

**Prevalence of MC4R deficiency in European population  
and their age-dependant penetrance in multi-generational pedigrees**

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*Objective.* Melanocortin-4 receptor (*MC4R*) deficiency is the most frequent genetic cause of obesity. However there is uncertainty regarding the degree of penetrance of this condition and the putative impact of the environment on the development of obesity in *MC4R* mutation carriers is unknown.

*Research Design and Methods.* We determined the *MC4R* sequence in 2,257 obese individuals and 2,677 non-obese controls of European origin and established the likely functional impact of all variants detected. We then included relatives of probands carriers and studied 25 pedigrees including 97 carriers and 94 non-carriers from 3 generations.

*Results.* 68% of the *MC4R* non synonymous mutations found in obese subjects result in a loss of function *in vitro*. They were found in 1.72% of obese vs. 0.15% of non-obese people ( $p=6.9 \times 10^{-10}$ ). Among the families, abnormal eating behaviour was more frequent in both *MC4R*-deficient children and adults than in non-carriers. While BMI was inversely associated with educational status in non-carrier adults, no such relationship was seen in *MC4R* mutation carriers. We observed a generational effect, with a penetrance of 40% in *MC4R*-deficient adults aged >52 years, 60% in 18-52 year-old adults and 79% in children. The longitudinal study of adult carriers showed an increasing age-dependent penetrance (37% at 20yrs vs. 60% at >40yrs).

*Conclusions.* We have established a robust estimate of age-related penetrance for *MC4R* deficiency and demonstrated a generational effect on penetrance, which may relate to the development of an “obesogenic” environment. It remains to be seen whether appropriate manipulation of environmental factors may contribute to prevent the development of obesity even in those strongly genetically predisposed to it.

The leptin-melanocortin axis controls human energy homeostasis and the melanocortin-4 receptor is a key player in its central regulation (1). Indeed, loss-of-function mutations in *MC4R* cause severe familial forms of obesity (2; 3) and infrequent gain-of-function polymorphisms have been associated with protection against obesity (4; 5). At least 72 non-synonymous mutations have been discovered so far, but some have no obvious functional consequences (6; 7), highlighting the importance of functional characterization of *MC4R* mutations in the determination of potential pathogenicity. MC4R is a membrane bound G-protein coupled receptor that activates adenylate cyclase. Loss-of-function mutations result in a partial or complete loss of function as measured by cAMP production. The majority of missense mutations in *MC4R* result in intracellular retention of the mutated protein, while some primarily affect ligand binding or ligand/receptor activation (8; 9). In some cases, the alteration of the basal activity of the receptor (8; 10) has been reported.

The prevalence of loss-of-function *MC4R* mutations ranges from 0.5% to 5.8% in childhood onset obesity (11-14). The contribution of *MC4R* mutations to late-onset obesity is still debated (13; 15-18). Obesity due to *MC4R* mutations has been extensively studied and while heterozygous loss-of-function mutations can clearly cause familial obesity, they can be found in individuals who are not obese (19). There is a need for reliable estimates of penetrance. Furthermore, no study has thoroughly assessed the effect of loss-of-function *MC4R* mutations in elderly subjects. Previous studies using part of our French cohort evidenced the first mutation in MC4R and demonstrated that most of them lead to an intracellular retention of the receptor (2; 13; 18).

Whilst hyperphagia is a key feature of MC4R deficiency, with increased food intake at an *ad libitum* test meal reported in severely

obese MC4R-deficient children (10), an apparent amelioration of obesity and food intake disturbances has been suggested in adulthood in some studies (6; 11). Obesity is a complex trait and *MC4R* mutations offer a unique opportunity to analyze the effects of both aging and shared environment on the evolution of body mass in this paradigm. In this extensive study of 2,257 unrelated obese subjects, 2,677 controls of European descent and 25 multigenerational pedigrees with several *MC4R* mutations carriers, we provide a comprehensive picture of the prevalence of this condition and of factors that determine the expression of the obesity phenotype and support previous observations reported in a German familial study (20).

## RESEARCH DESIGN AND METHODS

**Subjects.** The study protocol was approved by all local ethics committees and an informed consent was obtained from each subject before participating in the study, in accordance with the Declaration of Helsinki principles. For children younger than 18 years, an oral consent was obtained and parents provided written informed consent. We used the 90<sup>th</sup> and 97<sup>th</sup> BMI percentiles as thresholds for childhood overweight and obesity respectively, according to the recommendations of the European Childhood Obesity Group study (21) in a French reference population (22). The classes of adiposity in adult subjects were defined as: lean (BMI<27kg/m<sup>2</sup>), overweight (27≤BMI<30kg/m<sup>2</sup>), classI-II obese (30≤BMI<40kg/m<sup>2</sup>) and classIII obese (BMI≥40 kg/m<sup>2</sup>). Populations are described in table 1.

