Gestational diabetes is associated with an increased expression of miR-27a in peripheral blood mononuclear cells

Ognjen Radojičić[1], Zorana Dobrijević[2], Dragana Robajac[2], Nikola Gligorijević[2], Vesna Mandić Marković[3,1], Željko Miković[3,1], Olgica Nedić[2]

[1] University Clinic for Gynecology and Obstetrics "Narodni Front", Belgrade, Serbia

[2] University of Belgrade – Institute for the Application of Nuclear Energy, Belgrade, Serbia

[3] University of Belgrade – Medical School, Belgrade, Serbia

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Correspondence: Zorana Dobrijević, Department for Metabolism, Institute for the Application of Nuclear Energy (INEP), University of Belgrade, Belgrade, Serbia Tel: +381 11 2618 666, E-mail: zorana.dobrijevic@inep.co.rs, ORCID: 0000-0002-4652-7719

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Author Contributions

All authors contributed to the conception and design of the study. Material preparation, data collection and analysis were performed by Ognjen Radojičić, Zorana Dobrijević, Dragana Robajac, Nikola Gligorijević and Vesna Mandić Marković. Protocol optimization was conducted by Zorana Dobrijević, Dragana Robajac and Nikola Gligorijević. Olgica Nedić and Željko Miković supervised the study. The first draft of the manuscript was written by Ognjen Radojičić and Zorana Dobrijević and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Abstract

Background: Dysregulation of microRNA-based mechanisms is associated with various human pathologies, including gestational diabetes (GDM), nominating them as potential diagnostic and/or prognostic biomarkers of GDM.

Methods: The expression of miR-340-5p, miR-27a-3p and miR-222-3p in peripheral blood mononuclear cells (PBMCs) obtained from patients with GDM (n=42) and healthy controls (n=34) was evaluated, together with their correlation with clinical parameters of participants and their newborns. The expression of the selected microRNAs was quantified by qPCR, after the reverse transcription with microRNA-specific stem-loop primers.

Results: The expression of miR-27a-3p was significantly higher in patients with GDM, than in controls (P=0.036), whereas no significant difference between groups was found for the other two tested microRNAs. The expression level of miR-27a-3p in GDM patients was found to negatively correlate with the number of erythrocytes, concentration of haemoglobin, haematocrit and low- and high-density lipoprotein (LDL/HDL) ratio, and positively with the concentration of glycated haemoglobin (HbA1c). In the case of miR-222-3p, a negative correlation between its expression and the concentration of cholesterol, LDL and LDL/HDL ratio was found only in healthy pregnant women. The expression level of miR-340-5p negatively correlated with the erythrocyte count, haemoglobin concentration and haematocrit in GDM patients, as well as with the concentration of cholesterol, LDL and LDL/HDL ratio in healthy women.

Conclusions: The obtained results illustrate the potential of PBMC-derived microRNA miR-27a-3p to serve as a diagnostic biomarker of GDM. MiR-27a and miR-340, on the other hand, may help in assessing the metabolic status relevant for the pregnancy.

Key Points

- Among the tested microRNAs, only mMiR-27a-3p in peripheral blood mononuclear cells demonstrated the potential to differentiate healthy pregnant women and those with gestational diabetes.
- The expression level of miR-27a-3p exhibited correlation with a number of blood parameters relevant for the oxygen transport, glycaemic and lipid status.
- MiR-222-3p was not found to associate with any of the tested parameters in gestational diabetes, while the expression level of this microRNA was indicative of lipid status and iron status in healthy pregnant women.
- The expression of miR-340-5p correlates with the lipid parameters in both patients with gestational diabetes and the control group.

1. Introduction

According to the most recent data published by the International Diabetes Federation (IDF) in 2019, the worldwide prevalence of pregnancies affected with maternal hyperglycaemia was estimated to be 16%, and 83.6% of them were attributed to the gestational diabetes melitus (GDM) [1]. The data on GDM prevalence are alarming, considering the association of GDM with an increased risk of serious obstetric complications and adverse outcomes in both mothers and newborn [2-4]. Furthermore, women diagnosed with GDM and their offspring were found to be susceptible to the development of diabetes mellitus type 2 (T2DM) [5-7].

The diagnostic procedure for GDM currently relies on an oral glucose tolerance test (OGTT). However, protocols are not uniform, the test is time-consuming and usually uncomfortable for patients. while OGTT monitoring in the early stage of pregnancy has limited ability to detect individuals who are susceptible to the development of GDM [8, 9]. Therefore, the investigation of other biomarkers which can diagnose, predict or detect consequences of GDM has been intensified. The focus recently shifted to microRNAs which exert regulatory functions and their expression level may change in relation to metabolic disorders and/or inflammation [10].

MicroRNAs were evaluated as circulatory biomarkers of GDM in a number of studies which utilised plasma or serum samples [11, 12]. The reported results were heterogeneous, rarely replicated and sometimes even contradictory. Analyses of microRNAs as potential GDM biomarkers from the whole blood are relatively scarce, while peripheral blood mononuclear cells (PBMC) were recognized as potential source of diabetes-related microRNAs [13, 14]. Still, PBMC-derived microRNA molecules were not previously analysed as biomarkers of GDM, although there was an evidence of the usefulness of PBMC as representative cells for the

assessment of inflammatory status in diabetic disorders and obesity [15, 16]. Even though the procedure of PBMC extraction is more complex and time consuming compared to the protocols for obtaining plasma and serum, it results in a significantly different pool of microRNAs, providing the basis for the discovery of additional biomarkers [17]. Furthermore, PBMC-based approach is associated with a higher yield of total RNA and it enables the usage of less-sensitive and less-expensive quantification methods with much reduced need for pre-amplification step [18-20]. The quantification of microRNA from PBMC relies on the usage of endogenous small RNAs as reference genes, while in case of plasma or serum, there are difficulties in choosing adequate normalizing strategy, and the results are more influenced by the existence of low level of haemolysis [21-23]. Also, since PBMC-derived microRNAs originate from cells involved in immune response, it is expected that this microRNA pool would better reflect the inflammation status associated with diabetic conditions [15, 16].

