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Running head: MicroRNAs - redox status indicators in GDM

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Abstract

Background: Gestational diabetes (GDM) is the most common pregnancy-related metabolic disorder and a major risk factor for both mother and a child, during pregnancy and after delivery. Impairment of the anti-oxidative system acts as the driving force of the devastating consequences in GDM, while microRNA molecules are affected by disturbances related to (glyco)oxidative stress ((g)OS) and emerged as potential sensors and effectors of (g)OS-associated mechanisms. Aims: The aim of the present study was to evaluate the potential of OS-related microRNAs miR-27a, miR-222 and miR-340 from peripheral blood mononuclear cells (PBMCs) to serve as OS indicators of in GDM.

Methods and Results: MicroRNA quantification was conducted in samples of patients with GDM and normoglycemic controls (n=45 each). Levels of expression were tested for correlations with the activities of glutathione reductase (GR), superoxide dismutase (SOD) and catalase (CAT) and serum thiol content. MiR-27a demonstrated correlation with the expression of other two microRNAs. GR activity during pregnancy weeks 24-28 was increased in GDM, while thiol content showed an opposite direction of change. Similar results were acquired when comparing GDM patients with and without adverse pregnancy outcomes. The expression of tested microRNAs was positively correlated with the activity of GR in GDM group, while the correlation of the expression of miR-27a-3p and miR-340-5p with SOD activity and blood thiol content was negative.

Conclusions: Our findings support the relevance of redox dysregulation in mid-pregnancy for developing obstetric and neonatal complications. The results also qualify miR-27a-3p, miR-222-3p and miR-340-5p for redox status indicators in GDM.

Keywords: microRNA; pregnancy; glutathione reductase; thiol content; Superoxide dismutase; zinc.

Abbreviations

α2M - alpha-2-macroglobulin;

ALT – alanine aminotransferase;

AST – aspartate aminotransferase;

AUC - area under the curve;

BMI - body mass index;

CAT – catalase;

CRP – C-reactive protein;

DTNB - 2 mM 5,5’ – dithiobis (2-nitrobenzoic acid);

EDTA - ethylenediaminetetraacetic acid;

GDM – gestational diabetes;

(g)OS - (glyco)oxidative stress;

GR - glutathione reductase;

HbA1c – glycated haemoglobin;

HDL - high-density lipoprotein;

Hgb – hemoglobin;

HOMA-β - Homeostatic Model Assessment of Beta Cell Function;

HOMA-IR - homeostatic model assessment of insulin resistance;

IADPSG - International Association of Diabetes and Pregnancy Study Groups;

IFM – inflammation;

LDL - low-density lipoprotein;

LSM - lymphocyte separation medium;

NADPH - reduced nicotinamide adenine dinucleotide phosphate;

NBT - nitroblue tetrazolium;

OGC NF - University Clinic for Gynecology and Obstetrics “Narodni Front”;

OGTT – oral glucose tolerance test;

PBMCs - peripheral blood mononuclear cells;

PBS – phosphate buffered saline;

qPCR – quantitative polymerase chain reaction;

ROC - receiver-operating characteristic curve;

SD – standard deviation;

SOD – superoxide dismutase;

TG – triglycerides;

TIBC – total iron-binding capacity.

1. Introduction

Gestational diabetes mellitus (GDM) is generally defined as any degree of glucose intolerance diagnosed in pregnancy without known pre-existing diabetes [1]. It is one of the most common pregnancy complications and the most frequent metabolic disorder occurring during pregnancy, with the estimated average worldwide prevalence of around 14% of pregnancies [2]. These statistics are especially raising concern when considering an increasing incidence of GDM due to sedentary behavior and eating habits associated with modern lifestyle, as well the associated health risks affecting both mothers with GDM and their offspring. Namely, GDM frequently associates with severe obstetric and immediate neonatal complications, such as maternal preeclampsia, neonatal macrosomia, hypoglycemia, shoulder dystocia, disorders of amniotic fluid, cesarean delivery, preterm labor and fetal mortality. In the long-term, it also contributes to the development of metabolic disorders, associated cardiovascular disease and obesity in mothers and their children affected by diabetic pregnancy [1].

Research efforts targeted on molecular mechanisms, GDM etiology and the molecular basis of accompanying complications are mostly focused on the effect of hormonal (dys)regulation, insulin resistance and hyperglycemia [3]. On the other hand, accumulating evidence points out the importance of dysregulation in the immune system, inflammatory activation and the impairment of the anti-oxidative system as the driving force of

devastating consequences in GDM [3-5]. These processes and mechanisms are interconnected, systemic and with a great damaging potential, partially due to a severe pathogenic impact on fetal and extraembryonic membrane development, as well as on vascular function [5,6]. Therefore, investigation of molecular indicators of (glyco)oxidative stress ((g)OS) and the associated low-level inflammation (IFM) could contribute to better understanding of the major mechanism of GDM onset and the development of severe complications. Furthermore, reliable molecular indicators with such properties could aid in GDM risk assessment, diagnostic procedure, or even in the prediction or early detection of GDM-related complications and adverse outcomes.

Major disturbances in microRNA-related mechanisms were reported to associate with the continuous presence of (g)OS/IFM. Therefore, these small regulatory RNA molecules with distinctive chemical properties which make them suitable as potential biomarkers, emerged as potential sensors and effectors of (g)OS/IFM-associated mechanisms [7]. Characteristic GDM-related (g)OS/IFM signature could be associated with a specific profile of microRNA expression, enabling the usage of a defined microRNA panel for the discrimination of GDM vs. normoglycemic pregnancy, monitoring of the associated (g)OS/IFM as well as for GDM outcome prediction.

