

Dietary Behaviours May Modulate the Association Between Common MC4R Genetic Variants and Obesity and Its Comorbidities: The 1000PLUS Cohort Study.

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Research

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Abstract

Background: The *MC4R* gene harbours one of the strongest susceptibility locus for obesity, and its metabolic consequences. The objective of this study was to analyze whether dietary factors may attenuate the *MC4R* genotypes effects on obesity and its comorbidities.

Methods: In 819 adult participants, genotyped for *MC4R* SNPs (rs17782313, rs12970134, rs633265, rs1350341) the anthropometric measurements, total body fat, visceral (VAT) and subcutaneous adipose tissue (SAT), blood glucose, insulin, total-, LDL-, HDL-cholesterol, and triglycerides concentrations were assessed. The daily macronutrient intake was calculated based on the three-day food intake records, and daily physical activity based on the validated questionnaire. The ANOVA or Kruskal-Wallis tests were used, and multivariate linear regression models were developed to evaluate the effects of diet on obesity and related factors in various genotypes carriers.

Results: The CC genotype (rs17782313) carriers, being in the upper quantiles of protein intake, presented significantly higher VAT, VAT/SAT ratio, fasting blood glucose and triglyceride concentrations, and an increase of energy derived from proteins was associated with significantly higher BMI (Est. 5.74, $R^2=0.12$), body fat content (Est. 8.44, $R^2=0.82$), VAT (32.59, $R^2=0.06$), and VAT/SAT ratio (Est. 0.96, $R^2=0.05$). The AA genotype carriers (rs12970134) being in the upper protein intake quantiles presented higher BMI, body fat, SAT and VAT volume, waist circumference, fasting blood glucose, triglycerides and total cholesterol concentrations. Individuals carrying the AG and GG genotypes in the upper carbohydrate intake quantile had significantly lower body weight, waist circumference, insulin resistance and fasting insulin levels. An increase of energy derived from proteins by AA carriers was associated with significantly higher VAT (Est. 19.95, $R^2=0.06$) and VAT/SAT ratio (Est. 0.64, $R^2=0.05$).

Conclusions: Our findings provide evidence that associations of the common *MC4R* SNPs with obesity and its comorbidities is modulated by dietary intake, and may be useful for genome-customized diets for obesity prevention.

Trial registration. This trial was registered at www.clinicaltrials.gov as NCT03792685. Date of registration: 3Jan2019 (retrospectively registered), <https://clinicaltrials.gov/ct2/show/NCT03792685>.

Background

Due to its increasing prevalence worldwide, obesity has become a major global health challenge(1, 2). Obesity is a major risk factor for type 2 diabetes mellitus (T2DM)(3), hyperlipidaemia, hypertension, cardiovascular disease(4, 5), as well as some types of cancers(6). Given the increasing prevalence of obesity, an increase in the prevalence of these diseases can be expected(7), and therefore, the urgent actions are required to find more effective treatments.

Excessive diet energy intake and diminished physical activity contribute to the development of obesity, but other factors are likely involved(8). Large-scale genome-wide association studies (GWAS) and meta-

analyses have revealed many genetic loci associated with high body fat content(9). In addition, many single nucleotide polymorphisms (SNPs) in or near genes including the fat mass and obesity-associated (*FTO*) gene(10), melanocortin-4 receptor (*MC4R*) gene(11), peroxisome proliferator-activated receptor gamma (*PPAR γ*) gene(12) as well as other genes(13) have been correlated with body fat content, body fat distribution and waist and hip circumference. Genetic variations have been associated with weight gain due to larger amounts of consumed food(14, 15), deprivation of appetite and satiety regulation(16), as well as due to deprivation of energy and substrate utilization(17), and also the sedentary behaviors have been shown to be related to genetic factors(18). Previous studies from our group(19, 20) also identified associations between genetic factors and mostly postprandial metabolic disturbances that may lead to obesity and T2DM. Nevertheless, obesity should not be considered only to be a genetic disorder, because it is a multifactorial disorder influenced by the interaction between lifestyle and genetic factors(21).

The melanocortin-4 receptors are expressed in brain and they are part of the melanocortin pathway, which controls energy homeostasis(22, 23). Rare coding mutations in the *MC4R* gene have consistently been associated with monogenic obesity in humans(24–26); however, most cases of obesity result from polygenic and multifactorial interactions including SNPs in or near the *MC4R* gene. Some common genetic variants near the *MC4R* gene have been associated with increased fat mass content, body weight, obesity, and T2DM(27–30). In previous study we found that *MC4R* genetic variants are associated with body weight, body fat content and body fat distribution, as well as postprandial carbohydrate utilization(31). Melanocortin-4 receptors are expressed in several sites in the brain that have been implicated in central energy balance regulation(11). Activation of melanocortin system increases energy expenditure, insulin sensitivity and may influence food intake regulation(11, 32). Several studies have demonstrated that SNPs near the *MC4R* gene influence appetite(33) as well as energy level and dietary fat intake(28, 33); however, other studies have indicated that SNPs near the *MC4R* gene do not influence food intake(34) and may not have any impact on body weight(35). These conflicting results may be due to the fact that *MC4R* may be dependent on dietary intake: one study found that *MC4R* expression levels in peripheral blood cells (PBCs) in children were associated with the percentage of energy intake from carbohydrates and fat (36). Studies conducted thus far indicate that interactions between *MC4R* genetic variants and dietary factors play a significant role in the development of obesity and type 2 diabetes phenotypes, while highlighting the need for additional research on this topic(30). Furthermore, physical activity and sedentary behaviours have been shown to modify the association between *MC4R* variants and body mass index (BMI) in Chinese population(37), and may have an impact on metabolic changes, such as insulin sensitivity, in women with prior gestational diabetes mellitus, dependently on *MC4R* genotype(38). However, it is still unclear whether dietary factors may influence the relationship between some genetic variants and obesity. Detecting *MC4R* risk genotypes in individuals and modifying their diets accordingly based on genetic results may be an efficient strategy to prevent obesity and its comorbidities.

Methods

The aim of our study was to evaluate whether dietary factors can alter the impact of the *MC4R* gene on obesity and related comorbidities. This study is registered at www.clinicaltrials.gov as NCT03792685.

Participants and Study Design. A population-based sample from the 1000PLUS Cohort Study group was enrolled in this study, consisting of 1,549 Caucasian individuals of Polish origin (aged 18–79 y), recruited between 2007–2019 as described previously(20, 39, 40). For this analysis, we included subjects who did not have any endocrine, renal, hepatic and gastrointestinal disorders and were not taking any treatments (including dietary supplements, following any specific eating pattern or diet etc.) that might affect the results. Subjects arrived at the laboratory between 7.30-8.00 AM, after an overnight fast and study procedures, including demographic and anthropometric data collection, body composition and body fat distribution analysis, oral glucose tolerance test (OGTT), blood samples collection, genetic and biochemical analysis, daily physical activity and dietary intake, were assessed as described below.

Anthropometric measurements and body composition analysis. Weight and height were measured using a standardized method(41). All individuals underwent body weight and body composition analysis: total body fat content was assessed with bioelectrical impedance analysis (InBody 220, Biospace, Korea), and visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT) content was measured with bioelectrical impedance analysis (Maltron 920-2 BioScan, Maltron International Ltd, UK). Waist circumference was assessed midway between the lower rib and the iliac crest on the midaxillary line. Hip circumference was measured at the level of the widest circumference over the great trochanters.

OGTT performance. The OGTTs were performed according to the World Health Organization (WHO) recommendations using a 75 g oral glucose dose. All participants were instructed to fast for 8–12 h prior to the tests but not to restrict carbohydrate intake 3 days before the test. Glucose and insulin levels were measured at 0, 30, 60 and 120 minutes after glucose load. OGTT was performed in all study participants without a history of diabetes.

Blood collections and biochemical analysis. The fasting venous blood samples were collected for assessing plasma glucose, insulin, total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), triglyceride (TG) concentration and haemoglobin A1c (HbA1c) levels. Next, the OGTTs were performed. The specimen was drawn and prepared for testing in accordance with the instructions provided with the laboratory kit. The specimen was stored in accordance with the kit instructions until testing at –20°C/–80°C. An immunoradiometric assay (Insulin, IRMA, DiaSource, Belgium; Wallac Wizard 1470 Automatic Gamma Counter, PerkinElmer, Life Science, Turku, Finland) was used to measure insulin concentration. Concentration of plasma glucose was measured by the hexokinase enzymatic colorimetric assay (Cobas c111, Roche Diagnostics Ltd., Switzerland). Concentrations of triglycerides, total cholesterol, LDL and HDL were measured with an enzymatic colorimetric assay (Cobas c111, Roche Diagnostics Ltd., Switzerland). HbA1c was assessed using HPLC (high performance liquid chromatography; D-10 Hemoglobin Testing System, Bio-Rad Laboratoriuues Inc. Hercules, CA, USA by France, Bio-Rad, Marnes-la-Coquette).

Calculations. BMI was calculated using the following formula: body weight (kg) divided by height squared (m). Waist-hip ratio (WHR; in centimetres) was determined by dividing waist circumference by hip circumference. VAT/SAT ratio (in cubic centimetres of tissue) was calculated by dividing visceral adipose tissue by subcutaneous adipose tissue. Homeostatic model assessment of insulin resistance (HOMA-IR) was calculated using the following standard formula: (fasting plasma glucose concentration (mmol/L)) × (fasting insulin concentration (μU/mL))/22.5. The index for homeostatic model assessment of β-cell function (HOMA-B) was determined using the following formula: 20 × fasting insulin (μU/mL)/fasting glucose (mmol/mL) – 3.5. The metabolic equivalent (MET, min per week) was calculated using the following formula: (MET level) × (minutes of activity) × (events per week).

Daily physical activity and dietary intake analyses. Daily physical activity was estimated with a self-administered questionnaire (International Physical Activity Questionnaire-Long Form, IPAQ-LF), considered to be a valid metric to assess the levels of physical activity(42). The results of the questionnaire were used to calculate MET values; each participant was subsequently classified as having a low, moderate or high physical activity level.

Analyses of three-day food diaries were performed in a group of 662 subjects, because not all participants completed the 3-day food intake diary. To estimate the portion size of foods, participants were asked to compare their portion sizes with colour photographs for each portion size. In addition, participants were asked to weigh their food if possible. Daily energy, protein, fat and carbohydrate intake was analysed using Dieta 4 software (National Food and Nutrition Institute, Warsaw, Poland).

Genetic analysis. We genotyped previously identified *MC4R* SNPs in: rs17782313, rs12970134, rs633265, rs1350341. DNA was extracted from peripheral blood leukocytes using a classical salting out method. The SNPs were genotyped with TaqMan SNP technology from ready-to-use human assays library (Applied Biosystems, USA) using a high throughput genotyping system (OpenArray, Life Technologies, USA). SNP analysis was performed in duplicate according to the manufacturer's instructions. To detect possible false positive signals caused by contamination, a negative control consisting of a sample without a template was used.

Ethics. Study procedures were carried out in accordance with ethical standards on human experimentation as well as the guidelines laid down in the Helsinki Declaration of 1975, revised in 1983. The study protocol was approved by the local ethics committee of the Medical University of Bialystok (Poland), and written informed consent was obtained from all participants.

Statistical analysis. Numerical data were summarized with number of observations (N), arithmetic mean and standard deviation (SD). For categorical data, number of observations and percentage were presented. Study participants were divided into quantiles based on their average daily protein, carbohydrate and fat intake, using the median values of each three parameters to set the thresholds. The differences between selected parameters and dietary groups were then compared using an analysis of variance (ANOVA) or Kruskal-Wallis test for numerical variables, with Tukey or Dunn post-hoc tests with Holm p-value adjustment where appropriate. For categorical variables, a chi-squared test was used.

Multivariate linear regression models that included interactions between the *MC4R* genotypes and average daily protein, fat and carbohydrate intake groups as well as numerical variables were prepared. P-values were adjusted for age, sex, BMI (when applicable), total energy intake and average physical activity. Robust Huber-White standard errors (HC1) were calculated. Model fit was estimated using R-squared values plus adjusted R-squared values. Some of the models were optimized with stepwise backward elimination based on the AIC. The statistical significance level was set at 0.05 for all two-sided tests and multivariate comparisons. All calculations were prepared in R(43) (R version 3.6.2).

Results

This study included 819 subjects (47.5% men and 52.5% women) 18–79 years old. The mean age of participants was 42.1(± 14.5) years old. The mean BMI was 28.5(± 6.6) kg/m² (min. 15.6 kg/m², max. 56.5 kg/m²). Of the participants, 33.9% had a BMI < 25.00 kg/m², 34.5% were overweight with a BMI ≥ 25.00 and < 30.00 kg/m², and 31.6% were obese with BMI ≥ 30.00 kg/m². Based on criteria for diagnosing diabetes(44), 411 participants (50.2%) were identified as having prediabetes or diabetes. Of these individuals, 109 participants (13.3%) had previous history of prediabetes or diabetes, and 56 participants (6.8%) were being treated with anti-diabetic medications. Participants who received anti-diabetic drug therapy or lipid-lowering medications (47 individuals, 5.7% of participants), as potential confounders, were excluded from analysis.

The clinical characteristics of the participants, stratified by investigated genotypes, are presented in Tables 1–4. No significant deviation from Hardy Weinberg equilibrium was observed for any of the SNPs investigated in this study ($p > 0.05$). The frequencies of overweight/obesity and prediabetes/diabetes prevalence did not differ between genotypes (Table 1–4). We did not find any differences in dietary factors, physical activity level, or body fat distribution between carriers of the investigated genotypes (Table 1–4). Additionally, we did not observe significant differences in HbA1c, HOMA-IR, HOMA-B, triglycerides, total cholesterol, LDL cholesterol or HDL cholesterol levels (data not shown) among individuals carrying these SNPs.

