post-test.

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