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The association of leukocyte phosphatidylinositol 3-kinase delta overexpression with gestational diabetes mellitus (GDM)

Związek podwyższonej ekspresji leukocytarnej kinazy 3-fosfatydyloinozytolu delta z cukrzycą ciążową (GDM)

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Abstract

Introduction: An increasing body of evidence has linked diabetes to inflammation. The phosphatidylinositol 3-kinase delta (PI3-K delta), a member of the PI3K class IA family, has been implicated in the regulation of inflammation since it is predominantly expressed in leukocytes. To date, no information has been available on the relationship of leukocyte PI3-K delta with gestational diabetes mellitus (GDM). Therefore, the aim of this study was to investigate changes in leukocyte *PIK3CD* mRNA expression in GDM women and, in turn, to correlate them with anthropometric and metabolic parameters of patients. Additionally, an association between leukocyte mRNA expression of *PIK3CD* and Sirtuin 1 (*SIRT1*) was determined.

Material and methods: Blood samples from women with normal glucose tolerance (NGT; n = 43) and GDM (n = 132) at 24–33 weeks of gestation were collected. After isolating leukocytes from the blood, quantitative real time PCR (qRT-PCR) was performed to determine *PIK3CD* gene expression in these cells. Univariate regression analyses were used to assess an association of leukocyte *PIK3CD* mRNA level with clinical characteristics of patients as well as with leukocyte *SIRT1* mRNA expression.

Results: Leukocyte *PIK3CD* mRNA was increased by 1.98-fold in the GDM *v.* NGT subjects and inversely correlated with low-density lipoprotein cholesterol (LDL-C) and total cholesterol (TC) in diabetic pregnancy. There were also significant positive correlations of leukocyte *PIK3CD* mRNA with plasma glucose concentration at 2h of 75 g oral glucose tolerance test (OGTT) and *SIRT1* mRNA in the whole study population (both $P < 0.05$).

Conclusions: GDM is accompanied by leukocyte *PIK3CD* overexpression associated with reduced plasma LDL-C and TC levels, as well as with hyperglycaemia and elevated leukocyte *SIRT1* mRNA. **(Endokrynol Pol 2014; 65 (1): 17–24)**

Key words: *phosphatidylinositol 3-kinase delta (PI3-K delta); gestational diabetes mellitus (GDM); Sirtuin 1 (SIRT1)*

Streszczenie

Wstęp: Coraz więcej dowodów wskazuje na związek cukrzycy ze stanem zapalnym. Kinaza 3-fosfatydyloinozytolu delta(PI3-K delta), członek klasy IA rodziny kinaz 3-fosfatydyloinozytolu, ulega ekspresji głównie w leukocytach i bierze udział w regulacji odpowiedzi immunologicznej. Do tej pory brak jest informacji o związku leukocytarnej PI3-K delta z cukrzycą ciążową (GDM). Dlatego celem badania było określenie zmian w poziomie ekspresji *PIK3CD* mRNA w leukocytach kobiet z GDM i skorelowanie ich z antropometrycznymi i metabolicznymi parametrami pacjentek. Dodatkowo zbadano związek między poziomem ekspresji *PIK3CD* a sirtuiny 1 (*SIRT1*) w leukocytach pacjentek.

Materiał i metody: Próbki krwi pobrano od kobiet z prawidłową gospodarką węglowodanową (NGT; n = 43) oraz od kobiet z GDM (n = 132) w 24.–33. tygodniu ciąży. Po wyizolowaniu leukocytów z krwi, poziom ekspresji *PIK3CD* mRNA w tych komórkach określono metodą ilościowego RT-PCR. Korelacje między ekspresją *PIK3CD* a klinicznymi parametrami pacjentek i ekspresją *SIRT1* analizowano z wykorzystaniem regresji jednokrotnych.

Wyniki: Ekspresja *PIK3CD* była 1,98-krotnie wyższa w leukocytach kobiet z GDM niż w grupie kontrolnej ($P < 0.05$) i ujemnie korelowała ze stężeniem cholesterolu (TC) i frakcji LDL-C w grupie GDM. Stwierdzono również istotne statystycznie dodatnie korelacje między ekspresją *PIK3CD* a stężeniem glukozy w osoczu w 2. godzinie testu doustnego obciążenia 75-g glukozy (OGTT) i ekspresją *SIRT1* w całej badanej populacji pacjentek ($P < 0.05$).

