Energy intake and energy expenditure among children with polymorphisms of the melanocortin-3 receptor

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ABSTRACT

Background: Homozygosity for 2 protein-altering polymorphisms in the melanocortin-3 receptor gene (MC3R) coding sequence, C17A and G241A, has been reported to be associated with an obesity phenotype in children, yet how these polymorphisms affect energy homeostasis is unknown. Association between adult body weight and +2138InsCAGACC, another variant in the 3′ untranslated region of MC3R, has also been described.

Objective: The objective of this study was to examine associations of C17A + G241A and +2138InsCAGACC MC3R variants with children’s energy balance.

Design: Children aged 6–19 y were genotyped for MC3R C17A, G241A, and +2138InsCAGACC. Subjects underwent studies of energy intake from a 9835-kcal food array (n = 185), resting energy expenditure (REE) by using indirect calorimetry (n = 302), or total daily energy expenditure (TEE) by using doubly labeled water (n = 120). Linear regression was used to examine the associations between MC3R polymorphisms and the measures of energy balance.

Results: Body mass index and fat mass were greater in those with double homozygosity for C17A + G241A (P = 0.001). After accounting for covariates (including body composition), the number of minor C17A + G241A alleles was associated with significantly greater energy intake (β = +0.15, P = 0.02) but not altered REE or TEE. No significant associations were observed between +2138InsCAGACC and measures of either fat mass or energy balance.

Conclusions: C17A + G241A polymorphisms may be associated with pediatric obesity because of greater energy intake rather than because of diminished energy expenditure. +2138InsCAGACC does not appear to be associated with obesity or measures of energy balance in children.

INTRODUCTION

Melanocortin-3 receptors (MC3R) have been shown to play an important role in energy homeostasis in both mouse models (1–3) and human studies linking obesity to the chromosomal region 20q13 (4). Inactivation of both alleles of the MC3R gene in mice is associated with positive energy balance through several mechanisms, including energy intake, balance of substrate oxidation, and energy expenditure (1, 5). Early MC3R mutation screening and association studies in adult humans reported that single MC3R gene polymorphisms did not appear to be associated with body weight (6–9). However, several reports have suggested human MC3R or the MC3R gene locus (10–15) may affect body composition. We previously reported that the co-occurrence of homozygosity for 2 polymorphisms in the MC3R coding sequence, C17A (Thr6Lys; rs3746619) and G241A (Val81Ile; rs3827103), was associated with greater body mass, fat mass, body circumference measurements, insulin, and leptin, than for children homozygous for wild-type alleles at both of these loci (11); some (16, 17), but not all (15), subsequent studies have found similar associations in other samples. With the use of in vitro models, we and others have found that the combined C17A + G241A MC3R variant was partially inactive, with decreased signal transduction probably because of reduced receptor expression (11). Recent reports have also suggested that +2138InsCAGACC, a 6-base pair insertion 2138 base pairs downstream of the MC3R start codon, is associated with high body mass index (BMI; kg/m²) (15) and adiposity (10) among adults. To our knowledge, associations of the +2138InsCAGACC MC3R variant with body mass or adiposity among children have not been reported.

Given that the co-occurrence of C17A and G241A MC3R variants in children is associated with an obesity phenotype, our primary objective was to examine the associations between these...
MC3R polymorphisms and the principal components of energy balance. We conducted an analysis of genotypes with the use of available clinical research data to study the effect of double polymorphism C17A + G241A MC3R on energy intake, resting energy expenditure (REE), and total daily energy expenditure (TEE). We also evaluated associations of the +2138InsCAGACC MC3R variant with body mass, adiposity, and measures of energy balance in our pediatric sample. On the basis of the observed effect of MC3R inactivation in mice and the extant human data, we hypothesized that, compared with children who were homozygous for C17A and G241A or +2138InsCAGACC MC3R variants would show both greater energy intake and lower energy expenditure.

SUBJECTS AND METHODS

Subjects

Healthy children and adolescents (aged 6–19 y) participating in 3 nonintervention metabolic protocols (NCT00320177, NCT00001195, and NCT00001522; clinicaltrials.gov) and overweight children and adolescents participating in 2 weight-loss treatment studies (NCT00001723 and NCT00005669) were assembled into 3 samples to study associations between genotype and energy intake, REE, and TEE. Energy intake was assessed as part of the nonintervention studies only, whereas body composition and energy expenditure measures were collected as part of both the obesity intervention and the nonintervention studies. Nonintervention studies, by design, were enriched with overweight participants (BMI ≥95th percentile for age and sex) (18). When studied, none of the participants was undergoing weight-loss treatment. Nonintervention participants were recruited through posted flyers; mailings to parents in the Montgomery County and Prince Georges County, MD, school districts; and mailings to local family physicians and pediatricians requesting children willing to participate in studies investigating hormones and growth in youth. The obesity treatment-seeking youth were recruited through newspaper advertisements and letters to physicians practicing ≤60 miles of Bethesda, MD, for weight-loss studies involving medication. The intervention participants were overweight African American and white adolescents (aged 12–17 y; 50% African American) with ≥1 obesity-related medical comorbidity (19) and children aged 6–12 y of any race-ethnicity who had fasting hyperinsulinemia (20). Participants in treatment protocols were assessed at baseline, before the initiation of any weight-reduction therapy. None of the participants had medical conditions that would preclude accurate completion of study assessments or were taking medication affecting body weight when they were studied. All participants were weight stable (±5% body weight) before measurement. The Eunice Kennedy Shriver National Institute of Child Health and Human Development Institutional Review Board approved the clinical protocols. Each child provided written assent, and parents gave written consent for their participation.

