Gestational diabetes mellitus is associated with increased leukocyte peroxisome proliferator-activated receptor γ expression

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Abstract

Introduction: Peroxisome proliferator-activated receptor γ (PPARγ) is a ligand-activated transcription factor of the nuclear receptor superfamily that is involved in lipid and carbohydrate metabolism as well as inflammation; thereby it participates in metabolic diseases including diabetes. Although PPARγ expression has been observed in different tissues of diabetic patients, its level in leukocytes from subjects affected by gestational diabetes mellitus (GDM) has not yet been reported. This study aimed to investigate leukocyte *PPARG* expression in GDM patients at 24–33 weeks of gestation and, in turn, to correlate these alterations with anthropometric and metabolic parameters of patients.

Material and methods: Leukocytes were isolated from the blood of normal glucose tolerant (NGT; $n = 34$) and GDM ($n = 77$) pregnant women between 24 and 33 weeks of gestation. Leukocyte *PPARG* mRNA expression was determined by semi-quantitative polymerase chain reaction. Univariate correlation analysis was performed to investigate associations between *PPARG* expression and clinical characteristics of patients.

Results: Leukocyte *PPARG* mRNA level was significantly higher in GDM than NGT women ($p < 0.05$). In the whole study group, *PPARG* expression positively correlated with plasma glucose concentrations at 1 h ($r = 0.222$, $p = 0.049$) and 2 h ($r = 0.315$, $p = 0.020$) of 75 g oral glucose tolerance test (OGTT), and negatively correlated with plasma HDL cholesterol concentration ($r = –0.351$, $p = 0.010$).

Conclusions: The correlation between leukocyte *PPARG* overexpression and hyperglycaemia suggests that *PPARG* mRNA expression in these cells might be up-regulated in high-glucose conditions in GDM patients at 24–33 weeks of gestation.

Key words: gestational diabetes mellitus, insulin resistance, PPARγ, type 2 diabetes mellitus.

Introduction

Gestational diabetes mellitus (GDM), defined as glucose intolerance with onset or first recognition during pregnancy [1], is currently the most common metabolic abnormality occurring during pregnancy, affecting from 1% to 14% of all pregnancies depending on ethnic group and the diagnostic test employed [2]. The GDM is linked to numerous and serious complications for both mother and newborn. In the short term, mothers

with GDM are at an increased risk of delivering a macrosomic infant and developing preeclampsia [3], whereas their offspring are prone to neonatal hypoglycaemia, hyperbilirubinaemia, hypocalcaemia, respiratory distress syndrome, and polycythemia [4]. In the long term, GDM women are at a substantially increased risk of developing type 2 diabetes mellitus (T2DM) and cardiovascular disease after pregnancy [5, 6], and their offspring are at risk for the development of obesity and abnormal glucose metabolism during childhood, adolescence, and adulthood [7].

The GDM shares with T2DM some metabolic abnormalities, including insulin resistance and β cell dysfunction. Although the precise mechanisms underlying the pathophysiology of GDM are not fully understood, several factors contributing to diabetic pregnancy have been identified so far. Among them, disturbances in the insulin signalling pathway [8, 9], altered plasma adipokine levels [10, 11] as well as inflammation [12] and oxidative stress, resulting from overproduction of free radicals and/or defects in the antioxidant defences [13, 14], have been found to associate with GDM.

Peroxisome proliferator-activated receptor γ (known as PPARγ or NR1C3) is a ligand-activated transcription factor with a distinct tissue distribution and different biological functions ranging from lipid and carbohydrate metabolism to immune/inflammatory responses, and control of cell proliferation/differentiation [15–17]. The PPARγ interacts with a wide spectrum of natural ligands such as long-chain polyunsaturated fatty acids, prostaglandin (PG)J2 derivatives, and oxidized fatty acids, as well as of synthetic ligands such as glitazones (also known as thiazolidinediones, TZDs) [18, 19]. From the mechanistic point of view, PPARγ activates transcription of their target genes as a heterodimer with the 9-*cis* retinoic acid-activated retinoid X receptors (RXRs), which bind to the PPAR response element (PPRE) sequences located within the promoters of target genes (Figure 1) [20–22]. In the absence of a ligand, the heterodimer is associated with a co-repressor that inhibits transcription through the recruitment of histone deacetylases (Figure 1 A). Ligand binding to the heterodimer causes a conformational change in the protein structure of PPARγ resulting in dissociation of the co-repressor and recruitment of the co-activator and, in turn, transcriptional activation of target genes (Figure 1 B) [23, 24].