In our previous study, we evaluated the GDM-relevant biomarker properties of a set of microRNAs (miR-27a-3p, miR-222-3p, miR-340-5p) with the defined functions related to glucose metabolism and/or estrogen

secretion [8]. The expression of selected microRNAs in peripheral blood mononuclear cells (PBMCs) demonstrated diagnostic significance (in the case of miR-27a-3p), as well as relevance for monitoring the metabolic status of pregnant women [8]. Since all three microRNAs demonstrate an involvement in the regulation of antioxidative system and/or inflammation [9-14], in an extension of this previous research, we aimed at investigating their role as indicators of (g)OS/IFM in GDM, as well as their potential significance for the prediction of adverse outcomes in both normoglycemic pregnancies and GDM. We selected PBMCs as a relevant biological source of GDM-related microRNAs based on our previous findings [8], as well as due to their role in the immune response, direct exposure to reactive species from the circulation and relevance for inflammatory status assessment in obesity and diabetic disorders [15,16]. Additionally, we intended to evaluate the prognostic significance of the selected microRNAs, as well as biochemical parameters related to the redox status, by assessing their levels at 24-30 gestational weeks and by conducting a follow-up at delivery, which enables the evaluation of obstetric/neonatal complications.

2. Material and Methods

2.1. Recruitment of participants, data acquisition and sample collection

45 patients diagnosed with GDM and 45 healthy pregnant normoglycemic women were recruited in the period 2019 - 2021 at the

University Clinic for Gynecology and Obstetrics “Narodni Front” (OGC NF), Belgrade, Serbia. Potentially eligible participants gave written informed consent for participation, while the study was approved by the Ethics committee of OGC NF (Approval no. 05006-2019-4925) and conducted in accordance with the Helsinki Declaration of 1975. Since the present study is an extension of a previous analysis on the biomarker properties of miR-27a, miR-222 and miR-340 in GDM, the participant recruitment procedure, selection, sampling and data acquisition were described in detail in our previous publication [8].

The results of the 75 g oral glucose tolerance test (OGTT) were used for establishing GDM diagnosis according to the criteria proposed by the International Association of Diabetes and Pregnancy Study Groups (IADPSG) [17]. Pre-determined inclusion criteria for consecutively recruited study participants were the following: availability of 75 g OGTT results, diagnosis according to IADPSG criteria, pregnancy week 24-30 and singleton pregnancy. The exclusion criteria comprised history of metabolic, autoimmune, psychiatric, malignant diseases and other unrelated pregnancy pathologies. Peripheral blood of GDM patients and normoglycemic healthy controls was sampled in vacutainers supplied with EDTA as an anticoagulant, as well as in serum tubes, after overnight fasting.

Patients provided relevant information for clinical database as part of a survey, which included questions about basic clinical and epidemiological

data, including age, gestational age, smoking status, anthropomorphic characteristics, personal anamnesis and comorbidities, therapies, dietary supplementation usage, family history of diabetes. Other relevant data were obtained from medical records of consenting patients: observational, biochemical and hematological parameters related to glucose tolerance (OGTT results, fasting glucose concentrations at sampling, glycated hemoglobin - HbA1c, insulin), lipid status (concentrations of triglycerides, cholesterol, HDL and LDL), metabolism of redox-active and inflammation-related metal ions and corresponding binding proteins (concentrations of hemoglobin, iron, copper, zinc, ferritin, transferrin, total iron-binding capacity – TIBC, alpha-2-macroglobulin (α_2M), complete blood cell count), kidney and liver function (activities of transaminases, serum concentrations of albumin, total serum proteins, urea, creatinine and uric acid), inflammation (CRP, sedimentation rate, leucocyte count, fibrinogen), data on pregnancy/delivery complications and neonates' characteristics (gestational age at birth, neonates' weight and length, Apgar score – 1' and 5'). In both GDM patients and controls, adverse pregnancy outcomes were defined as spontaneous preterm labor, disorders of fetal growth (macrosomia or intrauterine fetal growth restriction), shoulder dystocia, oligohydramnios or polyhydramnios, and gestational hypertension.

2.2. Blood fractioning

After transportation in a cold chain regime, blood samples with EDTA were centrifuged at 400 g at 4 ^{o}C for 10 min, plasma fraction was removed,

buffy coats were collected, and the remaining erythrocytes were washed twice with saline at 3000 g and stored at -80°C. Each buffy coat was diluted with 10 mM phosphate buffered saline (PBS) pH 7.4 and carefully layered onto Lymphocyte separation medium (LSM, 1.077 g/ml, Capricorn Scientific, Ebsdorfergrund, Germany). Separation of PBMCs from other blood constituents was performed according to the adjusted version of manufacturer's instructions: centrifugation of diluted buffy coats layered on LSM was performed at 700 g with slow acceleration and deceleration brakes off (18-21 °C, 30 min) (Centrifuge 5804R, Eppendorf, Hamburg, Germany), the layer of PBMCs was collected, washed twice with PBS at 100 g (room temperature, 10 min) and once with Erythrocyte lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) (room temperature, 10 min). The resulting pellet was completely dissolved in TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) and stored at -80°C.

Serum sampling tubes were left at room temperature for 45 min in order to allow blood coagulation, a centrifugation step at 3000 g (10 min) was performed, serum samples were collected, aliquoted and stored immediately at -80°C.

2.3. Hematological and basic biochemical analyses

Hematological analyzer Cell-Dyn Emerald (Abbott Diagnostics, Chicago, USA) was used for assessing standard hematological parameters. Validated commercial kits and the corresponding instrumentation (Alinity I analyzer, Abbott Diagnostics, Chicago, USA; biochemical analyzer

Bosystems A25, Biosystems, Spain) were used for evaluating basic biochemical indicators of glucose and lipid status, liver and kidney function, and inflammation. Radioimmunoassay (RIA INSULIN (PEG), INEP, Belgrade, Serbia) was employed for measuring insulin concentration. Homeostatic model assessment for insulin resistance (HOMA-IR) was calculated based on the following equation: (fasting insulin × fasting glucose)/22.5. Additionally, Homeostatic Model Assessment of Beta Cell Function (HOMA-β) was calculated as (20 × fasting insulin)/(fasting glucose - 3.5). The assessment of both indices was based on the insulin concentration expressed in mU/L and fasting glucose values in mmol/L. A colorimetric assay based on bromo-PAPS (Biosystems, Spain) was employed for the evaluation of zinc concentration in serum, while copper concentration was assessed by bathocuproine assay (Sigma-Aldrich, USA). The concentration of α_2M in serum samples was determined by enzyme-linked immunosorbent assay (FineTest, Wuhan, China).