Three microRNAs were chosen for this study as they were previously reported to be related to glucose metabolism and/or estrogen secretion. MiR-340-5p was suggested as potential whole blood-based GDM biomarker, whose expression level in cultured lymphocytes was affected by glucose and insulin concentrations [24]. Since PBMC have higher proportion of lymphocytes than the whole blood, the between-sample fluctuations of miRNA-340-5p should be reduced. Apart from miR-340-5p, two other microRNAs abundantly expressed in PBMCs were chosen to be analysed in this study. MiR-27a-3p and miR-222-3p were selected taking into account their altered expression levels in GDM patients, compared to healthy women [11, 12], as well as for their regulatory role in glucose metabolism, including glucose uptake [25-27]. One of the proposed mechanisms of the action of miR-222 in GDM pathophysiology relies on the direct targeting of estrogen receptor, while estrogen signalization was also shown to affect the

expression of miR-27a [25, 28]. The involvement of hormonal-guided mechanisms in pregnancy-associated insulin resistance qualifies these microRNAs as candidates for GDM biomarker research. Additionally, glucose was found to affect the expression of these microRNAs in different experimental models [29, 30].

Since the data on microRNA expression in PBMCs of GDM patients are limited, we considered that performing quantification analysis of several selected microRNAs could contribute to the quest for novel GDM biomarkers, as well as to the understanding of the role of microRNAs in the dysregulation of metabolic processes associated with GDM. The aim of the study was to compare the expression of miR-340-5p, miR-27a-3p and miR-222-3p in PBMC obtained from patients with GDM and healthy pregnant women. The results of microRNA expression analyses were further related to clinical data on participants and their newborns in order to assess whether microRNA dysregulation is associated with the metabolic status or may have a prognostic significance.

2. Material and Methods

2.1. Recruitment of participants, data acquisition and sample collection

The study included 42 patients with GDM and 34 healthy pregnant women recruited in the period 2019 - 2020 at the University Clinic for Gynecology and Obstetrics "Narodni Front" (OGC NF), Belgrade, Serbia. Experiments were conducted with the freely-given explicit consent of the study participants, in accordance with the Helsinki Declaration of 1975 and with the approval of the Ethics committee of OGC NF (Approval no. 05006-2019-4925). Demographic and clinical data were obtained from patients' medical records, upon obtaining written informed consent from each participant. Participants' data relevant for this research included OGTT

results, biochemical and haematological results monitoring glucose control (concentrations of glucose, insulin, glycated haemoglobin, (HbA1c)), lipid status (concentrations of cholesterol, high- and low-density lipoproteins (HDL, LDL), triglycerides), iron metabolism (blood cell count, concentrations of haemoglobin, iron, ferritin, total iron-binding capacity, (TIBC)), liver function (activities of transaminases), kidney function (concentrations of urea, creatinine, uric acid, albumin), inflammation (sedimentation rate, C-reactive protein, CRP leucocyte count, fibrinogen) , as well as the information on the potential pregnancy or delivery complications, together with the birth weight of the newborn. Each participant provided basic information relevant for the correlation analyses and the exclusion assessment such as age, height and self-reported pre-pregnancy weight (for the calculation of the body mass index, BMI), gestational age, number of miscarriages, pregnancy terminations and childbirths, history of gestational or other type of diabetes, family history of diabetes, type of therapy. BMI of the newborn was calculated using the same formula as for mothers, except for pre-term infants, which were excluded from the correlation analyses related to this parameter.

Peripheral blood samples were collected from GDM patients and controls in EDTA-containing tubes between pregnancy weeks 24 and 30. All samples were obtained after an overnight fasting period. GDM diagnosis was based on the results of OGTT (with 75 g of glucose), according to criteria defined by the International Association of Diabetes and Pregnancy Study Groups (AIDPSG) [31]. The exclusion criteria for both GDM patients and controls included other pregnancy pathologies, as well as previously diagnosed metabolic diseases.

2.2. PBMC isolation

Each blood sample was centrifuged at 400 g at 4 ^{o}C for 10 min, the buffy coat was collected, diluted with 10 mM phosphate buffered saline (PBS) pH 7.4 and layered onto Lymphocyte separation medium (1.077 g/ml, Capricorn Scientific, Ebsdorfergrund, Germany). The separation of PBMCs was conducted according to the manufacturer's instructions, with several adjustments. The centrifugation in the separation step was performed at 700 g (slow acceleration, deceleration brakes off) at 18-21 ^{o}C for 30 min (Centrifuge 5804R, Eppendorf, Hamburg, Germany), the PBMC layer was collected and washed twice with PBS at 100 g at room temperature for 10 min. Erythrocyte lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) was used for the last washing of PBMC pellet, under the same conditions. The pellet was dissolved in TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) and stored at -80 ^{o}C.

2.3. RNA extraction and microRNA quantification

Total RNA extraction was performed by phase separation and precipitation, according to the manufacturer's instruction, while RNA concentration was measured spectrophotometrically (Epoch microplate spectrophotometer, BioTek Instruments – Agilent, Santa Clara, CA, USA). The extracted RNA was subjected to DNase treatment as recommended by the manufacturer. Briefly, 1 µg of total RNA dissolved in RNase-free water was treated with 1 U of Amplification grade DNase I (Sigma-Aldrich, Burlington, MA, USA) at 37 ^{o}C for 30 min, the reaction was stopped by adding the recommended amount of the supplied Stop Solution (Sigma-Aldrich, Burlington, MA, USA) and by incubating the reaction mixture at 75 ^{o}C for 10 min. Immediately after the DNAse treatment, RNA was reversely transcribed to complementary DNA (cDNA) using 100 U of RevertAid Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA) in a final volume of the reaction mixture of 20 µl, containing 1X of the supplied Reaction