433 unrelated obese children were recruited through a multimedia campaign run by the CNRS UMR8090 and 93 obese children were patients of Toulouse Children's Hospital. French obese adults were recruited by the CNRS UMR8090 and the Department of Nutrition of Paris Hotel Dieu Hospital. The

cohort includes 160 classI-II and 703 classIII unrelated obese individuals. Some of them have been used in previous studies (18), the other have been recruited under the same criteria between 1999 and 2003. We also sequenced 868 Swiss obese people, recruited after gastric surgery in Zurich (15). Their BMI has been documented before surgery.

2,302 control subjects were selected from the D.E.S.I.R. general prospective study (23). Selection criteria were BMI<27kg/m<sup>2</sup> and fasting glucose<5.6mmol/L at baseline and during the 9-year study follow-up; 375 unrelated white French adults with BMI<27kg/m<sup>2</sup> and normal glucose tolerance after an oral glucose tolerance test recruited at the CNRS UMR8090 were also used as control subjects. Cases and controls were not matched for age and gender. Their mean age at inclusion was 48y (22.6 kg/m<sup>2</sup>; 41% male).

**Sequencing.** We amplified the coding sequence of the *MC4R* gene from patient's genomic DNA in 3 overlapping fragments. PCR conditions and primers sequences are available upon request. Sequencing was performed using the automated ABI Prism 3730xl DNA sequencer in combination with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit 3.1 (Applied Biosystems). Sequences were assembled and analysed with Sequencher software.

**Functional Characterization of the New Mutations.** All mutant receptors were generated from the wild-type as previously described(24). Wild Type and mutant MC4-receptors were transiently transfected into HEK293 cells using Lipofectamine 2000TM (Stratagene), according to the manufacturer's instructions. One hundred nanogramms of wild-type or mutant receptors were cotransfected with 100ng of Bos-beta-galactosidase construct as a control of transfection efficiency.

The response of cells transfected with wild type and mutant MC4-receptors to the addition of  $\alpha$ -MSH was measured as

previously described (9). Beta-galactosidase assay was performed with remaining lysate from cAMP EIA as previously described. The amount of cAMP produced per well was calculated on the standard curve derived from EIA using GraphPad Prism 4.0, and corrected by beta-galactosidase absorbance to correct for transfection efficiency. The data was fitted to the sigmoidal dose-response curve using GraphPad Prism 4.0. Each experiment was conducted in duplicates at least 4 times. Mutations detected in this study were classed as "loss-of-function" if they showed evidence of impaired cAMP generation either in reported studies or in our investigations.

**Familial Study.** We sequenced *MC4R* in the voluntary relatives of all individuals carrying loss-of-function mutations. Pedigrees were available only for patients recruited by the CNRS UMR8090 unit. This first recruitment already included parents of obese individuals. Proband carriers were then contacted until 2006 for the extension of the study to other family members, as well as children who were too young to be legally included the previous times.

**Generation Determination.** To compare the effects of loss-of-function *MC4R* mutations between generations, we classified the relatives of obese MC4R-deficient probands according to the following thresholds: the end of childhood was determined by the age of 18 years and a generational criterion was defined by the average age when people became grandparents in our cohort: thus we classified adult parents as aged 18 - 52 years and grandparents as aged >52 years.

**Body Composition and Growth.** Proband and relatives have been similarly phenotyped and genotyped. Weight and height were measured during medical consultation and BMI was calculated (weight/ height<sup>2</sup>). The weights and heights of the children, at different ages from birth to the recruitment, were obtained from the child's health notebook when available. We then estimated

the age of the rebound as previously described (25). The adiposity rebound corresponds to the second rise in BMI curve that occurs usually between ages 5 and 7 years. Age of the obesity onset was defined as the age when BMI first exceeded the 97<sup>th</sup> percentile for gender and age.

**Eating Behaviour.** In obese adults, eating behaviour was assessed by the TFEQ (26), which evaluates the cognitive restraint of eating, disinhibition, and hunger. The range of scores for hunger was 1–14, for disinhibition 1–18, and for restraint 1–21. We transformed the criteria of Stunkard into binary traits using the mean of each criterion for the thresholds (7, 9 and 10.5 respectively).