Table 1
 Characteristics of participants stratified by rs17782313 genotypes.

rs17782313	C/C	C/T	T/T	p value
N (women/men)	30 (10/20)	275 (137/138)	504 (277/227)	
Genotype frequency	3.71%	33.99%	62.30%	> 0.05
BMI (kg/m ²)	29.8 (6.4)	29.0 (6.9)	28.1 (6.5)	0.049
BMI < 25.0 (kg/ m ²)	7 (23.3%)	83 (30.5%)	182 (36.4%)	0.186
BMI 25.0-29.9 (kg/ m ²)	9 (30.0%)	98 (36.0%)	170 (34.0%)	
BMI ≥ 30.0 (kg/ m ²)	14 (46.7%)	91 (33.5%)	148 (29.6%)	
Fat mass (kg)	29.2 (12.6)	27.8 (14.6)	26.6 (13.4)	0.322
Fat mass (%)	32.0 (7.7)	31.5 (10.0)	31.3 (9.5)	0.924
WHR	0.944 (0.100)	0.935 (0.087)	0.923 (0.088)	0.133
Visceral fat (cm ³)	151.9 (114.3)	115.9 (89.7)	101.7 (71.8)	0.264
Visceral fat (%)	42.5 (14.0)	38.1 (13.9)	36.2 (10.7)	0.107
Subcutaneous fat (cm ³)	179.8 (82.2)	167.7 (79.7)	167.0 (82.8)	0.693
Subcutaneous fat (%)	57.5 (14.0)	61.9 (13.9)	63.6 (11.2)	0.110
Visceral/subcutaneous fat ratio	0.847 (0.478)	0.736 (0.575)	0.623 (0.345)	0.105
Frequency of prediabetes or diabetes				
Yes	19 (61.3%)	148 (53.6%)	241 (47.4%)	0.118
No	12 (38.7%)	128 (46.4%)	267 (52.6%)	
Fasting blood glucose level (mg/dl)	104.5 (31.8)	98.2 (24.0)	94.7 (17.2)	0.037
Blood glucose level at 30' of OGTT (mg/dl)	151.1 (32.0)	148.8 (37.3)	145.2 (35.8)	0.217
Daily energy intake (kcal)	1575.1 (1017.8)	1825.0 (697.5)	1774.6 (689.4)	0.514
% of daily energy from protein	19.8 (2.4)	18.7 (4.3)	19.1 (5.1)	0.329
% of daily energy from fat	27.9 (9.0)	31.1 (6.7)	31.3 (7.9)	0.563
% of daily energy from carbohydrates	50.2 (9.3)	47.6 (8.2)	47.4 (8.9)	0.644

Data presented as mean and standard deviation (SD). OGTT, oral glucose tolerance test; WHR, waist-hip ratio.

rs17782313	C/C	C/T	T/T	p value
Daily physical activity level				
Low	2 (6.5%)	22 (8.0%)	35 (6.9%)	0.528
Moderate	10 (32.3%)	53 (19.2%)	109 (21.5%)	
High	19 (61.3%)	201 (72.8%)	364 (71.7%)	
Data presented as mean and standard deviation (SD). OGTT, oral glucose tolerance test; WHR, waist-hip ratio.				

Table 2
Characteristics of participants stratified by rs12970134 genotypes.

rs12970134	A/A	A/G	G/G	p value
N (women/men)	44 (18/26)	308 (157/151)	459 (251/208)	
Genotype frequency	5.43%	37.98%	56.59%	> 0.05
BMI (kg/m ²)	30.6 (6.8)	28.8 (6.8)	28.1 (6.5)	0.010
BMI < 25.0 (kg/ m ²)	8 (18.2%)	98 (32.1%)	166 (36.5%)	0.050
BMI 25.0-29.9 (kg/ m ²)	15 (34.1%)	105 (34.4%)	157 (34.5%)	
BMI ≥ 30.0 (kg/ m ²)	21 (47.7%)	102 (33.4%)	132 (29.0%)	
Fat mass (kg)	31.3 (13.9)	27.5 (14.1)	26.4 (13.6)	0.035
Fat mass (%)	33.7 (8.2)	31.6 (9.8)	31.1 (9.7)	0.209
WHR	0.949 (0.099)	0.936 (0.089)	0.921 (0.086)	0.021
Visceral fat (cm ³)	145.6 (111.7)	111.6 (82.6)	102.9 (75.0)	0.235
Visceral fat (%)	39.4 (15.3)	37.8 (13.1)	36.4 (10.9)	0.685
Subcutaneous fat (cm ³)	196.4 (74.5)	165.5 (77.2)	167.0 (85.2)	0.075
Subcutaneous fat (%)	60.6 (15.3)	62.2 (13.1)	63.5 (11.4)	0.693
Visceral/subcutaneous fat ratio	0.777 (0.527)	0.712 (0.533)	0.630 (0.355)	0.672
Frequency of prediabetes or diabetes				
Yes	26 (57.8%)	162 (52.3%)	221 (47.8%)	0.285
No	19 (42.2%)	148 (47.7%)	241 (52.2%)	
Fasting blood glucose level (mg/dl)	104.1 (33.1)	97.8 (22.2)	94.5 (17.4)	0.038
Blood glucose level at 30' of OGTT (mg/dl)	148.8 (33.4)	149.3 (36.3)	144.6 (36.4)	0.119
Daily energy intake (kcal)	1599.3 (887.3)	1831.3 (700.8)	1780.0 (683.7)	0.316
% of daily energy from protein	20.5 (4.1)	18.6 (4.1)	19.0 (5.2)	0.130
% of daily energy from fat	29.1 (7.4)	31.1 (7.1)	31.3 (7.7)	0.490
% of daily energy from carbohydrates	47.9 (8.3)	47.9 (8.4)	47.4 (8.8)	0.691
Data presented as mean and standard deviation (SD). OGTT, oral glucose tolerance test; WHR, waist-hip ratio.				

rs12970134	A/A	A/G	G/G	p value
Daily physical activity level				
Low	4 (8.9%)	21 (6.8%)	35 (7.6%)	0.623
Moderate	13 (28.9%)	61 (19.7%)	99 (21.4%)	
High	28 (62.2%)	228 (73.5%)	328 (71.0%)	
Data presented as mean and standard deviation (SD). OGTT, oral glucose tolerance test; WHR, waist-hip ratio.				

Table 3
 Characteristics of participants stratified by rs633265 genotypes.

rs633265	G/G	G/T	T/T	p value
N (women/men)	278 (151/127)	399 (213/186)	130 (59/71)	
Genotype frequency	34.45%	49.44%	16.11%	> 0.05
BMI (kg/m ²)	27.9 (6.3)	28.6 (6.8)	28.9 (6.4)	0.134
BMI < 25.0 (kg/ m ²)	100 (36.2%)	140 (35.4%)	32 (24.8%)	0.219
BMI 25.0-29.9 (kg/ m ²)	93 (33.7%)	132 (33.4%)	51 (39.5%)	
BMI ≥ 30.0 (kg/ m ²)	83 (30.1%)	123 (31.1%)	46 (35.7%)	
Fat mass (kg)	26.1 (12.4)	27.5 (14.8)	27.6 (13.1)	0.535
Fat mass (%)	31.1 (9.1)	31.7 (10.3)	31.1 (8.6)	0.786
WHR	0.924 (0.087)	0.928 (0.089)	0.936 (0.088)	0.474
Visceral fat (cm ³)	101.9 (68.5)	107.0 (82.0)	123.8 (95.4)	0.379
Visceral fat (%)	36.5 (10.9)	36.7 (12.2)	39.4 (13.9)	0.163
Subcutaneous fat (cm ³)	167.6 (81.4)	166.7 (80.9)	170.5 (84.3)	0.935
Subcutaneous fat (%)	63.5 (10.9)	63.1 (12.8)	60.7 (13.8)	0.169
Visceral/subcutaneous fat ratio	0.635 (0.368)	0.665 (0.484)	0.749 (0.471)	0.164
Frequency of prediabetes or diabetes				
Yes	132 (46.8%)	203 (50.8%)	73 (55.7%)	0.244
No	150 (53.2%)	197 (49.2%)	58 (44.3%)	
Fasting blood glucose level (mg/dl)	98.1 (21.2)	96.8 (22.6)	94.5 (16.7)	0.144
Blood glucose level at 30' of OGTT (mg/dl)	152.6 (38.7)	147.8 (36.1)	142.0 (34.7)	0.023
Daily energy intake (kcal)	1818.8 (740.8)	1796.5 (675.8)	1733.0 (668.6)	0.715
% of daily energy from protein	19.1 (5.3)	18.7 (4.5)	19.2 (4.5)	0.502
% of daily energy from fat	32.1 (8.1)	30.5 (7.1)	31.4 (7.2)	0.120
% of daily energy from carbohydrates	47.0 (9.3)	47.9 (8.4)	47.2 (8.0)	0.535
Data presented as mean and standard deviation (SD). OGTT, oral glucose tolerance test; WHR, waist-hip ratio.				

rs633265	G/G	G/T	T/T	p value
Daily physical activity level				
Low	15 (5.3%)	36 (9.0%)	9 (6.9%)	0.401
Moderate	66 (23.4%)	79 (19.8%)	26 (19.8%)	
High	201 (71.3%)	285 (71.2%)	96 (73.3%)	
Data presented as mean and standard deviation (SD). OGTT, oral glucose tolerance test; WHR, waist-hip ratio.				

Table 4
 Characteristics of participants stratified by rs1350341 genotypes.

rs1350341	A/A	A/G	G/G	p value
N (women/men)	127 (59/68)	390 (207/183)	274 (149/125)	
Genotype frequency	16.06%	49.30%	34.64%	> 0.05
BMI (kg/m ²)	29.0 (6.5)	28.7 (7.0)	27.9 (6.3)	0.128
BMI < 25.0 (kg/ m ²)	31 (24.6%)	136 (35.2%)	100 (36.8%)	0.196
BMI 25.0-29.9 (kg/ m ²)	51 (40.5%)	131 (33.9%)	91 (33.5%)	
BMI ≥ 30.0 (kg/ m ²)	44 (34.9%)	119 (30.8%)	81 (29.8%)	
Fat mass (kg)	27.7 (13.2)	27.4 (15.0)	26.1 (12.5)	0.484
Fat mass (%)	31.2 (8.6)	31.5 (10.3)	31.1 (9.1)	0.890
WHR	0.934 (0.089)	0.927 (0.088)	0.923 (0.087)	0.507
Visceral fat (cm ³)	124.9 (96.8)	106.1 (81.4)	101.7 (69.1)	0.386
Visceral fat (%)	39.1 (14.0)	36.7 (12.3)	36.6 (11.0)	0.294
Subcutaneous fat (cm ³)	173.3 (84.9)	165.3 (80.2)	165.8 (80.2)	0.734
Subcutaneous fat (%)	61.0 (13.9)	63.1 (12.9)	63.4 (11.0)	0.304
Visceral/subcutaneous fat ratio	0.741 (0.476)	0.669 (0.490)	0.638 (0.371)	0.296
Frequency of prediabetes or diabetes				
Yes	69 (53.9%)	195 (50.0%)	129 (46.7%)	0.385
No	59 (46.1%)	195 (50.0%)	147 (53.3%)	
Fasting blood glucose level (mg/dl)	97.4 (20.7)	95.9 (17.9)	94.3 (16.2)	0.198
Blood glucose level at 30' of OGTT (mg/dl)	152.4 (38.7)	148.3 (36.0)	142.0 (34.8)	0.021
Daily energy intake (kcal)	1741.1 (670.7)	1789.2 (674.1)	1823.0 (744.7)	0.742
% of daily energy from protein	19.2 (4.6)	18.7 (4.3)	19.1 (5.4)	0.604
% of daily energy from fat	31.4 (7.1)	30.5 (7.2)	32.1 (8.1)	0.097
% of daily energy from carbohydrates	47.2 (8.1)	48.0 (8.4)	47.0 (9.3)	0.476
Data presented as mean and standard deviation (SD). OGTT, oral glucose tolerance test; WHR, waist-hip ratio.				

rs1350341	A/A	A/G	G/G	p value
Daily physical activity level				
Low	8 (6.2%)	34 (8.7%)	15 (5.4%)	0.524
Moderate	27 (21.1%)	76 (19.5%)	62 (22.5%)	
High	93 (72.7%)	280 (71.8%)	199 (72.1%)	
Data presented as mean and standard deviation (SD). OGTT, oral glucose tolerance test; WHR, waist-hip ratio.				

Dietary intake. To study interactions between genetic factors and diet, participants were divided into two quantiles as follows: lower and upper quantiles of average protein intake (protein \leq 18% and $>$ 18% of total energy intake, respectively), lower and upper quantiles of average fat intake (fat \leq 30% and $>$ 30% of total energy intake, respectively), and lower and upper quantiles of carbohydrate intake (carbohydrates \leq 48% and $>$ 48% of total energy intake, respectively).

Associations between the rs17782313 polymorphism and obesity, its comorbidities, and dietary intake.

We found significant differences in BMI between genotypes of rs17782313 (Table 1). CC genotype carriers, who presented the highest BMI, also had the highest fasting blood glucose levels (Table 1). Further analysis showed that CC genotypes carriers had significantly lower subcutaneous fat content (Fig. 1A), higher visceral fat content (Fig. 1B), higher VAT/SAT ratio (Fig. 1C), higher fasting blood glucose (Fig. 1D) and triglyceride levels (Fig. 1E). Among participants in the upper protein intake quantiles ($>$ 18% of daily energy intake), we noted that CC genotype carriers had higher skeletal muscle mass (SMM) content (Fig. 2A) and lower subcutaneous fat content (Fig. 2B); however, they had higher visceral fat content (Fig. 2C), VAT/SAT ratios (Fig. 2D), fasting blood glucose (Fig. 2E) as well as triglyceride levels (Fig. 2F) levels. We could not analyse participants from the lower protein intake quantiles (\leq 18% of daily energy intake) due to too few genotype CC carriers in the lower quantiles. Comparing participants in the upper protein intake quantiles with participants in the lower protein intake quantiles revealed that higher protein intake was associated with higher total body fat content also in CT and TT genotype carriers (Fig. 3A). Additionally, the diet energy intake of individuals in the upper protein intake quantiles with CT and TT gene variants was significantly lower than the values of individuals from the lower quantiles of protein intake (Fig. 3B). Among participants in the upper fat intake quantiles ($>$ 30% of daily energy intake), carrying the TT genotype was associated with lower subcutaneous fat content (Fig. 3C) but higher visceral fat content (Fig. 3D) and higher VAT/SAT ratio (Fig. 3E). Carriers of the TT genotype in the upper carbohydrate intake quantiles ($>$ 48% of daily energy intake) had lower body weight (Fig. 3F), lower waist circumference (Fig. 3G), lower fasting insulin levels (Fig. 3H), as well as lower HOMA-IR (Fig. 3I) compared to those in the lower carbohydrate intake quantiles (\leq 48% of daily energy intake).

