Journal of Human Genetics (2014) 59, 307–313

www.nature.com/jhg

ORIGINAL ARTICLE

Association study of common polymorphisms in *MSRA*, *TFAP2B*, *MC4R*, *NRXN3*, *PPARGC1A*, *TMEM18*, *SEC16B*, *HOXB5* and *OLFM4* genes with obesity-related traits among Portuguese children

David Albuquerque[1], Clévio Nóbrega[2], Raquel Rodríguez-López[3] and Licínio Manco[1]

At least 52 genetic *loci* were associated with obesity-related traits. However, little is known about the genetic basis of obesity among children. This study aims to test whether 10 polymorphisms in obesity-related genes methionine sulfoxide reductase A (*MSRA*), transcription factor AP-2 beta (*TFAP2B*), melanocortin 4 receptor (*MC4R*), neurexin 3 (*NRXN3*), peroxisome proliferator-activated receptor gamma coactivator 1 alpha (*PPARGC1A*), transmembrane protein 18 (*TMEM18*), homolog of *S. cerevisiae* Sec16 (*SEC16B*), homeobox B5 (*HOXB5*) and olfactomedin 4 (*OLFM4*) are associated with the risk of obesity in Portuguese children. A total of 730 children aging from 6 to 12 years old, recruited randomly from public schools in Portugal, were analysed. Anthropometric measurements were obtained and children were classified into three phenotypic groups, normal weight ($n=256$), overweight ($n=320$) and obese ($n=154$), according to the International Obesity Task Force cutoffs. Polymorphisms were genotyped by allelic discrimination TaqMan assays. The *MC4R* rs12970134 polymorphism was nominally associated with body mass index (BMI) ($P=0.035$), BMI *Z*-score ($P=0.043$) and waist circumference ($P=0.020$), and borderline associated with weight ($P=0.053$). Near nominal associations were also found for the *PPARGC1A* rs8192678 polymorphism with weight ($P=0.061$), and for the *MSRA* rs545854 polymorphism with BMI ($P=0.055$) and BMI *Z*-score ($P=0.056$). Furthermore, logistic regression showed that *MC4R* rs12970134 and *TFAP2B* rs987237 were nominally, respectively, associated ($P=0.029$) and borderline associated ($P=0.056$) with the obese phenotype. This study highlighted the possible association of *MC4R*, *PPARGC1A*, *MSRA* and *TFAP2B* polymorphisms with several obesity-related traits in a sample of Portuguese children. The two significant associated *TFAP2B* rs987237 and *MC4R* rs12970134 polymorphisms showed an opposite direction of effect to that in the original reports.
Journal of Human Genetics (2014) **59,** 307–313; doi:10.1038/jhg.2014.23; published online 27 March 2014

Keywords: BMI; childhood obesity; polymorphisms; Portuguese children; weight

INTRODUCTION

The obesity phenotype has been increasing in the last decades and the causes for this complex disorder are thought to be related with an imbalance between energy intake and energy expenditure due to changes in lifestyles including exposure to an obesogenic environment.[1] Furthermore, it is estimated that the heritable predisposition to obesity may range from 40 to 70%.[2–4]

In <7 years, genome-wide association studies (GWASs) have successfully identified >50 genetic *loci*, which were unequivocally associated with obesity-related traits.[5] The first *locus* associated with obesity was the fat mass and obesity associated (*FTO*) gene by Frayling *et al.*,[6] which is the most replicated gene across the world, both in adult and in children samples,[7–9] including the Portuguese population.[10] Subsequently, several other studies emerged associating single-nucleotide polymorphisms (SNPs) in several genes across the genome, most of them in adults,[11,12] and a few in children.[13] Nevertheless, candidate and replication studies of obesity *loci* among different populations emerge as an important step to identify and clarify which variants are indeed associated with obesity. The frequency of obesity-susceptibility alleles varies between populations, and these allele distributions could be a consequence of population-specific obesogenic environments associated with specific demography and cultural histories. Replication studies are also relevant to determine which polymorphisms previously associated with obesity in adults are also linked in children, and, in a final instance, to better understand the complexity of obesity susceptibility.

In this study, a sample of Portuguese children was tested for the association of obesity and obesity-related quantitative traits with ten

[1]Department of Life Sciences, Research Centre for Anthropology and Health (CIAS), University of Coimbra, Coimbra, Portugal; [2]Center for Neurosciences and Cell Biology, University of Coimbra, Coimbra, Portugal and [3]Servicio de Análisis Clínicos, Consorcio Hospital General de Valencia, Valencia, Spain
Correspondence: Dr L Manco, Department of Life Sciences, Research Centre for Anthropology and Health, University of Coimbra, 3000 Coimbra, Portugal.
E-mail: lmanco@antrop.uc.pt
Received 12 November 2013; revised 6 February 2014; accepted 3 March 2014; published online 27 March 2014

polymorphisms in nine candidate genes including, methionine sulfoxide reductase A (*MSRA*), transcription factor AP-2 beta (*TFAP2B*), melanocortin 4 receptor (*MC4R*), neurexin 3 (*NRXN3*), peroxisome proliferator-activated receptor gamma coactivator 1 alpha (*PPARGC1A*), transmembrane protein 18 (*TMEM18*), homolog of *S. cerevisiae* Sec16 (*SEC16B*), homeobox B5 (*HOXB5*) and olfactomedin 4 (*OLFM4*).

MATERIALS AND METHODS

Study subjects

The original sample consisted of 1433 Portuguese children of European descent, aging between 6 and 12 years old, randomly selected from several public schools in the central region of Portugal.[14] From this original sample, three body mass index (BMI) groups, using age- and sex-specific BMI cutoffs provided by the International Obesity Task Force,[15] were attained in a total of 730 children, including: (1) 154 obese subjects (resulting from the BMI in adult's cutpoints $\geqslant 30\,kg\,m^{-2}$); (2) 320 overweight subjects (resulting from the BMI in adult's cutpoints between 25 and $29\,kg\,m^{-2}$); and (3) 256 lean controls randomly selected from the initial group of 959 children with BMI $<25\,kg\,m^{-2}$.