Wniosk: Cukrzycy ciążowej towarzyszy podwyższona ekspresja *PIK3CD* w leukocytach, która wiąże się z obniżonymi stężeniami LDL-C i TC w osoczu pacjentek oraz hiperglikemią i podwyższonym poziomem *SIRT1* mRNA w leukocytach. **(Endokrynol Pol 2014; 65 (1): 17–24)**

Słowa kluczowe: *kinaza 3-fosfatydyloinozytolu delta (PI3-K delta); cukrzyca ciążowa (GDM); sirtuina 1 (SIRT1)*

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Introduction

Gestational diabetes mellitus (GDM) is defined as carbohydrate intolerance that begins or is first recognised during pregnancy [1]. This metabolic disorder is an increasing health problem among pregnant women in the world that affects 1–14% of all pregnancies, depending on the diagnostic and screening criteria [1, 2]. GDM is associated with numerous and serious complications for both mother (i.e. preeclampsia, preterm delivery, Caesarean section, pregnancy-induced hypertension as well as elevated risk of developing type 2 diabetes and cardiovascular disease after pregnancy) and foetus (i.e. prematurity, macrosomia, hypoglycaemia, jaundice, respiratory distress syndrome, polycythemia, and hypocalcaemia) [3].

Insulin resistance and pancreatic β-cell dysfunction are considered as two major determinants of GDM development [2]. Although the pathophysiology of GDM is still obscure, defective insulin signalling in adipocytes, muscle, and placenta of GDM subjects appears to be one of the cellular mechanisms underlying insulin resistance during maternal diabetic pregnancy [4, 5]. Of insulin signalling components, dysregulation of phosphatidylinositol 3-kinase (PI3-K) from the class IA has been demonstrated as an essential disturbance linked to impaired insulin-stimulated glucose transport in these tissues during GDM [4, 5].

Class IA PI3-Ks are heterodimers consisting of a 110-kDa catalytic subunit and an 85-kDa regulatory subunit, which convert phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3). PIP3 is a second messenger in insulin signalling cascade that is required to propagate downstream signalling to protein kinase B (PKB)/Akt and/or atypical protein kinase C (PKC), and glucose transport. To date, three catalytic subunit isoforms, called PI3-K alpha, PI3-K beta and PI3-K delta, and encoded by three separate genes, denoted *PIK3CA*, *PIK3CB* and *PIK3CD* respectively, have been identified. The PI3-K alpha and PI3-K beta isoforms are ubiquitously expressed, whereas PI3-Kdelta expression predominates in leukocytes [6]. Although the expression profiles of these catalytic subunits have been extensively studied in many different cell types, their precise roles and contributions in metabolic disorders, including diabetes, are still poorly understood. In recent years, several studies have investigated metabolic functions of the PI3-K alpha and/or PI3-K beta isoforms in vivo. In this regard, an essential role of PI3-K alpha in insulin signalling as well as in the regulation of hepatic glucose and lipid metabolism has been shown [7]. Interestingly, no changes in the expression of PI3-K alpha and PI3-K beta have been detected in adipose tissues and skeletal muscles of GDM subjects [5]. Thus, these various observations regarding PI3-K alpha may be related at least in part to its tissue-specific effects.

Taking into account a substantial role of leukocytes in metabolic dysfunctions during diabetic pregnancy and, on the other hand, the fact that PI3-K delta is a crucial regulator of innate and adaptive immune responses that is predominantly expressed in leukocytes [8], the main objective of the present study was to investigate changes in leukocyte *PI3KCD* mRNA expression in the GDM subjects v.normal glucose tolerant (NGT) pregnant women and, in turn, to correlate them with clinical characteristics of patients. Moreover, we sought to determine the relationship between the expression of *PI3KCD* and Sirtuin 1 (*SIRT1*) in leukocytes of the GDM patients since SIRT1 has received significant attention as a key NAD+-dependent deacetylase linking caloric restriction to mammalian cell life span, insulin secretion, and glucose/lipid metabolism [9, 10].

Material and methods

Subject recruitment

One hundred and thirty-two GDM and 43 NGT pregnant women between 24 and 33 weeks of gestation were recruited for this study during their first visit at the Polish Mothers' Memorial Hospital Research Institute, Diabetological Medical Centre in Lodz, Poland. GDM was diagnosed if one or more plasma glucose levels were elevated during a 75 g, 2 h oral glucose tolerance test (OGTT), according to the Polish Diabetes Association (PDA; modified WHO criteria) [11]. The inclusion criteria were the following:

— diagnosis of GDM according to the criteria set by PDA,
— Caucasian ethnicity,
— age between 18 and 40 years,
— no family history of diabetes within first-degree relatives,
— no GDM in a previous pregnancy,
— absence of pre-pregnancy diabetes,
— absence of concomitant systemic disease (chronic or acute or infectious).