Because the TFEQ has not been validated in obese children, we used an in-house questionnaire administrated by a trained physician (27; 28). Three questions related to eating behaviour during or between meals identified binge eating, eating large amounts of food during meals and snacking, as binary phenotypes (yes or no).

**Statistical Analysis.** Comparison of means was done with unpaired Student's t-test and contingency table chi-square analysis, using SPSS 14.0 for Windows. When the sample size was <30, the statistical significance of the difference between groups was assessed by the Mann-Whitney U-test and the Kolmogorov-Smirnov Z-test for non parametric results. The comparison of prevalence has been tested with the Fisher's exact test, because of theoretical sample size <5. Quantitative traits were also analysed by a linear regression adjusted for age, gender and BMI. All reported P-values are two-sided. P-values of less than 0.05 were considered to indicate statistical significance. Generalized estimating equations with an exchangeable correlation matrix were used with STATA software, to take familial relationships into account when testing the effects of the mutations.

We used allelic frequencies in cases and controls from the initial screening, to estimate penetrances, that is the probability  $P_{Gi}$ , of a given a genotype  $G_i$  being affected, where  $G_1$  is the wild-type,  $G_2$  is the heterozygote and  $G_3$  is the mutant homozygote. From Bayes theorem,  $P_{Gi}$  can be written as  $P(A | G_i) = \frac{P(G_i | A) \times P(A)}{[P(G_i | A) \times P(A) + P(G_i | U) \times P(U)]}$ , where  $A$  stands for affected (obese),  $U$  for unaffected, and  $P(A)$  is the prevalence of being obese, here chosen to be 15% in the European population.

## RESULTS

**Initial Screening of Loss-of-Function MC4R Mutations. Mutation detection.** The baseline characteristics of the 1,731 obese adults, 526 obese children and 2,677 non-obese controls, all of European descent, are reported in Table 1. We considered as a mutation any rare variant (frequency <1%) found in the coding sequence. In this regard, T112M was thus considered as a mutation (12; 29) whereas the more frequent V103I and I251L have been excluded (4; 5; 30). Amongst the 2,257 obese subjects, we identified 36 different mutations (4 synonymous, 31 missense (6 new) and 1 frameshift) in 56 carriers (2.48%) (Tables 1 and 2; figure 1). Two adults were homozygous carriers for I301T and one patient was a compound heterozygous carrier for Y80X and I301T. In the control group, we identified 20 carriers of 15 different mutations (0.75%: 5 synonymous and 10 (5 new) missense). The prevalence rates are given in table 1. The enrichment of non synonymous mutations in the obese group compared to the controls is statistically significant (Fisher's exact test, 2.26%, vs. 0.52%,  $p=9.8 \times 10^{-8}$ ) but there was no difference in the prevalence of synonymous mutations between the two groups (0.22% vs. 0.22%,  $p=1$ ).

**Functional characterization.** We characterized all newly identified missense mutations, by testing the ability of mutated

receptors to generate cAMP in response to increasing concentrations of  $\alpha$ -MSH. Results are reported in figure 1 and table 2 (E<sub>max</sub> and EC<sub>50</sub> are given in supplemental table 1): S94N, F201L, P260Q and R305S showed a partial cAMP response to  $\alpha$ -MSH. D126Y and I289L showed a complete inability to signal to cAMP. We observed no effect on the cAMP response to increasing concentrations of  $\alpha$ -MSH for H76R, D146N, G231V, Y332C and Y332H. The other 30 mutations have been characterized previously in *in vitro* assays (references in table 2). Out of the 34 mutations observed in obese subjects, only 23 (68%) result in a loss-of-function *in vitro*. From this stage, we only analysed carriers of loss-of-function *MC4R* mutations.

**Prevalence of loss-of-function *MC4R* mutations.** We calculated the prevalence of loss-of-function *MC4R* mutations in our European population.

In obese children, the prevalence was doubled in case of family history of obesity compared to sporadic obesity (2.31% vs. 1.08%, respectively). But the difference was not significant, which showed the relative homogeneity of the cohorts and allowed the calculation of a general prevalence in children: altogether, 1.81% of obese children carried loss-of-function *MC4R* mutations. In adults, likewise, the prevalence was higher in probands of obese families compared to obese subjects in whom there was no family history of obesity (2.32% vs. 0.92%,  $p=0.02$ ). Moreover 1.96% of class III obese subjects harboured loss-of-function *MC4R* mutations compared to 0.65% in class I-II, but the difference was not significant ( $p=0.08$ ). The mean prevalence of loss-of-function *MC4R* mutations in adult obesity was then 1.61%. Altogether, the prevalence in obese children and adults was 1.72%, whereas 0.15% non obese individuals (mean age: 44 y, mean BMI=22.8 kg/m<sup>2</sup> compared to 22.6 kg/m<sup>2</sup> in the whole control population, 1 man and 3

women) carried a loss-of-function mutation (11-fold increase,  $p=6.9 \times 10^{-10}$ , table 1).