Buffer, 0.5 mM dNTPs and stem-loop primers (250 nM each) (Microsynth, Balgach, Switzerland). Sequences of the stem-loop primers for the analysed microRNAs, as well as for the small nuclear RNA RNU6-1, which was used as an internal control, are shown in Table 1, together with the corresponding references [32-36]. The following conditions were used for reverse transcription (Applied Biosystems 2720 Thermal Cycler - Thermo Fisher Scientific, Waltham, MA, USA): incubation at 16 ^{o}C for 30 min, at 42 ^{o}C for 30 min and at 85 ^{o}C for 5 min. The obtained cDNA was diluted 30-fold with nuclease-free water to be further used in quantitative real-time PCR (qPCR). Reaction mixtures for qPCR contained SYBR™ Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), 0.5 µM universal MiR-rv qPCR primer and 0.5 µM qPCR primer specific for miR-27a-3p, miR-222-3p, miR-340-5p or RNU6-1 (Microsynth, Balgach, Switzerland) (Table 1). Primer sequences were selected from the published articles [24, 27] or were self-designed. Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) was programmed for the temperature profile of qPCR reaction which consisted of the initial denaturation at 95 ^{o}C for 10 min and 40 cycles that included denaturation at 95 ^{o}C for 15 sec, followed by primer annealing/elongation step at 60 ^{o}C for 1 min. Ct values were automatically calculated using the Applied Biosystems 7500 Software v2.3 (Thermo Fisher Scientific, Waltham, MA, USA).

The delta-delta Ct method was used for the calculation of the relative expression of microRNAs and $2^{-\Delta\Delta Ct}$ values were compared between GDM group and controls to assess the fold-change. Ct values of the reference gene RNU6-1 were subtracted from Ct values of the target microRNAs to calculate ΔCt. ΔΔCt values were calculated by subtracting the average ΔCt value of the control group from ΔCt values corresponding to each sample.

2.4. Statistical analysis

Statistical software OriginPro 8.5.1 was used for the analysis of the clinical data and the data on microRNA expression. Kolmogorov-Smirnov test was used to assess the normality of the distribution of results, while F-test was used to assess the equality of variances in study groups. Two-tailed Student's T test was employed for paired comparisons of normally distributed results, while Mann-Whitney U test was applied to analyse the data found to significantly deviate from the normal distribution. *P* values <0.05 were considered to be statistically significant. The logistic regression was used to adjust the results for age and BMI of participants. MicroRNA expression results were correlated with clinical parameters using linear regression analysis. The results were expressed as Pearson correlation coefficients (r) and the corresponding *P* values. The adequacy of the sample size was calculated using the study power calculator available at URL: https://clincalc.com/stats/SampleSize.aspx [37].

3. Results

3.1. Characteristics of participants and pregnancy outcome

Data for women with GDM and healthy controls are listed in Table 2. As expected, pre-pregnancy BMI and glucose levels during OGTT were higher in GDM patients than in controls, whereas HDL level was lower. GDM patients had higher erythrocyte count, haemoglobin concentration, haematocrit and fibrinogen concentration. No significant difference between groups was seen in respect to the characteristics of newborns or obstetric complications (Table 2), although higher macrosomia rate was detected in controls. This finding could be explained by the higher pre-term labor rate in GDM group and/or strict dietetic and follow-up regime of participants with GDM.

3.2. MicroRNA expression in GDM patients and controls

The results of microRNA expression analyses are shown in Figure 1. There were no significant differences in the expression of miR-222-3p and miR-340-5p between GDM patients and controls (*P* values 0.244 and 0.179, respectively) (Figure 1b and 1c). However, the expression of miR-27a-3p was significantly higher in patients diagnosed with GDM, compared to healthy controls (*P*=0.036) (Figure 1a) and the result remained statistically significant even after adjusting for age and BMI (*P*=0.048). When ΔCt values were converted to fold change, the group of GDM patients had 1.54-fold higher level of miR-27a-3p, compared to the averaged expression of this microRNA in controls.

3.3. Correlation of microRNA expression with clinical parameters

MiR-27a-3p expression level in GDM patients was negatively correlated with the erythrocyte count, haemoglobin concentration and haematocrit (Table 3, Figure 2a, 2b and 2c; correlation coefficients ranged from -0.387 to -0.441), as well as with LDL/HDL ratio (Table 3, Figure 2d). A positive correlation, on the other hand, was determined between miR-27a-3p expression and HbA1c level (Table 3, Figure 2e). No correlation was seen in respect to miR-27a-3p in the control group (Table 3).

Contrary to the results on miR-27a-3p, correlations between miR-222-3p and clinical parameters of GDM patients remained statistically insignificant, while this microRNA was found to correlate with the mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) in controls. The negative direction of correlation was determined for miR-222-3p expression and total iron-binding capacity (TIBC), as well as for the level of miR-222-3p in PBMC and several parameters defining the lipid status of healthy pregnant women. On the other hand, the positive correlation was found in the same group for miR-222-3p and the level of serum creatinine and C-reactive protein (CRP) (Table 4).

MiR-340-5p expression level correlated negatively with the erythrocyte count, haemoglobin concentration and haematocrit in GDM patients. On the contrary, positive correlation was found between miR-340-5p and HDL concentration in the same group of patients. This microRNA presented some similarities to miR-222-3p, since the expression of miR-340-5p negatively correlated with the total cholesterol, LDL concentration and LDL/HDL ratio in samples obtained from healthy women (Table 5).

None of the analysed characteristics of the newborn correlated with the expression of these three microRNAs (Tables 3-5). As shown in Table 2, there were no statistically significant differences in the frequency of obstetric complications or in the newborn anthropometric characteristics between GDM patients and controls.