Study protocol

All participants came to the laboratory for a screening visit and then returned for separate visits to assess food intake, energy expenditure, or both, as specified by their primary research protocols. During the screening visit, a brief history and physical examination were conducted by a pediatric endocrinologist or nurse practitioner, eligibility was assessed, blood samples were obtained for MC3R genotyping, and body composition measures were collected. Food intake and energy expenditure measures were obtained as part of a series of experiments conducted between July 1996 and July 2008. Body composition data have been reported previously (11) on 154 of the 416 participants presented in the current study (44 of the energy intake study participants, 153 of the resting metabolic rate study participants, and 75 of the TEE study participants). Complete data from all experiments (food intake plus REE plus TEE) were available for only 33 subjects, necessitating construction of 3 cohorts.

MC3R genotyping

Each child supplied a whole-blood sample from which genomic DNA was isolated (LoFstrand Laboratories Ltd, Gaithersburg, MD, or QiAmp DNA Blood Maxi Kit, QiAGEN Inc, Valencia, CA). MC3R genotyping for C17A and G241A was accomplished by polymerase chain reaction amplification of the MC3R region containing both single nucleotide polymorphism loci from genomic DNA with primers 5′-AC-CTTCCCATCCTTTATTC-3′ and 5′-AGGGCAATTGAGCA-CACCCATT-3′, followed by restriction fragment-length polymorphism (RFLP) analysis of the amplicon with the use of HpCH4IV for C17A and BsaI for G241A, as previously described (11). MC3R genotyping for +2138InsCAGACC was accomplished by PCR amplification of the MC3R region containing this locus from genomic DNA with primers 5′-AC-CTAGTTAGGGTCATGGTATATTCT-3′ and 5′-AGGCCATTGAGCACCCATT-3′ followed by RFLP analysis of the amplicon with the use of PsiI (New England Biolabs, Ipswich, MA). All RFLP analyses were performed twice for each sample to confirm genotype assignments.

Body composition

Weight and height were measured in the postabsorptive state as described previously (21) with the use of calibrated instruments. BMI and BMI z scores were calculated, the latter according to the Centers for Disease Control and Prevention 2000 growth charts (22). Total body fat mass (FM) and fat-free mass (FFM) were determined by air-displacement plethysmography (Life Measurement Inc, Concord, CA), as previously described (21), or by dual-energy X-ray absorptiometry (Hologic, Bedford, MA), as previously described (23). For analysis of TEE data, FFM was derived from total body water measured by deuterium and oxygen dilution and calculated with age- and sex-adjusted values for the hydration factor of FFM (23). FM was then calculated by subtracting FFM from total body mass.

Self-selected food intake test meal

Before participating in the test meal, participants were acclimated to the laboratory food intake setting by consuming a high-calorie shake (787 kcal, 52% carbohydrate, 11% protein, 37% fat) during the screening visit. Those unable to consume at least half of the shake under these conditions were considered unable to acclimate and were deemed ineligible. For the test meal, children arrived in the morning of the test meal after an overnight
fast and were provided with a standard 280-kcal (74% carbohydrate, 7% protein, 19% fat) breakfast. Children remained at the National Institutes of Health Clinical Center for the next 6 hours, during which time they were observed to ensure they consumed no calorie-containing items. Participants were allowed to participate only in sedentary activities. In the afternoon, each child was presented with a multiple-item, 9835-kcal test meal designed to contain an array of palatable foods, as previously described (20).

Participants received prerecorded instructions to “Let yourself go, and eat as much as you want” and were given unrestricted time to consume their meal while viewing preselected episodes of a television show devoid of any references to food and from which the commercials had been removed. Subjects ate alone in a room free of food-containing stimuli. All food items presented were weighed to the nearest 0.1 g before and after the test session. Nutrient intakes were calculated with data from the US Department of Agriculture (USDA) National Nutrient Database for Standard Reference (release 16; USDA, Agricultural Research Service, Beltsville, MD) and manufacturer information, when available.

Resting energy expenditure

REE and nonprotein respiratory quotient (RQ) were assessed by open-circuit indirect calorimetry performed with the use of a respiratory metabolic cart (SensorMedics Corp, Yorba Linda, CA, or ParvoMedics, Sandy, UT). Before each test, the calorimeter was calibrated with the use of a reference gas mixture obtained from the manufacturer. Measurements were obtained from participants in the morning after a 12-h fast and a minimum 30-min rest period in a thermoneutral environment, as previously described (24). Subjects consumed their habitual diet (invariably reported as ≥35% energy from carbohydrates) during the week before they were studied.

Total daily energy expenditure

TEE was estimated with the use of the doubly labeled water stable isotope method as previously described (25, 26). Briefly, for baseline isotope measurements, baseline urine specimens were collected after an overnight fast. Subjects then ingested 0.25 g H218O/kg total body water (estimated by height, weight, and bioelectrical impedance analysis) and 0.14 g 2H2O/kg total body water. A fixed volume breakfast shake was consumed 1 h later. Urine samples were collected serially for 4 h after DLW administration. Subjects were then discharged to their home environments, and 2 spot urine samples were again collected 7 d later during 2 h to determine isotope elimination rates. Isotopic analysis and calculation of energy expenditure was performed according to previously described methods (27). REE was also measured in conjunction with TEE measurement with the use of the method described above. Activity energy expenditure (AEE) was calculated as follows: AEE = (0.94 × TEE − REE) (28, 29).

