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Oxidative stress biomarkers in fetal growth restriction with and without preeclampsia

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ABSTRACT

Introduction: Oxidative stress as observed in fetal growth restriction (FGR) and preeclampsia (PE) can be identified by decreased levels of systemic free thiols (FT) and increased levels of plasma ischemia-modified albumin (IMA), which may serve as biomarkers in maternal blood for pregnancy complications. We evaluate the performance of oxidative stress-associated potential biomarkers for FGR and PE, and their relationship with clinical characteristics.

Methods: A prospective clinical pilot study was performed in healthy controls and women with pregnancies complicated by severe FGR with or without PE. Blood samples were taken directly after inclusion and analyzed for FT; IMA; soluble FMS-like tyrosine kinase-1 (sFlt-1); placenta growth factor (PlGF); and biomarkers like leptin and soluble receptors for advanced glycation end products (sRAGE). Placentas were examined microscopically. Descriptive statistics and receiver operating characteristics statistics were performed.

Results: Mothers with both severe FGR and PE had significantly reduced FT levels ($p < 0.001$) and PlGF levels ($p < 0.001$), and increased levels of plasma IMA ($p < 0.05$), sFlt ($p < 0.001$), leptin ($p < 0.05$) and sRAGE ($p < 0.01$) compared to women with FGR only. Systemic FT levels were significantly inversely associated with blood pressure ($p < 0.01$) and plasma IMA ($p < 0.001$), leptin ($p = 0.01$) and sRAGE ($p < 0.001$). Systemic FT and leptin showed significant discriminative ability to differentiate mothers with both FGR and PE from mothers with uncomplicated pregnancies or pregnancies complicated by FGR only.

Discussion: There is a significant discriminative capacity of FT, IMA, leptin and sRAGE that harbor potential as biomarkers of pregnancies complicated by combined FGR and PE.

1. Introduction

Fetal growth restriction (FGR) occurs in up to 10% of all pregnancies [1] and relates to abnormal placentation or secondary changes leading to placental insufficiency [2,3] such as maternal vascular malperfusion (MVM). Placental insufficiency as mechanism of MVM is captured in the 'Great Obstetrical Syndromes' and 'placental syndrome' [4,5] and includes preeclampsia (PE). PE relates to FGR, mainly when it develops early (<32 weeks), due to the hypoxic pathways involved. Placental hypoxia results in vascular damage, inflammation, and high reactive oxygen species (ROS) production, leading to endothelial dysfunction and vasoconstriction [6]. Classical biomarkers for placental insufficiency include soluble FMS-like tyrosine kinase-1 (sFlt-1) and placenta growth factor (PlGF) [7]. An increased sFlt-1/PlGF ratio indicates PE and/or FGR [8,9]. Higher plasma levels of pro-inflammatory markers may also associate with oxidative stress in FGR. Trophoblasts and other

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placental cells secrete tumor necrosis factor-α (TNFα) [10], interleukin (IL)-1 [11] and IL-6 [12] which are elevated during hypoxia [13] and enhance endothelial dysfunction. Recent markers for pregnancies complicated with PE also include plasma leptin and receptors for advanced glycation end products (sRAGE) [14–17].

ROS can be scavenged by systemic and cellular free thiols (FT, sulfhydryl groups), which can be easily and reproducibly quantified to evaluate the extracellular redox state [18,19]. They are a reflection of the redox status as they capture the balance between total oxidant burden and antioxidant capacity. Lower levels of free thiols could function as biomarker for human pregnancy complications that are characterized by oxidative stress [18,20] such as PE [21]. Ischemia-modified albumin (IMA) is a systemic biomarker of local ischemia. Both higher and lower levels of IMA in cord blood of complicated pregnancies have been reported [20,22–27]. Increased IMA levels in cord blood correlate with abnormal umbilical artery Doppler findings [23,25]. Significantly higher levels of IMA are found in maternal blood during the first trimester of pregnancy in small fetuses [28]. Until now studies have not combined FT, IMA and classical biomarkers of placenta insufficiency in a cohort of FGR with and without PE.

We hypothesize that lower levels of free thiols, and higher levels of IMA associate with the development of PE and FGR, and reflect the increase in inflammatory mediators and disbalance in the sFlt-1/PlGF ratio due to hypoxia. We also hypothesize that FGR without PE shows less oxidative stress and therefore less abnormal values of biomarkers as the maternal endovascular inflammation characterizing PE does not occur.

We studied the relationship between oxidative stress biomarkers, placental histopathological- and clinical characteristics in FGR with and without PE.

2. Materials and methods

2.1. Study population

We included 48 women: 14 with severe FGR, 11 with both FGR and PE, and 23 healthy control pregnancies. Inclusion criteria were: maternal age (18–40 years), and a gestational age (GA) between 24 and 36 weeks. Severe FGR was defined as abdominal circumference (AC) or estimated fetal weight (EFW) below the 3rd percentile or absent end diastolic flow (AEDF, indicative of underlying fetal vascular stress). FGR patients with hypertension and without proteinuria were included when AC or EFW $<$ p3 or AEDF was present. Hypertension was defined as systolic blood pressure (SBP) $>$ 140 mmHg and diastolic blood pressure (DBP) $>$ 90 mmHg. Healthy control patients were included with an EFW between the 40th and 60th percentile. The study was approved by the Medical Ethical Evaluation Committee (METc 2016/423) of the University Medical Center Groningen.

2.2. Blood sample collection

Blood samples were collected at inclusion with and without anticoagulant (EDTA). EDTA samples were stored at 4 °C and blood without anticoagulant was kept for at least 1 h at room temperature to allow clotting. After centrifuging the plasma and serum samples were stored at −80 °C.

