CLINICAL STUDY

The expression of genes involved in NF- κ B activation in peripheral blood mononuclear cells of patients with gestational diabetes

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Abstract

Objective: In patients with obesity and type 2 diabetes, the changes in insulin resistance are associated with the changes in expression of genes involved in nuclear factor- κ B (NF- κ B) activation in peripheral blood mononuclear cells (PBMCs). As such studies have never been carried out in patients with gestational diabetes (GDM), in this study, we evaluated the expression of genes involved in NF- κ B activation and related to glucose metabolism in PBMCs obtained from pregnant women with GDM and normal glucose tolerance (NGT).

Design and methods: RT-PCR was performed in 60 pregnant women divided into three groups: GDM at the 1st visit, i.e. in the 24th–28th weeks of gestation (GDM1), NGT at the first visit and GDM in the 29th–32nd weeks (GDM2), and NGT at both visits. The tests were repeated 3 months *postpartum*.

Results: The GDM1 group had significantly higher *TLR2* ($P=0.024$), *TLR4* ($P=0.037$), *STAT1* ($P=0.027$), and *CX3CL1* ($P=0.017$) mRNA expression, whereas the GDM2 group showed markedly lower *TNFRSF1A* ($P=0.042$), *PPARG* ($P=0.018$), *STAT3* ($P=0.013$), and *CX3CL1* ($P=0.038$) mRNA expression in comparison with the NGT group. The women with NGT at the 1st visit who later developed GDM had significantly higher fasting glucose ($P=0.01$), HOMA-IR ($P=0.004$), and *TLR2* mRNA expression ($P=0.04$), as well as lower ISSI2 ($P=0.01$) and disposition indices, DI_{30} ($P=0.03$) and DI_{120} ($P=0.01$), than had the women who remained normoglycemic.

Conclusions: Our results suggest that elevated *TLR2* expression, as well as higher fasting glucose and lower compensation for increased insulin resistance, may represent early metabolic disturbances in the development of GDM.

European Journal of Endocrinology 168 419–427

Introduction

The nuclear factor- κ B (NF- κ B) is a primary regulator of inflammatory response, controlling both the innate and adaptive immune systems (1, 2). NF- κ B (NFKB1) proteins are present in the cytoplasm in association with inhibitory proteins known as inhibitors of NF- κ B (I κ Bs) (1, 2). After activation by a wide range of stimuli, such as tumor necrosis factor α (TNF α), interleukin 1 β (IL1 β), pathogens, T-cell activation signals, growth factors, and stress inducers, I κ B proteins become phosphorylated by I κ B-kinase β (IKBK β) and are subsequently degraded by the proteasome, which allows NF- κ B proteins to translocate to the nucleus and activate the transcription of a large number of genes, including pro-inflammatory cytokines, chemokines, and adhesion molecules (1, 2). Among various NF- κ B inducers, the toll-like receptors (TLRs) seem to be key initiators of innate immunity, recognizing certain

pathogen-associated lipopeptides, peptidoglycans, and other lipid moieties and then triggering a cascade of cellular signals, culminating in the activation of NF- κ B (3, 4, 5, 6).

In addition to host defense against foreign pathogens, recent studies implicate a role of NF- κ B (7, 8, 9, 10, 11) and TLR pathway (10, 12, 13, 14, 15, 16, 17, 18, 19, 20) in the pathogenesis of obesity- and type 2 diabetes-related insulin resistance and metabolic disturbances. Moreover, de Mello *et al.* (10, 11) demonstrated that in patients with the metabolic syndrome, the changes in insulin resistance were associated with the changes in the expression of genes involved in the NF- κ B signaling pathways in peripheral blood mononuclear cells (PBMCs). On the other hand, it has been shown that insulin suppresses TLR expressions (21) and NF- κ B binding activity (22) in PBMCs. Taken together, these findings strongly suggest that PBMCs are a target for insulin action and may be used as a convenient model to

study the relationship between low-grade inflammation and insulin resistance (10, 11, 22, 23, 24).