**Penetrance and Mode of Inheritance.** We evaluated the penetrance of the loss-of-function *MC4R* mutations in two ways: first, we calculated the proportion of carriers of loss-of-function mutations with obesity in our entire population: 63.5% for heterozygous and 94.6% for homozygous/compound heterozygous carriers, respectively, according to the Bayes theorem. We also assessed the penetrance of loss-of-function *MC4R* mutations in 25 French multigenerational non-consanguineous pedigrees where the proband was obese and carried a loss-of-function *MC4R* mutation (supplemental figure 1). The familial penetrance was 60% for heterozygous carriers. It was 100% in homozygous and compound heterozygous carriers.

For mutation I301T, we observed a co-dominant mode of inheritance: two homozygous unrelated individuals presented a BMI of 40.5 and 42.9 kg/m<sup>2</sup>, compared to a BMI of 31.78 kg/m<sup>2</sup> for a I301T heterozygous carrier. Moreover, this heterozygous individual was the father of two compound heterozygous carriers of I301T plus of Y80X with a BMI of 49.68 kg/m<sup>2</sup> and 54.43 kg/m<sup>2</sup>, respectively (supplemental figure 1). The mother carrying mutation Y80X had a BMI of 33.05 kg/m<sup>2</sup>. The third generation of this family included two overweight children of 10 and 14 years (BMI= 19.0 and 22.3 kg/m<sup>2</sup> respectively, BMI $\geq$ 90<sup>th</sup> percentile) carrying either the I301T or the Y80X mutations. The increase in the BMI in homozygous/compound heterozygous compared to heterozygous carriers was significant ( $p=0.008$ ).

We observed no impact of the severity of the mutations (table 2: partial activity vs. no activity) on the severity of the phenotype of the carriers: mean BMI for carriers of mutations leading to a receptor with a partial

activity was 33.62 vs. 31.45 kg/m<sup>2</sup> for carriers of one non functional receptor (p=0.413).

**Effects of Age on Obesity Phenotype in *MC4R* Deficiency.** We compared obesity-related phenotypes between carriers and their relatives for children (n=19 vs. 13) and adults (n= 78 vs. 81). Within the 25 pedigrees, as expected, in each generation, the mean BMI was higher in mutation carriers compared to relatives with a normal *MC4R* genotype (supplemental table 2). We observed a gender effect in the whole\_family sample (+4.3kg/m<sup>2</sup> in males and +8.7kg/m<sup>2</sup> in females (20))\_but the difference is not significant (p=0.19). Children probands were not bigger than their sibs carrying loss-of-function mutations (26.2 vs. 22.6 kg/m<sup>2</sup>, p=0.19) but the difference was significant in adults (45.02 vs. 31.93 kg/m<sup>2</sup>, p=0.007).

**Onset of obesity and evolution of BMI with age.** We obtained heights and weights of children at different ages from the healthbook. Although there was a trend towards earlier development of obesity in *MC4R* mutation carriers compared to obese subjects with a normal *MC4R* genotype (3.2 vs. 6.0 years), this was not statistically significant (p=0.11). The adiposity rebound (a strong predictor of the development of childhood obesity (22)) occurred 3 years earlier in children with *MC4R* mutations (2.0 vs. 5.2 years; p=0.006).

In adults, we calculated BMI at 20 years of age from self-reported heights and weights, and compared this to measured BMI at the age at examination (mean = 44 years). The mean reported BMI of loss-of-function *MC4R* mutation carriers at 20 years of age (BMI<sub>20</sub>) was 26.8 kg/m<sup>2</sup>. Probands were all obese at 20y; among the carrier-relatives, 22 subjects were normal weight at this age (BMI<90<sup>th</sup> percentile, 43%), 10 overweight (90<sup>th</sup> percentile ≤ BMI < 97<sup>th</sup> percentile, 20%) and 19 obese (BMI≥97<sup>th</sup> percentile, 37%). The BMI<sub>20</sub> was slightly but significantly lower in relatives with a normal *MC4R* genotype (24.21 kg/m<sup>2</sup>, p=0.04). The BMI of carriers of

loss-of-function *MC4R* mutations increased significantly during this timeframe compared to non-mutation carrier relatives (13.75 kg/m<sup>2</sup> vs. 6.18 kg/m<sup>2</sup>, p=1.66x10<sup>-5</sup>). Seven out of the 22 (32%) carriers that were non obese at 20 years showed late-onset obesity and 8 out of the 10 individuals (80%) who were overweight became obese.