4. Discussion

The aim of the present study was to evaluate differences in the expression of the selected PBMC-derived microRNAs between patients diagnosed with GDM and healthy pregnant women. In addition, we aimed to assess the potential correlation between the expression level of these microRNAs and certain clinical parameters of both mothers and their newborn. The main findings suggest that the upregulation of miR-27a-3p in PBMC is associated with GDM, as well as with several biochemical and haematological parameters related to the glycaemic and lipid status. Furthermore, the level of miR-340-5p correlated with erythrocyte characteristics in GDM, while it was also indicative of the lipid status in both GDM and healthy pregnant women. The expression of miR-222-3p correlated with parameters related to lipid metabolism.

MicroRNA-based regulatory mechanisms have been implicated in molecular pathogenesis of various human pathologies, including diabetes. Therefore, numerous studies were conducted to analyse the functional significance of microRNAs in cell signalling and

biochemical pathways associated with metabolic dysfunction in GDM and T2DM [10]. Furthermore, microRNAs were recognized as potential biomarkers of diabetes susceptibility, disease onset, progression, associated complications and adverse outcomes [12, 38, 39]. Liquid biopsy, as a minimally invasive method for obtaining easy-accessible biomarker pool, was implemented in GDM biomarker quest, which included the analyses of circulatory microRNAs from plasma or blood serum [11]. Approaches based on the analysis of microRNA content of PBMCs or the whole blood were less frequently employed. However, a study of Stirm et al. [24] identified miR-340-5p as a putative whole-blood GDM diagnostic biomarker regulated by glucose and insulin in lymphocytes. Since PBMCs are a blood fraction enriched in lymphocytes, we selected miR-340-5p from PBMCs for the analysis of its potential biomarker properties related to GDM and the associated clinical characteristics and pregnancy outcomes.

Another microRNA selected for this assessment was miR-222-3p, due to its functional significance in regulating glucose and lipid metabolism [25, 40, 41], as well as for being one of the most abundantly expressed microRNAs in PBMCs, and among the commonly deregulated microRNAs in both GDM and T2DM [12, 39, 42]. Since miR-27a-3p is also involved in a regulatory network of glucose and lipid metabolism [27, 43-45], we selected this microRNA for our candidate-based analysis of potential GDM biomarkers. Namely, the expression of glucose transporter 4 (GLUT4) was found to be influenced by this microRNA through its direct inhibitory effect on peroxisome proliferator-activated receptor gamma (PPAR-γ) or p38α mitogen-activated protein kinase (MAPK) [27, 43]. Furthermore, miR-27a-3p directly targets mRNAs encoding fatty acid synthase (FAS) and stearoyl-CoA desaturase 1 (SCD1) [44]. The selection of miR-27a-3p relied also on the finding of its altered expression in GDM [46] and on a report suggesting its shared expression in PBMC of GDM, T1DM and T2DM patients [47].

Our results showed an increased expression of miR-27a-3p in PBMCs of GDM patients, compared to healthy controls. This result could not be compared to previous findings, since differences in PBMC-derived microRNAs were not previously analysed in women with GDM and. controls. However, miR-27a-3p was not among the upregulated microRNAs in whole blood cells from GDM patients identified through microRNA profiling in the study of Stirm et al. [24]. On the other hand, there are published data on the correlation of miR-27a-3p expression level with the parameters of oxidative stress in serum [48] as well as with the levels of inflammatory cytokines [49], which are both associated with obesity and GDM clinical phenotype [50, 51]. The disagreement between our results and those of Stirm et al. [24] could be attributed to the differences in the source material, but also to potential confounders and the applied methodology. For instance, the number of samples subjected to RNA-sequencing in their screening phase was limited to 8 and miR-27a might have been left out from their selection of microRNAs with the highest fold-change which were included in the validation phase. Furthermore, BMI of the participants in their screening group was significantly higher than in our study, while a period of sampling (in terms of the pregnancy week) was much wider [24].

The expression of other two microRNAs, miR-222-3p and miR-340-5p, was not found to significantly differ between pregnant women diagnosed with GDM and healthy controls. Our results on miR-340-5p did not confirm the findings of Stirm et al. [24], which relied on the whole blood cell microRNA profiling and the subsequent validation of the results by qPCR. One should be aware that the type of sample material was not exactly matched with ours, since we used PBMC fraction of the whole peripheral blood. Additionally, in their study, GDM group and controls were matched for BMI, but this parameter was calculated as BMI during pregnancy, while we used pre-pregnancy weight for calculations. The significant difference in miR-340-5p

expression in lymphocytes due to GDM was reported upon the analysis of only 15 samples in each group, which is a relatively small population size. According to the provided data on their patients' characteristics, based on the SDs corresponding to mean values of the pregnancy week when sampling was conducted, healthy controls in screening group, as well as GDM patients in lymphocyte donor group, showed significant variation. Not only that this parameter was not matched with ours, but could also introduce the inter-individual variability associated with potential bias. Furthermore, taking into account the mean value and the associated SD, the question arises about the timing of OGTT. All mentioned specificities of two studies, together with differences in genetic background, could have contributed to the disagreements between our results and those published by Stirm et al. [24].

