

MC3R gene polymorphisms are associated with early childhood adiposity gain and infant appetite in an Asian population*

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Summary

Background: Polymorphic variants within human melanocortin-3 receptor gene (*MC3R*) gene have been associated with obesity. However, its influence on infancy and early childhood adiposity has not been reported before.

Objectives: We assessed associations between genotype at polymorphic sites within *MC3R* with early childhood adiposity and interaction with early childhood appetitive traits.

Methods: We studied 1090 singletons in an Asian mother-offspring cohort genotyped for *MC3R* and in a subgroup ($n = 422$) who had completed Child Eating Behaviour Questionnaires (CEBQ) at 12 months. Children were followed from birth to 48 months, and up to 10 measurements of body mass index and five measures of triceps and subscapular skin-folds were obtained.

Results: Independent of potential confounders, each additional *MC3R* minor allele copy was associated with greater body mass index standard deviation score [B{95% confidence interval}: 0.004 units/month {0.001,0.007}; $p = 0.007$], triceps [0.009 mm/month {0.001,0.02}; $p = 0.021$] and subscapular skin-fold [0.008 mm/month {0.002,0.01}; $p = 0.011$] gain velocity in the first 48 months. Each additional *MC3R* minor allele copy was also associated with increased odds of overweight [odds ratio {95% confidence interval}: 1.48{1.17–1.88}] and obesity [1.58{1.10–2.28}] in the first 48 months. Every additional copy of *MC3R* minor allele was positively associated with 'slowness-in-eating' appetitive trait [0.24 {0.06,0.39}, $p = 0.006$]; however, the relationship between 'slowness-in-eating' with adiposity gain was not statistically significant.

Conclusions: Our findings support the role of *MC3R* genetic variants in adiposity gain during early childhood.

Keywords: Childhood adiposity, *MC3R*, single nucleotide polymorphism.

Introduction

Overweight and obesity are commonly associated with increased risk of chronic diseases including type 2 diabetes and cardiovascular disease and present a massive public health challenge as they rapidly become a

worldwide epidemic (1,2). Non-syndromic or common obesity is often viewed as a complex, multifactorial condition where exposure to an 'obesogenic' environment, coupled with an underlying genetic susceptibility to excessive weight gain, causes an obese phenotype (3–5). Linkage studies in humans and mice (6,7) have reported

that polymorphisms within melanocortin-3-receptor (*MC3R*) locus are associated with susceptibility for obesity. Mouse models with genetic alterations that disrupt *MC3R* exhibited hypophagia, higher energy efficiency, hyperleptinemia and reduced linear growth accompanied by higher percentage of body fat without increased body weight compared with wild-type mice (8,9).

In humans, two case-control studies have independently demonstrated two missense *MC3R* variants, Thr6Lys (rs3746619) and Val81Ile (rs3827103), which are in near complete linkage disequilibrium (LD), were significantly associated with increased adiposity in childhood and exhibited reduced *in vitro* activity compared with wild-type *MC3R* (10,11). We have previously described that Singaporean obese children with Thr6Lys/Val81Ile variants exhibited significantly higher leptin levels, percentage body fat and insulin sensitivity (11). Thus, there is evidence for the role of *MC3R* polymorphism in variation of human adiposity, and its effects on childhood adiposity appear to be mediated by altered energy intake and possibly altered sensitivity to satiety (12,13). However, the age at which the variants start to alter phenotype is unknown. In this study, we assessed the association between *MC3R* coding variants rs3746619 and rs3827103 with adiposity and appetitive traits during early childhood in an Asian mother-offspring prospective cohort.

Materials and Methods

Study population

This study is embedded in Growing Up in Singapore Towards Healthy Outcomes (GUSTO) cohort study, designed to test specific hypotheses related to developmental pathways to cardio-metabolic disorders in Chinese, Malay and Indian participants in Singapore. The study has previously been described in detail elsewhere (14). Pregnant women in their first trimester were recruited from two major public hospitals with obstetric services, KK Women's and Children's Hospital and National University Hospital, between June 2009 and September 2010. Of 3751 screened, 2034 met eligibility criteria, and 1247 women were recruited, of which there were 1176 deliveries, and 1090 with complete umbilical cord genotype data available and 422 with completed Child Eating Behaviour Questionnaire (CEBQ) (Figure S1). The women gave informed consent to participate in the study, which was approved by both National Healthcare Group Domain Specific Review Board and SingHealth Centralized Institutional Review Board.

