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Clinical Spectrum of Obesity and Mutations in the Melanocortin 4 Receptor Gene

I. Sadaf Farooqi, M.D., Ph.D., Julia M. Keogh, B.Sc., Giles S.H. Yeo, Ph.D., Emma J. Lank, B.Sc., Tim Cheetham, M.D., and Stephen O'Rahilly, M.D.

ABSTRACT

BACKGROUND

Melanocortin 4 receptor (MC4R) deficiency is the commonest monogenic form of obesity. However, the clinical spectrum and mode of inheritance have not been defined, pathophysiological mechanisms leading to obesity are poorly understood, and there is little information regarding genotype–phenotype correlations.

METHODS

We determined the nucleotide sequence of the *MC4R* gene in 500 probands with severe childhood obesity. Family studies were undertaken to examine cosegregation of identified mutations with obesity. Subjects with MC4R deficiency underwent metabolic and endocrine evaluation; the results were correlated with the signaling properties of mutant receptors.

RESULTS

Twenty-nine probands (5.8 percent) had mutations in *MC4R;* 23 were heterozygous, and 6 were homozygous. Mutation carriers had severe obesity, increased lean mass, increased linear growth, hyperphagia, and severe hyperinsulinemia; homozygotes were more severely affected than heterozygotes. Subjects with mutations retaining residual signaling capacity had a less severe phenotype.

CONCLUSIONS

Mutations in *MC4R* result in a distinct obesity syndrome that is inherited in a codominant manner. Mutations leading to complete loss of function are associated with a more severe phenotype. The correlation between the signaling properties of these mutant receptors and energy intake emphasizes the key role of this receptor in the control of eating behavior in humans.

From the Genetics of Obesity Collaborative Group (T.C.) and the University Departments of Medicine and Clinical Biochemistry (I.S.F., J.M.K., G.H.S.Y., E.J.L., S.O.'R.), Cambridge Institute for Medical Research, Addenbrooke's Hospital, Cambridge, United Kingdom. Address reprint requests to Dr. O'Rahilly at the Cambridge Institute for Medical Research, Addenbrooke's Hospital, Cambridge CB2 2QQ, United Kingdom, or at sorahill@hgmp.mrc.ac.uk.

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Although changes in diet and exercise underlie the current global increase in the prevalence of obesity, there is considerable evidence of a substantial genetic contribution to the regulation of body weight.[1] Causative mutations underlying several recognizable pleiotropic obesity syndromes (e.g., Bardet–Biedl syndrome) have recently been identified, but in no case has a clear mechanistic link between the product of the mutant gene and disordered energy balance been clarified.[2] Study of strains of genetically obese mice has resulted in the discovery of several genes, mutations of which have subsequently been found to lead to severe human obesity. Deficiency of the adipocyte-derived hormone leptin results in obesity, hyperphagia, infertility, and impaired T-cell–mediated immunity in mice[3,4] and humans,[5,6] and the administration of leptin completely reverses all aspects of the phenotype in both species.[7-11] Proopiomelanocortin is regulated by leptin and is cleaved by prohormone convertases to yield α melanocyte-stimulating hormone.[12] Loss-of-function mutations in the proopiomelanocortin gene lead to obesity in mice and humans.[13,14] The actions of α melanocyte-stimulating hormone on the melanocortin 4 receptor (MC4R) lead to a decrease in food intake,[15] and mice with null mutations in *MC4R* have increased food intake, obesity, and hyperinsulinemia.[16]

We and others have identified mutations in *MC4R* in obese subjects.[17-19] However, the lack of clinical information has precluded a thorough description of the clinical syndrome or systematic examination of correlations between the genotype and the phenotype. Therefore, we screened 500 subjects with severe, early-onset obesity for mutations in *MC4R* and conducted clinical studies of those with mutations. We also characterized the in vitro function of mutant receptors and examined relations between molecular and clinical phenotypes.

METHODS

SUBJECTS

Subjects with severe obesity of early onset (before 10 years of age) were eligible for entry into the Genetics of Obesity Study (GOOS) and are referred to as probands. We recruited 750 subjects. Standard deviation scores for body-mass index (the weight in kilograms divided by the square of the height in meters) were calculated with the use of reference data from the United Kingdom population.[20] Among the probands, the mean (±SD) standard-deviation score for body-mass index was 4.2±0.8. To date, the first consecutive 500 unrelated probands have been examined for mutations in *MC4R*. Subjects with mutations in *MC4R* and their relatives were invited to the Wellcome Trust Clinical Research Facility at Addenbrooke's Hospital, Cambridge, United Kingdom.

All studies were approved by the Anglia and Oxford multiregional ethics committee. The clinical studies were performed after approval by the local–regional ethics committee of Cambridge. Each subject, or his or her parent in the case of children younger than 16 years, provided written informed consent (oral consent was obtained from the minors themselves). All clinical studies were conducted in accordance with the principles of the Declaration of Helsinki.