All clinical investigations were conducted in accordance with the guidelines of The Declaration of Helsinki and were approved by the Ethical Committee of the Medical University of Lodz (No. RNN/154/09/KB from 21.04. 2009). Informed consent was obtained from all participating subjects.

Anthropometric and biochemical measurements

Information on maternal age and pre-pregnancy weight were collected from medical records. The weight and height of patients during pregnancy were measured using standard methods, and body weight gain as

well as pre- and pregnancy body mass index (BMI), expressed as weight (before pregnancy or during pregnancy) divided by squared height (kg/m^2), were calculated. Gestational age was established based on the last menstrual period.

Blood samples were drawn after a 12 h overnight fast. Serum triglycerides (TGs), HDL-cholesterol (HDL-C) and LDL-cholesterol (LDL-C) levels were determined by enzymatic colorimetric methods using triglyceride GPO-PAP and the Total Cholesterol CHOD-PAP kits (Roche Diagnostics GmbH, Mannheim, Germany). The glycated haemoglobin (HbA_{1C}) was measured by a latex-enhanced turbidimetric immunoassay using specific monoclonal antibodies. The C reactive protein (CRP) concentration was determined by turbidimetric assay with the use of the cassette COBAS INTEGRA C-Reactive Protein (Latex) according to the manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany).

The biochemical assays were carried out with a COBAS INTEGRA analyzer (Roche, SA). Plasma insulin level was quantified using Elecsys insulin assay (Roche Diagnostics GmbH, Mannheim, Germany). Insulin resistance and β-cell function were estimated by the homeostasis model assessment, HOMA–IR and HOMA–B, respectively [12]:

$$\text{HOMA–IR} = [\text{fasting insulin } (\mu\text{U/mL}) \times \text{fasting glucose (mg/dL)}]/405$$

$$\text{HOMA–B} = [360 \times \text{fasting insulin } (\mu\text{U/mL})]/-[\text{fasting glucose (mg/dL)} - 63]$$

Leukocytes separation

Fresh anti-coagulated blood samples (10 mL) withdrawn from each patient were centrifuged at 3,000 rpm for 10 min at 4°C. The supernatants containing plasma were discarded, and 15 mL of red blood cell lysis buffer (NH_4Cl, $KHCO_3$, EDTA) was added to the leaving untouched packet cells (erythrocytes and leukocytes). After 30 min. of erythrocytes lysis in ice bath, aliquots were centrifuged at 4,000 rpm for 10 min at 4°C, and supernatants were discarded. The pellets containing leukocytes were washed twice with the phosphate-buffered saline (PBS), and total RNA was isolated as described below.

RNA extraction and real-time RT-PCR

Total RNA was extracted from leukocytes using a commercially available acid-phenol reagent (TriReagent, Sigma-Aldrich, USA) according to the manufacturer's instructions. RNA concentration and its purity were assessed by a LAMBDA 25 UV spectrophotometer (PerkinElmer, UK) at UV_{260} and $UV_{260}/_{280}$, respectively. 4 μg total RNA was reverse-transcribed using a $(dT)_{18}$ primer and RevertAid™ H Minus M-MuLV reverse transcriptase as described in the manufacturer's protocol (Fermentas, Lithuania). The cDNA was diluted ten-fold, and 2 μL cDNA was used to perform RT-PCR using Maxima™ SYBR Green/ROX qPCR Master Mix (2 ×) (Thermo Scientific, USA) and specific primers for *PIK3CD* (forward 5'–CAGCGACAACATCATGATCC–3'; reverse 5'–TCGTAGGTGAGGATGAATGG–3'), and for glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as a housekeeping gene (forward 5'–GGTGGTCTCCTCTGACTTCAACA–3'; reverse, 5'–GTTGCTGTAGCCAAATTCGTTGT–3'). Amplification was carried out on 7500 Real Time PCR System (Applied Biosystems, USA) with initial denaturation at 94°C for 3 min, followed by 24 cycles at 94°C for 30s, 56°C for 15s, 72°C for 30s, and a final extension at 72°C for 1 min. All samples were run in duplicate. Specificity of the product was assessed from the melting curve analysis. The threshold cycle (Ct) of each target product was determined, and ΔCt between target and endogenous control *GAPDH* was calculated as: $\Delta Ct = Ct^{(PI3KCD)} - Ct^{(GAPDH)}$. The expression changes between the GDM and the NGT groups were expressed as ΔΔCt and calculated in the following manner: $\Delta\Delta Ct = \Delta Ct^{(GDM)} - \Delta Ct^{(NGT)}$. The fold change value between the two groups was determined as $2^{-\Delta\Delta Ct}$ [13].

