



Impact of the Polymorphism Near *MC4R* (rs17782313) on Obesity- and Metabolic-Related Traits in Women Participating in an Aerobic Training Program

by

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The C/T polymorphism (rs17782313) mapped 188 kb downstream of the melanocortin-4 receptor gene (*MC4R*) shows a strong relationship with an increased body mass index (BMI) and the risk of type 2 diabetes. However, the information on polymorphism's potential modifying effect on obesity- and metabolic-related traits achieved through training is still unknown. Therefore, we decided to check if selected body measurements observed in physically active participants would be modulated by the genotype. The genotype distribution was examined in a group of 201 Polish women measured for chosen traits before and after the completion of a 12 week moderate-intensive aerobic training program. A statistically significant relationship between the glucose level and the genotype was identified ($p = 0.046$). Participants with CC and CT genotypes had a higher glucose level during the entire study period compared with the TT genotype. However, our results did not confirm the relationship between the C allele and an increased BMI or other obesity-related traits. Additionally, we did not observe a near *MC4R* C/T polymorphism x physical activity interaction. However, our results revealed that majority of obesity-related variables changed significantly during the 12 week training program. The effect sizes (d) of these changes ranged from small to medium ($d = 0.11-0.80$), whereas the largest effect ($d = 0.80$; i.e. medium) was reported for the fat mass content (FM). We found a relationship between the near *MC4R* C/T polymorphism and an increased glucose level, and it is thus a candidate to influence type 2 diabetes. Interestingly, after the 12 week training program, participants with the C (risk) allele with fasting hyperglycemia had a normal glucose level. Although, this change was not statistically significant, it shows an important trend which needs further investigation.

Key words: *MC4R*, gene x physical activity interaction, exercise, obesity-related traits.

Introduction

Frequent and regular physical activity has significant benefits for health, including reduction of the risk of cardiovascular diseases, diabetes, cancer, and improvement of mental health (Rankinen and Bouchard, 2008). Additionally, properly selected exercises improve body composition and help control weight. Promoting training programs, particularly in those subjects

who are genetically predisposed, is a significant step towards controlling the presently increasing epidemic of obesity (Ahmad et al., 2013; Li et al., 2010). Some studies have confirmed that physical activity is connected with a reduction of up to 40% in the genetic predisposition to obesity (Ahmad et al., 2013; Li et al., 2010). Understanding the genetic determinants of

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reactions occurring in the human body allows detailed and accurate prediction of the consequences of performed exercises. Studies on gene x physical activity interactions will help identify people who are expected to respond well or poorly to exercise, thus making training programs more efficient (possibility of accurate prediction of the training results) and safer (early prevention of possible overload or other injuries). Nevertheless, so far very few studies have been conducted with the focus on gene x lifestyle interactions (Ahmad et al., 2013; Franks, 2011; Li et al., 2010).

The melanocortin-4 receptor (MC4R) is a well known major regulator of food intake and energy expenditure (Hebebrand et al., 2010). The MC4R is a 332-amino-acid, which belongs to the family of seven trans-membrane G-protein-coupled receptors (GPCR) and is expressed in brain regions involved in regulation of food intake (Gantz et al., 1993; Mountjoy et al., 1994). The MC4R transduces its signal by integrating a satiety signal provided by its agonist α -melanocyte-stimulating hormone (α -MSH) and an orexigenic signal provided by its antagonist agouti-related protein (AGRP) (Lu et al., 1994). These ligands are expressed in distinct neuronal populations of the arcuate nucleus of the hypothalamus and are regulated by the adipocyte-secreted hormone, leptin, to control food intake and maintain long-term energy homeostasis (Schwartz et al., 2000). Thus, the MC4R gene has been considered as a genetic marker related to human obesity (Loos et al., 2008).