DETECTION OF MUTATIONS AND GENOTYPING

Genomic DNA was isolated from whole-blood lymphocytes, and the coding region of the *MC4R* gene was amplified by the polymerase chain reaction and sequenced as previously described.[17] To determine allelic frequency, we determined the *MC4R* sequence in 100 alleles from nonobese control subjects from the United Kingdom who were randomly selected from a local population-based cohort.[21]

STUDIES OF MUTANT RECEPTOR FUNCTION

Wild-type (normal) and mutant *MC4R*s were cloned into the mammalian expression vector pcDNA3 (Invitrogen) as previously reported[17] and transiently transfected into HEK293 cells with a luciferase reporter under the control of a promoter that was responsive to cyclic AMP (cAMP),[22] according to the manufacturer's protocols (Fugene Reagent, Roche Diagnostics). All transfections incorporated

Figure 1 (facing page). Mutations in the Melanocortin 4 Receptor (*MC4R*) Gene (Panel A) and in Vitro Function (Panel B).

Panel A shows the positions of the 24 different mutations and sequence variants identified. Mutations found in heterozygous form in obese probands are shown in orange, and mutations found in homozygous form in obese probands are shown in green. Some mutations were found in more than one proband, and the number of probands identified is indicated. Shown in blue are common sequence variants not considered to be of pathogenic importance. Panel B shows the mean (±SE) response of mutant and wild-type MC4Rs to the addition of increasing amounts of ligand, α melanocyte-stimulating hormone, shown on a logarithmic scale, in a cAMP-responsive luciferase reporter assay.

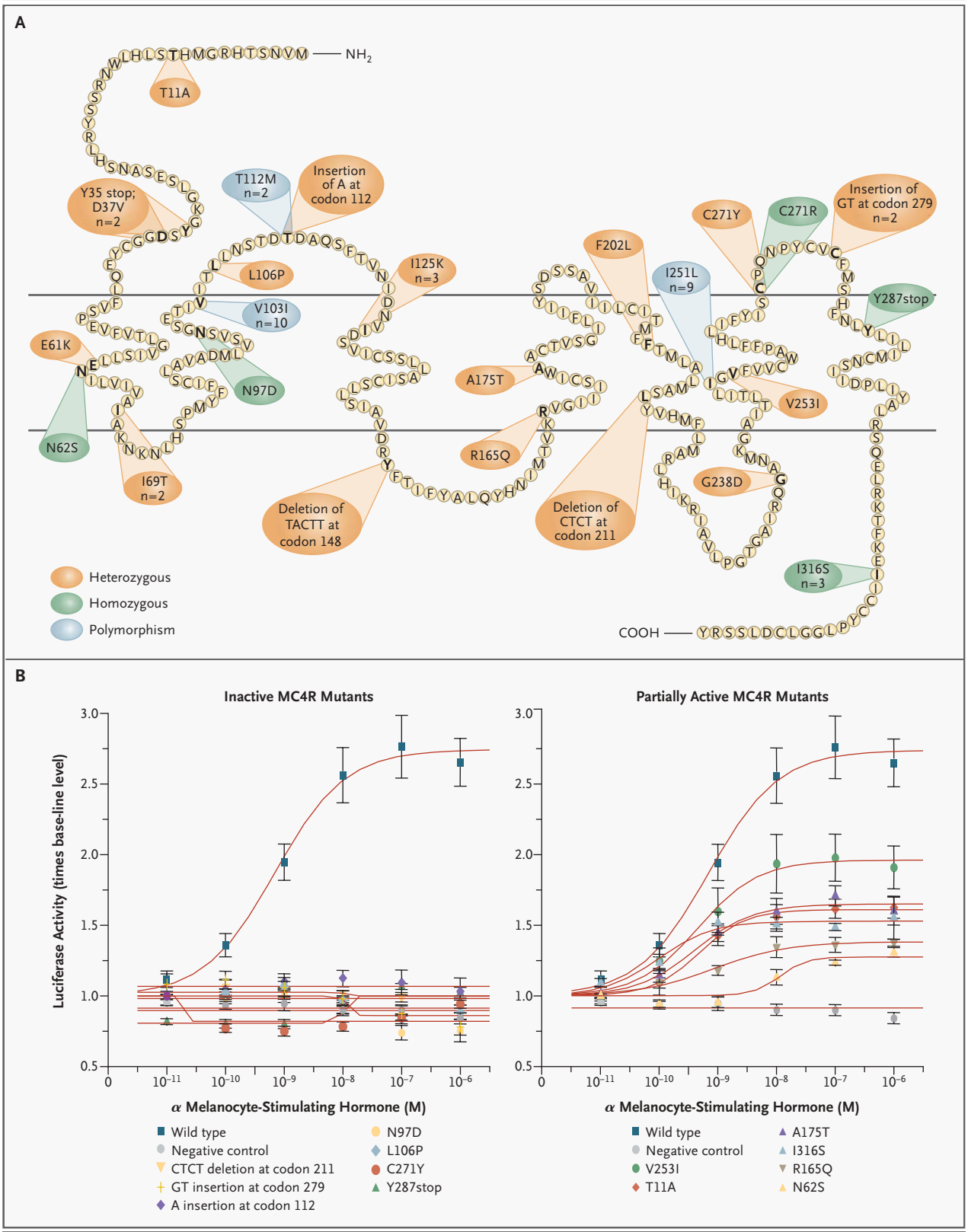


Table 1. Mutations in the Melanocortin 4 Receptor (*MC4R*) Gene Associated with Severe Obesity in Childhood and Loss of Function in Vitro.