2.3. Placental examination

Placentas were processed and evaluated according to the latest international criteria [29]. We evaluated maternal vascular malperfusion (MVM), fetal vascular malperfusion (FVM), chorioamnionitis (CA), villitis of unknown etiology (VUE), massive perivillous fibrin deposition (MPVFD), maternal floor infarction (MFI), chronic histiocytic intervillositis (CHIV), chorangiosis (CH) and fetal hypoxia (FH). Placental weight percentile [30] and maturation of the parenchyma was evaluated. The maturation of the placenta was compared to the maturation as to be expected for the given gestational age, according to the normal maturation stages described by Benirschke [31]. When maturation was seen matching a lower gestational age than given; it was marked as 'partly immature'. When maturation was seen matching a higher gestational age than given, it was marked 'party hypermature' if there was also some maturation as to be expected; or 'hypermature' if all the villi showed an increased maturation.

2.4. sFlt/PlGF

The sFlt-1 and PlGF plasma or serum concentrations were measured on a B·R·A·H·M·S KRYPTOR compact PLUS (Thermo Fisher, Amsterdam, the Netherlands) according to the manufacturer's instructions.

2.5. Free thiol and IMA measurements

Serum free thiol (FT) concentrations (triplicate) were measured as previously described [32]. Both intra- and interday coefficients of variation (CV) of the FT measurements were <10%. For plasma IMA detection (duplicate), we used a rapid colorimetric method [33]. The method was modified using the principles of the IMA-assay of the Szybio assay (Szybio Biotech, Wuhan, China) and Lee et al. [34] To adjust for total albumin, the IMA/albumin ratio was used [35]. Plasma albumin (g/L) was determined with a Cobas c501 analyzer using the bromocresol green colorimetric assay (Roche diagnostics GmbH, Germany).

2.6. Inflammatory biomarkers

To analyze multiple inflammatory biomarkers, magnetic Luminex assays (R&D systems, USA) were used. Plasma samples were diluted in a 1:2 dilution in Calibrator Diluent RD6-52 (R&D systems, USA), a micro particle cocktail (R&D systems, USA) was added, and samples were incubated on a shaker overnight (800 rpm, 4 °C). After washing, Biotin-Antibody Cocktail (R&D systems, USA) was added and incubated on a shaker (1hr, 800 rpm, 20 °C). After washing, streptavidin-PE (R&D systems, USA) was added and samples were incubated (30 min, 800 rpm, 20 °C). Samples were washed and analyzed with a Luminex 100/200 system (R&D systems, USA) with xPONENT software (R&D systems, USA). A standard curve was created on each plate with each concentration measured in duplicate.

2.7. Statistical analysis

Data analysis was performed using SPSS Statistics 25.0 software package (SPSS Inc., USA) and data visualization using GraphPad Prism version 9.1 (Prism software, San Diego, CA, USA). Demographic and clinical characteristics were presented as means (standard deviation, SD), medians [interquartile ranges, IQR], or proportions *n* with corresponding percentages (%). Assessment of normality was performed using normal probability plots (Q-Q plots) and by plotting histograms. Differences between groups were tested using either Mann-Whitney *U*-tests or Kruskal-Wallis tests in case of continuous variables, while nominal variables were statistically compared using chi-square test or Fisher's exact test, as appropriate. Bonferroni corrections were applied to account for multiple testing. Correlations between continuous variables were calculated using Spearman's rank correlation coefficients (ρ). In comparative analyses using both cytokines and redox parameters, Bonferroni-adjusted p-values were log-transformed to facilitate results interpretation. Associations between correlated parameters were visualized in scatter plots using smoothed curves. Smoothing was empirically applied using nonlinear regression with 1st order polynomial curves with $1/y^2$ weighting. Receiver operating characteristics (ROC) analyses were performed to evaluate discriminative ability of the biomarkers with regard to the study groups. ROC curves were visualized

and the area under the curve (AUC) was calculated as an overall measure of classification performance. ROC curves and AUC values were calculated using the nonparametric, tie-corrected trapezoidal approximation method. Two-tailed p-values $\leq$ 0.05 were considered statistically significant.

3. Results

3.1. Cohort characteristics

Clinical characteristics and outcomes (Table 1) are aligned to the developed Core Outcome Set for FGR studies [36]. Median gestational age at inclusion was 33.4 weeks in the FGR group, 29.4 weeks in the FGR + PE group and 33.0 weeks in the healthy control group, with a significant difference of p = 0.03 between the FGR and the FGR + PE group. Mean maternal age was 30.4 years (SD 3.9), and 5 women (10%) reported smoking during pregnancy. Twelve women (25%) had hypertensive complications during pregnancy. Mean maternal body mass index (pre-pregnancy) was 26.7 kg/m^2 (SD 6.6). The body mass index was highest in the FGR + PE group (mean 30.1 kg/m^2; SD 7.8) compared to 24.3 kg/m^2 (SD 5.1) and 25.4 kg/m^2 (SD 4.8) in the FGR and healthy control group, respectively (p = 0.09). Mean Apgar score at 5 min was 8.8 (SD 1.8).

3.2. Placenta histology

In total, 36 placentas of 48 women (75%) were evaluated. This was partly due to the fact that not all women gave birth in our hospital, and to the fact that in nightly hours there was not always someone available to transport the placenta to the pathology lab. However, there was no significant difference in numbers of placenta's missing between the groups (p = 0.353). Of the examined placentas, 22 (61%) had a fixed, trimmed weight below the 10th percentile [30] (Fig. 1, **panel A**); 18 (82%) in the groups with obstetric conditions (FGR±PE) and 4 (18%) in the controls. Ten placentas (28%) showed a maturation appropriate for gestational age; 4 (40%) in the groups with obstetric conditions and 6 (60%) in the control group. 15 placentas (42%) showed a partially immature maturation and 11 (31%) a (partially) hypermature maturation for gestational age (10 (91%) in the groups with obstetric conditions and 1 (9%) in the control group) (Fig. 1, **panel B**). All placentas with a hypermature maturation for gestational age (n = 6, 17%) were found in the FGR + PE group. Due to the low number of cases in each subgroup we decided not to look at statistical differences for placental weight and maturation. As for the microscopic lesions (Supplementary Table S1), some placentas showed multiple lesions. The most commonly observed lesion in the FGR only group was fetal vascular malperfusion (FVM) (n = 4, 33%) (Supplementary Fig. S1A), followed by villitis of unknown etiology (VUE) (n = 3, 25%). All placentas (n = 9) from the FGR + PE group showed maternal vascular malperfusion (MVM) (Supplementary Figs. S1C and S1D), in three cases combined with high grade FVM. The most common lesion in the control group was chorioamnionitis (CA, n = 11), of which five cases (45%) showed a fetal response (FR) (Supplementary Fig. S1B). Fetal chronic hypoxia, diagnosed by an increase in fetal nucleated red blood cells, was mostly seen in the FGR + PE group. In the total study population, massive perivillous fibrinoid deposition (MPVFD) and chronic histiocytic intervillositis (CHIV) were diagnosed (as a combination) only once in the FGR group. No delayed villous maturation (DVM) or maternal floor infarction (MFI) was observed. As for placental weight and maturation, we decided not to look at statistical differences for the histopathologic diagnosis between the subgroups, due to the low number of each.