Linear regression models (including possible covariates) showed a significant interaction between rs17782313 CC genotype and dietary factors. The increase of energy derived from proteins by CC carriers was associated with higher BMIs (Est. 5.74, $R^2 = 0.12$, $p = 0.03$), higher body fat content (Est. 8.44, $R^2 = 0.82$, $p = 0.001$), higher visceral fat content (32.59, $R^2 = 0.06$, $p < 0.001$), and higher VAT/SAT ratios (Est. 0.96, $R^2 = 0.05$, $p < 0.001$). These results were significant after adjustment for age, sex, BMI (when applicable) and total energy intake. Additionally, in the same group of CC carriers, we observed lower SMM content (Est. -9.97, $R^2 = 0.79$, $p = 0.001$) and lower subcutaneous fat content (Est. -32.59, $R^2 = 0.06$, $p < 0.001$).

Associations between the rs12970134 polymorphism and obesity, its comorbidities, and dietary intake.

We found significant differences in BMI, body fat mass, WHR and fasting blood glucose levels among rs12970134 genotypes (Table 2). Further analysis showed that AA genotype carriers had significantly higher BMIs (Fig. 4A), total body fat content (Fig. 4B), subcutaneous fat content (Fig. 4C), and waist circumferences (Fig. 4D). In participants in the upper protein intake quantiles, we found that AA genotype carriers had higher BMIs (Fig. 5A), percentage of total body fat content (Fig. 5B), subcutaneous fat content (Fig. 5C) and visceral fat volume (Fig. 5D), waist circumference (Fig. 5E), fasting blood glucose (Fig. 5F), triglyceride levels (Fig. 5G) and total cholesterol levels (Fig. 5H). We could not analyse participants in the lower protein intake quantiles due to too few AA genotype carriers. We also noted that AA carriers had higher BMI (Fig. 6A), body fat content (Fig. 6B), subcutaneous fat volume (Fig. 6C), hip circumference (Fig. 6D), fasting blood glucose (Fig. 6E) and triglyceride levels (Fig. 6F), mostly independent of dietary fat intake. Additionally, BMI (Fig. 7A), total body fat content (Fig. 7B), subcutaneous fat volume (Fig. 7C), fasting blood glucose (Fig. 7D) and triglyceride levels (Fig. 7E) were higher in AA genotype carriers, mostly independent from dietary carbohydrate intake.

A comparison of participants in the upper protein intake quantiles with those in the lower protein intake quantiles revealed higher total body fat percentage in participants in the higher protein intake quantiles also in GG genotype carriers (Fig. 8A). We could not analyse participants in the lower protein intake quantiles due to too few AA genotype carriers. The diet total energy intake of AG and GG participants in the upper protein intake quantiles was significantly lower than the diet total energy intake of individuals in the lower protein intake quantiles (Fig. 8B). GG genotype carriers in the upper protein intake quantiles had higher fasting insulin levels (Fig. 8C) and HOMA-IR values (Fig. 8D) compared to carriers of the same genotype but in lower protein intake quantiles. AG and GG genotype carriers in the upper fat intake quantiles had lower subcutaneous body fat (Fig. 8E) and higher visceral body fat content (Fig. 8F). AG and GG individuals in the upper carbohydrate intake quantiles had lower body weight (Fig. 8G), waist circumference (Fig. 8H), fasting insulin levels (Fig. 8I) as well as HOMA-IR values (Fig. 8J) compared to carriers of the same genotypes in the lower carbohydrate intake quantiles.

Linear regression models showed a significant interaction between genotype and dietary factors. The increase of energy derived from proteins by AA rs12970134 carriers was associated with lower SMM content (Est. -6.38, $R^2 = 0.79$, $p = 0.016$) and subcutaneous fat content (Est. -32.59, $R^2 = 0.06$, $p = 0.03$) but

higher visceral fat content (Est. 19.95, $R^2 = 0.06$, $p = 0.003$) and VAT/SAT ratio (Est. 0.64, $R^2 = 0.05$, $p = 0.006$). These results were significant after adjustment for age, sex, BMI and total energy intake.

Associations between the rs633265 polymorphism and obesity, its comorbidities, and dietary intake. We observed that carriers of the GG rs633265 genotype (Table 3) had the highest blood glucose levels at 30 minutes of OGTT; no other differences were observed among genotypes. Further analysis showed that GG carriers had significantly lower BMI values than TT carriers (Fig. 9A). Among participants in the lower protein intake quantiles, GG genotype carriers had lower blood glucose levels at 30 minutes (Fig. 10A) and 60 minutes (Fig. 10B) of OGTT, and lower HbA1c (Fig. 10C). Additionally, GG carriers in the lower carbohydrate intake quantiles had lower blood glucose levels at fasting (Fig. 10D) and 30 minutes of OGTT (Fig. 10E), lower HbA1c levels (Fig. 10F), and higher HOMA-B values (Fig. 10G).

Comparison of participants in the upper and lower protein intake quantiles showed that GG genotype carriers in the upper protein intake quantiles had higher percentages of total body fat content (Fig. 11A). In individuals in the upper protein intake quantiles, diet total energy intake was significantly lower compared to individuals in the lower intake quantiles (Fig. 11B). Additionally, the GG carriers in the upper protein intake quantiles had higher fasting insulin levels (Fig. 11C), HOMA-IR (Fig. 11D), volume of subcutaneous fat tissue (Fig. 11E), and waist circumference (Fig. 11F). Furthermore, body weight (Fig. 11G) and waist circumference (Fig. 11H) were lower when GG genotype carriers were stratified to the upper carbohydrate intake quantiles compared to carriers of the same genotype from lower quantiles, even if diets did not differ in total energy content (data not shown).

Association between the rs1350341 polymorphism and obesity, its comorbidities, and dietary intake. Similar to the previously described rs633265, AA rs1350341 carriers (Table 4) had the highest blood glucose levels at 30 minutes of OGTT; no differences were observed among other genotypes. Further analysis showed that GG carriers had significantly lower BMI values than individuals with the AA genotype (Fig. 12A). Among participants from the lower protein intake quantiles, we found that GG carriers had lower blood glucose levels at 30 minutes (Fig. 13A) and at 60 minutes (Fig. 13B) of OGTT, as well as lower HbA1c (Fig. 14C). Additionally, GG carriers from the upper fat intake quantiles and the lower carbohydrate intake quantiles had significantly lower blood glucose levels at 30 minutes of OGTT (Fig. 13D and Fig. 13E, respectively).

Comparison of participants in the upper and lower protein intake quantiles showed that GG carriers from the upper quantiles had higher BMI values (Fig. 14A) and higher percentage of body fat content (Fig. 14B). The diet total energy intake of individuals from the upper protein intake quantiles was significantly lower compared to the diet energy value of individuals from the lower quantiles of protein intake (Fig. 14C), independent of genotypes. Blood glucose levels at 120 minutes of OGTT were significantly higher in both GG and AA genotypes carriers (Fig. 14D) from the upper protein intake quantiles. GG carriers in the upper protein intake quantiles also had higher fasting insulin levels (Fig. 14E) and HOMA-IR (Fig. 14F). In addition, GG carriers in the upper protein intake quantiles had higher subcutaneous fat tissue volumes (Fig. 14G) as well as waist (Fig. 14H) and hip (Fig. 14I) circumferences.

Body weight (Fig. 14J), waist circumference (Fig. 14K) and muscle mass (Fig. 14L) were lower in GG carriers in the upper carbohydrate quantiles compared to carriers in the lower carbohydrate intake quantiles. Additionally, we observed lower skeletal muscle mass in AG and GG genotypes carriers in the upper carbohydrate intake quantiles (Fig. 14L).

Discussion

The central melanocortin system critically regulates energy balance by influencing food intake and energy expenditure. Moreover, the MC4R pathway seems to be involved in glycaemic homeostasis, independently of its effects on body weight and composition(22). This study demonstrated that the effects of *MC4R* genetic variants on body mass, body composition, as well as some comorbidities of obesity, depend on dietary factors. Results from previous studies have suggested that *MC4R* SNPs may influence dietary habits, which may explain the higher body mass and body fat content. However, our study participants did not differ by genotype in daily energy and diet macronutrient intake or daily physical activity levels. Since we did not observe these genotype differences, we can exclude the impact of different macronutrient intake on *MC4R* gene expression and activation of melanocortin pathways, as has been previously reported by Lauria et. al(36).

In the present study, we observed associations between SNPs in rs17782313 with BMI and fasting blood glucose levels. Moreover, we found that carriers of CC genotypes presented with lower subcutaneous fat content, higher visceral fat content and higher VAT/SAT ratio compared to TT genotype carriers. The metabolic consequences of these differences include higher fasting blood glucose and triglycerides levels, which we have also observed in this group. Our results are in line with a previous study by Qi et al. (28), who found that carrying a C-allele is associated with increased BMI and 14% increased risk of type 2 diabetes. Zobel et al.(45) also found associations between rs17782313 genotypes and BMI, but did not observe any differences in glucose homeostasis. In our previous study, consisting of participants from the same cohort group, we observed differences in body fat distribution without finding any differences in BMI between genotypes(31). These discrepancies may have arisen from differences in the groups chosen for analysis. Here, it is important to note that we choose our study group based on the availability of data to analyse in order to maximize the number of study participants included in every analysis. This prompted us to other factors that might influence our results. We did not observe any differences in daily dietary intake between genotypes, but we found metabolic disturbances that are significantly different in individuals from the upper dietary protein intake, so in those, whose daily percentage of energy intake from protein exceeds 18%. It is worth to notice that the mean energy value of diets from upper quantiles of protein intake was significantly lower compared to the lower intake quantiles across all studied genotypes examined. Our results are in contrast with findings from Qi et al.(28), who observed significant differences between rs17782313 genotypes in total energy, fat and protein intake, as well as association with BMI, which was independent of dietary intake. However, only women were included in that study, which may partially explain the different results. This is important to consider because there are both significant sex differences in dietary intakes as well as sexually dimorphic effects of diet on health(46). In another study, Hasselbalch et al.(34) did not observe any significant associations between these SNPs

near *MC4R*, food intake and food preferences. Actually, we could not exclude the genotype differences also in the participants in the lower protein intake quantiles, since we could not compare it due to too few CC genotype carriers in the lower protein intake quantiles group. Moreover, we have noted higher body fat mass content also among individuals carrying CT and TT genotypes in the upper protein intake quantiles, compared to the same genotypes in the lower quantiles. Therefore, we constructed linear regression models and found that the increase of percentage of energy derived from proteins in CC carriers of rs17782313 was associated with higher BMI and body fat content, as well as higher visceral fat content and VAT/SAT ratio. The distribution of body fat tissue is of crucial importance, since visceral adipocytes are more metabolically active and may lead to the insulin resistance (IR) development. VAT is associated not only with metabolic disturbances but also with all-cause mortality(47, 48). Moreover, higher VAT/SAT ratios, observed in AA genotype carriers, may be associated with increased metabolic and cardiovascular risk, independent from BMI and visceral fat content(49). Results from analyses of protective TT genotype carriers revealed that being in the upper quantiles of fat intake was associated with lower subcutaneous fat deposition but higher visceral fat content and VAT/SAT ratios. Intriguingly, carriers of the protective TT genotype in the upper carbohydrate intake quantiles had significantly lower body weight, waist circumference, fasting insulin levels, as well as HOMA-IR, what indicate higher insulin sensitivity of these individuals when in their diet more than 48% of energy was derived from carbohydrates. This is one of the most crucial observations of our study, which is salient in light of current interest in carbohydrate-restricted diets among the general population. Surprisingly, we did not observe any differences, which would be dependent on percentages of energy derived from dietary fat and carbohydrates in CC genotype carriers. It suggests that the impact of dietary fat and carbohydrates intake may not be crucial for the risk of obesity, in carriers of this high-risk genotype. The practical implication of these results may be that individuals with the CC genotype should avoid diets with > 18% of daily energy from protein, while the total dietary fat in the diets of TT genotype carriers should provide less than 30% of daily energy intake, and more than 48% of energy should be derived from carbohydrates. Ortega-Azorin et al.(50) found that adherence to the Mediterranean diet was effective in reducing risk of type 2 diabetes in carriers of the risk variant alleles of *MC4R* rs17782313. These observations may support our results if we consider that the Mediterranean diet is a predominantly plant-based diet, in which animal proteins (especially from meat) are consumed in very small amounts and come mostly from nuts, fish, and dairy sources(51, 52). Additionally, the Mediterranean diet includes fat comprising between 35% and 45% of energy intake, in which monounsaturated fatty acids (MUFAs) provide at least 50% of total fat content, mostly from the consumption of extra virgin olive oil(51).

The findings of our study also show that an AA genotype of rs12970134 was associated with increased BMI, body fat mass, WHR and fasting blood glucose levels in our participants. These observations are in line with our previous findings(31) as well as results from a study by Zobel et al.(45), who found that the A-allele was associated with obesity, morbid obesity and abdominal obesity. Moreover, AA genotype carriers following a diet with > 18% of total energy from protein not only had higher BMI, body fat mass, subcutaneous fat content and waist circumference but also higher visceral fat volume, higher fasting blood glucose, triglycerides and total cholesterol levels, even if the energy value of the diet from the upper

quantiles of protein intake was significantly lower compared to the diet from the lower quantiles of protein intake. Moreover, participants with the AA genotype had higher values of metabolic parameters, which were mostly independent from dietary carbohydrate and fat content. However, we also observed that GG genotype carriers from upper quantiles of protein intake had higher body fat percentages, higher fasting insulin levels and higher HOMA-IR values compared to the same genotype carriers from the lower quantiles of protein intake. This suggests that a diet providing > 18% of energy from protein may have disadvantageous effects independent from *MC4R* rs12970134 genotypes, promoting insulin resistance in individuals with the protective genotype even when the total energy intake is lower. Because we could not analyse these associations between AA genotypes from the lower quantiles of protein intake due to insufficient numbers, we constructed regression models which showed an increase of percentage of daily energy delivered from protein in AA genotype was associated with significantly lower skeletal muscle mass and subcutaneous fat mass content but higher visceral fat content and VAT/SAT ratios. The effects of visceral body fat deposition have been mentioned above, but in addition, reduction of skeletal muscle mass, may lead to development of type 2 diabetes (53), which we have previously reported on based on results from 5-years observation of our 1000PLUS Cohort Study group(39). In the present study, the AG and GG genotypes carriers had lower subcutaneous body fat and higher visceral body fat content when the percentage of daily energy from fat exceeded 30%. Koochakpoor et al.(30) noted that the risk of abdominal obesity increases across quantiles of total fat intake, but in A allele carriers, and this association was not significant for GG homozygotes participants. However, the authors also reported that the association of A allele carriers in rs12970134 with metabolic syndrome was modulated by saturated fatty acid intake, which was not analysed in our study and may help explain the different results obtained. Moreover, similar to the previously described loci near the *MC4R* gene, AG and GG genotype carriers in rs12970134 with a dietary intake of more than 48% of energy from carbohydrates had lower body weight, waist circumference, fasting insulin levels and HOMA-IR values compared to same genotype carriers from the lower quantiles of carbohydrate intake. Intriguingly, among the AA genotype carriers we did not observe any associations between investigated parameters and dietary carbohydrates content, suggesting that dietary carbohydrates may not affect the risk of obesity in high-risk genotype carriers. However, in carriers of the protective genotypes, dietary carbohydrate intake providing more than 48% of total energy may have even beneficial metabolic effects. Wang et al.(33) found that in overweight and obese children, rs12970134 is associated with appetite and beverage intake, which could indicate that rs12970134 SNPs may possibly increase adiposity by affecting eating behaviours. Nevertheless, in our study we observed higher BMI and body fat content without noting any differences in daily energy and macronutrient intake between genotypes.