The physical activity level (PAL) was calculated as the ratio of TEE to REE (30). Data for 3 individuals were excluded because of nonphysiologic TEE results (<500 kcal/d, n = 1, and >5500 kcal/d, n = 2).

Statistical analyses

Departure of genotype distributions from Hardy-Weinberg equilibrium was assessed by using chi-square analyses with 1 df. Linkage disequilibrium (LD) was assessed using the CubeX, cubic exact solutions program (31). Boys and girls were analyzed together, but African Americans and whites were analyzed separately, given the known differences in allele frequencies (9–11, 15).

Differences in study participant anthropometric and demographic characteristics were evaluated by analysis of variance and chi-square analyses for continuous and categorical variables, respectively. Post hoc comparisons were performed with Bonferroni’s adjustment for multiple comparisons. Linear regression analysis was performed to test the additive effects of each abnormal allele on each of the measures of energy balance. On the basis of our previous report showing an association between pediatric obesity and double homozygosity for C17A and G241A, and decreased functional activity in this double variant MC3R (11), genotype for these loci were coded based on the combined number of abnormal alleles among the 2 variants (an additive model): homozygous for wild-type alleles at both loci (Wt/Wt = 0), heterozygous for the variant alleles at one locus (Het/Wt or Wt/Het = 1), heterozygous for the variant alleles at both loci (Het/Het = 2), heterozygous for the variant alleles at one loci and homozygous for the variant alleles at the other loci (Het/Hom or Hom/Het = 3), and homozygous for the variant alleles at both loci (Hom/Hom = 4). For tabular and graphic representation of data, participants heterozygous for the C17A and G241A variant alleles for one or more locus (Het/Wt, Wt/Het, Het/Het, Het/Hom, or Hom/Het) were combined because there were so few cases in each group other than Het/Het. To better characterize the contribution of C17A and G241A variant alleles on intake and expenditure measures, formal haplotype analyses were also performed. The probabilities of haplotype pairs were estimated by SIMHAP (software version 1.0.2; Centre for Genetic Epidemiology and Biostatistics, The University of Western Australia). SIMHAP uses current estimation–maximization-based methods for the estimation of haplotypes from unphased genotype data (32) and allows testing for the association of haplotype with phenotypic data. Haplotypes with estimated frequencies <3% were excluded from analyses.

The effects of the +2138InsCAGACC variant on energy intake and expenditure measures were analyzed separately from the C17A + G241A variant. The +2138InsCAGACC MC3R genotypes were coded for the regression models as homozygous for the wild-type allele (Wt = 0), heterozygous for the +2138InsCAGACC variant (Het = 1), and homozygous for the +2138InsCAGACC variant (Hom + Ins = 2).

In addition to minor allele counts, age, sex, race (coded as African American compared with white), FM, and FFM were included simultaneously as independent variables in all regression analyses. Logarithmic transformations (log10) were applied to normalize body composition variables in regression analyses. Haplotype (C17A + G241A) association with energy intake and expenditure measures were examined with multiple adjustments for the covariates described above. All analyses were conducted with the use of SPSS for Windows (version 16.0; SPSS Inc, Chicago, IL) software, unless noted otherwise, and statistical significance was set at an level of 0.05. Mean (±SE) adjusted values generated by regression analyses on energy intake and expenditure measures are shown, as indicated in text, tables, and figure legends.
RESULTS

Hardy-Weinberg equilibrium, linkage disequilibrium, and haplotype frequencies

Data were available for a total of 416 children. Two subjects’ samples were not able to be genotyped for +2138InsCAGACC and were not included in the analyses. Genotype frequencies at C17A, G241A, and +2138InsCAGACC by race are shown in Table 1. Hardy-Weinberg equilibrium, by race, was observed for C17A, G241A, and +2138InsCAGACC. A small degree of LD was observed between +2138InsCAGACC and C17A \( (r^2 = 0.17) \), as well as G241A \( (r^2 = 0.17) \) among African American children, but not among white children \( (C17A: r^2 = 0.03; G241A: r^2 = 0.04) \). C17A and G241A were in strong LD among both race groups \( (African American: r^2 = 0.94, and white: r^2 = 0.83) \), supporting combination of these 2 variants in analyses. Two common haplotype pairs were estimated from C17A and G241A variants in African American \( (Thr Val: 0.5832 \pm 0.04; LysIle: 0.3938 \pm 0.04) \) and white \( (Thr Val: 0.8921 \pm 0.02; LysIle: 0.0774 \pm 0.0175) \) children.

Participant characteristics by MC3R genotypes

The clinical features of the 416 participants for whom clinical data were available are shown in Table 2 according to C17A + G241A genotype. No significant differences were observed in sex or age among groups. As previously described \( (11) \), African American children were most likely to carry the variant alleles \( (P < 0.001) \). Compared with double wild-type \( (Wt/Wt; n = 258) \) and heterozygous MC3R variant \( (Het; n = 126) \) children, double homozygous variant children \( (Hom/Hom; n = 32) \) had higher BMI, BMI z scores, and FM \( (all \ P < 0.001) \). Compared with Wt/Wt, FFM \( (P = 0.008) \) and FM expressed as a percentage of total body mass was significantly higher in Hom/Hom children \( (P = 0.001) \), whereas FFM expressed as a percentage of total body mass was correspondingly significantly lower in Hom/Hom children \( (P = 0.001) \).

