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# LEPTIN GENE, LEPTIN GENE POLYMORPHISMS AND BODY WEIGHT IN PREGNANT WOMEN WITH DIABETES MELLITUS TYPE I

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There have been several genetic causes of obesity discussed by past authors, among others leptin, that have provided information regarding signaling pathways in energy expenditure in humans. Genetic variants of the leptin gene and its receptor may influence body weight. Aim: To investigate the role of the leptin gene's polymorphism promotion region (2548 G/A) and the leptin gene receptor polymorphism (668 A/G) and its associations with body weight in pregnant women with type 1 diabetes (PGDM-1). Methods: 78 PGDM-1 were qualified to the study group (SG) which was divided into normal and over-weight individuals according to BMI criteria. The control group (CG) consisted of first trimester healthy pregnant women with normal body weight. Genetic variants of the leptin gene and its receptor were analyzed using PCR-RFLP assays. Within the SG, the following metabolic parameters were estimated: MBG, HbA1C, insulin dose, LDL, HDL, T-CHOL, creatinine, creatinine clearance and blood pressure. Results: There was a trend found among the majority of homozygous A and G variants in LEP -2548 G/A and LEPR 668 A/G in over-weight and obese individuals in comparison to normal-weight subjects (CG). There were no specific differences found in selected first trimester metabolic parameters in relation to patients' genotypes.

Key words: *leptin gene, leptin gene receptor, diabetes mellitus, body mass index (BMI), obesity*

## INTRODUCTION

Leptin (gr. *Leptos* – lean) is 16 kDa polipeptide hormone and a product of the obesity gene (ob gene), first described by Zhang in 1994 (1). This hormone is secreted mostly by adipose tissue. Leptin acts as a satiety factor – by inhibiting neuropeptide Y in the hypothalamus, provides a satiety signal with subsequent increase of energy expenditure and metabolic processes intensification. It also regulates the amount of fat tissue in organism, takes part in fertility processes and regulates blood pressure (2-4). The leptin gene is located on the long arm of chromosome 7 (7q31.3) and contains 3 exons and 2 introns. Several polymorphisms of the leptin gene were described, such as functional polymorphism V110M, promoting region polymorphism -188 C/A and -2548 G/A. Many authors have presented research which indicates that differences in the leptin genotype are associated with overweight individuals (5-8). Leptin acts *via* specific receptors located in adipose tissue, stomach, endometrium, liver, spleen, lungs, heart, ovaries and placenta. A high amountof leptin receptor was discovered in the hypothalamus, where leptin acts *via* negative loop feedback between its concentration in blood serum and its receptor expression on the cell surface (9-11). There are several isoforms of leptin receptor, known as long and short isoforms. In humans four isoforms were identified: 1165 amino-acid long isoform, responsible for leptin signaling and 3 alternative splicing short isoforms (12-14). The leptin receptor is a translation product of the leptin receptor gene (LEPR gene) located on chromosome 1 (1p31) which contains 20 exons (15). Both the leptin gene and its receptor gene are highly polymorphic. Several genotypic variants of investigated genes may affect leptin blood concentration and the biologic function of its receptor, thus influencing body weight. The most frequent leptin gene receptor polymorphisms are: functional -668 A/G and -109 A/G in exon 6, silent mutation -343 T/C and -1019 G/A. Pregnancy and diabetes are especially associated with body mass fluctuations.

The aim of the study was to investigate the frequency of occurrence of both the leptin gene and its receptor polymorphisms in pregnant women with type 1 diabetes mellitus (PGDM-1) and to estimate the association of several genotypes in normal weight and over-weight subjects. Moreover we aimed to evaluate the frequency of alleles in studied groups. Additionally, the relationship between metabolic parameters describing glycemic control and genotypes were evaluated.

## MATERIAL AND METHODS

78 Caucasian pregnant women having a single pregnancy with type 1 diabetes mellitus were qualified to the study group. All women were hospitalized in the Department of Obstetrics and Women Diseases in Poznań Poland. The study group was divided into 4 subgroups depending on the BMI value (16). The first subgroup consisted of 3 underweight subjects (body mass index- BMI $\leq$ 18,5 kg/m$^2$), second of 35 normal weight subjects with BMI between 18,5- 24,9 kg/m$^2$, the third

subgroup consisted of 27 subjects with BMI between 25 - 29,9 kg/m$^2$ and fourth subgroup consisted of 13 subjects with BMI ≥ 30 kg/m$^2$. The control group consisted of 34 healthy Caucasian women with a single pregnancies and with normal body weights (BMI ≤ 24, 9 kg/m$^2$).

