Placental Exosomes as Early Biomarker of Preeclampsia: Potential Role of Exosomal MicroRNAs Across Gestation

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Context: There is a need to develop strategies for early prediction of patients who will develop preeclampsia (PE) to establish preventive strategies to reduce the prevalence and severity of the disease and their associated complications.

Objective: The objective of this study was to investigate whether exosomes and their microRNA cargo present in maternal circulation can be used as early biomarker for PE.

Design, Setting, Patients, and Interventions: A retrospective stratified study design was used to quantify total exosomes and placenta-derived exosomes present in maternal plasma of normal (n = 32 per time point) and PE (n = 15 per time point) pregnancies. Exosomes present in maternal circulation were determined by nanoparticle tracking analysis. An Illumina TruSeq® Small RNA Library Prep Kit was used to construct a small RNA library from exosomal RNA obtained from plasma samples.

Results: In presymptomatic women, who subsequently developed PE, the concentration of total exosomes and placenta-derived exosomes in maternal plasma was significantly greater than those observed in controls, throughout pregnancy. The area under the receiver operating characteristic curves for total exosome and placenta-derived exosome concentrations were 0.745 ± 0.094 and 0.829 ± 0.077, respectively. In total, over 300 microRNAs were identified in exosomes across gestation, where hsa-miR-486-1-5p and hsa-miR-486-2-5p were identified as the candidate microRNAs.

Conclusions: Although the role of exosomes during PE remains to be fully elucidated, we suggest that the concentration and content of exosomes may be of diagnostic utility for women at risk for developing PE. (***J Clin Endocrinol Metab*** **102: 3182–3194, 2017)**

Each year, 10 million women worldwide develop preeclampsia (PE) and approximately 76,000 die of PE and related hypertensive disorders. The number of babies who die of these disorders is thought to be in the order of 500,000 per annum (1). PE and its related diseases are some of the most serious complications of pregnancy, with a prevalence of 3% to 5%, and are responsible for nearly 40% of premature births delivered before 35 weeks of gestation (2). PE is classically defined as a new onset of hypertension during the second half of pregnancy, but it has also been strongly associated with an increased risk of later-life death due to cardiovascular

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Abbreviations: ANOVA, analysis of variance; BMI, body mass index; GDM, gestational diabetes mellitus; NTA, nanoparticle tracking analysis; PBS, phosphate-buffered saline; PCA, principal component analysis; PE, preeclampsia; PI, pulsatility index; PLAP, placental alkaline phosphatase; PPV, posterior probability value; ROC, receiver operating characteristic; w/v, weight-to-volume ratio.

disease, independent of other risk factors (3–5). It is widely accepted that PE begins with an asymptomatic phase during the first trimester of gestation, characterized by a deficient trophoblast invasion and an incomplete spiral artery remodeling process. Both these processes contribute to an increase in oxidative stress and the development of systemic endothelial dysfunction leading to the characteristic clinical manifestation of PE in the later phases of the disease. Currently, there is no effective treatment to prevent the long-term consequences of PE, mainly because the pathophysiology is not well understood and is not detected early enough.

Recent studies have highlighted the utility of tissue-specific extracellular vesicles in the diagnosis of PE (6–8). Exosomes are extracellular vesicles that originate from the endosomal compartment with capacity to incorporate specific molecules in response to microenvironment milieu (9). Exosomes are small (40 to 100 nm), very stable (10) lipid bilayer nanovesicles that are formed by the inward budding of multivesicular bodies. Although little is known about the mechanism of packaging, exosomes contain a diverse array of signaling molecules that are released from the parent cell following the exocytotic fusion of multivesicular bodies with the cell membrane (11). Once exosomes are released into the extracellular space, some of these exosomes reach the systemic circulation to interact with distant tissues in the body. Thus, exosomes are involved in cell-to-cell communication. Exosomes present in blood represent a noninvasive biopsy of their tissue of origin and a new approach to the development of screening tests (9).