**Effect of generation on BMI and obesity penetrance.** Comparing the carriers of loss-of-function mutations to their relatives with a normal *MC4R* genotype in the 25 pedigrees, we observed a significant increase of the BMI of 6.78 kg/m<sup>2</sup> (26.37 vs. 33.15 kg/m<sup>2</sup>, p = 5.95x10<sup>-6</sup>).

Adult<52y carriers were 3 kg/m<sup>2</sup> heavier at 20 years of age than the previous generation at the same age (data not shown). The disease penetrance was 78% in children, 60% in adults<52y and 40% in adults>52y for obesity (supplemental table 3). The decrease in penetrance was significant between children and adults>52y (p=0.038). In non-carriers, the prevalence of obesity was the same in each generation (23%, supplemental table 3)

Although carrying a *MC4R* mutation was consistently associated with obesity in every generation, the risk of obesity in carriers was multiplied by 3.4 for children (p=10<sup>-100</sup>, Fisher's exact test), by 2.9 for adults<52y (p=10<sup>-84</sup>) and only 1.7 for adults>52y (p=10<sup>-11</sup>), compared to non-carrier individuals. This decrease in risk was both significant between adults>52y and <52y (p= 3.0x10<sup>-5</sup>) and between children and adults>52y (p=9.6x10<sup>-4</sup>; figure 2).

We looked at the genotype distribution of *FTO* rs1421085 (31) among the generations and found no differences between carriers and non-carriers, as well as between children, adults<52y and adults>52y (data not shown).

**Eating behaviour.** An in-house questionnaire distributed to children assessed 3 abnormal food intake behaviours. There were twice as many children who ate large amounts of food

during meals in the carrier group compared to their non-carrier relatives (61.1% vs. 37.5%;  $p=0.25$ ). No obese child was a binge-eater and snacking was equally frequent in the two groups.

In adults, we used the well established TFEQ questionnaire (26) and observed a significant increase of the disinhibition score in *MC4R* mutation carriers compared to non-carrier relatives (7.2 vs. 5.1,  $p=0.007$ , [supplemental table 2](#)). Moreover, the proportion of carriers harbouring a disinhibition rating above the mean score was 37% vs. 12% in non-carrier relatives ( $p=0.01$ ). The same was true for the hunger score: 31.0% of carriers above the mean vs. 10% in non-carrier subjects ( $p=0.047$ ). Using these methods, eating behaviour was not impaired in adult non obese carriers compared to their non-carrier relatives, suggesting that *MC4R*'s effects on eating behaviour may exhibit incomplete penetrance.

We observed a negative relationship between educational level and BMI in non-carrier adults ( $p=0.045$ ) within the families ( $N=81$ ), as previously reported (32), but no such correlation was observed for the 78 adult carriers of loss-of-function *MC4R* mutations ( $p=0.42$ , data not shown). Apart from BMI and reported eating behaviour, we observed no consistent difference for the other analysed clinical traits between carriers and non-carriers ([supplemental table 2](#)) in this study.

**Exclusion of the probands.** To exclude any recruitment bias, we performed the analyses of the same phenotypes without the probands. Effects of the mutations on BMI remained significant ( $+5.17 \text{ kg/m}^2$ ,  $p=1.37 \times 10^{-3}$ ); effects on the other clinical features seemed to remain as trends but we were no more able to observe the generational effect on penetrance ([supplemental table 4](#)).

## DISCUSSION

Mutations in *MC4R* associated with obesity have been extensively reported but the

true prevalence of this condition has been obscured by several factors. Firstly, the fundamental difference between loss-of-function, neutral and gain-of-function mutations in their major implication on weight has been ignored by several groups, and has led to conflicting and/or inconclusive results regarding the prevalence of *MC4R* mutations in different ethnic groups (14; 15). In this study, we highlighted that only 68% of non-synonymous mutations in *MC4R* result in a loss-of-function *in vitro* in obese subjects. Thus, the prevalence of loss-of-function *MC4R* mutations in our large sample set of obese subjects of European origin is 1.72%. Although our mode of recruitment of obese patients may have tended to overestimate the proportion of severe phenotypes the prevalence is consistent with that previously published in other obese cohorts (14; 18; 33-35). Our findings in the control cohort are consistent with other large population-based cohorts (6) such as the KORA-S4 study, where the prevalence of *MC4R* mutations was also 0.15%. It remains also possible that more subtle mutation effects - such as alteration of the response to other agonists or antagonists - have not been evidenced by us or by others (36), and that the prevalence of loss-of-function *MC4R* is slightly higher.

