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Association of *FTO* and *TMEM18* polymorphisms with overweight and obesity in the population of Polish children

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ABSTRACT: The objective of the study was to verify whether or not *FTO* rs9939609, rs9926289 and *TMEM18* rs4854344, rs6548238, rs2867125 variants are important risk factors for overweight and/or obesity in Polish children aged 6–16 (n=283). *FTO* rs 9939609 and rs9926289 exhibited a strong codominant obesity-predisposing effect of genotypes homozygous for minor alleles (OR=5.42, 95% CI: 2.04–14.39, $p=0.0006$). The important finding of the study is increased risk of overweight (OR=5.03, 95% CI: 1.15–21.93, $p=0.0306$) in individuals homozygous for the minor alleles rs4854344, rs6548238 and rs2867125 in the recessive inheritance model, while no other significant associations between *TMEM18* variants and risk of obesity were found. Given the identified interaction *TMEM18* genotype \times BMI category ($p=0.0077$), it seems that the effect of homozygous for the minor alleles may be compared to a “weight guard”, which significantly increases the risk of overweight, but not of obesity, because it promotes weight gain only up to the threshold of obesity. Conclusion: The proposed hypothetical effect (“weight guard”) of homozygous for the minor alleles in the *TMEM18* based on a rather small sample is a possible explanation of the effects of minor alleles, which minimize the risk of obesity.

KEY WORDS: body mass index, obesity- and overweight-associated gene, *FTO*, *TMEM18*, Polish population

Introduction

One of the genes thought to be associated with obesity is *FTO*, which is located on the long arm of chromosome 16 (16q12.2) and encodes 2-oxoglutarate-dependent nucleic acid demethylase, an enzyme present, among others, in the hypothalamic nuclei controlling appetite and energy expenditure (Gerken et al. 2007). Analyses of *FTO* rs9939609 polymorphism indicate its association with risk of type 2 diabetes mellitus and also with a strong predisposition to obesity, mostly through regulation of appetite and feeding preferences (Frayling et al. 2007; Field et al. 2007; Cecil et al. 2008; Wardle et al. 2008; Wardle et al. 2009). Other studies have shown that by influencing insulin sensitivity in the brain, the *FTO* gene participates in the differentiation of preadipocytes to mature adipose cells (adipogenesis) or has an effect on lipase in adipocytes, thus regulating fat tissue mass (Rampersaud et al. 2008; Wahlen et al. 2008). In turn, a study on another *FTO* polymorphism, that is, rs9926289, has shown that the gene may affect BMI in younger persons (under 55) through growth hormone (GH) and the insulin-like growth factor IGF1 associated with it (Roskopf et al. 2011). Interestingly, the two discussed SNPs seem to be closely linked in the *FTO* gene, and some authors confirm their joint effects (Frayling et al. 2007; Hotta et al. 2008), while studies on some other populations do not (Ohashi et al. 2007).

It has recently been proposed that other genetic determinants of BMI may include the rs2867125, rs4854344, rs6548238, and rs7561317 polymorphisms located within approximately 200 kb of *TMEM18*. The functional context of *TMEM18* and the effects of its

SNP variants have not been determined yet (Zhao et al. 2009; Holzapfel et al. 2010; Graff et al. 2012). However, it is known that the *TMEM18* gene encodes the highly conservative transmembrane protein 3TM, which occurs in the nuclear envelope and does not seem to regulate any brain centers controlling appetite, as opposed to the *FTO* and *MC4R* genes (Almen et al. 2010; Rask-Andersen et al. 2012). Some studies have indicated that the 4 SNPs located in the *TMEM18* region were in almost absolute linkage disequilibrium (Hotta et al. 2009; Zhao et al. 2009; Rask-Andersen et al. 2012).

Despite the increasing prevalence of abnormal BMI values in the Polish population, and especially in children, following the socioeconomic transformation of the 1990s (Kozieł et al. 2004; Kozieł 2005; Chrzanowska et al. 2007; Żądzińska et al. 2012), little research has been done on the genetic determinants of overweight and/or obesity in Polish children and adolescents, while the existing studies are devoted only to the *FTO* rs9939609 polymorphism (Luczynski et al. 2012). The objective of the present work is to determine (using case-control association analysis) whether or not *FTO* rs9939609, rs9926289 and *TMEM18* rs4854344, rs6548238, rs2867125 polymorphisms are important risk factors of overweight and/or obesity in the population of Polish children.

Materials and methods

Subjects

The study was approved by the Institutional Review Board of the University of Łódź (KBB-UŁ/I/10/2011). The study design included case-control analysis in-

volving anthropometric measurements and genetic tests of pupils from seven randomly selected primary schools in Łódź (a city of nearly 729,000 inhabitants in central Poland) located in all five districts of the city, as well as of children presenting with overweight and obesity at a public health care facility. According to the study design, groups of pupils with normal weight and excessive weight were identified (in consultation with the school nurse). Both in schools and in the health care facility, the children's parents or legal guardians were requested to express written consent for the procedures set forth in the study design.

In response to the requests, consent was obtained from the parents/legal guardians of 283 children (100%), including 130 boys (45.9%) and 153 girls (54.1%) aged 6–16 (mean age: 8.95 ± 1.53). Anthropometric measurements were conducted from October 2011 to April 2012, and genetic tests were concluded in December 2012.

Anthropometric measurements (body mass index)

Anthropometric measurements of body height and weight were conducted by researchers from the Department of Anthropology, University of Łódź, in accordance to the standard procedure developed by Martin (Knussmann 1988). Children were weighed in light sports apparel with an accuracy of 0.1 kg, and their height was measured using an anthropometer with an accuracy of 0.1 cm. The chronological age of each examined child was calculated as a difference between the examination date and the date of birth and expressed in the decimal system with an accuracy of 0.01 year. Based on individual body height and weight,

a body mass index was calculated for each examined child according to the formula $BMI = \text{body mass [kg]} / \text{height}^2 \text{ [m]}$.