A large body of evidence indicates that PPARγ is linked to T2DM since it participates in the regulation of glucose and lipid metabolism, adipocyte differentiation, and inflammation. In this respect, it has been shown that PPARγ modulates the expression of several adipocyte-specific genes involved in lipid synthesis and storage, insulin signalling, and adipokine production [25, 26] and, moreover, its activation by TZDs improves insulin sensitivity in insulin-resistant animal models and diabetic patients [27]. Several dominant-negative mutations in the human *PPARG* gene have also been found to cause partial lipodystrophy, increased insulin resistance, diabetes, and hypertension [28]. In addition to PPARγ function in glucose and lipid metabolism, this transcription factor has anti-inflammatory properties and its ligands have been demonstrated to suppress production of monocyte/macrophages inflammatory cytokines such as TNF-α, IL-6, and IL-1β through inhibiting the activity of transcription factors such as nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB), activator protein-1 (AP-1), and signal transducers and activators of transcription (STAT) [29, 30]. Although PPARγ is present in immune cells, including type B and T lymphocytes as well as monocytes/macrophages [31–33], its role in leukocytes from

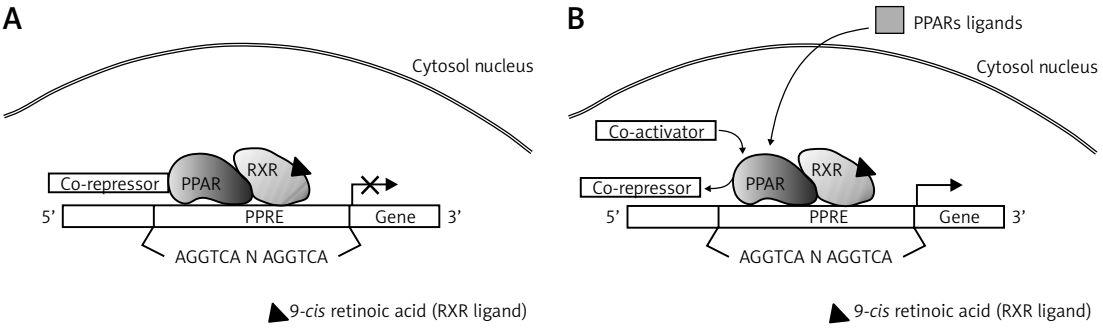

Figure 1. Transcriptional activity of PPARγ. The PPARγ regulates transcription of target genes as a heterodimer with the 9-*cis* retinoic acid-activated retinoid X receptors (RXRs), which bind to the PPAR response element (PPRE) sequences. The PPRE sequences are composed of two hexanucleotides (AGGTCA) separated by one nucleotide (this type of sequences is called DR-1) and located within the promoters of PPARγ target genes. **A** – In the absence of the PPARγ ligand, the heterodimer is associated with a co-repressor that inhibits transcription through the recruitment of histone deacetylases (HDACs). **B** – Ligand binding to the heterodimer causes a conformational change in the protein structure of PPARγ resulting in dissociation of the co-repressor and recruitment of the co-activator and, in turn, transcriptional activation of its target genes

GDM women remains largely unknown. Therefore, the objective of the present study was to investigate leukocyte *PPARG* mRNA expression in GDM and normal glucose tolerant (NGT) pregnant women at 24–33 weeks of gestation, and, in turn, to determine correlations between alterations in *PPARG* expression and anthropometric and metabolic parameters of patients.