The study protocol was approved by *Direção-Geral de Inovação e de Desenvolvimento Curricular*, the ethical Committee of the Portuguese Ministry of Education, and was conducted in accordance with the institutional and ethical guidelines of the University of Coimbra. Written informed consent was previously obtained from the children's parents.

Anthropometric measurements

All children underwent anthropometric measurements of height, weight, waist and hip circumference using a standardized protocol. Body weight (kg) and height (cm) were taken with participants dressed in lightweight clothing without shoes to determine the BMI. Waist circumference (WC) (cm) was measured midway between the lowest rib and the iliac crest, to the nearest 0.1 cm after inhalation and exhalation. Hip circumference (cm) was measured at the point over the buttocks yielding the maximum circumference. The BMI was calculated by dividing weight (in kg) by height (in m) squared ($kg\,m^{-2}$). Abdominal obesity was defined using the sex and age specific $\geqslant$90th WC percentile.[16]

Selection and genotyping of polymorphisms

Ten SNPs identified from the literature significantly associated with obesity or obesity-related traits in children of European origin were selected: rs17782313 and rs12970134 near *MC4R*, prominent in the literature; rs10146997 in *NRXN3*, rs8192678 in *PPARGC1A*, rs7561317 near *TMEM18* and rs10913469 in *SEC16B*, poorly studied; rs545854 in *MSRA* and rs987237 in *TFAP2B*, associated in adult's populations[11] but never replicated in children; and rs9299 in *HOXB5* and rs9568856 in *OLFM4*, recently associated with childhood obesity[13] but never replicated.

The genomic DNA was extracted from buccal cells using the PureLink® Pro 96 Genomic DNA Kit (Invitrogen Corporation, Carlsbad, CA, USA), according to the instructions of the manufacturer.

Samples were genotyped for all SNPs by allelic discrimination assays using TaqMan probes (Applied Biosystems, Foster City, CA, USA). All PCRs were done in a volume of 20 µl containing in 1× SsoFast Probes Supermix (Bio-Rad, Hercules, CA, USA), 0.5 µl of specific TaqMan SNP Genotyping Assays (20×) (Applied Biosystems) and about 40 ng of genomic DNA, according to the manufacturer's instructions. Thermal cycling conditions were 10 min at 95 °C, and 35 cycles each of 95 °C for 15 s and 60 °C for 1 min. The fluorescence was observed through a MiniOpticon real time PCR system (Bio-Rad). To assess genotyping reproducibility, 10% of random samples were selected and re-genotyped for all SNPs with 100% concordance.

Statistical analysis

The allelic and genotypic frequencies of all polymorphisms were estimated by direct counting. Hardy–Weinberg equilibrium probability values were achieved using an exact test.[17] Logistic regression under an additive genetic model, allowing for analysis of a binary outcome (a case–control *status*), was used to test obesity and overweight phenotype polymorphism associations, adjusted for age and sex, by calculating odds ratios (ORs) with 95% of confidence intervals (CIs) and *P*-values. For *MC4R* rs17782313 and rs12970134 polymorphisms, linkage disequilibrium (r^2) values and a case/control (normal vs obese) haplotype association were assessed. All these statistical analyses were done by using the set-based tests implemented on PLINK software v.1.07 (http://pngu.mgh.harvard.edu/purcell/plink/).[18] For each obesity-related quantitative parameters (BMI, BMI *Z*-score, weight and WC), the non-parametric Kruskal–Wallis test was used to evaluate differences among the three genotypes in all the polymorphisms. Normality of the data was assessed using the Kolmogorov–Smirnov test. This statistical analysis was performed using the SPSS software (statistical package for the social sciences for Windows, version 18.0., SPSS inc., Chicago, IL, USA). A significant *P*-value was considered below 0.005 (0.05/10) by applying a Bonferroni correction for multiple testing, and a *P*-value between 0.005 and 0.05 has been considered as nominally significant.

QUANTO, v.1.1 power calculator (http://hydra.usc.edu/gxe/) was used to estimate the power of association as a function of the frequency of the effect allele assuming an additive model.[19]

RESULTS

The anthropometric characteristics of the study subjects distributed by phenotype are shown in Table 1.

The genotyping success rate varied between 96.3 and 99.7%. The minor allele frequencies observed for all polymorphisms in the total sample were 15% for rs7561317-A (*TMEM18*), 16% for rs10913469-C (*SEC16B*), 36% for rs8192678-A (*PPARGC1A*), 18% for rs987237-G (*TFAP2B*), 16% for rs545854-C (*MSRA*), 12% for rs9568856-A (*OLFM4*), 18% for rs10146997-G (*NRXN3*), 32% for rs9299-C (*HOXB5*), 21% for 17782313-C (*MC4R*) and 22% for rs12970134-A (*MC4R*) (Table 2). These frequencies are in accordance with those found in the HapMap CEU population (http://www.ensembl.org/). Genotype distributions among the control group were in agreement with Hardy–Weinberg equilibrium for all the studied polymorphisms ($P>0.05$).

