

## Association of *FTO* gene with obesity in Polish schoolchildren

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**ABSTRACT:** The goal of the study was verification of fat mass and obesity-associated (*FTO*) gene polymorphisms as significant risk factors of obesity in the population of Polish children. Body mass index (BMI) and DNA were evaluated, where DNA was extracted from saliva, collected from 213 children at the age of 6–13 years.

DNA was genotyped by PCR (polymerase chain reaction) and HRM (high resolution melting) techniques, as well as by direct sequencing. Three (3) *FTO* polymorphisms were identified: rs9939609, rs9926289 and rs76804286, the last polymorphism located between the first two.

For the first time, absolute linkage disequilibrium (LD) of *FTO* gene rs9939609 and rs9926289 polymorphisms was confirmed in data for the Polish population ( $D' = 1$ ,  $r^2 = 1$ ). The lack of a complete dependence among the three single nucleotide polymorphisms (SNPs) of the *FTO* gene was a consequence of the concurrence of homozygotes with minor alleles A of rs9939609+rs9926289 of *FTO* (AA+AA) with major alleles of rs76804286 (GG).

A case-control association analysis for BMI in obese children ( $n = 51$ ), as compared to normal-weight children ( $n = 162$ ), was based on the effects of genotypes homozygous for the minor alleles of the studied SNPs in recessive and codominant inheritance models (assuming an independent effect of each genotype).

A comparison of children with normal BMI with obese children indicate a strong co-dominant effect of a genotype in homozygotes of minor alleles (AA+AA) of completely linked rs9939609+rs9926289 (OR at age  $8.89 \pm 1.54$  years = 4.87, 95% CI 1.81–13.12,  $p = 0.002$ ).

An almost five-fold increase of obesity risk in the examined children indicates that the genetic factors, associated with excessive body weight gain, exert stronger effects in the early period of ontogenetic development vs. puberty and adulthood. The role of genetic factors in predisposing to obesity declines with age.

**KEY WORDS:** body mass index, obesity-associated gene, rs76804286, rs9939609, rs9926289 (*FTO*), Polish population

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## Introduction

An epidemic of obesity in children is a national problem of utmost priority, with serious consequences for health, both in childhood and in adulthood. It is known that the etiopathogenesis of obesity includes a genetic component, as well as a set of environmental determinants (also those, regulating the prenatal environment) and behavioral factors, mostly linked to individual food consumption pattern and lifestyle. Despite the growing incidence of disorders in the weight-growth proportions, observed in the Polish population, especially among children after the social-economic transformation of the nineties in the previous century (among others, see Kozieł et al. 2004; Kozieł 2005; Chrzanowska et al. 2007; Chrzanowska and Suder 2010; Wronka et al. 2012; Żądzińska et al. 2012; Chrzanowska et al. 2013; Wronka 2013; Żądzińska and Rosset 2013), the genetic determinants of overweight and/or obesity in the population of Polish children and adolescents are very rarely studied (Luczynski et al. 2012).

The fat mass and obesity-associated (*FTO*) gene is one of the genes, associated with obesity pathogenesis. Genome-wide association studies have shown that *FTO* is associated with the obesity phenotype (Frayling et al. 2007; Scuteri et al. 2007). This gene is located on the long arm of chromosome 16 (16q12.2) and encodes a protein with the activity of 2-Oxoglutarate- and iron-dependent nucleic acid demethylase (II), which belongs to the superfamily of non-heme dioxygenases. This enzyme is present, among others, in the hypothalamus, namely in the appetite and energy expenditure controlling centre (Gerken et al. 2007). Single nucleotide polymorphisms (SNPs) were iden-

tified within the first intron of the gene, the SNPs being associated with excessive body weight gain. SNP rs9939609 lies within the first intron of the *FTO* gene and, based on the information from HapMap, is highly correlated ( $r^2 > 0.5$ ) with 45 additional SNPs within a 47-kb region that encompasses parts of the first two introns, as well as exon 2 of *FTO* (Frayling et al. 2007).

