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Original Article

Methylation Status of *CYP27B1* and *IGF2* Correlate to BMI SDS in Children with Obesity

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Keywords
DNA methylation · Childhood obesity · Insulin-like growth factor 2 · Vitamin D

Abstract
Objective: Worldwide increasing childhood obesity is due to interactions between environmental and genetic factors, linked together by epigenetic mechanisms such as DNA methylation. ***Methods:*** 82 obese children (>95th BMI percentile , age: 3–18 years) were included. Anthropometric data, metabolic parameters, 25-OH vitamin D (25OHD), and pubertal status were recorded, 24-hour blood pressure monitoring was performed. BMI standard deviation score (SDS) was calculated. Using candidate gene approach, obesity- (insulin-like growth factor 2 (*IGF2*), proopiomelanocortin (*POMC*)) and vitamin D metabolism-related genes (1-alfa-hydroxylase (*CYP27B1*), *VDR*) regulated by DNA methylation were selected. After isolating DNA from peripheral blood, bisulfite conversion, bisulfite specific polymerase chain reaction (BS-PCR), and pyrosequencing were carried out. ***Results:*** No significant correlation between 25-OHD and metabolic parameters and DNA methylation status, but a tendency of positive correlation between VDR methylation status and 25-OHD (r = 0.2053,p = 0.066) were observed. Significant positive correlations between BMI SDS and *CYP27B1* hypermethylation (r = 0.2371,p = 0.0342) and a significant negative correlation between *IGF2* hypomethylation and BMI SDS (r = –0.305,p = 0.0059) were found. ***Conclusions*** Rate of obesity shows correlation with DNA methylation. Hypomethylation of *IGF2* and hypermethylation of *CYP27B1* genes might positively influence the rate of BMI observed in obese children.

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Introduction

The prevalence of obesity has been increasing dramatically over the past years not only worldwide but in European countries as well. This condition tends to appear at younger ages, which indicates that the risk factors, comorbidities, and consequences of obesity start getting in action very early on in life [1]. The increase of body weight is also correlated with an increased risk of type 2 diabetes, cardiovascular diseases, cancer, and mortality [2]. The cause of obesity is complex and includes endogenous and exogenous factors. Researchers suggest that the main exogenous factor of the worldwide increasing weight gain is the 'obesogenic environment', which includes unfavorable changes in lifestyle and environment. At the level of individuals obesity is present when energy intake exceeds energy expenditure. But also endogenous factors can play a role in developing obesity. According to genomewide association studies (GWAS) more than 40 genetic variants have been associated with obesity and shifting in fat distribution, while gene-environment interaction is also proposed to be an important factor for causing obesity.

According to recent studies, obesity results mainly from interactions between environmental and genetic factors, which are linked together by epigenetic mechanisms [3, 4]. The so-called epigenetic marking or 'imprinting' affects gene function and expression without modifying the DNA sequence. Epigenetic marks are tissue-specific and include DNA methylation, histone modification and micro RNA pathways, and influence cell function as well as molecular and metabolic processes [3]. Some nutritional components are able to influence the expression of some of the key genes in obesity via epigenetic changes. DNA methylation is the most studied epigenetic control mechanism that results in alteration of gene functions [5]. In utero obesity-linked epigenetic modifications also result from potential impact of the environment and influence the phenotype of the infant and continue to effect the development of obesity from infancy to adulthood [4]. Lately obesity studies that focus on epigenetic factors are not only based on 'obesogenic environment' but on candidate gene approach (CGA) too. According to CGA, expression of insulin-like growth factor 2 (*IGF2*) and proopiomelanocortin (*POMC*) genes correlate directly with growth, obesity, and body composition. *IGF2* along with *H19* are imprinted genes controlling the regulation of growth and body composition. IGF2 triggers a major signal transduction pathway via activation of the IGF1 receptor, which mediates anabolic effects. Huang et al. [6] found that through increased DNA methylation of the *IGF2/H19* region and therefore decreased expression of IGF2 leads to more subcutaneous fat mass in young adults. *POMC* gene, which encodes an anorexogenic neuropeptide, plays a central role in body-weight regulation within the hypothalamus through regulation satiety and energy expenditure. According to Marco et al. [8] in obesity the hypothalamic signal transduction pathway might be impaired, since high fat diet treated rats with obesity showed hyper methylated *POMC* promoter area. Kuehnen et al. [7] and Marco et al. [8] found that hypermethylation of the intron 2 and exon 3 boundary region of *POMC* gene is associated with childhood obesity.