2.4. Measurement of the activity of antioxidant enzymes

Haemolysates were prepared by the addition of equal volume of sterile cold water to stored erythrocytes, centrifugation at 4500 rpm (15 min at 4^{o}C) (Centrifuge 5804R, Eppendorf, Hamburg, Germany) and isolation of the supernatant which was flash frozen in liquid nitrogen and stored at -80^{o}C. The activity of superoxide dismutase (SOD) was assessed spectrophotometrically according to the method described by Beauchamp and Fridovich [18]. Measurements were conducted in a 96-well plate, with

each well containing 175 µL of potassium phosphate buffer with 1 mM EDTA (100 mM, pH 7.5), 10 µL of methionine (13 µM), 8 µL of nitroblue tetrazolium (NBT) (75 µM), 6 µL of haemolysate thawed on ice and 1 µL of riboflavin (2 µM). Plates were incubated under the UV light for 30 min and each analysis was performed in triplicate, by measuring the absorbance at 560 nm (Epoch microplate spectrophotometer, BioTek Instruments – Agilent, Santa Clara, CA, USA) and by calculating ΔA (A_{blank} - A_{sample}). One IU of SOD activity was defined as a 50% inhibition of the SOD activity, while specific activity of SOD in tested samples was presented as IU/mg of haemoglobin.

The activity of glutathione reductase (GR) was measured spectrophotometrically according to the method described by Glatzle et al. [19]. Assay was performed in a 96-well plate format, with each well containing a reaction mixture of 60 µL of potassium phosphate buffer with 1 mM EDTA (100 mM), 10 µL of oxidized glutathione (2 mM), 10 µL of EDTA (0.5 M), 180 µL of sterile H_2O and 10 µL of the reduced nicotinamide adenine dinucleotide phosphate (NADPH) (1 mM) with added 20 µL of haemolysate (10x diluted in potassium phosphate buffer). The absorbance at 340 nm was recorded in triplicate reactions for 150 seconds by Epoch microplate spectrophotometer (BioTek Instruments – Agilent, Santa Clara, CA, USA). The activity of GR was calculated based on the extinction coefficient of NADPH at 340 nm, sample volume and a dilution factor, while specific activity of GR was expressed as nmol $NADP^+$/min/g of haemoglobin.

The activity of catalase (CAT) in blood samples was assayed spectrophotometrically according to a method described by Claiborn [20]. The absorbance at 240 nm was measured (each sample in triplicate) by a spectrophotometer (UV-1900i, Shimadzu, Kyoto, Japan) immediately after the addition of 22.5 µL of haemolysate sample to a mixture of 202.5 µL of potassium phosphate buffer with 1 mM EDTA (100 mM) and 22.5 µL of 50 mM H_2O_2 in a quartz cuvette. The activity of CAT was calculated based on the extinction coefficient of H_2O_2 at 240 nm, sample volume and a dilution factor, while the specific activity of CAT was expressed as µmol of the reduced H_2O_2/min/g of haemoglobin.

2.5. Serum thiol content

The thiol content of serum samples was measured spectrophotometrically in triplicate by Ellman's assay [21] in a 96-well plate format. Serum samples (30 µL) were thoroughly mixed with equal volumes of Ellman's reagent (2 mM 5,5' – dithiobis (2-nitrobenzoic acid) - DTNB) and 1 M Tris buffer (pH 8.0) and made up to 300 µL with sterile H_2O. Absorbance was measured at 412 nm (Epoch microplate spectrophotometer, BioTek Instruments – Agilent, Santa Clara, CA, USA) after a 30 min incubation against the sample blank. The thiol group content of serum samples was calculated based on the extinction coefficient value of the reagent.

2.6. RNA extraction and microRNA quantification

As previously described in detail in Radojičić et al. [8], total RNA was extracted from PBMCs lysed in TRIzol (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instruction. The remaining DNA contamination was removed from the extracted RNA by DNase treatment (Amplification grade DNase I, Sigma-Aldrich, Burlington, MA, USA) conducted according to the protocol recommended by the manufacturer. Reverse transcription (RT) was coupled with a DNAse treatment, and performed by incubating a reaction mixture of 20 µl (100 U of RevertAid Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA), 1X Reaction Buffer, 0.5 mM dNTPs, 250 nM stem-loop primers (Microsynth, Balgach, Switzerland)) at 16 ºC for 30 min, at 42 ºC for 30 min and at 85 ºC for 5 min. Diluted cDNA (30x) was used in a qPCR reaction mixtures containing SYBR™ Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), DNase-free water and 0.5 µM primers (Microsynth, Balgach, Switzerland). Primer sequences for both RT and qPCR are listed in previous publication of Radojičić et al. [8]. Temperature profile of qPCR reaction performed in a Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) was the following: 95 ºC for 10 min and 40 cycles of denaturation at 95 ºC for 15 sec and primer annealing/elongation at 60 ºC for 1 min. Applied Biosystems 7500 Software v2.3 (Thermo Fisher Scientific, Waltham, MA, USA) was used for the analysis of qPCR results and calculation of Ct values. RNU6-1was used as an internal reference, while relative expression was evaluated by using a

delta-delta Ct (ΔΔCt) method and $2^{-\Delta\Delta Ct}$ values were used for assessing the fold-change between GDM group and controls.

