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Common variants near *MC4R* are associated with general and visceral adiposity in European- and African-American Youth

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Abstract

Objective—Recent genome-wide association studies found common variants near the melanocortin 4 receptor (*MC4R*) gene associated with obesity. This study aimed to assess the influence of the identified single nucleotide polymorphisms (SNPs) rs17782313 and rs17700633 on general and visceral adiposity in European- and African-American youth.

Study design—In 1890 youth (49.1% European-American, 45.6% male, mean age 16.7 years), we examined the associations of the rs17782313 and rs17700633 with anthropometry, percent body fat (%BF), visceral adipose tissue (VAT) and subcutaneous abdominal adipose tissue (SAAT). Interaction of the SNPs with ethnicity or gender was investigated and haplotype analyses conducted.

Results—Rs17782313 was significantly associated with weight ($P=0.02$) and waist circumference ($P=0.03$) in all subjects, and with body mass index ($P=0.002$) in females. In females rs17700633 was significantly associated with %BF ($P=0.001$), VAT ($P<0.001$) and SAAT ($P<0.001$). Rs17700633 was significantly associated with fasting insulin and HOMA, but the significance attenuated after adjustment for %BF. These findings were confirmed by haplotype analysis. No significant interactions of the variants with ethnicity were found for any of these phenotypes.

Conclusions—The relatively large effect of these common variants near *MC4R* on general and visceral adiposity in childhood, especially in girls, could prove helpful in elucidating the molecular mechanisms underlying the development of obesity in early life.

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Keywords

obesity; genetics; ethnicity

Introduction

Obesity in childhood and adolescence is epidemic worldwide and is associated with several comorbidities such as hypertension, dyslipidemia and Type 2 diabetes^{1, 2}. Identification of genetic determinants of such a complex disease by linkage analysis or candidate-gene based association approach has only been modestly fruitful, despite extensive efforts. The emergence of the genome-wide association study (GWAS) approach has provided more clues to the allelic architecture of complex diseases and traits³.

Recently, Loos et al⁴ carried out a large-scale meta-analysis of GWAS data available for 16876 adult samples of European descent. A cluster of single nucleotide polymorphisms (SNPs) on chromosome 18q21 (55700-56400) was shown to be associated with body mass index (BMI) as a measure of general adiposity. This region seemed to contain at least two independent association signals (rs17782313 and rs17700633). The strongest association signal (rs17782313, $P=2.9\times 10^{-6}$) was mapped 188 kb downstream of the melanocortin 4 receptor (*MC4R*). Haplotype analysis for rs17782313 and rs17700633 in EPIC-Norfolk confirmed that rs17782313 drives the association. Loos et al⁴ also found that the effect of rs17782313 on BMI in children was about twice of that observed in adults, which indicated relevance for early-onset obesity characteristics. This finding may be an important clue for the design of functional studies of these variants. Another GWAS in adults of Indian Asian and European descent found that common variants near *MC4R* were associated with waist circumference and insulin resistance (IR)⁵. However, further replication studies in populations of different ethnic origin using better measures of adiposity are necessary to establish a definite relationship between these variants and obesity-related phenotypes, especially in children.

Thus, the aim of the current study is to assess whether the previously identified SNPs near *MC4R* (rs17782313 and rs17700633) by GWAS are associated with adiposity and insulin resistance in African-American (AA) and European-American (EA) youth available from the Georgia Cardiovascular Twin study⁶, the Lifestyle, Adiposity and Cardiovascular Health in Youths (LACHY) study⁷ and the Adiposity Prevention through EXercise (APEX) study⁸. Furthermore, the role of haplotypes of the two SNPs on adiposity and insulin resistance and the interaction of these 2 SNPs with ethnicity or gender are investigated. In addition to BMI and waist circumference, more accurate indices for general and visceral adiposity were used in our study, such as percent body fat (%BF) based on dual-energy X-ray absorptiometry (DXA), visceral adipose tissue (VAT) and subcutaneous abdominal adipose tissue (SAAT) measured by magnetic resonance imaging (MRI).

Subjects and Methods

Subjects

The present study included 1890 subjects from 3 cohorts, the Georgia Cardiovascular Twin study [(n=1179 twins, with 565 monozygotic (MZ) (282 pairs and 1 singleton) and 614 dizygotic (DZ) twins (279 pairs and 56 singletons)], the LACHY study (n=527, including 38 sib-pairs) and the APEX study (n=184, including 21 sib-pairs). All twins in the Georgia Cardiovascular Twin study were recruited from public middle and high schools in the Augusta, Georgia area and the cohort⁶, consisted of roughly equal numbers of AAs and EAs boys and girls (55.1% EA, 47.5% male, mean age [SD]: 18.1[3.7] years). All twin pairs were reared together and zygosity was determined by genotyping 5 standard microsatellite markers using buccal swabs or buffy coat DNA⁹. The LACHY study consisted of approximately equal numbers of EA and AA boys and girls (52.8% EA, 44.2% male) aged 14-18 years recruited from high schools in the Augusta, Georgia area⁷. In the APEX study, subjects were AA boys and girls only (37.5% male), aged 8 to 12 years recruited from local elementary schools. Subjects eligible for the study were only those that weighted <136.1kg and were not taking any medication known to affect body composition or fat distribution⁸. The criteria for classifying subjects as AAs or EAs using self-identification of ethnicity have been described previously¹⁰. Subjects in all the 3 studies were overtly healthy, free of any acute or chronic illness on the basis of parental reports and were taking no medication that could influence the results. The Institutional Review Board at the Medical College of Georgia approved the studies. Informed consent was obtained from all subjects and by parents if subjects were <18 years of age.