As such studies have never been carried out in patients with gestational diabetes (GDM), we hypothesized that the development of insulin resistance and hyperglycemia during pregnancy may influence the expression of genes encoding receptors of surface ligands and immune mediators involved in NF- κ B signaling pathway. To verify this hypothesis, we obtained PBMCs from women with GDM and normal glucose tolerance (NGT) during pregnancy and measured mRNA expression of genes: i) encoding receptors of surface ligands (*TLR4*, *TLR2*, TNF receptor superfamily member 1A (*TNFRSF1A*) and 1B (*TNFRSF1B*), and receptor for IL1 (*IL1R1*)) and mediators (*IKKB* and chemokine (C-C motif) ligand 5 (*CCL5*)) involved in NF- κ B signaling; ii) related to immunological and endothelial function and positively regulated by NF- κ B (*IL8*, *IL17* (*IL17A*), chemokine (C-X3-C motif) ligand 1 (*CX3CL1*), and intercellular adhesion molecule 1 (*ICAM1*)); iii) essential for cytokine receptor signaling (signal transducer and activator of transcription 1 (*STAT1*) and *STAT2*); iv) involved in inhibiting inflammatory response via negative regulation of several cytokine pathways (*IL10*, suppressor of cytokine signaling 1 (*Socs1*), *Socs2*, and *Socs3*), including NF- κ B signaling pathway (peroxisome proliferator-activated receptor- γ (*PPARG*)); and v) potentially positively (dipeptidylpeptidase-4 (*DPP4*)) or negatively (solute carrier family 27 fatty acid transporter, member 2 (*SLC27A2*)) regulated by insulin resistance (25, 26, 27).

Materials and methods

Study population

Sixty pregnant women attending the gynecological outpatient clinic of the Medical University of Białystok and tested for GDM with a 75 g 2-h oral glucose tolerance test (OGTT) between the 24th and 28th weeks of gestation were recruited. GDM was diagnosed according to the criteria of the Polish Diabetological Association, with the following threshold glucose levels: fasting ≥ 100 mg/dl (5.5 mmol/l), 1 h ≥ 180 mg/dl (10.0 mmol/l), and 2 h ≥ 140 mg/dl (7.8 mmol/l). The subjects with NGT at the first visit were tested again for GDM 4 weeks later. All tests were repeated 3 months after childbirth. Patients with abnormal glucose readings before pregnancy, as well as with pregnancy-induced hypertension, preeclampsia, and other complications (except GDM), were not included. All pregnancies were singletons. Written informed consent was obtained from all participants, and the protocol was approved by the Local Ethics Committee (Medical University of Białystok). On the basis of the OGTT, the patients were divided into three groups: GDM at the

first visit (GDM1, $n = 12$), NGT at the first visit and GDM 4 weeks later (GDM2, $n = 21$), and the patients with NGT at the first visit who remained so 4 weeks later (NGT, $n = 27$).

Analytical methods

The OGTT was performed after an overnight fast and blood samples were collected at 0, 30, 60, and 120 min after glucose ingestion. Plasma glucose concentration was measured using oxidase method (CORMAY, Lublin, Poland), serum insulin was assayed by immuno-radiometric method (Biosource Europe SA, Nlvelles, Belgium), and HbA1c was evaluated by a HPLC technique (Bio-Rad Laboratories). Total cholesterol, HDL-cholesterol, and triglyceride concentrations were measured by enzymatic methods (ANALCO-GBG, Warsaw, Poland). LDL-cholesterol concentrations were calculated using the Friedewald equation. Serum TNF α , soluble receptors sTNFR1 and sTNFR2, CCL5 (regulated on activation, normal T-cell expressed and secreted, RANTES), CX3CL1 (fractalkine), and IL10 concentrations were measured using commercial ELISA (Quantikine, R&D Systems, Minneapolis, MN, USA). Serum IL8 and IL17 concentrations were also assayed by commercial ELISA (Invitrogen and Biosource respectively).

Insulin sensitivity calculation

Insulin sensitivity was measured using the OGTT insulin sensitivity index of Matsuda & DeFronzo (IS_{OGTT}) (28), defined as $10\,000/\sqrt{((FPG \times FPI) \times (G \times I))}$, where FPG, fasting plasma glucose; FPI, fasting plasma insulin; G, mean glucose during the OGTT, and I, mean insulin (both calculated from glucose and insulin levels at 0, 30, and 120 min of the OGTT). As a measure of β -cell function, the insulin secretion sensitivity index-2 ($ISSI2$), defined as the product of i) insulin secretion measured by the ratio of the area under the insulin curve (AUC) to the area under the glucose curve and ii) insulin sensitivity measured by IS_{OGTT} , was used (29, 30, 31). The ratio of insulin AUC and glucose AUC during 0–30 min of the OGTT (AUC_{Ins30}/AUC_{Glu30}) as a surrogate index of the early-phase insulin release was also calculated (31). The trapezoidal method was used to calculate glucose and insulin AUC during the OGTT. Two disposition indices as the products of insulin sensitivity $\times$ insulin secretion ($DI_{30} = IS_{OGTT} \times AUC_{Ins30}/AUC_{Glu30}$ and $DI_{120} = IS_{OGTT} \times AUC_{Ins120}/AUC_{Glu120}$) were calculated as a measure of β -cell response to a given insulin sensitivity level (31). The homeostasis model assessment of insulin resistance (HOMA-IR) was also calculated as $FPG \times FPI/22.5$ (32).