Genetic variants within the MC4R coding region have been found to be associated with severe and common forms of obesity in humans (Hebebrand et al., 2010). Additionally, polymorphisms outside of the coding region have been associated with a predisposition to polygenic obesity. The non-coding variants of the MC4R probably influence MC4R expression, however, a molecular mechanism for the SNPs associations remains unclear (Evans et al., 2014). Meta-analyses of genome-wide association studies (GWAS) conducted in Caucasians revealed that the variant rs17782313 (C/T polymorphism) mapped 188 kb downstream of the gene (Loos et al., 2008), shows a strong relationship with an elevated body mass index (BMI) (Xi et al., 2012).

In addition to common forms of obesity, rs17782313 has also been associated with early-onset severe obesity (Meyre et al., 2009; Scherag et al., 2010). These associations have been replicated in multiple populations including children, adolescents and adults (Loos, 2012; Xi et al., 2012).

The minor allele (C) is associated with increased intake of total energy and dietary fat, and as a consequence higher prevalence of common human obesity (Qi et al., 2008). Each copy of the risk allele is associated with an increase in the BMI of ~ 0.22 kg/m² in adults (Loos et al., 2008). Moreover, the C allele was also related with a 14% increased risk of type 2 diabetes (Qi et al., 2008). Sex has repeatedly been shown to affect the association between rs17782313 and obesity markers, showing a more pronounced effect for female than male mutation carriers (Qi et al., 2008).

Taking into account the rs17782313 polymorphism's role in body weight homeostasis, we decided to examine whether selected traits observed in physically active participants would be modulated by the genotype. To test this hypothesis, we performed a genetic association study that aimed to detect the relationship between the near MC4R C/T genetic polymorphism and selected body measurements. Therefore, we studied the genotype distribution in a group of young Polish women measured for chosen body mass and body composition variables, as well as obesity-related metabolic traits before and after a 12 week training program to determine if there was an interaction between the genotype and training. In our opinion, research conducted on amateurs allows for better understanding of the genetic background of post-effort adaptation mechanisms. However, this knowledge can be used for the needs of competitive sports including the modeling of energy management and weight control in professional athletes representing endurance sport disciplines.

Methods

Ethics Statement

All the procedures followed in the study were approved by the Ethics Committee of the Regional Medical Chamber in Szczecin (Approval number 09/KB/IV/2011) and were conducted according to the principles of the World Medical

Association, Declaration of Helsinki and ethical standards in sport and exercise science research. Furthermore, the experimental procedures were conducted in accordance with the set of guiding principles for reporting the results of genetic association studies defined by the Strengthening the Reporting of Genetic Association studies (STREGA) Statement. All participants signed a consent form and were provided with written information concerning the study, providing all pertinent details (purpose, procedures, risks, and benefits of participation).

Participants

Two hundred and one Polish Caucasian women aged 21 ± 1 years (range 19–24) met the inclusion criteria and were selected for the study. None of these individuals had engaged in regular physical activity in the previous 6 months. They had no history of any metabolic or cardiovascular diseases. Participants were nonsmokers and refrained from taking any medications or supplements known to affect the metabolism. Prior to the beginning of the intervention, participants were asked to maintain a balanced diet of approximately 2000 kcal/day.

Physical exercise training protocol

Before the commencement of the training program, HR_{max} of each participant was evaluated using a continuous graded exercise test on an electronically braked cycle ergometer (Oxycon Pro, Erich JAEGER GmbH, Hoechberg, Germany) according to a previously described protocol (Kostrzewa-Nowak et al., 2015). The training period was preceded by a week-long familiarization stage, when the examined women exercised 3 times a week for 30 minutes, at an intensity of about 50% of their HR_{max} . After the week-long familiarization stage, the proper training program started. Training sessions were conducted in a sports hall; a platform was placed in front of the participants on which the instructor presented exercises based on knee bends, lunges, running, skipping and hopping and their combinations creating a choreography set. The individual heart rate (HR) of participants was registered with HR monitors to control the intensity of exercise. The participants were instructed to maintain previously indicated ranges of the HR or relative value of HR_{max} (maximum heart rate, %). Each training unit consisted of a warm-up (10 min), aerobic exercises

