INTRODUCTION

In pregnancy complicated by type 1 diabetes (T1DM), fetal growth is closely linked to metabolic control in the mother. It is also regulated by a series of hormones responsible for energy balance and intake. The most important substances related to these processes are proteins secreted by the adipose tissue: leptin, adiponectin, adipolin, visfatin, omentin, tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), resistin (1). Visfatin is a protein genetically encoded in chromosome 7, location between 7q21.1 and 7q31.33 (2). It was predominantly discovered as PBEF (pre-beta factor) growth factor, synthesized in liver, muscular cells and bone marrow (3), adipose tissue (4) and placenta. Visfatin activates the insulin receptor in a different site than insulin. This leads to a series of receptor phosphorylation (IRS-1, IRS-2), activation of mitogen-activated protein kinase (MAPK) and protein kinases (4). It is 52 kDa protein that has various functions in the human body, acting as a growth factor, cytokine and an enzyme (5).

The biological function of visfatin is still unclear. It was discovered as an insulin-mimetic and hypoglycemic factor and also triglyceride-stimulating agent. This adipocytokine has insulin-mimetic effects in mice and in cultured cells by binding to and activating the insulin receptor (6).

The role of visfatin in the course of pregnancy was studied in terms of gestational diabetes, pre-eclampsia, preterm labor, intrauterine growth restriction and fetal growth (7-13). It was found that women who subsequently develop preeclampsia had elevated concentration of serum visfatin in 11 – 13 weeks (14). Elevated serum visfatin may predict preeclampsia in late pregnancy (15). In the course of pregnancy, adipocytokines are also secreted by the placenta. High concentration of plasma visfatin was reported in pre-eclampsia but placental expression of this protein as well as VEGF was lower in this pregnancy complication (16, 17). Visfatin is up-regulated in hypoxic (13) and inflammatory conditions (19).

Several studies on visfatin in type 1 diabetic subjects revealed decreased fasting plasma visfatin levels in these patients as compared to healthy subjects. Oral glucose tolerance test does not affect plasma concentration of visfatin (20). Lower concentration of plasma visfatin in type 1 diabetic subjects was related to hyperglycemia and elevated HbA1c percentage (21, 22). It was reported that visfatin concentration can be lowered through physical activity in type 1 diabetic patients (22).

To the best of our knowledge there are no studies presenting the role of placental visfatin in type 1 diabetic women concerning its possible link to fetal growth. We hypothesize the possible role of placental visfatin, as insulin-mimetic and anabolic agent, in fetal overgrowth development. Thus, the aim
of the present study was to determine the expression of placental visfatin (NAMPT) in term placentas from T1DM women and to assess its link to neonatal birth weight (NBW) and glycemic control in the course of pregnancy.

MATERIAL AND METHODS

This is a retrospective analysis of 65 T1DM subjects (mean pregnancy duration at booking 10 wk, at delivery 37 wk) hospitalized in a tertiary level perinatal care unit in the Department of Obstetrics and Women’s Diseases between 2010 and 2014, who were selected and eligible for the study. The inclusion criteria were: type 1 diabetes, first trimester of pregnancy at booking. The exclusion criteria were - concomitant diseases i.e. maternal chronic hypertension, pregnancy-induced hypertension and preeclampsia in the course of pregnancy with intrauterine growth restriction (IUGR), other serious pregnancy complications requiring pharmacological treatment such as: threatened preterm labor and cholestasis. The study protocol obtained approval from the Ethics Committee of Poznan University of Medical Sciences. The studies were carried out in accordance with the Declaration of Helsinki. All participants were provided with clear and comprehensible medical information.

All women were offered a routine follow-up including at least three admissions to the Department and regular check-ups in our outpatient clinic, according to Polish recommendations (23, 24).

Moreover, all pregnant women were re-educated in intensive insulin therapy, optimal diet and blood glucose self-monitoring. Metabolic status was assessed. We collected data from general, obstetric and diabetic history, including age at onset, duration and the presence of vascular complications before pregnancy. During each visit in the Department or in the outpatient clinic (at least every two weeks), we collected data on glycemic profile and blood pressure. HbA1c concentration was estimated every 6 weeks. Once a trimester, all subjects had a retinal examination done and renal function checked.

All participants were treated with human insulin and/or insulin analogues following a basal-bolus protocol. The doses of insulin were adjusted according to glucose levels measured by way of self-monitoring. Target glucose values were set at 3.34 – 5.0 mmol/l for fasting glycaemia and less than 6.67 mmol/l for 2 hours postprandial glucose level. All biochemical parameters were analyzed in the Central Laboratory of the University Hospital.