Serum concentration of the following metabolic parameters were measured: MBG-mean blood glucose (mmol/l), $HbA_{1C}$ (%), LDL-cholesterol (mmol/l), HDL-cholesterol (mmol/l), total cholesterol (mmol/l), insulin dosage (Units), creatinin level (μmol/l), creatinin clearance (ml/min). The glucose level in serum of venous blood was determined by means of the enzymatic (heksokinase) method with the Roche Diagnostics laboratory reagents on Hitachi 912 analyzer.

The percentage of glycated hemoglobin ($HbA_{1c}$) in capillary blood was estimated using the Roche Diagnostics Tina-quant® Hemoglobin $A_{1C}$ II test.

The total serum cholesterol, HDL cholesterol and triglycerides levels were measured with the appropriate Roche Diagnostics reagents (Cholesterol CHOD-PAP, HDL-C plus and Triglycerides GPO-PAP respectively) on Hitachi 912 analyzer, and LDL cholesterol level was calculated using the formula: LDL cholesterol = total cholesterol – HDL cholesterol – TG/5.

The daily urine protein loss (g/24h), serum creatinine level (μmol/l), creatinine clearance (ml/min) were estimated by using Jaffe modified test.

## *Genetic analysis*

In all subjects (SG and CG), the leptin gene and leptin gene receptor polymorphisms (LEP 2548 G/A and LEPR 668 A/G respectively) were evaluated by Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP) assays. DNA was extracted from leucocytes using QIAamp DNA Blood mini KIT (Qiagen Inc. Germany).

PCR was performed for genotype analysis using PTC 100 Programmable Thermal Controller (MJ Research INC USA.). The following substrates were added: DNA sample, distilled water, $(NH_4)_2SO_4$ buffer, $MgCl_2$, dNTPs, primers, Taq DNA polymerase. PCR was performed in three stages: high temperature DNA denaturation (94°C), primers adding after temperature drop, essential replication (35 PCR cycles). The PCR product after digestion was transferred on 1,5% agarose gel (80V, 120 min) with TBE buffer (Sigma) and visualized in ultraviolet light.

As a next step, the PCR product was digested with specific restriction enzymes: *Cfo1* 5'...GC(G/A)CT…3' (Fermentas, Litwa) to identify genotypic variants within promoting region polymorphism of the leptin gene and *MspI* 5'…C/CGG…3' (EURx, Poland) to estimate polymorphisms of the leptin gene receptor. All procedures were performed according to the following manufacturer recommendations: digestion temperature 37°C (16 hours), and enzyme inactivation temperature 65°C (20 min.). The following genotypes were found, including -2548 G/A: heterozygote GA 242bp, 181bp, 61bp, homozygote GG 181bp, 61bp, mutated homozygote AA 242bp. Including LEPR 668 A/G: heterozygote AG 216pz, 134pz, 82pz, homozygote AA 216bp, mutated homozygote GG 134bp, 82bp. All molecular biology investigations were performed in the Molecular Biology Laboratory in Department of Perinatology and Women Diseases, Karol Marcinkowski University of Medical Sciences.

Moreover the patients' age, height (cm), first trimester weight (kg), systolic and diastolic blood pressure (mmHg) were determined.

## *Statistical analysis*

The frequency of observed genotypes and alleles are shown as a percentage of the whole group whereas expected values were estimated according to Hardy-Weinberg equilibrium. The statistical significance between genotypes in the study subgroups were estimated using the Chi-squared test. Differences between biochemical parameters in relation to genotypes were estimated by one-way

Anova and the post-hoc tests. Differences were acknowledged as statistically significant with p<0, 05. SPSS 16.0 for Windows was used for statistical analyses. To conduct the study, approval was granted by the Local Ethics Committee of the University.