Anthropometrics and body composition assessment

Height and weight were measured by standard methods using a wall-mounted stadiometer and a digital scale, respectively. BMI was calculated as weight/height² (kg/m²). Waist circumference (in cm) was measured twice at the center of the umbilicus over the T-shirt and the values were averaged. Skinfold thicknesses (i.e. triceps, subscapular, and suprailiac) were measured on the right side of the body with Lange calipers according to established protocols¹¹. Three sets of measurements for each skinfold were recorded and averaged. The inter-correlations were >99%. Measurements of skinfold thickness were available in 1888 subjects. BMI and the sum of the 3 skinfold thicknesses were used as measures of general adiposity, while waist circumference was used as a measure of central adiposity.

Biochemical assays

The blood was frozen quickly and glycolysis was immediately inhibited. Fasting glucose and insulin concentrations were measured at the NIDDK supported Clinical Nutrition Research Unit Core Laboratory at the University of Alabama. Glucose was measured in 10 µL of sera using an Ektachem DT II system (Johnson and Johnson Clinical Diagnostic, Rochester, NY). Insulin was assayed in duplicate 100-µL aliquots of serum by specific radioimmunoassay (Linco Research, Inc, St Charles, Mo). Cross-reactivity with proinsulin is <0.2%. Assay sensitivity was 3.41mU/mL. The intra- assay coefficient of variation was 3.7%. Fasting glucose and insulin were only available in a subsample of twins as twins coming on afternoon visits were not required to fast.

Based on fasting glucose and insulin we used the homeostasis model assessment (HOMA) 2 to calculate insulin resistance (HOMA2-IR) and β -cell function (HOMA2-%B) using a nonlinear computer model as specified in the HOMA2 software (<http://dtu.ox.ac.uk/homa>).

Dual-energy X-ray absorptiometry

In the LACHY study, %BF was measured using DXA (Hologic QDR-4500W, software version 6.0, Waltham, MA, USA). DXA provides reliable values for %BF⁷. Repeat measurements were performed using the QDR-4500W machine with 219 adolescents and the intraclass correlation coefficient (ICC) for %BF was found to be 0.99. For some subjects, DXA values were only available from the Hologic QDR-1000W, but not from the Hologic QDR-4500W model. For these individuals, %BF values were derived from prediction equations based on 284 youths who were assessed on both instruments, using linear regression; ethnicity, gender and QDR-1000W measurement were the predictor variables. The multiple R^2 value for %BF was 0.99¹². In the APEX study, all %BF measurements were obtained using a Hologic QDR-1000 (Waltham, MA) as previously described, the ICC for %BF was > 0.998 between two scans¹³. DXA calibration was done each day, as specified by the Hologic Company. DXA scans were not performed in the Georgia Cardiovascular Twin study.

Magnetic resonance imaging

In both the LACHY and APEX studies, VAT and SAAT was determined using MRI (1.5 T General Electric Medical Systems, Milwaukee, WI) as described previously¹⁴. Briefly, with subjects in the supine position, a series of five, 1-cm-thick, transverse images was acquired beginning at the inferior border of the fifth lumbar vertebra and proceeding toward the head. A gap is left between the slices to avoid cross-talk. To calculate volumes for VAT and SAAT, the cross-sectional area (cm²) from each slice was multiplied by the slice width (1 cm); the five individual volumes (cm³) were then summed as described previously¹⁵. VAT and SAAT were measured in the Department of Radiology on equipment dedicated to patient care. VAT and SAAT measures were obtained in those subjects who underwent testing on days when the MRI equipment was available for the research study. Eventually, VAT and SAAT measurements were available for 397 subjects, respectively. No measurements of VAT and SAAT were available in the Georgia Cardiovascular Twin study and males in the APEX study.