Table 1
Clinical characteristics and outcomes.

	FGR *(n = 14)*	FGR + PE *(n = 11)*	Controls *(n = 23)*
Mode of Birth			
-Spontaneous vaginal:	9 (64.3%)	2 (18.2%)	13/21 (61.9%)
-Assisted vaginal:	2 (14.3%)	0	2/21 (9.5%)
-Caesarian section:	3 (21.4%)	9 (81.8%)	6/21 (28.6%)
Fetal			
-Stillbirth	0	1 (9.1%)	0
-Livebirth	14 (100%)	10 (90.9%)	21/21 (100%)
Neonatal			
Gestational age at birth (weeks)	37.0 (2.5)	30.9 (3.1)	39.2 (1.5)
Preterm birth (<37 weeks gestation)	3 (21.4%)	10 (90.9%)	2/21 (9.5%)
Extremely preterm birth (<28 weeks gestation)	0	2 (18.2%)	0
Birthweight (g)	2168.6 (617.4)	1205.0 (423.2)	3390.7 (634.2)
Birthweight <10th percentile	11 (78.6%)	4/10 (40%)	3/21 (14.3%)
Birthweight <3rd percentile	7 (50%)	3/10 (30%)	1/21 (4.8%)
Need for mechanical ventilation			
-Additional oxygen only:	0	1 (9.1%)	0
-Mask ventilation:	2 (14.3)	5 (45.5%)	1/21 (4.8%)
-Endotracheal ventilation:	1 (7.1%)	2 (18.2%)	0
Necrotizing enterocolitis (Stage 2 or 3)	1 (7.1%)	1 (9.1%)	0
Hypoxic ischemic encephalopathy			
-IVH grade 1 or 2:	0	2 (18.2%)	0
-IVH grade 3 or 4:	1 (7.1%)	0	0
-PVL grade 1:	0	1 (9.1%)	0
-Other cerebral morbidity:	0	2 (18.2%)	0
Neonatal death	0	0	0

Data are presented in proportions n with corresponding percentages (%) or Standard Deviation (). Abbreviations: IVH: intraventricular hemorrhage. PVL: periventricular leukomalacia.

3.3. Classical biomarkers of oxidative stress are altered in pregnancy complications

The classical biomarkers for placenta insufficiency sFlt and PlGF, and the sFlt/PlGF ratio were compared between the three groups (Supplementary Table S2). sFlt showed a significant increase in the FGR + PE group compared to FGR only and healthy controls (p < 0.001). PlGF showed a significant decrease in the FGR + PE group compared to FGR only and healthy controls (p < 0.001). Lastly, the FGR + PE group showed a significant increase in the sFlt/PlGF ratio compared to FGR only and healthy controls (p < 0.001) (Fig. 3E).

3.4. Biomarkers of oxidative stress and metabolic inflammation are significantly altered in women with pregnancy complications

After correction for multiple comparisons, three biomarkers passed the significance threshold for -log-transformed adjusted p-values in comparisons of women with FGR + PE and healthy controls: FT, leptin, and the soluble form of receptor for advanced glycation end products (sRAGE) (Fig. 2). Serum FT levels were significantly lower in women with FGR + PE (median [IQR]: 177.9 [159.6; 194.2] μM) compared to women with only FGR (229.9 [219.2; 245.3] μM) and healthy controls (230.0 [20.7.7; 244.4] μM) (p < 0.001 for both) (Fig. 3A). Plasma levels of IMAR were significantly increased in women with FGR + PE (0.091 [0.085; 0.112]) compared to women with only FGR (0.082 [0.079; 0.088]) and healthy controls (0.084 [0.077; 0.089] (p < 0.05 for both) (Fig. 3B). Plasma leptin levels were significantly elevated in women with FGR + PE (47681 [20285; 74180] pg/mL) compared to women with only FGR (12583 [5538; 26475] pg/mL) and healthy controls (11294 [7454; 17107] pg/mL) (p < 0.05 and p < 0.01, respectively) (Fig. 3C). In addition, plasma levels of sRAGE were significantly higher in women with FGR + PE (1759 [1146; 2345] pg/mL) compared to healthy controls (799.1 [708.2; 893.7] pg/mL) (p < 0.01), though not compared to women with only FGR (929.4 [711.8; 1134] pg/mL) (p = 0.074) (Fig. 3D).

