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Differential expression of candidate circulating microRNAs in maternal blood leukocytes of the patients with preeclampsia and gestational diabetes mellitus

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ABSTRACT

Objectives: Preeclampsia (PE) is diagnosed in women presenting with new onset hypertension accompanied by proteinuria. Gestational diabetes mellitus (GDM) is the carbohydrate intolerance that can occur in pregnancy. Neutrophil activation is related to PE and GDM. Circulating microRNAs (miRs) are small, noncoding RNA molecules. The aim of this study was to verify the expression levels of three candidate miRs in blood leukocytes of the patients with PE, GDM, and PE-GDM compared to healthy controls.
Study design: We selected miR-21-3p, miR-155-5p, and miR-16-5p which have been associated with GDM and PE. Using real-time quantitative PCR, the expression levels of miR-21-3p, miR-155-5p, miR-16-5p were analyzed in PE (n = 23), GDM (n = 19), PE, and GDM (n = 9) compared to healthy controls (n = 28).
Main outcome measures: The relative expression of the target miR in patient samples was compared to the calibrator and the results were expressed as relative quantification values.
Results: There was a significant decrease in the expression levels of miR-21-3p in GDM and PE and miR-155-5p in PE group. No significant differences were observed in the expression levels of miRs in PE-GDM group. On receiving operator characteristic (ROC) analysis, areas under the curve (AUC) of the expression ratio of miR-21-3p in GDM was 0.73, and miR-21-3p, miR-155-5p in PE were 0.69 and 0.81, respectively.
Conclusions: Our findings indicated that decreased miR-21-3p and miR-155-5p expression levels are associated with PE and miR-21-3p levels are associated with GDM. Our study for the first time revealed that miR-21-3p, miR-16-5p and miR155-5p are not related to PE-GDM group.

1. Introduction

Preeclampsia (PE) is a syndrome of pregnancy characterized by hypertension and proteinuria after 20 weeks of gestation [1]. Insufficient remodeling of maternal spiral arterioles by trophoblast cells in the earliest stages of placental development, together with immune maladaptation establishes increasingly hypoxic conditions as feto-placental demands increase with advancing gestation [2]. However, the specific pathways leading to malfunction of the placenta and maternal endothelium are largely unknown [3]. Identifying the molecules that specifically change in plasma in patients with preeclampsia might be useful to identify biomarkers and the cause of preeclampsia [4].

Gestational diabetes mellitus (GDM) is a condition in which carbohydrate intolerance develops during pregnancy [5]. The pathophysiology of GDM is still not fully characterized. The inadequate β-cell adaptation to peripheral insulin resistance, characterizing second and third trimesters of gestation, is likely to be the main cause of GDM, even though the molecular mechanisms of such failure are mostly unknown [6].

The pathophysiologies of PE and GDM have been shown to be associated with each other [7]. It is unclear whether a common etiologic pathway underlies both PE and GDM. When compared to women with healthy pregnancies, researchers have identified many maladaptations to pregnancy that are present in both preeclampsia and GDM. These include endothelial dysfunction, angiogenetic imbalance, increased oxidative stress, and dyslipidemia [8]. However, studies to date have

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not had the power to compare biomarkers and risk factors among women with preeclampsia alone, GDM alone and preeclampsia with GDM.

MicroRNAs (miRs) are short (19–25 nucleotides), single stranded, and nonprotein-coding RNAs that regulate gene expression by binding to the 30-untranslated region of the target messenger RNAs (mRNAs) and function in diverse biological processes [9]. The identification of stable circulating miRs existing in plasma and serum has provided promise for minimally invasive biomarkers for disease prediction, diagnosis and prognosis [10]. Altered miRs profiles were reported in PE, suggesting that miRs may exert important functions in disease pathogenesis, and could be used as biomarkers of diagnosis and prognostic of PE [1]. Furthermore, previous studies have suggested that miRs are involved in GDM [11].

miR-21 has distinct expression profiles and plays a crucial role in various physiological and pathological processes such as cancer, type 2 diabetes, obesity, and fetal growth [12]. High levels of miR-21 may represent a common feature of pathological cell growth or cell stress [13]. miR-21 expression levels increase in rats on high-fat diet and in liver of type 2 diabetes patients and overexpression reduces maximal glucose induced insulin release in b-cells [14]. Furthermore, miR-21 was found to be associated with macrosomia in non-diabetic patients [12,15,16]. miR-155 has been defined as an inflammation-related miRNA [17]. miR-155 also was identified to be an essential regulator of endothelium-dependent vasorelaxation [18]. Among numerous proposed mechanisms involved in the pathogenesis of diabetes and its complications, nuclear factor kappa B (NF-κB) has been identified as a master switch [19]. It is believed that activation of NF-κB promotes the transactivation of the miR-155 gene that, in turn, can suppress the NF-κB activation by downregulation of the IκB kinases (IKKs) [20,21]. Studies have also shown that miR-155 could play an important role in the pathogenesis of type 2 diabetes and its complications [21,22]. miR-16 regulates the proliferation of cells [23]. miR-16 targets genes encoding the insulin receptor substrate (IRS) proteins 1 and 2. Upregulation of miR-16 will result in downregulation of IRS1 and IRS2, which might in turn lead to aberrant Wnt/β-catenin signaling and eventually diabetes [24].