Genotyping

DNA was extracted from buffy coats by using the QiaAmp DNA Blood Mini Kit (Qiagen, Valencia, CA) or from buccal swabs by using QuickExtract DNA Extraction Kit (Epicentre, Madison, WI). The rs17782313 and rs17700633 SNPs were genotyped by allelic discrimination Taqman assays (Applied Biosystems, Foster City, CA). PCR were performed in a 96-well format in a total of 5 μ l reaction volume using 10ng of genomic DNA and FAM/VIC dye labeled allelic probes with the Taqman Universal Fast Master mix and subjected to 95°C for 15 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min on an ABI 9800 Fast Thermocycler (Applied Biosystems, Foster City, CA). The Taqman assay plates were transferred to an ABI 7500 Fast Real Time PCR system in which the fluorescence intensity in each well of the plate was recorded and genotypes were analyzed

using Sequence Detection Software 1.3. Genotyping quality control procedures included genotyping 10% duplicates for accuracy checking and inclusion of both positive and non-template controls in each 96-well plate. Genotyping success rates were 97% for rs17700633 and 93% for rs17782313. Genotyping accuracy for the two SNPs, as determined by concordance between duplicates, was 100%.

Statistical Analyses

Main effects of SNPs on obesity-related phenotypes were tested using structural equation modeling with the statistical software Mx¹⁶. In this approach a model is specified for both the means and the variance-covariance matrices. In this approach a model is specified for both the means and the variance-covariance matrices. We adapted the model described previously¹⁷ to include MZ twin pairs, DZ twin pairs (or sib-pairs) and unpaired twins/singletons. This approach allows for non-independent observations in twin and family data. By modeling MZ and DZ (or sib-pair) covariances separately we accounted for their different degrees of relatedness. Each of the SNPs was analyzed separately in the combined data from all 3 cohorts. Effects of age, ethnicity, gender and cohort were regressed out for all variables before using the residuals in modeling in Mx. We only considered an additive model in testing for associations between the two SNPs and the phenotypes of interest based on the findings of Loos et al.⁴. Gender and ethnicity-specific effects of the SNPs were modeled as interactions of SNPs with gender and/or ethnicity using regression analyses within a generalized estimating equations (GEE) framework, which takes the non-independency of twin and family data into account and yields unbiased *P* values¹⁸.

In the APEX study, subjects were randomized to a physical activity intervention or control group. Obesity-related phenotypes were measured three times, at the baseline, mid- and end-point of the study. The measurement at the baseline, prior to randomization, was used for the analysis.

The haplotype trend regression (HTR) approach was used to test for associations of statistically inferred haplotypes with adiposity-related phenotypes as outlined by Zaykin et al.¹⁹. We adapted the HTR approach for the analysis of related subjects, such as twins, by replacing the linear regression with the GEE procedure²⁰. The HTR tests for the contribution of individual haplotypes taking into account the uncertainty of haplotype pair estimation by PHASE 2.0 software²¹, which was conducted separately in EAs and AAs. The most frequent haplotype was used as the reference haplotype with which effects of the other haplotypes were contrasted²².

Hardy-Weinberg equilibrium (HWE) and ethnic differences in allele and genotype frequencies were tested by a χ^2 test in only one member per family (i.e., twins or sib pairs), which was chosen randomly to prevent inflated significance. Pairwise linkage disequilibrium (LD) between the two SNPs was tested by calculating D' as well as r^2 . All variables except %BF were log transformed to obtain better approximations of the normal distribution. Analyses of SNP- ethnicity or gender interaction and the haplotype-phenotype association were performed using Stata 10 software (StataCorp, College Station, TX). A *P* value of 0.05 was considered to be statistically significant.

Results

Participant characteristics

Descriptive statistics for age, height, adiposity and insulin resistance related variables in combined data of the 3 studies are presented by ethnicity and gender in Table 1. Effects of ethnicity, gender and their interaction were tested using GEE with age and cohort identifier included as covariate. The mean age of the total sample was 16.2 years, with neither ethnicity nor gender difference. Many significant gender differences were observed, although some of these were limited to one ethnic group. Similarly, many of these significant differences were limited to either males or females as indicated in Table 1.

Allele and genotype frequencies

The allele and genotype distributions in EA and AA subjects were shown in Supplementary Table 1. The two SNPs were common in both ethnic groups with minor allele frequencies (MAF) 20%. As indicated in Supplementary Table 1, there were significant differences in allele and genotype frequencies of the rs17782313 between EA and AA subjects, but not in the frequencies of the rs17700633. Furthermore, the two SNPs were not found to be in strong LD ($D'=0.134$, $r^2=0.015$ for EA; $D'=0.394$, $r^2=0.111$ for AA). Both SNPs were in HWE in both ethnic groups according to Bonferroni corrected P -values ($0.05/4=0.0125$).