Statistically significant correlations between the expression of the analysed microRNAs and a number of clinical parameters related to participants were found. We found correlation between the lower expression level of miR-27a-3p and the higher values of erythrocyte characteristics in GDM group. Since the expression of this microRNA, erythrocyte count, haemoglobin concentration and haematocrit are increased in GDM patients, it seems paradoxical that the correlation between miR-27a-3p level and the values of these parameters is negative. Still, according to previous reports, the level of these parameters seem to increase during the pregnancy progression, with GDM being associated with higher values, compared to matched controls in the same stage of gestation [52, 53]. A difference in temporal variation of miR-27a-3p expression and erythrocyte-related parameters could be a possible explanation for this observation. Changes in miR-27a-3p expression during the gestation progression were not assessed in our study, as the sampling was conducted in a relatively narrow gestation age range. Since we did not assess the expression of miR-27a in red blood cells, we could not directly link

the observed correlation between PBMC miR-27a-3p expression and erythrocyte-related parameters with previously determined regulatory role of this microRNA in hematopoietic multipotent progenitor cell differentiation, including erythropoiesis [54-56]. The interpretation of the mentioned correlation in the context of miR-27a regulatory features is further complicated by the composition of PBMC fraction, since it consists of lymphocytes and monocytes, belonging to different lineages. Finally, the expression of miR-27a in PBMCs does not need to reflect the changes in the expression of this microRNA related to erythropoiesis, and the observed correlation could merely be the consequence of the activity of the same regulatory signal associated with GDM. An inverse correlation between the level of this microRNA and LDL/HDL ratio was also detected, but the statistical significance of this finding was marginal. More importantly, a direct correlation was found between miR-27a-3p and HbA1c levels in GDM patients. HbA1c is not considered to be a sensitive biomarker of GDM, but it remains one of the most significant indicators of the glycaemic status. Although the present analysis aimed to investigate a potential role of miR-27a-3p as a biomarker, without deeper functional implications, it would be useful to conduct future functional analyses on the effects of this microRNA on its predicted targets relevant for molecular pathogenesis of GDM.

As for miR-222-3p, significant correlations between its expression and clinical parameters were found only in the control group consisting of normoglycaemic women. Among the most significant correlations are those with parameters indicating the lipid status and iron status. Therefore, this microRNA could be one of the indicators of metabolic changes in normal pregnancy, unrelated to GDM. Its association with concentrations of blood cholesterol, LDL and LDL/HDL ration was negative, suggesting that better atherosclerotic indices are found in pregnant women with higher expression of miR-222 in their PBMCs. Previously, serum level of

this microRNA was found to inversely correlate with the isolated low HDL-C phenotype, whereas HDL is known to transport miR-222 within circulation [57, 58]. The expression of miR-222 in PBMC could possibly reflect the changes in the liver, where this microRNA has a crucial role in the regulation of lipid metabolism [59]. A direct correlation between the expression of miR-222-3p and MCV and MCH was observed, together with the negative correlation with TIBC, pointing out that lower miR-222-3p level could be an indicator of iron deficiency.

The expression of miR-340-5p was found to correlate with a number of parameters in both GDM patients and controls. An inverse correlation was found with erythrocyte characteristics in GDM group, whereas the positive correlation was found with HDL concentration. It is well known that these parameters differ between GDM patients and healthy pregnant women [52, 53, 60, 61]. However, Stirm et al. [24] did not evaluate these correlations, so we could not compare our results with any previous regarding miR-340-5p expression. The association of the expression of miR-340-5p and lipid parameters found in controls further supports the potential role of this microRNA as an indicator of lipid status in pregnancy. Higher expression of PBMC-derived miR-340-5p is associated with better atherosclerotic indices in both study groups.

4.1. Strengths and limitations

As far as we know, the present study is the first one evaluating the association between the expressions of miR-27a-3p, miR-222-3p and miR-340-5p in PBMCs with GDM. Our results illustrate the potential of PBMCs as an easily obtainable source of microRNAs with possible diagnostic capacity in GDM, as well as of microRNAs which could aid in assessing the metabolic status in pregnancy. The main limitation of our study is a relatively small sample size,

as well as and a small pool of microRNAs selected for the analysis. Therefore, our results need to be confirmed in a larger dataset, as well as in a study with a prospective design which would allow the assessment in several time points during pregnancy, enabling determination of the potential temporal variations in PBMC-microRNA expression. Even though we took into consideration a delivery and newborn characteristics, the evaluation of the potential association between these microRNAs and adverse pregnancy outcomes requires a much larger sample size. Further studies are also needed in order to identify the exact stimulus which affects the expression of these microRNAs in PBMC, especially miR-27a-3p, as well as to assess their potential functional properties related to GDM and/or pregnancy in general. Finally, the participants were recruited in a single medical centre and inclusion of women followed-up in other clinics would enable more reliable generalisation of the results at the population level.

5. Conclusions

According to our findings on three analysed PBMC-derived microRNAs derived from PBMCs, miR-27a-3p has a high potential to serve as a biomarker of GDM. MiR-222-3p and miR-340-5p, on the other hand, seem to have potential in assessing general metabolic changes in pregnancy, especially in lipid status and iron metabolism. However, further validation of the findings, on a much greater number of samples, is needed as it would enable a multivariate modelling to determine both independent and joint effects of various potentially predictive parameters, leading to a proposal of an algorithm for defining a GDM occurrence risk. The results reported in this work clearly illustrate the potential of PBMC-derived microRNAs as diagnostic biomarkers of GDM, or as biomarkers of metabolic changes related to GDM or with pregnancy in general.

Statements and Declarations

Compliance with Ethical Standards

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Conflict of Interest

The authors declare that they have no conflict of interest.

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" (05006-2019-4925).

Consent to participate

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

Data availability

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

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Figure 1. Differences in the expression levels of microRNAs between GDM patients and controls. Quantitative real-time PCR analysis of relative expressions of miR-27a-3p (A), miR-222-3p (B) and miR-340-5p (C) in GDM (n=42) and control (n=34) PBMCs. Data represent mean ± SD. Statistical significance was analysed by Student's T-test. $P < 0.05$ is indicated by asterisk.