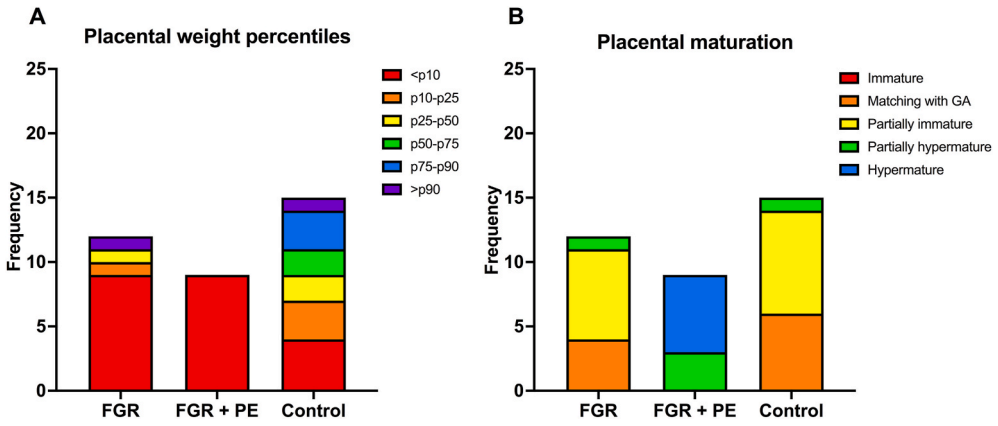

Fig. 1. Placental weight percentiles (A) and placental maturation (B). Data are presented in proportions *n*. Abbreviations: FGR, fetal growth restriction; PE, preeclampsia; GA, gestational age.

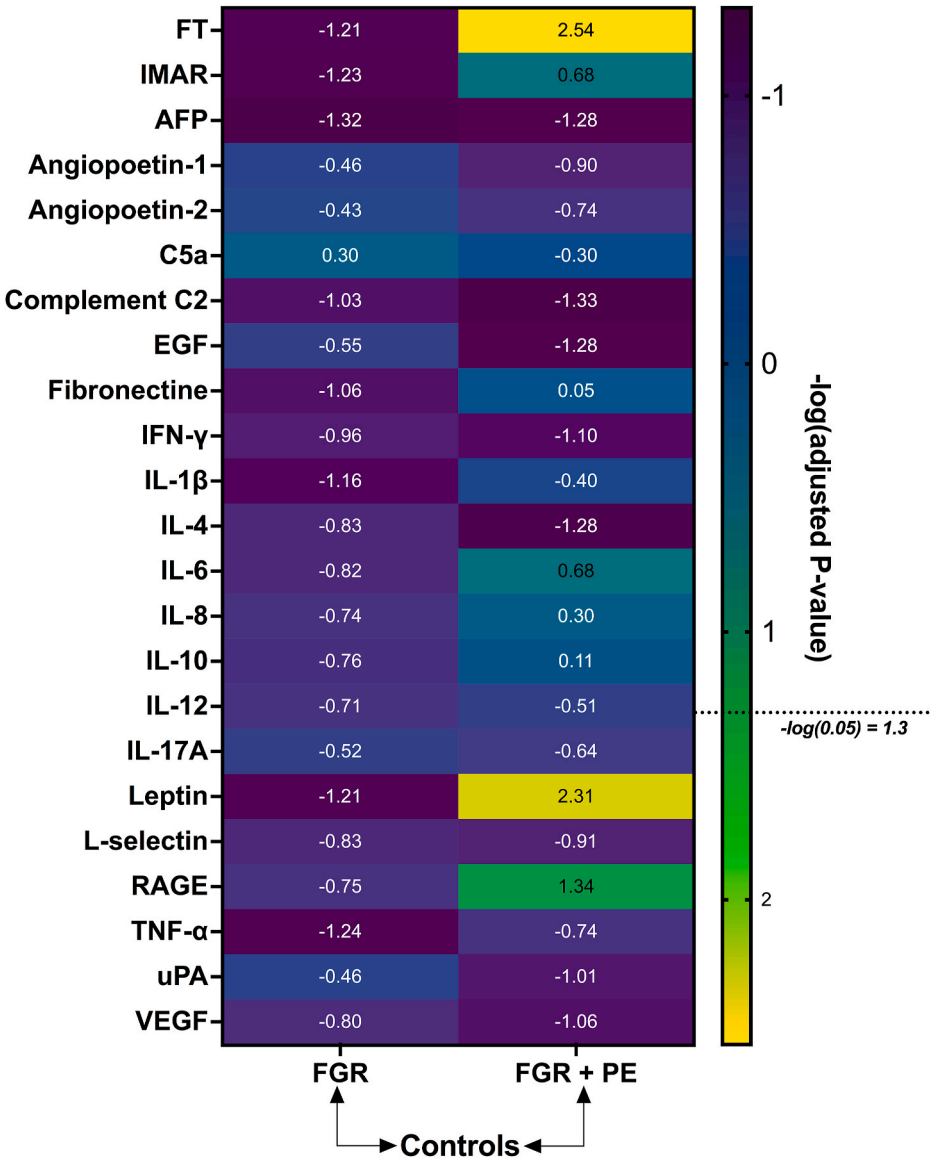

Fig. 2. Heatmap demonstrating -^{10}log-transformed Bonferroni-adjusted *P*-values for differences in serum biomarker levels between FGR vs. healthy controls (left panel) and FGR + PE vs. healthy controls (right panel). Values ≥ 1.3 indicate statistical significance after adjustment for multiple comparisons. Abbreviations: AFP, alpha fetoprotein; EGF, epidermal growth factor; FGR, fetal growth restriction; FT, total free thiols; IFN, interferon; IL, interleukin; IMAR, ischemia-modified albumin/albumin ratio; PE, preeclampsia; TNF, tumor necrosis factor; uPA, urokinase-type plasminogen activator; VEGF, vascular endothelial growth factor.