We analyzed the obesity-related quantitative traits BMI, BMI *Z*-score, weight and WC among different genotypes for each of the studied polymorphisms. The mean values for the anthropometric traits among the three different genotypes are detailed in Table 2. The *MC4R* rs12970134 major G-allele was found nominally associated

Table 1 General characteristics of the Portuguese children participants

		Phenotype distribution[a]		
	Total	*Normal*	*Overweight*	*Obese*
Subjects	730	256	320	154
Girls (%)	50.7	55.9	45.3	53.2
Age (years)	9.1 ± 1.7	8.6 ± 1.6	9.5 ± 1.6	9.0 ± 1.7
Height (cm)	136.2 ± 11.7	131.1 ± 11.1	139.5 ± 11.1	137.9 ± 10.6
Weight (kg)	37.2 ± 11.3	28.1 ± 6.6	40.2 ± 9.3	46.1 ± 11.0
BMI ($kg\,m^{-2}$)	19.6 ± 3.4	16.1 ± 1.5	20.3 ± 1.8	23.8 ± 2.5
BMI *Z*-score	0.93 ± 0.97	−0.15 ± 0.78	1.3 ± 0.23	1.99 ± 0.23
WC (cm)	67.2 ± 7.8	60.3 ± 4.5	68.9 ± 5.4	75.1 ± 6.6
HC (cm)	79.0 ± 10.3	70.4 ± 6.5	81.9 ± 8.0	87.1 ± 9.4
WHR	0.85 ± 0.06	0.86 ± 0.06	0.85 ± 0.06	0.87 ± 0.05

Abbreviations: BMI, body mass index; BMI Z-score, body mass index standard deviation score; WC, waist circumference; HC, hip circumference; WHR, waist-to-hip ratio.
Data are presented as mean ± standard deviation.
[a]Phenotype distribution was determined using age- and sex-specific BMI cutoffs provided by the International Obesity Task Force (IOTF).

Table 2 Minor allele frequencies and Hardy–Weinberg equilibrium test of the 10 studied polymorphisms in the sampled Portuguese children and their associations with obesity-related quantitative traits

								Genotype (mean ± s.d.)			
Polymorphism	*Chr.*	*Gene*	*Alleles*[a]	n	*MAF*	*HWE*	*No. 11/12/22*	*11*	*12*	*22*	P-*value*
rs10913469	1q25	*SEC16B*	C:T	728	0.16	0.479	20/186/522				
BMI (kg m^{-2})								19.6 ± 3.6	19.5 ± 3.5	19.6 ± 3.4	0.930
BMI *Z*-score								0.99 ± 0.9	0.92 ± 1.0	0.93 ± 1.0	0.902
Weight (kg)								37.5 ± 13.4	36.9 ± 11.9	37.0 ± 11.1	0.736
Waist C (cm)								67.9 ± 8.6	66.9 ± 8.3	67.3 ± 7.5	0.709
rs7561317	2p25	*TMEM18*	A:G	721	0.15	0.886	16/191/514				
BMI (kg m^{-2})								19.2 ± 3.6	19.2 ± 3.3	19.7 ± 3.5	0.141
BMI *Z*-score								0.81 ± 1.1	0.84 ± 0.9	0.97 ± 1.0	0.120
Weight (kg)								36.7 ± 13.1	36.3 ± 11.4	37.6 ± 11.2	0.306
Waist C (cm)								66.1 ± 7.7	66.3 ± 7.5	67.6 ± 7.8	0.121
rs8192678	4p15	*PPARGC1A*	A:G	703	0.36	1.000	89/323/291				
BMI (kg m^{-2})								20.0 ± 3.6	19.8 ± 3.4	19.4 ± 3.4	0.198
BMI *Z*-score								0.99 ± 1.0	0.97 ± 0.9	0.89 ± 1.0	0.359
Weight (kg)								38.9 ± 12.3	38.0 ± 11.3	36.1 ± 10.9	0.061
Waist C (cm)								68.9 ± 8.7	67.5 ± 7.6	66.6 ± 7.5	0.093
rs987237	6p12	*TFAP2B*	G:A	725	0.18	0.615	21/218/486				
BMI (kg m^{-2})								19.0 ± 2.9	19.3 ± 3.2	19.7 ± 3.5	0.392
BMI *Z*-score								0.87 ± 1.0	0.89 ± 1.0	0.94 ± 1.0	0.536
Weight (kg)								34.1 ± 9.4	36.8 ± 11.2	37.5 ± 11.5	0.353
Waist C (cm)								64.9 ± 7.8	66.6 ± 7.3	67.6 ± 7.9	0.130
rs545854	8p23	*MSRA*	C:G	717	0.16	0.334	22/187/508				
BMI (kg m^{-2})								18.3 ± 3.7	19.8 ± 3.6	19.7 ± 3.4	0.055
BMI *Z*-score								0.51 ± 1,1	0.96 ± 1.1	0.95 ± 1.0	0.056
Weight (kg)								34.3 ± 10.6	38.0 ± 11.7	37.1 ± 11.4	0.240
Waist C (cm)								65.1 ± 6.9	67.5 ± 8.3	67.3 ± 7.5	0.282
rs9568856	13q14	*OLFM4*	A:G	725	0.12	0.059	17/141/567				
BMI (kg m^{-2})								19.2 ± 3.6	19.9 ± 3.4	19.5 ± 3.4	0.374
BMI *Z*-score								0.92 ± 0.8	0.97 ± 0.9	0.92 ± 1.0	0.719
Weight (kg)								35.6 ± 12.2	38.1 ± 11.1	37.0 ± 11.4	0.286
Waist C (cm)								65.7 ± 8.4	68.0 ± 7.5	67.1 ± 7.8	0.248
rs10146997	14q31	*NRXN3*	G:A	716	0.18	0.798	21/212/483				
BMI (kg m^{-2})								19.1 ± 3.5	19.6 ± 3.3	19.7 ± 3.5	0.672
BMI *Z*-score								0.91 ± 1.0	0.92 ± 1.0	0.95 ± 1.0	0.579
Weight (kg)								34.3 ± 11.9	37.5 ± 11.3	37.4 ± 11.3	0.436
Waist C (cm)								64.6 ± 7.6	67.3 ± 7.7	67.5 ± 7.8	0.260
rs9299	17q21	*HOXB5*	C:T	727	0.32	0.672	78/313/336				
BMI (kg m^{-2})								19.5 ± 3.4	19.7 ± 3.6	19.5 ± 3.3	0.710
BMI *Z*-score								0.87 ± 1.0	0.94 ± 1.0	0.92 ± 0.9	0.751
Weight (kg)								37.3 ± 11.6	37.5 ± 11.3	37.0 ± 11.4	0.792
Waist C (cm)								66.9 ± 7.0	67.4 ± 7.9	67.2 ± 7.8	0.896
rs12970134	18q21	*MC4R*	A:G	729	0.22	0.163	29/266/434				
BMI (kg m^{-2})								18.1 ± 3.3	19.5 ± 3.5	19.7 ± 3.9	*0.035*
BMI Z-score								0.53 ± 1.0	0.89 ± 1.0	0.98 ± 0.9	*0.043*
Weight (kg)								32.5 ± 9.1	37.1 ± 11.3	37.7 ± 11.5	0.053
Waist C (cm)								63.6 ± 5.8	67.1 ± 7.6	67.6 ± 7.9	*0.020*
rs17782313	18q22	*MC4R*	C:T	716	0.21	0.432	28/247/441				
BMI (kg m^{-2})								18.6 ± 3.6	19.7 ± 3.4	19.6 ± 3.5	0.269
BMI *Z*-score								0.61 ± 1.1	0.95 ± 1.0	0.94 ± 1.0	0.257
Weight (kg)								34.4 ± 10.6	37.7 ± 11.0	37.2 ± 11.5	0.260
Waist C (cm)								65.6 ± 7.5	67.4 ± 7.3	67.3 ± 8.0	0.430