The other two SNPs in rs633265 and rs1350341 investigated in our study were associated with blood glucose levels at 30 minutes of OGTT. We have previously observed that these genetic variants are associated with body fat distribution without any differences in BMI and total body fat content(31). In our previously described dietary intervention sub-study, we also found differences in postprandial glucose utilization. Comparing genotypes revealed that GG genotypes of rs633265 carriers had significantly lower BMI values than homozygous carriers with the risk allele T. When we included dietary factors in the

analysis, GG genotype participants with daily protein intake lower than 18% of total energy had lower blood glucose levels at 30 and at 60 minutes of OGTT as well as lower HbA1c. Moreover, these participants also had lower fasting blood glucose levels, and 30 minutes of OGTT, lower HbA1c, and higher HOMA-B values when the percentage of energy derived from carbohydrates was less than 48%, indicating higher insulin sensitivity and better β -cell function. Carriers of the protective genotype GG had higher body fat percentages when > 18% of daily energy was derived from protein, even if the total energy intake in diets from the upper quantiles of protein intake was significantly lower compared to the lower quantiles. In GG individuals from the upper quantile of protein intake, we also observed higher fasting insulin levels, HOMA-IR, volume of subcutaneous fat tissue and waist circumference, which can indicate higher insulin resistance. Surprisingly, lower body weight and waist circumference were observed when more than 48% of energy of the GG genotype carriers' diet was derived from carbohydrates, compared to lower quantiles of carbohydrate intake.

We observed similar associations with protein intake in homozygous carriers of protective G allele in rs1350341. Moreover, carriers of GG genotype had lower blood glucose levels at 30 minutes of OGTT also when less than 48% of total energy was derived from carbohydrates, and when more than 30% of total daily energy was derived from fat. GG genotype carriers in the upper protein intake quantiles had higher BMI values and body fat percentages, even if the total energy content in diets from upper quantiles was significantly lower compared to the lower quantiles of protein intake. When > 18% of total energy intake of GG genotype individuals was derived from protein, participants had a higher volume of subcutaneous fat tissue, waist and hip circumference, as well as higher fasting insulin levels and HOMA-IR values, indicating lower insulin sensitivity. Body weight and waist circumference were lower when GG genotype carriers followed a diet with more than 48% of total energy coming from carbohydrates, compared with lower carbohydrate intake quantiles. Our previous studies showed that GG genotype carriers had significantly higher glucose utilization after high-carbohydrate meal intake(31). Taken together, we can hypothesize that a mechanism that protects these individuals from de novo lipogenesis and fat deposition may be at play not only after high-carbohydrate meal intake but also when carbohydrates provide more than 48% of daily energy. Similar to the previously described rs633265, we did not find any other studies that we could compare our results with, because we likely analysed associations between both rs633265 and rs1350341 and dietary factors for the first time.

In summary, recommendation to decrease dietary fat < 30% is appropriate for *MC4R* carriers of protective genotypes, but in carriers of *MC4R* risk genotypes dietary total fat intake does not seem to affect metabolic parameters. In individuals carrying the protective genotypes, better metabolic effects can be expected when these subjects follow diets of more than 48% of energy from coming carbohydrates, which does not seem to influence the impact of *MC4R* high-risk genotype variants on obesity and related comorbidities. A previous study by Butler et al.(54) suggested that *MC4R* regulates metabolic and behavioural responses to high-protein and low-fat intake. In our study participants, carriers of genotypes that predispose individuals to obesity seem to be affected by dietary proteins, and with increasing dietary protein intake the adverse effects may be induced, even if total energy intake is reduced. Additionally, Huang et al.(55) found that dietary protein intake significantly modifies the effects of *MC4R* on changes

in appetite. Furthermore, carriers of high-risk genotypes may experience greater increases in appetite and craving when consuming a high-protein weight-loss diet. Because our study population had a low number of risk variant carriers in the lower protein intake quantiles, our sample was too small to conduct analysis and obtain reliable results. However, we may hypothesize that decreasing dietary protein in this group may have beneficial metabolic effects. Nevertheless, we conclude that protein intake that provides > 18% of total energy may have negative metabolic consequences. On the other hand, the impact of protective genotypes investigated in rs633265 and rs1350341 may have more beneficial effects on obesity-related comorbidities, especially glucose homeostasis, if dietary protein is maintained at less than 18% of total energy intake. This is a crucial observation from our study, since dietary proteins are considered to be a highly satiating nutrient(56), which may also improve the glycaemic response by stimulating anorexigenic gastrointestinal peptides secretion (including glucagon-like peptide-1, GLP-1)(57). Since *MC4R* functions are affected by anorexigenic hormones, and GLP-1 receptor agonists (such as liraglutide) have been recommended as an effective treatment for the most common form of monogenic obesity caused by mutations in the *MC4R* gene(58), we might expect that exposure to high-protein diets may modulate *MC4R* metabolic effects. However, we could not expect that in this manner, although it has been observed in animal model that a low-protein diet is associated with significantly lower *MC4R* gene expression in PVN (paraventricular nucleus)(59).

Further studies are needed to investigate the possible mechanisms of noted differences, as well as studies investigating whether reducing dietary protein intake provides any metabolic benefits for people at high genetic risk of obesity, carrying a high-risk genetic variant of the *MC4R* gene. Nevertheless, it is important to note that some of these genetic variations are located close to non-coding regions of DNA, and potential associations between adiposity-related traits and non-coding variations are still not clear. Non-coding regions are very intriguing, but mechanisms for these associations have yet to be clarified(60, 61).

To the best of our knowledge, this is the first study to investigate interactions between these four common *MC4R* genetic variants and macronutrient intake as well as the effect of these relationships on obesity, body fat content and obesity-related comorbidities in the context of a large population study. Further studies are needed to determine whether our findings are generalizable to other ethnic groups. Even though the best models to test gene × environment interactions are randomized clinical trials, the number of participants in such studies are limited, and there is always a risk of false-positive findings. Therefore, replication in larger and more diverse populations is needed to verify findings. A major strength of our study is that it is based on a relatively large population-based sample, including women and men with a wide age range. The dietary information in this study was based on self-reported three-day diaries of food intake. This may be a major limitation in our study, because it has been demonstrated that people, especially obese individuals, tend to underreport their food intake(62). However, dietary questionnaires and diaries are the only tools that are currently available for large population studies. The other important limitation is that for the daily physical activity evaluation we could not use accelerometers, nevertheless, the long version of IPAQ questionnaire is a validated method to verify physical activity level, with a high reproducibility. In addition, advanced statistical methods are required to

dissect and analyse complicated relationships and interactions between factors such as macronutrient intake and genetic risk.

Conclusions

Our findings provide new insights into the role of common *MC4R* SNPs and diet interactions in determining risk of obesity and its metabolic comorbidities. Our results furthermore provide supportive evidence for personalized diet recommendations. Advances in this field are expected to open new methods for developing genome-customized diets for preventing and treating obesity and obesity-related comorbidities.

List Of Abbreviations

ANOVA, analysis of variance; BMI, body mass index; FTO, fat mass and obesity-associated; GLP-1, glucagon-like peptide-1; GWAS, genome-wide association studies; HbA1c, haemoglobin A1c; HDL, high-density lipoprotein; HOMA-B, homeostatic model assessment of β -cell function; HOMA-IR, homeostatic model assessment of insulin resistance; IR, insulin resistance; IPAQ-LF, International Physical Activity Questionnaire-Long Form; LDL, low-density lipoprotein; *MC4R*, melanocortin-4 receptor; MET, metabolic equivalent; MUFA monounsaturated fatty acid; OGTT, oral glucose tolerance test; *PPAR* γ , peroxisome proliferator-activated receptor gamma; PVN, paraventricular nucleus; SAT, subcutaneous adipose tissue; SNPs, single nucleotide polymorphisms; SD, standard deviation; SMM, skeletal muscle mass; TG, triglyceride; T2DM, type 2 diabetes mellitus; VAT, visceral adipose tissue; WHR, waist-hip ratio; WHO, World Health Organization.

Declarations

Ethics approval and consent to participate.

The study protocol was approved by the local ethics committee of the Medical University of Bialystok, Poland (R-I-002/35/2009), and written informed consent was obtained from all participants.

Consent for publication.

Not applicable.

Competing Interests.

The authors have declared that no competing interests exist.

Availability of data and materials

Data and materials used in this study are available from edyta.adamska@umb.edu.pl, on reasonable request.

Competing interest.

The authors have declared that no conflict of interest exists.

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Author Contributions.

EAP, MG, AK designed research; EAP, DB, JF, MM, UK, SP, MW, LS, DL conducted research; EAP, WB analyzed data or performed statistical analysis; EAP wrote paper; WB contributed to the writing of the manuscript; EAP, WB, MG, AK had primary responsibility for final content. All Authors agree with the manuscript's results and conclusions. All authors have read, and confirm that they meet, ICMJE criteria for authorship.

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Figures

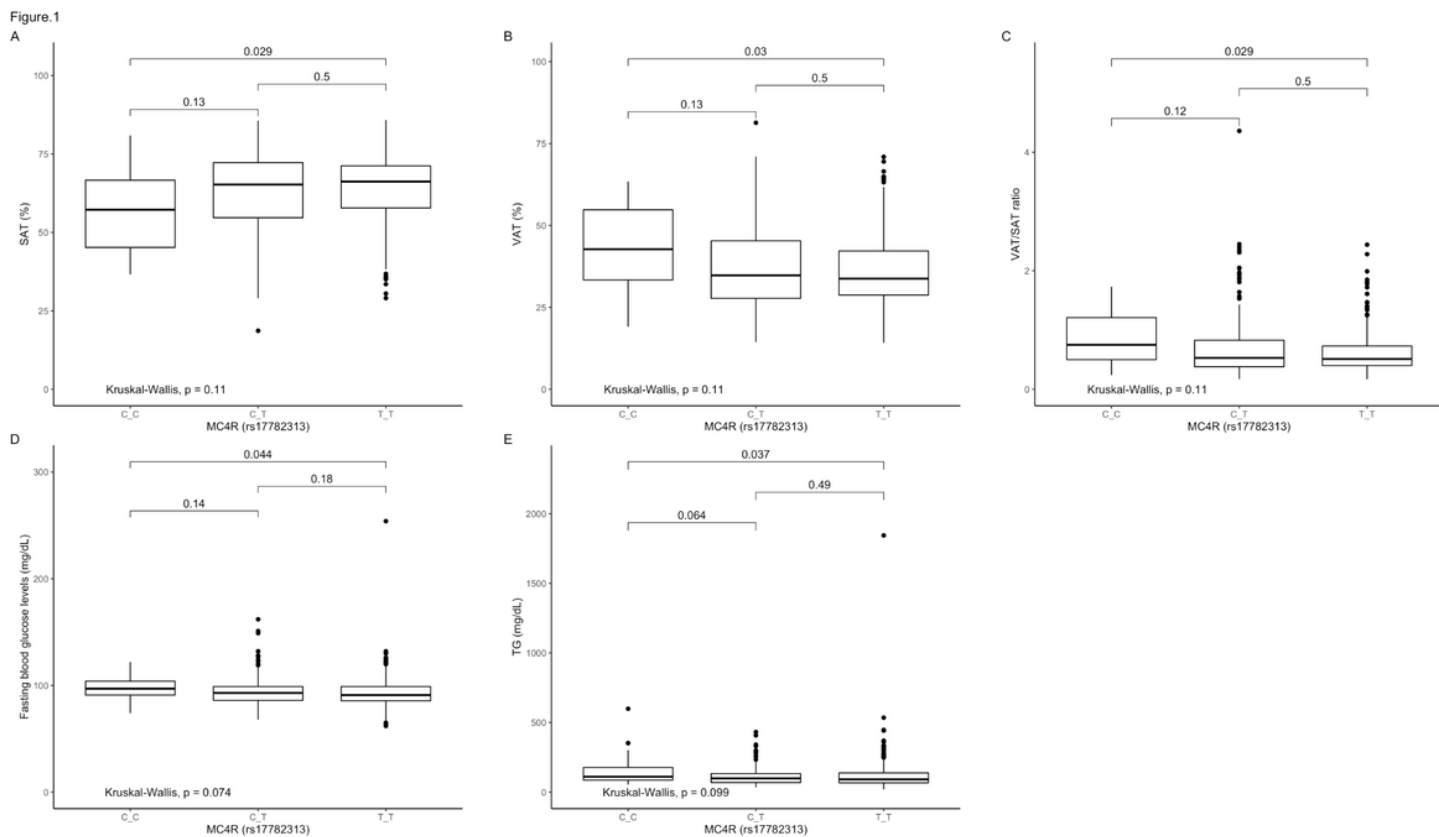


Figure 1

Association of MC4R rs17782313 genotypes with (A) SAT (%), (B) VAT (%), (C) VAT/SAT ratio, (D) fasting blood glucose levels (mg/dL) and (E) TG concentrations (mg/dL). SAT, subcutaneous adipose tissue; TG, triglycerides; VAT, visceral adipose tissue.