Associations between rs17782313 and obesity-related phenotypes

We found that each copy of the rs17782313 C allele was significantly associated with weight (per-allele effect of 2.45kg, $P=0.02$) and waist circumference (per-allele effect of 1.6cm, $P=0.03$) (Table 2). The explained percentages of variance were 0.49% and 0.52%, respectively. Significant interaction was found between rs17782313 and gender for BMI ($P_{\text{inter}}=0.04$), with significant association limited to females (per-allele effect of 1.2kg/m², $P=0.002$) with an explained percentage of variance of 1.17% (Table 2). Stratified analyses for weight and waist circumference revealed the same pattern, but the interactions did not reach significance (data not shown). The C allele carriers showed higher sum of skinfolds thicknesses compared to the TT allele carriers, but the difference did not reach statistical significance (Table 2). No significant associations were found between rs17782313 and fasting glucose, fasting insulin, HOMA2-%B or HOMA2-IR before or after adjustment for BMI (Table 2). No significant interactions between rs17782313 and ethnicity were observed for any of these obesity-related phenotypes ($P>0.05$), and no significant interactions between rs17782313 and gender were observed for any of these phenotypes except for BMI ($P>0.05$).

Associations between rs17700633 and obesity-related phenotypes

We found significant interactions between rs17700633 and gender for VAT ($P_{\text{inter}}=0.01$) and SAAT ($P_{\text{inter}}=0.02$), and a borderline significant interaction between rs17700633 and gender for %BF ($P_{\text{inter}}=0.06$), with the significant associations limited to females (Supplementary Table 2). In females, significant associations were revealed between rs17700633 and %BF ($P=0.001$), VAT ($P<0.001$) and SAAT ($P<0.001$), with per-allele effects of 1.5%, 16.0cm³ and 132.8cm³, and explaining 2.4%, 4.5% and 4.3% of the

variance, respectively (Fig.1, Supplementary Table 2). Rs17700633 was significantly associated with fasting insulin, HOMA2-%B and HOMA2-IR (Supplementary Table 2), However, after additional adjustment for BMI these significant associations attenuated to be borderline significant. When limited to the subjects with measurements of %BF available, whether before or after adjustment for %BF, rs17700633 was significantly associated with fasting insulin ($P=0.001$ and 0.03 , respectively), HOMA2-%B ($P=0.001$ and 0.02 , respectively) and HOMA2-IR ($P=0.001$ and 0.03 , respectively). We did not find any significant interactions between rs17700633 and ethnicity for any of these obesity-related phenotypes ($P>0.05$).

Associations between haplotypes and obesity-related phenotypes

Table 3 shows the inferred haplotype frequencies of the two SNPs and results of haplotype association tests. Haplotype frequencies were significantly different between EA and AA subjects ($P<0.001$). The TG haplotype was the most frequent haplotype and was used as the reference.

We found significant interactions between haplotype variation and gender for %BF, VAT and SAAT (overall $P_{\text{inter}} = 0.04, 0.02$ and 0.02 , respectively). Separate analyses in males and females showed that in females, haplotype variation was significantly associated with %BF ($P=0.007$), VAT ($P=0.01$) and SAAT ($P=0.003$) with haplotype 2 (TA) and haplotype 4 (CA) responsible for most of the effect in %BF, VAT and SAAT (Table 3). The explained percentages of variance in %BF, VAT and SAAT by modeling haplotypes in females were 2.8%, 5.5% and 5.7% respectively. Haplotype variation was also significantly associated with %BF ($P=0.01$) in males with haplotype 3 (CG) and haplotype 4 (CA) responsible for most of the effect. Haplotype 4 showed significantly higher fasting insulin ($P=0.03$) and HOMA2-IR ($P=0.02$) compared to the reference, but the difference was no longer significant after adjustment for BMI ($P=0.31$ and 0.26 , respectively) (data not shown). No significant interaction between haplotype variation and ethnicity for any of these obesity-related phenotypes were found ($P>0.05$).

Discussion

In the current study, the associations between rs17782313 and rs17700633 near *MC4R* and obesity-related phenotypes were investigated by both single locus and haplotype analyses in 1890 EA and AA youth from the Georgia Cardiovascular Twin, the LACHY and the APEX studies. To our knowledge, this is the first study to involve AAs as well as EAs to investigate the association of common variants near *MC4R* and obesity-related phenotypes. The main findings of this study were that rs17782313 was significantly associated with weight (per-allele effect=2.45kg) and waist circumference (per-allele effect=1.6cm) in total subjects with variances explained 0.49% and 0.52%, and BMI (per-allele effect=1.2kg/m²) in females with variance explained 1.17%, and rs17700633 was significantly associated with %BF (per-allele effect=1.5%), VAT (per-allele effect=16.0cm³) and SAAT (per-allele effect=132.8cm³) only in females, explaining 2.4%, 4.5% and 4.3% of the variance, respectively. No significant interactions between either of the SNPs and ethnicity for any of the obesity-related phenotypes were found. Compared to the most common haplotype, the

haplotype with minor allele of the rs17700633 showed significant association with %BF, VAT, SAAT in females and overall haplotype variation explaining up to 5.7% of the variance.