Exosomes are selectively packaged with signaling molecules such as microRNAs and proteins. MicroRNAs are small noncoding RNA fragments that are approximately 22 nucleotides in length (12) and regulate gene expression by binding to the 3′ end of the messenger RNA and modulating its translation. It has been established that exosomes can transfer microRNAs to target cells and influence their biological function (13). Several studies have reported dysregulation of circulating microRNAs in PE (14–22). A limitation of these studies has been the use of whole plasma or serum for analysis. Interestingly, approximately 60% of the circulating microRNA is associated with exosomes, and the microRNAs in exosomes are resistant to RNase treatment (23). Currently, there have been no studies that have assessed the microRNA profile in exosomes derived from PE pregnancies.

Recently, we used the well-established and validated method of buoyant density centrifugation to obtain an enriched exosome fraction that minimized the contamination of other extracellular vesicles (24–26). We established that placental-derived exosomes are present in the maternal circulation and that their concentration increases throughout the normal pregnancy (24, 26). Moreover, the total number of exosomes present in maternal plasma is approximately twofold greater in women between 11 to 14 weeks gestation who were subsequently identified as having gestational diabetes mellitus (GDM) (diagnosed between 22 and 28 weeks) than women who experienced a normoglycemic pregnancy (24). These data suggest that changes in the exosome concentration present in maternal circulation at early stages of pregnancy are a contributing factor in the development of pregnancy complications (*e.g.*, GDM and PE) later in gestation. The gestational age profile of exosome concentration (both total and placenta-derived) in the maternal circulation of presymptomatic women who subsequently develop PE later during pregnancy has not been investigated.

Previous *in vitro* studies demonstrated that exosomes released from placental cells and their content are regulated by oxygen tension (27–29). Hypoxia induces the release of exosomes from trophoblast cells, affecting both content and interaction with other cells. PE is associated with placental hypoxia that leads to insufficient remodeling of the spiral artery during the first 20 weeks of pregnancy (30). These observations support the putative utility of placenta-derived exosomes as an early pregnancy biomarker of a woman's risk of developing PE. To date, little is known about the exosome profile during PE pregnancies. In this study, we compare the total and placental exosomes between normal and PE pregnancies throughout gestation, from first to third trimester. In addition, we investigated the microRNA profiles within these exosomes across gestational age.

Subjects and Methods

Study group and samples

A prospectively collected, retrospectively stratified case control experimental design was used to establish pregnancy-associated change in exosome concentration in maternal blood obtained from normal and PE pregnancies. Women were recruited between January 2008 and December 2010 with informed written consent by research midwives from the Hospital Parroquial de San Bernardo, Santiago, Chile. Serial blood samples (BD Vacutainer® PLUS Tubes; EDTA) at 11 to 14 (early), 22 to 24 (mid), and 32 to 36 (late) weeks were collected, and women were prospectively followed as previously described (24). Samples from these patients were collected at three time points during pregnancy, and only women with a complete set of samples (*i.e.*, at early, mid, and late gestation from the same women) were included in this study (n = 47) (Supplemental Fig. 1). Both groups consisted of women with singleton gestation, and none of them took multivitamins or aspirin during pregnancy. Controls were healthy subjects without pregnancy

complications or chronic medical problems and did not differ in racial origin from PE patients. Gestational age at individual study visits and at delivery was calculated based on last menstrual period and confirmed by first trimester ultrasound.

Uterine artery Doppler, clinical variables, and pregnancy outcomes were recorded. Subjects were stratified into normal healthy pregnant women (n = 32; 3 samples during gestation, total samples = 96) and PE (n = 15, 3 samples during gestation, total samples = 45) patients, matched for gestational age. PE was defined as new-onset hypertension (blood pressure ≥140/90 mm Hg on two separate occasions at least 6 hours apart or blood pressure ≥160/110 mm Hg) and proteinuria (>300 mg/24 hours) after 20 weeks of gestation in previously normotensive women. Human plasma samples were obtained in accordance with the Declaration of Helsinki and approved by the Ethics Committee of The University of Queensland. Doppler ultrasound examinations of the umbilical artery (left and right arteries) were performed across pregnancy according to the methods described in our previously published studies (24, 26). Plasma samples were stored at −80°C until analyses. All experimental procedures were conducted within an ISO17025-accredited (National Association of Testing Authorities, Australia) research facility. All data were recorded within a 21 CRF part 11–compliant electronic laboratory notebook (LabArchives).