Performed analyses of the *FTO* rs9939609 polymorphism – indicating its association with the risk of type 2 diabetes mellitus – confirm the strong predisposition to obesity, mainly via the control of appetite and nutritional preferences (Field et al. 2007; Frayling et al. 2007; Cecil et al. 2008; Wardle et al. 2008a; 2009). Other studies demonstrate that the *FTO* gene, via its effects on insulin sensitivity in the brain cortex, takes part in the differentiation of preadipocytes to mature adipose cells (adipogenesis) or affects lipolysis in adipocytes, thus controlling the volume of adipose tissue (Rampersaud et al. 2008; Wahlén et al. 2008). In turn, performed analyses of rs9926289, another polymorphism, have indicated that the *FTO* gene may influence BMI values in younger subjects (below 55 years) via the growth hormone (GH) and its insulin-like growth factor (IGF1) (Roskopf et al. 2011). Both SNPs in the *FTO* gene seem to be closely related with each another, while and their combined actions may be confirmed (Frayling et al. 2007; Hotta et al. 2008), although certain results of studies, carried on various populations, do not confirm that observation (e.g., Ohashi et al. 2007; Dlouha et al. 2010; Bencova et al. 2012).

It is estimated that *FTO* polymorphism is responsible for 1% of BMI variations (Frayling et al. 2007). Regarding

the European population, the strongest effects have been demonstrated with the rs9939609 variant. Carriers of single allele A (associated with the risk of obesity), when compared with the carriers of TT genotype, are characterized by higher values of body weight, BMI, waist circumference and thickness of the subcutaneous fat layer. In AA homozygotes, this effect is even more pronounced. In the Asian population, where the incidence of the predisposing to obesity allele A is much lower, the effect of the rs9939609 variant turns out to be weaker (Chang et al. 2008).

In order to determine the age, from which the *FTO* rs9939609 relationship with BMI occurs, large birth cohorts were analyzed for which suitable measures were available from birth to early adolescence (Frayling et al. 2007). Those studies helped find out that the polymorphism was not related to changes in fetus development. A significant increase in the fat tissue volume was observed only in children at the age of 7 years and older. It has been shown that common variation in the *FTO* gene is reproducibly associated with BMI and obesity from childhood into old age. Other studies indicate growing effects of the *FTO* gene till the age of 20 years, followed by their steady decline (Hardy et al. 2010). Following other reports, the first effects the *FTO* variant may be observed already after two weeks from birth (Lopez-Bermejo et al. 2008).

The goal of our study was verification (by means of a case-control association analysis) of *FTO* gene rs9939609, rs76804286 and rs9926289 polymorphisms as significant risk factors for obesity occurrence in the population of Polish children.

## Materials and Methods

### Subjects

The study was approved by the Institutional Review Board of the University of Lodz (KBB-UŁ/1/10/2011). The study design included case-control analysis involving anthropometric measurements and genetic tests of pupils from seven randomly selected primary schools in Lodz located in all five districts of the city, as well as of children presenting with overweight and obesity at a public 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. The study consisted of a questionnaire concerning course of pregnancy and children's birth parameters, ethnicity and health. Due to incomplete questionnaire data, we analyzed a group of 213 pupils aged 6–13 (6.33–13.38), whose parents or guardians completed the questionnaire providing information. Based on information from the parents, it was determined that all children were ethnically Polish and none of them was born from a pregnancy complicated with maternal diabetes or suffered from diabetes. The study group was also free of genetic, metabolic, and other disorders that could be related to the children's body mass index (BMI). Anthropometric measurements were conducted from October 2011 to April 2012, and genetic tests were concluded in December 2012.

### Anthropometric measurements

Anthropometric measurements were done by workers of the Department of

Anthropology, University of Lodz following the standard procedure designed by Martin. The children were weighed in light sports apparel on medical scales with an accuracy of 0.1 kg. Body height was measured with an accuracy of 0.1 cm. On the basis of individual body height and weight measurements, the body mass index (BMI) was calculated for each examined child, following the formula:  $BMI = \text{body mass [kg]} / \text{body height squared [m}^2\text{]}$ .

BMI values were categorized according to the International Obesity Taskforce (IOTF) standards (Cole et al. 2000) using the LMSGrowth software (Pan and Cole 2011).