which constituted the main part of training (43 min), and stretching and breathing exercises (7 min). Aerobic training included two alternating types of exercises – low and high impact. A low impact style comprised movements with at least one foot on the floor at all times, whereas a high impact style included running, hopping, and jumping with a variety of flight phases (de Angelis, 1998). Music of variable rhythm and tempo was incorporated into both styles. A 12-week program of low-high impact aerobics was divided as follows: (i) 3 weeks including 9 training units, 60 min each, at about 50–60% of HR_{max} , tempo 135–140 BPM, (ii) 3 weeks comprising 9 training units, 60 min each, at 60–70% of HR_{max} , tempo 140–152 BPM, (iii) 3 weeks including 9 training units, 60 min each with the intensity of 65–75% of HR_{max} , tempo 145–158 BPM, and (iv) 3 weeks consisting of 9 training units, 60 min each with an intensity of 65–80% of HR_{max} , tempo 145–160 BPM. All 36 training units were administered and supervised by the same instructor.

Body Composition Measurements

All participants were measured for selected body mass and body composition variables before and after the completion of the 12-week training period. Body mass and body composition were assessed with the bioimpedance method using a Tanita TBF 300M electronic scale (Horton Health Initiatives, USA). The examined variables included: total body mass (kg), fat free mass (FFM, kg), fat mass percentage (FM, %), body mass index (BMI, $kg \cdot m^{-2}$), and total body water (TBW, kg).

Biochemical and Hematological Analyses

Fasting blood samples were obtained in the morning from the elbow vein. Blood samples from each participant were collected in two tubes. For biochemical analyses, a 4.9 mL S-Monovette tube with ethylenediaminetetraacetic acid (K 3 EDTA; 1.6 mg EDTA/mL blood) and separating gel (SARSTEDT AG & Co., Nümbrecht, Germany) were used. For complete blood count, a 2.6 mL S-Monovette tube with K 3 EDTA (1.6 mg EDTA/mL blood) (SARSTEDT AG & Co., Nümbrecht, Germany) was used. Blood samples for biochemical analyses were centrifuged $300 \times g$ for 15 minutes at room temperature in order to receive blood plasma. Biochemical and hematological analyses were performed before the

beginning of the training program and repeated after its completion. The analyses were performed immediately after the blood collection. Complete blood count, including white blood cells (WBC), red blood cells (RBC), haemoglobin (HGB), haematocrit (HTC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), and total platelet level (PLT) was obtained using a Sysmex K-4500 Haematology Analyzer (TOA SYSMEX, Kobe, Japan). All biochemical analyses were conducted using a Random Access Automatic Biochemical Analyzer for Clinical Chemistry and Turbidimetry A15 (BIO- SYSTEMS S.A., Barcelona, Spain). Blood plasma was used to determine the lipid profile: triglycerides (Tg), cholesterol (Chol), high-density lipoprotein (HDL) and low-density lipoprotein (LDL) concentrations. Plasma Tg and Chol concentrations were determined by a diagnostic colorimetric enzymatic method according to the manufacturer's protocol (BioMaxima S.A., Lublin, Poland). Manufacturer's declared intra-assay coefficients of variation (CV) of the method were < 2.5% and < 1.5% for the Tg and Chol determinations, respectively. HDL plasma concentration was determined using a human anti- β -lipoprotein antibody and colorimetric enzymatic method according to the manufacturer's protocol (BioMaxima S.A., Lublin, Poland). The manufacturer's declared intra-assay CV of the method was < 1.5%. Plasma concentrations of LDL were assessed using a direct method according to the manufacturer's protocol (PZ Cormay S.A., Lomianki, Poland). The manufacturer's declared intra-assay CV of the method was 4.97%. All procedures were verified using multiparametric control serum (BIOLABO S.A.S, Maizy, France), as well as control serum of normal level (BioNormL) and high level (BioPathL) lipid profiles (BioMaxima S.A., Lublin, Poland).