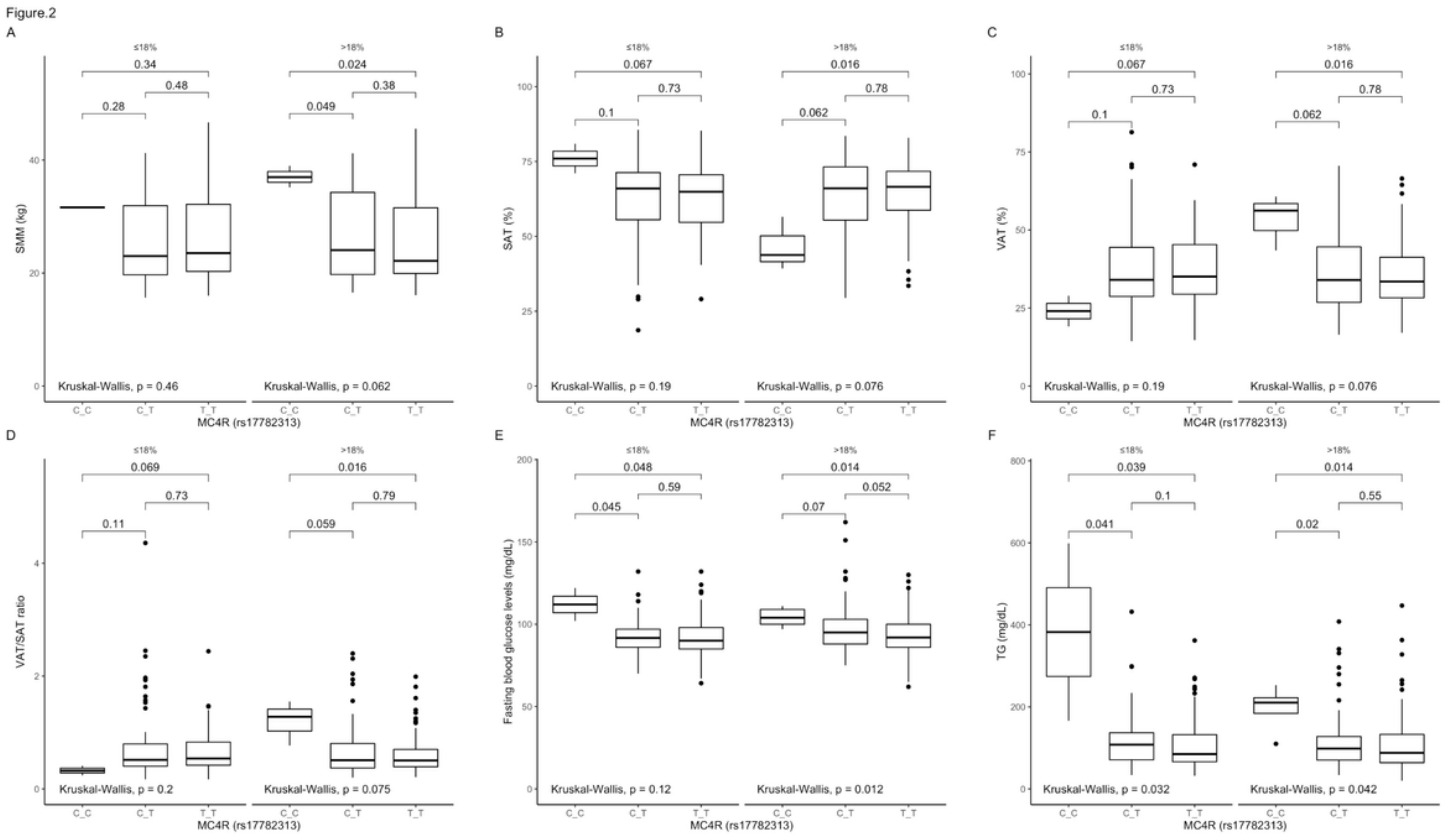


Figure 2

Association of MC4R rs17782313 genotypes with (A) SMM (kg), (B) SAT (%), (C) VAT (%), (D) VAT/SAT ratio; (E) fasting blood glucose levels (mg/dL); (F) TG concentrations (mg/dL), by dietary protein strata: $\leq 18\%$ and $> 18\%$ of total daily energy intake. SMM, skeletal muscle mass; SAT, subcutaneous adipose tissue; TG, triglycerides; VAT, visceral adipose tissue.

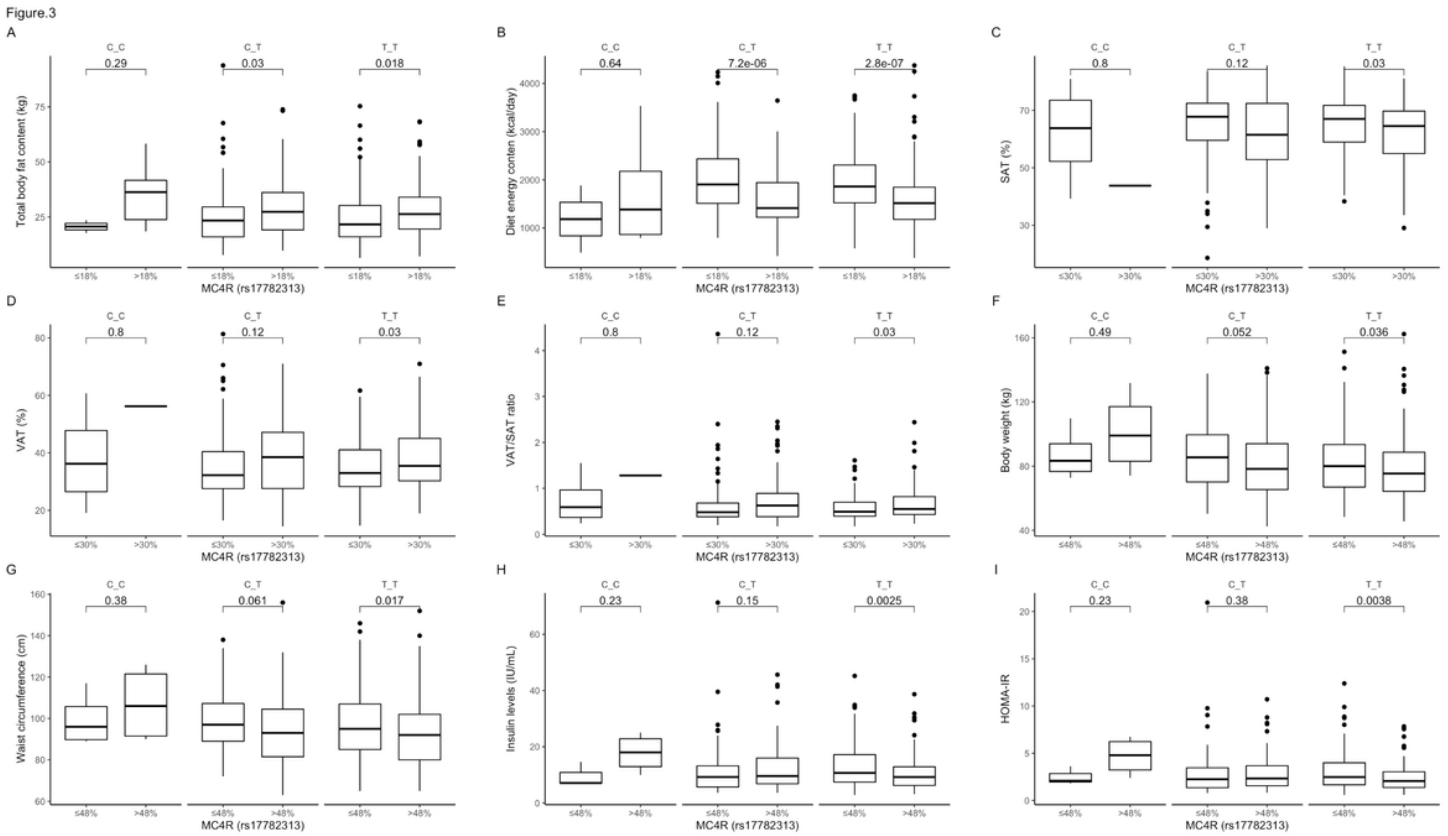


Figure 3

Association of dietary protein intake $\leq 18\%$ and $> 18\%$ of total daily energy intake with (A) total body fat content (kg) and (B) diet energy content (kcal/day), in MC4R rs17782313 genotypes carriers. Association of dietary fat intake $\leq 30\%$ and $> 30\%$ of total daily energy intake with (C) SAT (%), (D) VAT (%) and (E) VAT/SAT ratio, in MC4R rs17782313 genotypes carriers. Association of dietary carbohydrates intake $\leq 48\%$ and $> 48\%$ of total daily energy intake with (F) body weight (kg), (G) waist circumference (cm), (H) fasting insulin levels (IU/mL) and (I) HOMA-IR, in MC4R rs17782313 genotypes carriers. HOMA-IR, Homeostatic Models Assessment of Insulin Resistance; SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue.

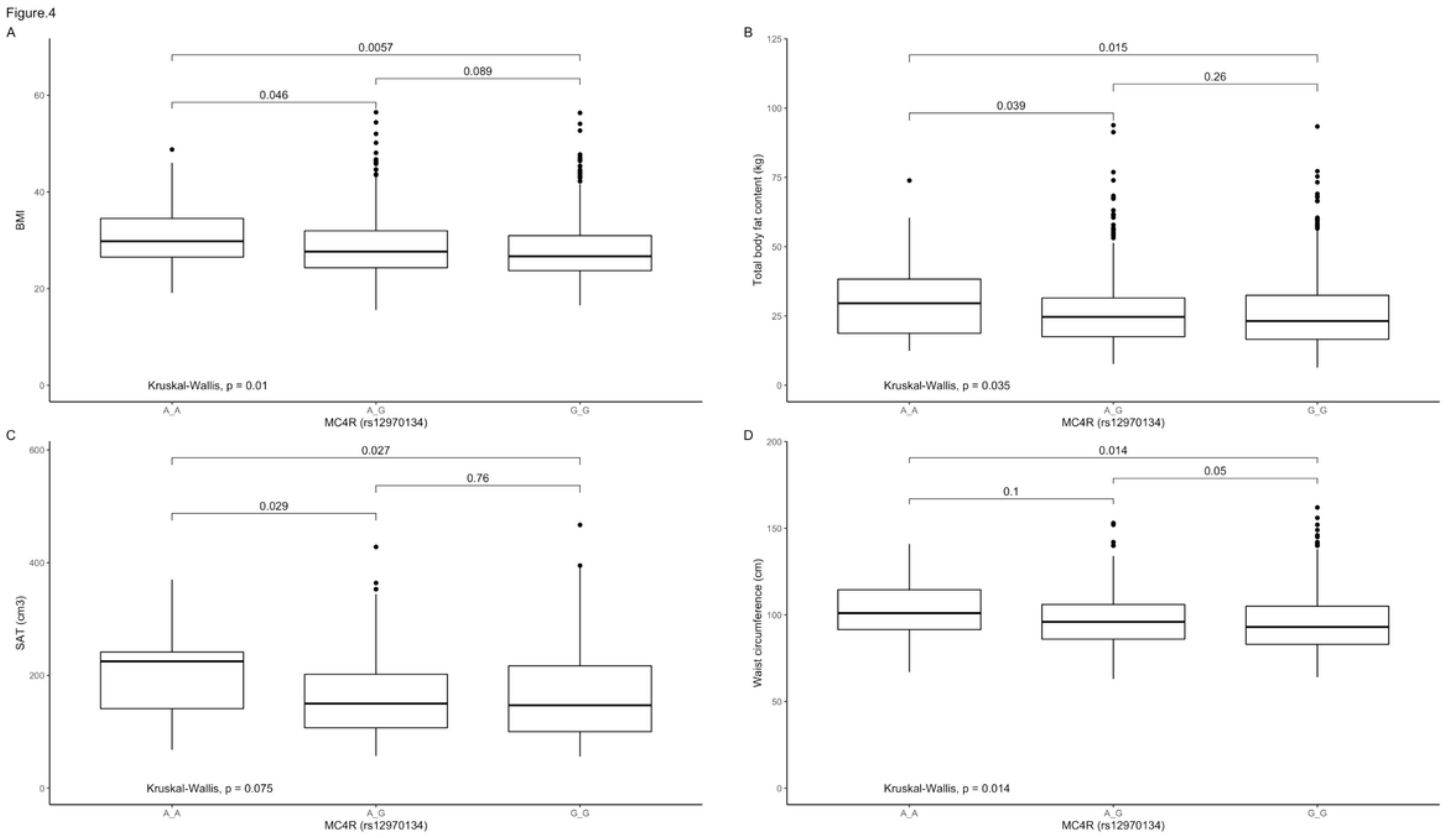


Figure 4

Association of MC4R rs12970134 genotypes with (A) BMI (kg/m²), (B) total body fat content (kg), (C) SAT (cm³) and (D) waist circumference (cm). BMI, Body Mass Index; SAT, subcutaneous adipose tissue.