Overweight and obesity were determined pursuant to the definitions of the International Obesity Task Force (IOTF) (Cole et al. 2000; Cole & Lobstein 2012), using LMSGrowth software (Pan & Cole 2011). The software classified the children into the following BMI categories: normal weight, overweight, and obesity. The classification used is based on the IOTF cut-offs, that is, BMI values defining the overweight threshold ($BMI \geq 25$) and obesity threshold ($BMI \geq 30$) for adults (18 years) adjusted to the children's age and sex (within the age range of 2 to 18 years).

Individual BMI [kg/m^2] values were standardised for age and sex according to the current BMI reference values for children and adolescents living in Łódź (Rosset et al. 2009), obtained using the LMS method (Cole & Green 1992). The z-scores for children's BMI were calculated from the L (the Box-Cox power transformation), M (the median) and S (the coefficient of variation) curves, using values appropriate for the children's age and sex, according to the formula (for $L \neq 0$): $Z = ((BMI / M)^L - 1) / (L \times S)$, where L, M, and S stand for children's age and sex.

Genetic tests (FTO and near TMEM18 SNPs)

All genetics tests were conducted by researchers from the Department of Molecular Biophysics, University of Łódź according to the methods described below. Samples of saliva for genetic testing were collected using an Oragene DNA Collection kit, OG-500 Tube Format, from DNA (Genotek, Canada) according to the manufacturer's instructions. The

samples were coded and stored at room temperature.

DNA isolation

Genomic DNA was extracted from 500 μL of saliva using a MagNaPure LC 2.0 Instrument with a MagNA Pure LC DNA Isolation Kit – Large Volume (Roche Diagnostics, USA) according to the manufacturer's instructions. The concentration of the isolated DNA was measured with a Quant-iT™ Broad-Range DNA Assay Kit (Life Technology, USA). All DNA samples were adjusted to the final concentration of 200 $\text{pg}/\mu\text{L}$ and stored at -30°C .

Primer design

All primers for amplification of selected SNPs were designed using the online Primer 3 software (Biology Workbench, <http://workbench.sdsc.edu>). Genotyping of *FTO* gene (rs9939609 and rs9926289) was performed by HRM method described elsewhere (Sitek et al. 2014). Genotyping of *TMEM18* gene (rs4854344, rs6548238, rs2867125) was performed by HRM method with primers which are presented in Table 1. Primer specificity was checked with Primer-BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast>).

PCR amplification

Five SNPs were genotyped on the CFX384 detection platform (BioRad, USA) using a PCR-HRM curve analysis assay which was validated by direct sequencing. Reactions were performed using White-Well Hard-Shell Thin-Wall 384-Well Skirted PCR Plates (BioRad, USA), PCR-HRM was conducted in 10 μL reaction volume,

which included 0.5 ng DNA template, 2 \times GoTaq® Hot Start Colorless Master Mix (Promega, USA), 10 \times LCGreen Plus Dye (Idaho Technology) and 0.25 μM of each primer set. Genotyping accuracy was evaluated using samples genotyped in duplicate. PCR was performed with initial denaturation at 95°C for 3 min, followed by 45 cycles of denaturation at 95°C for 15 s, and annealing at 60°C for 30 s. Following PCR amplifications, the samples were heated to 90°C for 1 min, and then rapidly cooled down to 40°C for 1 min.

HRM was carried out over the temperature range of $70\text{--}95^\circ\text{C}$, with an increment of 0.2°C every 10 s, and each amplicon cluster was determined using Precision Melt Analysis Software (BioRad, USA). For each of the examined SNPs, 15 samples representing all present clusters/genotypes were randomly chosen for sequencing to verify genotyping results (Genomed, Poland).

Statistical analysis

The number of carriers of the studied SNP genotypes in the study sample was the basis for determining frequencies of alleles and genotypes, whose distributions were analyzed for consistency with the Hardy-Weinberg law (Pearson's χ^2 test).

Analysis of linkage disequilibrium (LD) for the genotypes was done using the Haploview 4.2 software (<http://www.broadinstitute.org/haploview/haploview>).

Differences in the distributions of genotype frequencies between normal-weight vs. overweight and normal-weight vs. obese groups were evaluated using the codominant model (assuming an independent effect of each

Table 1. Primers used for the HRM method and for direct sequencing

Primers used for	Gene /nearby gene	Single nucleotide polymorphism	Forward primer (5' →3')	Reverse primer (5' →3')	Amplicon size bp
HRM method	TMEM18	rs4854344	TGTTTAGATACACACTCTCCACTGT	GATGGCTGTGCTGGAACTG	58
	TMEM18	rs6548238	ATGAACGAGAAATAGGCCCC	AAGGCAGAAAGTCCACAGC	70
	TMEM18	rs2867125	TTATCTGGAGCATGGAAAATAAGC	GGTTGCATTTATGGCAATATAAAA	78
direct	TMEM18	rs4854344	TGCCGATGAACTGAGTGTTGC	ACCATTTCTGGAACGTGGAG	638
sequencing	TMEM18	rs6548238	AACAGATTCATTCACACGATGC	AAACAGGGTTTCATCAAAGTTGG	650
	TMEM18	rs2867125	TTAATTTATGGGCATATTCCTGT	GTTACGTAGATGTTTCATACCTGCCG	700

genotype) and the recessive model, using Pearson's χ^2 test (with Yates' correction for discontinuity for 2×2 tables). For genotypes significantly correlated with BMI categories and having a distribution consistent with the Hardy-Weinberg law, the odds ratio (OR) for overweight or obesity was estimated (at 95% confidence interval, CI) in both inheritance models. Calculations were done using logistic regression analysis and multiple regression with binomial distribution and a logit link function. Based on the Akaike information criterion (AIC), multiple regression analysis revealed which model – codominant or recessive – better described the data. Correspondence analysis (CA), based on χ^2 for contingency tables, was used to examine associations between two qualitative variable categories – *FTO:TMEM18* SNP genotypes and BMI categories.