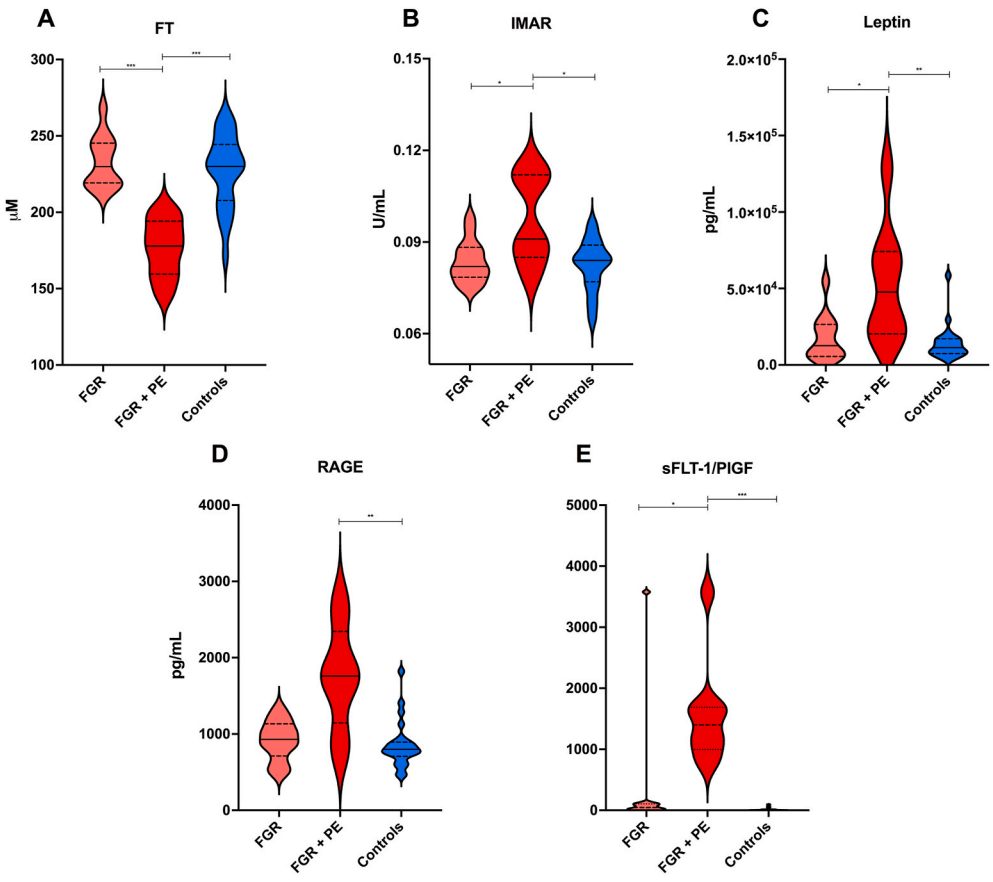

Fig. 3. (A–E): Biomarkers of oxidative stress and metabolic inflammation. Serum total free thiol (R–SH) levels are significantly decreased in mothers with FGR and concurrent PE (**A**), whereas plasma levels of ischemia-modified albumin (IMAR) are significantly increased in mothers with FGR and PE (**B**). Leptin levels in plasma are significantly higher in mothers with FGR + PE (**C**). Plasma levels of the soluble form of receptor for advanced glycation end products (RAGE) are significantly increased in mothers with FGR + PE compared to healthy controls (**D**). The sFlt/PlGF ratio is significantly increased in mothers with FGR + PE (**E**). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Abbreviations: FGR, fetal growth restriction; PE, preeclampsia; FT, total free thiols; IMAR, ischemia-modified albumin/albumin ratio; RAGE, receptor for advanced glycation end products.

3.5. Associations between biomarkers of oxidative stress and inflammation

All measured biomarkers of oxidative stress and inflammation were examined for interrelationships by computing a correlation matrix using Spearman's rank correlation coefficients (ρ) (Supplementary Fig. S2). Serum FT levels were significantly inversely correlated with IMAR ($\rho = -0.50$, $p < 0.001$), leptin ($\rho = -0.39$, $p = 0.01$) and sRAGE ($\rho = -0.57$, $p < 0.001$) (Fig. 4).

3.6. Associations between biomarkers of oxidative stress, blood pressure and proteinuria

Subsequently, serum FT levels were related to SBP and DBP in the study population (Fig. 5). Serum FT levels demonstrated a significant inverse association with systolic blood pressure (SBP, $\rho = -0.41$, $p < 0.01$, Fig. 5A). Similarly, with DBP, though less pronounced than with SBP, an inverse significant correlation was observed with serum FT ($\rho = -0.30$, $p < 0.01$, Fig. 5B). Lastly, patients with proteinuria showed a marked increase in IMAR levels (Fig. 5C) and a decrease in serum FT levels compared to non-proteinuric patients (Fig. 5D).

3.7. Discriminative accuracy of oxidative stress and inflammatory biomarkers regarding FGR and FGR + PE

Subsequently, the biomarkers of interest (FT, IMAR, leptin and sRAGE) were evaluated in terms of their discriminative accuracy to differentiate women with FGR or FGR + PE from healthy controls and FGR from FGR + PE (Fig. 6). None of the biomarkers showed significant discriminative ability to distinguish women with FGR from healthy controls (Fig. 6, panels A, D, G, J). In contrast, all of them demonstrated significant discriminative power to differentiate women with FGR + PE from healthy controls (Fig. 6, panels B, E, H, K) and FGR only (Fig. 6, panels C, F, I, L). For differentiation of FGR + PE from healthy controls,

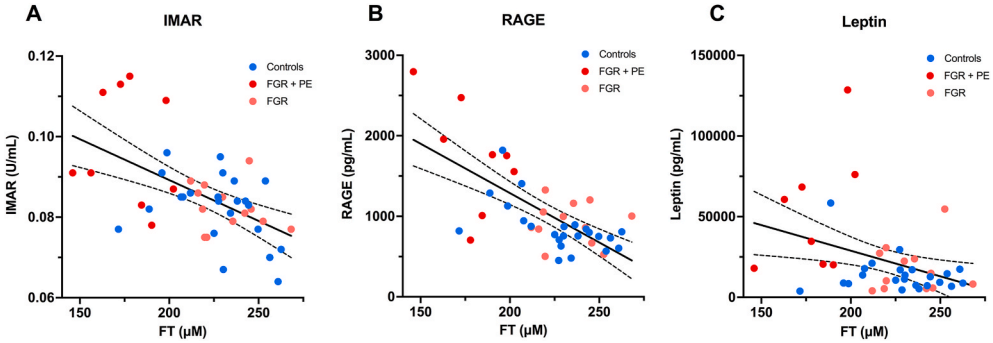

Fig. 4. (A–C): Associations between biomarkers of oxidative stress and inflammation. Serum FT levels were significantly inversely associated with IMAR (**A**). Similarly, serum FT levels were significantly inversely associated with the soluble form of receptor for advanced glycation end products (RAGE) and plasma leptin levels (**B–C**). Abbreviations: FGR, fetal growth restriction; PE, preeclampsia; FT, free thiols; IMAR, ischemia-modified albumin/albumin ratio; RAGE, receptor for advanced glycation end products.