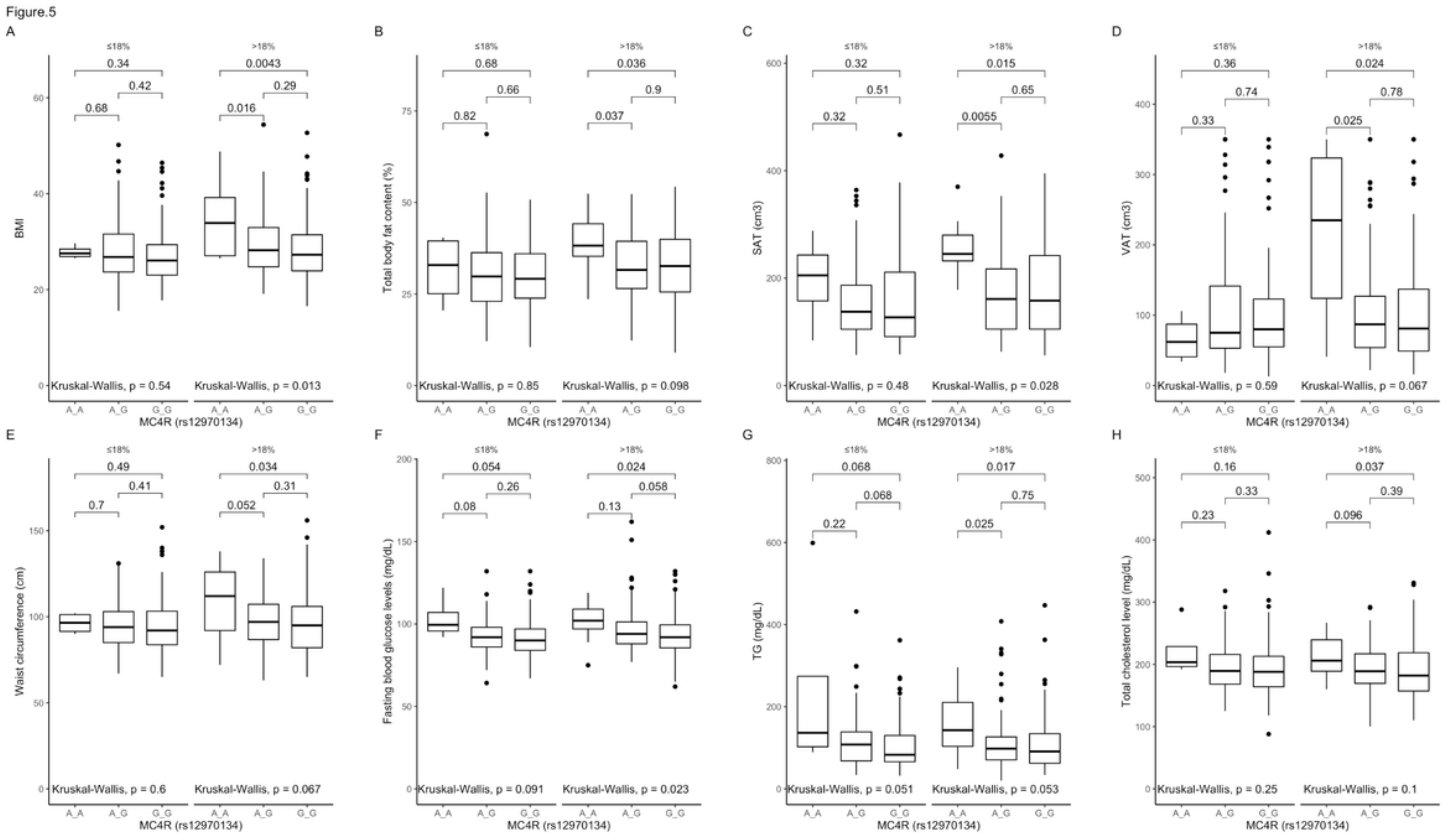


Figure 5

Association of MC4R rs12970134 genotypes with (A) BMI (kg/m²), (B) total body fat content (%), (C) SAT (cm³), (D) VAT (cm³), (E) waist circumference (cm), (F) fasting blood glucose levels (mg/dL), (G) TG concentrations (mg/dL) and (H) total cholesterol concentrations (mg/dL), by dietary protein strata: $\leq 18\%$ and $> 18\%$ of total daily energy intake. BMI, Body Mass Index; SAT, subcutaneous adipose tissue; TG, triglycerides; VAT, visceral adipose tissue.

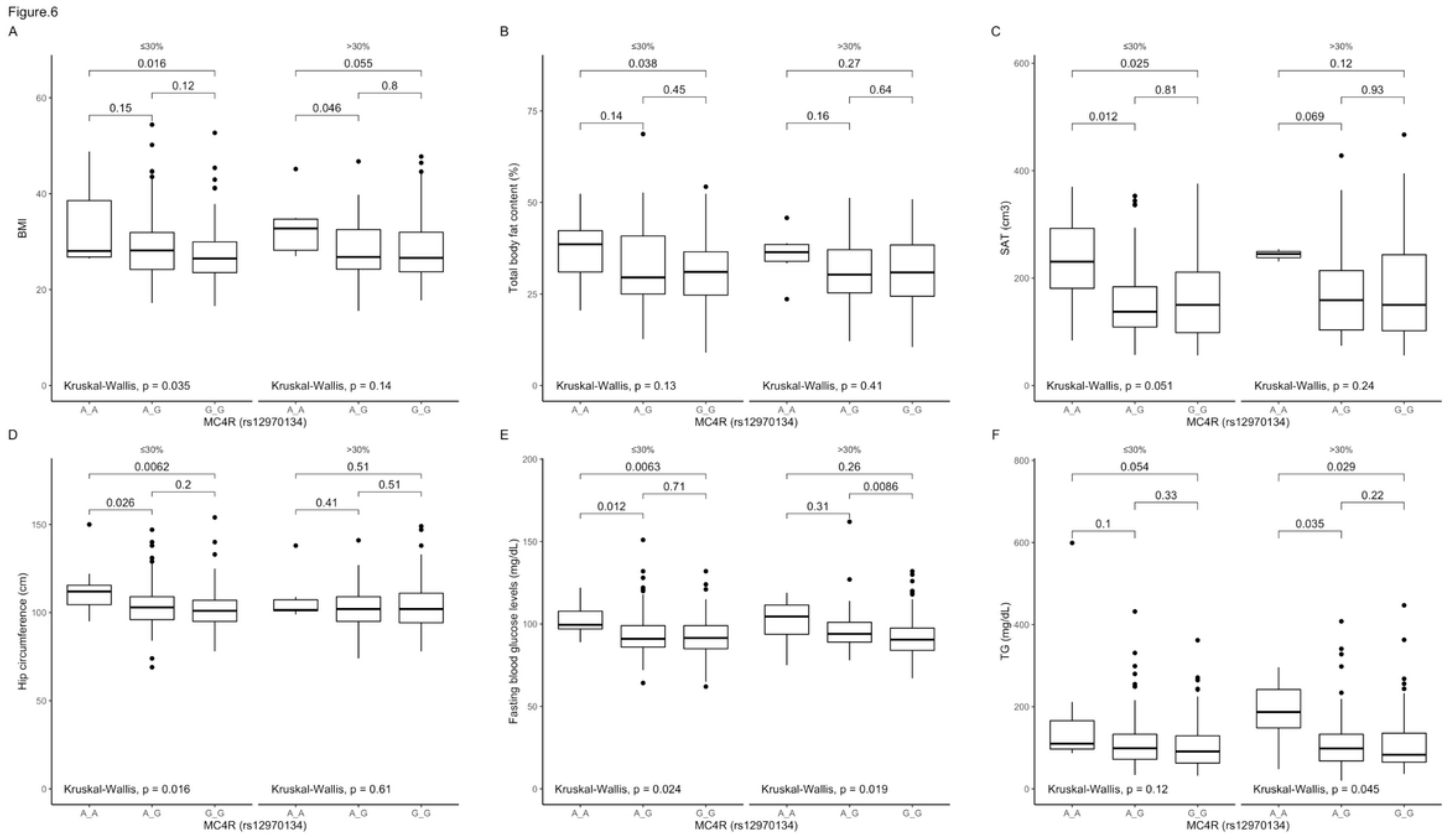


Figure 6

Association of MC4R rs12970134 genotypes with (A) BMI (kg/m²); (B) total body fat content (%); (C) SAT (cm³), (D) hip circumference (cm), (E) fasting blood glucose concentrations (mg/dL) and (F) TG concentrations (mg/dL), by dietary fat intake strata: $\leq 30\%$ and $> 30\%$ of total daily energy intake. BMI, Body Mass Index; SAT, subcutaneous adipose tissue; TG, triglycerides.

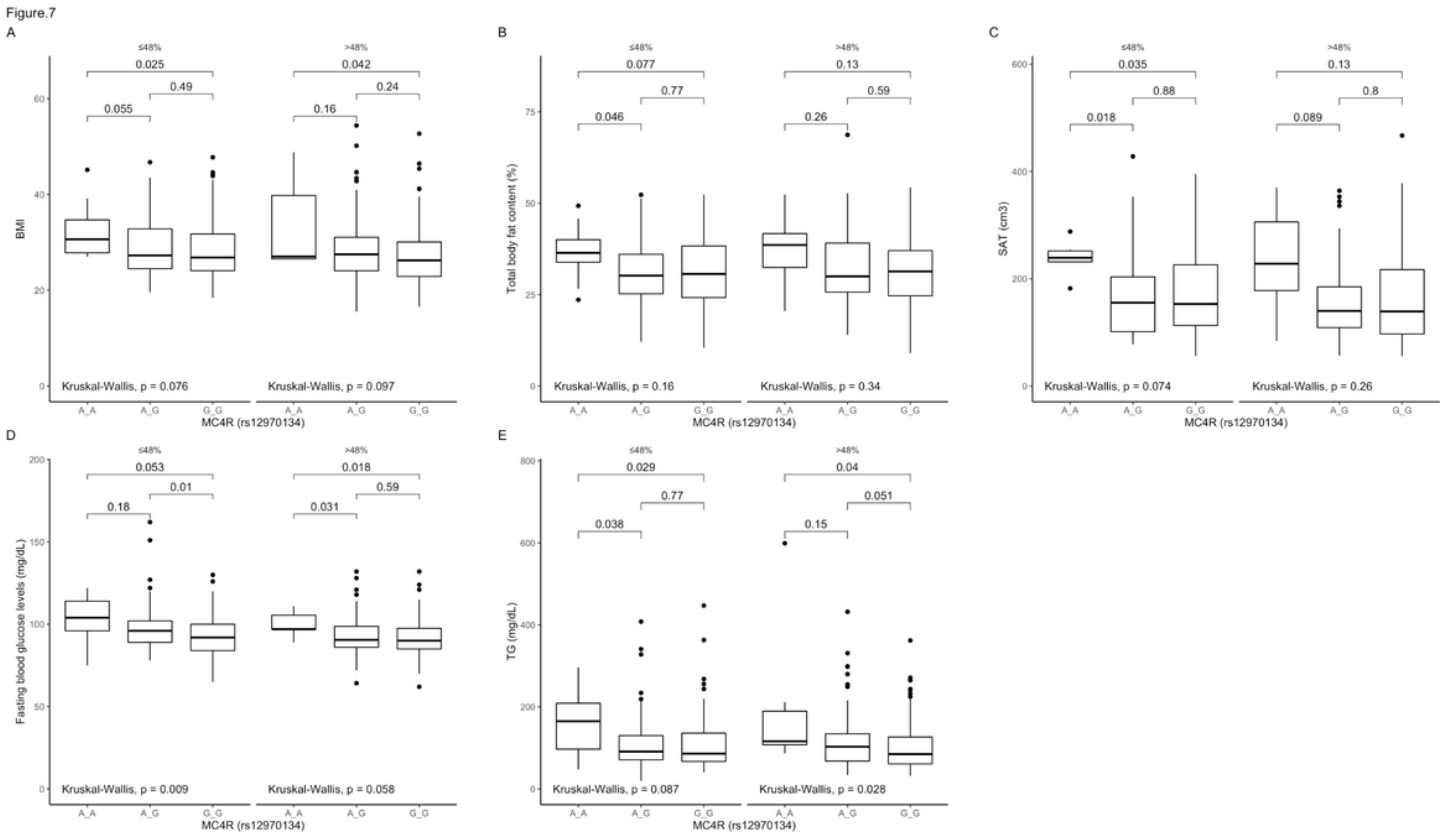


Figure 7

Association of MC4R rs12970134 genotypes with (A) BMI (kg/m²), (B) total body fat content (%), (C) SAT (cm³), (D) fasting blood glucose concentrations (mg/dL) and (E) TG concentrations (mg/dL), by dietary carbohydrates intake strata: $\leq 48\%$ and $> 48\%$ of total daily energy intake. BMI, Body Mass Index; SAT, subcutaneous adipose tissue; TG, triglycerides.