2.7. Statistical analysis

Statistical analysis of clinical data and microRNA quantification results was performed by using software JASP 0.19.0. (https://jasp-stats.org/). Normality of the distribution of results was assessed by employing Kolmogorov-Smirnov test and by the visual inspection of distribution graphs, while the equality of variances in study groups was assessed by F-test. Comparisons of normally distributed results (continuous variables) was conducted by two-tailed Student's T-test, while in cases of significant deviations from the normal distribution, Mann–Whitney U test was employed. The distributions of categorical variables were compared by χ2 test. *P* value of 0.05 was considered as a threshold for statistical significance. In order to evaluate the diagnostic/prognostic potential of tested microRNAs, receiver-operating characteristic (ROC) curves were plotted (ROC plotter software, https://www.rocplot.org/) [22] and the results were expressed as area under the curve (AUC) and the corresponding *P* values. Potential correlation between the expression of analyzed microRNAs and the values of biochemical, hematological and redox status-related parameters was evaluated based on the values of Pearson correlation coefficients (r) and the corresponding *P* values.

3. Results

3.1. Basic characteristics of study participants and microRNA expression levels

Basic information on anthropometric, clinical, biochemical and hematological characteristics of patients diagnosed with GDM and healthy normoglycemic controls are presented in Table 1. There were no statistically significant differences between groups regarding the age of pregnant women, gestational age at sampling and smoking status. On the other hand, patients with GDM and controls significantly differed in values of parameters known to be altered in GDM, such as BMI, glucose levels, indicators of lipid status and pancreatic β-cell function.

Table 1. Characteristics of study participants.

	GDM patients	Controls	*P* value
N	45	45	
Age (years) [a]	35.6±4.2	33.6±4.8	0.052
Smoking status (%)	20.4	17.8	0.75
Gestational age at sampling (weeks)	27.1±2.1	26.3±1.9	0.06
Gravidity, n (%)			
1	14 (31.1)	18 (40)	0.38
2	17 (37.8)	11 (24.4)	
≥3	14 (31.1)	16 (35.6)	
Parity, n (%)			
0	21 (46.7)	25 (55.6)	0.67
1	17 (37.8)	15 (33.3)	
≥2	7 (15.5)	5 (11.1)	
Family history of diabetes (%)	37.8	22.2	0.11
Anthropometric characteristics [a]			
Pre-pregnancy weight (kg)	71.3±13.4	66.9±8.3	0.08
Height (cm)	168.9±5.2	170.6±6.8	0.18
Weight gain (kg)	6.4±4.4	6.4±11.9	0.97
Pre-pregnancy BMI	25.0±4.9	23.0±2.6	**0.017***
Glycemic status [a]			
OGTT (mmol/L)			
0’	4.89±0.69	4.42±0.41	**0.0002**
60’	10.84±1.55	7.76±1.24	**1.3×10^{-16}**
120’	9.28±2.00	6.72±0.95	**$3.2\times10^{-}$**

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Fasting insulin (mU/L)	18.38±16.62	18.67±20.34	0.62
HOMA-IR	4.36±4.55	3.18±2.12	0.20
HOMA-β (%)	299.4±220.2	395.4±257.4	**0.017**
HbA1c (%)	4.77±0.20	4.70±0.24	0.26
Lipid profile [a]			
Triglycerides (TG) (mmol/L)	2.41±0.82	2.23±0.73	0.34
Cholesterol (mmol/L)	6.68±1.09	6.85±1.24	0.55
HDL (mmol/L)	1.85±0.40	2.17±0.50	**0.001**
LDL (mmol/L)	3.77±0.99	3.71±1.14	0.82
TG/HDL	1.42±0.63	1.14±0.64	0.09
LDL/HDL	2.12±0.66	1.76±0.65	**0.033**
Metal ions [a]			
Copper (µmol/L)	25.77±8.63	24.56±7.82	0.50
Zinc (µmol/L)	11.33±2.51	12.45±2.94	0.06
Iron (µmol/L)	15.40±5.75	16.51±8.04	0.51
Other biochemical parameters [a]			
Total proteins (g/L)	63.68±3.18	64.11±4.67	0.65
Albumin (g/L)	34.44±2.22	35.60±2.78	0.07
Urea (mmol/L)	3.11±1.04	2.97±0.81	0.53
Creatinine (µmol/L)	50.27±7.10	52.00±7.14	0.31
Uric acid (µmol/L)	235.66±50.54	216.71±36.82	0.08
CRP (mg/L)	6.95±8.86	6.26±4.12	0.41
AST (U/L)	17.46±6.85	17.86±5.56	0.80
ALT (U/L)	20.00±10.84	15.90±9.25	0.11
Fibrinogen (g/L)	3.99±0.88	3.29±0.75	**0.015**
Ferritin (µg/L)	18.93±11.42	17.70±13.85	0.34
TIBC (µmol/L)	60.85±11.34	60.07±10.08	0.74
Transferrin (g/L)	2.91±0.31	2.92±0.25	0.85
Complete blood count [a]			
Erythrocytes (10^{12} cells/L)	3.80±0.31	3.66±0.37	0.09
Hemoglobin (g/L)	116.27±8.44	109.11±9.05	**0.001**
Hematocrit	0.343±0.026	0.326±0.028	**0.008**
Sedimentation rate (mm/h)	33.89±16.24	33.03±12.94	0.81
Leukocytes (10^9 cells/L)	9.71±2.14	9.61±2.04	0.84
Thrombocytes (10^9 cells/L)	240.4±61.0	236.5±41.6	0.76
Granulocytes (10^9 cells/L)	6.95±1.85	6.83±1.73	0.78
Lymphocytes (10^9 cells/L)	2.11±0.40	2.19±1.13	0.69
Neonates' characteristics and obstetric complications [a]			
Weight (g)	3491.9±489.0	3612.9±498.3	0.36
Length (cm)	51.21±2.53	51.29±4.44	0.93
BMI	12.96±0.94	13.13±1.06	0.52