## RESULTS

*Figs 1-5* describes the frequency of observed genotypes and alleles of the leptin gene polymorphism (-2548 G/A) in the study subgroups and the control group in relation to BMI. With the increasing value of BMI in the study subgroups, there was a trend observed of G and A homozygous genotypes among the majority of SG subjects, however it did not reach statistical significance probably due to the

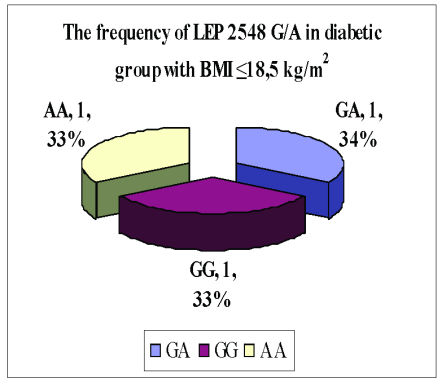


*Fig 1A*

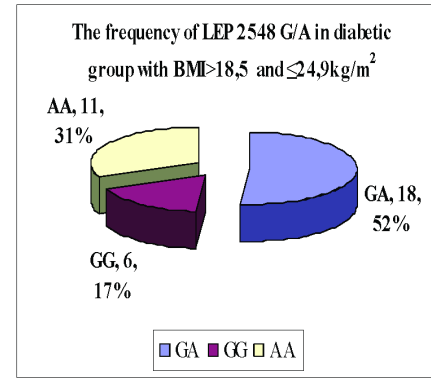


*Fig 2A*

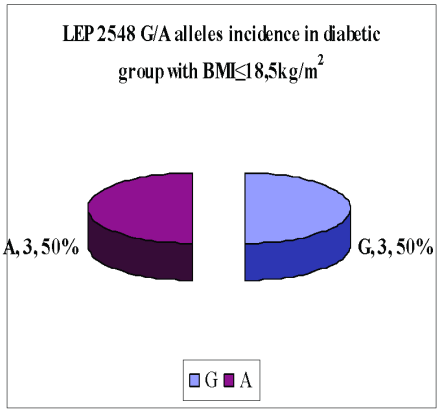


*Fig 1B*

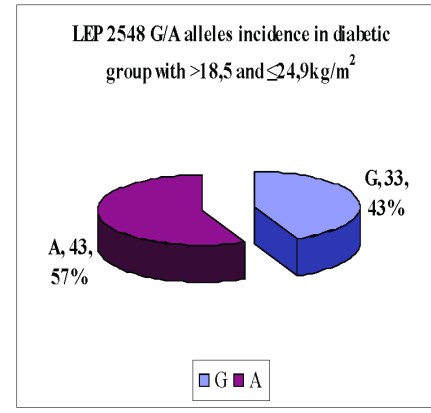


*Fig 2B*

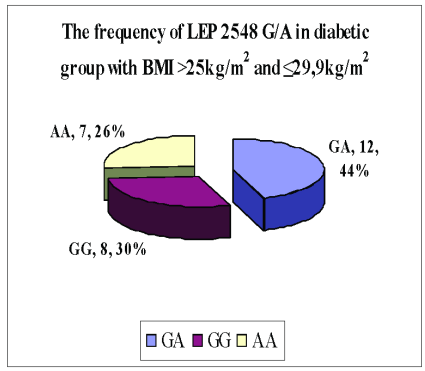


*Fig 3A*

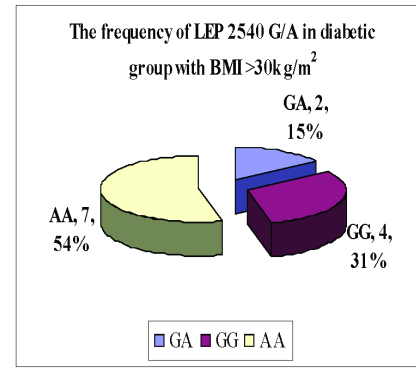


*Fig 4A*

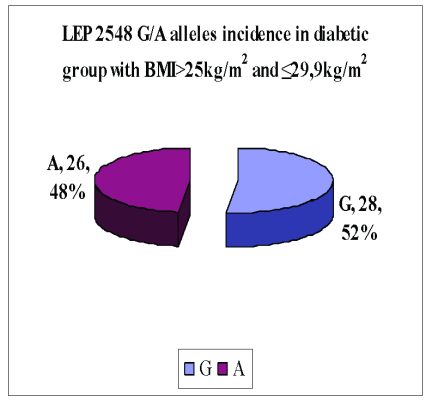


*Fig 3B*

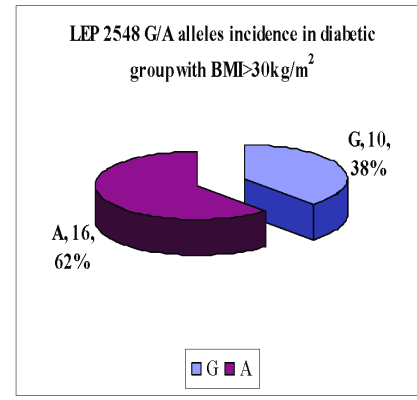


*Fig 4B*

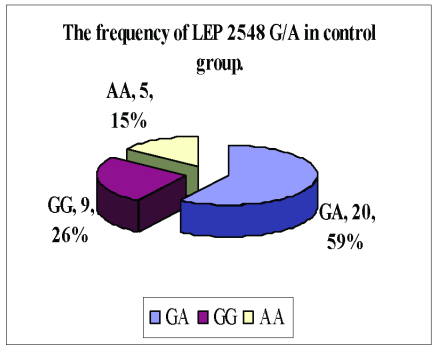


*Fig 5A*

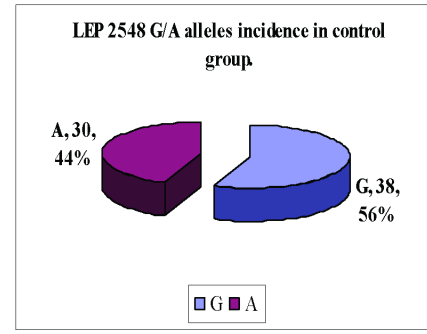


*Fig 5B*

*Figs 1AB-5AB.* Frequency of LEP 2548 G/A and its alleles in the study and control group in relation to BMI in first trimester of pregnancy (Chi-squared test).

low number of under- and over-weight subjects. In the control group which consisted of healthy lean individuals, a majority of GA heterozygous genotypes were observed, which corresponds to the frequency of heterozygous variants in normal weight, and diabetic subjects. There was a trend observed in the studied subgroups of a majority having allel A. Similar results were observed in relation to the frequency of occurrences of the leptin gene receptor polymorphism (668 A/G). With the increasing value of BMI in the first trimester, there was a trend to G and A homozygous majority genotypes noticed, however without statistical significance. In the control group, a majority of GA heterozygous genotypes were observed, which also corresponded to the frequency of this variant in the lean diabetic women. The above results are shown in *Figs 6-10.*

In the study group we analyzed the relationship between the observed genotypes and some biochemical parameters describing lipid and glycemic controls. We did not noticed statistical significant differences in the parameters in relation to observed