Abbreviations: BMI, body mass index; BMI Z-score, body mass index standard deviation; Chr., chromosome; HC, hip circumference; *HOXB5*, homeobox B5; HWE, Hardy–Weinberg equilibrium; MAF, minor allele frequency; *MSRA*, methionine sulfoxide reductase A; *n*, number of genotyped children; *NRXN3*, neurexin 3; *OLFM4*, olfactomedin 4; *PPARGC1A*, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; *SEC16B*, homolog of *S. cerevisiae* Sec16; *TFAP2B*, transcription factor AP-2 beta; *TMEM18*, transmembrane protein 18; waist C, waist circumference.
P-values were obtained using the Kruskal–Wallis test.
P-values nominally significant ($P<0.05$) are in italic.
[a]Alleles: Minor (1): Major (2).

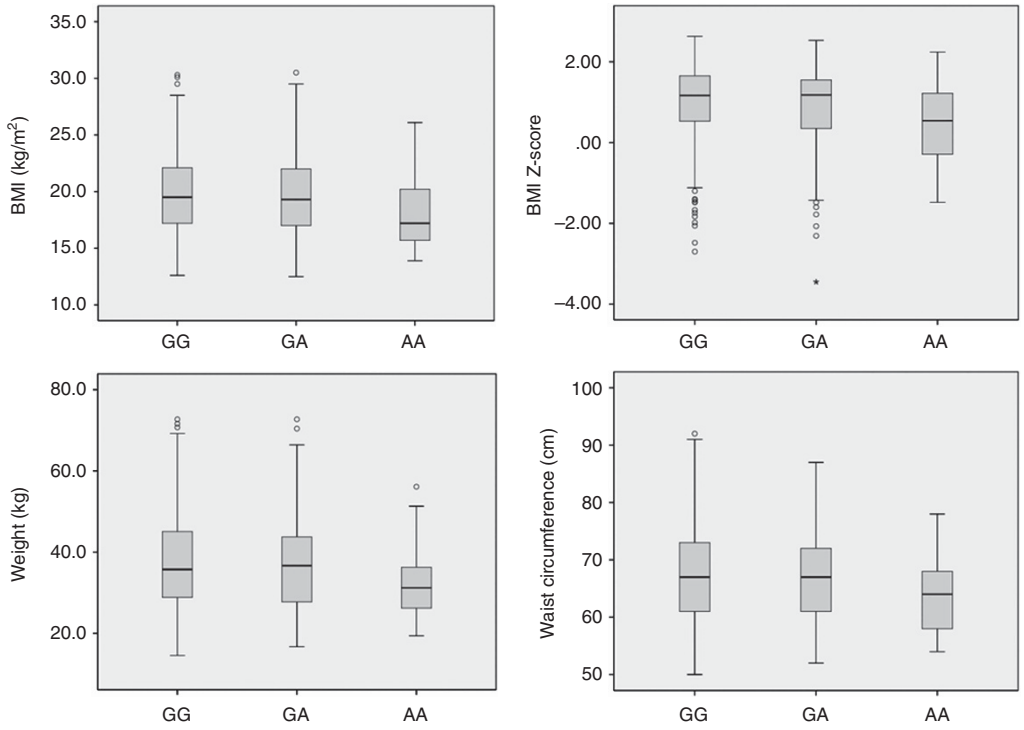

Figure 1 Box plot demonstrating the distribution of untransformed body mass index (BMI), BMI *Z*-score, weight and waist circumference within each genotype group of *MC4R* rs12970134 polymorphism. Each box represents the anthropometric traits values between the 25th and 75th quartiles, and the dark line within the boxes indicates the median values. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

with increase in BMI ($P=0.035$), BMI *Z*-score ($P=0.043$) and WC ($P=0.020$), and borderline associated with weight ($P=0.053$). Near nominal associations were also found for the *PPARGC1A* rs8192678 minor A-allele with weight ($P=0.061$) and WC ($P=0.093$), and for the *MSRA* rs545854 major G-allele with BMI ($P=0.055$) and BMI *Z*-score ($P=0.056$). After correction for multiple testing no statistically significant associations were found.