There is very limited research in the area of maternal circulating miR profiles in PE and GDM populations, and none that investigate the miRs in maternal blood leukocytes of the patients with both PE and GDM. Given their important biological roles, we selected three miRNAs (miR-21-3p, miR-155-5p, and miR-16-5p). The selected candidate miRNAs have previously been associated with GDM (miR-155 [25], -21 [25], and -16 [24,26]), PE (miR-21 [27] and-155 [27]). In the present study, we aimed to investigate miR-21-3p, miR-155-5p, miR-16-5p in maternal blood leukocytes of the patients with PE, GDM and both PE and GDM compared to healthy pregnant women as biomarkers for diagnosis.

2. Materials and methods

2.1. Ethics statement

The study was approved by the institutional review board of Istanbul Medical Faculty, Istanbul University. The research was conducted according to the ethical principles of the Helsinki Declaration (2013). The Declaration of Helsinki is the most widely accepted set of ethical principles for the protection of patients participating in medical research [28]. Written informed consent was obtained from every patient and control individual before blood was taken.

2.2. Study population

Study participants (pregnant women) were recruited from the prenatal obstetrics and gynecological (OB/GYN) clinic at Goztepe Training and Research Hospital affiliated to Istanbul Medeniyet University. Venous blood was obtained from women in the third trimester of their pregnancies. The pregnant women were followed from the third trimester of their pregnancy to delivery.

The patients were distributed into four groups: Group I comprising 23 pregnant women with PE, Group II made up of 19 pregnant women with GDM, Group III consisting of 9 women with both PE and GDM and Group IV (control) consisting of 28 pregnant women with no obstetrical or medical complications. Also, PE group (Group I) consisted of 6 patients with early-onset mild PE, 6 patients with early-onset severe PE, 5 patients with late-onset mild PE, and 6 patients with late- onset severe PE.

The diagnostic and the severity criteria for preeclampsia was made based on ACOG. Preeclampsia was defined by the onset of hypertension (blood pressure ≥ 140/90 mm Hg) and proteinuria (≥0.3 g of protein in the urine within a 24-hour period or a protein/ creatinine ratio of at least 0.3) during the second half of pregnancy (> 20 weeks). In the absence of proteinuria, the diagnosis of preeclampsia was made based on hypertension with any of the following: thrombocytopenia, impaired liver function, renal insufficiency, pulmonary edema, or cerebral or visual disturbances [29]. Preeclampsia occurring at less than 34 weeks of gestation was identified as early-onset disease, whereas PE that occurred at 34 weeks or later was labeled late-onset disease, irrespective of the gestational week at delivery [30]. Diagnosis of GDM was based on a 75-gram oral glucose tolerance test at 24 to 28 weeks' gestation, according to the International Association of Diabetes and Pregnancy Study Groups (IADPSG) criteria [31]. The diagnosis of GDM was made when 1 of the following plasma glucose values in the oral glucose tolerance test was met or exceeded: fasting plasma glucose 92 mg/dL (5.1 mmol/L), 1-hour plasma glucose 180 mg/dL (10.0 mmol/L) or 2-hour plasma glucose 153 mg/dL (8.5 mmol/L) [31]. Gestational age (GA) was determined from the last menstrual period and verified during the routine first-trimester ultrasound measurement of the fetal crown-rump length. Intrauterine growth restriction (IUGR) was defined as an estimated fetal weight < 10th percentile for GA. Fetal macrosomia status of the offspring was defined as baby's birthweight < 4000 g (no fetal macrosomia) or ≥4000 g (fetal macrosomia).

The exclusion criteria for all groups were as following: under 18 years of age, twin or multiple pregnancies, hemostatic abnormalities, chronic hypertension, any other confounding pathologies (including intrahepatic cholestasis of pregnancy, cardiovascular, autoimmune, renal and hepatic diseases), cancer, fetal congenital anomalies, intrauterine fetal death, pre-gestational diabetes.

