

The significance of epigenetic investigations in children with obesity

PhD thesis outlines

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I. Introduction

The prevalence of obesity has been increasing dramatically over the past years not only worldwide. 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. According to recent studies obesity results mainly from interactions between environmental and genetic factors, which are linked together by epigenetic mechanisms (e.g.: DNA methylation, histone modification, micro RNA pathways). According to candidate gene approach (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.

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. 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- α -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. Vitamin D through its nuclear receptor (VDR) regulates epigenetic pathways and transcription of a number of genes involved in regulating metabolism and cell proliferation. According to recent studies in adults low levels of circulating 25OHD are associated with increased fat mass, body mass index (BMI), mortality, type 2 diabetes, cardiovascular diseases, and also dyslipidemia. Therefore the aim of our study was to investigate whether there is a correlation between the rate of obesity (Body Mass Index (BMI) and Standard Deviation Score

(SDS) and the methylation status of genes related to vitamin D metabolism (*CYP27B1*, *VDR*) and metabolic status (*IGF2*, *POMC*) in children with obesity. We also aimed to estimate the prevalence of the most frequent obesity associated comorbidities in these obese, but otherwise healthy considered children.

Prader-Willi syndrome (PWS) is a rare complex genetic disease, and it's clinical symptoms alter with age. *Holm et al.* developed a diagnostic criteria score system, which aids the recognition of PWS in regard to the age of a child. The syndrome is due to loss of paternal expression of several genes on the long arm of chromosome 15, in the 15q11-q13 region. Paternal deletion of this gene region, which causes the most serious symptoms, can be found in approximately 65-75%, maternal uniparental disomy (mUPD) in 20-30% of cases, and 4-5% of the cases can occur sporadically or due to genomic imprinting center defects, while 1-2% of PWS results from balanced and unbalanced translocation. The suspicion of PWS diagnosis is based on age-specific clinical features and it should be confirmed by genetic analysis. According to the international literature, DNA methylation analysis of the promoter region of *SNRPN* locus is the most efficient way to start genetic investigation in patients with suspected Prader-Willi syndrome. The sensitivity of this method is 99%. In Western European countries and in the United States the most commonly used DNA methylation analysis methods are methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA). MS-MLPA is not only good for proving or excluding the diagnosis of PWS, but it also allows to determine the precise genetic background of the syndrome. The high resolution melting analysis (HRM) technique is also internationally approved and supported. Therefore, the aim of our study was to compare our altered, cost efficient, easily accessible, methylation sensitive HRM method with the most popular, expensive MS-MLPA.

II. Objectives

1. To prove that the obese but otherwise healthy considered children already develop obesity related laboratory abnormalities and comorbidities whereof they are assigned for medical check-up and hospital care.
2. To prove that since the vitamin D metabolism distinctly changes in obesity and lower vitamin D levels and insulin resistance often occur along with obesity, there is a connection between 25OHD3 vitamin blood levels and the rate of obesity in our patients.
3. To prove that since the gene of 1-alpha-hydroxylase, which is responsible for the serum level of active form of vitamin D, along with many other genes is part of the "obese epigenetic pattern", vitamin D mediates the epigenetic pattern in obesity and metabolic syndrome, and the DNA methylation status of genes that are involved in vitamin D activation and vitamin D receptor signaling (*VDR* and *CYPB1*) correlates with the rate of childhood obesity.
4. To prove that since the "obesity disposed stature" has many components and epigenetic mechanisms play an important role in its genetic transmission, the methylation status of metabolism related genes (*POMC*, *IGF2*) along with its consequences affect the rate of childhood obesity.
5. To prove the clinical use of Holm score system in cases of Prader-Willi syndrome and Prader-Willi like phenotype, and to map the DNA methylation pattern of *SNRPN* gene's promoter region.
6. To verify our self-designed DNA methylation based method for first-tier genetic diagnosis of Prader-Willi syndrome involving our patients with Prader – Willi syndrome.