Apgar score at 1 min	8.87±0.52	8.61±1.10	0.22
Preterm labor (%)	15.8	9.7	0.42
Macrosomia (%)	15.8	21.9	0.12
Polyhydramnion (%)	10.5	6.4	0.90

[a] Mean±SD,
*Statistically significant results are shown in bold

Abbreviations: GDM - gestational diabetes mellitus; BMI - body mass index; OGTT - oral glucose tolerance test; HOMA-IR - homeostatic model assessment of insulin resistance; HOMA-β - homeostatic model assessment of beta cell function; HbA1c – glycated hemoglobin; TG – triglycerides; HDL - high-density lipoprotein; LDL - low-density lipoprotein; CRP – C-reactive protein; AST – aspartate aminotransferase; ALT – alanine aminotransferase; TIBC – total iron-binding capacity.

Among the tested microRNAs, only miR-27a-3p displayed an association with GDM diagnosis (P = 0.03, fold change 1.5) (Fig 1a). According to the diagnostic ROC curve, this microRNA demonstrated a relatively modest diagnostic biomarker performance (AUC=0.613, P = 0.031), with a sensitivity of 0.756 and a specificity of 0.477 (Fig 1b). When GDM patients and normoglycemic controls were segregated according to pregnancy outcome, no significant differences in the expression levels of the analyzed microRNAs were found between the subgroups (Supplementary Fig 1).

Although the other two assessed microRNAs failed to demonstrate significant difference in the expression in PBMCs of GDM patients and controls, both of them showed a significant correlation with the expression of miR-27a-3p (Fig 2). These correlations were positive and were detected in both GDM group (Figs 2a and 2b) and in normoglycemic controls (Figs 2c and 2d). The acquired values of Pearson’s correlation coefficients ranged from 0.507 to 0.823, with the highest value corresponding to the correlation

between the expression of miR-27a-3p and miR-340-5p in PBMCs obtained from women with GDM (Fig 2d).

Since there was a statistically significant difference in pre-pregnancy BMI between GDM patients and controls, we stratified GDM group into subgroups according to overweight criterion (pre-pregnancy BMI≥25) and compared microRNA expression between these subgroups. The obtained results did not reach statistical significance for any microRNA tested (P = 0.828, P = 0.365 and P = 0.992 for miR-27a-3p, miR-222-3p and miR-340-5p, respectively) (Supplementary Fig 2).

3.2. Redox status in GDM patients and controls

When compared to healthy controls, GDM patients presented with an increased activity of GR in the circulation (P = 0.040), while the differences in the activities of SOD and CAT were statistically insignificant (Table 2). GDM also associated with a reduced concentration of thiol groups in serum samples (P < 0.001). Both GR activity and thiol content showed differences when comparing GDM pregnancies with the adverse and non-adverse outcomes (P = 0.042 and P = 0.043, respectively) (Table 3).

Table 2. Differences in values of redox status-related parameters in GDM group and controls.

	GDM patients		Controls		P value
	Mean	SD	Mean	SD	
GR (IU/mg Hgb)	0.20	0.16	0.14	0.09	**0.040***
SOD (IU/mg Hgb)	0.05	0.01	0.05	0.01	0.151
CAT (IU/mg Hgb)	96.42	44.93	91.51	38.42	0.630
SH (mmol/g proteins)	2.90	0.98	3.90	1.36	**<0.001**
Fe (µmol/L)	15.41	5.83	16.51	8.16	0.514
Cu (µmol/L)	25.77	8.73	24.56	7.92	0.499
Zn (µmol/L)	11.33	2.54	12.45	2.97	0.063

Ferritin (µg/L)	18.93	11.62	17.70	14.13	0.724
Hemoglobin (g/L)	116.27	8.56	109.11	9.18	**0.001**
TIBC (µmol/L)	60.85	11.48	60.07	10.21	0.745
Transferrin (g/L)	2.91	0.31	2.92	0.26	0.851
Albumin (g/L)	34.44	2.25	35.60	2.83	0.072
α_2M (g/L)	1.93	0.45	1.88	0.36	0.726
Zn/α_2M	6.24	1.84	6.94	2.36	0.258
Zn/Albumin	0.33	0.08	0.33	0.12	0.984
Cu/Albumin	0.72	0.20	0.67	0.24	0.382
Fe/Transferrin	5.35	2.05	5.36	3.27	0.991
Fe/Ferritin	0.90	0.28	1.54	1.57	0.340
Fe/Hemoglobin	0.13	0.05	0.15	0.07	0.234
Fe/Zn	1.40	0.58	1.34	0.74	0.749
Fe/Cu	0.66	0.29	1.09	1.74	0.600
Cu/Zn	2.44	1.24	2.17	1.11	0.301

*Statistically significant results are shown in bold

Abbreviations: GDM - gestational diabetes mellitus; Hgb – hemoglobin; GR – glutathione reductase; SOD – superoxide dismutase; CAT – catalase; SH – sulfhydryl (thiol) group; Fe – iron; Cu – copper; Zn – zinc; TIBC – total iron binding capacity; α_2M – alpha-2-macroglobulin; SD – standard deviation.

When it comes to redox-active and IFM-related metal ions, circulatory levels of iron and copper remained unchanged in GDM, compared to controls (Table 2). Zinc level, on the other hand, was lower in GDM group, with a statistical trend of significance observed as a $P = 0.06$. Furthermore, zinc concentration was lower in GDM patients with adverse pregnancy outcomes, compared to non-adverse outcome subgroup ($P = 0.037$) (Table 3). As for blood proteins acting as carriers of these metal ions, hemoglobin was increased in GDM, while the reduction of albumin level was close to statistical significance ($P = 0.07$) in this group (Table 2). Tested ratios of concentrations of metal ions, as well as metal ions and their corresponding carrier-proteins displayed no significant differences between GDM cases and controls (Table 2). Apart from zinc concentration, albumin concentration and the ratio of zinc:α_2M concentration varied between GDM

subgroups with different pregnancy outcomes (P = 0.018 and P = 0.034, respectively) (Table 3).