After the identification of the fat mass and obesity-associated (*FTO*) gene by the GWAS approach as the first locus harboring common variants for which there is widely replicated evidence of association with increased adiposity at the population level²³, Loos et al⁴ reported significant associations between common variants near *MC4R* and fat mass, weight and BMI in both children and adults. Across all 77228 adults of European descent, it was found that rs17782313 was strongly associated with BMI (per-allele effect $\approx 0.22\text{kg/m}^2$, $P=2.8\times 10^{-15}$). And in 5988 children aged 7-11 yrs, each additional copy of rs17782313 C was associated with a BMI difference twice of that observed in adults. In our cohorts of children and youth with an average age of 16.2 yrs, we observed that each additional C-allele of rs17782313 showed an increased weight of 2.45kg, which is much higher than the effect observed by Loos et al⁴, and is about twice of that observed in Danish²⁴. The explained percentages of variance by rs17782313 in weight was 0.49%, which were slightly higher than that of 0.30% Loos et al⁴ found in children aged 9. We also observed rs17782313 was significantly associated with waist circumference (per-allele effect=1.6cm). This is consistent with the results reported by Chambers et al⁵ that a common variant (rs12970134) near *MC4R* (150 kb downstream) in high LD with rs17782313 ($r^2=0.811$, $D'=0.957$ in CEU HapMap) was associated with waist circumference in 35-75 yrs aged males, with per-allele effect size of 0.88cm and the per-allele effect size of 0.67cm found in Danish adults²⁴. We found significant interaction between rs17782313 and gender for BMI, and the stratified analyses showed significant association between rs17782313 and BMI only in females (per-allele effect=1.2kg/m²). Findings on the interaction between rs17782313 and gender in previous studies are inconsistent^{25, 26}. In 3885 Swedish adults, rs17782313 showed significant interaction with gender ($P_{\text{inter}}=0.02$), with the association limited to females ($P=0.003$)²⁶, which is in line with our findings although the per-allele effect size of 0.4kg/m² was substantially lower than in our study of youth. On the contrary, a study in French adults found stronger association of rs17782313 with obesity and fat mass deposition in males than in females ($P=0.003$ and 0.03, respectively)²⁵. Further investigations are needed to ultimately clarify whether the effect of rs17782313 on obesity is modified by gender.

Loos et al found that the association between rs17700633 and BMI was less significant (per-allele effect $\approx 0.15\text{kg/m}^2$, $P=4.6\times 10^{-9}$)⁴. Its effect seemed to be dependent on that of rs17782313 since the association of rs17700633 with BMI did not reach statistical significance after conditioning on rs17782313⁴. We also observed that carriers of the A-allele of rs17700633 showed slightly higher BMI compared to GG allele carriers, but the association did not reach statistical significance. No significant interaction was found between rs17700633 and gender for BMI, weight, waist circumference or sum of skinfolds.

Recent studies have reported that rs17782313 was significantly associated with increased diabetes risk²⁷, and rs12970134, which is in high LD with rs17782313, was significantly associated with insulin resistance independent of adiposity⁵. However, in our study no significant associations between rs17782313 and fasting glucose, fasting insulin, HOMA2-

%B or HOMA2-IR were revealed neither before nor after adjustment for BMI. This finding is consistent with the non-significant association between fasting glucose, insulin and HOMA regardless of correction for BMI observed in 14940 Danes²⁴ and 343 Germans²⁸. The per-allele effect of HOMA2-IR we observed was far lower than that reported by Chambers et al⁵ (0.01 v.s 5.17). These differences in results may be explained by the different age category, and/or gender and ethnic compositions of our cohorts. We observed rs17700633 (LD with rs12970134: $D'=0.394$, $r^2=0.133$, in CEU HapMap) was significantly associated with fasting insulin, HOMA2-%B and HOMA2-IR after adjustment for age, gender and ethnicity. But after additional adjustment for BMI, the significant associations attenuated to be borderline or not significant. Since rs17700633 was significantly associated with %BF in our study, we further investigated the association between rs17700633 and fasting insulin, HOMA2-%B and HOMA2-IR adjusted for %BF. Interestingly, the associations attenuated, but remained significant. Thus we cannot exclude the possibility that rs17700633 has an independent effect on insulin function. Results obtained from the haplotype analyses further supported these findings. However, this is inconsistent with the non-significant association between rs17700633 and HOMA-IR regardless of adjustment for BMI in Danish adults²⁴. Whether there is significant association between rs17700633 and insulin resistance needs further investigation in populations of various ethnicities and different ages.

A major strength of our study is the involvement of AA as well as EA youth, and the investigation of a potential interaction of the two SNPs with ethnicity. We found the genotype distribution and MAF in EAs and AAs was significantly different for rs17782313, but not for rs17700633. Per-allele effect sizes in EAs and AAs were very similar. For example, for rs17782313, the per-allele effect of BMI was very similar in EA and AA females: 1.1kg/m^2 in EAs and 1.3kg/m^2 in AAs; for rs17700633, the per-allele effect of %BF in EA and AA females was also very similar: 1.3% in EAs and 1.5% in AAs. Our findings are slightly different from those of a recent case-control study in EA and AA youth²⁹, which revealed significant association between rs1087177 (LD with rs17782313: $r^2=1$ and 0.927 in CEU and YRI HapMap, respectively) and obesity in EA youth (OR=1.14, 95% CI: 1.00-1.29, $P=0.05$), but not in AA youth (OR=1.06, 95% CI: 0.94-1.18, $P=0.16$).