By studying 2,257 obese individuals and their family members (25 multigenerational pedigrees) and 2,677 non-obese controls, we identified 108 subjects who harboured loss-of-function *MC4R* mutations. This represents the largest group of *MC4R* mutation carriers studied up to now. Our family co-segregation data are consistent with a co-dominant mode of inheritance as reported previously (11). We found that *MC4R* loss-of-function mutations result in obesity in 63.5% of mutation carriers and this value is similar using two different ways of calculation (case-control study and pedigrees). Thus, the obesity phenotype associated with loss-of-function *MC4R* mutations exhibits variable penetrance.

On the contrary, some non-carrier relatives exhibit obesity. This may arise from the so-called phenomenon of “assortive mating” (*i.e.* obese people more often get married with other obese people).

This study establishes the first robust estimates of age-related penetrance for this condition. However, loss-of-function *MC4R* mutation adult carriers at 20 years of age displayed a low penetrance of obesity (37%). One explanation could be the difference in the environmental pressure between France, Switzerland and the UK during the eighties and an under-reported weight at 20 years in self-administered questionnaires, a recognised phenomenon especially in overweight/obese people (37). In some subjects, there was evidence for the development of obesity caused by *MC4R* mutations in adult life. Longitudinal familial analysis also provides evidence that the current “obesogenic” lifestyle may worsen the effect of loss-of-function *MC4R* mutations on childhood and adult energy but educational level does not positively impact on obesity risk among carriers. Non-obese carriers used as controls can develop obesity later in life, even if our controls have been selecting for stable weight <27kg/m<sup>2</sup> during 9 years; their mean age was 44 years at inclusion, so they are unlikely to become obese in the near future.

We also find that the penetrance of obesity in those carrying loss-of-function *MC4R* mutations seems to have increased when comparing grandparents, parents and children in multigenerational pedigrees. Of note, the prevalence of obesity was similar in each generation in wild-type carriers of the 25 pedigrees, showing that the environment alone can not explain such an effect.

We demonstrated that a common *FTO* variant does not influence severity or penetrance of obesity in our sample (data not shown). However, gender, age and generation impact penetrance and severity of obesity, which confirms previous observations made

by Dempfle et al. (20). *MC4R* mutation carriers born around the time of the Second World War were much less likely to be obese in childhood, compared to their offspring born in the 1960s and 70s and especially to the 1990s-2000s generation. It is plausible that the food constraints imposed in France during and after the Second World War (there was ticketing limited access to food until 1947) have delayed obesity, as suggested by the difference in BMI at 20 years amongst mutation carriers of different generations. Our results agree both with an oligogenic (20) and a co-dominant (11) mode of inheritance. We can not exclude that the increasing distant relationships between the family members could be a cause of the observed impact of age and generation on penetrance but direct effects of relationship distance, as well as other genetic and environmental factors, remain to be tested to confirm this assumption.

Given the age- and generation-related penetrance of obesity in carriers of functionally significant *MC4R* mutations, we show that environmental factors contribute to the development of obesity even in those strongly genetically predisposed to it. It remains to be seen whether timely manipulation of environmental factors may be able to prevent or delay the development of obesity or reduce its severity in subjects carrying loss-of-function *MC4R* mutations.

In conclusion, we have established the first robust estimates of age-related penetrance for this condition and demonstrated a generational effect on penetrance which may relate to the development of an “obesogenic” environment. In addition, educational level does not impact on obesity risk among carriers. *MC4R* deficiency may sometimes present as obesity developing in adult life. Our study also highlighted the importance of functional analysis in genetic diagnosis of this condition.

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**Conflict of Interest statement.** The authors declare that they have no competing financial interests

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**Table 1: Phenotypic description of the studied cohorts and results of the mutation screening**

The data are given in % of carriers in the cohort, in brackets are given the number of carriers. BMI of the Swiss individuals has been documented before surgery.