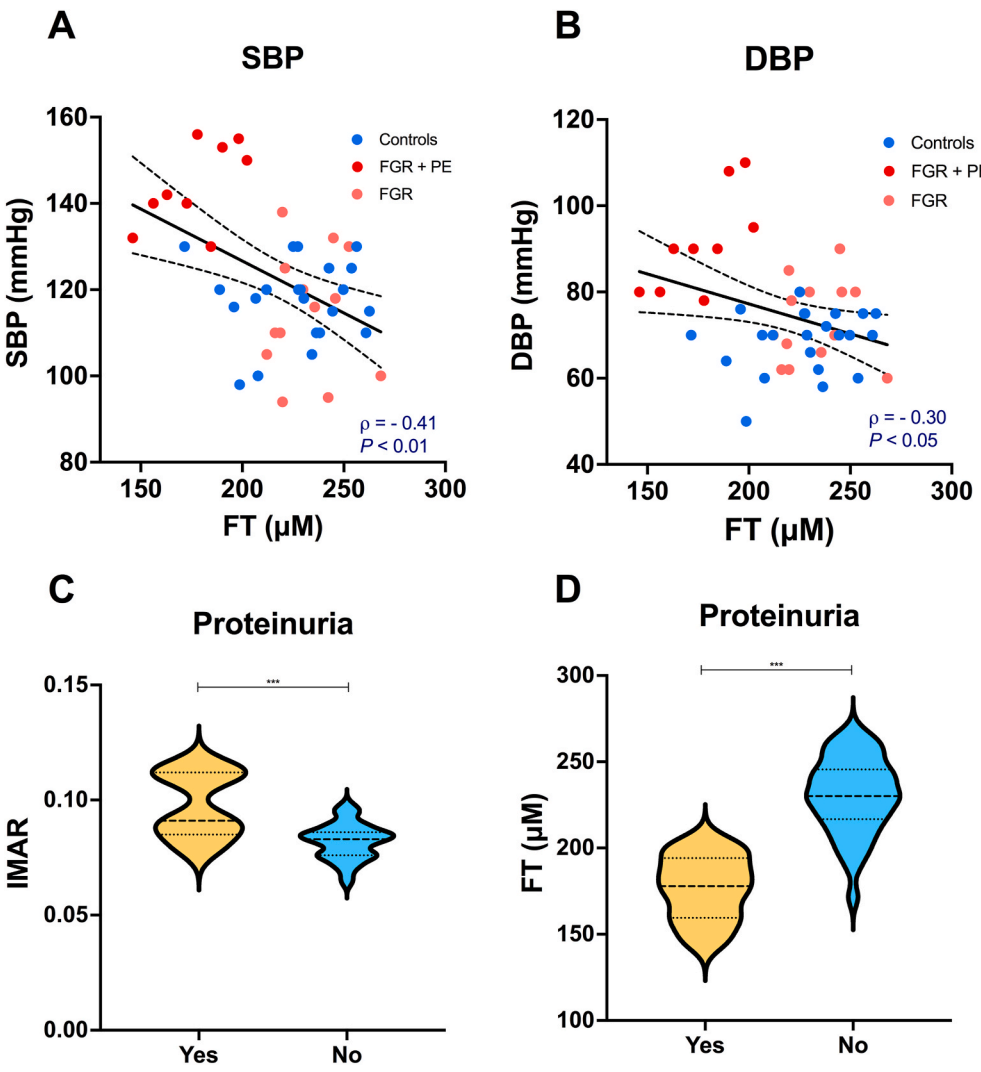

Fig. 5. (A–D): Associations between biomarkers of oxidative stress, blood pressure and proteinuria. Serum FT levels demonstrate a significant inverse association with SBP (**A**). Similarly, though less pronounced, serum FT levels show a significant inverse association with DBP (**B**). Mothers with proteinuria demonstrate a marked increase in IMAR levels compared to mothers without proteinuria (**C**). Mothers with proteinuria show a marked decrease in serum FT levels compared to mothers without proteinuria (**D**). Abbreviations: DBP, diastolic blood pressure; FT, free thiols; IMAR, ischemia-modified albumin/albumin ratio; SBP, systolic blood pressure.

serum FT (AUC = 0.94, p < 0.001, Fig. 6B) and leptin (AUC = 0.94, p < 0.001, Fig. 6K) levels showed the best discriminative capacity, whereas plasma IMAR (AUC = 0.80, p < 0.01, Fig. 6E) and sRAGE (AUC = 0.86, p < 0.01, Fig. 6H) levels showed less, though still reasonably good discrimination. Between women with FGR + PE and FGR only, serum FT level (AUC = 1.00, p < 0.001, Fig. 6C) showed the best discriminative capacity, whereas plasma IMAR (AUC = 0.80, p < 0.05, Fig. 6F), leptin (AUC 0.83, p < 0.05, Figure 6L) and sRAGE (AUC 0.87, p < 0.01, Fig. 6I) levels showed less, but still reasonably good discrimination.

4. Discussion

4.1. Principal findings

Serum FT levels are significantly reduced in mothers with combined FGR and PE, whereas plasma IMAR, leptin and sRAGE levels are significantly higher compared to mothers with uncomplicated pregnancies. According to existing literature, these differences are not attributable to the different gestational ages at which maternal blood was drawn, except for sRAGE in which sparse literature is contradicting [37–43]. The biomarker profile for women with only FGR is comparable to that of healthy controls, suggesting a different underlying pathological process compared to women with both FGR and PE.