BMI z-score values were used to assess BMI differences among the carriers of various SNP genotypes within the entire sample. As BMI z-score values were not normally distributed, the Kruskal–Wallis test was used for comparisons (post-hoc test: multiple comparisons for mean ranks). Analysis of BMI differences between the carriers of different genotypes within the examined groups of children (e.g., overweight vs. obese) required interaction testing. Thus, the generalized linear model (GLZ) was used for calculations on BMI [kg/m^2] values. As they were not normally distributed within the sample, the log-normal model was adopted for analysis. The dependent variable was BMI [kg/m^2] adjusted for age and sex, and the independent variables were SNP genotypes and BMI categories (normal weight, overweight, and obese). The expected results of the estimated model were interactions between geno-

types and BMI categories having a significant effect on mean BMI [kg/m^2] values. The models were verified by testing the normality of residual distributions. All calculations were done using STATISTICA 10.0 software (StatSoft Poland).

Results

Results of anthropometric measurements (BMI)

The study group consisted of BMI records [kg/m^2] for 283 children (100%). According to the International Obesity Taskforce (IOTF) classification, 168 (59.4%) children had normal weight, 59 (20.8%) were overweight, and 56 (19.8%) were obese. More girls than boys were characterized by normal weight (66.7% and 50.8%, respectively). A greater rate of obesity was observed in boys than in girls (28.5% and 12.4%, respectively, $p=0.0023$). The above data do not reflect the distribution of excessive body weight in the population of children inhabiting Łódź, but only characterize the sample in the present case-control association study.

Results of genetic tests (*FTO* and near *TMEM18* SNPs)

Analysis of linkage disequilibrium (LD) between *FTO* rs9939609 and rs9926289 genotypes revealed their complete dependence ($D'=1$, $r^2=1$). The frequency of allele A (the least common) was 48%. The distribution of genotypes AA, TA (GA rs9926289), and TT (GG) was 66 (23.3%), 140 (49.5%), and 77 (27.2%), respectively.

The results of LD analysis for the three studied *TMEM18* SNPs showed

the rs2867125 and rs6548238 genotypes to be dependent ($D'=1$, $r^2=0.98$), and the same was true for rs6548238 and rs4854344 ($D'=1$, $r^2=0.98$). The dependence of rs2867125 and rs4854344 was weaker ($D'=0.98$, $r^2=0.97$). The frequency of the minor alleles G (in rs4854344), T (rs6548238) and A (rs2867125) was 18% for all SNPs. In 281 children (99.3%), the distribution of genotypes was as follows: the major allele TT (rs4854344), CC (rs6548238) and GG (rs2867125) genotypes 190 (67.6%), TG, CT and GA heterozygotes 81 (28.8%) and GG, TT and AA homozygotes 10 (3.6%), respectively. A different genotype was observed only in two individuals (0.7%). One was heterozygous for rs4854344 (TG) and homozygous for the major alleles in the other SNPs; the other was heterozygous for rs2867125 (GA). As both individuals were of normal weight, they were not included in further analyzes ($n=281$). The distributions of the observed frequencies of all genotypes of the studied SNPs in the entire samples were consistent with the Hardy-Weinberg law ($p>0.05$).

Due to the above mentioned, the *TMEM18* and *FTO* polymorphisms are described as genotypic combinations in further statistical analysis – one SNP considered as the surrogate of other (rs9939609 for two SNPs *FTO* in high LD and rs4854344 *TMEM18* for three SNPs in high LD).

Genetic determinants of overweight and obesity

Differences in the frequency of genotypes between the group of normal-weight children (with normal BMI) and the group of overweight/obese children were evaluated using the codomi-

nant model (assuming an independent effect of each genotype) and the recessive model (Table 2).

Children with normal and abnormal weight differed significantly in the frequency of *FTO* genotypes ($p=0.0017$). Assuming a recessive inheritance model, carriers of the AA genotype (rs9939609) were characterized by a higher risk of obesity (OR=2.66, 95% CI: 1.35–5.25, $p=0.0046$). In turn, assuming a codominant model, the risk of obesity increased by a factor of 2.5 for TA heterozygous individuals (OR=2.71, 95% CI: 1.11–6.66, $p=0.0284$) and by a factor of more than 5 for AA homozygous individuals (OR=5.42, 95% CI: 2.04–14.39, $p=0.0006$). The lower AIC values indicate a better goodness of fit for the codominant model (244.69) than for the recessive model (248.14).

Overweight children differ from normal-BMI children in the frequency of *TMEM18* genotypes ($p=0.0493$). In the case of the recessive inheritance model (AIC=258.09), carriers of GG genotypes (rs4854344) in *TMEM18* are characterized by a higher risk of overweight (OR=5.03, 95% CI: 1.15–21.93, $p=0.0306$).

The observed significant correlations between the frequency of *FTO* polymorphisms and obesity in the studied children are confirmed by differences in BMI z-scores ($p=0.0029$) (Fig. 1). A multiple comparison test for the mean ranks of the groups compared (post hoc) showed that BMI for TA heterozygotes (median=0.67 (-0.05–1.81)) did not differ from BMI for TT homozygotes (0.27 (-0.07–1.22); $p=0.1279$) and AA homozygotes (1.26 (0.22–1.90); $p=0.1737$). A statistically significant difference was found between AA and TT homozygotes ($p=0.0019$). Polymorphisms within *TMEM18* did not

Table 2. Distribution of the studied *FTO* and near *TMEM18* SNP genotypes in normal-weight, overweight, and obese children with the odds ratio (OR) (n=283).