Statistical analysis

The distribution of analysed data was checked by the chi-square test. Differences between variables were calculated using the Student's *t* test (in the case of normally distributed data) or the Wilcoxon's test (if the variables were not normally distributed). The correlations were performed using the Spearman's rank correlation method. The data was expressed as mean ± standard deviation (SD). A P value < 0.05 was considered significant. Statistical analysis was performed using a commercially available statistical software package (Statistica version *8.0*, StatSoft, Poland, licence no AXAP911E504325AR-K).

Results

Subject characteristics

The clinical characteristics of 132 GDM and 43 NGT patients are given in Table I. In univariate analyses, women complicated by GDM were older and had significantly lower plasma HDL-C levels than the NGT group ($P < 0.05$). Fasting, 1-h and 2-h plasma glucose concentrations at 75 g OGTT as well as HbA_{1C} levels were significantly higher in the GDM women compared to the NGT controls ($P < 0.05$). No significant differences existed in gestational age and the parameters of maternal adiposity (i.e. pre- and pregnancy BMI, body weight gain), lipid metabolism (i.e. TGs, LDL-C and total cholesterol [TC]), inflammation (CRP), insulin resistance (i.e. insulin, HOMA-IR), and insulin secretion (HOMA-B) between the two groups ($P > 0.05$).

Table I. *Clinical characteristics of studied women*
Tabela I. *Charakterystyka kliniczna kobiet uczestniczących w badaniu*

Variable	NGT group (n = 43)	GDM group (n = 132)	*P*
Age (years)	29.0 ± 4.7	30.6 ± 4.9	0.035[a*]
Gestational age (week)	29.3 ± 2.4	29.7 ± 2.6	0.380[b]
Pre-pregnancy BMI [kg/m^2]	24.4 ± 4.5	25.2 ± 5.6	0.429[b]
Pregnancy BMI [kg/m^2]	27.9 ± 4.3	28.8 ± 5.7	0.446[a]
Body weight gain [kg]	9.7 ± 5.4	9.5 ± 5.7	0.653[b]
Fasting plasma TC [mg/dL]	257.8 ± 39.5	249.8 ± 46.8	0.330[a]
Fasting plasma TGs [mg/dL]	230.7 ± 76.5	232.1 ± 86.6	0.988[b]
Fasting plasma HDL-C [mg/dL]	77.0 ± 16.4	69.6 ± 18.5	0.021[a*]
Fasting plasma LDL-C [mg/dL]	142.5 ± 58.1	139.0 ± 46.5	0.843[b]
HbA_{1c} (%)	5.3 ± 0.4	5.5 ± 0.4	0.010[a*]
Fasting plasma glucose [mg/dL]	78.5 ± 8.2	90.2 ± 19.1	< 0.001[b*]
1-h plasma glucose [mg/dL]	154.5 ± 34.3	185.0 ± 31.6	< 0.001[b*]
2-h plasma glucose [mg/dL]	114.1 ± 20.5	163.0 ± 21.5	< 0.001[a*]
Insulin [μIU/mL]	5.3 ± 3.9	4.7 ± 3.2	0.573[b]
HOMA-IR	1.2 ± 1.0	1.6 ± 1.3	0.057[b]
HOMA-B	137.2 ± 99.8	115.8 ± 77.9	0.444[b]
CRP [mg/L]	4.3 ± 3.2	3.9 ± 3.0	0.653[b]

BMI — body mass index; CRP — C reactive protein; HDL — high-density lipoprotein; HOMA-B — homeostasis model assessment of β-cell function; HOMA-IR — homeostasis model assessment of insulin resistance; LDL — low density lipoprotein; TC — total cholesterol; TGs — triglycerides. Data represents mean ± SD.
[a]*P* value from Student's *t* test; [b]*P* value from Wilcoxon's test; [*]*P* value < 0.05 as statistically significant

Leukocyte PIK3CD mRNA expression and correlations

The changes in leukocyte *PIK3CD* gene expression in GDM (n = 132) *v.* NGT (n = 43) subjects were determined by RT-PCR. The *PIK3CD* mRNA level in these cells was significantly higher in the GDM group compared to the control group (0.29 ± 0.24 *v.* 0.15 ± 0.14, $P < 0.001$ as assessed by the non-parametric Wilcoxon's test) with a 1.98-fold up-regulation (Fig. 1).