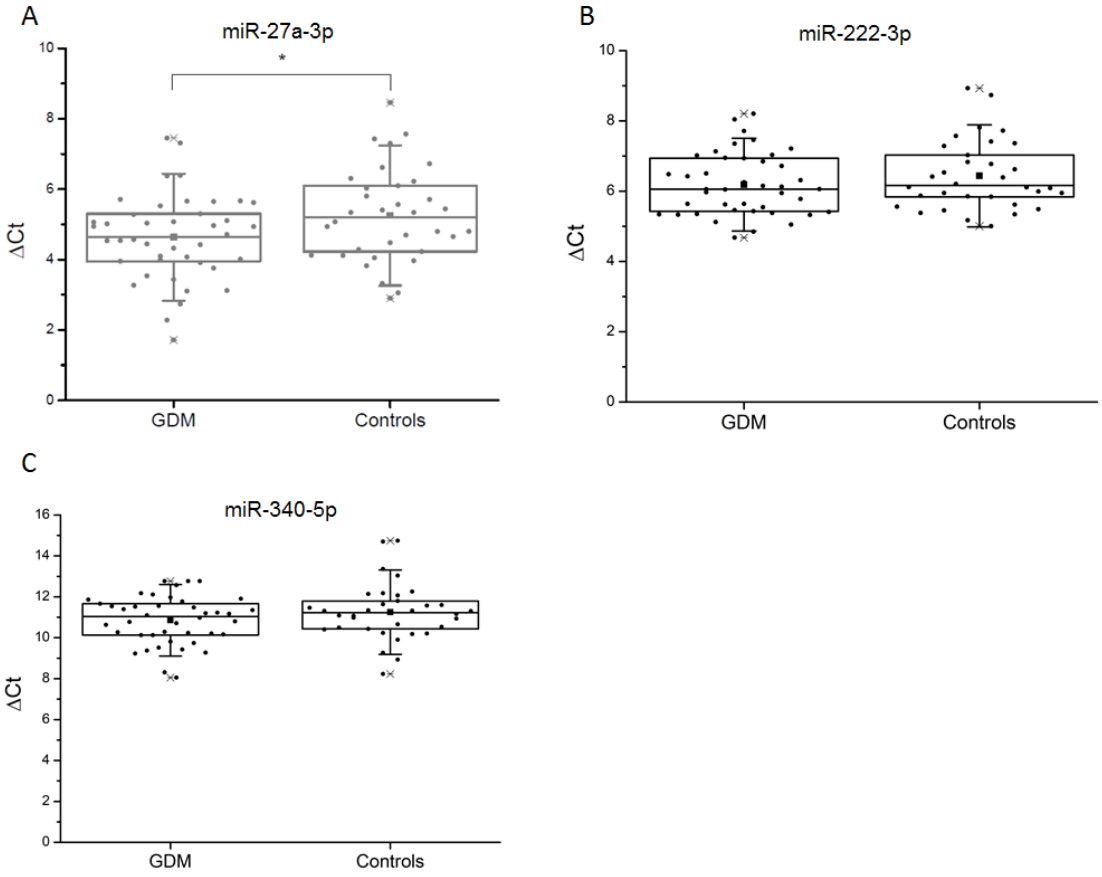

Figure 2. Correlations between the log(fold change) of miR-27a-3p expression and the logarithmically transformed values of clinical parameters of patients diagnosed with GDM: A) erythrocyte count, B) haemoglobin concentration, C) haematocrit, D) LDL to HDL ratio and E) HbA1c level. Pearson correlation coefficients (r) and the corresponding *P* values from the linear regression analysis are presented on each scatter plot.