III. Methods

III.1. Study population

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. Anthropometric data (height, weight, waist circumference, birth height and birth length), metabolic parameters (lipid profile, fasting blood sugar and insulin, data of oral glucose tolerance test, thyroid stimulating hormone), vitamin D, serum calcium and parathormone levels, 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.

In our study according to genetic diagnosis of Prader-Willi syndrome we retrospectively examined 17 clinically PWS suspected (clinical symptoms: muscle hypotonia, poor sucking in infancy, hypogonadism, mild to moderate mental retardation, behavioural problems, childhood-onset obesity and hyperphagia) underaged proponds. We collected anamnestic data focusing on perinatal issues, development, anthropometric parameters, age at examination, major and minor features of diagnostic criteria of PWS proposed by Holm et al.

III.2. Genetic analysis

III.2.1. Methods used in obese and in PWS suspected patients

III.2.1.1. DNA isolation and bisulfite treatment

We collected peripheral blood samples for DNA analysis from each patient. In each case genomic DNAs were extracted from peripheral whole blood using High Pure PCR Template Preparation Kit, and bisulfite conversion was performed using EZ DNA Methylation™ Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. Concentrations of bisulfite converted DNA samples were estimated by NanoDrop spectrophotometer using 'ssDNA' measurements.

III.2.1.2. Bisulfite-specific PCR (BS-PCR)

We used BS-PCR to estimate the DNA methylation level of *POMC*, *IGF2*, *CYP27B1*, *VDR* genes in obese patients and *SNRPN* (Small Nuclear Ribonucleoprotein-Associated Protein N) in PWS suspected cases.

In silico CpG island prediction was performed by CpG Plot EMBOSS Application (<http://www.ebi.ac.uk/Tools/emboss/cpgplot/index.html>). Bisulfite-specific PCR (BS-PCR) reactions were performed using primers designed with PyroMark Assay Design software (SW 2.0, Qiagen Inc., Valencia, CA, USA) 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>). BS-PCR reactions were performed using AmpliTaq Gold 360 Master Mix (Life Technologies, Carlsbad, CA, USA), LightCycler® 480 ResoLight Dye (Roche Applied Science, Basel, Switzerland), primers at 0.2 µM final concentrations, bcDNA samples (20-40 ng bcDNA/reaction) in 15 µl final volume. The final

concentration of MgCl₂ was 2.5 mM except for the *VDR* and *POMC*, where it was 1.5 mM and for *SNRPN* where it was 3.5 mM. Real-time PCR amplification was carried out with the following thermocycling conditions on the LightCycler® 480 System: 95°C for 10 min, then 95°C for 30 seconds, 60°C with 0.4°C decrease/cycle for 30 seconds, 72°C for 30 seconds for 10 touchdown cycles, followed by amplification at 95°C for 30 seconds, 56°C for 30 seconds, and 72°C for 30 seconds in 50 cycles. Following completion of the PCR thermal cycling, high resolution melting (HRM) analysis began with denaturation at 95°C for 1 minute, cool down to 40°C, and hold for 1 minute, 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, Basel, Switzerland). MS-HRM curve data were retrieved with the LightCycler® Gene Scanning software (Roche Applied Science, Basel, Switzerland).

In order to calibrate our MS-HRM assays, artificially methylated DNA samples were mixed with unmethylated 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.

III.2.2. Methods used in obese patients

III.2.2.1. PyroMark Q24 sequencing

We defined the exact DNA methylation level of CpG islands of metabolism related *POMC* and *IGF2* and vitamin D metabolism related *CYP27B1* and *VDR* using pyrosequencing.

Providing single-base resolution information about the methylation state of a CpG island in bisulfite converted DNA 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., Valencia, CA, USA) 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., Valencia, CA, USA) using PyroMark Gold Q24 Reagents (Qiagen Inc., Valencia, CA, USA) 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., Valencia, CA, USA) specific sequencing primers designed by PyroMark Assay Design software, SW 2.0 (Qiagen Inc., Valencia, CA, USA) in order to cover CpG sites in the amplicons. Sequencing results were analyzed using the PyroMark Q24 software v2.0.6 (Qiagen Inc., Valencia, CA, USA).