The clinical features of these participants according to the +2138InsCAGACC genotype are shown in Table 3. No significant differences were observed across all participants in age, sex, race, or measures of body mass or body composition by +2138InsCAGACC genotype. The previously reported \( (11) \) associations between C17A + G241A genotype and body composition \( (among \ n = 154) \) were confirmed in the additional individuals that were not studied previously \( (n = 262) \). BMI, BMI z scores, and FM and FM expressed as a percentage of total body mass, all \( P \leq 0.01 \).

Energy intake according to C17A + G241A genotype and estimated haplotypes

The clinical features of the 182 participants in the Self-Selected Food Intake study were similar to those of the larger sample described above \( (see Supplemental Table 1a under “Supplemental data” in the online issue) \). No significant differences were observed in sex or age between groups. African American children were most likely to carry the variant C17A and G241A alleles \( (P < 0.001) \). Compared with double wild-type \( (Wt/Wt; n = 115) \) children, double homozygous variant children \( (Hom/Hom; n = 13) \) had higher BMI, BMI z scores, FM \( (all \ P < 0.001) \), and FFM \( (P = 0.01) \). The clinical features of heterozygous MC3R variant \( (Het; n = 54) \) children were between Wt/Wt and Hom/Hom children.

In the regression model for energy intake, sex \( (standardized coefficient, \beta = -0.22, P < 0.001) \), age \( (\beta = -0.22, P = 0.03) \), FFM \( (\beta = 0.72, P < 0.001) \), and number of C17A and G241A variant alleles \( (\beta = 0.15, P = 0.02) \) all served as significant contributors to variation in energy intake \( (model \ adjusted \ R^2 = 0.45, P < 0.001) \); Table 4, analysis of all subjects). Although the Hom/Hom sequence variants were particularly prevalent among African American children, race was not a significant contributor to variation in energy intake \( (\beta = 0.07, P = 0.34) \), and no significant genotype by race interaction was observed \( (\beta = 0.11, P = 0.07) \). Analysis of energy intake among African American children and white children each showed \( \beta \) coefficients of comparable magnitude \( (\beta = 0.13, and \ beta = 0.13, respectively) \), although in these smaller subgroup analyses, the number of C17A and G241A variant alleles was not a significant contributor to variation in energy intake \( (P = 0.20, and \ P = 0.08, respectively, Table 4, analysis restricted to white subjects and analysis restricted to African American subjects) \). The number of C17A and G241A variant alleles did not contribute to the proportions of energy consumed from protein, fat, or carbohydrate during the meal \( (all \ P > 0.27 for genotype contribution) \).

Secondary analyses predicting energy intake with all heterozygous MC3R variants combined showed results similar to the original model, with the number of C17A and G241A variant alleles \( (\beta = 0.13, P = 0.04) \) remaining a significant contributor to variation in energy intake \( (model \ adjusted \ R^2 = 0.45, P < 0.001) \). Mean \( (\pm SE) \) adjusted predicted energy intakes generated by these analyses found that Hom/Hom children exhibited greater energy intake than did Wt/Wt children and that the energy consumed during the meal by Het children was intermediate to that of Hom/Hom and Wt/Wt children \( (Hom/Hom: 2082 \pm 144 \ kcal; Het: 1735 \pm 53 \ kcal; Wt/Wt: 1492 \pm 41 \ kcal; Figure 1) \). Time to complete the test meal was similar between the 3 groups (analysis of variance; \( Wt/Wt: 27.5 \pm 1.1 \ min; Het: 30.6 \pm 2.2 \ min; \) and Hom/Hom: 30.8 \pm 4.2 \ min; \( P = 0.32) \).

Analysis of energy intake among only the African American children showed mean adjusted energy intakes relatively unchanged from the group as a whole \( (Hom/Hom: 2243 \pm 210; Het: 2162 \pm 212) \).
TABLE 2
Participant characteristics according to C17A + G241A variant alleles

| Race            | Wt/Wt (n = 258) | Het (n = 126) | Hom/Hom (n = 32) | P2  |
|-----------------|-----------------|---------------|-----------------|-----|
| African American| 23.6 (19, 29)   | 69.8 (61, 77) | 84.4 (68, 94)   | <0.001 |
| White           | 76.4 (71, 81)   | 30.2 (23, 39) | 15.6 (6, 32)    |     |
| Sex, female     | 53.9 (48, 60)   | 54.0 (46, 63) | 65.6 (48, 80)   | 0.44 |
| Age (y)         | 12.5 ± 3.0a     | 12.9 ± 3.0    | 13.2 ± 3.4      | 0.23 |
| BMI (kg/m²)     | 28.3 ± 10.5a    | 31.7 ± 11.2b  | 37.2 ± 11.7b    | <0.001 |
| BMI z score     | 1.5 ± 1.1a      | 1.9 ± 1.0b    | 2.3 ± 0.8c      | <0.001 |
| Fat mass (kg)   | 27.9 ± 21.9a    | 33.5 ± 23.3b  | 42.9 ± 23.8b    | <0.001 |
| Fat-free mass (kg) | 42.3 ± 15.7a  | 46.3 ± 16.3b  | 49.7 ± 14.1b    | 0.008 |
| Fat-free mass (% total mass) | 35.0 ± 13.8a | 37.9 ± 12.9b  | 43.5 ± 11.3b   | 0.001 |
| Fat-free mass (% total mass) | 64.9 ± 13.8a | 61.9 ± 12.9b  | 56.3 ± 11.3b  | 0.001 |

1 Hom/Hom, homozygous for the C17A + G241A variant alleles; Het, heterozygous for the variant alleles at one or both loci; Wt/Wt, homozygous for wild-type alleles at both loci. Values in the same row with different superscript letters are significantly different, P < 0.05 (post hoc multiple pairwise comparisons with Bonferroni’s correction).
2 Chi-square tests for categorical variables and ANOVAs for continuous variables were used to compare adolescents by race, sex, age, and BMI.