Antenatal data

Socio-demographic and health data (e.g. age, education and gestational diabetes) were captured

during recruitment visits and at 26–28 weeks of gestation. Maternal ethnicity was self-reported, and homogenous paternal ethnic background was an eligibility criteria; ethnicity was later confirmed by genotype analysis. Gestational age (GA) was determined by dating ultrasonography in first trimester and reported in completed weeks.

Infant anthropometry measurements

Measurements of weight and length, adopted from standardized protocols (15), were obtained at birth and at 3, 6, 9, 12, 15, 18, 24, 36 and 48 months of age. Weight was measured to the nearest gramme using calibrated scales [SECA 334 Weighing Scale {birth to 18 months}; SECA 803 Weighing Scale {24 to 48 months}, SECA Corp, Hamburg, Germany]. Recumbent length (from birth to 18 months) was measured from the top of the head to soles of feet using an infant mat (SECA 210 Mobile Measuring Mat) to the nearest 0.1 cm. Standing height (between 18 and 48 months) was measured using a stadiometer (SECA stadiometer 213) from the top of participant's head to his or her heels. For reliability, all measurements were taken in duplicates and averaged. Two skin-folds (triceps and subscapular) were measured using standardized protocols (15) at birth and at 18, 24, 36 and 48 months in triplicates using Holtain skin-fold callipers (Holtain Ltd, Crymych, UK) on the right side of the body, recorded to the nearest 0.2 mm. Anthropometric training and standardization sessions were conducted quarterly (once every 3 months), and observers were trained to obtain anthropometric measurements that, on average, were closest to the values measured by a master anthropometrist. Assessment of reliability was estimated by inter-observer technical error of measurement and coefficient of variation (16) (Table S1).

Genotyping of melanocortin-3-receptor variants

Genotyping was performed on DNA extracted from frozen umbilical cords using Illumina omniexpress + exome array platform, which type the most frequent single nucleotide polymorphisms (SNPs) genome-wide from all three HapMap phases (17), capturing the greatest amount of common SNP variation. DNA hybridization to arrays and scanning was performed by Expression Analysis Inc. Data were processed in GenomeStudio Genotyping Module™. Genotyping calls were made by the GenCall software, which incorporates a clustering (GenTrain) and calling algorithm (Bayesian model). GenCall

score of each SNP probe and call rate of each sample are generated. Genotypes with GenCall scores less than 0.15 were considered missing. There were no poorly performing samples as defined by low sample call rates or GenCall Scores.

Only nine variants within *MC3R* were genotyped in the array, and the frequencies and positions of these SNPs are described in Table S2. Two variants (rs3746619 and rs3827103) were polymorphic in our study population, representing missense polymorphisms 17C>A (Thr6Lys) and 241G>A (Val81Ile), respectively. LD, in the form of r^2 , was estimated using Haploview (18). The two variants exhibited strong LD in each ethnic group ($r^2 > 0.9$) (Figure S2), indicating no big difference across ethnicities. Thus, we discuss results for rs3746619 as the representative variant for *MC3R*, as it exhibited the highest minor allele frequency (Table S2).

Infant feeding and appetitive traits

Mothers were asked about infant milk-feeding using questionnaires, based on 24-h recall, at house visits when infants were 3, 6, 9 and 12 months of age. In accordance with World Health Organization guidelines (19), feeding practices were classified into exclusive, predominant, partial breastfeeding and formula-feeding. In our data collection, both direct breastfeeding and expressed breastmilk intake were classified as breastfeeding.

Appetitive traits were measured using CEBQ (20), which is a self-report completed by mothers. This was sent to all mothers by postal service prior to 12-month visit. Questionnaires distributed were in English unless a preference for another language (Mandarin, Malay or Tamil) was expressed, in which translated versions were distributed. CEBQ relates to a period when the child was predominantly fed solid food, with each questionnaire item answered using a 5-point Likert frequency scale (1 = never, 2 = rarely, 3 = sometimes, 4 = often and 5 = always). Only 422 children had questionnaires that were completed and returned by mothers.