2.3. Sample collection and RNA isolation

Maternal non-fasting peripheral blood samples were collected from cases and controls during prenatal care in Goztepe Training and Research Hospital affiliated to Istanbul Medeniyet University into 10 mL Vacuette K_2EDTA tubes (BD, Franklin Lakes, NJ). Gestational age at blood drawn were 33.6 ± 4.6, 33.5 ± 3.6, 32.6 ± 3.5, and 33 ± 4.1 weeks for PE, GDM, PE and GDM, and control group, respectively. Gestational age at sampling was not significantly different between groups ($p < 0.893$). After collected into two hours, each blood sample was treated with 2 volumes of Gey's solution (155 mM NH4Cl, 10 mM KHCO3 in DEPC treated distilled water) to lyse the red blood cells (twenty minutes at 4 °C). White blood cells (leukocytes) were pelleted via centrifugation for 10 min at 450 × g at 10 °C. The supernatant was discarded and pellets washed second time in Gey's solution as equal volume of the blood. Samples were washed with 1 × Phosphate Buffered Saline (PBS), centrifuged at 450 × g for 5 min at 4 °C, homogenized in 800 µl TRIzol (Invitrogen, Carlsbad, CA) reagent on ice and stored at −80 °C until RNA extraction. The lysate was transferred to a new tube with 160 µl cold chloroform, mixed and centrifuged at 12,000 × g for 20 min in 4 °C centrifuge. The upper aqueous phase was transferred to a new microcentrifuge tube and 1.5 vol of 100% isopropanol was added and mixed. The tubes were

Table 1
Patient demographics, clinical and biochemical characteristics.

Index Characteristics	Preeclampsia (n = 23) Mean ± SD	GDM (n = 19) Mean ± SD	Preeclampsia and GDM (n = 9) Mean ± SD	Control Group (n = 28) Mean ± SD	P value
Age (years)	29.8 ± 5.9	30.4 ± 4.6	31.1 ± 5.8	28.1 ± 5.8	0.4
Gestational age at sampling (weeks)	33.6 ± 4.6	33.5 ± 3.6	32.6 ± 3.5	33 ± 4.1	0.893
Smoking n (%)	1(4.3)	2(10.5)	–	1(3.6)	0.612
Pre-Pregnancy BMI, kg/m^2	27.0 ± 6.5	26.4 ± 3.8	27.6 ± 6	23.6 ± 2.9	0.02*
BMI (kg/m^2)	31.0 ± 5.8	30.7 ± 4.1	32.2 ± 5	27.1 ± 2.8	0.001*
Nulliparous n (%)	8(34.8)	6(31.6)	1(11.1)	8(28.6)	0.608
Systolic blood pressure at sampling (mmHg)	154.3 ± 15.9	106.8 ± 16.7	142.2 ± 4.4	105.4 ± 8.8	0.0001*
Diastolic blood pressure at sampling (mmHg)	95.4 ± 14.9	62.1 ± 9.2	90.0 ± 0.0	62.8 ± 7.6	0.0001*
Total protein	6.3 ± 0.6	6.7 ± 0.5	5.9 ± 0.7	6.6 ± 0.5	0.006*
Albumin	3.2 ± 0.4	3.4 ± 0.7	3.2 ± 0.4	3.5 ± 0.2	0.059
AST (IU/l)	69.5 ± 199.3	18.6 ± 10.1	41.3 ± 52.7	14.8 ± 4.3	0.302
ALT (IU/l)	36.0 ± 124.5	19.3 ± 14.7	68.7 ± 128.8	11.4 ± 5.9	0.326
LDH (IU/l)	343.8 ± 405.7	171.9 ± 25.4	224.6 ± 65	175.5 ± 50	0.036
Creatinine (mg/dl)	0.6 ± 0.12	0.4 ± 0.6	0.5 ± 0.7	0.5 ± 0.06	0.0001*
Hemoglobin (g/dl)	12.1 ± 2.2	11.8 ± 1.1	11.1 ± 1.0	11.6 ± 1.9	0.538
Hematocrit (%)	35.4 ± 4.4	35.5 ± 3.1	33.6 ± 2.4	33.9 ± 3.8	0.302
White blood cell count ($\times 10^3$/μl)	12.5 ± 4.4	10.6 ± 3	9.5 ± 3	10 ± 2	0.027
Platelet count ($\times 10^3$/μl)	216.2 ± 57.5	215.2 ± 39.3	171 ± 92.7	214.3 ± 57.2	0.219
HbA1c (%)	4.9 ± 0.4	5.3 ± 0.4	5.5 ± 0.1	4.8 ± 0.3	0.0001*
Fasting glucose (mg/dl) at sampling	72.9 ± 8.2	87.9 ± 8.1	87.2 ± 11.8	74.3 ± 6.4	0.0001*
1-h glucose (mg/dl) at sampling	121.5 ± 11.1	135 ± 19.1	134.4 ± 19	111.3 ± 24.3	0.0001*
2-h glucose (mg/dl) at sampling	97 ± 11.3	126.7 ± 19.5	122.1 ± 18.7	99.6 ± 11.9	0.000
Fetal macrosomia n (%)	2(8.7)–	3(15.8)	–	2(7.1)	0.371
IUGR n (%)	2(8.7)	–	–	2(7.1)–	0.669
GA at delivery, weeks	34.6 ± 4.4 (n = 15)	38.6 ± 1.5 (n = 19)	36.2 ± 2.1 (n = 9)	39.3 ± 1.1 (n = 26)	0.0001*
Infant birth Weight, g	2165.7 ± 1127.8	3254.7 ± 477.1	2493.9 ± 756.6	3354.1 ± 461.2	0.0001*
Fetal sex (male) n (%)	7(46.7)	10(52.6)	5(55.6)	15(57.7)	0.922