REE was measured in 302 children and adolescents. The clinical features of these participants according to genotype were for the most part similar to those observed for participants in the Self-Selected Food Intake study (see Supplemental Table 1b under “Supplemental data” in the online issue), although this cohort of children was somewhat younger and had greater BMI and FM than participants in the Self-Selected Food Intake study (all P < 0.01).

In the regression model of REE, sex (β = 0.16, P < 0.001), race (β = −0.15, P < 0.001), age (β = −0.30, P < 0.001), FM (β = 0.18, P = 0.01), and FFMI (β = 0.99, P < 0.001) all served as significant contributors to variation in REE (model adjusted R² = 0.82, P < 0.001), whereas the number of C17A and G241A variant alleles was not a significant contributor to variation in REE (P = 0.70). Secondary regression analyses predicting REE with heterozygous MC3R variants combined showed results similar to the original model (model adjusted R² = 0.82, P < 0.001, with P = 0.60 for number of C17A and G241A variant alleles). Mean (±SE) adjusted REE of Hom/Hom children (178 ± 49 kcal/d; n = 27) was comparable to that of Wt/Wt children

TABLE 3
Participant characteristics according to +2138InsCAGACC polymorphism

| Race            | Wt (n = 222) | Het (n = 167) | Hom (n = 25) | P2  |
|-----------------|--------------|---------------|--------------|-----|
| African American| 44.6 (38, 51) | 41.3 (35, 49) | 28.0 (14, 48) | 0.27 |
| White           | 55.4 (49, 62) | 58.7 (51, 66) | 72.0 (52, 86) |     |
| Sex, female     | 55.9 (49, 62) | 51.5 (44, 59) | 68.0 (48, 83) | 0.27 |
| Age (y)         | 12.4 ± 3.2a   | 13.0 ± 2.9    | 12.3 ± 3.2    | 0.13 |
| BMI (kg/m²)     | 29.8 ± 11.2   | 30.7 ± 11.4   | 27.1 ± 8.0    | 0.29 |
| BMI z score     | 1.6 ± 1.2     | 1.7 ± 1.0     | 1.6 ± 1.1     | 0.62 |
| Fat mass (kg)   | 30.6 ± 22.9   | 31.9 ± 23.6   | 23.8 ± 15.7   | 0.26 |
| Fat-free mass (kg) | 42.6 ± 15.4  | 45.6 ± 16.4   | 41.1 ± 16.1   | 0.06 |
| Fat mass (% total mass) | 37.0 ± 13.6  | 36.2 ± 13.7   | 34.2 ± 13.5   | 0.57 |
| Fat-free mass (% total mass) | 62.9 ± 13.6  | 63.3 ± 13.6   | 65.8 ± 13.5   | 0.57 |

1 Wt, homozygous for wild-type alleles; Het, heterozygous for +2138InsCAGACC; Hom, homozygous for +2138lnsCAGACC alleles. Samples for 2 subjects were not able to be genotyped for +2138lnsCAGACC.
2 Chi-square tests for categorical variables and ANOVAs for continuous variables were used to compare adolescents by MC3R coding sequence variant +2138lnsCAGACC.
3 Categorical value percentage; 95% CI, extended Wald, in parentheses (all such values).
4 Continuous variable mean ± SD (all such values).
(1625 ± 32; n = 166) and Het children (1713 ± 49; n = 109). In the regression model for RQ, age (β = −0.31, P = 0.001), but not number of C17A and G241A variant alleles (P = 0.13), was a significant contributor to variation in RQ (model adjusted \( R^2 = 0.16, P < 0.001 \)). Regression analysis examining the association between estimated haplotypes and REE yielded results similar to genotype analyses (model adjusted: \( R^2 = 0.79, P < 0.001 \); haplotype: \( P = 0.64 \)).