Genetic Analyses

The buccal cells donated by the subjects were collected in Resuspension Solution (GenElute Mammalian Genomic DNA Miniprep Kit, Sigma, Germany) using sterile foam-tipped applicators (Puritan, USA). DNA was extracted from the buccal cells using a GenElute Mammalian Genomic DNA Miniprep Kit (Sigma, Germany) according to the manufacturer's

protocol. All samples were genotyped in duplicate using an allelic discrimination assay on a StepOne Real-Time Polymerase Chain Reaction (RT-PCR) instrument (Applied Biosystems, USA).

Statistical Analyses

Allele frequencies were determined by gene counting. A chi-square test was used to examine the Hardy-Weinberg equilibrium. To determine the influence of the near *MC4R* C/T polymorphism on training response, the 2 × 2 mixed-design ANOVA was used. The Kolmogorov-Smirnov test was applied to check for data normality. The level of statistical significance was set at $p < 0.05$. Additionally, to determine effect sizes (d), difference in means was divided by standard deviation of the difference between means. For interpretation of the meaningfulness of the findings, effect sizes according to Cohen's d were calculated and interpreted as follows: 0.2 to <0.6, small; 0.6 to <1.2, medium; 1.2 to <2.0, large; 2.0 to <4.0, very large; and ≥ 4.0 , extreme large (Hopkins et al., 2009).

Results

The near *MC4R* genotypes were in Hardy-Weinberg equilibrium. In order to examine the hypothesis that the near *MC4R* C/T polymorphism modulated training response, we conducted a mixed 2 × 2 ANOVA with one between-subject factor (the near *MC4R* genotype: TT vs CC+CT) and one within-subject factor (time: before training vs after training) (Table 1). Except for TBW, Chol, and LDL, all variables changed significantly during training (d ranged from small to medium, 0.11 to 0.80), however, none of the variables changed significantly across the near *MC4R* genotypes (genotype × training interaction). The main effect of genotype was found for glucose, $p = 0.046$ (Table 1). We did not identify a relationship between the genotype and body height ($p = 0.726$).

insert Table 1 here

Discussion

Recently, in two GWAS, the common polymorphism rs17782313 near *MC4R* gene was shown to be associated with increased obesity risk and type 2 diabetes (Loos et al., 2008; Thorleifsson et al., 2009). However, it still remained to be established whether the described relationship of

this polymorphism with a higher BMI is related to the MC4R's role in obesity, or whether it has an independent role in regulation of body weight. Of interest is that this SNP is not only associated with a higher BMI, but also with increased height, which is consistent with the phenotype of individuals with mutations within the MC4R coding region (Loos et al., 2012). We hypothesized

that rs17782313 may be a marker of the casual genetic variant in a regulatory region of the MC4R. This is consistent with observations that regulatory regions can be several hundred kilobases away from the affected gene (Chambers et al., 2008).

Table 1

The near MC4R genotypes and response to training (two-way mixed ANOVA)