The relationship between obesity and lower vitamin D levels has not been cleared yet. Vitamin D insufficiency and deficiency has become an issue worldwide. 25-hydroxy vitamin D (25OHD) is the dominant circulating form of vitamin D and tends to be a useful indicator of the actual vitamin D status [9]. The activation and metabolism of 25OHD is a complex process, which also includes the cytochrome P450 enzymes in the kidney and liver, e.g. the activating enzyme 1-alfa-hydroxylase (CYP27B1) in the kidney. The activity and the expression of the genes encoding these enzymes are also modulated by epigenetic pathways such as DNA methylation [10]. Vitamin D through its nuclear receptor (VDR) regulates epigenetic pathways and transcription of a number of genes involved in regulating metabolism and cell proliferation [11, 12]. According to recent studies in adults, low levels of circulating 25OHD are associated

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with increased fat mass, BMI, mortality, type 2 diabetes, cardiovascular diseases, and also dyslipidemia [13–18]. The expression of vitamin D metabolism-related genes and vitamin D receptor genes has been investigated in several studies focusing on cancer but rarely in patients with metabolic problems, especially in children suffering from obesity [19].

Therefore, the aim of our study was to investigate whether there is a correlation between the rate of obesity (BMI and BMI standard deviation score (SDS)) and the methylation status of genes related to vitamin D metabolism (*CYP27B1, VDR*) and metabolic status (*IGF2, POMC*) in Hungarian children with obesity.

Material and Methods

Subjects

A total of 82 (40 boys and 42 girls) children were included in the study with age-and sex-specific BMIs above the 95th percentile, who were otherwise healthy and aged 3–18 years. Our probands were examined and followed up at the 2nd Department of Pediatrics, Semmelweis University, Budapest, Hungary.

Anthropometric Data

Anthropometric data (height, weight, waist circumference, birth height, and birth length), metabolic parameters (lipid profile, fasting blood sugar, insulin, data of oral glucose tolerance test, and thyroid stimulating hormone), vitamin D, serum calcium and parathormone levels as well as pubertal status were recorded, and a 24-hour blood pressure monitoring was carried out. BMI SDS was calculated in each case in order to estimate the relative degree of overweight. Patients' history was taken focusing on mother's weight gain during pregnancy, perinatal issues, development, eating habits, and lifestyle. We collected peripheral blood samples for DNA analysis from each patient.

The study was approved by the Semmelweis University Regional and Institutional Committee of Science and Research Ethics, and a written informed consent was provided in each case.

DNA Isolation

Genomic DNAs were extracted from peripheral whole blood using QIAamp DNA Blood Mini KIT (250) (Qiagen Inc., Valencia, CA, USA) according to the manufacturers' instructions.

Quantitative Analysis of the Isolated DNA Samples

Concentrations of isolated nucleic acid samples were measured with NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Bisulfite Conversion

Bisulfite conversion was performed using EZ DNA Methylation™ Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. Based on NanoDrop spectrophotometer measurements, quantity of input DNA isolated from peripheral blood was 1 µg. Bisulfite-converted DNA (bcDNA) samples were eluted in 10 µl according to protocol. Concentrations of bcDNA samples were estimated by NanoDrop spectrophotometer using 'ssDNA' measurements.

Bisulfite-Specific PCR

In silico CpG island prediction of *CYP27B1, VDR, IGF2* and *POMC* genes was performed by CpG Plot EMBOSS Application (*www.ebi.ac.uk/Tools/emboss/cpgplot/index.html*). For island prediction gene's start codon ± 5–10 kb region was used. In order to select best target regions ENCODE DNA methylation data was analyzed by the UCSC Genome Browser (*https://genome.ucsc.edu*; fig. 1). Bisulfite-specific PCR (BS-PCR) reactions were performed using primers designed with PyroMark Assay Design software (SW 2.0; Qiagen Inc.) to be specific for CpG regions in order to amplify the methylated sequence of the bcDNA samples. PCR primers in the opposite direction of sequencing primers were biotin-labelled (table 1). Primer specificities were tested in silico by BiSearch software (*http://bisearch.enzim.hu*). Primers' target regions were designed to cover all methylation sites that play role in gene regulation. In case of *IGF2*, after in silico testing primers targeting CpG sites in promoter region did not turn out to be specific. To avoid bias in analysis, specific primers targeting other CpG regions of *IGF2* were used. BS-PCR reactions were performed using AmpliTaq