Type of Mutation	Amino Acid Change	Family No.	No. of Subjects Phenotyped	Degree of in Vitro Function
Heterozygous				
Frame shift	Insertion of A at codon 112	1	3	No activity
Frame shift	Deletion of CTCT at codon 211	2	2	No activity
Frame shift	Insertion of GT at 279	3, 4	4	No activity
Missense	I125K	5, 6	2	No activity
Missense	C271Y	7	7	No activity
Missense	T11A	8	0	Partial activity
Missense	R165Q	9	0	Partial activity
Missense	A175T	10	2	Partial activity
Missense	I316S	11	5	Partial activity
Homozygous				
Deletion	Null	12	6	Not studied
Nonsense	Y287stop	13	0	No activity
Missense	N97D	14	5	No activity
Missense	N62S	15	9	Partial activity
Missense	C271R	16	0	Not studied
Missense	I316S	17	6	Partial activity

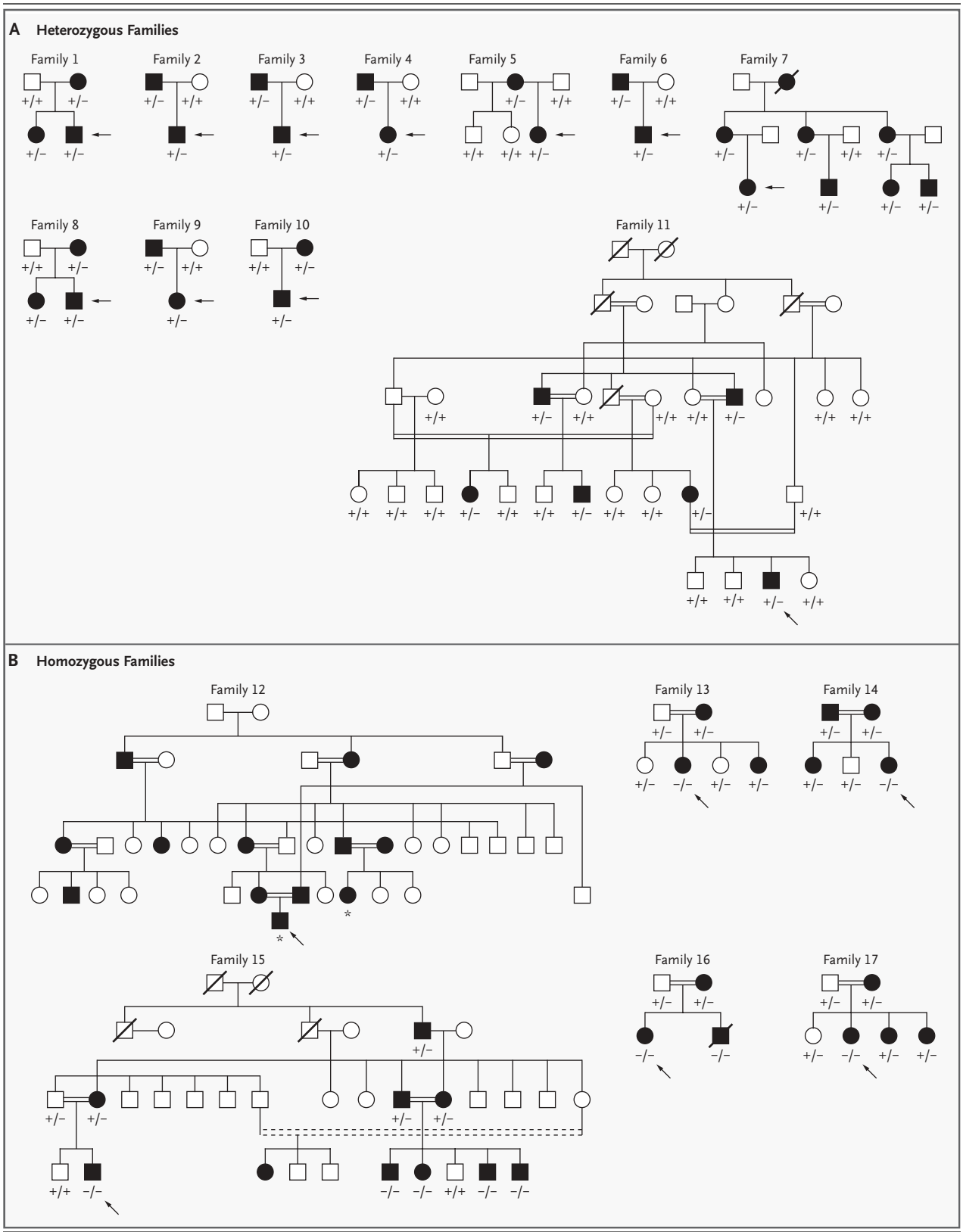
a pRL-CMV plasmid (Promega), which constitutively expresses *Renilla* luciferase and controls for the efficiency of transfection. Cells were deprived of serum, and various concentrations of α melanocyte-stimulating hormone (Bachem) were added. After the cells were harvested, luciferase activity was determined with a luciferase assay system (Promega). Activation of MC4R increases intracellular cAMP, which stimulates the expression of luciferase. Each experiment was conducted in quadruplicate; values are given as means ±SE. Data were analyzed and curves were fitted with the use of Origin software (OriginLab).