Genotype distributions of obesity-related parameters for the *MC4R* rs12970134 polymorphism, which showed the highest statistical significant associations with the obesity-related traits, are detailed in Figure 1. Homozygotes for the minor A-allele have the lowest value distributions for all the analyzed quantitative parameters (BMI, BMI *Z*-score, WC and weight).

Logistic regression analysis, in an additive model, revealed that *MC4R* rs12970134 major G-allele was nominally associated with the obesity risk (OR = 1.477; $P=0.029$) and that *TFAP2B* rs987237 major A-allele and *TMEM18* rs7561317 major G-allele are near nominally associated with the risk of obesity (OR = 1.455; $P=0.056$; and OR = 1.416; $P=0.092$, respectively) (Table 3). Only the *PPARGC1A* rs8192678 polymorphism was found nominally associated with the overweight phenotype (OR = 1.297; $P=0.041$) (Table 3).

We further investigated the difference in the genotype distribution between cases and controls for abdominal obesity by logistic regression analysis. In the total of 730 children, 112 revealed abdominal obesity (using sex and age specific $\geqslant$90th WC percentile). From the 10 polymorphisms studied, only *TMEM18* rs7561317 (major G-allele) showed nominal association with increased abdominal obesity (OR = 1.589; 95% CI, 1.02–2.50; $P=0.042$).

The haplotype analysis for the two *MC4R* rs17782313 and rs12970134 polymorphisms, located at a distance of 33.5 Kb and in high linkage disequilibrium ($r^2=0.74$), revealed that the common TG haplotype was associated with the risk of obesity ($P=0.043$) (with frequency of 81.2% in the obese group vs 75.0% in the control group).

DISCUSSION

Understanding the genetic basis of obesity in children could be used as a first step to develop possible preventive measures. Recent GWASs have identified many (>50) different genetic variants conferring susceptibility to obesity.[20] However, the modest association with the obesity risk observed for most variants implies that replication studies in different populations are required to detect and confirm such signals of association, eliminating false positives that may arise by chance or systematic bias.

Focused on 10 polymorphisms across the genome (located in or near the *MSRA*, *TFAP2B*, *NRXN3*, *PPARGC1A*, *TMEM18*, *SEC16B*, *HOXB5*, *OLFM4* and *MC4R* genes), previously associated by GWAS with obesity-related outcomes in populations of European origin, we conducted a genetic association study to investigate their role in the susceptibility of obesity in a sample of Portuguese children. Using obesity-related quantitative traits to assess whether the genotypes predict the trait value, we identified the *MC4R* rs12970134 *loci* nominally associated with several obesity-related traits, and two *loci* (*PPARGC1A* and *MSRA*) near nominally associated with at least one anthropometric parameter (Table 2). In addition, using logistic regression analyses, the *MC4R* rs12970134 polymorphism was found nominally associated ($P=0.029$) with the risk of obesity and the *TFAP2B* rs987237 polymorphism showed borderline significant association ($P=0.056$) with the obese phenotype. Thus, this study highlights these four polymorphisms as potential genetic markers of the obesity phenotype in this Portuguese children sample.

Table 3 Allele frequencies of the 10 studied polymorphisms in the Portuguese children, and their associations with risk of obesity among phenotypic groups

SNP ID/ Gene	Allele	Normal[a]	*Obese* vs *normal*			*Overweight* vs *normal*		
			Obese[a]	OR (CI 95%)	P-value	Overweight[a]	OR (CI 95%)	P-value
rs10913469	T	0.855	0.847	1.058 (0.71–1.57)	0.780	0.825	1.154 (0.83–1.59)	0.387
SEC16B T>C	C	0.145	0.153			0.175		
rs7561317	G	0.825	0.869	0.706 (0.47–1.06)	0.092	0.850	0.829 (0.60–1.13)	0.248
TMEM18 G>A	A	0.175	0.131			0.150		
rs8192678	G	0.678	0.642	1.176 (0.86–1.59)	0.293	0.619	1.297 (1.00–1.66)	*0.041*
PPARGC1A G>A	A	0.322	0.358			0.381		
rs987237	A	0.804	0.856	0.687 (0.46–1.01)	0.056	0.817	0.915 (0.67–1.23)	0.559
TFAP2B A>G	G	0.196	0.144			0.183		
rs545854	G	0.820	0.836	0.960 (0.65–1.41)	0.838	0.847	0.878 (0.63–1.20)	0.425
MSRA G>C	C	0.180	0.164			0.153		
rs9568856	G	0.882	0.866	1.156 (0.75–1.77)	0.504	0.884	0.985 (0.68–1.41)	0.935
OLFM4 G>A	A	0.118	0.134			0.116		
rs10146997	A	0.818	0.860	0.731 (0.49–1.08)	0.119	0.808	1.07 (0.79–1.45)	0.645
NRXN3 A>G	G	0.182	0.140			0.192		
rs9299	T	0.679	0.654	1.122 (0.83–1.51)	0.453	0.687	0.962 (0.74–1.23)	0.762
HOXB5 T>C	C	0.321	0.346			0.312		
rs12970134	G	0.749	0.815	0.677 (0.47–0.96)	*0.029*	0.782	0.829 (0.63–1.09)	0.183
MC4R G>A	A	0.251	0.185			0.218		
rs17782313	T	0.781	0.817	0.799 (0.55–1.14)	0.221	0.780	1.005 (0.75–1.33)	0.972
MC4R T>C	C	0.212	0.183			0.220		

Abbreviations: BMI, body mass index; CI, confidence interval; *HOXB5*, homeobox B5; *MC4R*, melanocortin 4 receptor; *MSRA*, methionine sulfoxide reductase A; *NRXN3*, neurexin 3; *OLFM4*, olfactomedin 4; OR, odds ratio; *PPARGC1A*, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; SNP ID, single-nucleotide polymorphism identification; *SEC16B*, homolog of *S. cerevisiae* Sec16; *TFAP2B*, transcription factor AP-2 beta; *TMEM18*, transmembrane protein 18.
Logistic regression was used to compare genotype distribution. *P*-values (asymptotic *P*-value for t-statistic) shown are for an additive model and are adjusted for age and sex. OR is shown for the minor allele.
P-values significant ($P<0.05$) are in italic.
[a]Phenotypic groups were determined using age- and sex-specific BMI cutoffs provided by the International Obesity Task Force (IOTF).