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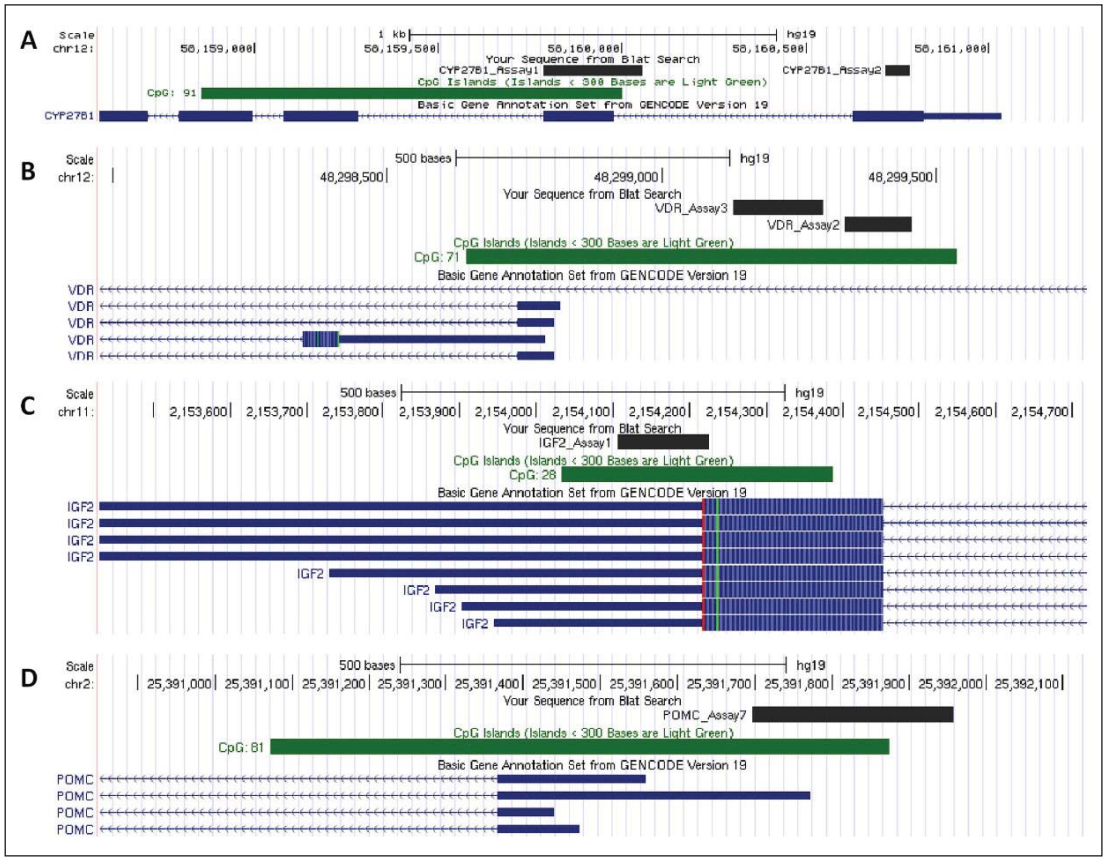

Fig. 1. Genes' loci and locations of bi-sulfite sequenced target regions. Investigated genes (labeled with lighter blue). CpG islands (location labeled with dark green) and bi-sulfite sequenced target regions (labeled with dark blue) presented with UCSC Genome Browser. (*https://genome.ucsc.edu*). **A** *CYP27B1* **B** *VDR* **C** *IGF2* **D** *POMC*. chr = Chromosome; Assay = bisulfite sequenced target region; *CYP27B1* = 1-alfa-hydroxylase gene; *VDR* = vitamin D receptor gene, *IGF2* = insulin-like growth factor gene; *D* = proopiomelanocortine gene.