Variable	CC+CT (n = 78)		TT (n = 123)		Genotype	Training	Genotyp e x Training
	Before training	After training	Before training	After training			
Body mass (kg)	60.6 ± 8.3	59.6 ± 8.0	60.8±7.4	60.1±7.4	<i>p</i> = 0.726	<i>p</i> < 0.001*	<i>p</i> = 0.219
	d = -0.65		d = -0.41				
BMI (kg·m ⁻²)	21.5 ± 2.3	21.2 ± 2.3	21.7±2.4	21.5±2.4	<i>p</i> = 0.436	<i>p</i> < 0.001*	<i>p</i> = 0.246
	d = -0.66		d = -0.37				
FM (%)	23.9 ± 6.0	22.2 ± 6.3	24.0±5.2	22.7±5.3	<i>p</i> = 0.710	<i>p</i> < 0.001*	<i>p</i> = 0.246
	d = -0.80		d = -0.53				
FFM (kg)	45.6 ± 3.5	46.1 ± 3.4	45.8±3.1	46.2±3.2	<i>p</i> = 0.718	<i>p</i> < 0.001*	<i>p</i> = 0.439
	d = 0.48		d = 0.27				
TBW (kg)	33.4 ± 2.6	33.3 ± 4.3	33.6±2.6	33.9±2.4	<i>p</i> = 0.346	<i>p</i> = 0.447	<i>p</i> = 0.306
	d = -0.01		d = 0.20				
Chol (mg/dl)	167 ± 24	168 ± 30	170±27	168±26	<i>p</i> = 0.659	<i>p</i> = 0.698	<i>p</i> = 0.391
	d = 0.04		d = -0.09				
Tg (mg/dl)	76.4 ± 25.8	85.0 ± 38.5	80.0±34.5	83.3±31.0	<i>p</i> = 0.824	<i>p</i> = 0.012*	<i>p</i> = 0.254
	d = 0.24		d = 0.11				
HDL (mg/dl)	66.1 ± 13.7	61.0 ± 13.3	64.0±12.2	60.2±12.2	<i>p</i> = 0.371	<i>p</i> < 0.001*	<i>p</i> = 0.367
	d = -0.44		d = -0.38				
LDL (mg/dl)	86.1 ± 21.0	101.8 ± 101.6	89.5±21.5	90.7±22.4	<i>p</i> = 0.476	<i>p</i> = 0.067	<i>p</i> = 0.119
	d = 0.16		d = 0.07				
Glucose (mg/dl)	79.8 ± 11.0	76.8 ± 10.5	76.81±9.2	74.80±10.1	<i>p</i> = 0.046*	<i>p</i> = 0.001*	<i>p</i> = 0.525
	d = -0.24		d = -0.22				

Mean ± standard deviation; p values for main effects (genotype and training) and genotype x training interaction; d – effect size; BMI – body mass index; FM – fat mass percentage; FFM – fat free mass; TBW – total body water; Chol – cholesterol; Tg – triglycerides; HDL – high-density lipoprotein; LDL – low-density lipoprotein

Discussion

Previous studies confirm a strong association between the polymorphism near *MC4R* gene and body weight (Haupt et al., 2009; Loos et al., 2008). However, results of the present study did not confirm this relationship of the C allele with increased body weight, BMI or other obesity-related traits. Connection between increased height and the polymorphism was also not found in our study. In fact, the small effect size (per-allele effect = 0.005) of this molecular marker with human obesity implied the need of extremely large-scale data to establish some specific common variants (Kuo et al., 2011). Probably, our study had limited power to detect minor SNP effects on an increased BMI.

On the other hand, we identified a statistically significant association between the glucose level and the genotype. Participants with CC and CT genotypes had a higher glucose level during the entire study period compared with the TT genotype. Our results showed that 6.5% of individuals with the risk genotype, but no one with the TT genotype, had fasting hyperglycemia. Their glucose level ranged from 106 to 118 mg/dl. Pre-diabetes, based on glycaemic variables above normal, but below diabetes thresholds, constitutes a high-risk state for diabetes with high conversion rates and there is accumulating evidence implying that damage on kidneys and nerves already exists at the pre-diabetic stage (Tabak et al., 2012). Interestingly, after the 12 week training program their glucose level was normal, and it ranged from 85 to 93 mg/dl. Although, this change was not statistically significant, it shows a medically important trend, which needs further experimental investigations. Our results may suggest that SNP rs17782313 influences the regulatory region of the *MC4R*, which is a key regulator of energy balance and can affect food intake and energy expenditure through central melanocortin neuronal pathways (Balthasar et al., 2005). Alterations in expression of the *MC4R* may affect glucose utilization and insulin sensitivity (Nogueiras et al., 2007).