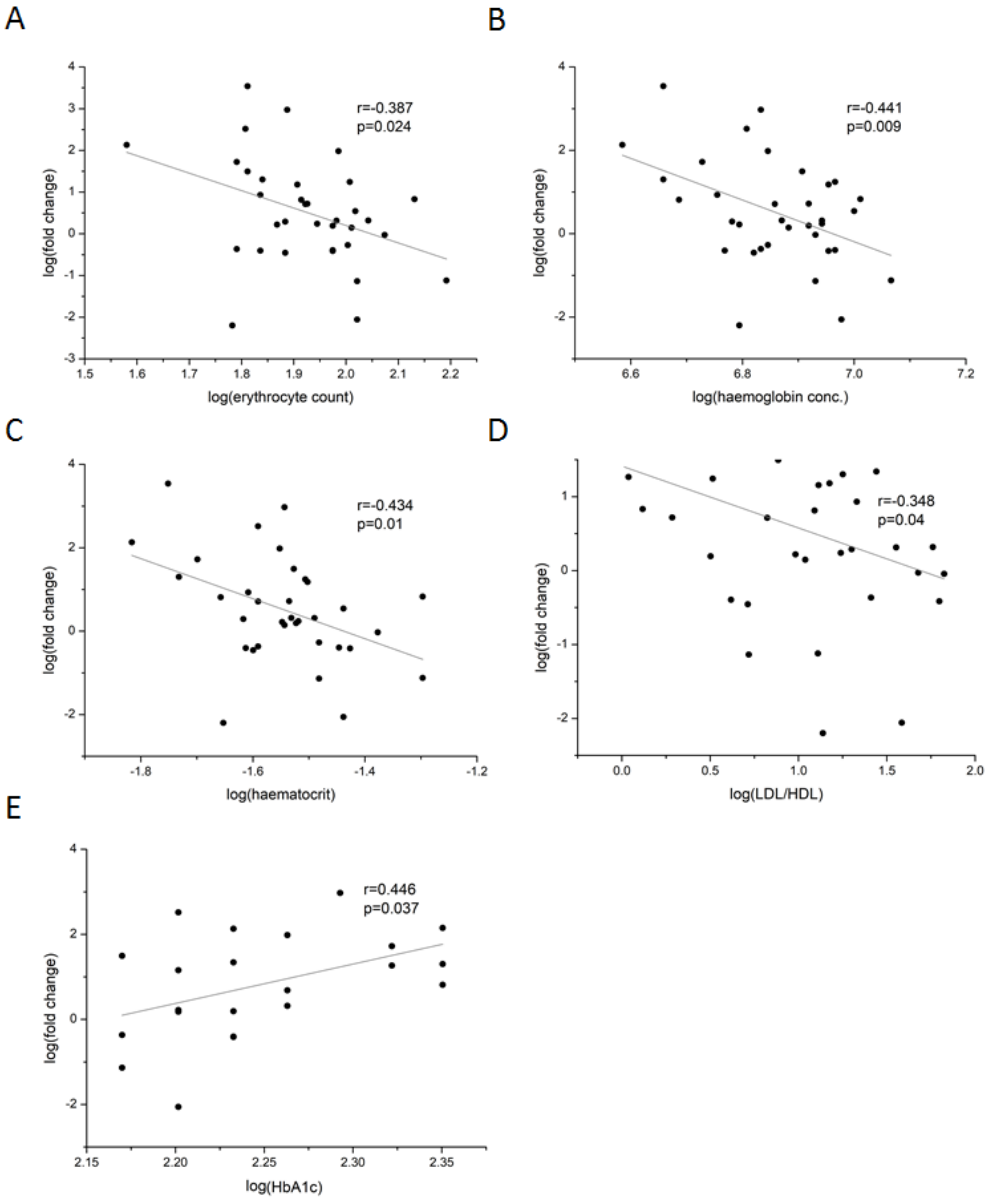

Table 1. Primer sequences.

	Primer	Sequence	Ref.
RT-PCR	RNU-6-RT	5’-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAAATATGG-3’	Popov et al., 2015 [23]
	miR-27a-RT	5’-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGCGGAA-3’	Xu et al., 2018 [24]
	miR-222-RT	5’-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACCCAG-3’	Yao et al., 2014 [25]
	miR-340-RT	5’-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAATCAG-3’	Zhang et al., 2019 [26]
qPCR	Universal miR-rv	5’- CCAGTGCAGGGTCCGAGGTAT-3’	Xu et al., 2018 [24]
	RNU6-fw	5’-GCGGTCGCAAGGATGACACG-3’	-
	miR-27a-fw	5’-CGGCGGTTTCACAGTGGCTAAG-3’	Xu et al., 2018 [24]
	miR-222-fw	5’-CGGCGGTAGCTACATCTGGCTA-3’	Jang et al., 2016 [27]
	miR-340-fw	5’-CGGCGGCTTATAAAGCAATGAGA-3’	-

Table 2. Characteristics of study participants.

	GDM patients	Controls	*P* value
N	42	34	
Age (years) [a]	35.6±4.3	33.9±5.1	0.12
Smoking status (%)	19	12.1	0.42
Gestational age at sampling (weeks)	26.7±2.13	25.9±1.75	0.06
Gravidity, n (%)			
1	14 (33.3)	12 (35.3)	0.66
2	15 (35.7)	9 (26.5)	
≥3	13 (31)	13 (38.2)	
Parity, n (%)			
0	21 (50)	17 (50)	0.80
1	14 (33.3)	13 (38.2)	
≥2	7 (16.7)	4 (11.8)	
Family history of diabetes (%)	35.7	23.5	0.25
Pre-pregnancy weight (kg) [a]	71.4±13.9	67.6±7.9	0.18
Height (cm) [a]	168.8±5.29	171.7±5.6	**0.03***
Weight gain (kg) [a]	6.5±4.4	8.3±3.2	0.053
Pre-pregnancy BMI	25.1±5.0	22.9±2.5	**0.02**
Glycaemic status [a]			
OGTT (mmol/l)			
0’	4.87±0.69	4.47±0.42	**0.0027**
60’	10.8±1.54	7.77±1.25	**1e-13**
120’	9.25±1.93	6.6±0.98	**5e-10**
Fasting insulin (mU/l)	18.09±17.16	19.46±22.46	0.18
HOMA-IR	4.29±4.71	3.26±2.38	0.32
HbA1c (%)	4.75±0.19	4.71±0.24	0.54
Lipid profile [a]			
Triglycerides (TG) (mmol/l)	2.32±0.67	2.19±0.78	0.49
Cholesterol (mmol/l)	6.69±1.12	6.92±1.23	0.46
HDL (mmol/l)	1.85±0.4	2.16±0.5	**0.0036**
LDL (mmol/l)	3.81±1.0	3.69±1.2	0.70
TG/HDL	1.34±0.52	1.11±0.66	0.15
LDL/HDL	2.12±0.68	1.76±0.69	0.052
Other biochemical parameters [a]			
Total proteins (g/l)	63.68±3.23	63.63±4.37	0.96
Albumin (g/l)	34.26±2.12	35.3±2.90	0.17
Urea (mmol/l)	3.18±1.03	2.97±0.88	0.40
Creatinine (µmol/l)	50.62±7.29	51.82±7.45	0.54
Uric acid (µmol/l)	231.76±48.72	216.81±40.07	0.21
CRP (mg/l)	6.63±8.76	6.38±3.74	0.18
AST (U/l)	17.53±6.97	18.00±6.01	0.79
ALT (U/l)	20.00±11.02	16.33±9.78	0.20
Fibrinogen (g/l)	3.99±0.91	2.92±0.71	**0.0033**
Iron (µmol/l)	15.42±5.69	16.73±6.35	0.42
Ferritin (µg/l)	19.30±11.83	18.74±13.10	0.89
TIBC (µmol/l)	60.85±11.84	61.98±11.5	0.68
Complete blood count [a]			
Erythrocytes (10^{12} cells/l)	3.81±0.30	3.62±0.32	**0.03**
Haemoglobin (g/l)	116.38±8.77	109.00±8.87	**0.0028**
Haematocrit	0.344±0.027	0.325±0.028	**0.013**

Sedimentation rate (mm/h)	34.15±16.87	33.91±14.12	0.96
MCV (fl)	90.33±3.65	90.00±5.54	0.80
MCH (pg/cell)	30.6±1.52	30.20±1.90	0.38
MCHC (g/l)	338.65±9.80	335.44±8.40	0.20
Leucocytes (10^9 cells/l)	9.67±2.19	9.32±1.73	0.52
Thrombocytes (10^9 cells/l)	240.65±60.66	237.84±40.31	0.84
Granulocytes (10^9 cells/l)	8.68±10.27	6.63±1.59	0.26
Lymphocytes (10^9 cells/l)	2.11±0.41	2.28±1.27	0.54
Newborn characteristics and obstetric complications [a]			
Weight (g)	3347.1±576.7	3492.8±553.4	0.34
Length (cm)	51.11±2.52	51.72±2.22	0.35
BMI	12.96±0.97	13.05±1.12	0.77
Apgar score at 1 min	8.86±0.54	8.75±0.43	0.43
Preterm labor (%)	15.4	8.0	0.30
Macrosomia (%)	17.1	24.0	0.51
Polyhydramnion (%)	11.4	8.0	0.66

[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; HbA1c – glycated haemoglobin; 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; MCV – mean corpuscular volume; MCH – mean corpuscular haemoglobin; MCHC – mean corpuscular haemoglobin concentration.