Abbreviations: GDM, gestational diabetes mellitus; SD, standard deviation; BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, Lactate dehydrogenase; IUGR, intrauterine growth restriction; GA, Gestational Age.
Data presented as mean ± SD and compared using unpaired *t*-test *p < 0.05.

centrifuged (12,000 × g, 10 min in 4 °C) to collect total RNA pellet after overnight −20 °C incubation. The RNA pellets were washed in cold 75% ethanol and centrifuged (7500 × g, 5 min in 4 °C) and re-suspended in 50 μl nuclease-free water after air-drying for 10–15 min. The concentration and quantity of total RNA were measured at 260 nm and 280 nm (A260/280) using a Nanodrop 1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE).

2.4. Quantification of candidate miRs

Real-time quantitative PCR was performed to measure the expression levels of microRNAs with the miScript Primer Assays (Qiagen, Valencia, CA, USA). Reverse transcription of RNA was performed on 1 μg of RNA using the miScript II RT kit (Qiagen). Prepare a master mix that contains 5× miScript RT Buffer (4 μl), miScript Reverse Transcription Mix (1 μl), and RNase-free water to bring reactions to final volume of 20 μl. Also include template RNA (1 μg) in the master mix. Incubate the samples for 60 min at 37 °C followed immediately by an incubation for 5 min at 95 °C. The cDNA samples were diluted in nuclease-free water as 40× and stored at −20 °C. The cDNA samples were amplified using the miScript SYBR® Green PCR kit (Qiagen), the provided miScript Universal Primer (reverse primer) and a small RNA specific primer (forward primer). Primers were ordered from the miScript Primer Assay catalogue for hsa-miR-21-3p (Assay ID: MS00009086), hsa-miR-16-5p (Assay ID: MS00031493), hsa-miR-155-5p (Assay ID: MS00031486), RNU6 (Assay ID: MS00033740). 10x miScript Primer Assays were solved in 550 μl TE buffer, pH 8.0. The PCR reactions were performed in a final volume of 20 μl, and a total of 0.1 ng cDNA was used per reaction. The reactions were performed in duplicate in 96-well plates on a LightCycler480 instrument (Roche, San Francisco, CA) using manufacturer-recommended cycling conditions: 95 °C for 15 min with a ramping rate of 4.4 °C/s, 45 cycles of 94 °C for 15 s, 55 °C for 30 s, 70 °C for 30 s with a ramping rate of 1.0 °C/s, and a final melt-curve analysis.

Threshold cycle number was used to calculate the relative expression between samples. We used the ΔΔCt (cycle threshold) method in which relative expression = $2^{-\Delta\Delta Ct}$, where ΔΔCt = (ΔCt of test sample) − (ΔCt of calibrator). The data were normalized using RNU6 as endogenous control. The arithmetic mean Ct of the control samples (n = 28) was used as the calibrator. The relative expression of the target miR in patient samples was compared to the calibrator and the results were expressed as relative quantification (RQ) values.

2.5. Statistical analysis

Comparison of miR expression levels between patient samples and controls were conducted by using RQ values and results were expressed as mean and standard deviation (S.D.). The miRNA expression levels were assessed using the Shapiro-Wilk test, which did not conform to a normal distribution. Therefore, miRNA expression levels were compared using non-parametric tests. Mann Whitney *U* test was used for RQ values (relative expression levels) between two groups and Kruskal-Wallis test was used for multiple comparisons among four groups. Power analysis was determined using the G*Power statistics software [32]. Receiver operating characteristic (ROC) curve analysis was applied to find the best cut off values of miRs for diagnosing the patients with GDM and/or PE. Non-parametric Spearman correlation was used for calculating the correlation between RQ values and continuous variables in groups. All statistical analyses were performed using SPSS 14.0 (SPSS Inc., Chicago, IL, USA). A p value of < 0.05 was considered

statistically significant.