Gold 360 Master Mix (Life Technologies, Carlsbad, CA, USA), LightCycler® 480 ResoLight Dye (Roche Applied Science, Basel, Switzerland), primers at 0.2 µmol/l final concentrations, and bcDNA samples (20–40 ng bcDNA/reaction) in 15 µl final volume. The final concentration of $MgCl_2$ was 2.5 mmol/l except for the *VDR* and *POMC*, where it was 1.5 mmol/l. Real-time PCR amplification was carried out with the following thermocycling conditions on the LightCycler 480 system: 95 °C for 10 min, 95 °C for 30 s, 60 °C with 0.4 °C decrease/cycle for 30 s, 72 °C for 30 s for 10 touchdown cycles, followed by amplification at 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s in 50 cycles. Following completion of the PCR thermal cycling, high resolution melting (HRM) analysis began with denaturation at 95 °C for 1 min, cool down to 40 °C, and hold for 1 min, then continuous warm up to 95 °C with 20 acquisition/°C rate during melting curve fluorescence acquisition. Cp values and normalized melting curves were retrieved after data preprocessing using the LightCycler 480 software release 1.5.0 (Roche Applied Science). MS-HRM curve data were retrieved with the LightCycler® Gene Scanning software (Roche Applied Science).

In order to calibrate our MS-HRM assays, artificially methylated DNA samples were mixed with non-methylated standard samples after bisulfite conversion resulting in different DNA methylation ratios (0%, 10%, 25%, 50%, 75%, 100%) and analyzed by MS-HRM. The average methylation level of all blood samples was estimated by two experts independently by visually comparing melting peak curves with those of standard samples. In the course of data processing the four parallel methylation values were verified, outliner values were excluded.

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Table 1. Target genes, assays and forward, biotinylated reverse primers for bisulfite-specific PCR and sequencing primers for pyrosequencing

Target gene and assay	Forward primer	Reverse primer (biotinylated)	Sequencing primer
CYP27B1 Assay 1	GGTTTTTGGGGGTAGAGAAGAT	CTCCCTATTCCCAAACCCAATCAA	GGGGGTAGAGAAGATTTA
CYP27B1 Assay 2	AGAGGGGTTTGGGATGTT	AACCCTCAAATACCCCTCCAAAATATTCCAT	GGGATGTTTGTTAAGTT
VDR Assay 2	GGATTAGGGATTAGGGAAGTTGAGATTTA	TACTACTACAAAACCCCAAAAAACTCAACCTAA	AGATTTAGTTTTTTTGGGTGA
VDR Assay 3	ATTTTAATTTGTGGGATTAGGTTGAGT	TAATCCAAAATACAACCCCCCACCCTTCCTAC	TGGAGTTTTGTAGTAGTAATAGG
IGF2 Assay1	GGGATTGGGTTAGGAGAAGT	CCCCCCCAAAAATAACCAACAAT	GGGTTAGGAGAAGTTTTA
POMC Assay 7	GTTGGAAAGGGGTTGGAATTAGTA	ACACCCACAAAACCACTCCTAACTTCTAC	TTTAGGAAGAATTTAATTATGGAT

CYP27B1 = 1-alfa-hydroxylase gene, *VDR* = *v*itamin D receptor gene; *IGF2* = insulin-like growth factor gene; *POMC* = proopiomelanocortin gene.

PyroMark Q24 Sequencing

Providing single-base resolution information about the methylation state of a CpG island, we used direct sequencing. After bisulfite treatment and BS-PCR, all cytosines are converted to thymines except for those originally methylated. Qiagen PyroMark System (Qiagen Inc.) pyrosequencing technology was applied to analyze DNA methylation of BS-PCR. The read length that can be analyzed with the then available PyroMark chemistry was limited to 100 bp. Pyrosequencing was performed on a PyroMark Q24 instrument (Qiagen Inc.) using PyroMark Gold Q24 Reagents (Qiagen Inc.) according to the manufacturer's recommendations. Purification and subsequent processing of the biotinylated single-stranded DNA were performed in a run by applying (Qiagen Inc.) specific sequencing primers designed by PyroMark Assay Design software, SW 2.0 (Qiagen Inc.) in order to cover CpG sites in the amplicons. Sequencing results were analyzed using the PyroMark Q24 software v2.0.6 (Qiagen Inc.). To define DNA methylation status the average methylation percentage of a gene region was estimated by pyrosequencing.