Gene SNP	Genetic model	Genotype	Normal BMI			Overweight			Obesity		
			n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	OR	95% CI	p-value ^a
<i>FTO</i> rs9939609	codominant	TT	55 (32)	15 (25)	1	1	7 (13)	1			
		TA	84 (51)	27 (46)	1.18	1.18	0.57, 2.42	0.6532	2.71	1.11, 6.66	0.0284
		AA	29 (17)	17 (29)	2.15	2.15	0.94, 4.94	0.0698	5.42	2.04, 14.39	0.0006
	recessive		p-value ^b	0.1501			0.0017				
		TT or TA	139 (83)	42 (71)	1	1	36 (64)	1			
<i>TMEM18</i> ^c rs4854344	codominant	AA	29 (17)	17 (29)	1.94	1.94	0.97, 3.89	0.0602	2.66	1.35, 5.26	0.0046
		TT	113 (68)	37 (63)	1	1	40 (71)	1			
		TG	50 (30)	17 (29)	1.04	1.04	0.53, 2.03	0.9117	0.79	0.39, 1.59	0.5077
	recessive	GG	3 (2)	5 (8)	5.09	5.09	1.15, 22.52	0.0310	1.88	0.30, 11.80	0.4966
		TT or TG	163 (98)	54 (92)	1	1	54 (96)	1			
		GG	3 (2)	5 (8)	5.03	5.03	1.15, 21.93	0.0306	2.01	0.32, 12.49	0.4503
			p-value ^b	0.0493			0.8036				

Abbreviations: *FTO* rs9939609 considered as the surrogate of rs9926289 (genotypes: GG, GA or AA, respectively) in high linkage disequilibrium (LD); *TMEM18* rs4854344 considered as the surrogate of rs6548238 (CC, CT or TT, respectively) and rs2867125 (GG, GA or AA, respectively) in high LD.

^a Univariate logistic regression p-value; ^b Pearson χ^2 p-value; ^c n=281.

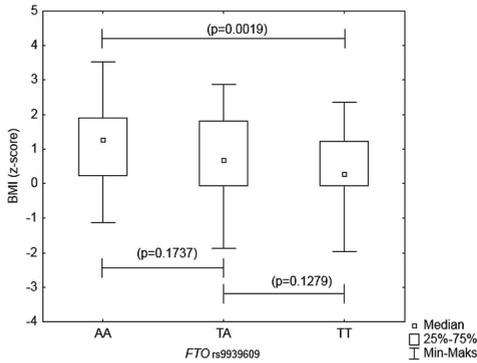


Fig. 1. Association between BMI z-scores and *FTO* rs9939609 genotypes. Differences were tested with the Kruskal-Wallis test; *p*-values are for multiple (two-way) comparisons.

have a significant effect on BMI z-scores ($p=0.0955$).

Putative explanation of the effect of *TMEM18* SNP genotypes on risk of overweight

To elucidate the nature of the association between the studied *TMEM18* SNPs and BMI, we analyzed the number of carriers with random combinations of *TMEM18* and *FTO* SNPs in the studied sample. Correspondence analysis (CA) made it possible to graphically display associations between all combinations of SNP allele variants in both genes (row profile) with BMI categories (column profile). Fig. 2 shows points perfectly representing (quality=1) the total measure of dispersion (inertia=100%) of these two profiles in a two-dimensional coordinate system. The first dimension (66% of total inertia) shows the greatest distance between the group of normal-weight individuals (to the left of 0) and those with overweight of obesity (to the right). In turn, the second dimension (34%) separates overweight

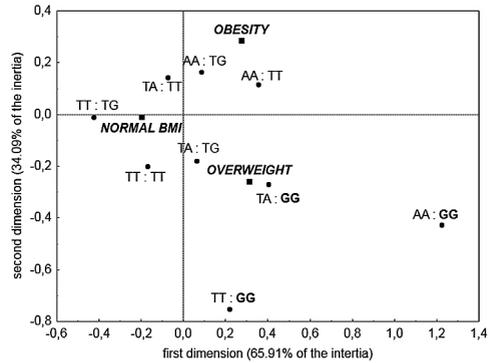


Fig. 2. Correspondence analysis (CA) – plot of two-dimensional coordinates for the row profile (number of carriers of *FTO* rs9939609: *TMEM18* rs4854344 genotypes) and the column profile (number of children with normal BMI, overweight, or obesity). *TMEM18* minor allele homozygotes are marked in bold.

individuals (below) from obese ones (above).

Overweight or obesity (dimension 1) is relatively more often associated with the minor allele homozygotes of *FTO* rs9939609 (AA) (to the right) than the major allele homozygotes (TT), which are more frequent on the side of normal body weight (to the left). In turn, in differentiating overweight from obesity (dimension 2), of greater importance is the *TMEM18* allele variant than the effects of the *FTO* heterozygote or AA homozygote. The minor allele *TMEM18* homozygotes (GG) shown in the figure are relatively more often associated with overweight than obesity. The question arises whether or not the higher risk of overweight in individuals homozygous for the *TMEM18* SNP minor alleles is indirectly the effect of *FTO* alleles A, which predispose to obesity. Co-occurrence with the *FTO* SNP AA homozygote precludes normal body weight, while co-occurrence with the TT homozygote precludes obesity, but not overweight (Table 3). Among the minor allele homozygotes for the *TMEM18* SNP,

Table 3. Log-normal model (GLZ) results for the dependent variable BMI [kg/m²] adjusted for the age and sex of the examined children and for the independent variables: *FTO* and *TMEM18* SNP genotypes and BMI category (n=281).

Independent variables	Tests of all effects <i>p</i> -value ^a	Evaluations of parameters		
		β^b	95% CI	<i>p</i> -value ^b
BMI category	<0.0001			
normal BMI vs. obesity		-0.192	-0.218, -0.166	<0.0001
overweight vs. obesity		0.015	-0.006, 0.037	0.1676
<i>FTO</i> rs9939609 genotype	0.6758			
<i>TMEM18</i> rs4854344 genotypes	0.0034			
TT vs. GG		0.009	-0.009, 0.027	0.3313
TG vs. GG		-0.025	-0.045, -0.005	0.0128
BMI category \times <i>TMEM18</i> rs4854344 genotypes	0.0077			
normal BMI vs. obesity \times TT vs. GG		-0.033	-0.060, -0.006	0.0159
normal BMI vs. obesity \times TG vs. GG		-0.012	-0.040, 0.017	0.4316
overweight vs. obesity \times TT vs. GG		-0.009	-0.032, 0.015	0.4715
overweight vs. obesity \times TG vs. GG		0.012	-0.014, 0.039	0.3677

Abbreviations: *FTO* rs9939609 considered as the surrogate of rs9926289 (genotypes: GG, GA or AA, respectively) in high LD; *TMEM18* rs4854344 considered as the surrogate of rs6548238 (CC, CT or TT, respectively) and rs2867125 (GG, GA or AA, respectively) in high LD.