3. Results

3.1. Subject characteristics

We selected blood samples third-trimester of maternal for this prospectively planned miRs expression levels study. Blood samples from 23 women with PE group, 19 women with GDM group, 9 women with PE and GDM group and 28 healthy pregnant women as control group were assessed. There were no significant differences in mean maternal age, gestational age at sampling and fetal gender among the study groups. The demographics, clinical and biochemical characteristics of pregnant women are listed in Table 1. Corticosteroids were used in 10 of 23 preeclamptic cases and 4 of 9 cases of women with PE and GDM cases at less than 34 weeks of gestation and 2 of 23 preeclamptic cases, 3 of 9 cases of women with PE and GDM between 34 0/7 weeks and 36 6/7 weeks of gestation to accelerate lung maturation (one or maximally two doses of betamethasone administered intramuscularly 24 h apart). Peripheral blood samples were collected from 3 of 10 cases of women with PE at less than 34 weeks of gestation before first dose of betamethasone administration. The remaining 16 cases of women with PE and both PE and GDM were treated with one or maximally two doses of betamethasone before blood sampling.

3.2. Expression analysis of miRs in pregnant women with preeclampsia and GDM

We compared miR-21-3p, miR-155-5p and miR-16-5p expression levels in maternal blood leukocytes among groups. There was at least 80% ($\alpha = 0.05$) power to detect an effect size of 0.4 in Kruskal-Wallis test of four study groups and an effect size of 0.8 in Mann Whitney *U* test between two groups. The fold change value was derived from RQ values where miRs expression levels were normalized by RNU6 expression levels. The expression levels of miR-21-3p are shown in Fig. 1-a for PE, GDM, PE and GDM, and control groups.

There was a significant decrease in the expression levels of miR-21-3p in the GDM (RQ value = 0.67 ± 0.54, p = 0.008) and PE group (RQ value = 1.12 ± 1.85, p = 0.02) compared to the control group (RQ value = 1.31 ± 1.13). Moreover, no significant differences were observed between the expression levels of the PE-GDM (RQ value = 1.55 ± 1.52, p = 0.958). miR-155-5p expression levels for the PE, GDM, PE-GDM, and control groups are shown in Fig. 1-b. There was a significant decrease in the expression levels of miR-155-5p in the PE group (RQ value = 0.54 ± 0.59, p = 0.0001) compared to the control group (RQ value = 1.29 ± 0.97). Nevertheless, no significant differences were observed between the expression levels of the PE-GDM (RQ value = 1.46 ± 1.65, p = 0.808), GDM (RQ value = 0.98 ± 0.79, p = 0.111) groups and compared to the control group (RQ value = 1.29 ± 0.97). Although no associations were found between PE-GDM and GDM groups compared to control group, the statistically significant difference in miR-155 levels was found among four groups in Kruskal-Wallis test (p = 0.001).

Fig. 1-c indicates the expression levels of miR-16-5p of PE, GDM, PE-GDM and control groups. There was no significant difference in the expression levels of miR-16-5p in the PE (RQ value = 21.75 ± 37.03, p = 0.098), GDM (RQ value = 3.14 ± 5.43, p = 0.429) and PE-GDM (RQ value = 11.78 ± 26.74, p = 0.284) group compared to the control group (RQ value = 1.29 ± 0.97). But also, statistically borderline difference was found among four groups in Kruskal-Wallis test (p = 0.098).

3.3. ROC analysis

In order to study the diagnostic accuracy of miR-21-3p, miR-155-5p and miR-16-5p in maternal blood leukocytes as biomarkers for PE, GDM and PE-GDM ROC curve was calculated (Fig. 2). The value of the area under the ROC curve (AUC) between 0.5 and 1 is considered having diagnostic significance. On ROC analysis, miR-21-3p and miR-155-5p reflected obvious separation between the PE and control group, with areas under the curve of 0.69 (95%CI: 0.53–0.84), 0.81 (95%CI 0.68–0.93), respectively; with cut-offs > 0. 56 and > 0.80, respectively; sensitivity of 52.2% and 87%, respectively; and specificity of 89.3% and 67.9%, respectively. Furthermore, The AUC of the expression ratio of miR-21-3p in GDM was 0.73 (95% CI 0.58–0.88); with cut-off > 0.55; sensitivity of 52.6% and 89.3% specificity (Fig. 2a–c).

3.4. Correlation of miR expression levels with clinical parameters

The correlation analyses were performed to verify the associations of blood pressures and glucose levels and the three miRs: miR-21-3p, miR-155-5p and miR-16-5p in four groups, separately. A significantly positive correlation was observed between miR-16-5p, miR-155-5p and miR-21-3p levels each other in the control group (respectively, r = 0.836, r = 0.569, r = 0.640, p < 0.05; Fig. 3a–c). Furthermore, we found positive correlation between diastolic blood pressure and miR-21-3p (r = 0.528, p = 0.004; Fig. 3d) and miR-155-5p (r = 0.430, p = 0.022; Fig. 3e) expression levels in the control group. Also, we found significantly strong positive correlation between miR-155-5p and fasting glucose levels (r = 0.543, p = 0.016; Fig. 3f), but did not a positive correlation with blood pressures in the GDM group. In other hand, a negative correlation was observed between miR-16-5p and fasting glucose levels (r = -0.732, p = 0.039; Fig. 3g) in GDM-PE group. In addition, systolic and diastolic blood pressures and blood glucose levels were not correlated to expression of miR-16-5p, miR-155-5p and miR-21-5p in the PE groups.