Another major strength of the study is the use of more accurate measurements of general (%BF) and visceral adiposity (VAT) as well as SAAT. BMI is a convenient and low cost measure of adiposity, but it does not distinguish between the contributions of muscle and fat mass leading to misclassification of some individuals regarding adiposity status³⁰. Previous studies have also indicated that body composition is a primary determinant of health and a better predictor of mortality risk than BMI³¹. Thus, in our study, we assessed body composition by utilizing DXA and MRI for obtaining the best possible estimates of %BF, VAT and SAAT. No statistically significant associations were revealed between rs17782313 and %BF, VAT or SAAT. The lack of association between rs17782313 and %BF is not in agreement with Loos et al's study⁴ where this variant was associated with increased %BF with a per-allele effect of 0.10 Z-score units ($P=3.0\times 10^{-5}$) in 5281 children. The lack of association between rs17782313 and VAT is consistent with the findings in Germans²⁸. However, we did find that the rs17700633 A-allele was significantly associated with

increased %BF, VAT and SAAT in females, but not with anthropometric measures in spite of larger sample sizes. This discrepancy in the findings between the two studies could be partly due to differences in population characteristics, such as age, gender or ethnic composition, as well as environmental exposures. Furthermore, in line with the single SNP results, the haplotype analyses also showed significant interactions between gender and haplotype variation for %BF, VAT and SAAT. The haplotype analyses showed that in females, haplotypes with minor allele of rs17700633 were significantly associated with higher %BF, VAT and SAAT compared to the most common haplotype. And of interest is that haplotype TA carrying the minor allele of rs17700633 appeared to have a stronger effect on %BF, VAT and SAAT than the haplotype CG carrying the minor allele of rs17782313, which suggests that rs17700633 drives these associations.

Several limitations of our study need to be mentioned. In the Georgia Cardiovascular Twin study, no measurements of %BF, VAT and SAAT were available since DXA scans and MRI examinations were not performed. Meanwhile, in the twin study, fasting glucose and insulin were only available from part of the subjects since fasting was not requested for twins examined in the afternoon. Another limitation is that sexual maturation was not assessed in the twin cohort and could not be incorporated as a covariate.

The two SNPs assessed in the current study (rs17782313 and rs17700633) are located on chromosome 18 near *MC4R* (109-188 kb downstream of the coding sequence). *MC4R* is a seven transmembrane G-protein coupled receptor highly expressed in hypothalamic nuclei, which regulates energy homeostasis³². Rare functional mutations of *MC4R* are associated with hyperphagia and hyperinsulinaemia, and are the leading cause of monogenic severe childhood-onset obesity³³, *MC4R* thus represents a compelling biological candidate. Moreover, experimental studies have shown that *MC4R* has a key role in food intake and energy expenditure through functionally divergent central melanocortin neuronal pathways³⁴. Although rs17782313 and rs17700633 are located within a ~190kb recombination interval between *MC4R* and *PMAIP1* (a putative hypoxia-inducible factor, alpha-regulated proapoptotic gene encoding the protein Noxa), it is less plausible that *PMAIP1* is a candidate gene for weight regulation. Furthermore, although there is no direct functional evidence relating these variants to *MC4R* expression yet, the disproportionate BMI effect size in children compared to adults, which is consistent with the early-onset obesity characteristic of rare *MC4R* mutations, indicate that these variants resemble phenotypic patterns characteristic of rare, severe coding mutations in *MC4R*. Collectively, these findings could indicate that the two variants assessed in the current study might be markers of causal genetic variants in the regulatory region of *MC4R*, despite the relatively large distance between these SNPs and *MC4R*.

In summary, we replicated in a large sample of youth the significant association between rs17782313 and BMI reported in GWAS, but effects were limited to females. Significant associations of rs17700633 with %BF, VAT and SAAT were observed in females only. Moreover, these effects were similar for EAs and AAs. The relatively large effect of these common variants near *MC4R* with obesity-related phenotypes in childhood, especially in girls, could prove helpful in elucidating the molecular mechanisms underlying the development of obesity in early life.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

GWAS	genome-wide association study
SNPs	single nucleotide polymorphisms
MC4R	melanocortin 4 receptor
AA	African-American
EA	Europe-American
LACHY	Lifestyle, Adiposity and Cardiovascular Health in Youths
APEX	Adiposity Prevention through EXercise
BMI	Body Mass Index
%BF	percent body fat
DXA	dual-energy X-ray absorptiometry
VAT	visceral adipose tissue
MRI	magnetic resonance imaging
MZ	monozygotic
DZ	dizygotic
HOMA	homeostasis model assessment
ICC	intraclass correlation coefficient
IR	insulin resistance
HOMA2-%B	homeostasis model assessment 2 β -cell function
GEE	generalized estimating equations
HTR	haplotype trend regression
HWE	Hardy-Weinberg equilibrium
LD	linkage disequilibrium
MAF	minor allele frequencies
FTO	fat mass and obesity-associated

CI confidence interval

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Note:

- (1) VAT and SAAT are presented as geometric mean and 95%CI adjusted for age, ethnicity and cohort;
- (2) %BF is presented as arithmetic mean and 95%CI adjusted for age, ethnicity and cohort;
- (3) P values represent significance of the additive model, adjusted for age, ethnicity and cohort.