Univariate correlation analyses with the use of the Spearman rank test were performed to investigate whether the leukocyte *PIK3CD* expression is related to clinical characteristics of subjects given in Table I. As indicated in Table II, *PIK3CD* mRNA inversely correlated with fasting plasma levels of LDL-C ($r = –0.303$, $P = 0.006$) and TC ($r = –0.259$, $P = 0.019$) in the GDM group and the entire study population. There was also a significant positive correlation between the leukocyte *PIK3CD* mRNA and the plasma glucose concentration at 2 h of 75 g OGTT in the whole study population ($r = 0.262$, $P = 0.001$) (Fig. 2). In contrast, no correlations were observed between *PIK3CD* expression and other clinical parameters of subjects.

To further study the relationship between the leukocyte *PIK3CD* expression and GDM, we examined the association between the leukocyte expression levels of *PIK3CD* and *SIRT1*. This correlation was rendered possible by the fact that *SIRT1* mRNA level was recently determined by our group in the same samples of patients with GDM and NGT as those used in the

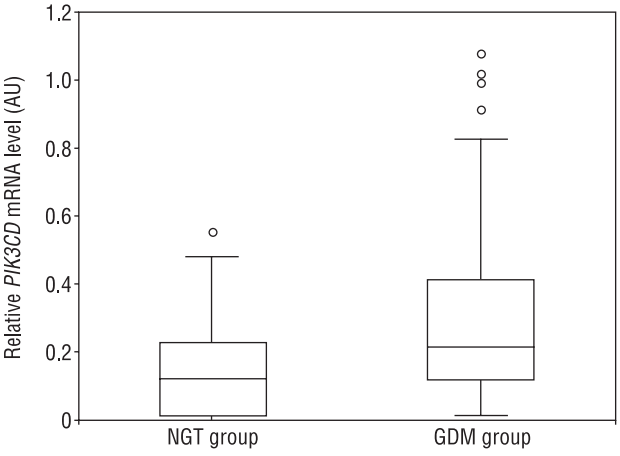

Figure 1. *Boxplots of PIK3CD mRNA expression (AU — arbitrary units) in the NGT (n = 43) and GDM (n = 132) groups. Middle line: median; box: interquartile range; whisker: range (excluding outliers). P < 0.001 compared to the NGT group by the Wilcoxon's test*

Rycina 1. *Wykresy pudełkowe ekspresji PIK3CD mRNA (AU – jednostki arbitralne) w grupach NGT (n = 43) i GDM (n = 132). Środkowa linia: mediana; pudełko: zakres międzykwartylowy; wąsy: zakres wartości nieodstających. P < 0,001 jak porównano z grupą NGT*

Table II. *Correl ations of PIK3CD mRNA expression with clinical parameters of patients*
Tabela II. *Korelacje ekspresji PIK3CD mRNA z klinicznymi parametrami pacjentek*

Variable	NGT		GDM		NGT + GDM	
	r	P	r	P	r	P
Age (years)	–0.166	0.299	0.038	0.665	0.048	0.529
Gestational age (week)	0.122	0.677	–0.039	0.856	0.084	0.609
Pre-pregnancy BMI [kg/m^2]	0.005	0.975	–0.064	0.472	–0.026	0.736
Pregnancy BMI [kg/m^2]	–0.015	0.929	–0.050	0.574	–0.020	0.795
Body weight gain [kg]	0.008	0.962	–0.022	0.799	–0.047	0.546
Fasting plasma TC [mg/dL]	–0.279	0.122	–0.259	0.019*	–0.281	0.002*
Fasting plasma TGs [mg/dL]	–0.265	0.143	–0.055	0.621	–0.102	0.281
Fasting plasma HDL-C [mg/dL]	–0.031	0.866	–0.017	0.877	–0.090	0.341
Fasting plasma LDL-C [mg/dL]	–0.257	0.155	–0.303	0.006*	–0.278	0.003*
HbA_{1c} (%)	0.083	0.624	0.008	0.926	0.067	0.392
Fasting plasma glucose [mg/dL]	0.132	0.416	–0.063	0.490	0.082	0.301
1-h plasma glucose [mg/dL]	0.151	0.358	–0.131	0.194	0.059	0.491
2-h plasma glucose [mg/dL]	0.257	0.114	0.020	0.826	0.262	0.001*
Insulin [μIU/mL]	–0.165	0.368	–0.171	0.135	–0.166	0.084
HOMA-IR	0.039	0.831	–0.161	0.179	–0.014	0.887
HOMA-B	0.000	1.000	–0.159	0.192	–0.140	0.175
CRP [mg/L]	–0.162	0.352	–0.049	0.640	–0.078	0.381

r- and *P*-values are given. Abbreviations as in Table I. *Significant correlation as assessed by Spearman's correlation method