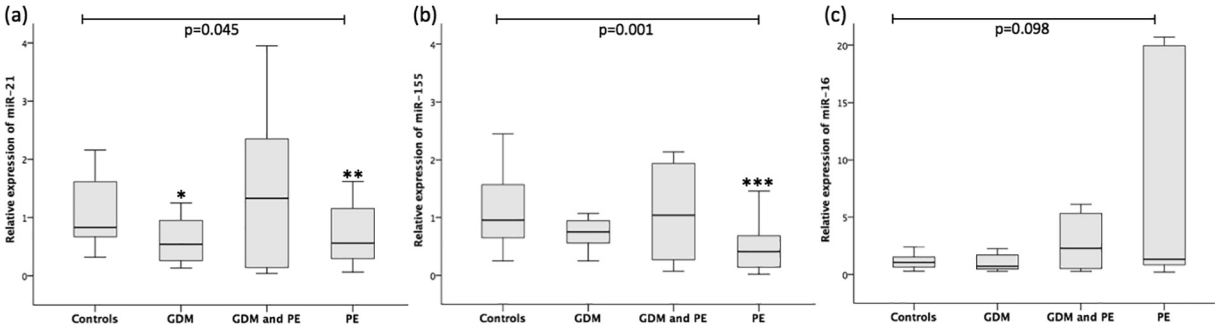

Fig. 1. Box-plots of relative miR-21 (a), miR-155 (b), and miR-16 (c) expression levels in healthy control, gestational diabetes (GDM), GDM and preeclampsia (PE), and PE samples. Kruskal-Wallis test was used to compare the difference of the miRs expression levels among four groups (p = 0.045, p = 0.001, and p = 0.098, respectively). Two-sided Mann-Whitney *U* test was used to compare the difference between the patient group and control group (Statistically significant p-values are shown; *p = 0.008, ** p = 0.02, and *** p = 0.0001). The box-plots are presented as mean ± standard deviation.

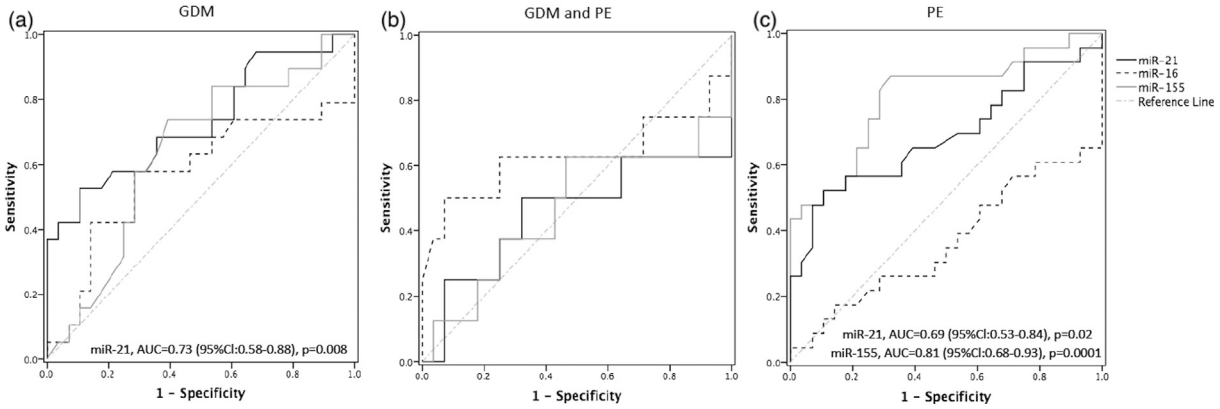

Fig. 2. Receiver operating curve (ROC) analysis for candidate miRs between preeclampsia (PE), gestational diabetes (GDM) and PE-GDM patients and healthy controls. (a) Area under the curve (AUC), 0.73; sensitivity, 52.6%; specificity, 89.3% for miR-21 in GDM; (c) AUC, 0.69; sensitivity, 52.2%; specificity, 89.3% for miR-21 in PE; AUC, 0.81; sensitivity, 87%; specificity, 67.9% for miR-155 in PE.

4. Discussion

Leukocytes from the nonspecific or innate immune system are important in normal pregnancy, as they promote successful implantation, also be involved in the pathophysiology of pregnancy disorders [33,34]. Immune-system alterations are associated with the origin of PE. Proinflammatory cytokines, neutrophil activation, and endothelial dysfunction, are also related to the pathophysiology of PE [35,36]. In addition, GDM was found in relation with overt neutrophil activity [37]. To better address the role of miRs in PE and GDM, we examined circulating miRNAs from maternal blood leukocytes. The present study is pioneer one in this aspect.