Chambers et al. (2008) confirmed a relationship between the glucose metabolism and SNPs localized near the *MC4R* gene. In this genome-wide association study, 318,237 SNPs connected with insulin resistance were analyzed. The authors described a BMI-independent

association of rs12970134 with insulin resistance and related phenotypes. Our results also show that rs17782313 is connected with the glucose level, what contrasts with the findings of Haupt et al. (2009) who did not find a relationship between the SNP and glucose metabolism. Kuo et al. (2011) also did not observe a relationship between the investigated polymorphism and glucose metabolism. However, the authors identified an interaction between rs2229616 also localized near the *MC4R* and the insulin level. In another study, rs17782313 was associated with an increased insulin level, but not with the glucose level (Vogel et al., 2011).

In the present study, we did not observe the near *MC4R* C/T polymorphism x physical activity interaction. However, our results revealed that variables such as body mass, BMI, FM, FFM, Tg, HDL, and glucose changed significantly during the 12 week training program. There were small changes in FFM ($p < 0.001$; $d = 0.48$), Tg ($p = 0.012$; $d = 0.24$), HDL ($p < 0.001$; $d = -0.44$), and glucose level ($p = 0.001$; $d = -0.24$). Additionally, significant changes in body mass ($p < 0.001$; $d = -0.65$), BMI ($p < 0.001$; $d = -0.66$), and FM ($p < 0.001$; $d = -0.80$) were noticed. The finding that physical activity is connected with a reduction in obesity-related traits is still a significant observation. A limitation of the lifestyle study is that the results were driven by a relatively small sample size and a short time of intervention. Thus, replication in similarly phenotyped and larger cohorts is required to fully determine the effect of the SNP on changes in body composition.

Our results may be supported by the previous study which did not show an association between the polymorphism and selected body composition measurements in 242 participants undergoing a 9-month lifestyle intervention (Haupt et al., 2009). In a study performed on 111,421 adults of European descent, Ahmad et al. (2013) analyzed 12 loci connected with obesity-related traits and also did not reveal evidence of the polymorphism rs17782313 x physical activity interactions, although the impact of *FTO* and *SEC16B* genes on changes in the BMI in a group undergoing lifestyle intervention was shown. In contrast to our study, Li et al. (2010) genotyped 12 SNPs in obesity-susceptibility loci including rs17782313 in a group of 20,430 European subjects, and showed that the genetic

predisposition to an increased BMI and obesity was attenuated by a physically active lifestyle.

The study has several limitations that should be mentioned. The failure to detect a gene \times physical activity interaction in our study may reflect the influence of population-specific characteristics such as high overall physical activity levels and relatively low body mass in the studied population, a small sample size or the effect of age (Li et al. 2010). In addition, obesity is a polygenic trait, as a result the genetic marker analysed independently is likely to make only a limited contribution to the obesity phenotype: it seems more likely that such status depends on the simultaneous presence of multiple such variants.

In summary, we identified a statistically significant relationship between the glucose level and genotype, what is an important observation from a medical point of view. Participants with CC and CT genotypes had a higher glucose level during the entire study period compared with the TT genotype. However, we did not confirm the effect of the near *MC4R* C/T polymorphism (rs17782313) on the higher BMI or other obesity-related traits. Although selected body mass and body composition variables, as well as obesity-

related metabolic variables changed significantly during the 12 week training program, we found no evidence of an interaction between the investigated polymorphism and physical activity in chosen variables. However, we would like to point out that promoting physical activity, particularly in those subjects who are genetically predisposed to obesity, is an important step towards controlling the currently increasing obesity epidemic. More experimental studies are needed to explain the association between the glucose level and genotype, as well as to establish the gene \times physical activity interaction. Research conducted on amateurs allows for better understanding of the genetic basis of post-effort adaptation mechanisms. Yet, these results constitute an excellent source of information, which after thorough analysis and replication can be used for the needs of competitive sports. In the future, the results described in this article can be used for the purpose of modeling the energy management and weight control in professional athletes representing mostly endurance sport disciplines.

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