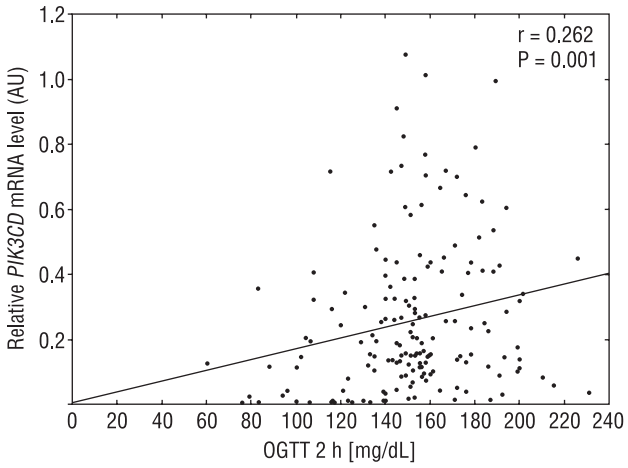

Figure 2. *The positive correlation of leukocyte PIK3CD mRNA expression (AU — arbitrary units) with 2-h OGTT glucose plasma concentration in the entire study group of pregnant women (NGT + GDM; n = 145)*

Rycina 2. *Dodatnia korelacja między ekspresją PIK3CD mRNA (AU — jednostki arbitralne) w leukocytach a stężeniem glukozy w osoczu w 2 godzinie OGTT w grupie wszytkich kobiet ciężarnych uczestniczących w badaniu (NGT + GDM; n = 145)*

present study. The leukocyte *SIRT1* mRNA level was significantly increased in the GDM group (n = 109) *v.* the NGT group (n = 36) (0.93 ± 0.73 *v.* 0.58 ± 0.57, $P = 0.014$ as assessed by the non-parametric Wilcoxon's test) [manuscript in preparation]. The Spearman test revealed the positive correlation between the expression of *PIK3CD* and *SIRT1* in the NGT ($r = 0.451$, $P = 0.006$) and GDM groups ($r = 0.250$, $P = 0.009$), as well as in the entire study population of pregnant women (n = 145; $r = 0.350$, $P < 0.001$) (Fig. 3).

Discussion

In the current study, to investigate a potential significance of PI3-K delta in GDM, we evaluated leukocyte *PIK3CD* mRNA expression in the GDM *v.* NGT groups and its correlation with known parameters of lipid and glucose metabolism as well as indices of insulin resistance and inflammation.

A group consisting of 132 women complicated by GDM and another 43 NGT pregnant women at 24–33 weeks of gestation was included in the study. The GDM women were older and had significantly lower HDL-C and higher HbA_{1c} and glucose levels compared to the NGT subjects. Of note is that the NGT pregnant women had slightly greater HOMA-B and CRP values than the GDM subjects, even though the differences between the groups did not reach statistical significance. In regard to the HOMA-B index, evidence exists for its significant reduction in GDM *v.* NGT women [14, 15], suggest-

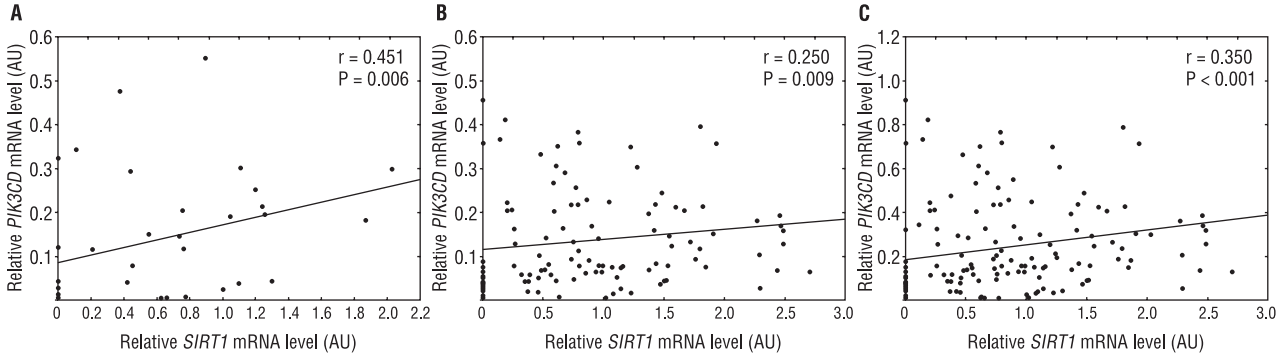

Figure 3. *The positive correlations between the expression of PIK3CD and SIRT1 (AU — arbitrary units) in leukocytes of pregnant women with (***A***) NGT (n = 36) and (***B***) GDM (n = 109), and (C) the entire study group of patients (NGT + GDM; n = 145)*