Isolation of exosomes from maternal circulation

Exosomes were isolated from plasma as previously described (24–26) (Supplemental Fig. 2). In brief, plasma was diluted with an equal volume of phosphate-buffered saline (PBS) (pH 7.4) and centrifuged at 2000*g* for 30 minutes at 4°C (Sorvall® high-speed microcentrifuge, fixed rotor; Thermo Fisher Scientific, Asheville, NC). The 2000*g* supernatant fluid was then centrifuged at 12,000*g* for 45 minutes at 4°C (Sorvall® high-speed microcentrifuge, fixed rotor). The resultant supernatant fluid (2 mL) was transferred to an ultracentrifuge tube (Beckman; 10 mL) and centrifuged at 100,000*g* for 2 hours (Sorvall® T-8100 fixed ultracentrifuge rotor).

The 100,000*g* pellet was suspended in PBS (10 mL) and filtered through a 0.22-μm filter (Steritop™; Millipore, Billerica, MA) and then centrifuged at 100,000*g* for 2 hours. The 100,000*g* pellet was resuspended in 500 μL PBS and layered on the top of a discontinuous iodixanol gradient containing 40% (weight-to-volume ratio [w/v]), 20% (w/v), 10% (w/v), and 5% (w/v) iodixanol and centrifuged at 100,000*g* for 20 hours. Solutions were made by diluting a stock solution of OptiPrep™ (60% [w/v] aqueous iodixanol from Sigma-Aldrich). An exosome-containing fraction (density, 1.12 to 1.19 g/mL) was collected, diluted with PBS, and centrifuged at 100,000*g* for 2 hours at 4°C. Finally, the pellet containing the enriched exosome population was resuspended in 50 μL PBS. Exosomes were characterized by measuring the size distribution, detecting TSG101 (ab125011) expression, and examining morphology using nanoparticle tracking analysis (NTA), Western blot, and transmission electron microscopy, respectively.

Quantification of total exosomes and placenta-derived exosomes

The concentrations of total and placental exosomes in maternal circulation were quantified using NTA (NanoSight) and placental alkaline phosphatase (PLAP) enzyme-linked immunosorbent assay kits as previously described (25, 26). PLAP is a syncytiotrophoblast-specific marker. Therefore, exosomes derived from placental origin are positive for PLAP.

Exosomal RNA isolation

Exosomal RNA was extracted using the miRNeasy Mini Kit (Qiagen, Brisbane, Australia) and TRIzol LS Reagent (Life Technologies, Brisbane, Australia) as the lysis solution. The manufacturer's protocol was followed, with slight modifications in accordance with a protocol used previously (23). A spectrophotometer (SPECTROstar Nano Microplate Reader; BMG LABTECH) was used to quantify RNA concentration. Following a cleanliness check and blank measurement using RNase-free water, 2 uL of sample was pipetted onto each microdrop well on an LVis plate. RNA concentration was measured using MARS Data Analysis microplate reader software.

Small RNA next-generation sequencing

Sequencing libraries were generated using the Illumina TruSeq® Small RNA Library Prep Kit, according to the manufacturer's instructions. A total of 100 to 300 ng of exosomal RNA was used as input for library preparation. These RNA samples were barcoded by ligation with unique adaptor sequences to allow pooling of samples in groups of 24. Subsequently, these ligated samples were reverse transcribed, polymerase chain reaction amplified, and size selected using gel electrophoresis. Finally, the DNA libraries were eluted from the gel pieces overnight in 200 μL nuclease-free water. The elution containing the pooled DNA library was further processed for cluster generation and sequencing using the Illumina NextSeq 500 high-output kit (75 cycles) and the Illumina NextSeq 500 sequencing platform, respectively.