**TABLE 4**

Linear regression model predicting energy intake

| Variable                              | B ± SE  | Standardized β coefficient | t     | P       |
|---------------------------------------|--------|---------------------------|-------|---------|
| Analysis of all subjects              |        |                           |       |         |
| Sex, F = 0                            | −292.81 ± 83.74 | −0.22                    | −3.50 | 0.001   |
| Age, y                                | −51.00 ± 23.40  | −0.22                    | −2.18 | 0.03    |
| Race, AA = 1                          | 92.28 ± 96.22   | 0.07                     | 0.96  | 0.34    |
| Fat-free mass, kg                     | 3284.41 ± 505.56| 0.72                     | 6.50  | <0.001  |
| Fat mass, kg                          | −80.36 ± 131.40 | −0.04                    | −0.61 | 0.54    |
| No. of C17A + G241A minor alleles     | 83.59 ± 36.23   | 0.15                     | 2.31  | 0.02    |
| Analysis restricted to white subjects |        |                           |       |         |
| Sex, F = 0                            | −337.35 ± 95.01 | −0.29                    | −3.55 | 0.001   |
| Age, y                                | −43.17 ± 25.31  | −0.21                    | −1.71 | 0.09    |
| Fat-free mass, kg                     | 2811.70 ± 524.79| 0.71                     | 5.36  | <0.001  |
| Fat mass, kg                          | −152.40 ± 148.51| −0.87                    | −1.03 | 0.31    |
| No. of C17A + G241A minor alleles     | 87.80 ± 48.91   | 0.13                     | 1.80  | 0.08    |
| Analysis restricted to African American subjects | | | | |
| Sex, F = 0                            | −208.35 ± 164.06| −0.14                    | −1.27 | 0.21    |
| Age, y                                | −63.22 ± 52.07  | −0.24                    | −1.21 | 0.23    |
| Fat-free mass, kg                     | 4202.48 ± 1175.65| 0.81                     | 3.58  | 0.001   |
| Fat mass, kg                          | −51.97 ± 266.90 | −0.03                    | 0.20  | 0.85    |
| No. of C17A + G241A minor alleles     | 76.70 ± 59.10   | 0.13                     | 1.30  | 0.20    |

1 AA, African American. Linear regression analysis was performed to test the association between the number of C17A + G241A minor alleles, along with associated covariates, and energy intake (kcal). Logarithmic transformations (log10) were applied to normalize fat mass and fat-free mass. Race was included as a covariable in the combined regression analysis. White, model adjusted: \( R^2 = 0.42, P < 0.001 \). African American, model adjusted: \( R^2 = 0.41, P < 0.001 \). Combined, model adjusted: \( R^2 = 0.45, P < 0.001 \).

**TEE according to C17A + G241A genotype and estimated haplotypes**

The clinical features, according to C17A + G241A genotype, of the 120 children and adolescents in whom TEE was assessed were generally similar to participants described above (see Supplemental Table 1c under “Supplemental data” in the online issue). The TEE study participants were, on average, younger than those in the Self-Selected Food Intake and RIE studies and had greater BMI z scores than the Self-Selected Food Intake study group but lower BMI z scores than the RIE study group (all \( P < 0.01 \)). The relatively smaller group of Hom/Hom children (n = 9) had significantly higher BMIs and BMI z scores than did Wt/Wt children (n = 74). The clinical features of Het children (n = 37) were between Wt/Wt and Hom/Hom children. Differences in FM were marginally statistically significant (\( P = 0.05 \)), whereas FM showed statistically nonsignificant differences.

In the regression model of TEE, sex (β = −0.13, P = 0.03), race (β = −0.19, P = 0.002), and FFM (β = 0.99, P < 0.001) all served as significant contributors to variation in TEE (model adjusted \( R^2 = 0.70, P < 0.001 \)). A trend was observed toward the significance of age (β = 0.14, P = 0.06) as a contributor to TEE; however, the number of C17A and G241A variant alleles was not a significant contributor to variation in TEE (P = 0.59). Secondary regression analyses predicting TEE with heterozygous MC3R variants combined showed results similar to the original model (model adjusted \( R^2 = 0.70, P < 0.001 \), with \( P = 0.78 \) for number of C17A and G241A variant alleles). Mean adjusted TEE generated by these analyses have shown comparable TEE among these children according to their C17A + G241A genotype (Figure 2A). Regression analysis examining the association between estimated haplotypes and TEE yielded results similar to genotype analyses (model adjusted: \( R^2 = 0.71, P < 0.001 \); haplotype: \( P = 0.52 \)).

Because the calculation of TEE depends on the total body water value derived from isotope dilution, regression analysis in a smaller cohort of the children was also carried out with only those subjects for whom FFM and FM determined by dual-energy X-ray absorptiometry had been performed within 1 mo of the TEE study. Regression analysis, including only those participants for whom these data were available (60% of the TEE participants), showed results similar to the original model (model adjusted \( R^2 = 0.61, P < 0.001 \) with \( P = 0.42 \) for number of C17A and G241A variant alleles).

REE results among TEE study participants were similar to those of the larger RIE study group described above, such that the number of C17A and G241A variant alleles was not a significant contributor to variation in REE (P = 0.68; model adjusted \( R^2 = 0.80, P < 0.001 \), or RQ (P = 0.16; model adjusted \( R^2 = 0.06, P = 0.05 \)). Likewise, neither AEE (model adjusted \( R^2 = 0.38, P < 0.001 \); Figure 2B) nor PAL (model adjusted \( R^2 = 0.07, P = 0.02 \)) was significantly influenced by the number
Different letters indicate significant differences (\(b \leq 0.001\)), FM (\(P=0.05\) determined by post hoc multiple pairwise comparisons with Bonferroni’s correction.

Energy intake according to +2138InsCAGACC genotype

Among participants in the Self-Selected Food Intake study, no significant differences in age, sex, race, BMI, BMI \(z\) score, or FM by +2138InsCAGACC genotype were observed (see Supplemental Table 3a under “Supplemental data” in the online issue). A significant FM (\(P=0.05\)) difference by +2138InsCAGACC genotype was observed in this cohort. In the regression model of energy intake, sex (\(\beta=-0.21, P=0.001\)), race (\(\beta=0.13, P=0.03\)), age (\(\beta=-0.20, P=0.05\)), and FFM (\(\beta=0.74, P<0.001\)) all served as significant contributors to variation in energy intake (model adjusted \(R^2=0.44, P<0.001\)), whereas the presence of +2138InsCAGACC alleles was not a significant contributor to variation in energy intake (\(P=0.32\)). Mean adjusted energy intakes generated by these analyses are presented in Supplemental Table 3a.