Material and methods

Subject recruitment

A total of 111 Caucasian pregnant women between 24 and 33 weeks of gestation were recruited for this study at the Polish Mother's Memorial Hospital Research Institute in Lodz, Poland. Among them, 77 subjects had GDM and 34 had NGT. The 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 criteria set by WHO (modified) [34]. The inclusion criteria were the following: Caucasian ethnic background, age range between 18 and 40 years, no family history of diabetes in first-degree relatives, no GDM in a previous pregnancy, absence of any form of pre-pregnancy diabetes, absence of concomitant systemic disease (chronic or acute or infectious), not taking insulin or oral hypoglycaemic medications, no control by diet and exercise before the overnight fast.

All clinical investigations were conducted in accordance with the guidelines in the Declaration of Helsinki and were approved by the Bioethics Committee for Research on Humans at the Medical University in Lodz (No. RNN/154/09/KB from 21.04.2009). Informed consent was obtained from all participating subjects.

Anthropometric and biochemical measurements

Patients gave information on their maternal age and pre-pregnancy weight. The weight and height of patients during the third trimester of pregnancy were measured using standard methods, and both body weight gain and pre-pregnancy body mass index (BMI) expressed as weight before pregnancy divided by height squared (kg/m^2) were calculated.

Blood samples were drawn after a 12 h overnight fast. Serum triglycerides (TGs), and HDL and LDL cholesterol levels were determined by enzymatic colorimetric methods with 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. Concentrations of CRP were determined by turbidimetric assay using the cassette COBAS INTEGRA C-Reactive Protein (Latex) according to the manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany).

The above-mentioned biochemical assays were carried out with a COBAS INTEGRA analyzer (Roche, SA). Plasma insulin was quantified using Elecsys insulin assay (Roche Diagnostics GmbH, Mannheim, Germany). Insulin resistance and β-cell function were estimated by homeostasis model assessment (HOMA-IR and HOMA-B, respectively [35]: HOMA-IR = [fasting insulin (μU/ml) × fasting glucose (mg/dl)]/405 and HOMA-B = [360 × fasting insulin (μU/ml)]/[fasting glucose (mg/dl) – 63]. To assess insulin sensitivity, the quantitative insulin sensitivity check index (QUICKI-IS) was calculated as follows: QUICKI = 1/[(log(I0) + log(G0), where I0 is the fasting plasma insulin (μU/ml) and G0 is the fasting blood glucose concentration (mg/dl) [36].

Leukocytes separation

Fresh anticoagulated blood samples (10 ml), withdrawn from each patient, were centrifuged at 3000 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 erythrocyte lysis in an ice bath, aliquots were centrifuged at 4000 rpm for 10 min at 4°C, and supernatants were discarded. The pellets containing leukocytes were washed twice with phosphate-buffered saline (PBS).

Gene expression assay

Total RNA was extracted from leukocytes using a commercially available acid-phenol reagent (TriReagent, Sigma-Aldrich, USA) according to the manufacturer's procedure. RNA concentration and its purity were assessed by a LAMBDA 25 UV spectrophotometer (PerkinElmer, UK) at UV_{260} and $UV_{260/280}$, respectively. Samples were stored at –80°C until use.

Reverse transcription was performed for 1 h at 42°C in a total volume of 20 μl with 4 μg of total RNA per sample, 1.0 mM deoxynucleotide triphosphates (dNTPs), reverse transcriptase buffer (5x), 20 U of RNase inhibitor, 0.5 μg $(dT)_{18}$ primer and 200 U of RevertAid™ H Minus M-MuLV reverse transcriptase (Fermentas, Lithuania). The resulting cDNA was subjected to polymerase chain reaction (PCR).