Figure 1.

Association between rs17700633 and %BF, VAT and SAAT

Table 1

General characteristics of study subjects

	N	European-American				African-American				Ethnicity <i>P</i> (<i>a/b</i>)	Gender <i>P</i> (<i>a/b</i>)
		Males		Females		Males		Females			
		N	Mean ± SD	N	Mean ± SD	N	Mean ± SD	N	Mean ± SD		
Age, years ^c	458	17.4±3.2	469	17.8±3.4	403	16.0±4.0	560	15.8±4.5	0.40	0.16	
Height(cm)	458	173.5±8.8	469	162.4±6.9	403	168.1±14.7	560	157.8±11.6	0.74	<0.001	
Weight(kg)	458	70.2±18.2	469	60.4±15.2	403	67.0±22.0	560	62.1±21.5	** <0.001	<0.001	
BMI(kg/m ²)	458	23.1±5.1	468	22.7±4.8	403	23.2±5.5	558	24.4±6.7	** <0.001	### <0.001	
Waist circumference (cm)	457	80.5±13.0	468	75.0±12.5	403	76.0±13.5	560	75.8±15.1	<0.001	### <0.001	
Suprailiac skinfold (mm)	458	14.9±9.3	469	18.6±9.5	403	14.6±11.4	560	20.8±12.3	0.95	<0.001	
Subscapular skinfold (mm)	457	13.3±7.9	469	16.6±8.3	403	14.5±9.1	559	20.4±11.3	** <0.001	<0.001	
Triceps skinfold (mm)	458	13.1±7.2	468	20.5±7.3	403	13.3±8.4	560	21.6±9.8	0.69	<0.001	
Sum of skinfolds(mm)	458	41.3±23.1	468	55.6±23.5	403	42.4±27.7	559	62.8±31.9	** <0.001	<0.001	
Body fat(%)	133	18.7±7.8	145	29.4±7.1	168	20.0±10.0	265	29.4±8.8	0.24	<0.001	
Visceral adipose tissue(cm ³)	65	95.8±56.2	73	111.9±55.6	71	67.1±49.9	188	95.5±72.1	* <0.001	<0.001	
Subcutaneous abdominal adipose tissue(cm ³)	65	621.4±551.9	73	899.1±514.9	71	612.7±667.3	187	987.9±819.0	0.53	<0.001	
Fasting glucose(mmol/L)	162	5.3±0.5	176	5.0±0.5	190	5.2±0.6	283	5.0±0.5	0.47/0.94	<0.001/<0.001	
Fasting insulin(pmol/L)	160	100.6±55.6	170	94.0±57.9	184	102.9±55.7	282	131.2±76.5	*** <0.001	### <0.001	
HOMA2-%B	157	131.4±48.0	170	137.3±49.9	184	132.7±49.8	280	170.9±62.4	*** <0.001	### <0.001	
HOMA2-IR	157	1.9±1.0	170	1.7±1.0	184	1.8±1.0	280	2.3±1.3	*** <0.001	### <0.001	

BMI=body mass index; SD=standard deviation; HOMA2-%B=homeostasis model assessment 2 β -cell function; HOMA2-IR=homeostasis model assessment 2 insulin resistance.^a: adjusted for age and cohort;^b: adjusted for age, cohort and BMI^c: Number of subjects with phenotype and genotype data

* significant only in males

** significant only in females

significant only in European-American
significant only in African-American.