We examined placentas from 65 T1DM subjects. Placental tissue (women aged 18 – 40; 37 – 40 wk) was obtained immediately after placenta delivery, cleaned from amniotic membranes and maternal decidua, rinsed in saline, snap-frozen in liquid nitrogen and stored at –80°C until assayed. Placenta was weighed immediately after the labor.

To examine the role of placental visfatin/NAMPT in fetal development, the study group was divided into 3 subgroups according to neonatal birth weight (NBW) as defined in the Fenton Chart: subgroup 1 included T1DM mothers with SGA infant - neonatal birth weight < 10 percentile (SGA, small for gestational age), subgroup 2 included T1DM mothers with AGA infants - neonatal birth weight between 10 – 90 percentile (AGA, appropriate for gestational age), subgroup 3 included T1DM mothers with LGA infants - neonatal birth weight exceeding 90 percentile (LGA, large for gestational age, macrosomic) - as also previously designed in our study protocol on placental VEGF expression in the same group of women (25, 26).

RNA extraction and cDNA synthesis

Total cellular RNA was isolated from the placenta tissue using 1 ml TriPure Isolation Reagent (Roche, Germany) and homogenized according to the manufacturer’s protocol. The concentrations and the purity of RNA were determined by measuring the absorbance at 260 and 320 nm in a spectrophotometer (BioPhotometer Eppendorf, USA). RNA concentration was calculated according to the formula - RNA concentration (µg/ml) = OD260 x40xR (R – solution rate). RNA samples were stored at –80°C. Complementary DNA was synthesized from 2 µg of total RNA in a total volume of 20 µl using the Transcriptor First Strand cDNA Synthesis Kit (Roche) with PTC 200 DNA Engine Gradient Cycler (MJ Research, Inc) in a laminar column with ice. cDNA synthesis was performed in 50°C for 35 minutes. The obtained cDNA transcripts were stored at –20°C or used directly for the real-time quantitative PCR (RT-PCR) (21).

Real-time PCR

The level of mRNA expression was analyzed using RT-PCR. The RT-PCR conditions used for Visfatin (NAMPT) and GAPDH were as follows: Visfatin (NAMPT) - Forward: GACGCCAGCAAGGAATTTTGTTAC, Reverse: AGCTTTTGTGACCTTGCaACTTCT (21).
Mixture content: 3.5 µl RNAse free water, 0.25 µl starter Forward (final concentration 0.5 µM), 0.25 µl starter Reverse (final concentration 0.5 µM), 5 µl LightCycler®480 SYBR Green I Master, 10 × concentrated, 1 µl cDNA. RT-PCR conditions: 35 cycles, primary denaturation 95°C, 20 s, annealing 56°C, 15 s, extension 72°C, 15 s. Glyceraldehyde-3-phosphate dehydrogenase (GADPH) - Forward: GAA GGT GAA GGT CGG AGT C, Reverse: GAA GAT GGT GAT GGG ATT TC.
Mixture content: 3.5 µl RNAse free water, 0.25 µl Forward (final concentration 0.5 µM), 0.25 µl Reverse (final concentration 0.5 µM), 5 µl LightCycler®480 SYBR Green I Master, 10 × concentrated, 1 µl cDNA. RT-PCR conditions: 35 cycles, primary denaturation 95°C, 20 s, annealing 56°C, 15 s, extension 72°C, 15 s. RT-PCR was carried out using a LightCycler TM Instrument (Roche, Germany) and a LightCycler DNA Master SYBR Green I kit (Roche, Germany) according to the manufacturer’s protocol. The data were evaluated with LightCycler®480 Basic Software. RT-PCR procedures were performed in the Institute of Natural Fibers and Medicinal Plants, Poznan, Poland.

Statistical analysis

We performed a statistical analysis using Statistica 12.0 for Windows. All data are presented as mean ± standard deviation (SD). The normality of data distribution was checked with the Kolmogarov-Smirnoff test. As the distribution of variables met the criteria for normal distribution, we compared the placental visfatin/NAMPT gene expression using the analysis of variance (ANOVA) for more than two groups, and a post-hoc test (Fisher least significant difference (LSD)) to specify the groups in which the differences were significant. Pearson rank and multiple regression model (MRM) were used to establish significant correlations and variables. Statistical significance was established at P < 0.05 for all comparisons.