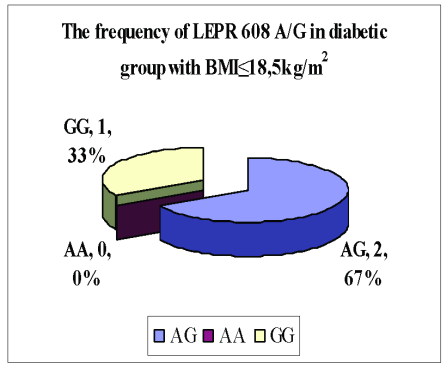


*Fig 6A*

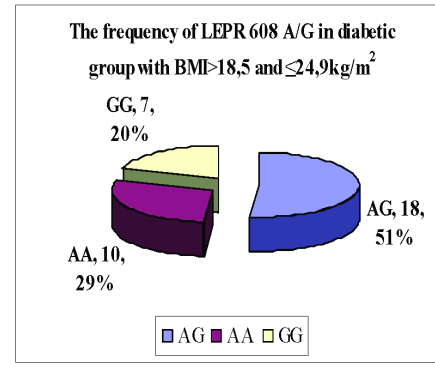


*Fig 7A*

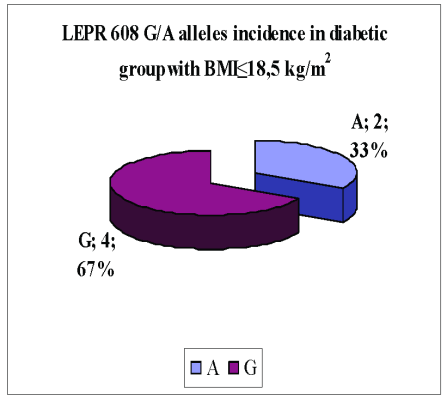


*Fig 6B*

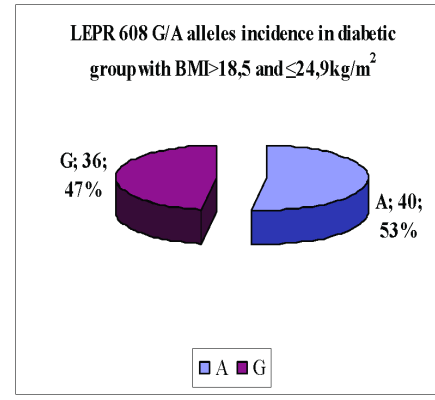


*Fig 7B*

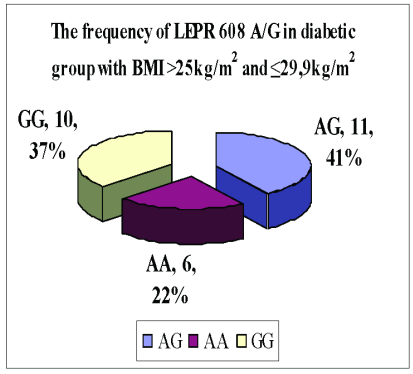


*Fig 8A*

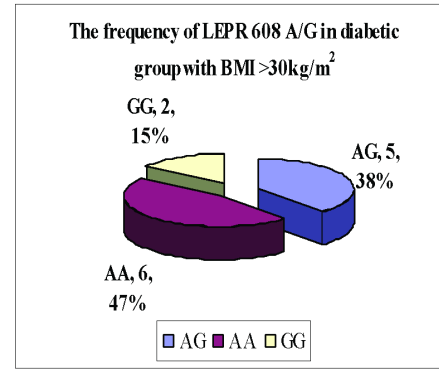


*Fig 9A*

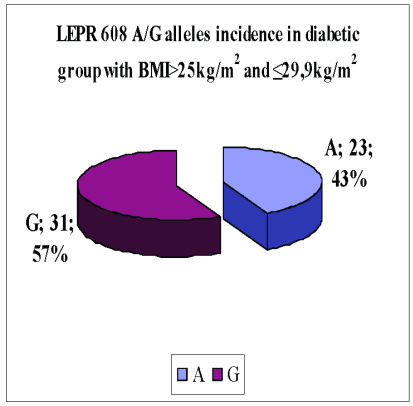


*Fig 8B*

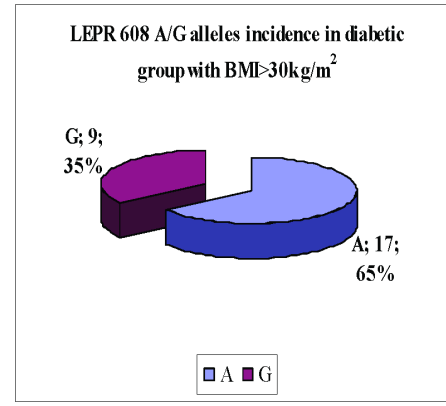


*Fig 9B*

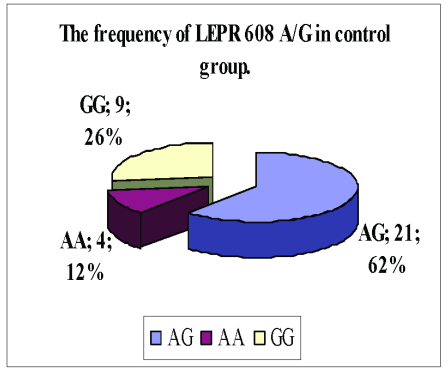


*Fig 10 A*

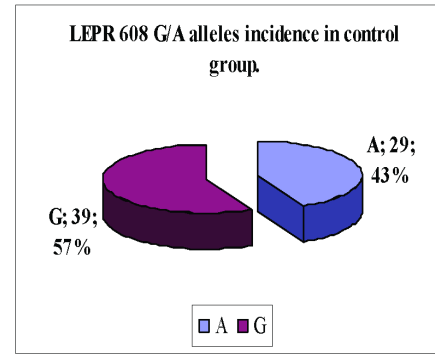


*Fig 10 B*

*Figs 6AB-10AB*. Frequency of LEPR 608 A/G and its alleles in the study and control group in relation to BMI in first trimester of pregnancy (Chi-squared test).

genotypes of the leptin gene polymorphism and leptin gene receptor polymorphism. The relationships between these parameters are shown in *Table 1* and *2*.