The *MC4R* gene is known to be the most common cause of monogenic obesity in extreme childhood obesity,[5] but also its flanking genomic region has been the third strongest implicated in polygenic obesity. The expression of *MC4R* is restricted to the hypothalamus involved in food intake regulation.[21] Until now, several variations of this gene were established with BMI and/or WC, showing an independent role in body variation. Polymorphisms rs12970134 and rs17782313, located 154 kb and 188 kb, respectively, downstream of the *MC4R* gene, were found associated with obesity and obesity-related traits in several studies in Asian and European populations, both in children and in adults.[22–27] In the present study, nominal significant associations were found between *MC4R* rs12970134 and BMI, BMI *Z*-score and WC ($P<0.05$), as also with the risk of obesity ($P=0.029$). However, our findings do not replicate previous reports that show the minor A-allele associated with increased risk in BMI and WC in children of European[22,24–26,28] or Asian[27] descent. Instead, in the present study, Portuguese children showed the minor rs12970134 A-allele significantly associated with lower BMI ($P=0.035$), BMI *Z*-score ($P=0.043$), WC ($P=0.020$), and also with a lower risk for the obesity phenotype (OR = 0.677; $P=0.029$). For this polymorphism, the minor A-allele frequency was 18.5% in the obese group vs 25.1% in the control group. For the second *MC4R* polymorphism, rs17782313, previous studies showed the C-allele associated with childhood obesity (increasing BMI in $\pm 0.22\,kg\,m^{-2}$),[23] however, in the Portuguese children, no statistical association was found with any obesity-related trait. The haplotype analysis showed the *MC4R* rs17782313/rs12970134 TG haplotype associated with the risk of obesity, confirming the potential role of rs12970134 major G-allele in the etiology of obesity in our sample of Portuguese children.

A genome-wide association scan meta-analysis conducted by Lindgren *et al.*[29] found that the G-allele of the *MSRA* rs545854 polymorphism was associated with WC ($P=8.9\times10^{-9}$) in adults. Bille *et al.*[11] also found significant association between this polymorphism and WC (OR = 1.08; $P=0.02$). In our study, nominal borderline significant associations with BMI ($P=0.055$) and BMI *Z*-score ($P=0.056$) were observed. The biological function between *MSRA locus* and adiposity remains unclear.[29]

PPARGC1A is a transcriptional co-activator that has been implicated in the regulation of genes involved in energy metabolism.[30] The Gly482Ser missense mutation (rs8192678), predicted by a G-to-A transition at position +1564 in exon 8 of the *PPARGC1A* gene, was found associated with obesity indices in middle-aged women of a cross-sectional Austrian population[30] and with abdominal obesity in Chinese adults.[31] In our study, near nominal associations were obtained with weight ($P=0.061$) and WC ($P=0.093$) for the 482Ser variant.

The molecular function of the *TMEM18* gene product is to bind DNA to suppress transcription; it could be differently expressed in the hypothalamus and is possibly involved in the regulation of feeding.[32]

Polymorphism rs7561317, located about 22 kb downstream of *TMEM18*, is the second best associated *locus* with BMI after *FTO* gene.[5] A GWAS conducted by Thorleifsson *et al.*[33] found that the rs7561317 GG genotype increases BMI in $\pm 0.70\,kg\,m^{-2}$ and is associated with obesity. In the present study, significant associations were not found between any obesity-related trait and the *TMEM18* rs7561317 polymorphism; however, our findings are directionally consistent with previous studies conducted in children and adolescents, as the rs7561317 major G-allele was found marginally associated with the risk of obesity (OR = 0.706; $P = 0.092$).

The *TFAP2B* gene is suggested to be involved in global adipocyte response to positive energy balance.[29] The minor G-allele of rs987237 polymorphism was previously found associated with increased WC ($P = 1.9 \times 10^{-11}$) and BMI ($P = 7.0 \times 10^{-12}$) in a meta-analysis of 16 GWASs within adults of European ancestry;[29] and also in children it was found associated with increased WC ($P = 3.5 \times 10^{-2}$) and BMI ($P = 0.06$).[28] Our data in Portuguese children do not replicate previous findings while we observed a nominal borderline significant association of the major rs987237 A-allele with the risk of obesity (OR = 0.692; $P = 0.056$).

In the present study, the major *MC4R* rs12970134-G and *TFAP2B* rs987237 A-alleles showed, respectively, nominal and near-nominal significant associations with the risk of obesity, but the direction of effect was reverse when comparing with that in the original reports. Despite opposite direction on the effect of a risk allele is highly unlikely, this was observed in several studies including between different populations[34] but also inside a same population.[35] For the *TFAP2B* rs987237 polymorphism, the original significant association was found in adults,[29] and to the authors knowledge, this study with Portuguese participants is the first replication report involving children. Therefore, the opposite direction of association for rs987237-A risk allele could be due to differences between children and adults regarding natural physiological differences.