Isolation of PBMCs, RNA extraction, and cDNA synthesis

PBMCs were isolated from anticoagulated peripheral blood using Histopaque-1077 gradient (Sigma-Aldrich)

according to the manufacturer's instructions and were stored in -80°C until assayed. Total RNA was isolated using the RNeasy Mini Kit (Qiagen) following the manufacturer's protocol. Total RNA concentration was determined using NanoDrop ND-1000 spectrophotometer (NanoDrop 1000, Thermo Scientific, Wilmington, DE, USA). cDNA synthesis was performed using High Capacity cDNA RT Kit (Life Technologies) in the MJ Research Thermal Cycler (Model PTC-200, USA).

Quantitative real-time RT-PCR

RT-PCR was performed with TaqMan Low-Density Array chemistry (TLDA, Life Technologies). The reaction mixture consisted of 50 μl cDNA and 50 μl Master Mix (TaqMan Gene Expression Master Mix, Life Technologies). Each sample was transferred to the fill port of a TaqMan Array Micro Fluidic Card and run on the 7900HT Fast Real-Time PCR System (Life Technologies). The thermal profile of the reaction was as follows: 2 min at 50°C , 10 min at 94.5°C , followed by 40 cycles of 30 s at 97°C , and 1 min at 59.7°C . β -Actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as an endogenous control.

Statistical analysis

The differences in gene expression between the groups studied were calculated by REST (Relative Expression Software Tool) 2009, taking into account qPCR efficiency and fold changes of threshold cycle (C_T) values relative to the control (33). Other data were analyzed by the Statistica 8.0 for Windows Software (StatSoft, Inc., Tulsa, USA). Before analysis, data were tested for normality of distribution using the Shapiro–Wilk test. Differences between the groups were compared by Mann–Whitney U test, and relationships between variables were tested by Spearman's correlations. One-way ANOVA with Bonferroni's correction was used to compare the differences in gene expression during the course of pregnancy. P value <0.05 was regarded as statistically significant.

Results

Characteristics of the groups studied

The clinical characteristics of the groups studied are summarized in Table 1. The patients diagnosed as having GDM at the 1st visit (GDM1) had significantly higher fasting ($P=0.04$) and post-load glucose levels ($P<0.0001$), higher insulin at 120 min of the OGTT ($P=0.04$), higher triglyceride concentration ($P=0.04$), as well as markedly lower IS_{OGTT} ($P=0.04$), ISSI2 ($P=0.001$), DI_{30} ($P=0.002$), and DI_{120} ($P=0.001$) values than had the women with NGT.

The women with NGT in the 2nd trimester but diagnosed as having GDM after the second OGTT (GDM2) at the 1st visit had significantly higher fasting glucose ($P=0.01$), insulin ($P=0.006$), HbA1c ($P=0.01$), triglycerides ($P=0.003$), and HOMA-IR ($P=0.004$), as well as lower HDL-cholesterol ($P=0.01$), ISSI2 ($P=0.01$), DI_{30} ($P=0.03$), and DI_{120} ($P=0.01$), than had the women who remained normoglycemic throughout pregnancy.

The same patients at the 2nd visit, when they were classified as having GDM (GDM2), had significantly higher fasting ($P=0.02$) and post-load glucose levels ($P<0.0001$), respectively, higher insulin levels at 0 and 120 min of the OGTT ($P=0.03$ and $P=0.004$ respectively), higher HOMA-IR ($P=0.001$), as well as markedly lower IS_{OGTT} ($P=0.003$), ISSI2 ($P=0.0002$), DI_{30} ($P=0.0009$), and DI_{120} ($P=0.0002$) values, than had the women with NGT. Three months *postpartum*, there were no significant differences in anthropometric or metabolic variables between the patients with and without GDM in pregnancy.

Serum IL levels in patients with and without GDM

At the 1st visit, the women with NGT who later developed GDM (GDM2) had significantly lower serum IL10 concentration ($P=0.04$) than had the patients with NGT throughout pregnancy (Table 2). The same patients also showed markedly higher IL8 and lower IL17 levels but the differences were not significant, probably due to large variations among individuals. There were no significant differences in the serum levels of other ILs and chemokines studied between the women with and without GDM both during and after pregnancy (Table 2).