RESULTS

There was no significant difference in: maternal age, diabetic history (disease onset and duration), first trimester parameters such as maternal BMI at booking, HbA1c at booking, total cholesterol, triglycerides, HDL, LDL and GFR at booking.
between the studied groups. In our study, mean maternal diurnal glucose concentrations at first trimester were increased in mothers of LGA infants than those in the other two groups. The above data is presented in Table 1.

We found no significant differences in third trimester maternal parameters such as BMI, GFR, week of delivery and neonatal status after delivery - umbilical arterial and venous pH. The women who delivered LGA neonates had the highest 3rd trimester mean diurnal glycaemia and HbA1c percentage (poorest metabolic control). T1DM LGA women also had the highest LDL concentration at delivery. The lowest placental expression of visfatin was noted in the T1DM LGA group but the placental mass was the highest. The above data is presented in Table 2.

We discovered that T1DM women, who delivered LGA neonates, had the highest placental mass, but the placental expression of visfatin was found to be the lowest. In the next stage of the present study, we attempted to check which of the maternal factors may affect the placental visfatin expression in this group of women. All third-trimester biochemical parameters were taken for the calculation, as the explanatory variables, in multiple regression model (MRM): mean diurnal glycaemia at delivery, HbA1c at delivery, total cholesterol, triglycerides, LDL and HDL at delivery.

The best-fitted MRM adjusted for placental visfatin expression in T1DM LGA subjects revealed significant impact of 3rd trimester HbA1c on placental visfatin expression. 3rd trimester mean blood glucose as well as 3rd trimester lipid profile had no impact on placental visfatin expression, after adding these variables to the developed MRM. We also revealed significant negative correlation between placental visfatin and maternal HbA1c percentage - Pearson rank R: 0.08667654, P < 0.034. The above data are presented in Table 3.

**DISCUSSION**

Diabetes type 1 still remains a major risk factor for poor perinatal outcome. Diabetes-related complications such as microangiopathy and pregnancy-induced hypertension are still associated with increased perinatal mortality rate in these women.

It is widely known that in the course of diabetic pregnancy, fetal growth is regulated by several maternal factors, including glycemic control and lipid profile, maternal nutritional status, pregnancy weight gain, energy balance/intake and the presence of concomitant disorders influencing placental function, such as hypertension or preeclampsia (28-31). Since it also known that even in well-controlled diabetes, feto-maternal complications may occur, in our previous studies we attempted to assess the possible role of placenta-secreted proteins: leptin and VEGF in the fetal overgrowth development (25, 29).

Visfatin maternal plasma concentrations increase during pregnancy. Alterations in visfatin level occur in women with pregnancy complications: preterm labor, pre-eclampsia and gestational diabetes mellitus (7).

Visfatin expression was discovered in the majority of human tissues, suggesting its multipotential role in human development (5). It was also reported that visfatin has an insulin-mimetic effect, therefore its placental expression may have a role in contributing to fetal growth in diabetic pregnancy. Little is known about visfatin placental expression in type 1 diabetic pregnancy.

In our study, we documented low placental visfatin expression in women with poor metabolic control in the third trimester of diabetic type 1 pregnancy. Our data are consistent with other studies on cord visfatin, but in gestational diabetes. Oncul et al. documented low visfatin concentration in cord blood in gestational diabetes subjects with increased insulin resistance (32).