In our study we found that the expression levels of miR-155-5p and miR-21-3p were decreased in PE and also miR-21-3p were decreased in GDM compared with healthy pregnant women. To our knowledge, this study is the first study to evaluate the expression of miRs in maternal blood leukocytes of patients with both PE and GDM. Our study showed that miR-16-5p, miR-155-5p and miR-21-3p expression levels in maternal blood leukocytes were not statistically different in patients with PE and GDM compared to with healthy controls. Although our sample size is limited in this study, our results add new information to the data from previous studies regarding the relationship between PE, GDM and miRs.

Clinical studies examining the effect of PE on the circulating miR-21-3p has thus far been limited. Lasabova et al. showed overexpression of placental miR-21 in women with PE [38]. In contrast, it has been shown that during PE-complicated pregnancy, miR-21 was highly decreased in placental tissue at 20 weeks of gestation when compared with normal pregnancies [39]. Li et al. analyzed plasma of mild PE cases using SOLID sequencing for the expression of miR-21. They found that miR-21 expression was upregulated in PE [40]. Similarly, Jairajpuri et al suggested that miR-21 was upregulated in plasma of preeclamptic women compared to the control women, and plasma of women with severe PE compared to mild PE [27]. Data from the literature on miR-21 in PE are inconsistent. In our study, we showed that the expression level of miR-21-3p in maternal blood leukocytes was found to be decreased in patients with PE, compared to healthy controls.

Studies investigating association between miR-21 and gestational diabetes is sparse. miRs have been demonstrated to be involved in

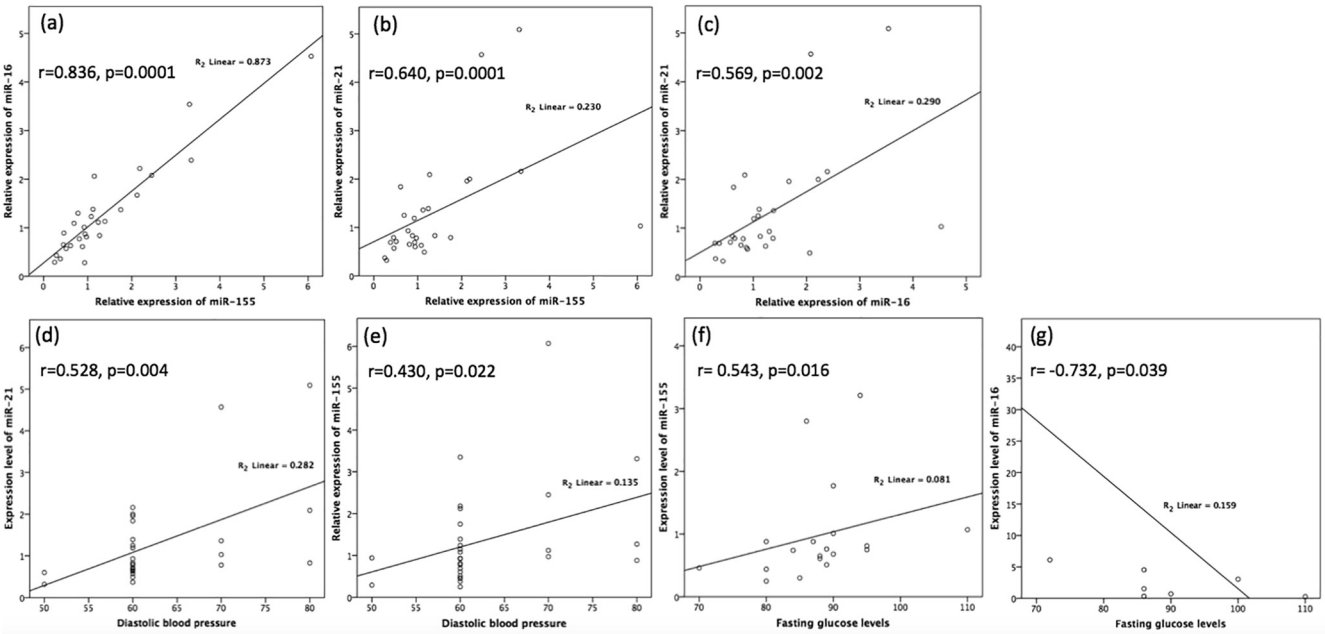

Fig. 3. Correlations between the expression level of miRs and clinical parameters. Bivariate scatterplots indicated as (a–e) for healthy control group, as (f) for gestational diabetes (GDM) group and as (g) for GDM-PE group. Data were analyzed using a Spearman correlation test. The line of linear best fit was shown.

multiple sides of beta-cell function and differentiation, both in normal and diabetic conditions, as well as in beta- cell compensatory process during pregnancy [41]. Wander et al. showed that associations of plasma levels of miR-21-3p with GDM were observed among overweight/obese but not lean women [25]. Also, in sex-stratified analyses, associations of miR-21-3p with subsequent risk of GDM were present only among mothers bearing male offspring [25]. Our results showed that the expression level of miR-21-3p was found to be decreased in patients with GDM. Although, miR-21-3p was found to be down-regulated in patients with PE and GDM, interestingly the expression level of miR-21-3p was not statistically significant in patients with both PE and GDM.