^a*p*-value for Wald statistics; ^bbeta coefficient from the generalized linear model for the variable indicated, which is the log of the mean change in BMI [kg/m²] and *p*-value for beta.

the overweight group (with “pre-obesity”) is the most numerous, and, at the same time, the most differentiated in terms of *FTO* SNP genotypes.

How can one interpret the fact of increasing the risk of overweight, but not obesity? Is the risk of obesity modified in individuals homozygous for the minor alleles of the studied *FTO* and *TMEM18* SNPs?

To address this question, we decided to examine BMI differentiation within the normal-weight, overweight, and obese categories taking into consideration the children’s genotypes. Table 4 presents the results of the log normal model (GLZ) for the dependent variable BMI [kg/m²] adjusted for the age and sex of the examined children and for the independent variables: SNP genotypes in *FTO* and *TMEM18* and the BMI category. Using the BMI category in the analysis led

to identification of a significant correlation between BMI and the *TMEM18* SNP genotypes (*p*=0.0034). The *FTO* genotypes, which have a significant effect on BMI (z-scores) for the entire sample, are not significant within particular BMI categories (*p*=0.6758). The significant role of the *TMEM18* SNP genotype is due to its interaction (*p*=0.0077) with the BMI category, which is obviously associated with BMI values [kg/m²] (*p*<0.0001).

An interaction indicates that one variable has a different effect at different levels of another variable. In this case, this concerns the mean BMI in carriers of GG and TT *TMEM18* SNP genotypes in the obese category as compared to the normal-weight category. Obese individuals with the GG genotype had the lowest mean BMI within their category, while normal-weight individuals with this genotype had the high-

Table 4. Log-normal model (GLZ) predictions for mean BMI [kg/m²] values and standard deviations for carriers of *FTO* and *TMEM18* SNP genotypes by BMI category calculated for the mean values of accompanying variables (sex, age and *FTO* or *TMEM18* SNP genotypes) in particular BMI categories (n=281); confidence interval estimates for population means are given (CI=±95%).

Gene/SNP	BMI category	Genotype	n	Mean	Standard deviation	95% CI
<i>FTO</i> rs9939609	Normal BMI	AA	29	16.58	0.02	15.99, 17.19
		TA	84	16.28	0.01	15.89, 16.67
		TT	53	16.33	0.01	15.87, 16.81
	Overweight	AA	17	20.06	0.02	19.38, 20.77
		TA	27	20.94	0.01	20.35, 21.55
		TT	15	20.63	0.02	19.84, 21.44
	Obesity	AA	20	25.08	0.01	24.37, 25.82
		TA	29	25.13	0.01	24.49, 25.80
		TT	7	24.07	0.02	22.96, 25.23
<i>TMEM18</i> rs4854344	Normal BMI	TT	113	16.38	0.01	16.10, 16.67
		TG	50	16.18	0.01	15.78, 16.59
		GG	3	17.83	0.05	16.20, 19.63
	Overweight	TT	37	20.66	0.01	20.20, 21.13
		TG	17	20.39	0.02	19.71, 21.10
		GG	5	20.91	0.03	19.74, 22.15
	Obesity	TT	40	25.55	0.01	25.09, 26.02
		TG	14	23.67	0.02	22.94, 24.41
		GG	2	23.68	0.04	21.71, 25.82

Abbreviations: *FTO* rs9939609 considered as the surrogate of rs9926289 (genotypes: GG, GA or AA, respectively) in high LD; *TMEM18* rs4854344 considered as the surrogate of rs6548238 (CC, CT or TT, respectively) and rs2867125 (GG, GA or AA, respectively) in high

est BMI ($p=0.0159$) (Fig. 3). In other words, the minor allele (GG) *TMEM18* homozygotes are the fattest among normal-weight children, but the leanest among obese children. Thus, the effect of this *TMEM18* SNP genotype, which is the least common in the study group, may be likened to a weight guard, which significantly raises the risk of overweight, but not obesity, because it is conducive to higher body mass within the “pre-obesity” limits. As can be seen, the low number of carriers of the minor allele (GG) genotype in the studied *TMEM18* SNPs leads to considerable width of the adopted confidence interval (±95%) estimated for the population mean (Table 5). To make this interval

narrower, it would be necessary to increase the sample size.

The mean BMI in carriers *FTO* SNP genotypes do not exhibit interactions with BMI categories in the study sample. The levels of differences between the highest and lowest values of BMI do not vary significantly across the compared groups of normal-weight, overweight, and obese children ($p=0.1314$).

Discussion

Meta-analysis of studies on the *FTO* rs9939609 polymorphism involving over 38,000 Europeans has revealed that adult AA homozygous carriers (accounting for 16% of the population) weigh more by

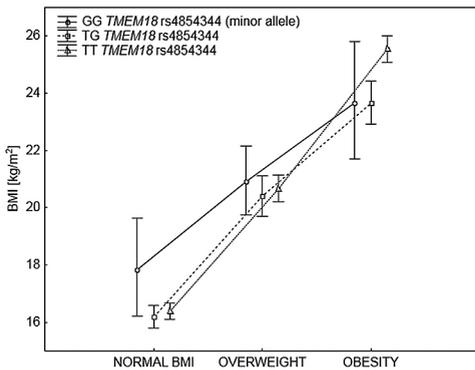


Fig. 3. Log-normal model (GLZ) predictions for mean BMI [kg/m²] values for carriers of *TMEM18* rs4854344 genotypes calculated for the mean values of accompanying variables (sex, age, and *FTO* rs9939609 genotypes). Error bars represent confidence interval estimates ($\pm 95\%$) for population means. A significantly lower mean BMI value was found for minor allele homozygotes (GG) as compared to major allele homozygotes (TT) in the obese vs. normal-weight children group ($p=0.0159$).