Individual BMI [ $\text{kg/m}^2$ ] values were standardized for age and sex according to the current BMI reference values for children and adolescents (aged 6.50 – 19.49 years) living in Lodz (Rosset et al. 2009), obtained using the LMS method (Cole and Green 1992). The z-scores for children's BMI were calculated 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

Saliva specimens 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 spec-

imens were coded and stored at room temperature. The collected material was tested for 3 polymorphisms: rs76804286, rs9939609, rs9926289 (*FTO*). All of these single-nucleotide polymorphisms (SNPs) exhibited an allele distribution consistent with the Hardy–Weinberg law in the studied material treated as a whole (cases+controls).

### DNA isolation

Genomic DNA was extracted from 500  $\mu\text{L}$  of saliva, collected with an OG–500 Oragene DNA Kit (DNA Genotek, Canada), using a MagNaPure LC 2.0 instrument with an 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 by 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  $-30^\circ\text{C}$ .

### Primer design

All primers for the amplification of 3 SNPs were designed using the online software Primer 3 (Biology Workbench, <http://workbench.sdsc.edu>) (Table 1). The specificity of primers was checked on Primer-BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast>).

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

Primers used for	Gene	Single nucleotide polymorphism	Forward primer (5'→3')	Reverse primer (5'→3')	Amplicon size bp
the HRM method	<i>FTO</i>	rs9939609 rs76804286 rs9926289	CATCAGTTATGCAT- TTAGAATGTCTG	AGAGTAACAGAGAC- TATCCAAGTGC	95
direct sequencing	<i>FTO</i>	rs9939609 rs76804286 rs9926289	TGGTTTCAGAGGCTTGT- GTG	GCCCAAGGATGGTGT- TTCTA	695

### PCR amplification

Three 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  $\mu$ L reaction volume, which included 0.5 ng DNA template, 2 $\times$ GoTaq<sup>®</sup> Hot Start Colorless Master Mix (Promega, USA), 10 $\times$ LCGreen Plus Dye (Idaho Technology) and 0.25  $\mu$ M 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 70–95°C, with an in-

crement of 0.2°C every 10 s, and each amplicon cluster was determined using Precision Melt Analysis Software (BioRad, USA) (Figure 1). For tested 3 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  $\chi^2$  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. obese groups were eval-

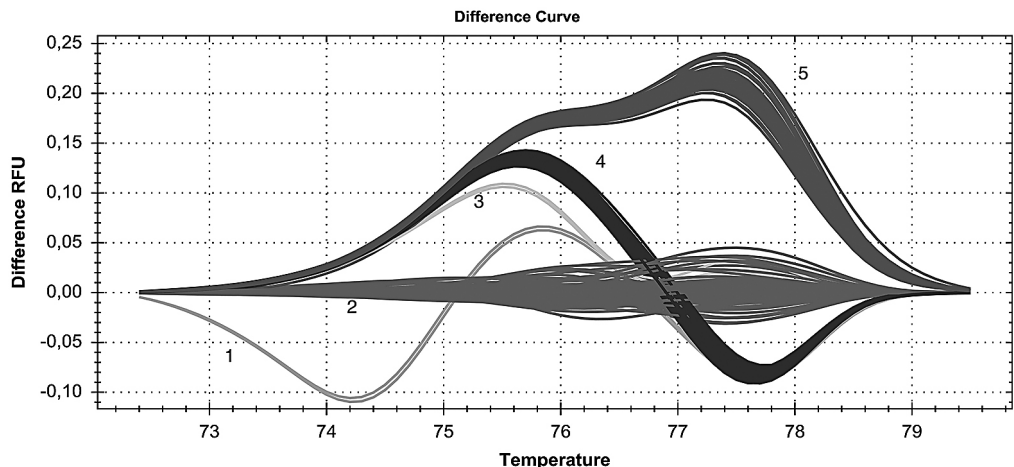


Fig. 1. Sample results of HRM analyses for studied *FTO* SNPs. Genotypes of three examined SNPs (rs9939609, rs76804286, rs9926289): 1 – TA, GA, GA; 2 – TA, GG, GA; 3 – TT, GA, GG; 4 – AA, GG, AA; 5 – TT, GG, TT

uated using the codominant model (assuming an independent effect of each genotype) and the recessive model, using Pearson's  $\chi^2$  test (with Yates' correction for discontinuity for  $2 \times 2$  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.