Table 3. Correlations of miR-27-3p expression with clinical parameters of GDM patients and healthy controls.

Group of participants		Pearson correlation coefficient	*P* value
GDM patients			
	Lipid profile		
	Cholesterol	-0.021	0.905
	HDL	0.284	0.068
	LDL	-0.179	0.304
	LDL/HDL	-0.348	**0.040***
	Glycaemic profile		
	Fasting glucose	0.025	0.875
	Fasting insulin	0.058	0.715
	HbA1c	0.446	**0.037**
	Other biochemical parameters		
	Creatinine	-0.106	0.551
	CRP	0.075	0.673
	TIBC	-0.084	0.597
	Complete blood count		
	Erythrocyte count	-0.387	**0.024**
	Haemoglobin	-0.441	**0.009**
	Haematocrit	-0.433	**0.010**
	Newborn characteristics		
	Birth weight	-0.075	0.699
	BMI	-0.293	0.123
Controls			
	Lipid profile		
	Cholesterol	-0.383	0.060
	HDL	-0.105	0.554
	LDL	-0.365	0.073
	LDL/HDL	-0.201	0.335
	Glycaemic profile		
	Fasting glucose	0.136	0.443
	Fasting insulin	0.001	0.996
	HbA1c	-0.220	0.380
	Other biochemical parameters		
	Creatinine	0.262	0.187
	CRP	0.188	0.338
	TIBC	-0.320	0.065
	Complete blood count		
	Erythrocyte count	-0.241	0.246
	Haemoglobin	-0.087	0.679
	Haematocrit	-0.062	0.768
	Newborn characteristics		
	Birth weight	-0.355	0.096

	BMI	-0.374	0.080

* Statistically significant results are shown in bold

Table 4. Correlations of miR-222-3p expression with clinical parameters of GDM patients and healthy controls.

Group of participants		Pearson correlation coefficient	*P* value
GDM patients			
	Lipid profile		
	Cholesterol	-0.115	0.511
	HDL	0.028	0.860
	LDL	-0.218	0.208
	LDL/HDL	-0.203	0.242
	Glycaemic profile		
	Fasting glucose	0.106	0.504
	Fasting insulin	-0.025	0.875
	Other biochemical parameters		
	Creatinine	0.097	0.585
	CRP	-0.023	0.897
	TIBC	-0.031	0.845
	Complete blood count		
	Erythrocyte count	-0.269	0.124
	Haemoglobin	-0.313	0.070
	Haematocrit	-0.246	0.161
	Newborn characteristics		
	Birth weight	-0.112	0.563
	BMI	-0.027	0.889
Controls			
	Lipid profile		
	Cholesterol	-0.421	**0.036***
	HDL	0.161	0.363
	LDL	-0.512	**0.009**
	LDL/HDL	-0.472	**0.018**
	Glycaemic profile		
	Fasting glucose	-0.118	0.506
	Fasting insulin	-0.322	0.060
	Other biochemical parameters		
	Creatinine	0.452	**0.018**
	CRP	0.390	**0.040**
	TIBC	-0.343	**0.044**
	Complete blood count		
	Erythrocyte count	-0.198	0.323
	Haemoglobin	-0.103	0.624
	Haematocrit	-0.121	0.565
	MCV	0.526	**0.007**
	MCH	0.495	**0.012**
	Newborn characteristics		
	Birth weight	-0.080	0.624
	BMI	-0.108	0.717

* Statistically significant results are shown in bold

Table 5. Correlations of miR-340-5p expression with clinical parameters of GDM patients and healthy controls.

Group of participants		Pearson correlation coefficient	*P* value
GDM patients			
	Lipid profile		
	Cholesterol	0.108	0.537
	HDL	0.409	**0.007***
	LDL	-0.017	0.923
	LDL/HDL ratio	-0.239	0.167
	Glycaemic profile		
	Fasting glucose	0.015	0.925
	Fasting insulin	0.059	0.711
	Other biochemical parameters		
	Creatinine	-0.069	0.698
	CRP	0.072	0.686
	TIBC	-0.101	0.524
	Complete blood count		
	Erythrocyte count	-0.494	**0.003**
	Haemoglobin	-0.480	**0.004**
	Haematocrit	-0.462	**0.006**
	Newborn characteristics		
	Birth weight	-0.041	0.833
	BMI	-0.263	0.168
Controls			
	Lipid profile		
	Cholesterol	-0.483	**0.014**
	HDL	0.188	0.287
	LDL	-0.571	**0.003**
	LDL/HDL	-0.587	**0.002**
	Glycaemic profile		
	Fasting glucose	-0.153	0.388
	Fasting insulin	-0.087	0.625
	Other biochemical parameters		
	Creatinine	0.238	0.232
	CRP	0.188	0.338
	TIBC	-0.184	0.298
	Complete blood count		
	Erythrocyte count	-0.360	0.071
	Haemoglobin	-0.103	0.617
	Haematocrit	-0.121	0.556
	Newborn characteristics		
	Birth weight	-0.254	0.221
	BMI	-0.191	0.360

* Statistically significant results are shown in bold