Principal component analysis with Varimax normalized rotation was used to reduce the 35-item CEBQ to seven subscales, which were the same as the original CEBQ (20). Questions with reverse scales were first reverse-scored, and a factor loading cut-off of 0.5 was applied. Four subscales measured food-approach (food responsiveness, enjoyment of food, emotional over-eating and desire to drink), whilst three measured food-avoidant behaviours (emotional under-eating, satiety responsiveness and slowness in eating) (21). Internal reliability for each derived

subscale was calculated using Cronbach's alpha reliability coefficients, with coefficients ranging from 0.6 to 0.9, indicating a moderate-to-good internal reliability of the subscales in our study cohort (Table S3).

Statistical analyses

Descriptive statistics were reported as means and standard deviations for continuous variables and percentages for categorical variables. Infant body mass index (BMI) was calculated as infant weight/(infant height)². Age-specific and gender-specific standard deviation scores (SDS) were calculated for BMI, referencing the local Singapore population (22). Overweight and obesity status was defined as having a BMI above 85th (i.e. BMI SDS > 1.036) and 95th percentile (i.e. BMI SDS > 1.645), respectively (22). We examined the longitudinal effect of *MC3R* on BMI SDS, triceps and subscapular skin-folds trajectory using linear mixed effects models, which accounts for correlation between repeated measures on the same individual and allows for incomplete outcome data, assuming that they are missing at random (23). Maximum likelihood was the method of estimation, and an unstructured working covariance matrix for random effects parameters was chosen. The Akaike information criterion statistic facilitated model selection, and final models included linear, quadratic and cubic terms for children's ages and age-allele interaction to estimate change in BMI SDS, triceps and subscapular skin-folds over time associated with each additional copy of *MC3R* minor allele. Besides the fixed effect of age, we allowed for a random intercept and linear slope for age. There was no evidence of significant differences in maternal health and socio-demographic factors across offspring *MC3R* genotype (Table S4); hence, only breastfeeding duration, ethnicity and birthweight-for-GA were included as covariates.

Generalized estimating equations (GEE) were used to estimate the association between *MC3R* and odds of overweight or obesity in the first 48 months longitudinally, adjusting for ethnicity, breastfeeding duration and birthweight-for-GA. Similar with linear mixed effects models, GEE models are often used for repeated measures data as it account for within-subject correlation between observations across time and allows for missing outcome data. In all models, potential effect modifications by ethnicity were investigated by adding the interaction term of *MC3R* with ethnicity to the fully adjusted model. We also analysed associations between *MC3R* with the seven CEBQ subscales at 12 months using multivariable linear regression with Bonferroni-corrected multiple

testing. In all analyses, an additive genetic model was used, where genotypes were coded as having 0, 1 or 2 copies of the minor allele. All analyses were performed using Stata version 13.0 (Statacorp College Station, TX, USA).

Results

Offspring clinical characteristics are shown in Table 1. No significant differences in birthweight, gender, skin-folds at birth and *MC3R* genotype were observed across the three ethnic groups. We noted significant differences in GA ($p=0.038$), breastfeeding duration ($p<0.001$) and offspring BMI SDS at birth ($p=0.001$) across the three ethnic groups.

Figures 1A–C illustrates the modelled BMI SDS, triceps and subscapular skin-folds trajectory, respectively, in the first 48 months of life for *MC3R*. We observed an increase in BMI SDS, triceps and subscapular skin-folds velocity across age for each additional copy of *MC3R* minor allele ($p=0.007$, 0.021 and 0.011, respectively), with an estimated rate of increase of 0.004 units/month (95% CI: 0.001, 0.007) for BMI (Fig. 1A), 0.009 mm/month (95% CI: 0.001, 0.02) for triceps (Fig. 1B) and 0.008 mm/month (95% CI: 0.002, 0.01) for subscapular skin-folds, respectively (Fig. 1C), independent of potential confounders (i.e. breastfeeding duration, ethnicity and birthweight-for-GA). These differences in adiposity across *MC3R* genotype began to manifest significantly from 24 months of age (BMI: [B{95%

CI]: 0.09{0.01,0.17}; $p=0.025$], triceps: [0.18 {0.05,0.32}; $p=0.007$] and subscapular skin-folds: [0.19{0.07,0.32}; $p=0.002$]). In addition, we noted no significant interactions between *MC3R* and ethnicity, indicating that there were no ethnic-specific associations in the relationship between *MC3R* and adiposity.