Rycina 3. *Dodatnie korelacje między ekspresją PIK3CD a SIRT1 (AU – jednostki arbitralne) w leukocytach kobiet ciężarnych z (***A***) NGT (n = 36), (***B***) GDM (n = 109) oraz całej badanej grupie pacjentek (NGT+ GDM; n = 145)*

ing an impairment in basal insulin secretory capacity during diabetic pregnancy that could be due to β-cell dysfunction partially resulting from β-cell apoptosis [16]. In the case of the inflammatory marker CRP, a non-specific acute phase reactant that is primarily produced by the liver in response to inflammatory stimuli [17], its positive association with GDM has been reported [18, 19]. However, there is one study demonstrating that maternal plasma CRP level is not related to GDM, but rather correlates significantly with pre-pregnancy obesity [20]. Since the NGT and GDM groups were similar in terms of pre-pregnancy BMI in our study, it seems likely that the higher CRP concentration in the NGT subjects was not due to differences in adiposity. However, we cannot exclude the possibility that there were temporal variations in CRP levels during the day between normal and diabetic pregnancy [21].

The novel finding of our study was a 1.98-fold increase in leukocyte *PIK3CD* mRNA level in the GDM women compared to the NGT pregnant controls in the third trimester of pregnancy, suggesting that GDM could affect alteration in PI3-K delta at its gene expression level. To date, most studies on the significance of PI3-K in GDM have concerned functional characteristics of a p85 regulatory subunit [22, 23] and some have focused on evaluating the expression of PI3-K alpha and PI3-K beta catalytic subunits in muscle, adipose, and placental tissues of diabetic pregnant women [4, 5]. However, their exact biological functions in the pathophysiology of GDM still remain obscure.

Oxidative stress, defined as excessive formation and/or insufficient removal of reactive oxygen species (ROS) and reactive nitrogen species (RNS), has been implicated in the ageing process as well as in the pathogenesis of various diseases, including cancer, cardiovascular diseases (atherosclerosis, ischaemic heart disease, hypertension, cardiomyopathies, cardiac hypertrophy, congestive heart failure), cataract, rheumatoid arthritis, neurodegenerative diseases (Alzheimer's and Parkinson's diseases), and diabetes [24, 25]. GDM has been shown to associate with enhanced oxidative stress [26], leading to increased inflammation, activation of metalloproteinases, and increased circulating levels of vascular molecules such as E-selectin and vascular cell adhesion molecule 1 (*VCAM-1*) [27]. Thus, a close relationship exists between oxidative stress, low-grade inflammation, and atherosclerosis in GDM. Since it has been shown that *in vitro* oxidative stress induces PI3-K delta signalling in monocytes and macrophages, and both PI3-K delta expression and signalling are increased in the lungs of patients with chronic obstructive disease (COPD) [28] where oxidative stress and inflammation are the main events in the pathogenesis of this disease, we cannot rule out the possibility that the leukocyte *PIK3CD* overexpression in the GDM women observed in our study might be related to enhanced oxidative stress and/or inflammation in the diabetic subjects. Although markers of oxidative stress and pro-inflammatory cytokines that could confirm the above hypothesis have not been determined in this study, the positive linear correlation between leukocyte *PIK3CD* mRNA and 2 hour post-glucose plasma level found in the present study seems to support, at least partially, this concept. The available data suggests that hyperglycaemia induces oxidative stress through several metabolic mechanisms, including the polyol pathway, formation of advanced glycation endproducts (AGEs), activation of PKC, the hexosamine pathway, and enhanced ROS production in the mitochondria [27]. Thus, hyperglycaemia-induced oxidative stress might be one of the mechanisms underlying the leukocyte *PIK3CD* overexpression in the GDM women. On the other hand,

it is likely that the increased *PIK3CD* mRNA level in the diabetic patients could be due to inflammation state induced directly by hyperglycaemia [29, 30]. According to this assumption, it has been reported that PI3-K delta affects different cellular functions of leukocytes, and its inactivation leads to impaired inflammatory and immune responses [8]. Since there is a close relationship between oxidative stress and inflammation, it is also possible that hyperglycaemia stimulates oxidative stress-mediated inflammation that is accompanied by the up-regulation of leukocyte *PIK3CD* mRNA in the GDM women. However, the sequence of events by which hyperglycaemia might affect leukocyte *PIK3CD* overexpression under diabetic conditions requires further detailed studies.