Gene expression in PBMCs from the patients with and without GDM

At the 1st visit, the patients from the GDM1 group had significantly higher TLR2 ($P=0.024$), TLR4 ($P=0.037$), STAT1 ($P=0.027$), and CX3CL1 ($P=0.017$) mRNA expression than had the women from the NGT group (Table 3). The women with NGT at the 1st visit but diagnosed as having GDM after the second OGTT (GDM2) had significantly higher TLR2 mRNA expression compared with the subjects with NGT throughout pregnancy (32.0 (24.2–34.0) vs 9.4 (6.8–28.2) AU, $P=0.04$).

At the 2nd visit, women from the GDM2 group showed significantly lower TNFRSF1A ($P=0.042$), PPARG ($P=0.018$), STAT3 ($P=0.013$), and CX3CL1 ($P=0.038$) mRNA expression in PBMCs in comparison with the NGT group. Three months *postpartum*, the women with previous GDM had significantly higher IL1R1 ($P=0.028$), IKBKB ($P=0.018$), and SLC27A2

Table 1 Clinical and biochemical characteristics of the groups studied. Data are shown as medians (interquartile range).

	2nd trimester				3rd trimester			3 months postpartum		
	NGT	GDM2	GDM1	NGT	NGT	GDM2	NGT	NGT	GDM1 + GDM2	
<i>n</i>	27	21	12	27	27	21	27	27	33	
Age (years)	30.5 (29–35)	31 (24–31)	34 (31–34)	31 (29–34)	31 (29–34)	31 (27–32)	31 (29–35)	31 (29–35)	32 (27–35)	
Gestational age (weeks)	26 (24–27)	26 (26–28)	25.5 (25–27)	30 (29–32)	30 (29–32)	30 (29–32)	NA	NA	NA	
Prepregnancy BMI (kg/m ²)	24.7 (21.7–26.6)	25.9 (24.2–29.4)	24.5 (22.6–27.1)	24.0 (21.0–26.6)	24.0 (21.0–26.6)	24.7 (23.2–28.6)	24.0 (21.0–26.6)	24.0 (21.0–26.6)	24.6 (22.9–29.5)	
Current BMI (kg/m ²)	28.1 (25.6–29.5)	28.1 (27.3–31.6)	27.9 (25.6–31.7)	28.1 (25.8–30.7)	28.1 (25.8–30.7)	28.8 (27.1–31.4)	24.2 (21.9–27.3)	24.2 (21.9–27.3)	24.3 (21.9–27.0)	
Birth weight (g)	4.2 (4.0–4.4)	4.6 (4.4–4.7) [§]	4.8 (4.1–5.2) [§]	4.2 (3.9–4.8)	4.2 (3.9–4.8)	4.7 (4.3–4.8) [§]	3475 (3220–3700)	3475 (3220–3700)	3340 (2950–3700)	
Glucose 0' (mmol/l)	6.4 (5.6–6.8)	6.6 (6.4–7.2)	8.7 (8.0–9.2) [*]	6.3 (5.7–7.3)	6.3 (5.7–7.3)	8.2 (8.1–8.5) [*]	4.8 (4.4–5.0)	4.8 (4.4–5.0)	4.9 (4.5–5.4)	
Glucose 120' (mmol/l)	71.0 (38.0–107.5)	116.3 (97.2–132.6) [†]	85.5 (71.1–155.9)	74.0 (36.4–107.9)	74.0 (36.4–107.9)	126.0 (97.0–142.7) [§]	5.0 (4.4–6.4)	5.0 (4.4–6.4)	5.7 (4.6–6.5)	