Target gene and Assay	Forward primer	Reverse primer (biotin labeled)	Sequencing primer
CYP27B1 Assay1	GGTTTTGGGGGT AGAGAAGAT	CTCCCTATCCCAA CCCAATCAA	GGGGGTAGAG AAGATTA
CYP27B1 Assay2	AGAGGGGTTGGG ATGTT	AACCCTCAAATACC CTCCAAAATATTCCA T	GGGATGTTTGT TAAGTT
VDR Assay2	GGATTAGGGATTA GGGAAGTTGAGAT TTA	TACTACTACAAAACC CCAAAAACTCAACC TAA	AGATTTAGTTT TTTTGGGTGA
VDR Assay3	ATTTAAATTGTGG GATTAGGTTGAGT	TAATCCAAAATACAA CCCCCACCCTTCCTA C	TGGAGTTTGT AGTAGTAATAG G
IGF2 Assay1	GGGATTGGGTTAG GAGAAGT	CCCCCCAAAATAA CCAACAAT	GGGTTAGGAG AAGTTTA
POMC Assay7	GTTGGAAAGGGT TGGAATTAGTA	ACACCCACAAAACCA CTCCTAACTTCTAC	TTTAGGAAGAA TTTAATTATGG AT
SNRPN Assay1	GAGGGAGTTGGGA TTTTGT	AATAACCCCTCCCCA AACTATCTCT	

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

While performing BS-PCR we used self-designed forward and reverse primers, while performing pyrosequencing specific sequencing primers were used. In case of VDR and CYP27B1 genes we designed more Assays in order to cover the entire promoter region of the genes. CYP27B1: 1- α -hydroxylase gene, VDR: vitamin D receptor gene, IGF2: insulin like growth factor gene, POMC: proopiomelanocortin gene

III.2.3. Methods used in PWS suspected patients

In patient with the clinical diagnosis of PWS with methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) the copy number (e.g. deletion) and the methylation status (e.g. imprinting defect or mUPD) of the target 15q11-q13 region was determined in one step.

For further distinguishing mUPD and imprinting defect microsatellite analysis (MSA) was used.

III.2.3.1 Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA)

The SALSA ME028 (lots B2-0413 and B2-0811) MS-MLPA mix was purchased from MRC-Holland (Amsterdam, Netherlands). The probe mix contained 32 probes specific for the Prader-Willi/Angelman syndrome chromosome 11p15 region. Of these, 5 were MS-MLPA probes, specific for an imprinted sequence and contained a recognition site for the methylation sensitive HhaI enzyme. In addition, 14 MLPA probes located outside the PWS/AS region were added as control probes for copy-number quantification, as well as two MS-MLPA control probes for complete HhaI digestion in the MS-MLPA reaction. We used a total of 100 ng of genomic DNA for each sample tested. After 16 h of hybridization at 60 C°, samples were split equally into two aliquots. The first aliquot was subjected to ligation only, whereas the second one was subjected to ligation plus enzymatic digestion. Ligation, enzymatic digestion and PCR amplification were performed according to the manufacturer's instructions. PCR products (1µl) from each tube were mixed with 1µl of internal size standard and 20µl of deionized formamide and were injected into an ABI-3100 genetic analyzer (Applied Biosystems, Life Technologies) to analyse our data.

III.2.3.2. Microsatellite analysis (MSA)

MSA was performed using five markers within the critical region 15q11-q13 (D15S541, D15S817, D15S128, D15S1234 and D15S822), after multiplex PCR. PCRs were performed with Qiagen Multiplex PCR Kit (Qiagen) in 25µL reaction volume containing 100ng DNA and 2µM of each primer. Amplification was performed with an initial denaturation of

15 min at 95°C, followed by 30 to 35 cycles each of 30 sec at 94°C, 90 sec at 58°C, and 90 sec at 72°C with an extension at 72°C for 10 min and finally cooling to 4°C.