Additionally, we observed that every additional copy of *MC3R* minor allele contributed to an increase in proportion of overweight (Figure S3) and obesity (Figure S4) at 12, 24, 36 and 48 months of age. The longitudinal GEE model illustrated that *MC3R* increased the risk of being overweight ($p=0.001$) or obese ($p=0.014$) in the first 48 months, with each additional copy of *MC3R* minor allele increasing odds of overweight by 1.48 times (95%CI: 1.17–1.88) and obesity by 1.58 times (95%CI: 1.10–2.28) after adjustment for potential confounders (Table 2). Similarly, we noted no significant interactions between *MC3R* and ethnicity, indicating no ethnic-specific associations in the relationship between *MC3R* with overweight or obesity in children.

We additionally analysed association of *MC3R* with each CEBQ subscale for those with completed CEBQ questionnaires ($n=422$). This subgroup tended to be Chinese with at least 12 years of education, longer breastfeeding duration, lower BMI, longer gestational duration and higher birthweight compared with those who did not complete the questionnaire (Table S5); thus, these variables were included as potential confounders in subsequent analyses. Independent of these potential confounders, we

Table 1 Clinical characteristics of offspring

Offspring	Chinese ($n=617$)	Malay ($n=276$)	Indian ($n=197$)	Total ($n=1090$)	p value
Gestational age (weeks)	38.4 ± 1.4	38.1 ± 1.3	38.2 ± 1.6	38.3 ± 1.4	0.038
Gender					0.917
Male	324 (52.5)	149 (54.0)	105 (53.3)	578 (53.0)	
Female	293 (47.5)	127 (46.0)	92 (46.7)	512 (47.0)	
Birthweight (kg)	3.1 ± 0.4	3.1 ± 0.4	3.0 ± 0.5	3.1 ± 0.4	0.081
Birth adiposity measures					
Body mass index standard deviation score	−0.28 ± 1.37	−0.03 ± 1.32	−0.49 ± 1.38	−0.25 ± 1.37	0.001
Triceps skin-folds (mm)	5.4 ± 1.2	5.5 ± 1.3	5.2 ± 1.2	5.4 ± 1.2	0.052
Subscapular skin-folds (mm)	5.0 ± 1.2	5.0 ± 1.2	4.8 ± 1.1	4.9 ± 1.2	0.141
Any breastfeeding duration					<0.001
<6 months	254 (53.8)	153 (77.3)	86 (60.6)	493 (60.7)	
≥6 months	218 (46.2)	45 (22.7)	56 (39.4)	319 (39.1)	
<i>MC3R</i> rs3746619					0.069
CC	385 (62.4)	150 (54.3)	104 (52.8)	639 (58.6)	
AC	199 (32.3)	108 (34.1)	78 (39.6)	385 (35.3)	
AA	33 (5.3)	18 (6.5)	15 (7.6)	66 (6.1)	

Numbers represent mean ± SD or n (%). $^{\#}P$ value for continuous variables by one-way analysis of variance; for categorical variables by chi-square analysis