REE measures according to +2138InsCAGACC genotype

In the REE cohort, no significant differences were observed in age, sex, race, BMI, BMI \(z\) score, or body composition by +2138InsCAGACC genotype. However, significant age (\(P=0.03\)) and FFM (\(P=0.03\)) differences by +2138InsCAGACC genotype were observed in this cohort (see Supplemental Table 3b under “Supplemental data” in the online issue).

In the regression model predicting REE, sex (\(\beta=0.17, P<0.001\)), race (\(\beta=-0.15, P<0.001\)), age (\(\beta=-0.30, P<0.001\)), FM (\(\beta=0.18, P<0.001\)), and FFM (\(\beta=0.99, P<0.001\)) all served as significant contributors to variation in REE (model adjusted \(R^2=0.82, P<0.001\)), whereas the presence of +2138InsCAGACC alleles was not a significant contributor to variation in REE (\(P=0.40\)). Mean adjusted REE generated by these analyses are presented in Supplemental Table 3b. Age (\(\beta=-0.31, P=0.002\)) was the only significant contributing factor to variation in RQ (model adjusted \(R^2=0.16, P<0.001\)).

FIGURE 1. Mean (±SE) adjusted predicted ad libitum energy intake as measured with a multiple-item, 9835-kcal afternoon food array. Regression analyses found that, in addition to sex, age, and fat mass (FM), the number of C17A and G241A variant alleles were significant contributors to variation in energy intake (\(\beta=0.13, P=0.04\); model adjusted \(R^2=0.45, P<0.001\)). Hom/Hom indicates homozygous for the C17A + G241A variant MC3R alleles (n = 13); Het, heterozygous for the variant MC3R alleles (n = 54); and Wt/Wt, homozygous for wild-type MC3R alleles at both loci (n = 115). Different letters indicate significant differences (\(P<0.05\)) determined by post hoc multiple pairwise comparisons with Bonferroni’s correction.

Energy intake according to +2138InsCAGACC genotype

In the TEE cohort, no significant differences were observed in age, sex, BMI, BMI \(z\) score, or body composition by +2138InsCAGACC genotype. However, a significant race (\(P=0.03\)) difference by +2138InsCAGACC genotype was observed in this cohort (see Supplemental Table 3c under “Supplemental data” in the online issue).

In the regression model predicting TEE, sex (\(\beta=0.14, P=0.03\)), race (\(\beta=-0.17, P=0.002\)), age (\(\beta=-0.15, P=0.04\)), and FFM (\(\beta=1.0, P<0.001\)) all served as significant contributors to variation in TEE (model adjusted \(R^2=0.70, P<0.001\)), whereas the presence of +2138InsCAGACC alleles was not a significant contributor to variation in TEE (\(P=0.94\)). Mean adjusted TEE generated by these analyses are presented in Supplemental Table 3c. The +2138InsCAGACC genotype also showed no significant relations with variation in resting metabolic rate, RQ, AEE, or PAL among these children (all \(P>0.60\) for +2138InsCAGACC genotype contribution; data not shown).

FIGURE 2. A: Mean (±SE) adjusted predicted total daily energy expenditure (TEE), estimated with the use of doubly labeled water, in children and adolescents. B: Mean (±SE) adjusted daily activity energy expenditure (AEE). Regression analyses found that age, race, and body fat mass, but not the number of C17A and G241A variant alleles (\(P>0.60\)), were significant contributors to TEE and AEE. How/Hom indicates homozygous for the C17A + G241A variant alleles (n = 9); Het, heterozygous for the variant MC3R alleles (n = 37); and Wt/Wt, homozygous for wild-type alleles at both loci (n = 74).

TEE measures according to +2138InsCAGACC genotype

DISCUSSION

We previously reported that co-occurrence of homozygous C17A and G241A MC3R missense sequence variants was associated with an obesity phenotype in children and adolescents and that the combination of these 2 polymorphisms decreased MC3R expression and signal transduction (11). In the current study, we sought to examine the associations of these variants with alterations in energy balance. On the basis of mouse data suggesting that both energy intake and metabolic efficiency may...
be altered in MC3R<sup>−/−</sup> mice (1, 5), we anticipated that we might find evidence for differences in both energy intake and energy expenditure among children bearing partially inactivating MC3R sequence variants. We found that ad libitum energy intake of an afternoon buffet meal was significantly greater in those with double homozygosity for the C17A and G241A MC3R variants than for those having wild-type MC3R alleles. We also found that TEE, measured by using doubly labeled water, and REE, measured by using indirect calorimetry, did not differ significantly according to MC3R genotype.

Lee et al (16) recently reported additive effects of C17A and G241A MC3R variants for adiposity, leptin concentrations, and insulin resistance indexes among a small group of obese Chinese children. These 2 variants were also associated with higher first-phase insulin secretion, but not BMI, in Finnish adults (13). The extent to which our findings in children are applicable to adult obesity is unclear, given that such associations have not been observed in adults of European descent (6–9) or in African American adults (15). As appears to be the case for other body composition-affecting genes, it is possible that the effects of MC3R variation may be easier to detect among children, in whom there has been less time for the obesogenic environment and for genes that primarily affect adult body composition to act.