The genotyping of three loci outside the PWS/Angelman region (D15S144, D15S1007, and D15S642) allowed to distinguish a deletion from uniparental disomy (UPD), that is, uniparental inheritance within the PWS/Angelman region together with biparental inheritance outside this region identifies a deletion, and the presence of uniparental inheritance both within and outside the critical region reveals UPD. For MSA, fluorescence-tagged PCR products were analyzed using an ABI 3100 automatic capillary genetic analyzer and GeneScan and Genotyper software (Applied Biosystems).

III.3. Statistical analysis

To characterize the examined parameters in obese patients descriptive statistics (mean \pm SD) were used. 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.

In Prader-Willi syndrome suspected cases due to low item number statistical analysis was not performed.

IV. Results

IV.1. Describing the metabolic status and the prevalence of obesity related comorbidities in obese children

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: 12.5 ± 3.1 , boys: 12.9 ± 2.6). After comparing anthropometric and metabolic status of girls and boys, we found that according to BMI SDS girls tend to be more than boys with obesity (4.5 ± 0.3 vs. 3.9 ± 0.2 , $p = 0.1571$), the mother's weight gain during pregnancy was more in case of boys' than in girls' (13.7 ± 2.5 vs 11.9 ± 1.5 , $p = 0.5488$), and glutamate pyruvate transaminase (GPT) and alkaline phosphatase (ALP) levels were significantly lower in girls than in boys (28.1 ± 3.8 vs. 18.23 ± 1.3 , $p=0.0228$; 230.3 ± 16.4 vs. 184.2 ± 15.6 , $p=0.0452$).

27 of 82 children with obesity had either hypertension (according to 24-hour blood pressure monitoring (ABPM), 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 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. 9 of the 55 patients without hypertension, impaired glucose tolerance and dyslipidemia presented isolated hyperuricemia (data not shown). In 36 cases in 43,9% of our probands) at least one obesity linked comorbidity was present, while 46 patients did not present any signs of comorbidities.

IV.2. The relation of vitamin D and the rate of obesity

There was no significant correlation between 25OH vitamin D levels and DNA methylation status of metabolism related (*POMC*, *IGF2*) and D vitamin metabolism related (*CYP27B1*) genes. There was a tendency of

positive correlation of VDR methylation status and vitamin D levels ($r=0.2053$, $p=0.066$).

IV.3. Correlations between the rate of obesity and the methylation status of genes (*VDR*, *CYP27B1*) related to vitamin D metabolism

The vitamin D metabolism related *VDR* gene's DNA methylation did not show significant correlation with obesity ($r=-0.06579$, $p=0.5595$), while the methylation level of *CYP27B1*, which is responsible for the activity of 1- α -hydroxylase enzyme, showed a significant positive correlation with the rate of obesity ($r=0.2371$, $p=0.0342$). Therefore, the greater the rate of obesity was, the greater DNA methylation was found in the target gene region.

IV.4. Correlations between the rate of obesity and the methylation status of metabolism related genes (*POMC*, *IGF2*)

We expressed the rate of obesity in BMI SDS and examined its correlation with DNA methylation of the metabolism related genes (Pearson correlation analysis). The methylation status of the anorexigenic pathway mediator *POMC* gene did not show correlation with the rate of obesity ($r=0.2321$, $p=0.1334$), while the growth mediator *IGF2* gene's methylation status showed significant negative correlation with BMI SDS ($p=0.0059$, $r= -0.305$). Therefore the greater the rate of obesity was, the lower DNA methylation was found on the *IGF2* gene.

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 and 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). According to our final model we could statistically prove a relation between BMI SDS and the methylation status of *CYP27B1* and *IGF2* genes.