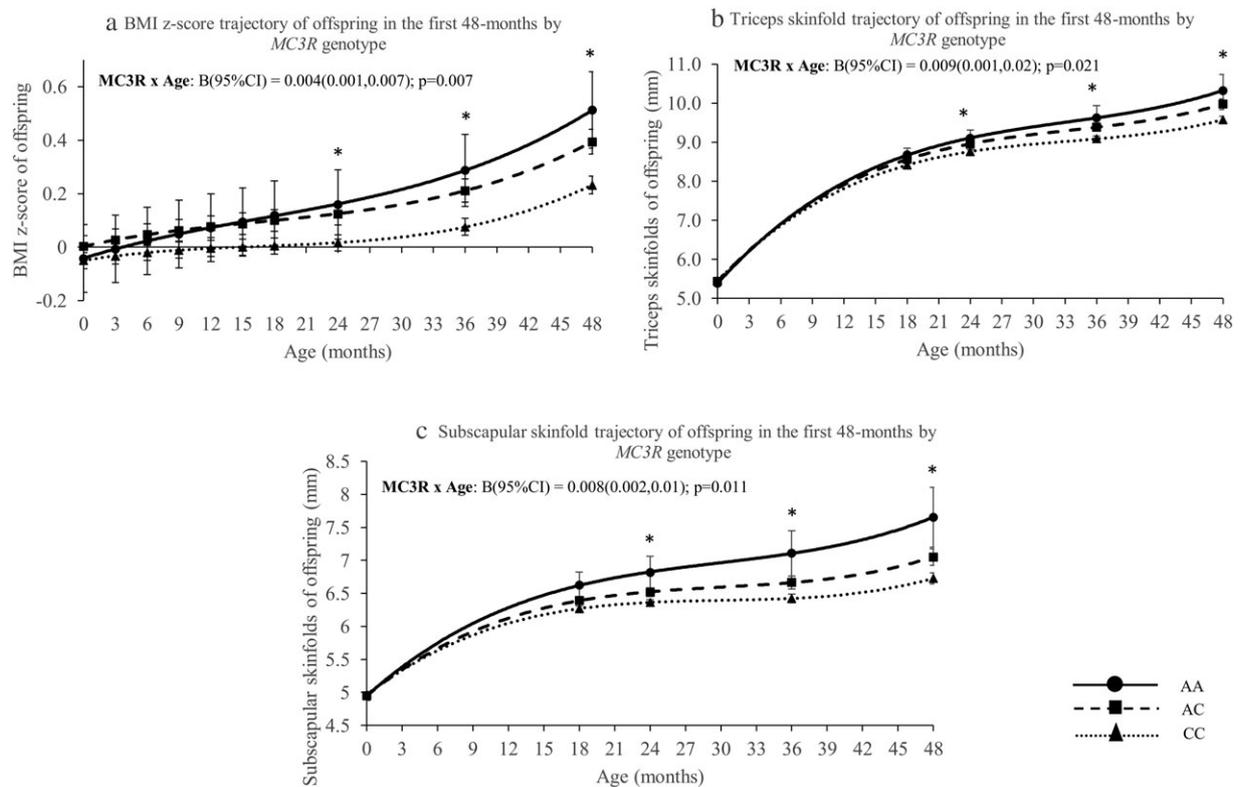


Figure 1 (A) Body mass index standard deviation score, (B) triceps skin-folds and (C) subscapular skin-folds trajectory in the first 48 months by offspring *MC3R* genotype. Adjusted for ethnicity, breastfeeding duration and birthweight-for-gestational age. Dotted line = CC genotype; Dashed line = AC genotype; Solid line = AA genotype. * $p < 0.05$ for every additional copy of minor allele; *MC3R*, melanocortin-3-receptor.

Table 2 Estimated odds of child overweight and obesity in the first 48 months by longitudinal logistic regression using GEE model, according to offspring *MC3R* minor allele and other characteristics

	Odds ratio	95% Confidence interval		P value
		Lower bound	Upper bound	
Overweight				
rs3746619 (per A allele)	1.48	1.17	1.88	0.001
Birthweight-for-GA (per unit z-score increase)	1.62	1.39	1.88	<0.001
Malay (vs. Chinese)	2.99	1.60	5.57	0.001
Indian (vs. Chinese)	2.69	1.31	5.53	0.007
Breastfeeding duration (per month increase)	0.98	0.95	1.01	0.126
Age (per year increase)	1.03	0.94	1.13	0.531
Obesity				
rs3746619 (per A allele)	1.58	1.10	2.28	0.014
Birthweight-for-GA (per unit z-score increase)	1.68	1.36	2.08	<0.001
Malay (vs. Chinese)	2.85	1.65	4.93	<0.001
Indian (vs. Chinese)	2.63	1.48	4.67	0.001
Breastfeeding duration (per month increase)	0.96	0.92	1.01	0.106
Age (per year increase)	1.00	0.99	1.01	0.808