	N (%male)	Mean age (SD)	Mean BMI (SD)	Mutations	Synonymous	Non synonymous	Non synonymous Loss-of- function
<b>controls</b>	2,677 (41)	48 (11)	22.6 (2.7)	0.75 (20)	0.22 (6)	0.52 (14)	0.15 (4)
<b>children</b>	526 (47)	10 (4)	-	2.66 (14)	0.38 (1)	2.47 (13)	1.81 (11)
Lille	433 (49)	11 (3)	-	2.30 (10)	0.00 (0)	2.31 (10)	2.31 (10)
Toulouse	93 (36)	5 (2)	-	4.30 (4)	1.08 (1)	3.23 (3)	1.08 (1)
<b>French adults</b>	863 (30)	44 (14)	41.8 (8.8)	3.24 (28)	0.23 (2)	3.01 (26)	2.32 (20)
classI-II	160 (36)	48 (15)	36.8 (2.9)	2.50 (4)	0.00 (0)	2.50 (4)	1.25 (2)
classIII	703 (24)	44 (12)	47.0 (7.2)	3.41 (24)	0.28 (2)	3.13 (22)	2.56 (18)
<b>Swiss adults</b>	868 (23)	43 (11)	43.2 (7.3)	1.61 (14)	0.23 (2)	1.38 (12)	0.92 (8)
classI-II	297 (23)	42 (11)	36.3 (3.2)	0.67 (2)	0.34 (1)	0.34 (1)	0.34 (1)
classIII	571 (23)	43 (10)	46.8 (6.2)	2.10 (12)	0.18 (1)	1.93 (11)	1.23 (7)
<b>all adults</b>	1,731 (28)	46 (13)	42.3 (8.4)	2.43 (42)	0.23 (4)	2.19 (38)	1.61 (28)
classI-II	457 (33)	47 (14)	34.9 (3.1)	1.31 (6)	0.21 (1)	1.09 (5)	0.65 (3)
classIII	1,274 (24)	45 (12)	47.4 -6.9)	2.82 (36)	0.26 (3)	2.59 (33)	1.96 (25)
<b>All obese</b>	2,257	-	-	2.48 (56)	0.22 (5)	2.26 (51)	1.72 (39)

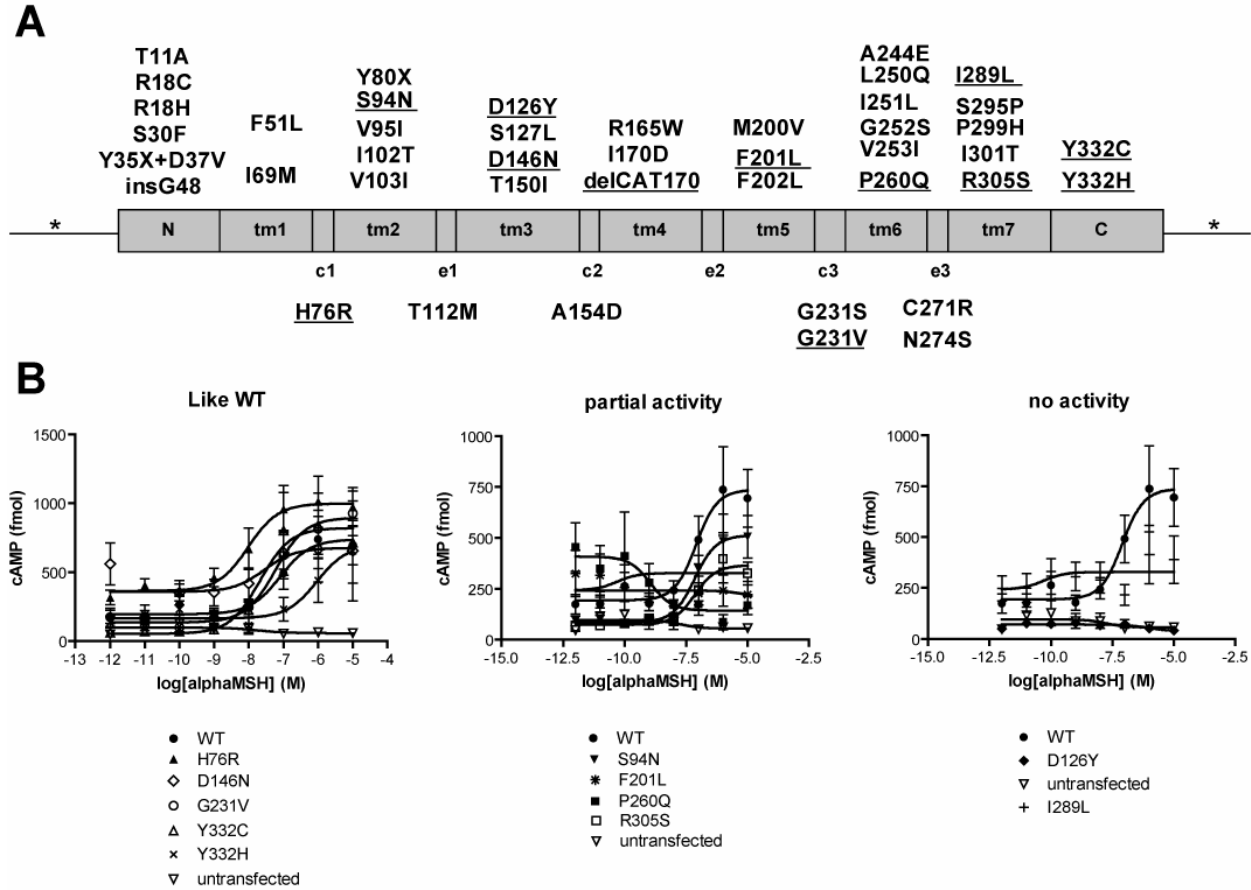
**Figure 1: Mutations identified in MC4R in the screening of 5,620 subjects**