The PCR amplification of PPARγ cDNA was performed in a 20 μl reaction mixture containing 1 U of polymerase *Taq*, 0.2 mM dNTPs, 1 μM each primer, *Taq* polymerase buffer (10x), and 1 μl of RT cDNA product. Amplification of PPARγ cDNA (471-bp fragment) was performed for 29 cycles (denaturation at 94°C for 30 s, annealing at 56°C for 15 s, and

extension at 72°C for 30 s) by using forward primer: 5' GTGCAGGAGATCACAGAGTATG 3' and reverse primer 5' GTGTTCCGTGACAATCTGTCTG 3'. Each PCR reaction was run in duplicate on a Thermocycler TPersonal 48 (Biometra, Germany). The amplification products were analysed on 1.2% agarose gels stained with ethidium bromide, and quantified by densitometry with the Gelix One 220 program (Biostep GmbH, Germany). The housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), was used to normalize reactions and for calculations of the *PPARG*/*GAPDH* ratio.

Statistical analysis

Results are shown as mean value ± standard deviation (SD). Differences between the two groups, including clinical characteristics and expression data, were assessed by Wilcoxon's test. Relationships between leukocyte *PPARG* expression and each anthropometric and metabolic parameter value were determined by the nonparametric test of Spearman's rank correlation coefficient. Value of $p < 0.05$ was considered as statistically significant in all analyses. Statistical analysis was performed using a statistical software package (Statistica version 8.0, StatSoft, Poland, license no. AXAP911E504325AR-K).

Results

Characteristics of NGT and GDM pregnant subjects

The clinical characteristics of 77 women with GDM and 34 NGT pregnant controls, including their anthropometric and metabolic parameters, are shown in Table I. Plasma glucose concentrations at 0 h, 1 h, and 2 h of 75 g OGTT as well as the HbA_{1c} levels were, as expected, significantly higher in the GDM patients compared with the NGT pregnant women ($p < 0.05$). There were no statistically significant differences between studied groups regarding plasma lipids, CRP, and insulin resistance/sensitivity ($p > 0.05$).

Leukocyte *PPARG* mRNA expression and its correlations with clinical characteristics of patients

We then examined leukocyte *PPARG* mRNA expression in the GDM versus NGT pregnant women. For this purpose, total RNA was extracted from

Table I. Clinical characteristics of all participants involved in the study

Parameter	NGT group (n = 34)	GDM group (n = 77)	Value of p
Age [years]	29.0 ±4.4	29.8 ±4.6	0.388
Pre-pregnancy BMI [kg/m^2]	23.7 ±3.9	25.1 ±6.0	0.458
Pregnancy BMI [kg/m^2]	27.4 ±3.8	28.8 ±5.9	0.230
Body weight gain [kg]	10.7 ±5.6	9.9 ±5.9	0.402
Total cholesterol [mg/dl]	257.9 ±47.7	269.7 ±39.5	0.326
TGs [mg/dl]	224.0 ±65.8	262.6 ±83.5	0.093
HDL-cholesterol [mg/dl]	78.3 ±13.0	70.2 ±23.3	0.147
LDL-cholesterol [mg/dl]	145.4 ±68.3	156.0 ±51.5	0.170
HbA_{1c} (%)	5.2 ±0.4	5.4 ±0.4	0.036*
Glucose 0 h [mg/dl]	77.0 ±6.8	90.9 ±17.9	< 0.001*
Glucose 1 h [mg/dl]	152.7 ±33.9	189.4 ±32.7	< 0.001*
Glucose 2 h [mg/dl]	115.6 ±18.8	163.9 ±21.2	< 0.001*
Insulin [µlU/ml]	5.2 ±3.9	3.5 ±2.9	0.089
HOMA-IR	1.0 ±0.8	1.6 ±1.5	0.211
HOMA-B	120.4 ±94.4	99.9 ±76.0	0.573
QUICKI-IS	0.4 ±0.1	0.4 ±0.2	0.966
CRP [mg/l]	4.2 ±3.4	3.7 ±2.9	0.805

*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, QUICKI-IS – quantitative insulin sensitivity check index, TG – triglycerides. Data represent the mean ± SD. *Values of p < 0.05 as compared to control as assessed by Wilcoxon's test.*

their leukocytes, and *PPARG* expression was measured by a semi-quantitative RT-PCR as described in Material and methods. The amplification products of *PPARG* (upper gel) and of the control *GAPDH* (lower gel) were viewed on an ethidium bromide stained electrophoresis gel as shown in Figure 2 A. The *PPARG* mRNA expression, calculated as the *PPARG*/*GAPDH* ratio, showed a significant increase in the GDM group compared with the control group (0.72 ±0.40 vs. 0.52 ±0.19, $p < 0.05$) (Figure 2 B).