Although no prior published data relate the presence of the +2138InsCAGACC MC3R variant to phenotypic data in children or adolescents, Matsuoka et al (15) recently reported that heterozygosity (but not homozygosity) for +2138InsCAGACC was associated with a significantly greater BMI among African American adults. The results of Matsuoka et al (15) support an earlier study among French-Canadian adults that found that homozygosity for +2138InsCAGACC was associated with increased FM and percentage body fat in nonobese individuals and decreased total abdominal fat among obese individuals (10). We were unable to identify any consistent association of this variant with pediatric BMI, body FM, or disruptions in measures of energy balance, although a non-significant trend toward an increased FFM among heterozygotes was observed in the full cohort. Our results are consistent with other association studies in adults showing no association between the +2138InsCAGACC polymorphism and differences in energy expenditure (10) or glucose homeostasis (10, 15). Thus, the physiologic explanation for the observed associations of +2138InsCAGACC with adult obesity remains unclear.

Inactivation of both alleles of the MC3R gene in mice is associated with positive energy balance through several mechanisms, including alterations in energy intake, energy expenditure, and balance of substrate oxidation (1, 5, 33, 34). Two groups have suggested MC3R knockout animals may have diminished physical activity (5, 34). There are also some conditions in which MC3R knockout mice have exhibited significantly increased food intake (1, 35, 36). We found a positive relation between the number of abnormal C17A + G241A alleles and energy intake, such that Hom/Hom children consumed approximately 38% more energy during an afternoon test meal than did Wt/Wt children after adjusting for relevant variables, including body composition. Because energy intake, REE, and TEE were not assessed together or measured in all participants, the energy intake results cannot be directly compared with the results of either energy expenditure measure. These findings do, however, suggest that the association between human MC3R C17A and G241A homozygous variants and pediatric obesity is perhaps a result of altered regulation of eating behavior.

Our investigation showed no apparent differences in REE or substrate oxidation based on MC3R genotype. Rutanen et al (13), who studied adults who were the offspring of individuals with type 2 diabetes, recently reported no differences in REE, but they observed low rates of lipid oxidation, high rates of glucose oxidation, and elevated free fatty acids in a combination of subjects with heterozygous and homozygous C17A and G241A MC3R variants compared with those with wild-type MC3R alleles. Although the sample size of Hom/Hom subjects in their study (n = 5) was insufficient to analyze independently, their combined group of subjects carrying C17A and G241A MC3R variants did not exhibit significant differences in BMI, body composition, or waist circumference than did others in their cohort. Metabolic obesity, consisting of increased body fat, impaired free fatty acid oxidation, hyperinsulinemia, and hyperleptinemia, has been observed in MC3R knockout mice fed a high-fat diet (34). Similar to the rodent models, we also reported that Hom/Hom children were significantly heavier, had more body fat, had greater plasma leptin and insulin concentrations, and had greater insulin resistance than did Wt/Wt or Het children (11).

Findings from the present study are limited because measures of energy intake and energy expenditure were each assessed in convenience samples of children enrolled in different studies that varied in terms of age, sex, and race. Differences in participant characteristics across the 3 groups reflect the availability of data at the time of analysis. The observation that double homozygosity for the C17A + G241A MC3R sequence variants is particularly prevalent in African Americans is consistent with other previous reports (9, 11, 15). It is possible that the small number of white Hom/Hom participants in the current study may have introduced bias because of population stratification and that intake could be higher in African American children independent of the occurrence of MC3R sequence variants. Race was not a significant main effect in these analyses, but the sample size was insufficient to study the interaction of race and genotype or to perform a meta-analysis of the associations with food intake and energy expenditure in children of different race. Subgroup analysis of energy intake among only the African American or white participants found that the mean differences in intake were not statistically different but were comparable in magnitude to those found in the entire study sample. Finally, we cannot rule out the possibility that the positive findings in the present study were due to undetermined variables, such as linkage disequilibrium between C17A + G241A MC3R sequence variants and another nearby gene locus on chromosome 20.

One strength of the present study is that energy intake was assessed by an established model of actual food intake under controlled conditions (20) rather than through reliance on self-reports of intake (37, 38). The extent to which our results are generalizable outside of laboratory conditions remains to be determined. We did not assess whether Hom/Hom children had increased energy intake at other meals or whether they compensated for their greater consumption at the test meal by reducing intake at subsequent meals. In addition, the pattern of the children’s usual food consumption (ie, size and number of meals per day, etc) was not determined in this study. Further studies of children carrying minor MC3R alleles in considerably larger samples are required to confirm these observations.
In summary, +2138insCAGACC was not significantly associated with obesity or measures of energy balance in children. However, children homozygous for MC3R C17A and G241A showed greater body fat and, in analyses accounting for body composition, greater energy intake in the laboratory setting than did MC3R wild-type children. REE, TEE, and PALs were all found to be comparable among children regardless of MC3R genotype. Should these findings be confirmed in other studies, they support the hypothesis that an increase in energy intake, rather than a decrease in energy expenditure, may be a significant contributing factor to the obesity phenotype observed in children with partially inactivated MC3R resulting from C17A and G241A polymorphisms.

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