Odds ratios represent the odds of being overweight or obese in the first 48 months. GA, gestational age; GEE, generalized estimating equations; *MC3R*, melanocortin-3-receptor.

observed an association between *MC3R* with the 'slowness-in-eating' subscale only [B{95% CI}: 0.24 {0.06,0.39}; $p=0.006$] (Table S6). However, within the sample size studied, 'slowness-in-eating' was not associated with child's gain in adiposity in the first 48 months [-0.0001 SDS units/month { $-0.003, 0.003$ }; $p=0.966$] (data not shown).

Discussion

In this study, we have identified novel associations between *MC3R* with early childhood adiposity gain in the first 48 months of life, with the differences in adiposity across *MC3R* genotype manifesting as early as 24 months of age. To our knowledge, this is the first study to assess the relationship between *MC3R* and adiposity at such an early age. Additionally, we demonstrated that every additional *MC3R* minor allele was associated with increasing odds of child overweight and obesity in the first 48 months. Earlier reports (10,11,24) have documented associations of the same *MC3R* polymorphism with adiposity in older, obese children, whilst other studies (24–26) identified similar relationships in adult populations. We have thus extended these findings to show that *MC3R* associated with adiposity in early childhood, further supporting the role of *MC3R* minor allele variants in determining early childhood adiposity.

Our findings are consistent with other studies from different populations that have independently described associations between *MC3R* variants with childhood adiposity. A recent GWAS study conducted on Hispanic children had reported nominal association of *MC3R* variants with childhood obesity (27). Feng *et al.* (10) showed that children who were homozygous for Thr6Lys and Val81Ile variants were significantly heavier, had more body fat, greater plasma leptin and insulin concentrations and greater insulin resistance than children who were wild-type or heterozygous for these variants. These homozygous variants were also only observed amongst children who were at risk of overweight (BMI \geq 85th percentile), or obesity (BMI \geq 95th percentile). We also described similar observations in a population of obese Singaporean children, whereby obese children with Thr6Lys and Val81Ile variants exhibited significantly higher leptin levels and percentage body fat, highlighting that *MC3R* may be a predisposing factor for excessive body weight gain in children (11). However, other studies have reported null findings between *MC3R* risk variants with childhood obesity. A recent study (12) found insufficient evidence of significant association between childhood obesity and common *MC3R* variants, including the rs3827103

variant. Another study on a group of Polish children (28) showed that the common *MC3R* rs3827103 polymorphism was widely distributed amongst obese and control cohorts, suggesting that its predisposing effect to obesity may be rather unlikely. Potential causes for the discrepancy between studies could be epistatic interactions between *MC3R* alleles and other loci, which may be differential between populations. Studies that have shown an effect of *MC3R* have been conducted in those of African (10), Asian (11) or Hispanic (27) populations, whilst studies which failed to show a significant effect have been mostly conducted in European populations (28,29). Data from HapMap have identified that *MC3R* minor allele frequencies are more frequent in Hispanics, Asians and Africans (HapMap Data Rel 28 Phase II+III, dbSNP b126). Thus, the contribution of these *MC3R* variants in predisposing to increased adiposity may vary significantly from population to population, and the alleles may be differentially penetrant in different populations or cultures with different food customs.

Interestingly, we also reported positive associations between *MC3R* with 'slowness-in-eating' at 12 months, consistent with findings from a Chilean cohort study, where obese boys carrying risk *MC3R* variants exhibited higher scores for 'slowness-in-eating' compared with those without (12), indicating that children with risk *MC3R* variants were perceived by their mothers to be eating more slowly compared with those without the risk allele. This is in contrast with current literature that have reported children with risk *MC3R* variants having higher energy intake (13) and children with food-approach behaviours exhibiting greater weight gain (30). Our findings, however, appear to be consistent with phenotypes observed in *MC3R* knockout mice, which exhibited hypophagia compared with wild-type littermates and were unusually susceptible to high fat diet-induced obesity, partly explained by physical inactivity (8,9). These mouse studies indicated that the mechanism in which *MC3R* variation leads to increased body fat was not increased food intake, but increased feed efficiency. Taken together, we postulate that children with risk *MC3R* variants may be eating more but taking a longer time to complete meals, which mothers ultimately perceive as 'slowness-in-eating'. Unfortunately, our study lacks data on child energy intake; thus, we were unable to test this hypothesis.