Since the majority of homozygous genotypes were found in the group of patients who were noted to be overweight and obesity, we evaluated the relationship between biochemical parameters in these groups. We found statistically significant differences in concentrations of triglycerides, HDL-cholesterol and creatinine clearance between analyzed subgroups of the diabetic pregnant women *(Table 3)*.

We also studied whether there was any difference in the presence of studied metabolic parameters and the combination of possible genotypes including both the leptin gene and the leptin gene receptor polymorphisms. Since there are 3 possible genotypes including LEP -2548 G/A (GA, GG and AA) and 3 possible genotypes including LEPR 668 A/G (AG, AA, GG), we established 9 groups of patients in the diabetic group (*e.g*. LEP GA heterozygote and LEPR AA homozygote or LEP GA heterozygote and LEPR GG homozygote *etc*.), depending on genetic configuration. The same was performed in the control group. In the study group, no specific genotype was significantly related to obesity. We also did not find statistically significant differences in any of the studied biochemical parameters between these subgroups. In the control group no specific genotype was dominantly observed.

## DISCUSSION

Mutations in the leptin gene and the leptin gene receptor may lead to extreme obesity, this was found to be true in mice by Chen *et al.* (17), and in humans by

*Table 1*. Metabolic parameters and observed genotypes in leptin gene (LEP 2548 G/A) in first trimester pregnant women with type 1 diabetes mellitus.

| Parameter | Heterozygote GA (mean±SD). N=33 | Homozygote GG (mean±SD) N=19 | Homozygote AA (mean±SD) N=26 | p-value* |
|---|---|---|---|---|
| Body mass index (kg/m$^2$) | 23.1±3.1 | 24.0±5.0 | 24.8±6.0 | NS |
| MBG (mmol/l) | 6.27±1.73 | 5.93±1.07 | 6.16±1.49 | NS |
| $HbA_{1C}$ (%) | 8.1±1.9 | 7.8±1.3 | 7.2±1.5 | NS |
| LDL (mmol/l) | 2.26±0.70 | 2.08±0.53 | 2.07±0.62 | NS |
| HDL (mmol/l) | 1.88±0.46 | 1.84±0.61 | 1.90±0.54 | NS |
| T-CHOL (mmol/l) | 4.48±1.00 | 4.32±0.54 | 4.45±0.98 | NS |
| Triglycerides (mmol/l) | 0.79±0.24 | 1.05±1.00 | 1.05±0.58 | NS |
| Insulin dose (Units/24h) | 42.6±17.1 | 43.8±14.5 | 47.1±23.1 | NS |
| Systolic BP (mmHg) | 106±13 | 108±16 | 105± 12 | NS |
| Diastolic BP (mmHg) | 65±7 | 66±10 | 64±10 | NS |
| Creatinine (µmol/l) | 56.5±31.1 | 55.7±26.5 | 47.7±6.2 | NS |
| Creatinine clearance (ml/min) | 133.4±66.9 | 124.7±41.1 | 126.5±31,1 | NS |

**-One-way Anova, post-hoc tests – Levene, Scheffe, Tukey*

*Table 2*. Metabolic parameters and observed genotypes in leptin gene receptor ( LEPR 668 A/G) in first trimester pregnant women with type 1 diabetes mellitus.