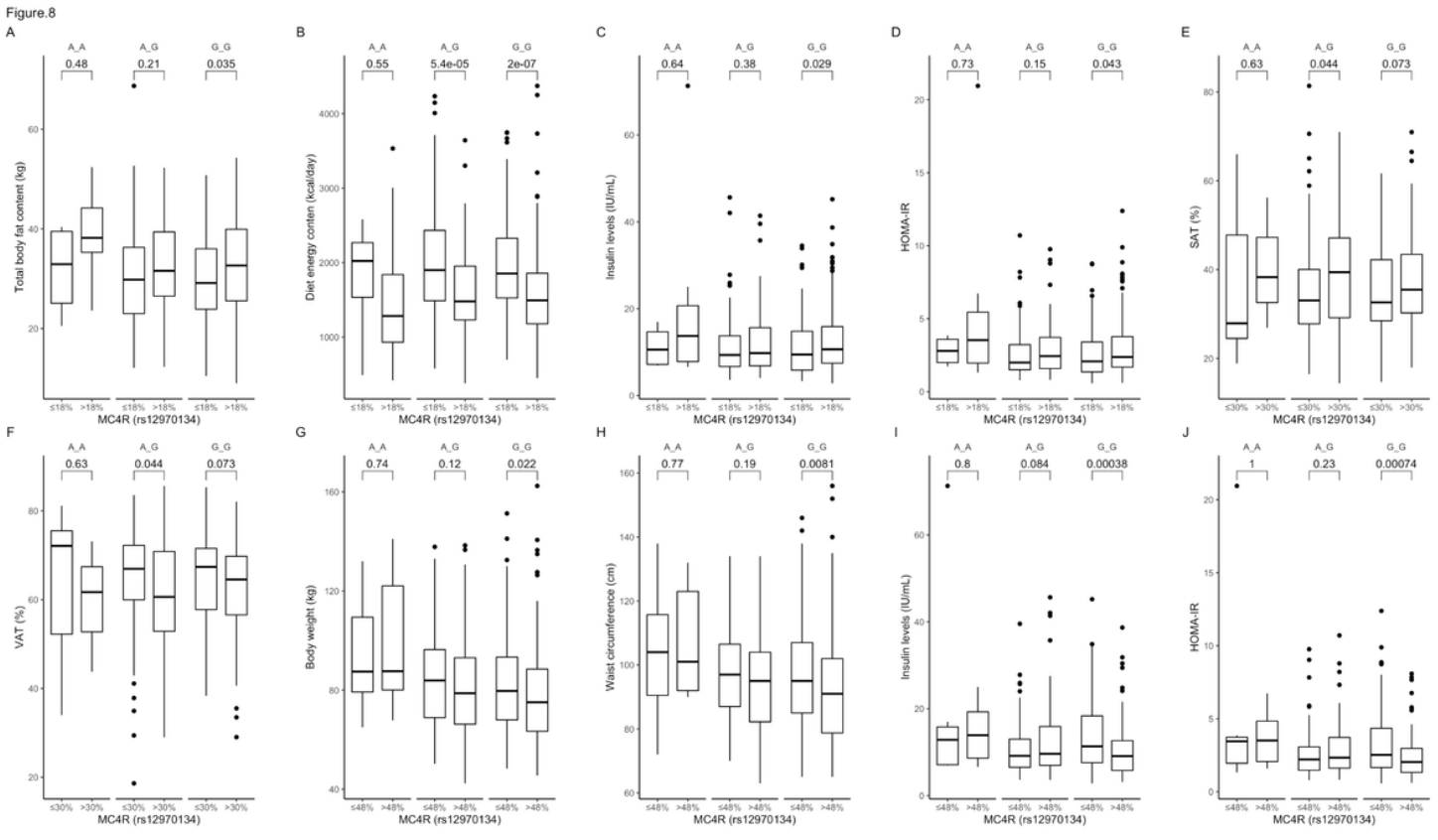


Figure 8

Association of dietary protein intake $\leq 18\%$ and $> 18\%$ of total daily energy intake with (A) total body fat content (kg), (B) diet energy content (kcal/day), (C) fasting insulin levels (IU/mL) and (D) HOMA-IR, in MC4R rs12970134 genotypes carriers. Association of dietary fat intake $\leq 30\%$ and $> 30\%$ of total daily energy intake with (E) SAT (%) and (F) VAT (%), in MC4R rs12970134 genotypes carriers. Association of dietary carbohydrates intake $\leq 48\%$ and $> 48\%$ of total daily energy intake with (G) body weight (kg), (H) waist circumference (cm), (I) fasting insulin levels (IU/mL) and (J) HOMA-IR, in MC4R rs12970134 genotypes carriers. HOMA-IR, Homeostatic Models Assessment of Insulin Resistance; SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue.

Figure.9

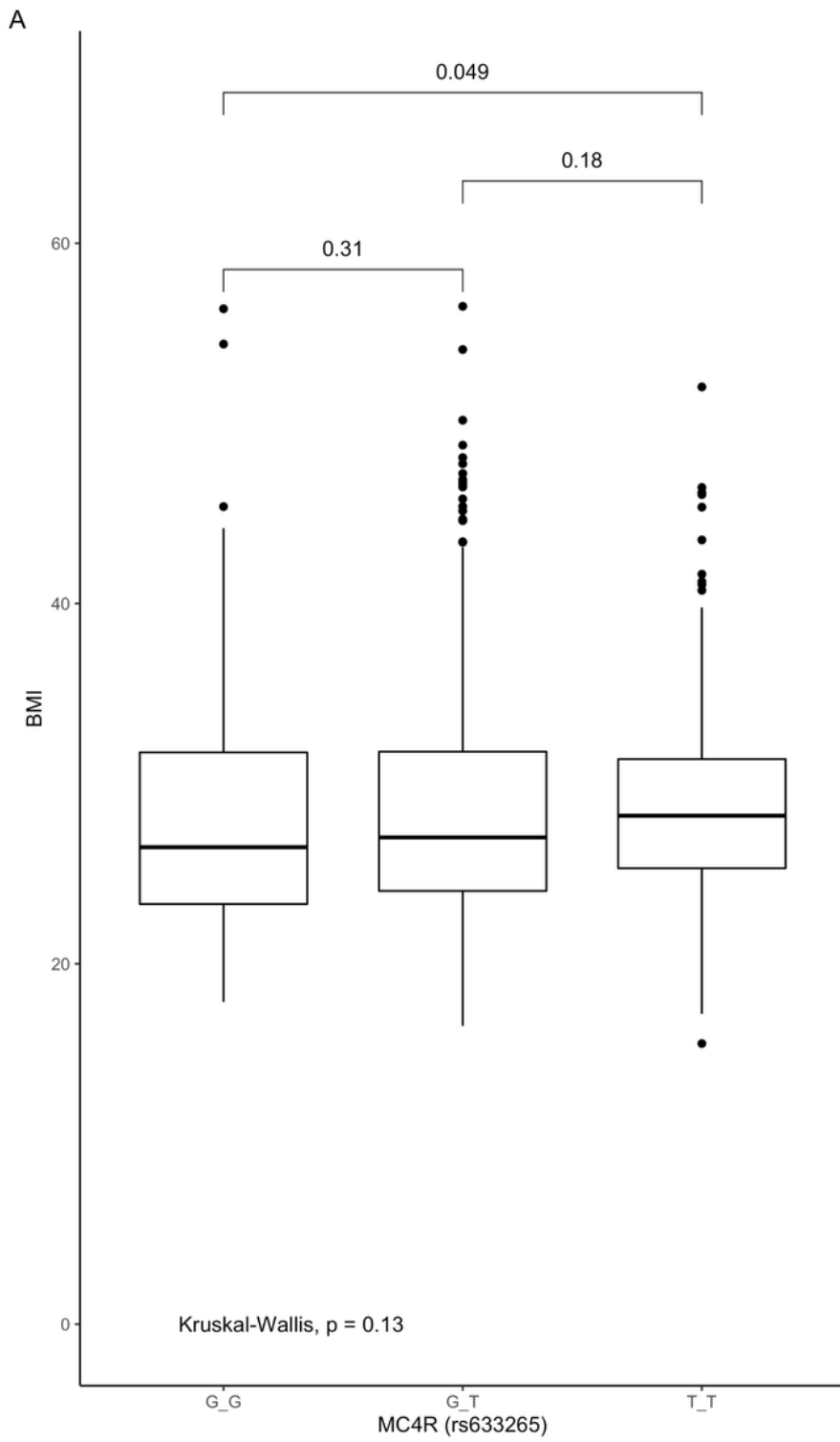


Figure 9

Association of MC4R rs633265 genotypes with (A) BMI (kg/m²). BMI, Body Mass Index.

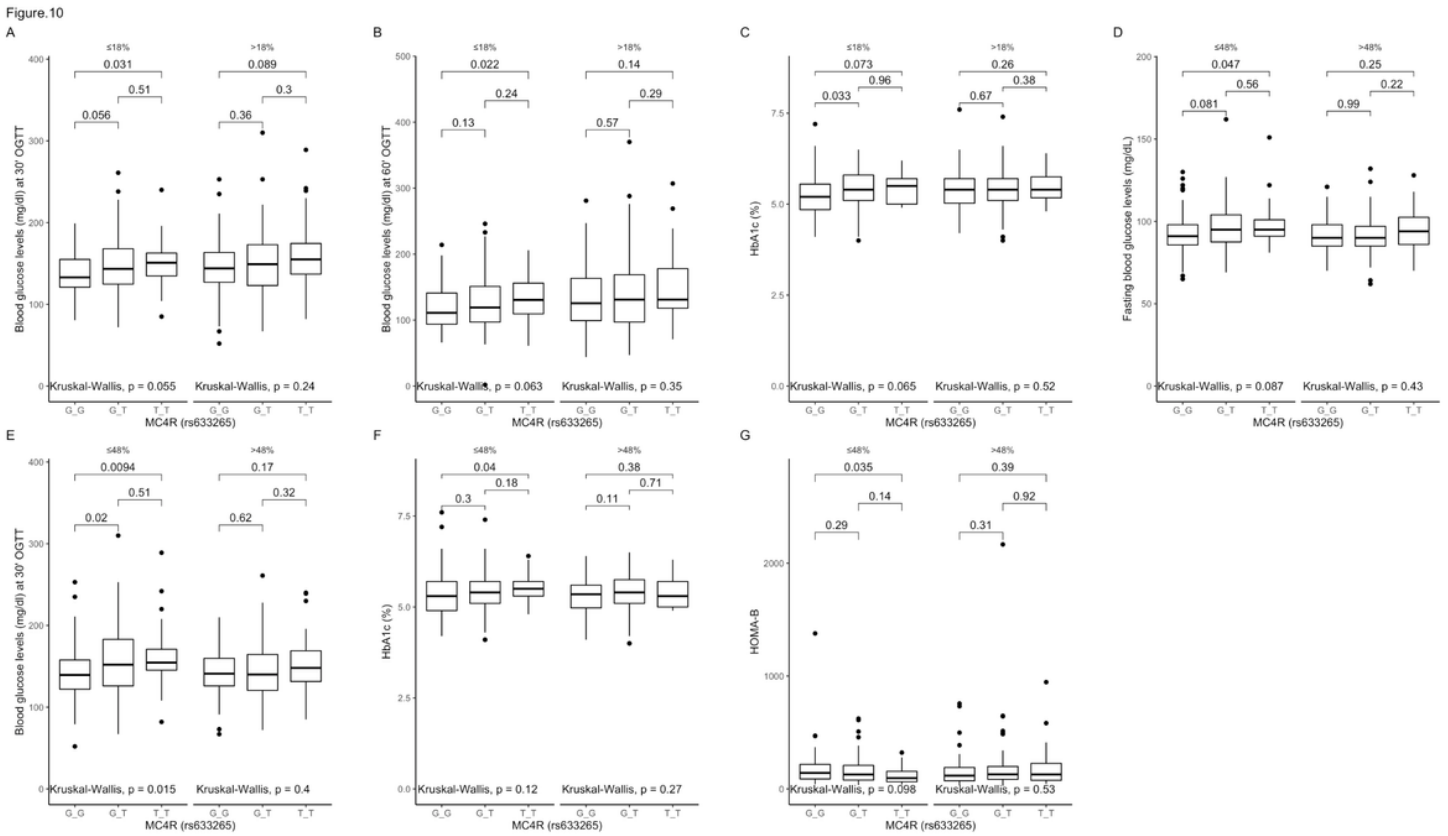


Figure 10

Association of MC4R rs633265 genotypes with (A) blood glucose levels (mg/dL) at 30 minutes of OGTT, (B) blood glucose levels (mg/dL) at 60 minutes of OGTT and (C) HbA1c levels (%), by dietary protein intake strata: $\leq 18\%$ and $> 18\%$ of total daily energy intake. Association of MC4R rs633265 genotypes with (D) fasting blood glucose levels (mg/dL), (E) blood glucose levels (mg/dL) at 30 minutes of OGTT, (F) HbA1c levels (%) and (G) HOMA-B (%), by dietary carbohydrates intake strata: $\leq 48\%$ and $> 48\%$ of total daily energy intake. HOMA-B, Homeostatic Models Assessment of β -cell function; OGTT, Oral Glucose Tolerance Test.

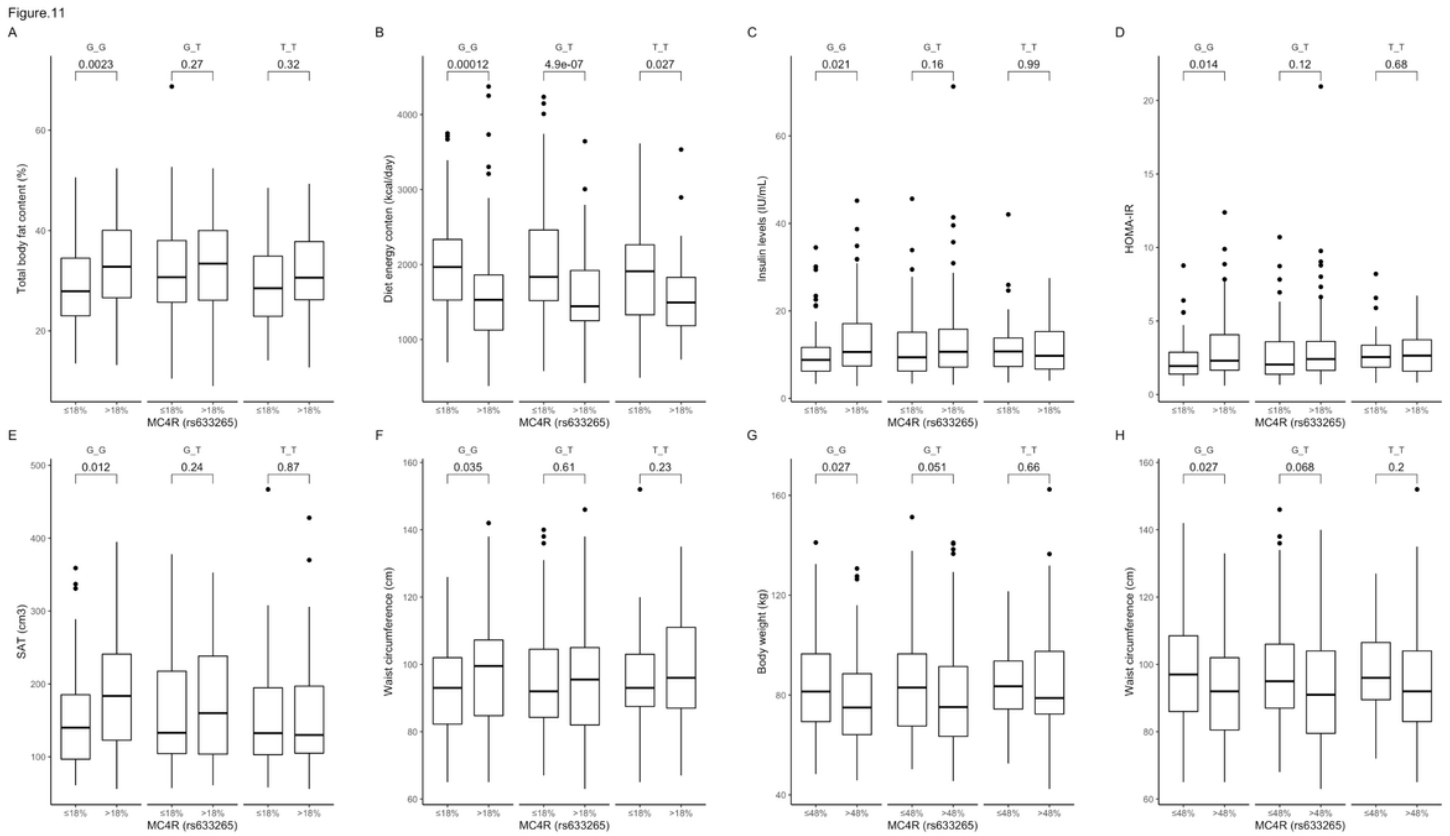


Figure 11

Association of dietary protein intake $\leq 18\%$ and $> 18\%$ of total daily energy intake with (A) total body fat content (%), (B) diet energy content (kcal/day), (C) fasting insulin levels (IU/mL), (D) HOMA-IR, (E) SAT (cm³) and (F) waist circumference (cm), in MC4R rs633265 genotypes carriers. Association of dietary carbohydrate intake $\leq 48\%$ and $> 48\%$ of total daily energy intake with (G) body weight (kg) and (H) waist circumference (cm), in MC4R rs633265 genotypes carriers. HOMA-IR, Homeostatic Models Assessment of Insulin Resistance; SAT, subcutaneous adipose tissue.