Kim et al. demonstrated that miR-155 levels were significantly higher in maternal plasmas of women with PE than in healthy pregnant women. In this study, miR-155 has been found involving in NF-kB dependent miR-155/eNOS pathway in the pathogenesis of PE [42]. Gan et al. was found that miR-155 were upregulated in the serum of PE pregnancies by a case–control study compared with healthy pregnancies [43]. However, the expression levels of miRs in plasma/serum may not completely consistent with that in maternal blood leukocytes. Our study findings suggest that there was a significant decrease in the expression levels of miR-155 in the PE group compared to the control group. Moreover, studies investigating the expression levels of miR-155 have shown that miR-155 expression increased in the preeclamptic placentas, as compared with the healthy controls [17,44].

In the literature, evidence linking circulating miRs in pregnancy and GDM, a condition that has similar pathophysiologic features to type 2 diabetes is scarce. Wander et al. demonstrated that circulating early–mid-pregnancy miR-155-5p was not associated with GDM in quantile normalized analyses using all participants, but it was associated with GDM when they restricted the quantile normalized analysis to pregnancies with male fetuses only [25]. However, we did not see differences in miR-155-5p expression levels between PE- GDM and GDM alone case compared to the control group, although miR-155-5p was associated with PE. Also, we found significantly strong positive correlation between miR-155-5p and fasting glucose levels in the GDM group. Larger studies are needed to precisely identify the role of miR-155 in GDM.

While Hu et al. revealed up-regulation of miR-16-5p in severe preeclamptic placentas; Hromadnikova et al. indicated no statistical significance in miR-16-5p gene expression levels in preeclamptic placental tissues which is consistent with our findings. [45,46]. The differences in miR expression levels may be attributed apart from the where the sampling site within the placenta and sample handling. Our analysis showed that the expression level of miR-16-5p was not statistically significant in patients with PE and PE-GDM compared to the control group. However, the miR level in tissue was not completely consistent with that in maternal blood leukocytes.

Zhu et al. have found that hsa-miR-16-5p was upregulated in plasma samples of women at 16–19 weeks of pregnancy, before GDM was diagnosed using next-generation sequencing technology [24]. But, the expression of those plasma mRs is uncertain during the entire progress of pregnancy, especially when GDM is diagnosed on OGTT at 24–28 weeks of pregnancy. Cao et al. suggested that plasma miR-16-5p was significantly upregulated at different times during pregnancy in GDM compared with healthy women [26]. With regard to miR-16-5P, our data are inconsistent with the studies of Zhu et al. [24] and Cao et al. [26]. We did not see differences in miR-16-5p levels between GDM, PE-GDM and healthy control. In other hand, a negative correlation was observed between miR-16-5p and fasting glucose levels in GDM-PE group. The discrepancies in the expression levels of miR-16-5p might be explained by different experimental and technological approaches. It is also known that possible involvement of miR-16 in the modulation of circadian rhythms in leukocytes [47]. In the present study, peripheral blood samples were collected from cases independently of circadian cycle.

The study had some limitations. The sample size was small; thus, further research involving a larger population size is needed to confirm clinical utility. We used a candidate-miR approach, which might exclude important miRs related PE and GDM. Recent studies suggest that global profiling of miRs and their target genes using an integrated 'omics' approach will be of greatest benefit [48].

5. Conclusion

We revealed for the first time, circulating miR profiles in the maternal blood leukocytes of pregnant women with PE, GDM and PE-GDM as compared to normal pregnancies. Our findings provide evidence for decreased miR-21-3p and miR-155-5p expression levels are associated with PE and also, miR-21-3p levels are associated with GDM. Moreover, our study revealed that miR-21-3p, miR-16-5p and miR155-5p were not differentially expressed in PE-GDM group compared to healthy controls. We suggest further studies to confirm and extend our findings.

Conflict of interest

None.

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Author contributions

Participated in study design: SD, HS, MH, AT, EKB. Conducted studies: SD, HS (carried out genetic experiments), MH and AT (provided blood samples, collected clinical data), EKB (supervised the experiments, analyzed data). Drafted the manuscript: SD, HS, MH, AT, EKB. All authors read and approved the final manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.preghy.2019.04.004.

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