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). All calculations were done using STATISTICA 10.0 software (StatSoft Poland).

## Results

According to the IOTF classification, 162 (76.1%) children had normal weight, 51 (23.9%) were obese (Table 2). More girls than boys were characterized by normal weight (84.6% and 65.6%, respectively). A greater rate of obesity was observed in boys than in girls (34.4% and 15.4%, respectively,  $p=0.001$ ). The above data do not reflect the distribution of excessive body weight in the population of children inhabiting Lodz, but only characterize the sample in the present case-control association study. Children with obesity were slightly older ( $9.86 \pm 2.19$  years) than children with normal body weight ( $8.58 \pm 1.11$  years). The higher proportion of boys was found in the obese group (65.6%) than in the control group (34.4%). However, these differences do not affect the objective of this study because the IOTF standard accounts for children's age and sex in BMI classification.

Analysis of linkage disequilibrium (LD) between *FTO* rs9939609 and rs9926289 genotypes revealed their complete dependence ( $D'=1.0$ ,  $r^2=1.0$ ). The frequency of allele A (the least common)

Table 2. Characteristics of the examined children

Variable	Total	Normal-weight (control group)	Obese (case group)
Number <i>n</i> (%)	213 (100)	162 (76.1)	51 (23.9)
Boys <i>n</i> (%)	96 (45.1)	63 (65.6)	33 (34.4)
Girls <i>n</i> (%)	117 (54.9)	99 (84.6)	18 (15.4)
Age (years) Mean $\pm$ SD	8.89 $\pm$ 1.54	8.58 $\pm$ 1.11	9.86 $\pm$ 2.19
BMI (z score) <sup>a</sup> Median (interquartile range)	0.34 (0.20–0.97)	0.14 (–0.41–0.50)	2.21 (2.04–2.39)

Abbreviations: SD – standard deviation

<sup>a</sup> Standardized using the LMS parameters developed on the basis of a population study of children inhabiting Lodz (Rosset et al. 2009)

was 47%. The distribution of genotypes AA, TA (GA in rs9926289), and TT (GG) was 46 (22%), 107 (50%), and 60 (28%), respectively. Between the completely dependent rs9939609+rs9926289 (which are consistently given in this order in the present paper), there is rs76804286. The frequency of allele A (the least common) was 2%. There was not a single AA homozygote for rs76804286 in the entire sample. The distribution of the GG and GA genotypes was 205 (96%) and 8 (4%), respectively. LD analysis shows that the dependence between the rs76804286 and rs9926289 genotypes and between the rs76804286 and rs9939609 genotypes ( $D'=1.0$ ,  $r^2=0.017$ ) was smaller (the  $r^2$  values are lower). All double AA homozygotes in rs9939609+rs9926289 were found to be also GG homozygotes in rs76804286 (100%). The distributions of the observed frequencies of all genotypes of the studied SNPs in the

entire samples were consistent with the Hardy–Weinberg law ( $p>0.05$ ).

Differences in the frequency of genotypes between the group of normal-weight children and the group of obese children were evaluated using the codominant model (assuming an independent effect of each genotype) and the recessive model (Table 3). Children with normal and abnormal weight differed significantly only in the frequency of *FTO* rs9939609+rs9926289 genotypes ( $p<0.011$ ). Assuming a recessive inheritance model, carriers of the AA+AA genotype were characterized by a higher risk of obesity (OR=2.61, 95% CI 1.29–5.30,  $p=0.008$ ). The risk of obesity increased by a factor of 2.5 for AA+AA homozygous as compared to the reference group – heterozygous or the major allele homozygous (TA+GA and TT+GG). In turn, assuming a codominant model, the TA+GA genotype in *FTO* was not found

Table 3. Associations of single nucleotide polymorphisms with BMI (categorized according to the International Obesity Task Force)