Table 3. Differences in values of redox status-related parameters in GDM patients and controls with and without adverse pregnancy outcomes.

	GDM patients					Controls				
	Adverse		Non-adverse		*P* value	Adverse		Non-adverse		*P* value
	Mean	SD	Mean	SD		Mean	SD	Mean	SD	
GR (IU/mg Hgb)	0.23	0.14	0.14	0.08	**0.042***	0.14	0.11	0.12	0.07	0.500
SOD (IU/mg Hgb)	0.049	0.006	0.049	0.014	0.865	0.047	0.011	0.056	0.007	**0.014**
CAT (IU/mg Hgb)	91.31	32.22	103.21	54.11	0.483	93.74	42.61	89.10	38.96	0.778
SH (mmol/g prot.)	2.57	0.83	3.30	0.97	**0.043**	3.70	1.10	4.37	1.54	0.217
Fe (µmol/L)	15.16	6.35	14.40	4.14	0.701	15.43	6.66	15.24	6.76	0.946
Cu (µmol/L)	26.24	6.96	24.33	6.18	0.391	23.49	8.98	24.13	6.65	0.821
Zn (µmol/L)	10.54	2.17	12.30	2.61	**0.037**	13.00	3.34	11.99	2.35	0.348
Ferritin (µg/L)	16.67	7.67	20.40	14.09	0.490	22.42	9.00	13.81	9.24	0.173
Hemoglobin (g/L)	117.00	9.81	117.68	6.63	0.818	113.91	17.08	106.44	7.22	**0.013**
TIBC (µmol/L)	63.19	12.33	61.50	8.78	0.633	61.12	6.98	60.49	11.74	0.880
Transferrin (g/L)	2.88	0.35	2.90	0.27	0.867	2.91	10.79	2.94	0.26	0.813
Albumin (g/L)	33.22	1.79	35.31	2.17	**0.018**	35.89	0.31	36.42	2.48	0.637
α_2M (g/L)	2.00	0.29	1.88	0.50	0.545	1.87	2.67	1.89	0.33	0.892
Zn/α_2M	5.29	1.11	6.83	1.89	**0.034**	7.99	0.44	6.41	1.78	0.152
Zn/Albumin	0.31	0.07	0.35	0.08	0.181	0.36	3.12	0.34	0.06	0.526
Cu/Albumin	0.82	0.26	0.68	0.15	0.097	0.61	0.12	0.63	0.14	0.883
Fe/Transferrin	5.89	2.43	5.08	1.81	0.339	5.20	0.27	5.34	2.56	0.891
Fe/Ferritin	0.95	0.24	0.88	0.32	0.670	1.22	2.36	1.41	0.88	0.690
Fe/Hemoglobin	0.13	0.05	0.12	0.04	0.629	0.14	1.00	0.14	0.06	0.843
Fe/Zn	1.50	0.61	1.23	0.48	0.218	1.21	0.06	1.28	0.66	0.788
Fe/Cu	0.62	0.31	0.62	0.20	0.990	1.58	0.66	0.71	0.40	0.271
Cu/Zn	2.64	1.06	2.08	0.74	0.073	2.01	2.86	2.16	0.96	0.698

*Statistically significant results are shown in bold

Abbreviations: GDM - gestational diabetes mellitus; Hgb – hemoglobin; GR – glutathione reductase; SOD – superoxide dismutase; CAT – catalase; SH – sulfhydryl (thiol) group; Fe – iron; Cu – copper; Zn – zinc; TIBC – total iron binding capacity; α_2M – alpha-2-macroglobulin; SD – standard deviation.

3.3. Correlations between microRNA expression and redox status-related parameters

All three tested microRNAs demonstrated a correlation between their expression in PBMCs of patients diagnosed with GDM and the values of redox status-related parameters (Figs 3 and 4). The expression of miR-27a-3p, miR-222-3p and miR-340-5p was positively correlated with the activity of GR in GDM group (Fig 3a-c). Furthermore, the correlation of the expression of miR-27a-3p and miR-340-5p with SOD activity and blood thiol content was negative in GDM group (Fig 3). These correlations were not found in the control group. Also, none of the analysed microRNAs showed correlations with the activity of CAT (Supplementary Fig 3).

Correlation tests of miR-27a-3p expression and concentrations of metal ions and the corresponding carrier-proteins resulted in statistically insignificant values (Supplementary Fig 4). Similar findings were obtained for the analyses that included data on miR-340-5p. The correlation between the expression of miR-222-3p in GDM patients and the plasma level of zinc and α_2M did not reach the statistical significance (r = -0.218 and r = 0.289, respectively) (Fig 4). However, the expression level of this microRNA in PBMCs obtained from women with GDM negatively correlated with the zinc/α_2M ratio (r = -0.418, P = 0.034) (Figure 4).

4. Discussion

GDM is one of the most common pathologies occurring in pregnancy and frequently associates with complications that affect both the mother and the fetus/newborn [1]. These complications may impact the health of the affected individuals in short-term, as well as in long-term, which positions GDM as a globally relevant health threat and as a source of substantial medical costs [23]. Since the diagnosis is established relatively late in pregnancy, based on the results of OGTT, which is uncomfortable and time-consuming, the pursuit for novel clinically relevant diagnostic and predictive biomarkers of GDM is imperative in order to improve the clinical management of this disease [24,25]. The identification and validation of relevant indicators of GDM with a strong potential for discovering pregnant women with an increased risk of GDM could substantially improve diagnostic protocols and result in earlier detection of women needing intervention related to glucose intolerance [26]. Furthermore, the assessment of predictive biomarkers in the context of adverse effects of GDM and the occurrence of obstetric and neonatal complications is invaluable for broadening the knowledge on the molecular basis of disease progression, introduction of novel therapeutics and for selection of patients requiring closer monitoring and therapeutic approaches other than dietary intervention [26-29].