| Parameter | Heterozygote AG (mean ± SD) N=36 | Homozygote GG (mean ± SD) N=20 | Homozygote AA (mean ± SD) N=22 | p-value* |
|---|---|---|---|---|
| Body mass index (kg/m$^2$) | 23.6±4.4 | 23.0±3.5 | 25.1±5.6 | NS |
| MBG (mmol/l) | 6.54±1.76 | 5.77±1.05 | 6.30±1.42 | NS |
| $HbA_{1C}$ (%) | 8.1±1.9 | 7.8±1.3 | 7.2±1.5 | NS |
| LDL (mmol/l) | 2.26±0.56 | 4.46±1.14 | 2.07±0.62 | NS |
| HDL (mmol/l) | 1.88±0.46 | 1.84±0.61 | 1.90±0.54 | NS |
| T-CHOL (mmol/l) | 4.50±1.00 | 4.32±0.55 | 4.45±0.85 | NS |
| Triglycerides (mmol/l) | 0.79±0.23 | 1.03±0.23 | 1.05±0.58 | NS |
| Insulin dose (Units/24h) | 45.2±20.4 | 47.9±16.2 | 39.4±17.1 | NS |
| Systolic BP (mmHg) | 106±15 | 107±11 | 105±13 | NS |
| Diastolic BP (mmHg) | 66±8 | 65±9 | 65±4 | NS |
| Creatinine (µmol/l) | 56.7 ±30.1 | 55.7±26.5 | 47.7±6.3 | NS |
| Creatinine clearance (ml/min) | 133.4±66.9 | 127.4±41.1 | 126.5±31.1 | NS |

**-One-way Anova, post-hoc tests – Levene, Scheffe, Tukey*

*Table 3*. Metabolic parameters in the study group according to patients' BMI value

| Parameter | BMI<18,5 (kg/m$^2$) N=3 | BMI 18,5-24,9 (kg/m$^2$) N=35 | BMI 25-29,9 (kg/m$^2$) N=27 | BMI>30 (kg/m$^2$) N=13 | p-value* |
|---|---|---|---|---|---|
| MBG (mmol/l) | 7.55±0.29 | 6.14±1.04 | 6.11±1.93 | 6.14±2.02 | NS |
| $HbA_{1C}$ (%) | 7.5±2.3 | 7.7±1.5 | 8.1±1.9 | 7.4±1.6 | NS |
| LDL (mmol/l) | 1.97±0.27 | 2.15±0.55 | 2.20±0.75 | 2.32±0.71 | NS |
| HDL (mmol/l) | 2.48±0.04 | 1.94±0.52 | 1.82±0.53 | 1.31±0.32 | 0.0000 |
| T-CHOL (mmol/l) | 4.81±0.73 | 4.47±0.84 | 4.48±1.17 | 4.55±0.73 | NS |
| Triglycerides (mmol/l) | 0.79±0.02 | 0.81±0.03 | 0.84±0.4 | 2.00±1.2 | 0.0000 |
| Insulin dose (Units/24h) | 37.0±20.8 | 43.9±19.6 | 48.2±18.3 | 37.1±11.2 | NS |
| Systolic BP (mmHg) | 96±5 | 103±12 | 109±12 | 114±21 | NS |
| Diastolic BP (mmHg) | 56±5 | 63±7 | 68±8 | 70±13 | NS |
| Creatinine (µmol/l) | 48.9±13.7 | 55.1±24.2 | 53.6±29.1 | 42.5±19.8 | NS |
| Creatinine clearence (ml/min) | 108±1.41 | 55.1±24.2 | 133±42 | 213±89 | 0.05 |

**-One-way Anova, post-hoc tests – Levene, Scheffe, Tukey*

Clement *et al*. (18). They found, that the presence of a homozygous mutations in the leptin gene (homozygotes ob/ob) and also homozygous mutations in the leptin gene receptor (homozygotes db/db) were associated with early onset of extreme obesity due to hyperphagia, poor energy expenditure and severe insulin-resistance (17, 18). These studies gave rise to the idea that several genotypes of the leptin gene and its receptor may lead to obesity.

The leptin gene and the leptin gene receptor are polymorphic, which means that in different subjects, differences in genetic variants may be found. Investigations involving these polymorphisms were conducted in relations to obesity in different populations. Wang *et al*. (5) performed a study on 200

Taiwanese subjects with extreme obesity, where the relationship between obesity and several polymorphism among the promoting region of the leptin gene -2548 G/A and the leptin gene receptor 668 A/G was analyzed. They found, that the homozygous variants of the LEP -2548 G/G were strongly associated with the development of extreme obesity, whereas no specific association between obesity and the leptin gene receptor polymorphism was discovered (5).

Several studies conducted in Caucasian and Afro-American populations discovered that common leptin gene polymorphisms in the flanking region -2548 G/A may affect the level of circulating leptin in humans, which was also associated with an increase in birth weight that depending on gender. It was found that allele A was related to an increase in female size for gestational age, while the G allele was associated with decreased male birth in the Caucasian population. Among African-Americans, the A allele was associated with a decrease in the umbilical cord leptin in females and with an increase in the cord leptin in males (19).