Gene/SNP	Genetic model	Genotype	Normal-weight n (%)	Obese n (%)	OR (95% CI)	<i>p</i> value <sup>a</sup>
<i>FTO</i> rs76804286	codominant	GG	156 (96)	49 (96)		
		GA	6 (4)	2 (4)		
		<i>p</i> value <sup>b</sup>	0.726			
<i>FTO</i> rs9939609 +rs9926289	codominant	TT+GG	53 (33)	7 (14)	1	
		TA+GA	81 (50)	26 (51)	2.43 (0.98, 6.03)	0.054
		AA+AA	28 (17)	18 (35)	4.87 (1.81, 13.12)	0.002
		<i>p</i> value <sup>b</sup>	0.004		AIC=229.47	
	recessive	TT+GG TA+GA	134 (83)	33 (65)	1	
		AA+AA	28 (17)	18 (35)	2.61 (1.29, 5.30)	0.008
		<i>p</i> value <sup>b</sup>	0.011		AIC=231.60	

Abbreviations: SNP – single nucleotide polymorphism; OR – odds ratio; CI – confidence interval; AIC – Akaike information criterion

<sup>a</sup> Univariate logistic regression *p*-value

<sup>b</sup> Pearson  $\chi^2$  *p*-value (with Yates' correction for 2 × 2 tables)

to be a risk factor for obesity, although it was close to statistical significance (OR=2.43, 95% CI: 0.98, 6.03,  $p=0.054$ ) and the risk of obesity increased by a factor of nearly 5 for AA+AA homozygous individuals (OR=4.87, 95% CI 1.81–13.12,  $p=0.002$ ) as compared to the reference group – the major allele homozygous (TT+GG). The lower AIC values indicate a better goodness of fit for the codominant model (229.47) than for the recessive model (231.60).

The observed significant correlations between the frequency of *FTO* polymorphisms and obesity in the studied children are confirmed by differences in BMI z-scores ( $p=0.024$ ) (Figure 2). A multiple comparison test for the mean ranks of the groups compared (post hoc) showed that statistically significant difference ( $p=0.019$ ) was found between AA+AA (median (interquartile range): 0.79 (–0.08 to 2.11)) and TT+GG homozygotes (0.18 (–0.17 to 0.53)). BMI for TA+GA heterozygotes (0.40 (–0.28 to 1.33)) did not differ from BMI for TT+GG homozygotes ( $p=0.451$ ) and AA+AA homozygotes ( $p=0.255$ ).

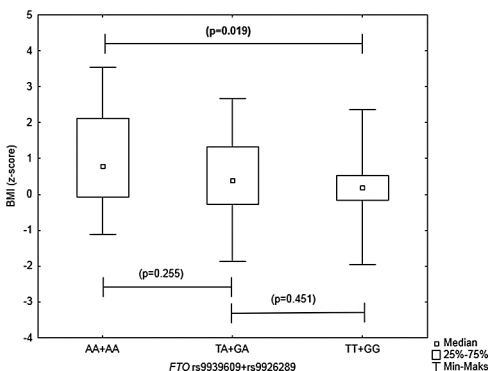


Fig. 2. Association between BMI z-scores and *FTO* rs9939609+rs9926289 genotypes. Differences were tested with the Kruskal-Wallis test;  $p$ -values are for multiple (two-way) comparisons

## Discussion

A metaanalysis of studies on the *FTO* gene rs9939609 polymorphism, based on data from more than thirty-eight (38) thousand Europeans, has demonstrated that the adult carriers of AA homozygotes (16% of the population) are characterized by higher body weight (by approximately 3 kg) and higher risk of overweight (OR=1.38) or obesity (OR=1.67) vs. the carriers of TT genotype (Frayling et al. 2007). Numerous studies in various populations and age groups confirm in unison the significant relationship of A allele (especially of AA genotype) with elevated BMI values, as well as with increased values of other anthropometric features, i.e., total body weight, waist circumference of the thickness of skin/fat folds (among others, Frayling et al. 2007; Scuteri et al. 2007; Cecil et al. 2008; Tan et al. 2008; Wardle et al. 2008b; 2009; Graff et al. 2012). In turn, the relationship of the *FTO* gene rs9926289 polymorphism with BMI was confirmed in studies on the genetically isolated population of Sardinia (Scuteri et al. 2007) and the Chinese and the Malayan populations in Singapore (Tan et al. 2008). In all those studies a strong linkage disequilibrium of rs9926289 and rs9939609 was unequivocally confirmed. These results are not surprising since both polymorphisms are within 47 kb of the *FTO* region, the SNPs of which are highly correlated, what has been confirmed by the analyses, based on as many as 15 *FTO* SNPs located within the same LD block of approximately 50 kb in the Japanese population (Hotta et al. 2008). In our studies, a full correlation of the *FTO* gene rs9939609 and rs9926289 polymorphisms ( $D'=1.0$ ,  $r^2=1.0$ ) was for the first time confirmed in the population of Polish children.