Univariate correlation analysis using the Spearman rank order correlation coefficient was performed to investigate whether leukocyte *PPARG* expression in the GDM women was associated with their clinical characteristics given in Table I. The *PPARG* mRNA expression positively correlated with plasma glucose concentrations at 1 h ($r = 0.222$, $p = 0.049$) and 2 h ($r = 0.315$, $p = 0.020$) of 75 g OGTT in the whole study group (Table II, Figure 3). Moreover, there was a negative correlation between leukocyte *PPARG* mRNA expression and plasma HDL cholesterol level ($r = -0.351$, $p = 0.010$) (Table II). No correlations were observed between leukocyte *PPARG* expression and other clinical parameters of patients ($p > 0.05$).

Discussion

The role of PPARγ in normal placental development and trophoblast differentiation and invasion in gestational tissues has been established [37, 38]. However, there is very limited knowledge about the significance of this transcription factor in human diabetic pregnancy, partially due to the poor availability of metabolic tissues from pregnant women. Therefore, to circumvent the invasive and non-ethical procedures involved in taking metabolic tissue samples from pregnant women, we used their leukocytes (including granulocytes, monocytes, and lymphocytes) as an alternative experimental cell model for investigating changes in *PPARG* mRNA expression. It has been reported that PPARγ is expressed in various cell types of the immune system, such as macrophages [31], B lymphocytes [32] and T lymphocytes [33]. Since PPARγ is involved in glucose and lipid metabolism in T2DM, it could be considered as an important molecular factor participating in these metabolic pathways during diabetic pregnancy. Indeed, decreased *PPARG* expression has been observed in subcutaneous adipose tissue from obese GDM women, suggesting that this change might be part of the molecular mechanism to accelerate fat catabolism and thereby ensure fetal nutrition in

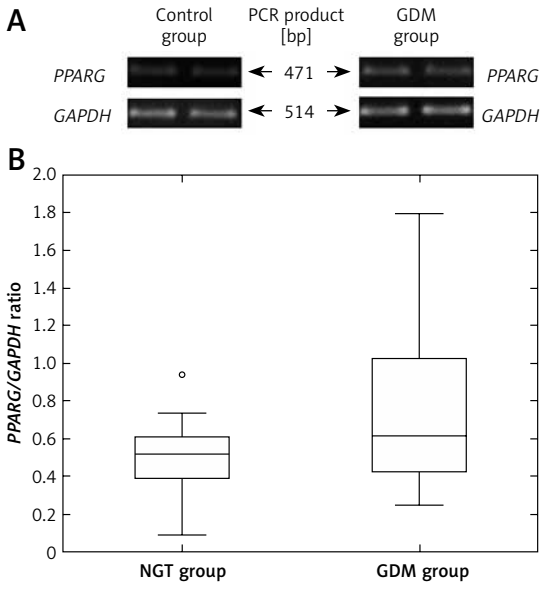

Figure 2. Leukocyte PPARG expression in the NGT and GDM pregnant subjects. **A**. Representative agarose gel electrophoresis for the PCR products of PPARG (upper gel) and the control GAPDH (lower gel). **B**. Box-plot graphs in leukocyte PPARG mRNA expression normalized to GAPDH in the NGT ($n = 34$) and GDM ($n = 77$) pregnant women. Median values are presented. Differences between the groups were analysed by Wilcoxon's test. *Value of $p < 0.05$

Table II. Univariate correlations between leukocyte *PPARG* mRNA expression and clinical characteristics of patients in the whole study group