BODY COMPOSITION, GROWTH, AND ENERGY BALANCE

Anthropometry and whole-body dual-energy x-ray absorptiometry to determine body composition were performed as previously described.[17] Bone mineral density z scores were calculated with use of the data set of the third National Health and Nutrition Examination Survey.[23] The subjects' resting metabolic rate was measured by indirect calorimetry[17] after they had slept for eight hours at the clinical research facility. After adjustment for body composition, the resting metabolic rate was compared with that predicted according to age- and sex-specific equations.[24,25] Semiquantitative assessment of eating behavior was undertaken in children under 16 years of age. The children were given a 4300-kcal meal at breakfast after an overnight fast, the contents were covertly weighed before the meal and after the children finished eating, and total energy intake and nutrient composition were calculated.[10] Energy intake was expressed per kilogram of lean body weight as a simple means of comparing intake among subjects of different ages and body sizes, since no method of adjustment for age or sex has been validated.

Figure 2 (facing page). Pedigrees of 11 Families with a Proband Who Was Heterozygous for a Melanocortin 4 Receptor (*MC4R*) Mutation (Panel A) and 6 Families with a Homozygous Proband (Panel B).

Pedigrees are shown for the 17 families in which family members were available for genotyping. Squares denote male family members; circles female family members; slashes family members who have died; open symbols unaffected family members; and solid symbols family members with early-onset obesity, defined as weight above the 98th percentile and onset of obesity before 10 years of age; and arrows indicate the proband in each family. Known genotypes are indicated below each symbol: minus signs indicate mutations in *MC4R* on one allele, and plus signs indicate a normal *MC4R* genotype on one allele; thus, heterozygotes are depicted as +/–, and homozygotes as –/–. In two cousins in Family 12 (indicated by the asterisks), the *MC4R* gene could neither be amplified by the polymerase chain reaction nor detected by Southern blotting; they are likely to be homozygous for a null mutation in *MC4R* and were included as such in the phenotypic studies, as were their consanguineous parents, who are presumed to be heterozygous for a deletion of *MC4R*.



METABOLIC AND ENDOCRINE STUDIES

Blood samples obtained while the subjects were fasting were analyzed for leptin, lipids, glucose, insulin, thyrotropin, free thyroxine, corticotropin, insulin-like growth factor I, follicle-stimulating hormone, luteinizing hormone, estradiol, and testosterone with the use of standard assays. In addition, 24-hour urine free cortisol was measured in some subjects.

STATISTICAL ANALYSIS

Clinical data are expressed as means ±SD, and in vitro data are expressed as means ±SE. Differences between groups were compared with use of the unpaired Student's t-test. All reported P values are two-sided. P values of less than 0.05 were considered to indicate statistical significance.

RESULTS

DETECTION OF MUTATIONS AND FUNCTIONAL ANALYSIS

We identified mutations in 29 of the 500 probands (5.8 percent) that were not found in 100 alleles from randomly selected nonobese subjects (Fig. 1A). Three variants — V103I, I251L, and T112M — were found in obese and control subjects; these variants have been shown to have no effect on MC4R signaling and were not studied further. Signaling properties of mutant receptors were examined (Fig. 1B). Whereas all of the frame-shift mutations and some of the missense mutations resulted in complete loss of signaling, some of the missense mutations encoded receptors with residual ability to generate cAMP in response to ligand (Fig. 1B and Table 1).

MODE OF INHERITANCE

All relatives of the probands were genotyped at *MC4R* by direct nucleotide sequencing. *MC4R* mutations identified in heterozygous probands segregated with early-onset obesity with 100 percent penetrance (Fig. 2A). In six families the proband was homozygous for a mutation in *MC4R* (Fig. 2B), including one (Family 12) in which the proband appeared to be homozygous for a deletion of *MC4R*. In these families all 12 homozygotes became severely obese at an early age; in contrast, the prevalence of early-onset obesity was only 68 percent among heterozygous subjects (17 of 25). Heterozygotes from these families were less obese than their homozygous relatives, as indicated by mean (±SD) body-mass index standard-deviation scores of 1.92±1.6 and 4.64±1.2, respectively (P=0.001). These findings indicate that the obesity resulting from mutations in *MC4R* is associated with a codominant mode of inheritance.