The analytic method of polymorphism occurrence, applied in our study and based on differences in the melting profiles of obtained PCR products, allowed for an identification and evaluation of the *FTO* rs76804286 polymorphism, located between the entirely conjugated rs9939609 and rs9926289 polymorphisms. As it may be suspected, the lack of complete dependence between the 3 studied *FTO* SNPs may result from the fact that the sample did not contain any AA homozygotes for rs76804286. Perhaps, a larger sample will allow to verify our very preliminary hypothesis of the co-occurrence of the minor A allele in rs76804286 only with the T+G major allele variant in the completely linked *FTO* rs9939609+rs9926289.

The other compatibility with results of other authors is the association of the AA+AA homozygote for rs9939609+rs9926289 with significantly increased BMI values (z-scores) as compared to homozygotes of the major alleles TT+GG ( $p=0.019$ ), while the mean BMI of TA+GA heterozygous individuals was not affected significantly. Similar results were obtained from studies of 968 Polish children from various regions of the country, aged 4 to 18 years (the mean age:  $14.01 \pm 3.24$  years) (Luczynski et al. 2012). It should, however, be emphasized that the codominant model is better adapted to data in the estimation of obesity risks vs. the recessive model, what would not confirm the concept of inheritance of the *FTO* rs9939609 polymorphism in the population of Polish children, the concept being postulated in the quoted studies (Luczynski et al. 2012). In the codominant model, used in our study, the odds ratio for obesity was nearly 5-fold higher for AA+AA homozygous individuals (OR at age

6–13 years=4.87, 95% CI 1.81–13.12,  $p=0.002$ ). This result is much higher than the data for the European population (Frayling et al. 2007) as well as for Polish children (OR at age 4–18 years=1.97, 95% CI 1.29–3.00,  $p=0.002$ ) (Luczynski et al. 2012). It seems that it may be related to the age of the studied subjects, as analyses, carried out in various populations, demonstrate that many of the genetic variant know to be associated with BMI have stronger role during childhood and adolescence than during adulthood (e.g., Hardy et al. 2010; Graff et al. 2012; Rask-Andersen et al. 2012). According to the authors, during adulthood, the obesogenic environment may have a relatively higher influence on body weight than the genetic factors. The results, obtained in those studies and indicating a stronger role of genetic factors in children in the early period of ontogenesis (the mean age:  $8.89 \pm 1.54$  years) – seem to confirm the quoted reports. Taking into account a verification of the results for the Polish population, we have planned to undertake an effort towards increasing the study material for the population of children, as well as to submit the BMIs of adult Poles to analysis, together with presentation of study results, concerning correlations among the other known genes, associated with the risk of obesity and to evaluate the interactions among gene polymorphisms, birth parameters and the socioeconomic status in the context of estimating the probability of overweight and/or obesity.

## Conclusions

Regarding the Polish population of children, linkage disequilibrium was confirmed between the analyzed *FTO* SNPs, which means that the rs9926289 is asso-

ciated with a BMI in the examined sample in exactly the same way as rs9939609. Minor allele homozygotes, rs9939609 and rs9926289, are associated with an increased predisposition to obesity.

The obtained results suggest a greater role of genetic factors in children at an early stage of ontogeny (for children by the age of  $8.89 \pm 1.54$  years) than in adolescence and adulthood.

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### Author contribution

AS served as principal investigator for the research, performed statistical analysis, analyzed data; IR performed statistical analysis, analyzed data; DS designed and carried out genetic laboratory analysis and interpreted the results; 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 conflict of interest in the research.

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