Taking into account the results indicating the strong association between GDM and (g)OS/IFM [4], as well as the well-documented changes in patterns of microRNA expression associated with human pathologies, we

selected microRNAs with known (g)OS/IFM-related functions for the analysis of their possible biomarker properties in GDM and for the assessment of their role as indicators of the redox status at the time of diagnosis, all in the context of potential predictive significance. Due to their desirable chemical properties and the relatively high abundance in liquid biopsy samples, these microRNAs were selected for evaluation in our previous GDM-related study [8], which demonstrated their relevance as indicators of lipid and glucose status in pregnancy and encouraged our further evaluation of biomarker properties of miR-27a-3p, miR-222-3p and miR-340-5p relevant for GDM. In the present study, we also aimed to assess the prognostic significance of redox status-related parameters, which were evaluated for their correlation with the expression of these microRNAs in PBMCs of pregnant women obtained during gestational weeks 24-30.

The evaluation of discriminatory properties of selected microRNAs in the current enlarged study group provided results consistent with our previous findings [8], which suggested that the expression of miR-27a-3p in GDM was higher than in controls. However, the upregulation was merely 1.5-fold and the discriminatory potential of this microRNA in terms of GDM diagnosis is modest, making it a relatively poor classifier. Even though the median expression of other two microRNAs (miR-222-3p and miR-340-5p) was higher in GDM, compared to controls, the observed differences remained statistically insignificant. However, the same direction of changes in the expression of all three tested microRNA molecules is consistent with

the finding suggesting that there is a significant correlation in the expression of miR-27a-3p with the expression of both miR-222-3p and miR-340-5p. This may explain the previously observed roles of all three microRNAs as indicators of lipid and iron status in pregnancy [8]. The established correlations exist in both GDM patients and in controls, which implies that all three (g)OS/IFM-related microRNAs may be coregulated in pregnancy, regardless of the glycemic status. Furthermore, correlation coefficients found for the GDM group were higher than in controls, which may arise from a more prominent effect of stimuli present in hyperglycemic pregnancy on the expression of these microRNAs, resulting in a tighter coregulation. For all three microRNAs, OS was associated with an increase in the expression in different tissues, which supports the mentioned hypothesis, since the inherent property of pregnancy is an increased susceptibility to OS, while hyperglycaemia further induces the production of reactive species [9,30,31].

In line with well documented relation between hyperglycemia and OS [32], we determined differences regarding the values of the redox status parameters in GDM compared to normoglycemic pregnancy. An increased activity of GR and lower serum thiol content in GDM patients suggest that protective antioxidant response is intensified to counteract the increasing damaging effects of the reactive species. Similarly, GDM patients with adverse pregnancy outcomes differed from a subgroup without significant obstetric/neonatal complications in respect to both of these redox

parameters, with the same direction of change as in the GDM vs. controls comparison. This observation implies that the oxidative damage is even more pronounced in GDM pregnancies resulting in later complications and supports the basic hypothesis that the adverse outcomes of GDM may be related to the persistent presence of an increased level of OS in women with poorer antioxidative response. Additionally, the reduction in serum zinc level confirmed similar trends as the mentioned parameters of OS in terms of GDM vs. controls distinction and adverse vs. non-adverse outcome prediction in GDM pregnancies. This is consistent with our previous finding [33] and may be related to anti-inflammatory role of zinc ions, which is relevant for the interconnection of IFM and OS [34]. Furthermore, both albumin level and α_2M/zinc ratio were lower in GDM patients with adverse pregnancy outcome, which may be dependent on zinc ion bioavailability.

The expression of all three tested microRNAs was found to correlate with the activity of GR in GDM, while miR-27a-3p and miR-340-5p displayed a correlation of their expression with the serum thiol content. This observation is in line with the finding suggesting a significant correlation between the expression of all three microRNAs. Furthermore, it supports the hypothesized relevance of the dysregulation of these microRNAs in GDM, since GR activity and thiol content are significantly disrupted in our GDM cohort, compared to controls. Although the difference in SOD activity between GDM patients and controls did not reach the statistical significance, lower values were found in GDM group and negatively

correlated with the expression of miR-27a-3p and miR-340-5p. Differences in the activities of antioxidative enzymes and in the direction of correlation with the expression of microRNAs are a consequence of complex regulations of the antioxidant response and its pathology-related specificities. For instance, miR-27a-3p directly targets NRF2 mRNA, but the effect of reactive species could be both stimulatory and inhibitory on the expression of this microRNA [9,35]. The expression of Nrf2-controlled antioxidant enzymes was reduced in diabetic embryopathy through miR-27a-dependent mechanism [9] while enzymes involved in glutathione homeostasis are also identified as targets of miR-27a-driven regulation [36]. On the other hand, miR-222-3p did not demonstrate the correlation with the expression of SOD, even though *SOD2* is identified as its direct target. This observation might be related to the contribution of other SOD enzymes to the total SOD activity, as well as to the effects of other OS-responsive regulators on SOD enzyme production. When it comes to miR-340-5p, mRNA for NRF2 was confirmed as a direct target of regulation. The presence of reactive species, however, is a signal that induces both the expression of NRF2 and its negative regulator miR-340-5p [37,38].