Insulin 0' (pmol/l)	519.0 (350.7–786.7)	462 (336–629)	749.4 (600.4–869.7) [§]	499.5 (296.6–760.0)	499.5 (296.6–760.0)	824.3 (517.1–1124.9) [†]	78.7 (36.6–116.6)	78.7 (36.6–116.6)	85.3 (46.7–105.2)	
Insulin 120' (pmol/l)	4.7 (4.5–4.9)	5.0 (4.8–5.1) [§]	4.8 (4.5–4.9)	5.2 (4.7–5.6)	5.2 (4.7–5.6)	5.0 (4.9–5.3)	210.5 (141.2–351.7)	210.5 (141.2–351.7)	197.0 (147.4–366.8)	
HbA1c (%)	5.19 (3.29–7.89)	3.80 (2.77–4.38)	3.36 (2.14–4.72) [§]	3.84 (3.21–8.75)	3.84 (3.21–8.75)	2.62 (2.07–3.50) [†]	5.3 (4.9–5.5)	5.3 (4.9–5.5)	5.1 (4.8–5.4)	
ISOGTT (mU/l, mg/dl)	366.2 (287.1–413.7)	243.4 (211.3–292.7) [§]	205.7 (152.9–251.4) [†]	366.6 (275.7–446.3)	366.6 (275.7–446.3)	233.4 (180.8–267.0) [†]	6.69 (4.53–9.17)	6.69 (4.53–9.17)	5.458 (4.27–8.51)	
ISSI2	52.3 (32.9–59.6)	44.8 (41.5–67.9)	46.7 (31.4–57.9)	42.2 (32.6–60.6)	42.2 (32.6–60.6)	49.7 (35.4–65.2)	221.8 (191.6–355.6)	221.8 (191.6–355.6)	218.5 (176.4–246.5)	
AUC _{ins30} /AUC _{gluco30} (pmol/mmol)							32.2 (29.7–44.5)	32.2 (29.7–44.5)	34.3 (23.7–46.7)	
DI ₃₀	235.8 (182.2–265.9)	190.8 (156.7–229.9) [§]	133.1 (97.7–207.2) [†]	214.3 (170.4–313.2)	214.3 (170.4–313.2)	135.6 (123.1–168.8) [*]	193.9 (149.2–253.1)	193.9 (149.2–253.1)	168.1 (125.5–222.6)	
DI ₁₂₀	365.9 (287.3–413.8)	243.5 (211.5–292.9) [§]	205.7 (152.7–251.4) [†]	366.6 (276.1–446.4)	366.6 (276.1–446.4)	233.1 (180.8–267.2) [*]	50.5 (28.0–78.1)	50.5 (28.0–78.1)	53.6 (27.3–66.8)	
HOMA-IR (mU/l, mmol/l)	1.83 (1.02–3.02)	3.27 (2.74–3.97) [†]	2.71 (1.94–4.99)	2.0 (0.93–3.30)	2.0 (0.93–3.30)	3.87 (2.65–4.39) [†]	2.31 (1.17–3.79)	2.31 (1.17–3.79)	2.57 (1.50–3.43)	
Total cholesterol (mmol/l)	5.8 (5.5–6.8)	6.5 (6.0–7.3)	6.5 (5.5–6.9)	6.0 (5.2–7.0)	6.0 (5.2–7.0)	7.0 (6.4–8.0)	4.0 (3.6–4.8)	4.0 (3.6–4.8)	4.6 (4.3–5.5)	
HDL-cholesterol (mmol/l)	1.6 (1.4–1.8)	1.8 (1.8–2.2) [§]	1.9 (1.7–2.1)	1.7 (1.4–1.9)	1.7 (1.4–1.9)	1.8 (1.6–2.0)	1.7 (1.4–1.9)	1.7 (1.4–1.9)	1.7 (1.4–1.8)	
LDL-cholesterol (mmol/l)	3.4 (3.0–4.4)	3.3 (3.1–4.6)	3.6 (2.5–3.9)	3.6 (2.6–4.4)	3.6 (2.6–4.4)	4.1 (3.6–5.1)	2.2 (1.8–2.9)	2.2 (1.8–2.9)	2.5 (2.2–3.1)	
Triglycerides (mmol/l)	1.6 (1.4–1.9)	2.5 (2.0–2.9) [†]	2.3 (1.7–3.0) [§]	2.2 (1.7–2.7)	2.2 (1.7–2.7)	2.5 (2.1–3.1)	0.96 (0.72–1.09)	0.96 (0.72–1.09)	0.83 (0.64–1.03)	