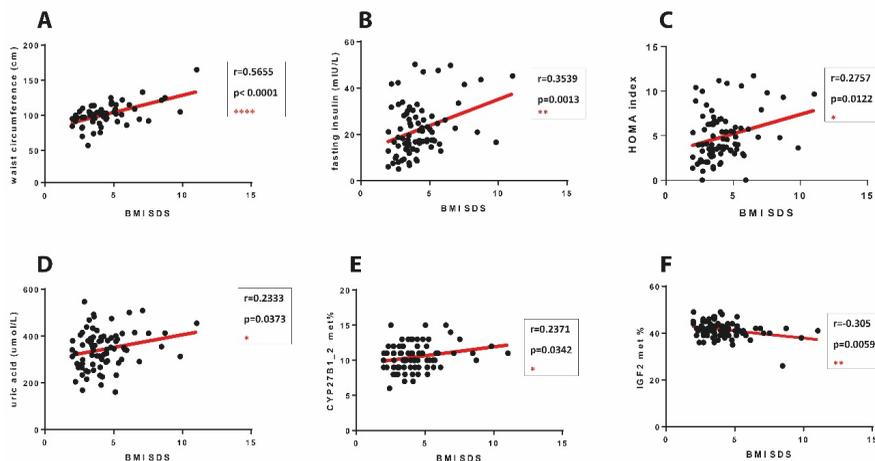


Figure 1. Significant correlations of BMI SDS and antropometric, metabolic parameters and methylation status

BMI SDS: body mass index standard deviaton score, *HOMA:* Homeostasis Model, *CYP27B1:* 1-alfa-hydroxylase gene

IV.5. Proving the clinical significance of Holm score system in patients with Prader- Willi syndrome and in patients with Prader-Willi like

While comparing the results of genetic analysis with the registered Holm scores, patients with genetically proved PWS had higher scores, than Prader-Willi like patients, where PWS genetically could not be confirmed.

IV.6. The results of our self-designed method for firstier diagnosis of Prader-Willi syndrome

We examined 17 PWS suspected patients' DNA with self-designed HRM and widespread MS-MLPA methods. We confirmed PWS in 6 cases. For further analysis in the positive cases MSA was performed, which revealed that one patient had deletion, two patients had mUPD in the investigated *SNRPN* (15q11-q13) region. One patient had either mUPD or deletion but for further analysis no MSA could be performed due to unavailable parental DNA samples.

The results of HRM and MS-MLPA methods did not differ in any cases (Figure 2.).

IV.7. Limitations

Although our research has reached its aims, there were some unavoidable limitations. First, because genomic DNA was extracted from peripheral whole blood, which includes many different cell types and 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 to confirm our results further investigation on larger population is needed.

A small number of patients were involved in the Prader-Willi syndrome genetic diagnosis study, future larger, prospective studies could better define the efficiency of our method.