Parameter	Value	
	r	*p*
Age [years]	–0.093	0.505
Pre-pregnancy BMI [kg/m^2]	0.183	0.069
Pregnancy BMI [kg/m^2]	0.186	0.183
Body weight gain [kg]	–0.185	0.064
Total cholesterol [mg/dl]	–0.007	0.961
TGs [mg/dl]	0.216	0.116
HDL cholesterol [mg/dl]	–0.351	0.010*
LDL cholesterol [mg/dl]	–0.030	0.827
HbA_{1c} (%)	–0.011	0.940
Glucose 0 h [mg/dl]	0.168	0.108
Glucose 1 h [mg/dl]	0.222	0.049*
Glucose 2 h [mg/dl]	0.315	0.020*
Insulin [μlU/ml]	–0.063	0.606
HOMA-IR	0.115	0.907
HOMA-B	–0.061	0.647
QUICKI-IS	–0.003	0.977
CRP [mg/l]	–0.037	0.739

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

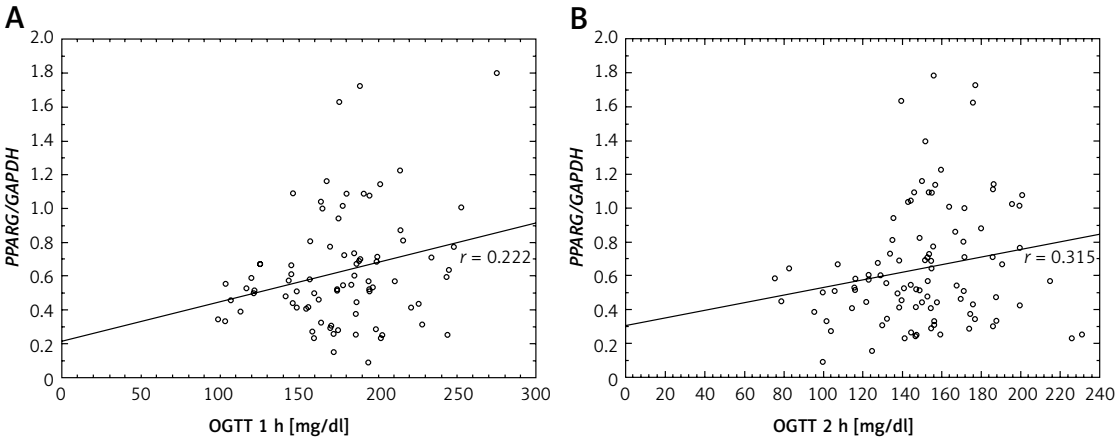

Figure 3. Positive correlations between leukocyte PPARG mRNA expression and plasma glucose concentrations at 1 h (**A**) and 2 h (**B**) of 75g OGTT ($p < 0.05$) in the whole study group

late gestation [39]. These findings contradict our results that showed significantly higher leukocyte *PPARG* mRNA expression in the GDM than NGT pregnant women in late gestation. These discrepancies may partly result from tissue-specific differences in *PPARG* expression (adipocytes versus leukocytes) as well as differences in patient selection criteria (obese GDM women versus non-obese GDM) [39]. In the current study, no differences were observed in adiposity (i.e. pre-pregnancy and pregnancy BMI, body weight gain) between the GDM and NGT groups (Table I).

Next, we found that leukocyte *PPARG* mRNA expression was positively correlated with plasma glucose concentrations at 1 h and 2 h of 75 g OGTT in the whole study group, suggesting that hyperglycaemic conditions might up-regulate maternal leukocyte *PPARG* expression during the diabetic state. In accordance with this hypothesis, a significant increase in PPARγ expression has been demonstrated in placentas of diabetic pregnant mice with severe hyperglycaemia [40]. However, since there are also reports showing down-regulation of placenta PPARγ expression under mild hyperglycaemia in streptozotocin-induced diabetic rats and in gestational diabetic women [41, 42], further studies on the determination of PPARγ expression under different degrees of hyperglycaemic conditions in different species and cells should be performed to elucidate these discrepancies. The study by Panchapakesan *et al.* [43] demonstrating PPARγ up-regulation in the proximal tubular cell line HK-2 as a consequence of exposure to high glucose supports this line of research.