All genes studied in this work are considered as candidates for the risk of developing obesity. Most of them are involved in homeostasis and energy metabolism, nevertheless the casual effect of these polymorphisms in the pathogenesis of obesity remains unclear. In the present study with Portuguese children, only the *MC4R* rs12970134 polymorphism showed nominal significant association ($P < 0.05$) with obesity and most of obesity-related traits. In a previous study for the *FTO* gene using the same cohort of individuals,[10] significant associations were also found between the rs9939609 minor A-allele and increased risk for several anthropometric traits, including weight ($P = 0.019$), BMI ($P = 0.018$), BMI *Z*-score ($P = 0.011$) and WC ($P = 0.016$), in concordance with reports worldwide. Thus, both *loci FTO* and *MC4R* appear to have a key role in the obesity phenotype in Portuguese children. Most of the analyzed polymorphisms in this present study showed no nominal effects in obesity-related traits, but this difference with the previous findings may be due partly to the sample size, which may have been insufficient to replicate the original findings. The estimated power of association observed ranges from 6 to 72%, but at least for *MC4R* rs12970134 polymorphism, the variant most associated with obesity in our sample, the obtained power (72%) is close to values ($\geqslant 80\%$) commonly considered as sufficient to detect genetic variant interaction effects.

In conclusion, among the 10 *loci* reported in this study, polymorphisms in or near *MC4R*, *PPARGC1A*, *MSRA* and *TFAP2B* genes could be assumed to have a role in the risk of obesity in this population sample of Portuguese children. While we could not replicate the original findings concerning the direction effect of the *MC4R* rs12970134 and *TFAP2B* rs987237 risk alleles our results deserve confirmatory studies in other populations. Moreover, our data may show that the polymorphisms provided here could have a modest role in the obesity etiology in children, at least when comparing with the *FTO* gene, suggesting the existence of other unknown *loci* involved in the obesity susceptibility. Our replication study could also have public health significance while genes playing an essential role in energy homeostasis, such as *PPARGC1A* or *TFAP2B*, suggested by our data as obesity-related genes, may be used as targets for obesity treatment. Further investigations in the near future regarding genetic associations and functional roles of these polymorphisms should be helpful to confirm its implication in the development of obesity and if they could be attractive targets for therapeutic agents.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We are very grateful to the children who participated in our study, children's parents, teachers, school staff and Inês Costa for the English review. This work was performed with the support of *Fundação para a Ciência e Tecnologia* (FCT) (PEst-OE/SADG/UI0283/2011). David Albuquerque as a PhD grant (SFRH/BD/68774/2010) from FCT.

1 Garver, W. Gene-diet interactions in childhood obesity. *Curr. Genomics* **12,** 180–189 (2011).
2 Allison, D. B., Kaprio, J., Korkeila, M., Koskenvuo, M., Neale, M. C. & Hayakawa, K. The heritability of body mass index among an international sample of monozygotic twins reared apart. *Int. J. Obes. Relat. Metab. Disord.* **20,** 501–506 (1996).
3 Faith, M. S., Pietrobelli, A., Nuñez, C., Heo, M., Heymsfield, S. B. & Allsion, D. B. Evidence for independent genetic influences on fat mass and body mass index in a pediatric twin sample. *Pediatrics* **104,** 61–67 (1999).
4 Silventoinen, K., Rokholm, B., Kaprio, J. & Sørensen, T. I. The genetic and environmental influences on childhood obesity: a systematic review of twin and adoption studies. *Int. J. Obes.* **34,** 29–40 (2010).
5 Loos, R. J. Genetic determinants of common obesity and their value in prediction. *Best Pract. Res. Clin. Endocrinol. Metab.* **26,** 211–226 (2012).
6 Frayling, T. M., Timpson, M. J., Weedon, M. N., Zeggini, E., Freathy, R. M., Lindgren, C. M. *et al.* A common variant in the FTO gene is associated with body mass index and predisposes to childhood and adult obesity. *Science* **316,** 889–894 (2007).
7 Chang, Y. C., Liu, P. H., Lee, W. J., Chang, T. J., Jiang, Y. D., Li, H. Y. *et al.* Common variation in the fat mass and obesity-associated (FTO) gene confers risk of obesity and modulates BMI in the Chinese population. *Diabetes* **57,** 2245–2252 (2008).
8 Grant, S. F. A., Li, M., Bradfield, J. P., Kim, C. E., Annaiah, K., Santa, E. *et al.* Association analysis of the FTO gene with obesity in children of Caucasian and African ancestry reveals a common tagging SNP. *PLoS One* **3,** e1746 (2008).
9 Rodríguez-López, R., González-Carpio, M., Serrano, M. V., Torres, G., García de Cáceres, M. T., Herrera, T. *et al.* Association of FTO gene polymorphisms and morbid obesity in the population of Extremadura. *Endocrinol. Nutr.* **57,** 203–209 (2010).
10 Albuquerque, D., Nóbrega, C. & Manco, L. Association of FTO polymorphisms with obesity and obesity-related outcomes in Portuguese children. *PLoS ONE* **8,** e54370 (2013).
11 Bille, D. S., Banasik, K., Justesen, J. M., Sandholt, C. H., Sandbæk, A., Lauritzen, T. *et al.* Implications of central obesity-related variants in LYPLAL1, NRXN3, MSRA, and TFAP2B on quantitative metabolic traits in adult Danes. *PLoS One* **6,** e20640 (2011).
12 Rouskas, K., Kouvatsi, A., Paletas, K., Papazoglou, D., Tsapas, S., Lobbens, S. *et al.* Common variants in FTO, MC4R, TMEM18, PRL, AIF1, and PCSK1 show evidence of association with adult obesity in the Greek population. *Obesity* **20,** 389–395 (2012).
13 Bradfield, J., Taal, H. R., Timpson, N., Scherag, A., Lecoeur, C., Warrington, E. *et al.* A genome-wide association meta-analysis identifies new childhood obesity loci. *Nat. Genet.* **44,** 526–532 (2012).
14 Albuquerque, D., Nóbrega, C., Samouda, H. & Manco, L. Assessment of obesity and abdominal obesity among Portuguese children. *Acta. Med. Port.* **25,** 169–173 (2012).
15 Cole, T. J., Bellizzi, M. C., Flegal, K. M. & Dietz, W. H. Establishing a standard definition for child overweight and obesity worldwide: international survey. *Br. Med. J.* **320,** 1–6 (2000).
16 Cook, S., Weitzman, M., Auinger, P., Nguyen, M. & Dietz, W. H. Prevalence of a metabolic syndrome phenotype in adolescents: findings from the third National Health and Nutrition Examination Survey, 1988-1994. *Arch. Pediatr. Adolesc. Med.* **157,** 821–827 (2003).