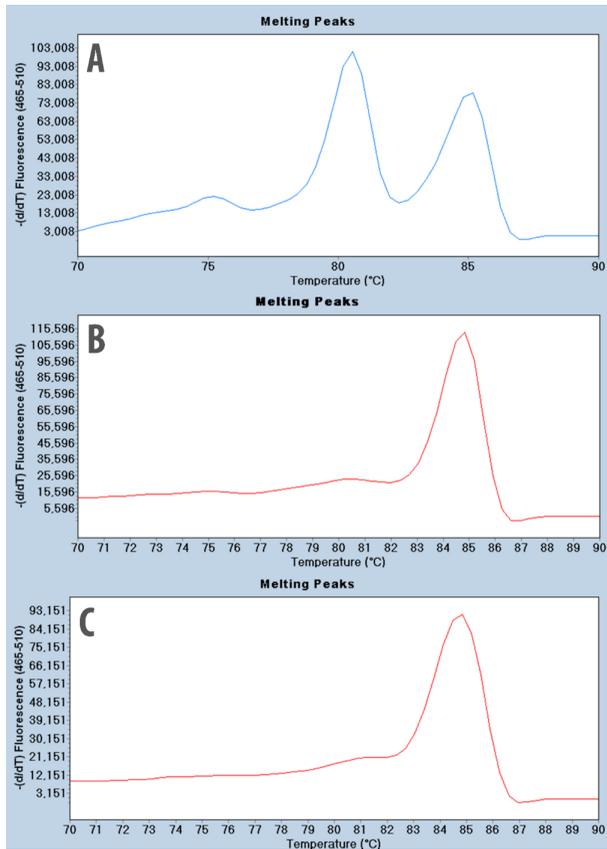


Figure 2. Methylation curves from HRM analysis of the SNRPN loci. *A:* Two peaks (unmethylated paternal: $T_m = 80.63 \pm 0.24^\circ\text{C}$, methylated maternal: $T_m = 84.97 \pm 0.35^\circ\text{C}$) can be observed in the normal sample, one of the minor peaks may indicate LIS1 loci ($T_m = 76.0^\circ\text{C}$)*, the smallest peak may stem from melting of a heteroduplex of paternal and maternal SNRPN products, *B:* deletional PWS ($T_m = 84.48 \pm 0.99^\circ\text{C}$), *C:* non-deletional PWS ($T_m = 84.5 \pm 0.6^\circ\text{C}$). No paternal (unmethylated) peak can be observed in PWS patients. T_m = melting temperature

V. Conclusions

1. In the course of our research we found that by the time obese children are assigned to medical check-up and hospital treatment due to their obesity, many of them already present some kind of laboratory abnormalities or obesity related comorbidities. According to our results hypertension and dyslipidemia individually were present in 18.29-18.29% and carbohydrate metabolism disorder was present in 13.41% of the cases. 9 of the 55 patients without hypertension, impaired glucose tolerance and dyslipidemia presented isolated hyperuricemia. 36 children presented at least one of the above mentioned comorbidities, which is 43.9% of the examined children.
2. We could not prove the fact, that since the vitamin D metabolism distinctly changes is obesity and lower vitamin D levels often occur along with obesity, there is a connection between 25OHD3 vitamin blood levels and the rate of obesity in our patients.
3. The DNA methylation status of *CYP27B1* gene, which plays an important role in activating vitamin D, showed significant positive correlation with the rate of childhood obesity. Therefore *CYP27B1* could be part of the "obese epigenetic pattern". Vitamin D through epigenetic regulation, within this through DNA methylation could get involved in the pattern that is present in obesity and in metabolic syndrome. We could not find any correlations between vitamin D receptor (VDR) gene's methylation status and the rate of obesity.
4. We could prove a negative correlation between the methylation status of metabolism, growth and body composition affecting IGF2 gene and the rate of childhood obesity, therefore IGF2 could play a major role in childhood obesity. The "obesity disposed stature" has many components, epigenetic mechanisms play role in it's genetic transmission. We could

not find any correlations between the methylation status of anorexigenic *POMC* gene and the rate of childhood obesity.

5. We could prove the clinical use of Holm criteria in patients with Prader-Willi syndrome and with Prader-Willi-like phenotype by genetically proved PWS patients scoring higher, than patients with Prader-Willi like phenotype without the genetic diagnosis of PWS. When using HRM method the methylation pattern of *SNRPN* gene loci squarely differed in PWS patients compared to non- PWS patients.

6. Our self-designed high resolution melting point analysis method (HRM) can be used for first-tier diagnosis in cases of suspected Prader-Willi syndrome. In the course of validation we compared HRM results with the precise, widespread methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) method's results. In each case the results were the same.

According to our research, the hypomethylation of *IGF2* and hypermethylation of *CYP27B1* genes might positively influence the rate of obesity in obese children. 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. Using our self-designed primers and altered bisulfite-specific PCR conditions, high-resolution melting analysis appears to be a simple, fast, reliable and effective method for primarily proving or excluding clinically suspected Prader-Willi syndrome cases. According to our results HRM could be used as a rapid, first-tier genetic analysis, in case MS-MLPA is not available due to technical or financial difficulties. We claim, that using Holm's diagnostic criteria score system to establish the clinical diagnosis of Prader-Willi syndrome could be helpful to decide, which patients should undergo further genetic investigation.

VI. Bibliography

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