CLINICAL PHENOTYPE OF MC4R DEFICIENCY

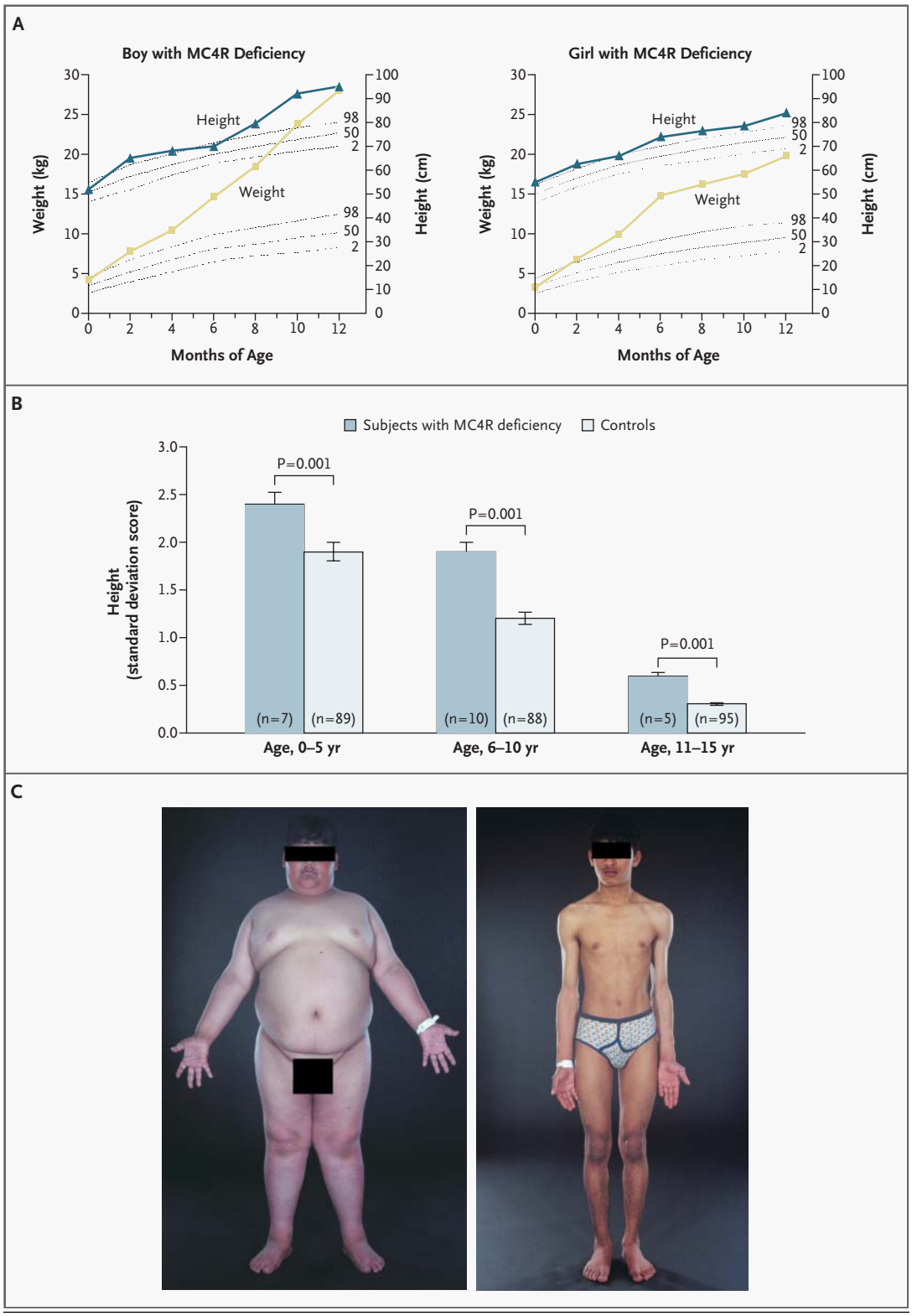
Clinical Presentation, Body Composition, and Growth

The clinical phenotype of MC4R deficiency was studied in 12 probands and 39 of their relatives with early-onset obesity who had mutations in *MC4R*. This group consisted of 42 heterozygotes and 9 homozygotes. The mean body-mass index standard-deviation score for the 51 subjects was 3.14±1.61, with a mean score of 2.79±1.38 among heterozygotes and 4.81±1.63 among homozygotes. Body weight deviated from predicted United Kingdom reference percentiles in the first year of life in over 80 percent of those for whom early growth charts were available (Fig. 3A). At all ages, but particularly during the first five years, the standard-deviation scores for height of children with MC4R deficiency were greater than those of obese children without *MC4R* mutations (Fig. 3B). Serum concentrations of insulin-like growth factor I were appropriate for age in all instances (data not shown).