Statistical Analysis

We used paired Student's t test, Pearson correlation analysis, and linear regression models to investigate the connection between the DNA methylation status, vitamin D levels and the metabolic parameters. Statistical analysis was performed by GraphPad Prism 6 (2015 GraphPad Software, Inc., La Jolla, CA, USA) and SPSS Version 20.0 (2011 IMB Corporation, Armonk, NY, USA).

Results

While analyzing data one proband showed extreme weight (159.2 kg) and BMI SDS (16.38) values. To avoid data distortion, the patient was considered as outlier and got excluded from further examination.

At the time of inclusion, 33 of 42 girls and 27 of 40 boys were in puberty, the average age of the two genders was close (girls: 12.52 ± 3.10 years, boys: 12.94 ± 2.69 years). After comparing anthropometric and metabolic status of girls and boys, we found that according to BMI SDS girls tend to be more obese than boys (4.51 ± 0.29 vs. 3.96 ± 0.24; p = 0.1571), the mother's weight gain during pregnancy was more pronounced in boys' than in girls' (13.67 ± 2.51 vs. 11.94 ± 1.47; p = 0.5488), and glutamate pyruvate transaminase (GPT) (28.10 ± 3.813 vs.18.23 ± 1.335, p = 0.0228) and alkaline phosphatase (ALP) (230.3 ± 16.41 vs. 184.2 ± 15.589; p = 0.0452) levels were significantly lower in girls than in boys (supplementary table 1, available at *http://content.karger.com/ProdukteDB/produkte.asp?doi=477462*).

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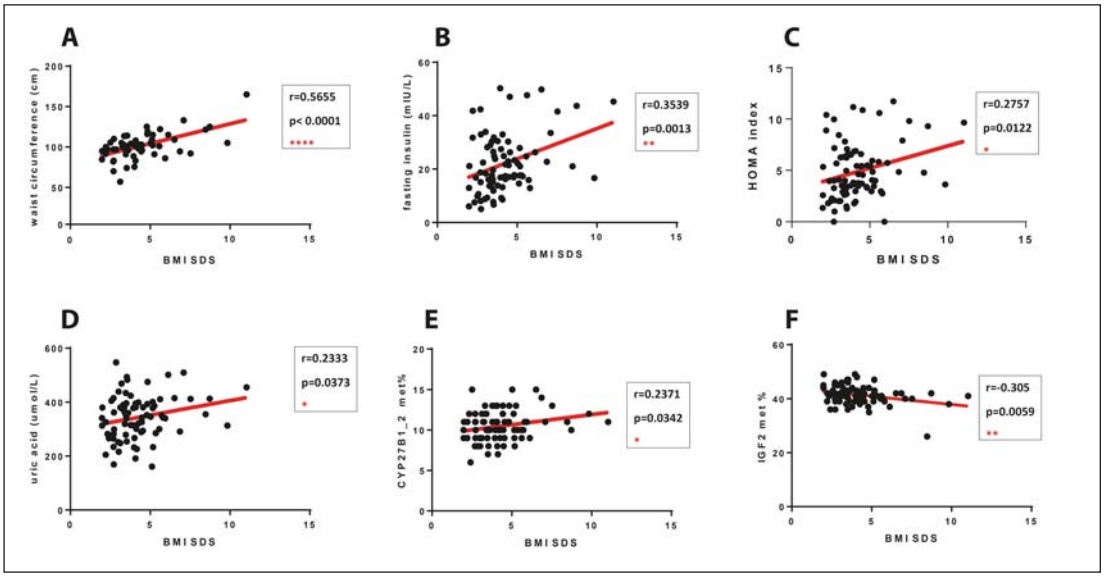

Fig. 2. Significant correlations of BMI SDS and anthropometric, metabolic parameters and methylation status. Significant positive correlations between BMI SDS and waist circumference (**A**) (r = 0.5655, p < 0.0001), fasting insulin level (**B**) (r = 0.3539, p = 0.0013), HOMA index (**C**) (r = 0.2757,p = 0.0122) , uric acid levels (**D**) (r = 0.2333, p = 0.0373) , CYP27B1 methylation status (**E**) (r = 0.2371, p = 0.0342). Assessment IGF2 methylation status and BMI SDS (**F**) showed significant negative correlation (r = –0.305, p = 0.0059). BMI SDS = BMI standard deviation score, HOMA: Homeostasis model; *CYP27B1* = 1-alfa-hydroxylase gene; *IGF2* = insulin-like growth factor gene.