Table 2

Associations between rs17782313 and obesity-related phenotypes

Variables	No. TT/CT/CC	Mean (95%CI)			χ^2 (a/b)	P (a/b)	Variance (%)
		TT	CT	CC			
Height(cm)	934/620/98	164.8(164.3-165.3)	164.7(164.1-165.3)	167.1(165.5-168.8)	1.36	0.24	-
Weight(kg)	934/620/98	61.9(61.0-62.8)	63.5(62.3-64.8)	66.8(63.5-70.2)	5.82	0.02	0.49
BMI(kg/m ²)	932/619/98	22.5(22.2-22.8)	23.2(22.8-23.6)	23.8(22.7-24.8)	5.22	-	-
males ^c	427/270/45	22.5(22.1-22.9)	23.1(22.6-23.6)	22.5(21.2-23.9)	0.05	0.82	-
females ^c	505/349/53	22.6(22.2-23.0)	23.3(22.7-23.8)	25.0(23.5-26.6)	9.44	0.002	1.17
Waist circumference (cm)	934/619/98	75.0(74.3-75.6)	76.3(75.4-77.2)	78.2(75.8-80.8)	4.73	0.03	0.52
Suprailiac skinfold(mm)	934/620/98	14.0(13.6-14.5)	14.7(14.1-15.4)	15.4(14.0-17.0)	2.79	0.10	-
Subscapular skinfold(mm)	933/620/97	14.0(13.6-14.5)	14.7(14.1-15.4)	15.5(14.0-17.0)	3.14	0.08	-
Triceps skinfold(mm)	934/620/98	15.4(15.0-15.9)	16.2(15.6-16.9)	16.7(15.1-18.4)	2.88	0.09	-
Sum of skinfolds(mm)	933/620/98	44.3(42.9-45.7)	46.9(45.0-48.9)	48.3(43.9-53.1)	3.20	0.07	-
Body fat(%)	378/275/47	23.2(22.4-24.0)	24.8(23.8-25.9)	23.0(21.3-24.9)	2.26	0.13	-
Visceral adipose tissue(cm ³)	209/162/25	72.6(66.6-79.1)	86.6(78.5-95.6)	73.8(60.5-90.1)	3.64	0.06	-
Subcutaneous abdominal adipose tissue(cm ³)	208/162/25	581.0(515.1-655.3)	706.0(612.2-814.2)	649.7(537.2-785.7)	2.82	0.09	-
Fasting glucose(mmol/L)	449/299/51	5.1(5.0-5.1)	5.1(5.0-5.1)	5.1(5.0-5.2)	0.10/0.02	0.75/0.89	-
Fasting insulin(pmol/L)	439/293/49	97.8(93.5-102.3)	97.1(90.8-103.8)	98.2(86.1-112.0)	0.01/0.57	0.94/0.45	-
HOMA2-%B	436/291/49	141.2(137.2-145.2)	139.1(133.5-144.9)	139.7(128.0-152.5)	0.02/0.59	0.88/0.44	-
HOMA2-IR	436/291/49	1.8(1.7-1.9)	1.7(1.6-1.9)	1.8(1.5-2.0)	0.01/0.41	0.95/0.52	-

BMI=body mass index, HOMA2-%B=homeostasis model assessment 2 β -cell function; HOMA2-IR=homeostasis model assessment 2 insulin resistance; df=degrees of freedom; *P*-values represent significance of the additive model (per-allele effect); Significant associations (*P* 0.05) are indicated in bold.

All variables are presented as geometric means and 95%CI adjusted for age, ethnicity, gender and cohort, except that %BF is presented as arithmetic mean and 95%CI.

%variance=100* (the difference in R-squared value of the regression model including the SNP compared to the base model).

a: adjusted for age, ethnicity, gender, cohort identifier;

b: adjusted for age, ethnicity, gender, cohort identifier and BMI

^c these variables are presented as geometric means and 95%CI adjusted for age, ethnicity and cohort.

Table 3

Haplotype analyses for rs17782313 and rs17700633 with obesity-related phenotypes

Haplotype	Frequency (%) EA/AA	male		female	
		β (SE)	P	β (SE)	P
%BF					
1(TG)	61.3/52.8	–	–	–	–
2(TA)	16.7/21.2	–3.6(2.0)	0.07	3.9(1.7)	0.02
3(CG)	9.5/16.9	–5.5(2.2)	0.01	2.3(2.0)	0.25
4(CA)	12.5/10.1	4.2(2.1)	0.05	4.7(2.0)	0.02
Overall P Value/Variance (%)		–	0.01/3.1	–	0.007/2.8
VAT(cm³)					
1(TG)	61.3/52.8	–	–	–	–
2(TA)	16.7/21.2	–0.3(0.2)	0.16	0.4(0.2)	0.03
3(CG)	9.5/16.9	–0.1(0.2)	0.53	0.2(0.2)	0.49
4(CA)	12.5/10.1	0.2(0.2)	0.22	0.5(0.2)	0.04
Overall P Value/Variance (%)		–	0.28	–	0.01/5.5
SAAT(cm³)					
1(TG)	61.3/52.8	–	–	–	–
2(TA)	16.7/21.2	–0.1(0.2)	0.58	0.6(0.2)	0.001
3(CG)	9.5/16.9	–0.1(0.3)	0.69	0.5(0.2)	0.03
4(CA)	12.5/10.1	0.1(0.3)	0.70	0.4(0.2)	0.04
Overall P Value/Variance (%)		–	0.91	–	0.003/5.7

%BF=% body fat; VAT=visceral adipose tissue, SAAT= subcutaneous abdominal adipose tissue, EA: European-American, AA: African-American.

P values: adjusted for age, ethnicity and cohort.