17 Wigginton, J. E., Cutler, D. J. & Abecasis, G. R. A Note on Exact Tests of Hardy-Weinberg Equilibrium. *Am. J. Hum. Genet.* **76,** 887–893 (2005).
18 Purcell, S., Neale, B., Todd-Brown, K., Thomas, L., Ferreira, M. A., Bender, D. *et al.* PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am. J. Hum. Genet.* **81,** 559–575 (2007).
19 Gauderman, W. & Morrison, J. *QUANTO 1.1: A Computer Program for Power and Sample Size Calculations for Genetic-Epidemiology Studies.* http://hydra.usc.edu/gxe
20 Berndt, S. I., Gustafsson, S., Mägi, R., Ganna, A., Wheeler, E., Feitosa, M. F. *et al.* Genome-wide meta-analysis identifies 11 new loci for anthropometric traits and provides insights into genetic architecture. *Nat. Genet.* **45,** 501–512 (2013).
21 Mountjoy, K. G., Mortrud, M. T., Low, M. T., Simerly, R. B. & Cone, R. D. Localization of the melanocortin-4 receptor (MC4-R) in neuroendocrine and autonomic control circuits in the brain. *Mol. Endocrinol.* **8,** 1298–1308 (1994).
22 Chambers, J. C., Elliott, P., Zabaneh, D., Zhang, W., Li, Y., Froguel, P. *et al.* Common genetic variation near MC4R is associated with waist circumference and insulin resistance. *Nat. Genet.* **40,** 716–718 (2008).
23 Loos, R. J., Lindgren, C. M., Li, S., Wheeler, E., Zhao, J. H., Prokopenko, I. *et al.* Common variants near MC4R are associated with fat mass, weight and risk of obesity. *Nat. Genet.* **40,** 768–775 (2008).
24 Zhao, J., Bradfield, J. P., Li, M., Wang, K., Zhang, H., Kim, C. E. *et al.* The role of obesity-associated loci identified in genome wide association studies in the determination of pediatric BMI. *Obesity* **17,** 2254–2257 (2009).
25 Scherag, A., Jarick, I., Grothe, J., Biebermann, H., Scherag, S., Volckmar, A. L. *et al.* Investigation of a genome wide association signal for obesity: synthetic association and haplotype analyses at the melanocortin 4 receptor gene locus. *PLoS One* **5,** e13967 (2010).
26 Vogel, C. I., Boes, T., Reinehr, T., Roth, C. L., Scherag, S., Scherag, A. *et al.* Common variants near MC4R: exploring gender effects in overweight and obese children and adolescents participating in a lifestyle intervention. *Obes. Facts* **4,** 67–75 (2011).
27 Dwivedi, O. P., Tabassum, R., Chauhan, G., Kaur, I., Ghosh, S., Marwaha, R. K. *et al.* Strong influence of variants near MC4R on adiposity in children and adults: a cross-sectional study in Indian population. *J. Hum. Genet.* **58,** 27–32 (2013).
28 den Hoed, M., Ekelund, U., Brage, S., Grontved, A., Zhao, J. H., Sharp, S. J. *et al.* Genetic susceptibility to obesity and related traits in childhood and adolescence: influence of loci identified by genome-wide association studies. *Diabetes* **59,** 2980–2988 (2010).
29 Lindgren, C. M., Heid, I. M., Randall, J. C., Lamina, C., Steinthorsdottir, V., Qi, L. *et al.* Genome-wide association scan meta-analysis identifies three loci influencing adiposity and fat distribution. *PLoS Genet.* **5,** e1000508 (2009).
30 Esterbauer, H., Oberkofler, H., Linnemayr, V., Iglseder, B., Hedegger, M., Wolfsgruber, P. *et al.* Peroxisome proliferator-activated receptor-gamma coactivator-1 gene locus: associations with obesity indices in middle-aged women. *Diabetes* **51,** 1281–1286 (2002).
31 Weng, S. W., Lin, T. K., Wang, P. W., Chen, I. Y., Lee, H. C., Chen, S. D. *et al.* Gly482Ser polymorphism in the peroxisome proliferator-activated receptor gamma coactivator-1alpha gene is associated with oxidative stress and abdominal obesity. *Metabolism.* **59,** 581–586 (2010).
32 Jurvansuu, J. & Goldman, A. Obesity risk gene TMEM18 encodes a sequence-specific DNA-binding protein. *PLoS One* **6,** e25317 (2011).
33 Thorleifsson, G., Walters, G. B., Gudbjartsson, D. F., Steinthorsdottir, V., Sulem, P., Helgadottir, A. *et al.* Genome-wide association yields new sequence variants at seven loci that associate with measures of obesity. *Nat. Genet.* **41,** 18–24 (2009).
34 Wang, X., Tang, N. L., Leung, C. C., Kam, K. M., Yew, W. W., Tam, C. M. *et al.* Association of polymorphisms in the Chr18q11.2 locus with tuberculosis in Chinese population. *Hum. Genet.* **132,** 691–695 (2013).
35 Fukuda, H., Imamura, M., Tanaka, Y., Iwata, M., Hirose, H., Kaku, K. *et al.* Replication study for the association of a single-nucleotide polymorphism, rs3746876, within KCNJ15, with susceptibility to type 2 diabetes in a Japanese population. *J. Hum. Genet.* **58,** 490–493 (2013).