27 of 82 children with obesity had either hypertension (according to 24-hour blood pressure monitoring), carbohydrate metabolism disorder (according to oral glucose tolerance test – impaired glucose tolerance), and/or dyslipidemia (elevated cholesterol level). One patient had all three of the above mentioned comorbidities mentioned above and also presented elevated uric acid level. According to our results, hypertension and dyslipidemia individually was present in 18.29–18.29% and carbohydrate metabolism disorder was present in 13.41% of the cases. Nine of the 55 patients without hypertension, impaired glucose tolerance, and dyslipidemia presented isolated hyperuricemia (data not shown). 46 patients did not present any signs of comorbidities (supplementary fig. 1, available at *http://content.karger.com/ProdukteDB/produkte.asp?doi=477462*).

There was no significant correlation between the 25-OH vitamin D levels and metabolic parameters and DNA methylation status. There was a tendency of positive correlation of *VDR* methylation status and vitamin D levels (supplementary table 2, available at *http://content.karger.com/ProdukteDB/produkte.asp?doi=477462*).

Significant positive correlations between BMI SDS and waist circumference (r = 0.5655, p < 0.0001), fasting insulin level (r = 0.3539, p = 0.0013), Homeostasis Model Assessment (HOMA) index (r = 0.2757, p = 0.0122) , uric acid levels (r = 0.2333, p = 0.0373) as well as *CYP27B1* methylation status (r = 0.2371, p = 0.0342) were found. *IGF2* methylation status and BMI SDS showed significant negative correlation (r = –0.305, p = 0.0059). While performing statistical analysis, excluding these potential outliers did not modify the significance of the found correlations (supplementary table 3, available at *http://content.karger.com/ProdukteDB/produkte.asp?doi=477462*) (fig. 2.)

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To further analyze the possible effects of DNA methylation status of *CYP27B1* and *IGF2* genes and 25OH vitamin D on the BMI SDS, linear regression was performed. Because it is already known that IGF2 is associated with growth and body composition, vitamin D can be associated with higher BMI, and CYP27B1 converts 25OH vitamin D to the active form 1,25(OH)2 vitamin D, we included these variables into our analyses. We entered variables stepwise with the linear regression function of SPSS. If a variable did not improve model fit, it was excluded from the model (backward elimination).

Model 1 included *CYP27B1*, *IGF2* methylation status, and vitamin D level as variables. *CYP27B1* and *IGF2* methylation status were significantly associated with BMI SDS while vitamin D level did not show significant association (supplementary table 4, available at *http://content.karger.com/ProdukteDB/produkte.asp?doi=477462*). Vitamin D did not improve model fit; therefore, it was excluded from the final model (adjusted R^2 = 0.96 vs. 0.98). Model 2 accounted for 9.8% of the variance and was statistically significant ($F(2,78) = 5.246$ $p = 0.007$) (supplementary table 5, available at *http://content.karger.com/ProdukteDB/produkte.asp?doi=477462*).

Discussion

The prevalence of obesity among children has been rising enormously over the past decades [20]. Children suffering from obesity tend to have higher risk for several obesity-related problems such as high blood pressure, dyslipidemia, and type 2 diabetes mellitus during adolescence and later on during adulthood [21, 22]. They are also more likely to remain obese as adults [23, 24]. Several studies have suggested that imprinting and methylation can play role in weight control and obesity [25–28]. According to our results, there were significant positive correlations between BMI SDS and waist circumference, fasting insulin or glucose levels, HOMA index, uric acid level as well as higher blood pressure. 18.29% of our patients had either hypertension or impaired carbohydrate metabolism, while dyslipidemia was present in 13.41% and hyperuricemia in 10.97% of the cases. Altogether, 43.9% of these children with obesity already presented at least one of the main metabolic and cardiovascular risks of obesity. Our findings support the hypothesis that with early onset obesity the metabolic consequences of this condition start getting in action very early on in life [1].