Dual-energy x-ray absorptiometry was used to examine body composition in 48 subjects. Although the mean percentage of body fat among carriers of *MC4R* mutations was clearly abnormal at 42.9±8.6 percent (normal range, 15 to 25), the relative contribution of fat-free mass to overall weight was greater in these subjects than in subjects with leptin deficiency who had a similar body-mass index (mean fat mass, 57.0 percent).[11] Thus, MC4R deficiency is characterized by an increase in both fat and lean mass. Homozygotes had a higher mean percentage of body fat than heterozygotes (49.5±4.7 percent vs. 41.6±6.6 percent, P=0.01). The characteristic

Figure 3 (facing page). Growth (Panels A and B) and Body Composition (Panel C) in Subjects with Melanocortin 4 Receptor (MC4R) Deficiency.

Panel A shows growth charts for two children with MC4R deficiency during the first year of life, as compared with normal reference values in the United Kingdom (2nd, 50th, and 98th percentiles). Panel B shows mean (±SD) standard-deviation scores for height at different ages in subjects with MC4R deficiency and obese subjects with a normal *MC4R* genotype who were matched for age and body-mass index. Panel C shows a 9-year-old boy who was homozygous for a mutation in *MC4R* (left-hand side) and his 16-year-old brother, who had a normal genotype (right-hand side).



appearance of a child with MC4R deficiency — severely obese and tall, with increased fat-free mass as well as fat mass — is illustrated in Figure 3C.

We have previously reported that subjects with MC4R deficiency have increased bone mineral density and bone mineral content.[17] These extended clinical studies confirmed those observations: 37 of 44 subjects with MC4R deficiency (84 percent) had a bone mineral density z score of more than 1 (mean, 1.7±0.9).

Energy Balance

All subjects had a history of increased appetite, particularly in childhood. The energy consumed by carriers of *MC4R* mutations at an ad libitum meal was three times that of their unaffected siblings, after adjustment for lean body mass (mean, 36.4±8.4 kcal per kilogram of lean mass vs. 11±1.9 kcal per kilogram of lean mass; P=0.001). In general, older children (those 11 to 15 years of age) were less hyperphagic and ate less at the test meal (Fig. 4A). The resting metabolic rate of subjects with *MC4R* mutations was similar to that predicted on the basis of age- and sex-specific equations after correction for lean body mass ($r^2=0.84$ for adults, $r^2=0.94$ for boys, and $r^2=0.70$ for girls) (see Supplementary Appendixes 1 and 2, available with the full text of this article at http://www.nejm.org).

Metabolic and Endocrine Function

All subjects with MC4R deficiency were euglycemic, but plasma insulin concentrations were significantly elevated as compared with those in obese subjects matched for age, standard-deviation score for body-mass index, and sex who did not have *MC4R* mutations (Fig. 4B). There was also an age-dependent effect: children had higher plasma insulin concentrations than adults with MC4R deficiency (data not shown).

Serum lipid concentrations and urinary 24-hour free cortisol excretion were within normal ranges, and serum leptin concentrations were appropriate for fat mass (data not shown). All subjects had free thyroxine concentrations in the normal range. Four subjects had a slight elevation in thyrotropin, and one a subnormal concentration of thyrotropin (see Supplementary Appendix 3, available with the full text of this article at http://www.nejm.org). Gonadotropin secretion, concentrations of sex steroids, and secondary sexual characteristics were appropriate for age in affected children (data not shown). None of the adults reported a history of infertility, males did not report decreased erectile function or decreased libido, and all females of reproductive age had regular menstrual cycles.

RELATION BETWEEN GENOTYPE AND CLINICAL PHENOTYPE

To examine whether functional properties of particular mutant MC4Rs might influence the clinical phenotype, standard-deviation scores for body-mass index and height, energy intake at the test meal, and fasting plasma insulin concentrations were compared in subjects with complete loss of function of MC4R and those with a partial loss of function. Since the phenotype appears to change with age, only children (those younger than 16 years) were compared. The 23 subjects who were heterozygous for nonfunctional mutant receptors were more obese than the 22 with partially functioning mutant receptors (body-mass index standard-deviation score, 3.3±1.1 vs. 1.9±1.3; P=0.005). For each phenotype, subjects with a mutation resulting in complete loss of function in vitro were more severely affected (Table 2). For variables that did not appear to be affected by MC4R deficiency, such as resting metabolic rate per kilogram of lean mass, there was no correlation between genotype and phenotype. Figure 4C shows the results of the test meal in heterozygotes with partially functioning receptors and those with inactive receptors; for comparison, results are also shown for two children with congenital leptin deficiency before and after leptin therapy.