NGT, normal glucose tolerance; GDM1, gestational diabetes mellitus in the 2nd trimester; GDM2, NGT in the 2nd trimester and GDM in the 3rd trimester; ISOGTT, OGTT insulin sensitivity index; ISSI2, insulin secretion sensitivity index; HOMA-IR, homeostasis model assessment of insulin resistance; DI, disposition index; differences between NGT and GDM were significant at * $P < 0.0001$, † $P < 0.001$, ‡ $P < 0.01$, and § $P < 0.05$ by Mann-Whitney U test.

Table 2 Serum levels of interleukins and chemokines studied. Data are shown as medians (interquartile range).

	2nd trimester			3rd trimester			3 months postpartum		
	NGT	GDM2	GDM1	NGT	GDM2	GDM1	NGT	GDM1 + GDM2	
n	27	21	12	27	21	27	27	33	
TNF α (pg/ml)	1.16 (0.97–1.49)	0.98 (0.73–1.03)	0.93 (0.85–1.02)	1.20 (1.04–1.52)	1.00 (0.77–1.30)	1.25 (1.0–1.92)	1.25 (1.0–1.92)	1.15 (0.97–1.31)	
sTNFR1 (ng/ml)	1.76 (1.60–2.06)	1.78 (1.70–1.91)	1.61 (1.54–2.13)	1.89 (1.69–2.14)	2.0 (1.72–2.25)	1.60 (1.24–1.66)	1.60 (1.24–1.66)	1.42 (1.35–1.63)	
sTNFR2 (ng/ml)	3.50 (3.13–3.91)	3.08 (2.71–3.26)	3.40 (3.16–3.59)	3.29 (3.01–3.73)	3.38 (2.94–4.02)	2.41 (2.36–2.99)	2.41 (2.36–2.99)	2.40 (2.14–2.86)	
CCL5 (ng/ml)	67.6 (45.6–87.1)	57.6 (51.6–75.9)	65.9 (42.2–74.6)	71.8 (54.8–93.2)	70.2 (62.5–83.3)	76.4 (60.5–103.4)	76.4 (60.5–103.4)	74.4 (63.3–88.3)	
CX3CL1 (ng/ml)	0.82 (0.65–0.95)	0.81 (0.73–1.13)	0.98 (0.85–1.08)	1.11 (0.78–1.15)	0.88 (0.67–1.02)	0.97 (0.84–1.26)	0.97 (0.84–1.26)	1.11 (0.81–1.34)	
IL8 (pg/ml)	1.24 (0.93–2.48)	2.06 (1.50–2.36)	1.44 (1.15–2.17)	1.92 (1.66–2.53)	2.39 (1.81–2.80)	3.85 (2.12–4.72)	3.85 (2.12–4.72)	4.0 (2.75–5.25)	
IL10 (pg/ml)	0.43 (0.19–0.75)	0.19 (0.11–0.34)*	0.25 (0.12–0.46)	0.42 (0.14–0.59)	0.34 (0.21–0.55)	0.34 (0.22–0.53)	0.34 (0.22–0.53)	0.25 (0.19–0.92)	
IL17 (pg/ml)	14.5 (2.7–26.2)	4.59 (3.96–7.54)	14.7 (10.4–17.4)	2.73 (2.07–22.60)	3.67 (2.08–4.59)	17.9 (14.4–76.4)	17.9 (14.4–76.4)	9.9 (2.7–16.4)	

NGT, normal glucose tolerance; GDM1, gestational diabetes mellitus in the 2nd trimester; GDM2, NGT in the 2nd trimester and GDM in the 3rd trimester; TNF α , tumor necrosis factor α ; sTNFR1, soluble TNF α receptor-1; CCL5, chemokine (C-C motif) ligand 5; CX3CL1, chemokine (C-X3-C motif) ligand 1; the difference between NGT and GDM2 was significant at * $P < 0.05$ by Mann–Whitney U test.

($P = 0.021$) mRNA expression than had the women with NGT in pregnancy (Table 3).

Changes in PBMC gene expressions during and after pregnancy

In patients with NGT, there was a significant increase in *TLR4* ($P = 0.03$), *CX3CL1* ($P = 0.02$), *STAT1* ($P = 0.04$), and *STAT3* ($P = 0.015$) mRNA expression in the 3rd trimester, as well as a significant decrease in *STAT1* ($P = 0.01$) and *PPARG* ($P = 0.006$) mRNA expression 3 months after childbirth (Fig. 1A, B and C).

In the GDM2 group, *CCL5* ($P = 0.04$), *IKBKB* ($P = 0.02$), *DPP4* ($P = 0.01$), *IL17A* ($P = 0.04$), and *SOCS2* ($P = 0.04$) mRNA expression decreased significantly in the 3rd trimester compared with the 2nd trimester values. Three months after childbirth, *CCL5* and *DPP4* mRNA expressions were significantly higher ($P = 0.04$ and $P = 0.02$ respectively), whereas *STAT1* mRNA expression was markedly lower ($P = 0.03$) than in the 3rd trimester (Fig. 1D, E and F).

Correlations between gene expression and other variables

In patients with GDM, there were negative correlations between HOMA-IR and *DPP4* expression ($R = -0.43$, $P = 0.03$), AUC_{Ins30}/AUC_{Glu30} , and *STAT3* expression ($R = -0.47$, $P = 0.03$), as well as positive correlations between IS_{OGTT} and *DPP4* expression ($R = 0.42$, $P = 0.04$), IS_{OGTT} and *IL17A* expression ($R = 0.57$, $P = 0.03$), and between *CX3CL1* serum level and *CX3CR1* expression in PBMCs ($R = 0.57$, $P = 0.02$).