Recent studies observed that increased BMI is associated with low circulating 25OH vitamin D levels, but no evidence was proven for elevated 25OH vitamin D lowering either weight or BMI [29]. Increased VDR function is associated with increased body fat and unfavorable lipid profile in mice [30]. Interestingly, our results did not show any significant correlations between 25OH vitamin D and BMI SDS, metabolic status-describing parameters, blood pressure increase or *VDR* methylation status. Therefore, we could not confirm the association between lower vitamin D levels and unfavorable metabolic profiles [31]. However, the increased methylation of the gene of the 25OH vitamin D activating CYP27B1 enzyme was associated with higher BMI SDS. Lower CYP27B1 expression in subcutaneous adipose tissue in adults with obesity was found by Wamberg et al. [10]. These findings indicate that 1,25(OH)2 vitamin D, the active form of vitamin D, might be reduced in individuals with obesity – in adults and in children as well.

IGF2 is an important regulator of growth, obesity, and body composition. In young adults, *IGF2* hypermethylation was associated with greater subcutaneous adiposity, but not with BMI, weight, height, waist circumference or visceral adiposity [6]. According to Chen et al. [32], in women suffering from obesity *IGF2* methylation level was lowest in subcutaneous adipose tissue when compared with that in visceral adipose tissue or muscle. While studying Mexican-American lean and obese children Hernandez-Valerio et al. [33] found that children

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with lower *IGF2/H19* methylation had higher birth weights than did children with higher methylation. Heijmans et al. [34] stated a negative association between *H19* methylation and body size. Supporting this, our data also show significant negative correlation between *IGF2* methylation and increased BMI SDS. Therefore, we suggest that the lower *IGF2* and *H19* methylation status tend to have an impact not only on birth weight but later in childhood on the level of obesity described by BMI SDS.

According to our results, the methylation status of the *POMC* gene, which encodes an anorexogenic neuropeptide and plays key role in body weight regulation within the hypothalamus, did not show any correlations with BMI SDS. This might be due to the fact that CpG islands within one gene can be differently methylated and the primer used in our study targeted the gene's exon 1 region, while Kuehnen et al. [7] found association with childhood obesity and the hypermethylation of the intron 2 and exon 3 boundary region of *POMC* gene.

To analyze the possible effects of DNA methylation status of *CYP27B1* and *IGF2* genes and 25OH vitamin D on BMI SDS we preformed linear regression. *CYP27B1* and *IGF2* methylation status were significantly associated with BMI SDS, while vitamin D level did not show any significant association, which suggests that hypomethylation of *IGF2* and the hypermethylation of *CYP27B1* might positively influence the rate of BMI observed in children with obesity. This can be explained by the fact that hypomethylation of the *IGF2* gene might lead to increased expression of IGF2, which is responsible for growth and somatic development, and is associated with increased visceral fat mass [6]. Higher DNA methylation of the *CYP27B1* gene can lead to decreased enzyme activity and lower levels of active 1,25(OH)2 vitamin D. According to experimental studies, 1,25(OH)2 vitamin D has an active role in adipose tissue by modulating inflammation, adipogenesis, and adipocyte secretion [29]. We speculate that potential lower active vitamin D and increased IGF2 levels alter adipose tissue function and metabolism towards the direction of increasing BMI SDS.

To our knowledge, this is first study examining the methylation status of genes related to vitamin D metabolism related (*CYP27B1* and *VDR*) and to metabolic status (*IGF2* and *POMC*) in children with obesity. Although our research has reached its aims, there were some unavoidable limitations. First, because genomic DNA was extracted from peripheral whole blood includig many different cell types, we cannot provide correction for cell composition. Since DNA methylation is tissue-specific, adipocytes might show different methylation patterns than blood cells. Second, considering the epigenetic contribution to the pathogenesis of obesity, besides DNA methylation, other epigenetic pathways could influence the level of obesity. Therefore, further epigenetic researches are needed on this topic. Third, due to our relative small sample size and the seasonal and ethnic variation in vitamin D levels, conformation of our results by further investigations on larger populations are needed.

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Author Contribution Statement

All authors contributed equally to this work. D.T., A.Sz. and O.D.Á. designed the study; O.D.Á., B.P. and P.H. performed experiments; A.L., D.T. and O.D.Á. collected and analyzed data; O.D.Á. and D.T. wrote the manuscript; P.H., B.P., D.T. and A.Sz. gave technical support and conceptual advice.

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Disclosure Statement

The authors declare no conflict of interest.

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