Further interesting findings in the current study are the inverse correlations of leukocyte *PIK3CD* mRNA expression with plasma LDL-and TC levels in the GDM group. An elevated plasma LDL-C is currently believed to be a crucial pathogenic factor in the development of atherosclerosis, and statin treatment has been shown to decrease LDL-C level by 20–30% and reduce the incidence of coronary artery disease by 20–30% [31]. An increased LDL susceptibility to oxidation under oxidative stress conditions is one of the most important mechanisms in the development of atherosclerosis [32]. The oxidised LDL (ox-LDL) is taken up through macrophages scavenger receptor pathways, leading to the formation of cholesterol-loaded foam cells, essential components of atherosclerotic plaques [32]. A link between oxidative events and the process of atherosclerosis during GDM has been found. In this regard, an increased LDL susceptibility to oxidation in pregnancies complicated by diabetes has been demonstrated [33]. Moreover, GDM has been shown to increase a patient's risk of atherosclerosis and coronary heart disease [34]. From these considerations, the relationship between leukocyte *PIK3CD* overexpression under hyperglycaemic conditions and decreased plasma LDL-and TC levels in the GDM group observed in our study suggests that the PI3-K delta isoform could be a potential negative regulator of LDL-C and cholesterol metabolism in diabetic subjects, which would protect against atherosclerosis. However, the underlying mechanisms by which PI3-K delta could affect plasma LDL-C level in diabetic patients need to be fully elucidated.

Over the past years, SIRT1 has been considered as an essential modulator of glucose and lipid homeostasis in different metabolic tissues, including liver, skeletal muscle, and white adipose tissue, as well as insulin secretion by pancreatic β cells and inflammation [9, 10]. Therefore, establishing whether SIRT1 is related to PI3-K delta in GDM was of great interest to us. We were able to correlate leukocyte mRNA levels of *PIK3CD* with *SIRT1* in the same GDM and NGT subjects since leukocyte *SIRT1* mRNA expression has also been determined in our laboratory [manuscript in preparation]. Interestingly, we found that *SIRT1* and *PIK3CD* mRNA levels positively correlated with each other in the GDM subjects as well as in the NGT controls, suggesting the existence of a close relationship between them during normal and diabetic pregnancy. The reasons for these associations are currently unknown, but may reflect a protective role of SIRT1 against oxidative stress, which has been implicated in both normal and complicated pregnancy [27, 35]. In keeping with this, several studies *in vitro* and *in vivo* have revealed the protective effect of SIRT1 up-regulation in some cell types against oxidative stress. For example, Alcendor et al. [36] demonstrated that cardiac-specific Sirt11 overexpression at low to moderate levels (up to 7.5-fold) in transgenic mice protected their heart from oxidative stress through an increase of catalase expression via the forkhead box O proteins (FoxO)-dependent mechanism. Interestingly, a high level (12.5-fold) of Sirt1 increased apoptosis and hypertrophy and decreased cardiac function, thereby stimulating the development of cardiomyopathy [36]. A recent study in rodent models has also supported a protective function of cardiac-specific Sirt1 overexpression against oxidative stress [37]. In proximal tubular cell lines, Sirt1 was up-regulated with the enhancement of catalase expression under ROS-stimulated conditions [38]. Thus, it is possible that, at least partially, leukocyte *SIRT1* overexpression in normal and diabetic pregnancy might protect against oxidative stress, but further study will be necessary in this field to address this hypothesis. Moreover, the positive associations of SIRT1 with PI3-K delta during human normal pregnancy and pregnancy complicated by GDM also remain to be established.

Conclusions

In the past few years, the list of identified molecular factors linked to diabetic pregnancy has greatly expanded, although it is still far from complete [39, 40]. Since no published reports are accessible on a relationship of leukocyte *PIK3CD* mRNA with GDM, we conducted the present study to address this gap.

Our data shows that leukocyte *PIK3CD* mRNA is overexpressed in GDM women in the third trimester of gestation and its expression level positively associates with 2 hour post-glucose plasma concentration as well as with leukocyte *SIRT1* gene expression in the whole study population.

The study also identifies the inverse correlations between leukocyte *PIK3CD* mRNA expression and plasma LDL-C and TC levels in the GDM group which should be analysed in greater detail to provide a new

insight into the role of PI3-K delta in lipid metabolism during diabetic pregnancy.

The major limitation of our study is the lack of measurements of markers of oxidative stress and pro-inflammatory cytokines which probably would help to clarify the functional significance of the above-mentioned associations. Therefore, additional studies are certainly needed to confirm our present findings in the context of the link between leukocyte PI3-K delta and oxidative stress and inflammation during GDM. Moreover, whether an elevated leukocyte *PIK3CD* mRNA expression persists during the postpartum period after pregnancy complicated by GDM is also unknown and worthy of further investigation.

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