In patients with NGT, there were negative correlations between AUC_{Ins30}/AUC_{Glu30} and *TLR4* expression ($R = -0.78$, $P = 0.03$) and between AUC_{Ins30}/AUC_{Glu30} and *STAT1* expression ($R = -0.78$, $P = 0.03$), as well as positive correlations between DI_{30} and *IKBKB* expression ($R = 0.85$, $P = 0.02$), DI_{120} and *IKBKB* expression ($R = 0.82$, $P = 0.02$), and DI_{120} and *IL17A* expression ($R = 0.82$, $P = 0.03$).

Discussion

The most interesting finding of our study was a significant increase in *TLR2* mRNA expression in the PBMCs obtained from the pregnant women who were normoglycemic at the time of sampling but later developed GDM. The expression of *TLR2* and *TLR4* mRNA was also elevated in the patients diagnosed as having GDM in the 2nd trimester; however, 4 weeks later, the difference was not significant, probably as a result of an increase in TLR expressions in the healthy pregnant women. To the best of our knowledge, there are no other data concerning TLR expressions in GDM subjects, but our results can be explained by the observation made by Dasu *et al.* (13), who demonstrated

Table 3 Fold changes of relative gene expression in PBMCs obtained from women with gestational diabetes in comparison with healthy pregnant women. Differences between groups were calculated by REST 2009 Software. Relative gene expression of the respective gene in the healthy pregnant group = 1.

Gene	2nd trimester, GDM1		3rd trimester, GDM2		3 months <i>postpartum</i> , GDM1+GDM2	
	ExpR	P value	ExpR	P value	ExpR	P value
<i>TNFRSF1A</i>	1.114	0.714	0.684	0.042	1.035	0.702
<i>TNFRSF1B</i>	1.327	0.163	0.776	0.125	1.105	0.323
<i>IL1R1</i>	0.690	0.422	0.659	0.483	1.430	0.028
<i>TLR2</i>	2.022	0.024	0.863	0.524	1.279	0.178
<i>TLR4</i>	1.875	0.033	0.697	0.105	1.134	0.178
<i>ICAM1</i>	1.427	0.299	0.734	0.071	0.959	0.686
<i>CCL5</i>	1.085	0.810	0.797	0.156	1.242	0.180
<i>IKBKB</i>	0.997	0.994	0.920	0.558	1.284	0.018
<i>DPP4</i>	1.777	0.214	0.844	0.558	1.236	0.183
<i>PPARG</i>	0.800	0.419	0.556	0.018	0.945	0.701
<i>IL8</i>	1.494	0.553	0.860	0.683	0.762	0.508
<i>IL10</i>	1.121	0.689	0.860	0.640	1.297	0.301
<i>STAT1</i>	1.670	0.027	0.748	0.096	0.992	0.933
<i>STAT3</i>	1.401	0.127	0.676	0.013	1.263	0.060
<i>IL17A</i>	0.558	0.206	1.044	0.925	1.640	0.169
<i>CX3CL1</i>	2.134	0.017	0.533	0.038	0.720	0.191
<i>CX3CR1</i>	1.169	0.628	0.763	0.194	1.271	0.158
<i>SOCS1</i>	0.505	0.371	1.309	0.565	1.033	0.854
<i>SOCS2</i>	1.630	0.152	0.905	0.608	1.131	0.450
<i>SOCS3</i>	0.596	0.381	0.891	0.755	0.864	0.323
<i>SLC27A2</i>	1.157	0.708	0.719	0.238	1.447	0.021

ExpR, expression ratio.

that inflammatory effects of high glucose may be at least in part mediated through TLR activation as high glucose induces a marked increase in *TLR2* and *TLR4* expression in human monocytes, resulting in NF- κ B activation and proinflammatory cytokine secretion. However, recently, it has been shown that moderate postprandial glucose excursions are not able to increase TLR expressions and that the major mediators of TLR activation may be free fatty acids and specific macronutrients such as saturated fat (17, 34, 35). It is also relevant that insulin exerts an opposite, i.e. suppressive, effect on TLR expressions in PBMCs (21) so that insulin deficit or insulin resistance would be expected to result in TLR expression upregulation. Indeed, *TLR2* and *TLR4* expressions have been shown to be increased in the skeletal muscle and adipose tissue of type 2 diabetic subjects (12, 19), as well as in the monocytes obtained from the patients with both type 1 (36) and type 2 diabetes (14), and the metabolic syndrome (15).