Findings suggesting the correlation between the expression of miR-222-3p and α_2M/zinc ratio may indicate the effect of zinc signalization on the expression of miR-222-3p, as a part of network involved in zinc ion-orchestrated inflammatory reactions. Previous findings postulated *TNFAIP3*, *SOCS1*, *SOCS3* and *IRF-2* mRNAs as targets of miR-222-3p, while

there is also evidence on the effect of zinc on the expression of these regulators of cytokine signalling [12,39,40].

Although we found significant differences in the redox status at 24-30 gestational weeks between patients with adverse and non-adverse outcomes in GDM, the expression of the evaluated microRNAs did not differentiate GDM patients according to pregnancy outcome. Therefore, the activity of GR and thiol content demonstrated improved discriminatory capacity, compared to the expression of miR-27a-3p, miR-222-3p and miR-340-5p, despite of their correlations.

Even though the main limitation of the present study is its relatively small sample size, the intention was to conduct a pilot research on the topic which is generally neglected. Despite obvious constraints regarding the generalization of the results due to sample size and the number of analyzed microRNAs, the obtained findings clearly support the relevance of redox dysregulation in mid-pregnancy in developing obstetric and neonatal complications. Furthermore, the evaluated OS/IFM-related microRNAs demonstrate correlations of their expression with the values of redox status parameters relevant for GDM. Since these correlations were only found in GDM group, the results qualify miR-27a-3p, miR-222-3p and miR-340-5p for redox status indicators in GDM, rather than in healthy pregnancy. However, our results require confirmation in a much larger dataset, preferably in a prospective study with several time points for follow-up and sampling, which should enable the recruitment of a larger cohort of patients with

adverse pregnancy outcomes, stratification according to a specific obstetric/neonatal complication, as well as definition of temporal variations in both GDM-associated redox patterns and microRNA expression. This should also clarify the changes in the maternal PBMCs' expression of microRNA after the childbirth and form basis for the long-term evaluation of biomarker potential for predicting metabolic disturbances in both mothers and their offspring after GDM pregnancies. In order to address the open questions about the relation between microRNA expression and the presence of OS/IFM in GDM, the results of microRNA quantification should be interpreted in the context of IFM profile, while mechanistic studies would be crucial for confirming such relation and biomarker relevance, as well as for pointing out the crucial stimuli associated with GDM which are potentially connected to the regulation of microRNA expression in PBMCs. Future investigations should also include the determination of the placenta-PBMCs relation in the context of OS/IFM-related microRNA expression and placental development and function in GDM.

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Statements and Declarations

Compliance with Ethical Standards

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

Ana Penezić: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing - original draft. Ognjen Radojičić: Conceptualization, Investigation, Formal analysis, Writing - original draft. Jovana Stevanović: Investigation, Formal analysis, Writing - review &

editing. Dragana Robajac: Conceptualization, Validation, Writing - review & editing. Goran Miljuš: Investigation, Validation, Writing - review & editing. Miloš Šunderić: Investigation, Formal analysis, Validation, Writing - review & editing. Daniela Ardalić: Investigation, Validation, Writing - review & editing. Vesna Mandić-Marković: Conceptualization, Investigation, Writing - review & editing, Validation, Supervision. Željko Miković: Conceptualization, Investigation, Writing - review & editing, Supervision. Olgica Nedić: Conceptualization, Validation, Supervision, Project administration, Funding acquisition, Writing - review & editing. Zorana Dobrijević: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing - original draft, Validation, Supervision. All authors read and approved the final manuscript.

Ethics approval

This study was conducted in accordance with the Helsinki Declaration of 1975 and with the approval of ethics committee of University Clinic for Gynecology and Obstetrics “Narodni Front” (March 18, 2019; No. 05006-2019-4925).

Consent to participate

Written informed consent was obtained from all individual participants included in the study.

Consent to publish

Not applicable.

Data availability

The data supporting the findings of this study are available from the corresponding author, upon reasonable request.

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Figure Captions

Fig 1 Diagnostic properties of miR-27a-3p in GDM. A) Relative expression of miR-27a-3p in GDM vs. normoglycemic controls; ΔCt values are presented on y-axis, boxes represent medians with interquartile ranges, while whiskers represent min and max values; statistical significance was analyzed by Student's T-test; $P < 0.05$ is indicated by an asterisk. B) ROC curve for diagnostic performance of miR-27a-3p in GDM; AUC value with the corresponding P value is presented within the graph

Fig 2 Correlations between the expression (ΔCt values) of microRNAs in PBMC samples: A) miR-27a-3p and miR-222-3p in GDM patients; B) miR-

27a-3p and miR-340-5p in GDM patients; C) miR-27a-3p and miR-222-3p in controls; D) miR-27a-3p and miR-340-5p in controls. Pearson's correlation coefficient (r) and the corresponding *P* value from the regression analysis are presented within each scatter plot

Fig 3 Correlations between the expression (-ΔCt values, or ln(fold change)) of microRNAs in PBMC samples and the activities of antioxidant enzymes. Log2-transformed values of GR activity in GDM patients are presented on y-axis within plots labeled A-C displaying the correlation with the expression of miR-27a-3p, miR-222a-3p and miR-340-5p, respectively. Correlations between the log2-transformed values of SOD activity in GDM patients and the expression levels of miR-27a-3p, miR-222a-3p and miR-340-5p, are presented within plots D-F, respectively. The results of correlation analyses between log2-transformed values of serum thiol content and the expression of miR-27a-3p, miR-222a-3p and miR-340-5p, are presented within plots G-I, respectively. Pearson's correlation coefficient (r) and the corresponding P value from the regression analysis are presented within each scatter plot

Fig 4 Correlations between the expression (-ΔCt values, or ln(fold change)) of miR-222-3p in PBMC samples and the Zn/α_2M ratio. Log2-transformed values of A) zinc, B)α_2M concentration and C) Zn/α_2M ratio are presented on y-axis within correlation plots. Pearson's correlation coefficient (r) and the corresponding *P* value from the regression analysis are presented within each scatter plot