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Table 1. Maternal characteristics and studied parameters at first trimester in studied groups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>T1DM SGA subjects N = 19&lt;sup&gt;a&lt;/sup&gt;</th>
<th>T1DM AGA subjects N = 30&lt;sup&gt;b&lt;/sup&gt;</th>
<th>T1DM LGA subjects N = 16&lt;sup&gt;c&lt;/sup&gt;</th>
<th>P-value&lt;sup&gt;*&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age (years)</td>
<td>28 ± 5</td>
<td>29 ± 5</td>
<td>25 ± 4</td>
<td>NS</td>
</tr>
<tr>
<td>T1DM duration (years)</td>
<td>14 ± 5</td>
<td>13 ± 5</td>
<td>14 ± 5</td>
<td>NS</td>
</tr>
<tr>
<td>Maternal age at onset of the T1DM (years)</td>
<td>15 ± 7</td>
<td>21 ± 11</td>
<td>16 ± 9</td>
<td>NS</td>
</tr>
<tr>
<td>BMI at booking (kg/m²)</td>
<td>23.9 ± 4.6</td>
<td>20.9 ± 10.3</td>
<td>23.1 ± 5.7</td>
<td>NS</td>
</tr>
<tr>
<td>Mean diurnal glycaemia at booking (mmol/l)</td>
<td>5.83 ± 0.94</td>
<td>4.34 ± 0.80</td>
<td>6.71 ± 2.50</td>
<td>&lt;0.05&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>HbA1c at booking (%)</td>
<td>7.1 ± 1.4</td>
<td>7.2 ± 1.0</td>
<td>7.7 ± 1.7</td>
<td>NS</td>
</tr>
<tr>
<td>Total cholesterol at booking (mmol/l)</td>
<td>4.81 ± 1.01</td>
<td>4.25 ± 1.45</td>
<td>4.12 ± 0.69</td>
<td>NS</td>
</tr>
<tr>
<td>Total triglycerides at booking (mmol/l)</td>
<td>1.12 ± 0.65</td>
<td>0.83 ± 0.34</td>
<td>0.82 ± 0.25</td>
<td>NS</td>
</tr>
<tr>
<td>HDL at booking (mmol/l)</td>
<td>2.17 ± 0.59</td>
<td>1.61 ± 0.49</td>
<td>1.69 ± 0.32</td>
<td>NS</td>
</tr>
<tr>
<td>LDL at booking (mmol/l)</td>
<td>2.66 ± 0.84</td>
<td>2.40 ± 0.88</td>
<td>2.11 ± 0.58</td>
<td>NS</td>
</tr>
<tr>
<td>GFR at booking (ml/min)</td>
<td>121.70 ± 44.23</td>
<td>121.69 ± 71.11</td>
<td>160.19 ± 61.22</td>
<td>NS</td>
</tr>
</tbody>
</table>

<sup>a</sup>ANOVA, post-hoc LSD; <sup>b</sup>C versus A and B; <sup>c</sup>T1DM, type 1 diabetes; BMI, body mass index; HDL, high density lipoprotein, LDL, low density lipoprotein, GFR, glomerual filtration rate
Placental expression of visfatin was studied in gestational diabetes subjects. In the paper from China, the authors reported no significant differences in placental visfatin expression as compared to healthy subjects, but they observed a negative correlation with glycemic control, which is consistent with our data (33). Another study from Ma revealed increased placental and adipose tissue visfatin expression in gestational diabetes subjects (34). One more study on placental visfatin in GDM subjects from Telejko et al. found no significant differences between diabetic and healthy subjects, but revealed several specific correlations with interleukin-6 and tumor necrosis factor-alpha (35).

In our study, we found that the lowest placental visfatin was present in women who delivered macrosomic neonates and these women had also poor metabolic/glycemic control in the course of pregnancy. We speculate, on the one hand, that the prolonged maternal hyperglycemia reflected by HbA1C percentage down-regulates placental visfatin expression. On the other hand, as a result of hyperglycemia, low level of placental visfatin - an insulin-mimetic agent, may indirectly lead to fetal macrosomia. We conclude that poor metabolic control in the course of type 1 diabetic pregnancy, as a possible cause of low placental visfatin expression, can be a modulator of fetal overgrowth in diabetic pregnancy. This was documented in our MRM (R-0.9167861, B-0.346688, P-0.014). This hypothesis was also supported in the study of Shang et al. who found low umbilical concentration of visfatin in macroscopic neonates (36). Cord blood visfatin concentrations were increased in LGA and IUGR neonates (37).

There is also evidence that the first trimester maternal BMI correlated negatively with cord blood visfatin and fetal birth-weight. These data were presented in the study by Valsamakis et al. (38). In our study, women who delivered macrosomic infants also had high BMI at booking.

The study by Bilski et al. revealed a significant decrease in plasma visfatin concentrations after exercise intervention (the Wingate test) in non-pregnant subjects. Moreover, an increase in visfatin concentration in sedentary, but not exercise series, was observed after test meals application (39).

Another study by Kiec-Klimczak et al. revealed an elevated concentration of visfatin in non-pregnant subjects with high GLP-1 level after oral glucose and lipid tolerance tests (40).

One important limitation of our study, as mentioned in our paper on placental VEGF expression, is that there is no evidence and no study on exogenous insulin (doses/administration) these patients are treated with, and its potential impact on placental expression of different substances (25). We speculate that increased doses of exogenous insulin in poorly controlled hyperglycemic women, may down-regulate placental visfatin expression and indirectly lead to fetal macrosomia. This will be the aim of another study conducted in our center.

To conclude our data may suggest that down-regulated placental visfatin and 3rd trimester hyperglycemia may have a role in fetal overgrowth in type 1 diabetic pregnancy but more studies need to be conducted to support this hypothesis.