Although the reasons for elevated leukocyte *PPARG* expression under high-glucose conditions in the GDM patients are unclear, we cannot exclude the possibility that this change might protect against hyperglycaemia-induced oxidative stress, which appears when there is an imbalance between generation of reactive oxygen species (ROS) and its clearance by the antioxidant defences [44]. It is now well established that under hyperglycaemic conditions in diabetes, ROS are produced during glucose autoxidation (generation of hydroxyl radicals), the polyol pathway (an increase in superoxide anion radical level), and formation of advanced glycation end products (AGEs) as a consequence of a non-enzymatic glucose reaction with proteins [45]. In recent years, several studies have observed the association between GDM and an increased level of oxidative stress [13, 14]. Additionally, a growing body of evidence indicates that TZD PPARγ agonists exert anti-oxidative effects in various cells, including leukocytes [46, 47]. Taking into account these findings, it is reasonable to assume that leukocyte *PPARG* overexpression under hyperglycaemia in the GDM women might be a regulatory adaptation of the maternal organism to increased oxidative stress during diabetic pregnancy. However, further studies are clearly needed to investigate whether elevated leukocyte *PPARG* expression under high-glucose conditions could affect the expression of genes involved in oxidative stress in these cells.

The GDM affects lipid and lipoprotein metabolism; however, there are conflicting results regarding alterations in circulating lipoprotein profiles between the GDM and NGT women [48]. Among them, increased triglycerides and decreased HDL cholesterol are often detected in GDM [49]. With respect to HDL cholesterol, several studies have revealed that its low plasma level in both non-diabetic and diabetic subjects constitutes an independent risk factor for coronary heart disease [50–53]. Although current knowledge concerning the precise mechanisms underlying a reduced HDL cholesterol concentration in diabetes is far from complete, there is evidence that the impairment of reverse cholesterol transport (RCT), i.e. the process by which cholesterol is removed from peripheral cells and transported to the liver for metabolism

and excretion in the bile, is related, at least in part, to this abnormality [54]. The PPARγ has been implicated in RCT as a key regulator of expression of the gene encoding the membrane-associated ATP binding cassette transporter-A1 (ABCA1) [55]. It has been shown that activation of the liver X receptor-α (LXRα) by PPARγ upregulates *ABCA1* expression, resulting in an increase of apo-A1-mediated cholesterol efflux from macrophages [55, 56]. These data suggest that PPARγ could mediate anti-atherogenic effects by enhancing cholesterol efflux from macrophages by ABCA1. In the light of these findings, the inverse correlation between leukocyte *PPARG* mRNA expression and plasma HDL-cholesterol level found in the present study was rather surprising. The reasons for this association are currently unclear, but we cannot rule out the possibility out that increased *PPARG* expression could be linked to increased HDL removal in terms of hepatic cholesterol metabolism and biliary excretion, resulting in a low plasma HDL cholesterol. However, to explore this highly speculative hypothesis, further complex studies are required in this field. On the other hand, a positive correlation between leukocyte expression of *PPARG* and *ABCA1* in GDM subjects also remains to be proven.

In conclusion, we demonstrated for the first time that leukocyte *PPARG* mRNA expression was significantly elevated in the GDM women between 24 and 33 weeks of gestation and it was positively associated with hyperglycaemia, implying that *PPARG* expression in these cells might be up-regulated in high-glucose conditions. Surprisingly, our data also showed an inverse correlation between *PPARG* mRNA expression and plasma HDL cholesterol concentration. Although more studies performed on a larger population of women are required to confirm our results, these findings serve as take-off data for future research to clarify the role of leukocyte PPARγ in GDM and to elucidate the cascade intracellular events linking its overexpression with hyperglycaemia as well as with low plasma HDL cholesterol level in GDM women. Furthermore, leukocytes seem to be a convenient experimental cellular model for studying GDM since there is a serious ethical problem linked to any invasive method used to obtain metabolic tissue samples from pregnant women.

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Conflict of interest

The authors declare no conflict of interest.

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