about 3 kg than carriers of the TT genotype and are at greater risk of overweight (OR=1.38) and obesity (OR=1.67) (Frayling et al. 2007). Numerous studies on different populations and age groups confirm a significant correlation of allele A (and especially the AA genotype) with elevated BMI values and with higher values of other anthropometric traits such

as total body fat mass, waist circumference, and skinfold thickness (Frayling et al. 2007; Scuteri et al. 2007; Cecil et al. 2008; Tan et al. 2008; Wardle et al. 2008; Willer et al. 2009; Graff et al. 2012). In turn, the association of *FTO* rs9926289 with BMI has been confirmed by a study on the genetically isolated population of Sardinia (Scuteri et al. 2007) as well as on the Chinese and Malay populations inhabiting Singapore (Tan et al. 2008), in which rs9926289 and rs9939609 were found to be in strong linkage disequilibrium. These results are not surprising, because both of these polymorphisms are located within the 47 kb region of *FTO*, in which SNPs are highly correlated with each other. This has been corroborated by analysis of as many as 15 *FTO* SNPs located within the same LD block of approximately 50 kb in the Japanese population (Hotta et al. 2008). Our study revealed complete dependence between *FTO* rs9939609 and rs9926289 ($D'=1.0$, $r^2=1.0$) for the first time in the population of Polish children.

Another finding from our study that is consistent with reports of other authors is the association of the AA homozygote for rs9939609 and rs9926289 with significantly increased BMI val-

Table 5. Number of carriers of different *FTO* rs9939609 and *TMEM18* rs4854344 genotypes in normal-weight, overweight, and obesity categories ($n=281$).

<i>FTO</i> rs9939609	<i>TMEM18</i> rs4854344	Normal BMI n (%)	Overweight n (%)	Obesity n (%)
AA	TT	17 (10)	11 (19)	13 (23)
	TG	12 (7)	4 (7)	6 (11)
	GG	-	2 (3)	1 (2)
TA	TT	60 (36)	14 (24)	22 (39)
	TG	22 (13)	11 (19)	6 (11)
	GG	2 (1)	2 (3)	1 (2)
TT	TT	36 (22)	12 (20)	5 (9)
	TG	16 (10)	2 (3)	2 (3)
	GG	1 (1)	1 (2)	-

ues (z-scores) as compared to homozygotes of the major alleles (TT and GG, respectively) ($p=0.0019$), while the mean BMI of heterozygous individuals was not affected significantly. Similar results were obtained in a study of 968 Polish children living in different parts of the country and aged 4 to 18 years (mean age: 14.01 ± 3.24 years) (Luczynski et al. 2012). However, it should be emphasized that, based on the AIC, we found that in estimating risk of obesity the codominant model has a better fit to the dataset than the recessive model, which is in contrast to the model of *FTO* rs9939609 inheritance in the population of Polish children postulated by Luczynski (2012). In our study, in the codominant model the odds ratio for obesity was over 2.5-fold higher for heterozygous individuals (OR=2.71, 95% CI: 1.11–6.66, $p=0.0284$) and over 5-fold higher for AA homozygous individuals (OR=5.42, 95% CI: 2.04–14.39, $p=0.0006$) than in TT homozygous individuals. This result is much higher than data for the European population (Frayling et al. 2007) as well as for Polish children (Luczynski et al. 2012). This may be related to the age of the subjects as analyzes conducted on different populations show that many of the genetic variants known to be associated with BMI have a stronger role during childhood and adolescence than during adulthood (Hardy et al. 2010; Graff et al. 2012; Rask-Andersen et al. 2012). Some authors propose that in adulthood an obesogenic environment may have a greater influence on body mass than genetic factors. Our results, which show the stronger role of genetic factors in children at an early stage of ontogenesis (mean age: 8.95 ± 1.53 years) seem to corroborate the above conclusion. Other authors have reported that a significant

relationship of *FTO* rs9939609 with BMI and obesity in a cohort of Chinese children aged 6–18 years may change from childhood to adolescence, and the association did not appear until children reached 12–14 years (Zhang et al. 2014).

As it was already mentioned, genome-wide association studies have shown a strong association of the rs2867125, rs4854344, rs6548238, and rs7561317 polymorphisms located near *TMEM18* (within approximately 200 kb) with weight and BMI in adult Europeans (Thorleifsson et al. 2009; Willer et al. 2009). Other authors have reported a significant relationship of rs2867125 and rs4854344 with BMI in a cohort of American children of European origin aged 0–18 (Zhao et al. 2009). Subsequent studies on BMI and other morphological traits based on case-control association analysis have confirmed the risk contribution of the *TMEM18* locus to obesity for the adult population (Holzapfel et al. 2010), also outside Europe (Hotta et al. 2009), as well as for children (Almen et al. 2010; Graff et al. 2012; Rask-Andersen et al. 2012).

The fact that these SNPs located near *TMEM18* are in almost absolute linkage disequilibrium has been consistently reported many publications, including a study of Japanese adults based on analysis of rs4854344, rs6548238, rs2867125, and rs7561317 (not studied by us) polymorphisms ($D'>0.99$, $r^2>0.95$) (Hotta et al. 2009); a study of American children of European origin involving rs2867125, rs4854344, and rs7561317 ($r^2=1$) (Zhao et al. 2009); and a study of Greek children based on rs6548238 and rs4854344 ($r^2=0.98$) (Rask-Andersen et al. 2012). Our material (a sample of 283 children) allowed us to confirm for the first time complete dependence between all 3 SNPs

(rs4854344, rs6548238 and rs2867125) in 281 Polish children (99.3% of the sample).