DISCUSSION

In this large study, we found that 5.8 percent of subjects with severe obesity commencing in childhood had pathogenic mutations in *MC4R*. Thus, MC4R deficiency represents the commonest known monogenic obesity disorder. The lower prevalence reported in some studies may be explained by the differences in prevalence in certain ethnic groups,[26] but it may also reflect the later onset and reduced severity of obesity of subjects in these studies.[27] The great majority of subjects thus far described have been heterozygotes, with only one homozygote[17] and one compound heterozygote[18] reported. We identified five additional homozygous probands, allowing us to examine the mode of inheritance in a more detailed manner. We found complete penetrance of early-onset obesity in heterozygous probands and found that homozygous probands were more obese than heterozygotes in these families. Thus, codom-

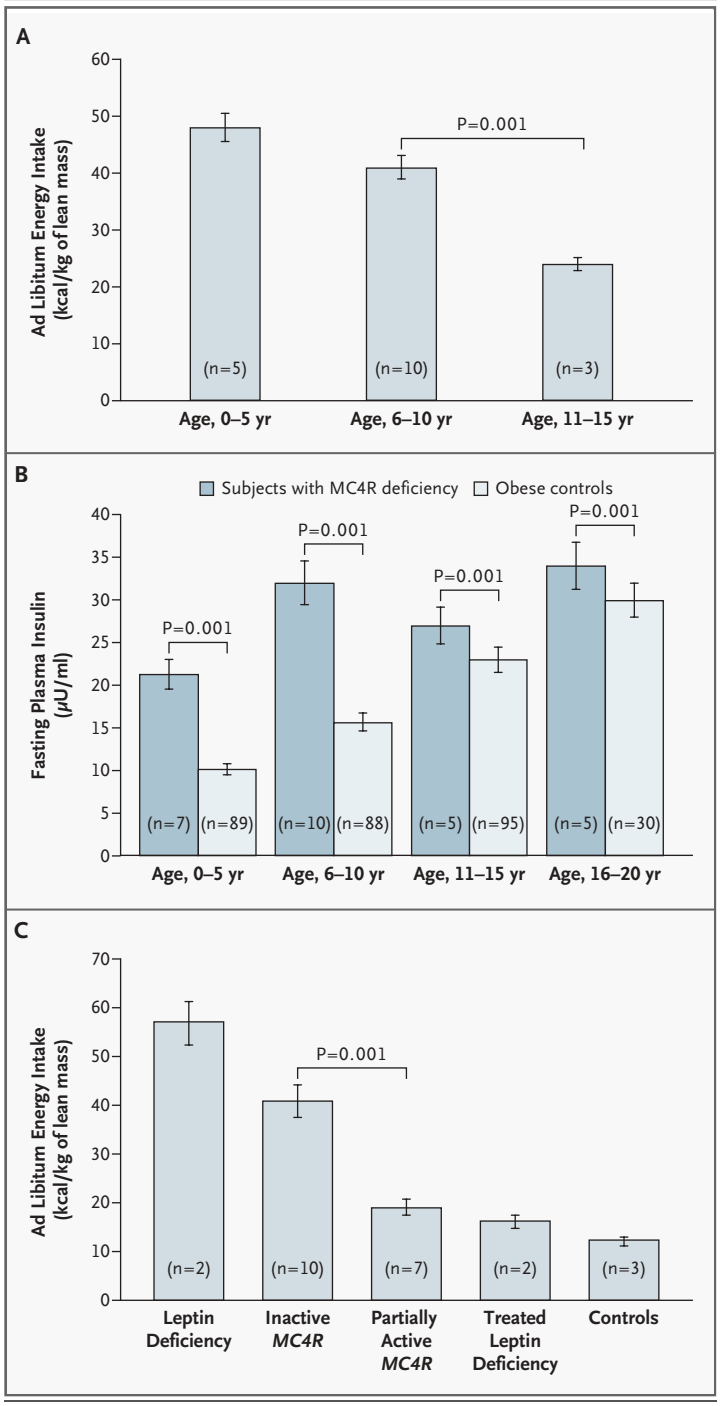


Figure 4. Effects of Age and in Vitro Function of the Mutant Melanocortin 4 Receptor (MC4R) on Mean (±SD) ad Libitum Energy Intake (Panels A and C) and Plasma Insulin Concentrations (Panel B).

Panel A shows the ad libitum energy intake at a 4300-kcal test meal in subjects with MC4R deficiency at different ages. Only data for subjects who were heterozygous for *MC4R* mutations resulting in complete loss of function are shown. Panel B shows plasma insulin concentrations in subjects with MC4R deficiency at different ages, as compared with subjects from the cohort with a normal *MC4R* genotype who were matched for age, sex, and body-mass index standard-deviation scores. Panel C shows the mean energy intake at a 4300-kcal test meal (adjusted for lean body weight in kilograms) in subjects who were heterozygous for *MC4R* mutations resulting in complete or partial loss of function in vitro. Mean energy intake in two children with leptin deficiency (before and after treatment) and in family members with no mutations at the *MC4R* locus is shown for comparison. It is not possible to match subjects with leptin deficiency for age with subjects who have MC4R deficiency, since only four subjects with leptin deficiency have been described to date, two of whom were studied here; there appears to be no age-related change in food intake in leptin deficiency.[11]

inance is the most appropriate descriptor for the mode of inheritance, a finding supported by the pattern of inheritance of obesity seen in heterozygous and homozygous *Mc4r* knockout mice.