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**Table 2. Maternal characteristics and studied parameters at delivery in studied groups.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>T1DM SGA subjects N = 19*</th>
<th>T1DM AGA subjects N = 30#</th>
<th>T1DM LGA subjects N = 16^</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean diurnal glycaemia at delivery (mmol/l)</td>
<td>5.34 ± 0.99</td>
<td>4.81 ± 1.25</td>
<td>5.52 ± 0.91</td>
<td>&lt;0.05^</td>
</tr>
<tr>
<td>HbA1C at delivery(%)</td>
<td>5.7 ± 0.9</td>
<td>6.2 ± 1.7</td>
<td>7.1 ± 1.5</td>
<td>&lt;0.05^</td>
</tr>
<tr>
<td>BMI at delivery (kg/m²)</td>
<td>29.1 ± 8.1</td>
<td>28.1 ± 6.3</td>
<td>24.9 ± 5.6</td>
<td>NS</td>
</tr>
<tr>
<td>Total cholesterol at delivery (mmol/l)</td>
<td>5.26 ± 1.12</td>
<td>5.11 ± 0.33</td>
<td>5.62 ± 0.87</td>
<td>NS</td>
</tr>
<tr>
<td>Total triglycerides at delivery (mmol/l)</td>
<td>1.89 ± 1.65</td>
<td>1.91 ± 0.55</td>
<td>1.92 ± 0.76</td>
<td>NS</td>
</tr>
<tr>
<td>HDL at delivery (mmol/l)</td>
<td>1.95 ± 0.56</td>
<td>2.71 ± 0.62</td>
<td>2.18 ± 0.82</td>
<td>NS</td>
</tr>
<tr>
<td>LDL at delivery (mmol/l)</td>
<td>3.18 ± 0.91</td>
<td>3.03 ± 0.87</td>
<td>3.92 ± 0.53</td>
<td>&lt;0.05^</td>
</tr>
<tr>
<td>GFR at delivery (mL/min)</td>
<td>90.11 ± 51.20</td>
<td>92.71 ± 38.24</td>
<td>116.21 ± 45.26</td>
<td>NS</td>
</tr>
<tr>
<td>Neonatal birth weight (g)</td>
<td>2450 ± 340</td>
<td>3430 ± 260</td>
<td>4420 ± 230</td>
<td>&lt;0.05^</td>
</tr>
<tr>
<td>Gestational age at delivery (weeks)</td>
<td>37 ± 2</td>
<td>37 ± 1</td>
<td>38 ± 1</td>
<td>NS</td>
</tr>
<tr>
<td>pH umbilical artery</td>
<td>7.29 ± 0.10</td>
<td>7.21 ± 0.18</td>
<td>7.21 ± 0.14</td>
<td>NS</td>
</tr>
<tr>
<td>pH umbilical vein</td>
<td>7.25 ± 0.10</td>
<td>7.28 ± 0.12</td>
<td>7.28 ± 0.15</td>
<td>NS</td>
</tr>
<tr>
<td>Placental visfatin/NAMPT</td>
<td>1.09 ± 0.95</td>
<td>0.87 ± 0.67</td>
<td>0.76 ± 0.05</td>
<td>&lt;0.05^</td>
</tr>
<tr>
<td>Placental mass</td>
<td>510 ± 130</td>
<td>648 ± 130</td>
<td>790 ± 130</td>
<td>&lt;0.05^</td>
</tr>
</tbody>
</table>

*ANOVA, post-hoc LSD; ^C versus B; #A versus C; /B versus C; *significant in all groups; ^A versus C, B versus C; *significant in all groups; T1DM, type 1 diabetes; BMI, body mass index; HDL, high density lipoprotein; LDL, low density lipoprotein; GFR, glomerular filtration rate; NAMPT, nicotinamide phosphoribosyltransferase.

**Table 3. MRM and Pearson rank in T1DM LGA subjects with 3rd trimester HbA1C as explanatory variable.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>MRM R-value</th>
<th>MRM B-value</th>
<th>MRM P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>3rd trimester HbA1C</td>
<td>0.9167861</td>
<td>0.346688</td>
<td>0.014</td>
</tr>
<tr>
<td>Parameter</td>
<td>Pearson R-value</td>
<td>Pearson P-value</td>
<td></td>
</tr>
<tr>
<td>3rd trimester HbA1C versus visfatin/NAMPT</td>
<td>R-08667654</td>
<td>–</td>
<td>0.034</td>
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</tbody>
</table>
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