**a. Localisation of the identified mutation according to MC4R conformation**

The transmembrane domains are indicated as tm1-7, cytoplasmic loops c1-3, and extracellular loops e1-3. Mutations identified are listed according to the domain in which they are located. Underlined mutations are new.

**b. Functional characterization of the new non synonymous mutations: cAMP EIA**

The graphs indicate the response of mutant and wild-type MC4R to the addition of a logarithmic increase of  $\alpha$ -MSH. Each point represents the mean  $\pm$  SE of at least 4 independent experiments performed in duplicates.



**Table2: Summary of the mutations, name, carriers and functional characterization.**  
In brackets are the BMI of the carriers.

Mutation	Number of obese carriers (BMI)	Number of non-obese carriers (BMI)	Functional characterization	Ref
<b>Known</b>				
T11A	1 (45.61)		Like WT	[(36)]
R18C	1 (52.59)		Partial	[(33)]
R18H	1 (40.65)		Partial	[(33)]
S30F		4 (21.92; 20.10;20.80;20.40)	Like WT	[(36)]
Y35X+D37V	2 (32.42; 53.67)		No activity	[(29)]
InsG48	1 (41.52)		No activity	[(18)]
F51L	2 (42.52;50.68)		Like WT	[(15)]
I69M	1 (45.51)		No activity	[(17)]
Y80X	2 (32.51; 49.68 compound htz I301T)		No activity	[(17)]
V95I	1 (42.78)		No activity	[(33)]
I102T	2 (35.25;41.09)		Partial	[(17)]
T112M	7 (37.17;20.34;33.21; 47.58;31.35;39.97;53.93)	1 (24.70)	Partial	[(10)]
S127L	2 (36.35;27.22)		Partial	[(13)]
T150I	2 (47.87;49.94)		Partial	[(10)]
A154D	2 (42.62;39.51)		Like WT	[(19)]
R165W	2 (50.86;66.95)	1 (24.00)	Partial	[(10)]
I170V	3 (33.67;49.53;21.90)		Partial	[(10)]
M200V	2 (47.62;45.00)		Like WT	[(6)]
F202L		1 (22.90)	Like WT	[(34)]
G231S	1 (41.62)		Partial	[(17)]
A244E	1 (26.28)		Like WT	[(36)]
L250Q	1 (58.82)		Partial	[(13)]
G252S	1 (49.78)		Partial	[(36)]
V253I	1 (34.47)		Like WT	[(10)]
C271R	1 (28.03)		Like WT	[(11)]
N274S	1 (40.34)		Like WT	[(36)]
S295P	1 (42.19)	2 (25.50;24.80)	Like WT	[(17)]
P299H	3 (23.30;26.45;36.8)		No activity	[(13)]
I301T	3 (42.91hzmz; 40.47hzmz;31.78htz)		Partial	[(13)]
<b>New</b>				
H76R	1 (30.84)		Like WT	-
S94N	1 (67.86)		Partial	-
D126Y	1 (42.65)		No activity	-
D146N	1 (19.20)		Like WT	-
Del170V	1 (22.22)		Not tested	-
F201L		1 (22.30)	partial	-
G231V		1 (24.70)	Like WT	-
P260Q	2 (23.30;25.88)		partial	-
I289L		1 (20.30)	No activity	-
R305S	1 (34.31)		Partial	-
Y332C		1 (20.13)	Like WT	-
Y332H		1 (23.00)	Like WT	-

**Figure 2: Distribution of individuals according to BMI:** we used the official threshold of BMI: percentiles 90 and 97 to discriminate lean, overweight and obese people (21) within the 97 carriers and 81 non-carrier relatives from intra-familial study. Above the bars are given the absolute sample sizes of each subgroups