The major alleles of rs2867125 (G), rs6548238 (C), rs4854344 (T), and rs7561317 (G) have been consistently shown to increase the risk of obesity and/or imply higher BMI values in Swedish children (Almen et al. 2010), Greek children (Rask-Andersen et al. 2012), American adolescents of various ethnic backgrounds (Graff et al. 2012), and the adult Japanese population (Hotta et al. 2009). There are also some less known results showing that the minor alleles of rs2867125 (A), rs4854344 (G), and rs7561317 (A) decrease BMI values in American children of European origin aged 0–18 (Zhao et al. 2009), while the minor allele of rs6548238 (T) has the same effect on the adult German population (Holzapfel et al. 2010). In this respect, of special importance are the results of a study on 12,462 adults (from the population-based MONIKA/KORA Augsburg study), which proved for the first time that risk of obesity decreased by 14% per minor allele (T) of rs6548238 (Holzapfel et al. 2010). According to a study on non-diabetic adult Swedes, differences in weight between individuals homozygous for the major and minor alleles of rs6548238 were predominantly associated with adipose accumulation, rather than with non-adipose tissue (e.g., bone, muscle, and organ tissue) (Renstrom et al. 2009).

Our study involved not only obese, but also overweight, children, and focused on the effects of minor allele genotypes using the codominant and recessive models. The *TMEM18* SNPs did not exhibit a correlation with obesity in the examined children. The most significant result was an increased odds ratio for

overweight (OR=5.03, 95% CI: 1.15–21.93, $p=0.0306$) in homozygous carriers of the minor alleles rs4854344 (G), rs6548238 (T) and rs2867125 (A) in the recessive inheritance model.

The results obtained in further analyzes are not inconsistent with the results for the effects of the minor or major alleles of the near-*TMEM18* SNPs because they indicate that individuals homozygous for the major alleles have a higher BMI than those homozygous for the minor alleles, but in our opinion this observation concerns only obese children (Fig. 3).

The underlying cause of the higher risk of overweight in individuals homozygous for all the three minor alleles in the sample of Polish children is not known, but it may be associated with a higher BMI also in normal weight carriers. The paradox of the effect of minor allele homozygotes is conveyed in the proposed concept of a “weight guardian,” as body mass growth seems to be promoted up to the obesity threshold (within the limits of “pre-obesity”).

The hypothetical effect of genotypes homozygous for minor alleles in the *TMEM18* region proposed by us on the basis of a rather small sample is one of the possible explanations for the effects of minor alleles, which, in contrast to major alleles, minimize the risk of obesity. The obesity-prevention effect of *TMEM18* minor allele homozygotes can be easily verified by case-control analysis involving a group of individuals with diagnosed overweight, in addition to obese individuals. A new approach to the study material may elucidate the functional context of *TMEM18* and the effects of its SNP variants. In order to verify the results for the Polish population, we intend to extend the study material for the pop-

ulation of children and also analyze the BMI of adult Poles.

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Authors' contributions

IR and DS equally contributed to this work. IR served as principal investigator for the research, performed statistical analysis, analyzed data; DS served as principal investigator for the research, designed and carried out genetic laboratory analysis and interpreted the results, analyzed data; AS performed statistical analysis, analyzed data; MM carried out laboratory analysis; LO-N served as principal investigator for the research; EŻ conceived the concept, designed and performed the research project, analyzed data. All authors were involved in drafting the manuscript and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflicts of interest regarding this paper.

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References

- Almen MS, Jacobsson JA, Shaik JH, Olszewski PK, Cedernaes J, Alsio J, et al. 2010. The obesity gene, TMEM18, is of ancient origin, found in majority of neuronal cells in all major brain regions and associated with obesity in severely obese children. *BMC Med Genet* 11(58).
- Cecil JE, Tavendale R, Watt P, Hetherington MM, Palmer CN. 2008. An obesity-associated FTO gene variant and increased energy intake in children. *N Engl J Med* 359(24):2558–66.
- Chrzanowska M, Koziel S, Ulijaszek SJ. 2007. Changes in BMI and the prevalence of overweight and obesity in children and adolescents in Cracow, Poland, 1971–2000. *Econ Hum Biol* 5(3):370–8.
- Cole TJ, Bellizzi MC, Flegal KM, Dietz WH. 2000. Establishing a standard definition for child overweight and obesity worldwide: international survey. *BMJ* 320(7244):1240–3.
- Cole TJ, Green PJ. 1992. Smoothing reference centile curves: the LMS method and penalized likelihood. *Stat Med* 11(10):1305–19.
- Cole TJ, Lobstein T. 2012. Extended international (IOTF) body mass index cut-offs for thinness, overweight and obesity. *PediatrObes* 7(4):284–94.
- Field SF, Howson JM, Walker NM, Dunger DB, Todd JA. 2007. Analysis of the obesity gene FTO in 14,803 type 1 diabetes cases and controls. *Diabetologia* 50(10):2218–20.
- Frayling TM, Timpson NJ, Weedon MN, Zeggini E, Freathy RM, Lindgren CM, et al. 2007. A common variant in the FTO gene is associated with body mass index and predisposes to childhood and adult obesity. *Science* 316(5826):889–94.
- Gerken T, Girard CA, Tung YC, Webby CJ, Saudek V, Hewitson KS, et al. 2007. The