Identification, normalization, and statistical analysis of microRNAs

Initially, raw FASTQ files were processed to remove barcode and adaptor sequences. Subsequently, this file was analyzed using the miRDeep2 program to identify known microRNAs (31). The miRDeep2 algorithm requires a genomic index and microRNA database to perform analysis. The human genome (version 19) prebuilt index was obtained from the bowtie website (http://bowtie-bio.sourceforge.net/index.shtml). The microRNA reference database (version 20) was obtained from the miRBase website (www.mirbase.org) (32). Subsequently, raw counts and corresponding microRNAs underwent normalization and statistical analysis using the DESeq2 package in R (33). This package uses the median ratio method for normalization and applies a generalized linear model for differential expression. Statistical analysis and significance were calculated using a Wald test, corrected for multiple testing using the Benjamini and Hochberg procedure. An adjusted P value of <0.01 was designated as statistically significant. Sequencing data have been deposited in the Gene Expression Omnibus database (GSE94721).

Gene target and gene ontology analysis

Gene target identification for microRNAs was performed using the CyTargetLinker application in Cytoscape. Firstly, candidate microRNAs identified from the sequencing data were imported into Cytoscape. A total of three microRNA gene target databases (MicroCosm, miRTarBase, and TargetScan) were allocated to Cytoscape for analysis. Subsequently, gene targets were filtered to select for those that have been identified to be

targeted by the same microRNA within at least two databases. These filtered genes were subjected to gene ontology analysis using the BiNGO application in Cytoscape. Statistics were performed using a hypergeometric test and corrected for multiple testing using the Benjamini and Hochberg procedure. An adjusted P value of <0.05 was designated as statistically significant, where the increasing color of the gene ontology node (from yellow to orange) is proportional to increasing statistical significance, whereas a white gene ontology node is not statistically significant.

Statistical analysis and power

Data are presented as mean ± standard deviation, with n = 32 (controls) and n = 15 (PE = cases) different patients per group (*i.e.*, early, mid, and late gestation; total samples for PE group = 45 and for the normal group = 96). The effects of gestational age on plasma exosome number and exosomal PLAP concentrations were assessed using repeated-measures two-way analysis of variance (ANOVA), with the variance partitioned between gestational age and condition. Thus, gestational age was treated as an independent factor. Statistical differences between groups were identified by *post hoc* analyses (Bonferroni tests) to compare between PE and normal pregnancies. For two-group analyses (paired data), Student *t* tests were used to assess statistical difference. Linear regression analysis was used to assess the relationship between continuous variables [*i.e.*, between pulsatility index (PI), placental weight, and glucose level with exosomes]. Statistical significance was defined as a P value of <0.05. Statistical analyses were performed using Stata 11 (StatCorp, College Station, TX) and Prism 6 (GraphPad, La Jolla, CA).

With respect to total exosome number, the experimental designs described previously, at a significance level of $\alpha = 0.01$, with size effect of 1.74 and control-to-PE sample ratio of 2.1 achieve a power of 0.88. With respect to total placenta-derived exosomes, the experimental designs described previously, at a significance level of $\alpha = 0.01$, with size effect of 2.36 and control-to-GDM sample ratio of 2.1 achieve a power of 0.92. A multivariate modeling approach was used to develop a proof-of-principle classification model for PE. LogitBoost regression analysis was used for modeling within the Weka package (version 3.6) (34). The implemented classification algorithm reported a predicted posterior probability value [(PPV); *i.e.*, the likelihood that a sample came from a woman with PE] for each patient sample. PPV values were used to generate the receiver operating characteristic (ROC) curve, and the area under the curve was calculated within the Weka analysis package.