4.1.1. Placenta pathology

Since the maturation of the placenta is related to the status of oxygenation over the syncytiovascular membrane and the oxygen supply from the maternal blood, it is no surprise that all placentas of women with both FGR and PE showed a (partially) hypermature maturation. Maternal vascular malperfusion (MVM), a hallmark of hypoxia and PE was also observed in all FGR and PE placentas. In contrast, placentas with solitary FGR and controls had virtually no MVM lesions. Solitary FGR could result from a variety of placental lesions, not only as a result of ischemia/hypoxia, but also by inflammation.

4.1.2. Serum FT and plasma IMAR levels

Serum FT levels were significantly reduced in pregnancies complicated with FGR and PE compared to mothers with uncomplicated pregnancies and mothers with FGR only. Our findings are compatible with previous studies [21,44,45]. Serum FT levels were also inversely related to SBP and DBP as well as reduced in mothers with proteinuria. Increased BP and proteinuria are pro-inflammatory conditions associated with endothelial activation. This may result in increased production of reactive species. IMAR levels were higher in mothers with PE and FGR, compared to mothers with uncomplicated pregnancies and FGR only. This is well in line with the assumption that an ischemic organ changes oxidizes albumin, causing a systemic increase. A recent meta-analysis found contradicting results, however, based on a random-effects model it was concluded that plasma IMAR

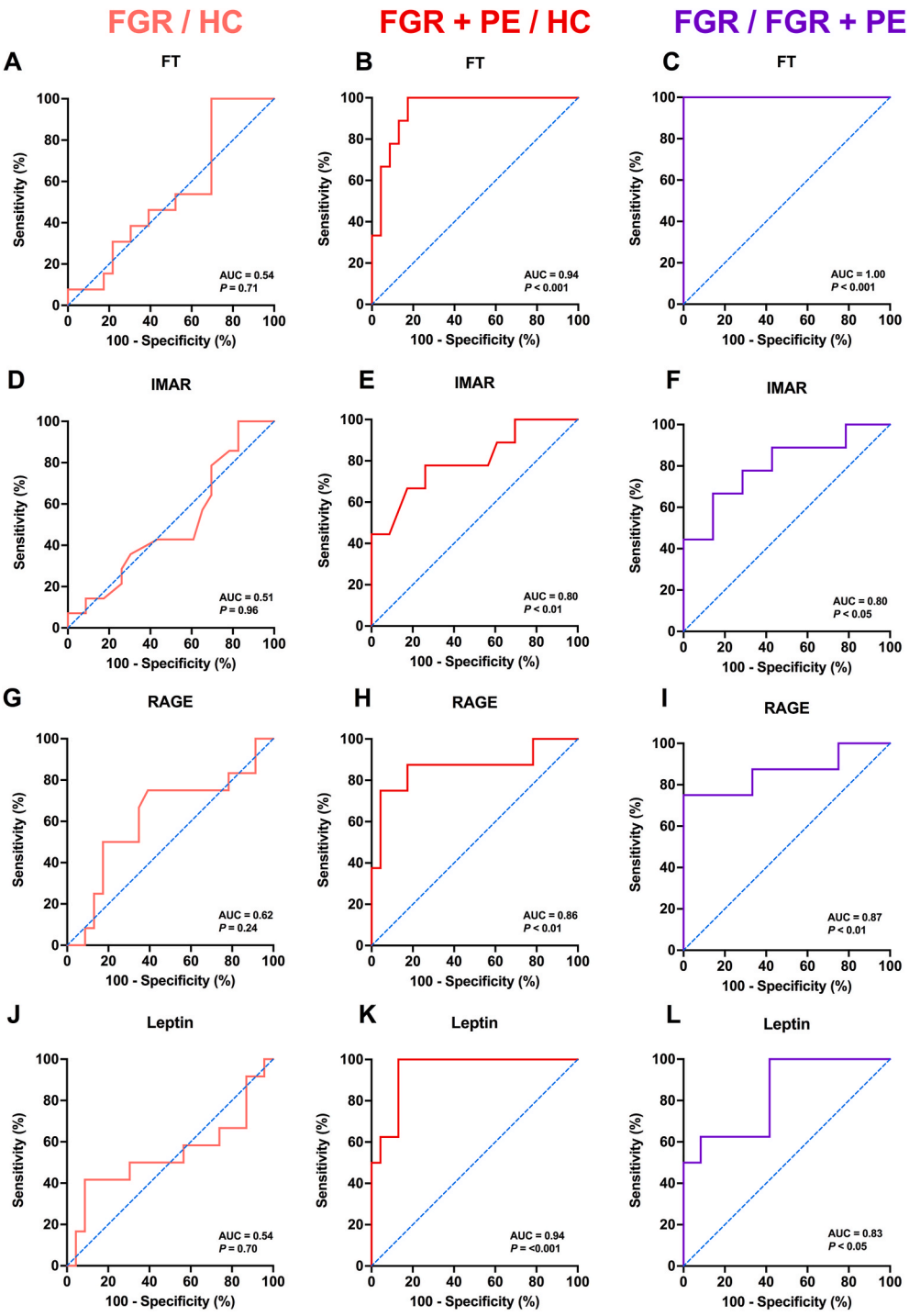

Fig. 6. (A–L): Discriminative accuracy of oxidative stress and inflammatory biomarkers. Discriminative capacity of serum and plasma biomarkers of oxidative stress (A–F) and metabolic inflammation (G–L) regarding fetal growth restriction (FGR) compared to healthy controls (HC) (left column); FGR combined with preeclampsia (FGR + PE) compared to HC (middle column); and FGR compared to FGR + PE (right column). Abbreviations: Abbreviations: AUC, area under the curve; FGR, fetal growth restriction; PE, preeclampsia; HC, healthy controls; R–SH/FT, total free thiols; IMAR, ischemia-modified albumin; RAGE, receptor for advanced glycation end products.

concentrations were indeed significantly higher in patients with PE compared to healthy controls [46]. Serum FT levels were inversely related to IMAR, compatible with results in literature [47]. Together, both findings in serum FT and plasma IMAR levels reflect the hypoxic status of pregnancies complicated with FGR and PE, compared to pregnancies complicated by FGR only and healthy controls.