Obesity is the result of an imbalance between food intake and energy expenditure resulting in the storing of energy as fat, in most cases due to specific eating patterns. The study conducted by de Krom *et al*. (20) on a large study population was focused on identifying whether carriers of a common leptin receptor and cholecystokinine gene polymorphisms are genetically predisposed to obesity. It was found that common genetic variations of the leptin gene receptor are related to specific snacking behavior, whereas the carriers of cholecystokinine polymorphisms had an increased risk of eating enlarged meal sizes. Also in the Australian population of obese women, Pro1019Pro leptin gene receptor polymorphism was associated with longitudinal increases in body weight, fat mass and body mass index. It was also discovered that individuals homozygous for the A allele at this locus had a greater susceptibility to gain body weight. No association was found in relation to variation in the beta-3 adrenergic receptor Trp64Arg, tumor necrosis factor-alpha promoter, or leptin genes in non-obese or obese women. This conclusion confirms the hypothesis that in most of the cases the leptin gene receptor can be involved in the development of obesity (21). In the Spanish population, the leptin gene receptor variants (Q223R) can also be associated with obesity, rather than a -2548 G/A leptin gene polymorphism (6). Similar results were obtained for Brazilian population (7)

Studies including lipid profile were carried in normal and over-weight populations. Van der Vleuten *et al*. discovered the presence of homozygous genotypes rather than heterozygous for the leptin gene receptor may be associated with familiar combined hyperlipidemia (22).

In our study, we found that with the increasing value of BMI in first trimester of pregnancy complicated by diabetes mellitus type 1, increased the tendency for the presence of homozygous A and G genotypes in both the leptin gene and the leptin gene receptor. It is worth mentioning that it may be necessary to recruit more patients into study, especially those who were noted to be under and

overweight in order to reinforce our findings. Since our results did not reach statistical significance, we may be able to conclude that specific trends were discovered. Our data correspond with results reached by other investigators as mentioned above. In the group of lean diabetic women in comparison to lean healthy subjects, no specific differences in the frequency of observed genotypic variants were noted. In both groups a majority of heterozygous variants were found, which may suggest that obesity and being overweight may be associated with homozygous variants of the leptin gene and its receptor.

We also tried to find association between investigated polymorphisms and biochemical parameters describing glycemic control, lipid profile, the blood pressure value and other parameters presented in table 1 and 2. We observed that in first trimester of pregnancy there are no specific or statistically significant differences in the studied parameters in these groups of women, however when analyzing the whole groups of patients with different BMI, where the trend to homozygous variant was discovered, the statistical differences among lipid parameters were found.

In world-wide literature, there is only one study describing the influence of the leptin gene receptor polymorphism (Q223R, 668 A/G) on body weight gain during first trimester pregnancy, the levels of circulating leptin and fetal body weight. This study performed was performed on on 455 women of different races having healthy pregnancies and showed no significant association with maternal BMI in early pregnancy and with fetal body weight (23). There are however some studies describing the role of leptin and changes of its concentrations and leptin polymorphisms during normal pregnancy and complicated by pre-eclampsia and gestational diabetes (24-26). There are no studies describing the possible role of the selected leptin gene and its receptor polymorphisms on body weight gain during pregnancy complicated by diabetes mellitus type 1. Thus our study allows us the possibility to monitor this group of diabetic women during pregnancy, and their weight gain changes, and correlate them with defined leptin polymorphisms as well as with changes of serum leptin concentrations that will be monitored in relation to the studied polymorphisms. The possible role of those factors will also be analyzed.

Since a trend to an increased number of homozygotic variants in both the leptin gene and the leptin gene receptor in over-weight subjects were found, it is necessary to include more patients to the study to confirm this result. It is also necessary to conduct the study in the same groups of patients during the whole term of the pregnancy, to establish if genetic variants of the investigated polymorphisms are associated with elevated body weight in the second and third trimester. This will also let us confirm the association between genetic variants and glycemic controls, lipid control, and the development of specific diabetic complications during pregnancy. It has also been found that several endocrine complications related to pregnancy are associated with multi-genetic factors, therefore, in every subject, it is necessary, to take under consideration

simultaneously both investigated genes and its polymorphisms as risk factors for the development of these complications and to avoid considering it separately.

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