Results

Patient cohort data

All women were nonsmokers and had singleton pregnancies without intrauterine infection. Women in the PE group were 4 years older, had a greater body mass index (BMI) (20%) and PI (35%, during first and second trimesters), and delivered babies of lower birth weight (9.6%) than women in the control group (Table 1). No statistically significant differences in gestational age at delivery, mode of delivery, or fetal sex ratio were identified (Table 1).

Exosome isolation and characterization

The characteristics of exosomes isolated and purified using a well-established and validated method (35, 36) are presented in Fig. 1. NTA identified particles with a diameter of 40 to 130 nm [Fig. 1(a–c)]. Transmission electron microscopy identified vesicles with a cup shape morphology that is characteristic of an exosome [Fig. 1(d)]. Furthermore,

Table 1. Clinical Characteristics of Patients and Newborns

	Normal (n = 32)	PE (n = 13)
Maternal variables		
Age (years)	25 ± 1.2 (18–36)	29 ± 1.6 (18–40)[a]
Weight (kg)	64 ± 2.2 (46–93)	76 ± 4.6 (53–124)[a]
Height (cm)	158 ± 1.0 (150–168)	157 ± 1.5 (149–168)
BMI (kg/m^2)	25 ± 0.8 (20–37)	30 ± 1.4 (22–38)[b]
Type of delivery		
Vaginal	19	5
Caesarean	13	8
Early gestation (weeks)	12 ± 0.1 (11–14)	12 ± 0.2 (11–14)
Mid gestation (weeks)	23 ± 0.1 (21–24)	23 ± 0.2 (21–24)
Late gestation (weeks)	33 ± 0.11 (31–34)	32 ± 0.18 (32–34)
Gestational age at delivery (weeks)	39 ± 0.15 (38–41)	38 ± 0.27 (37–39)
Uterine artery Doppler (PI, right and left uterine artery)		
Early gestation	1.48 ± 0.12 (0.54–2.8)	2.0 ± 0.23 (0.88–4.0)[a]
Mid gestation	0.89 ± 0.05 (0.37–1.5)	1.2 ± 0.11 (0.52–1.9)[b]
Late gestation	0.62 ± 0.04 (0.39–1.3)	0.79 ± 0.09 (0.35–1.33)
Newborn variables		
Placental weight (g)	603 ± 16 (434–758)	600 ± 31 (418–780)
Fetal weight (g)	3524 ± 71 (2830–4830)	3369 ± 223 (2340–3800)[a]
Fetal height (cm)	50 ± 0.25 (48–54)	50 ± 0.25 (48–54)
Fetal sex (male/female)	13/19	7/6

[a] $P < 0.05$.

[b] $P < 0.001$.