4.1.3. Plasma mediators of pregnancy complications

In our cytokines multiplex analysis, we found leptin and sRAGE to be significantly different in the FGR and PE group compared to mothers with uncomplicated pregnancies. Leptin is secreted predominantly by adipose tissue and is involved in the pathogenesis of several disorders of reproduction and gestation [48]. Increased leptin levels are associated with higher BMI [49,50]. In our population, however, the BMI of the three groups were comparable. Previous studies have shown a significant increase in plasma leptin levels in women with PE compared to healthy controls [14,15]. It has also been suggested that plasma leptin levels increase prior to clinical symptoms of PE [15,48]. On the other hand, Doster et al. found no association between leptin levels and PE [51]. For FGR only, a significant increase in maternal plasma leptin levels has been found [52]. However, we demonstrated lower, not significantly different leptin levels in FGR only, indicating that maternal plasma leptin is a controversial marker for discriminating FGR.

RAGE is a multiligand member of the immunoglobulin superfamily

of receptors [53,54] and is expressed on the surface of various cells, including trophoblasts of placental villi [16]. RAGE binds to advanced glycation end products (AGEs) which are formed from reducing sugars and amino groups of proteins, lipids and nucleic acids [55]. Binding of AGEs to RAGE results in oxidative stress. Increased expression of RAGE protein has been found in preeclamptic placentas, whereas placentas of FGR complicated pregnancies showed a decrease in RAGE protein expression [16]. An increase in RAGE expression in trophoblasts of placentas of preeclamptic women has been found compared to healthy controls [56]. Since AGE and RAGE are fairly new investigated markers, not much is known about the soluble form of RAGE (sRAGE) in maternal plasma. In healthy pregnancy, no difference in sRAGE level was seen between second and third trimester of pregnancy [39] However, another study found significant differences in sRAGE levels between the second and third trimester in healthy pregnancies; with higher levels in second trimester [38] Also, a significant increase of sRAGE in maternal plasma in pregnancies complicated by PE compared to healthy controls has been found [17]. This is confirmed in our study, in which we demonstrated an increase of RAGE in maternal plasma in pregnancies complicated by FGR and PE compared to healthy controls and to FGR only. No studies of sRAGE in maternal plasma in pregnancies complicated by FGR only are known. Interestingly, sRAGE is also increased in the amniotic fluid of women with pregnancies complicated by intraamniotic infection and inflammation [57]; indicating sRAGE to be an inflammatory marker as well as a hypoxic marker.

4.2. Clinical implications

FGR and PE are often considered similar pathologies. FGR has a higher incidence than PE [1,58] and is more difficult to diagnose, especially when fetal size is within normal range [59]. Predictive or diagnostic markers are needed. In this study we did not find a marker that is indicative of FGR only. We demonstrated that in addition to classical biomarkers for oxidative stress, both serum FT and leptin levels had significant discriminative ability to differentiate mothers with both FGR and PE from mothers with uncomplicated pregnancies and from mothers with FGR only. In addition, plasma IMAR and sRAGE levels showed reasonable, though less prominent discriminative capacity, but none of the analyzed biomarkers could differentiate mothers with FGR from mothers with uncomplicated pregnancies. Since the FGR only biomarker profile in our study equals that of healthy controls, this also indicates a different underlying pathological process. This is important as the underlying placental pathology (MVM) in FGR is often considered to be equal to the underlying MVM lesion in PE. Unfortunately, this further complicates our search for relevant biomarkers in FGR only.

4.3. Research implications

The findings in this pilot study can be used to validate the findings in prospectively collected (cohort) samples prior to development of PE and FGR to determine their potential role in disease prediction. In future studies, larger patient groups are needed to confirm our findings. With a larger sample size, it could also be possible to determine approximate cut-off values for the various biomarkers to predict the likelihood of developing FGR and/or PE in pregnant women.

4.4. Strengths and limitations

This pilot study has limited inclusions. Nevertheless, we do think that the prospectively collected data can help guide future marker analysis for FGR. We did not take samples from the study participants prior to clinical onset of their pregnancy complications. As such, we were not able to compare current data of the affected mothers to their previous health status or use the markers as a prediction tool; we do however appreciate the potential option. Another limitation of the present study includes the absence of mothers with PE, but without FGR, to further differentiate between the systemic redox status of PE and FGR separately in concert with their placental lesions. Unfortunately, it was impossible for us to include these patients in our clinical setting, as we do not have specialized medium care for neonates between 32 and 35 weeks of gestation and PE in patients admitted to our hospital manifests rather early during pregnancy and is thus usually combined with FGR. Late onset PE is associated with larger rather than smaller fetuses but was not anticipated at this study's inclusion process [60]. Furthermore, the small sample size of this study warrants careful interpretation of the data. The small sample size across the subgroups did not provide sufficient statistical power to reliably adjust for confounding factors like the moment (gestational age) of sampling. Also, because of a relatively small number of collected placentas in each subgroup, we were not able to look at differences for placental weight and maturation, or to select only cases with one placental lesion each. Therefore, to compare the amount of oxidative stress as reflected by IMAR and FT levels to a specific placental lesion was not possible.

5. Conclusions

Serum free thiols and plasma ischemia-modified albumin were decreased and increased in patients with FGR + PE, respectively. Similarly, we showed that leptin and sRAGE were significantly increased in the same group, as compared to healthy controls and pregnancies complicated by FGR only. Our results were strengthened by confirmation that these individual biomarkers also correlated significantly with each other. More longitudinal studies with larger sample sizes are warranted, both to test the potential pitfalls of our study, and to further disentangle the interplay between the placenta, hypoxia and inflammatory processes. These further studies could also add in determining the prognostic value of these biomarkers. In this respect, our identified biomarkers harbor considerable potential in these future studies and our findings can be used to study their potential role in pregnancy disease prediction.

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Declaration of competing interest

None.

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Appendix A. Supplementary data

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