Figure.12

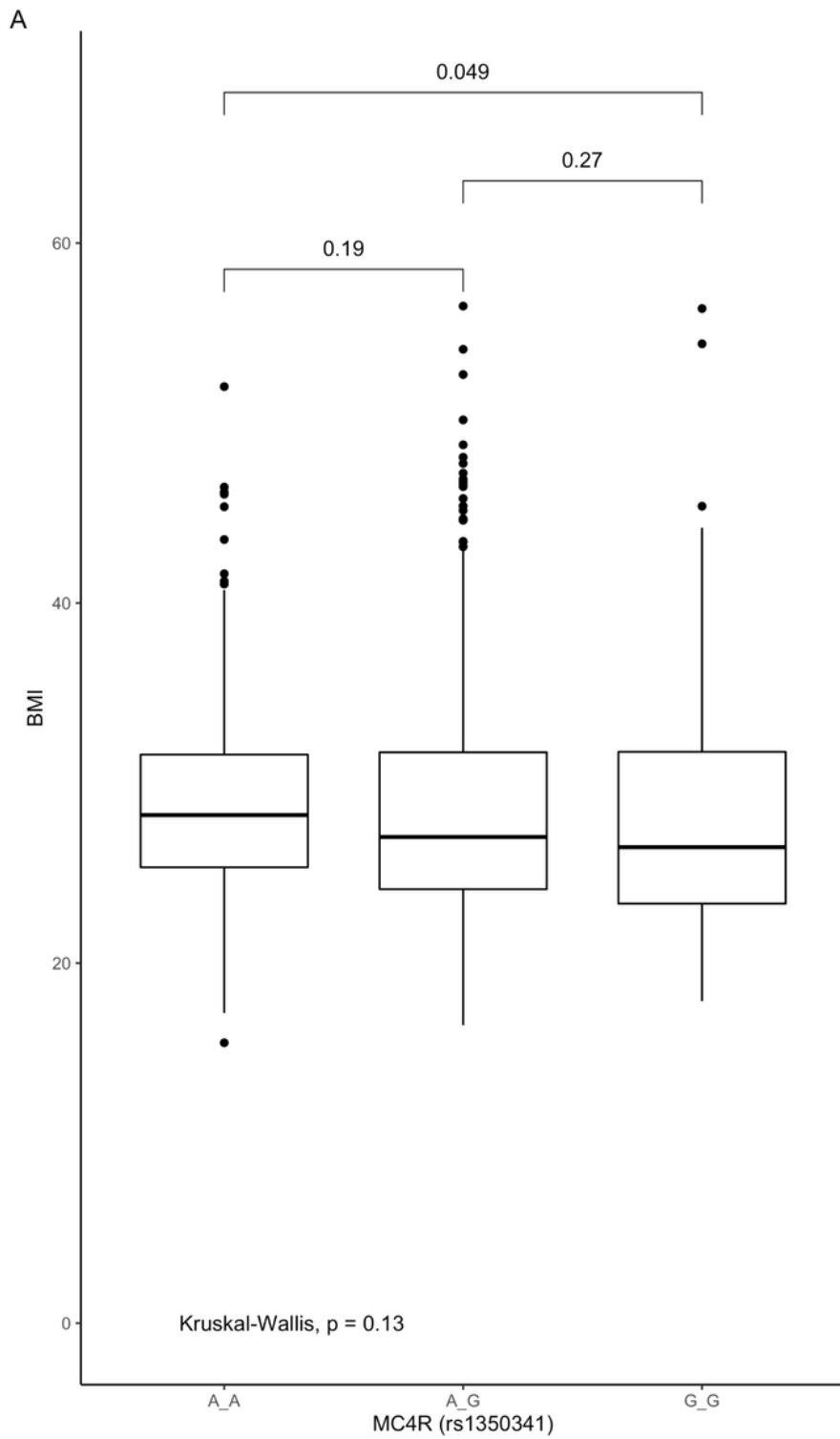


Figure 12

Association of MC4R rs1350341 genotypes with (A) BMI (kg/m²). BMI, Body Mass Index.

Figure.13

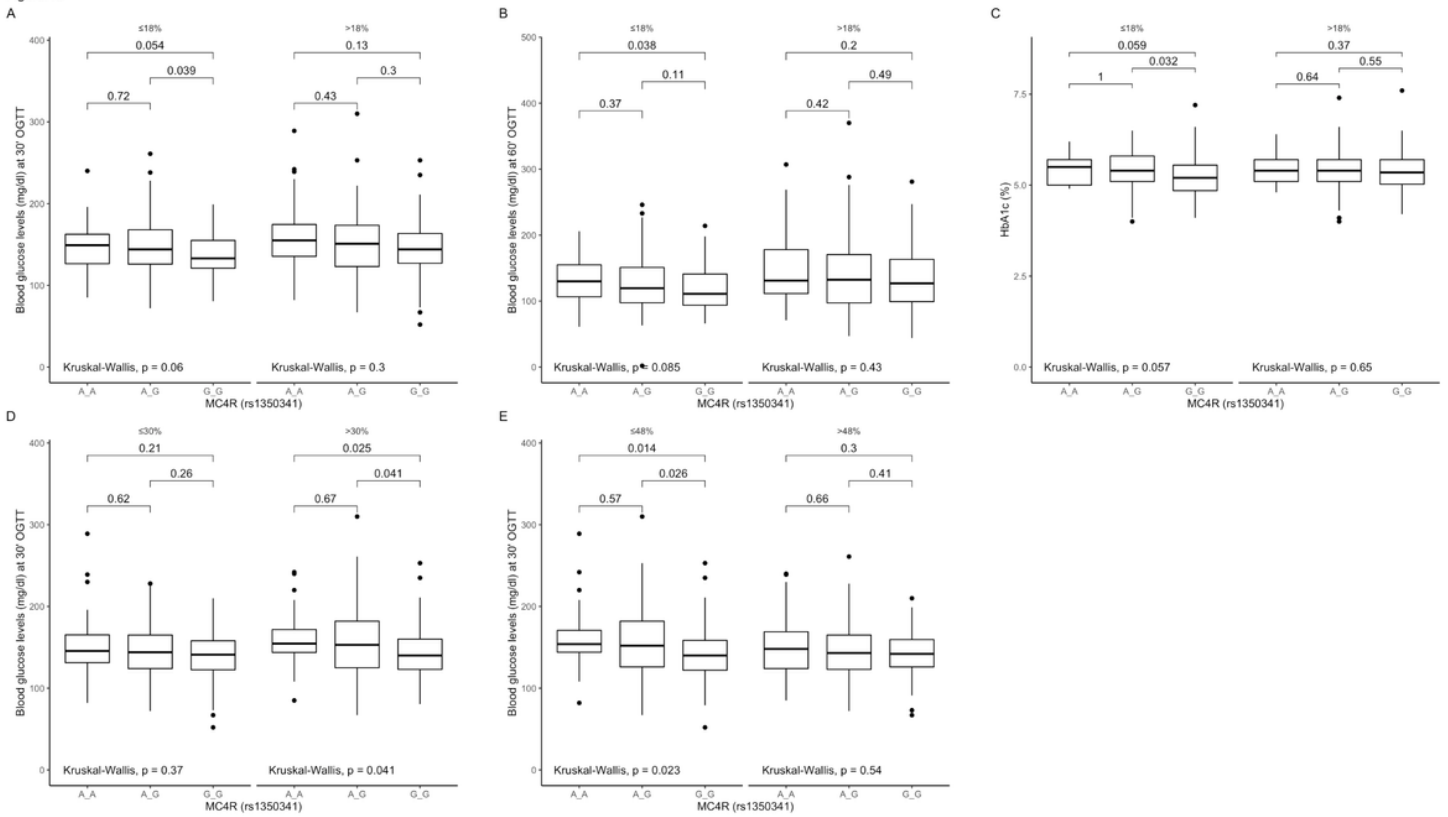


Figure 13

Association of MC4R rs1350341 genotypes with (A) blood glucose levels (mg/dL) at 30 minutes of OGTT, (B) blood glucose levels (mg/dL) at 60 minutes of OGTT and (C) HbA1c levels (%), by dietary protein intake strata: $\leq 18\%$ and $> 18\%$ of total daily energy intake. Association of MC4R rs1350341 genotypes with (D) blood glucose levels (mg/dL) at 30 minutes of OGTT, by dietary fat intake strata: $\leq 30\%$ and $> 30\%$ of total daily energy intake. Association of MC4R rs1350341 genotypes with (E) blood glucose levels (mg/dL) at 30 minutes of OGTT, by dietary carbohydrates intake strata: $\leq 48\%$ and $> 48\%$ of total daily energy intake. OGTT, Oral Glucose Tolerance Test.

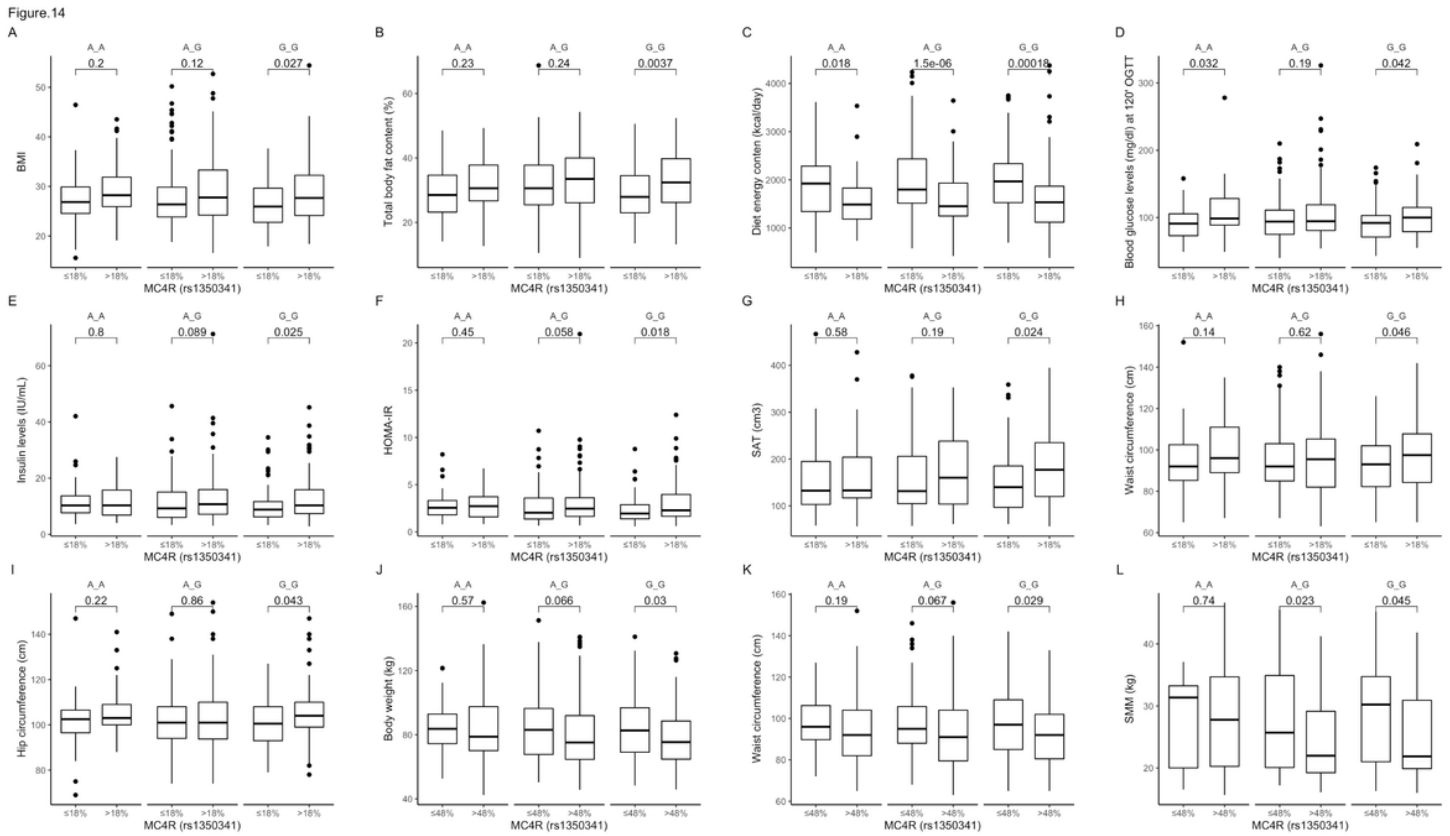


Figure 14

Association of dietary protein intake $\leq 18\%$ and $> 18\%$ of total daily energy intake with (A) BMI (kg/m³); (B) total body fat content (%); (C) diet energy content (kcal/day); (D) blood glucose levels (mg/dL) at 120 minutes of OGTT; (E) fasting insulin levels (IU/mL); (F) HOMA-IR; (G) SAT (cm³), (H) waist circumference (cm) and (I) hip circumference (cm), in MC4R rs1350341 genotypes carriers. Association of dietary carbohydrates intake $\leq 48\%$ and $> 48\%$ of total daily energy intake with (J) body weight (kg); (K) waist circumference (cm) and (L) SMM (kg) in MC4R rs1350341 genotypes carriers. BMI, Body Mass Index; HOMA-IR, Homeostatic Models Assessment of Insulin Resistance; OGTT, Oral Glucose Tolerance Test; SAT, subcutaneous adipose tissue; SMM, skeletal muscle mass.

Supplementary Files

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