The next finding in our study was an upregulation of *CX3CL1* mRNA expression in the women with GDM in the 2nd trimester compared with the healthy pregnant subjects. However, in the 3rd trimester, *CX3CL1* mRNA expression rose markedly in the patients with NGT, probably as a result of a physiological increase in insulin resistance during pregnancy, and it was even higher than in the patients with late-onset GDM. *CX3CL1* (fractalkine) is a structurally unique chemokine that signals through a single known receptor *CX3CR1* and acts either as a soluble chemotactic factor or as a membrane-anchored adhesion molecule for circulating

leukocytes (37, 38), thereby modulating monocyte recruitment and adhesion to adipocytes (39, 40) and vascular endothelium (41). Although no data concerning *CX3CL1* expression in women with GDM are available so far, both animal and human studies suggest that *CX3CL1*–*CX3CR1* system is implicated in the pathogenesis of atherosclerosis, obesity, insulin resistance, and type 2 diabetes (38, 39, 40, 41).

We also showed that 3 months *postpartum*, women with the history of GDM had significantly higher expression of some genes involved in the activation of NF- κ B and upregulated in obesity and insulin resistance, in particular *IL1R1* and *IKBKB* (4, 7, 11). Interestingly, the expression of *SLC27A2* – a gene associated with lipid biosynthesis and fatty acid degradation, which may be an early marker of overweight development (42) – was also elevated in these patients compared with women with NGT.

This study also showed that the patients with NGT in the 24th–28th weeks of gestation, who later developed GDM, had significantly higher fasting glucose, insulin, HbA1c, and HOMA-IR, as well as lower ISSI2 and DIs, than had the patients who remained normoglycemic. Although the pathogenesis of GDM is still far from clear, it is believed that it results from a limitation in pancreatic β -cell reserve and an inadequate insulin secretion unable to meet increasing tissue insulin demands (43). As a decrease in β -cell function is to some degree masked by physiological insulin resistance during pregnancy, the DI has been developed to adjust for compensatory high insulin response to a decreased

Figure 1 Changes in PBMC gene expression during and after pregnancy in the patients with normal glucose tolerance (A, B and C) and those who were normoglycemic in the 2nd trimester but later developed GDM (D, E and F): white bars, 2nd trimester; gray bars, 3rd trimester; black bars, 3 months *postpartum*; data are shown as medians with interquartile ranges. Gene expression was normalized to β -actin and *GAPDH*. The differences are significant at * $P < 0.05$ and ** $P < 0.01$ by one-way ANOVA.

insulin sensitivity (31). As it was demonstrated by Qvigstad *et al.* (44), DI seems to be markedly lower in GDM subjects and this reduction may be noticeable at the beginning of the 2nd trimester when the patients are classified as glucose tolerant. In our study, the patients with both earlier and late onset of GDM also demonstrated a significant decrease in the surrogate markers of β -cell function, although an index of the early-phase insulin release (AUC_{Ins30}/AUC_{Glu30}) did not differ markedly between the women with and without GDM. The patients who developed GDM in the 3rd trimester also had significantly higher HOMA-IR, which emphasizes the role of increased insulin resistance against the background of enormous tissue insulin demands in the development of late-onset GDM. Our findings are consistent with the results of other studies (44, 45, 46) showing unchanged AUC_{Ins}/AUC_{Glu} index, but an inadequate insulin compensation for decreased insulin sensitivity in women with GDM. We should also mention that the number of patients with a normal OGTT result at the 1st visit, but later diagnosed as having GDM, was much higher than expected. Although this study was part of a large genetic research program and the group studied might not reflect an incidence of GDM in the general population, our results suggest that the number of women with late-onset GDM may be underestimated.

Our results were limited by the fact that we did not measure NF- κ B activity in PBMCs and that among all analyzed genes involved in NF- κ B signaling pathway, only increased TLR expressions seemed promising as a potential early marker of low-grade inflammation in pregnant women. However, the validity of this finding needs to be confirmed in larger studies with more statistical power and with the analysis of TLR protein expressions in PBMCs.

In conclusion, our results showed quite a different pattern of gene expression in PBMCs obtained from the patients who developed GDM before and after the 28th week of gestation. Furthermore, our findings suggest that an increase in *TLR2* expression, as well as higher fasting glucose and lower compensation for increased insulin resistance, may represent very early metabolic disturbances, which may be noticeable before the clinical onset of GDM.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

The study was supported by grant no. N N407 141937 from the State Committee for Scientific Research.

Author contribution statement

M Kuzmicki and B Telejko performed statistical analyses and wrote the manuscript; N Wawrusiewicz-Kurylonek, D Lipinska, J Pliszka, and J Wilk processed the samples, analyzed, and prepared the data; A Zielinska and J Skibicka prepared the data and performed statistical analyses; J Szamatowicz and A Kretowski designed the experiments and interpreted the data; and M Gorska contributed to the interpretation of the data and revised the article. All authors approved the final version of the manuscript.

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Received 30 July 2012

Revised version received 15 November 2012

Accepted 10 December 2012