- obesity-associated FTO gene encodes a 2-oxoglutarate-dependent nucleic acid demethylase. *Science* 318(5855):1469–72.
- Graff M, North KE, Mohlke KL, Lange LA, Luo J, Harris KM, et al. 2012. Estimation of genetic effects on BMI during adolescence in an ethnically diverse cohort: The National Longitudinal Study of Adolescent Health. *Nutr Diabetes* 2:e47.
- Hardy R, Wills AK, Wong A, Elks CE, Wareham NJ, Loos RJ, et al. 2010. Life course variations in the associations between FTO and MC4R gene variants and body size. *Hum Mol Genet* 19(3):545–52.
- Holzappel C, Grallert H, Huth C, Wahl S, Fischer B, Doring A, et al. 2010. Genes and lifestyle factors in obesity: results from 12,462 subjects from MONICA/KORA. *Int J Obes (Lond)* 34(10):1538–45.
- Hotta K, Nakamura M, Nakamura T, Matsuo T, Nakata Y, Kamohara S, et al. 2009. Association between obesity and polymorphisms in SEC16B, TMEM18, GNPDA2, BDNF, FAIM2 and MC4R in a Japanese population. *J Hum Genet* 54(12):727–31.
- Hotta K, Nakata Y, Matsuo T, Kamohara S, Kotani K, Komatsu R, et al. 2008. Variations in the FTO gene are associated with severe obesity in the Japanese. *J Hum Genet* 53(6):546–53.
- Knussmann R. 1988. *Anthropologie. Handbuch der vergleichenden Biologie der Menschen. Band I. Wesen und Methoden der Anthropologie.* Stuttgart.
- Kozieł S, Welon Z, Bielicki T, Szklarska A, Uliaszek S. 2004. The effect of the economic transition on the body mass index of conscripts in Poland. *Econ Hum Biol* 2(1):97–106.
- Kozieł S. 2005. What is the risk for overweight children of being overweight at the age of 18 years? The Wrocław Growth Study. *Przegląd Antropologiczny – Anthropol Rev* 68: 43–52.
- Łuczyński W, Zalewski G, Bossowski A. 2012. The association of the FTO rs9939609 polymorphism with obesity and metabolic risk factors for cardiovascular diseases in Polish children. *J PhysiolPharmacol* 63(3):241–8.
- Ohashi J, Naka I, Kimura R, Natsuhara K, Yamauchi T, Furusawa T, et al. 2007. FTO polymorphisms in oceanic populations. *J Hum Genet* 52(12):1031–5.
- Pan H, Cole T. 2011. LMS growth a Microsoft Excel add-in to access growth references based on the LMS method. (Version 2.74).
- Rampersaud E, Mitchell BD, Pollin TI, Fu M, Shen H, O'Connell JR, et al. 2008. Physical activity and the association of common FTO gene variants with body mass index and obesity. *Arch Intern Med* 168(16):1791–7.
- Rask-Andersen M, Jacobsson JA, Moschonis G, Chavan RA, Sikder MA, Allzen E, et al. 2012. Association of TMEM18 variants with BMI and waist circumference in children and correlation of mRNA expression in the PFC with body weight in rats. *Eur J Hum Genet* 20(2):192–7.
- Renstrom F, Payne F, Nordstrom A, Brito EC, Rolandsson O, Hallmans G, et al. 2009. Replication and extension of genome-wide association study results for obesity in 4923 adults from northern Sweden. *Hum Mol Genet* 18(8):1489–96.
- Rosset I, Roślak M, Grabowska J, Borowska-Strugińska B, Lorkiewicz W, Sitek A, et al. 2009. The state of physical development of children in Łódź. In Żądzińska E (Ed.), *Dziecko łódzkie 2004. Normy rozwoju biologicznego.* (pp. 39–142). Łódź: Wydawnictwo Uniwersytetu Łódzkiego. (in polish)
- Roszkopf D, Schwahn C, Neumann F, Bornhorst A, Rimmbach C, Mischke M, et al. 2011. The growth hormone--IGF-I axis as a mediator for the association between FTO variants and body mass index: results of the Study of Health in Pomerania. *Int J Obes (Lond)* 35(3):364–72.
- Scuteri A, Sanna S, Chen WM, Uda M, Albai G, Strait J, et al. 2007. Genome-wide association scan shows genetic variants in the FTO gene are associated with obesity-related traits. *PLoS Genet* 3(7):e115.

- Sitek A, Rosset I, Strapagiel D, Majewska M, Ostrowska-Nawarycz L, Żądzińska E. 2014. Association of FTO gene with obesity in Polish schoolchildren. *Anthropol Rev* 77(1):33–44.
- Tan JT, Dorajoo R, Seielstad M, Sim XL, Ong RT, Chia KS, et al. 2008. FTO variants are associated with obesity in the Chinese and Malay populations in Singapore. *Diabetes* 57(10):2851–7.
- Thorleifsson G, Walters GB, Gudbjartsson DF, Steinthorsdottir V, Sulem P, Helgadóttir A, et al. 2009. Genome-wide association yields new sequence variants at seven loci that associate with measures of obesity. *Nat Genet* 41(1):18–24.
- Wahlen K, Sjolín E, Hoffstedt J. 2008. The common rs9939609 gene variant of the fat mass- and obesity-associated gene FTO is related to fat cell lipolysis. *J Lipid Res* 49(3):607–11.
- Wardle J, Carnell S, Haworth CM, Farooqi IS, O’Rahilly S, Plomin R. 2008. Obesity associated genetic variation in FTO is associated with diminished satiety. *J Clin Endocrinol Metab* 93(9):3640–3.
- Wardle J, Carnell S, Haworth CM, Plomin R. 2008. Evidence for a strong genetic influence on childhood adiposity despite the force of the obesogenic environment. *Am J Clin Nutr* 87(2):398–404.
- Wardle J, Llewellyn C, Sanderson S, Plomin R. 2009. The FTO gene and measured food intake in children. *Int J Obes (Lond)* 33(1):42–5.
- Willer CJ, Speliotes EK, Loos RJ, Li S, Lindgren CM, Heid IM, et al. 2009. Six new loci associated with body mass index highlight a neuronal influence on body weight regulation. *Nat Genet* 41(1):25–34.
- Żądzińska E, Rosset I, Kozieł S, Nawarycz T, Borowska-Strugińska B, Lorkiewicz W, et al. 2012. Frequency of under- and overweight among children and adolescents during the economic transition in Poland. *Homo* 63(3): 216–32.
- Zhang M, Zhao X, Cheng H, Wang L, Xi B, Shen Y, et al. 2014. Age- and sex-dependent association between FTO rs9939609 and obesity-related traits in Chinese children and adolescents. *PLoS One* 9(5):e97545.
- Zhao J, Bradfield JP, Li M, Wang K, Zhang H, Kim CE, et al. 2009. The role of obesity-associated loci identified in genome-wide association studies in the determination of pediatric BMI. *Obesity (Silver Spring)* 17(12):2254–7.