Strengths of our study include the prospective design with high follow-up rate in an Asian population. To date, there are no published studies relating *MC3R* genetic variants with adiposity in the first

48 months of life; thus, our study provides useful and informative data on this relationship. There are, however, limitations to consider. Firstly, GUSTO was not designed to detect associations between gene variants and adiposity, and thus may not be sufficiently powered to detect the association. However, analyses between *MC3R* with various measures of adiposity were conducted using longitudinal models, making use of repeated measurements for each individual over a period of time, which would subsequently result in increased power. Our study genotyped only nine of the most common *MC3R* variants, but many more variants exist and not all were genotyped. Secondly, although all analyses were adjusted for potential confounders of adiposity, we could not rule out possible confounding by population stratification at higher resolution than ethnicity. Thirdly, this study used anthropometry as indicators of adiposity, but lacks more detailed measures of body composition, such as dual x-ray absorptiometry or air displacement plethysmography, thus unable to further distinguish if these *MC3R* genetic variants were associated with fat mass or fat-free mass. Lastly, CEBQ was only a self-report measure by mothers on child's appetite, which may not be a direct reflection of the child's energy intake. Furthermore, the relatively low response rate of CEBQ completers (those with completed CEBQ questionnaire), which represented only 40% of the sample, may lack statistical power to answer the hypothesis of relation between 'slowness-in-eating' and adiposity. The results thus need to be taken as exploratory, with a need for further replication and research.

In summary, this study has provided evidence of significant association between *MC3R* variants with early childhood adiposity in an Asian population. Our study provided clues for the relationship of *MC3R* on early childhood adiposity and highlighted the plausible role of *MC3R* susceptibility alleles on weight regulation pathways at an early age. Follow-up studies with larger numbers would be necessary to examine if these variants would continue to influence adiposity at later ages with more detailed measures of body composition and to address the role of *MC3R* in human weight regulation and pathogenesis of obesity.

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Conflict of Interest statement

IMA, MTT, ALT, JDH, PLQ, CFFM, SES, SMS, KK, NL, FY, and YSL have nothing to declare. PDG, KMG and YSC have received reimbursement for speaking at conferences sponsored by companies selling nutritional products. They are part of an academic consortium that has received research funding from Abbot Nutrition, Nestec and Danone.

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Clinical Trial Registration

This study is registered under the Clinical Trials identifier NCT01174875; <http://www.clinicaltrials.gov/ct2/show/NCT01174875?term=GUSTO&rank=2>

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Inter-observer reliability of anthropometric measurements

Table S2. Allele frequency and Hardy-Weinberg test of the 9 SNPs genotyped at the MC3R locus in the GUSTO population

Table S3. Factor loadings for all items of the Children Eating Behaviour Questionnaire (CEBQ) and Cronbach-alpha scores for each subscale

Table S4. Clinical characteristics of mothers by offspring MC3R genotype

Table S5. Characteristics of participants who completed the CEBQ questionnaire compared to those who did not complete the CEBQ questionnaire

Table S6. Association between MC3R with childhood appetitive traits at 12-months of age.

Figure S1. Flowchart for GUSTO study recruitment and eventual study sample

Figure S2. Linkage disequilibrium (r^2) between the two MC3R variants

Figure S3. Percentage of offspring who are overweight in the first 48-months by offspring MC3R rs3746619 genotype. P-values indicate test of trend for proportion of overweight with every additional copy of the minor allele. White bars = CC genotype; Grey bars = AC genotype; Black bars = AA genotype.

Figure S4. Percentage of offspring who are obese in the first 48-months by offspring MC3R rs3746619 genotype. P-values indicate test of trend for proportion of obese with every additional copy of the minor allele. White bars = CC genotype; Grey bars = AC genotype; Black bars = AA genotype.