However, although all homozygotes in the families of homozygous probands were severely obese, only 68 percent of heterozygotes were obese, differences that cannot be explained by the in vitro function of these mutations. Since all homozygous probands were of Indo–European origin, the penetrance of MC4R mutations may vary in different ethnic groups. Given the large number of potential influences on body weight, it is not surprising that genetic and environmental modifiers will have major effects in some pedigrees. Such effects may also explain differences in the severity of the clinical phenotype observed in other populations.

MC4R deficiency is characterized by an increase in lean body mass and bone mineral density, increased linear growth, hyperphagia, and severe hyperinsulinemia. Most of these features are seen in *Mc4r* knockout mice, suggesting the preservation of the relevant melanocortin pathways between rodents and humans. We confirmed that ad libitum energy intake was greatly increased in children with MC4R deficiency as compared with their unaffected siblings. This finding was consistent with their reported food-seeking behavior in the free-living situation. However, all subjects with MC4R deficiency, including those who were homozygous for a deletion of MC4R, had a lower ad libitum food intake than those with leptin deficiency (Fig. 4C), suggesting that some of the inhibitory effects of leptin

Table 2. Correlations between Genotype and Phenotype in Children with Melanocortin 4 Receptor (MC4R) Deficiency.*

Characteristic	Heterozygotes		Homozygotes	
	Inactive MC4R (N=14)	Partially Active MC4R (N=10)	Inactive MC4R (N=3)	Partially Active MC4R (N=4)
Body-mass index standard-deviation score	3.9±0.5†	2.3±0.3	6.2±1.5†	4.1±0.8
Height standard-deviation score	1.9±0.4†	0.4±0.1	3.1±0.4†	2.5±0.2
Bone mineral density z score	1.9±0.5†	1.3±0.6	—	2.9±0.4
Energy intake (kcal/kg of lean mass)	44.0±5.1†	20.3±1.9	35.2±1.7†	20.3±1.3
Plasma insulin (μU/ml)	27±4.4	25±3.8	31±3.1	28±4.0

* Plus–minus values are means ±SD.
† P≤0.05 for the comparison with partially active MC4R.

on food intake may be mediated by other neuropeptides.

We found no evidence of a major deficit in basal energy expenditure in subjects with MC4R deficiency, although *Mc4r* knockout mice have a 10 percent reduction in basal oxygen consumption.[28] This may reflect a true species difference or subtle defects in human energy expenditure, which may be detectable only when energy homeostasis is perturbed. In *Mc4r* knockout mice, overfeeding with a high-fat diet leads to increased feed efficiency and is associated with a failure to increase diet-induced thermogenesis, suggesting that MC4R has a key role in adaptive thermogenesis.[29]

All obese subjects with MC4R deficiency had severe hyperinsulinemia. Severe hyperinsulinemia, which appears before the onset of hyperphagia or obesity in *Mc4r* knockout mice, can be blocked by the administration of an α-adrenergic blocker, suggesting a role for the central melanocortin pathways in activating sympathetic drive to the pancreas.[30,31] Whether MC4R directly regulates insulin secretion in humans is yet to be determined. The severe, early hyperinsulinemia may contribute to the increased linear growth associated with MC4R deficiency, since no evidence of excessive secretion of growth hormone has been found in either rodents or humans.

The development of puberty and fertility were normal in subjects with MC4R deficiency, in contrast to findings in obese subjects with mutations in leptin, leptin receptor, or PC-1,[6,32,33] suggesting that the effects of leptin on reproductive function are not mediated by MC4R. Male subjects with MC4R deficiency did not report decreased erectile function, whereas pharmacologic MC4R activation in mice has been reported to increase erections and sexual behavior.[34] We found no clear evidence of an effect on the thyroid axis, which is consistent with the normal thyroid function of *Mc4r* knockout mice.[16]

There was an age-related decrease in hyperinsulinemia, which parallels the apparent amelioration of hyperphagia that seems to occur with adulthood in these subjects. As yet there is no explanation for our observation that the phenotype becomes less prominent with age.

Finally, we found evidence of a correlation between the in vitro function of mutant MC4Rs and the severity of the clinical phenotype. All aspects of the phenotype were more severe in those with complete loss as opposed to partial loss of function of MC4R, and this finding appeared to be consistent for both heterozygotes and homozygotes. Our findings suggest that the regulation of body weight in humans is sensitive to variations in the amount of functional MC4R. The correlation between the in vitro properties of a neuropeptide receptor and a measure of a complex human behavior such as food intake is striking and perhaps unique. Our data provide compelling confirmation of the critical role of MC4R in the control of eating behavior and fat mass in humans.

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APPENDIX

Other members of the Genetics of Obesity Collaborative Group are as follows: Drs. R. Stanhope, B. Houlsby, D. Matthews, P. Clayton, E. Crowne, R. Cooke, N. Lingham, B. Adler, M. Rossitor, S. Shakil, S. Sawhney, B. Nauriah, and G. Butler.

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