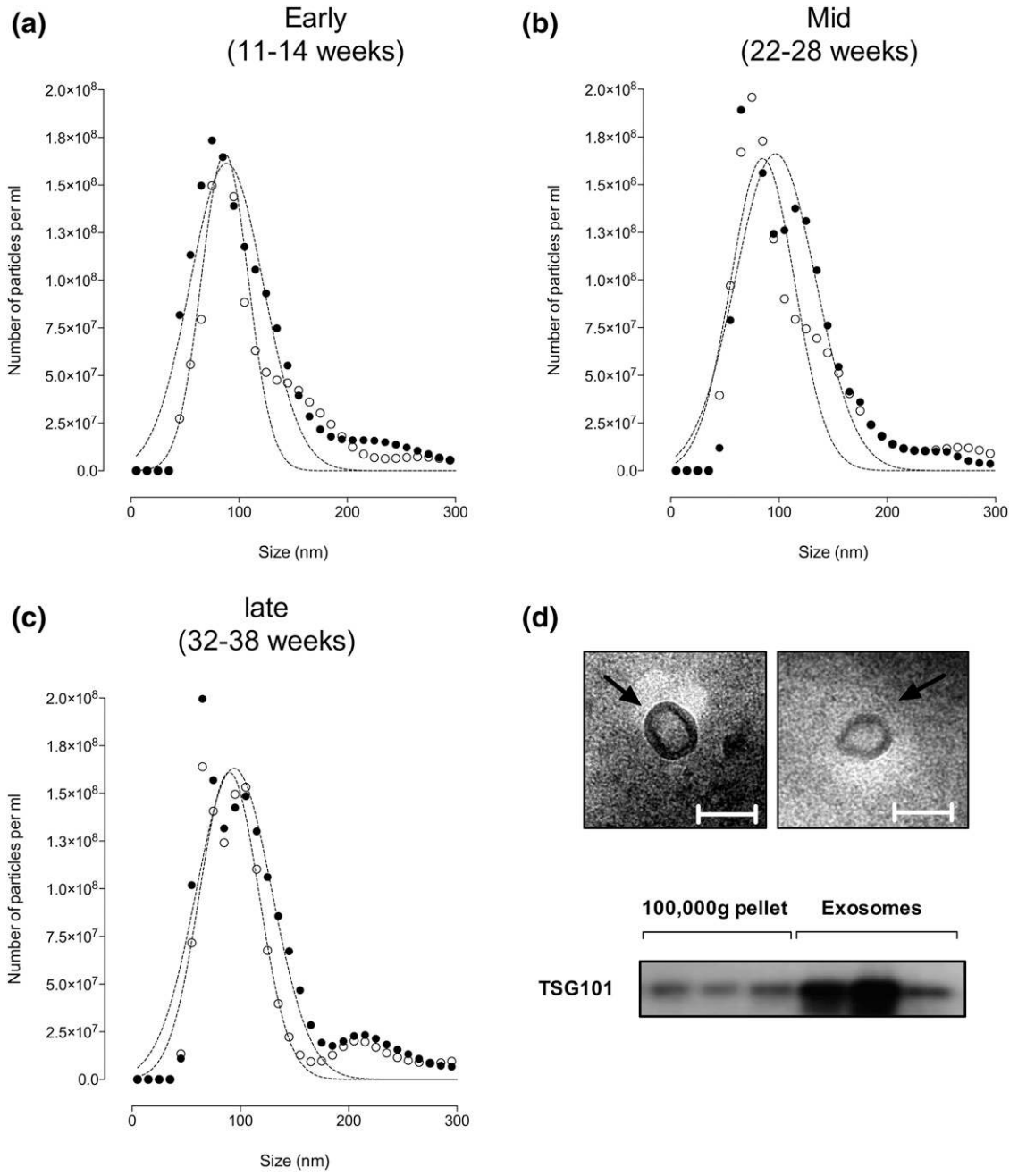

Figure 1. Characterization of exosome from PE pregnancies. Exosomes were isolated from maternal plasma during early (*i.e.*, 11 to 14 weeks), mid (*i.e.*, 22 to 28 weeks), and late (*i.e.*, 32 to 38 weeks) gestation by differential and buoyant density centrifugation from normal (white circles) and PE (black circles) pregnancies. (a–c) Representative exosomes enriched fraction size distribution isolated from maternal circulation across the pregnancy using a NanoSight NS500 instrument. (d) Representative electron micrograph of exosome fractions (pooled enriched exosome population from fractions 4 to 8). (e) Representative Western blot for exosome enriched marker TSG101. Scale bar = 100 nm.

exosomes were positive for exosomal protein marker TSG101 [Fig. 1(e)] (37). No significant differences between the NTA characteristics of exosomes isolated from normal and PE pregnancies were identified (Supplemental Table 2).

Effect of gestational age and clinical status on maternal plasma exosome concentration

To determine the effect of gestational age and clinical status on exosome concentration in maternal plasma during pregnancy, exosome-containing fractions were pooled and the total number of exosomes was determined by NTA (Fig. 2). The concentration of exosome-associated PLAP in maternal plasma was determined by enzyme-linked immunosorbent assay and presented in Fig. 2.

In presymptomatic women who subsequently developed PE, the concentration of exosomes in maternal plasma was significantly greater than that observed in controls throughout pregnancy [Fig. 2(a)]. The concentration of

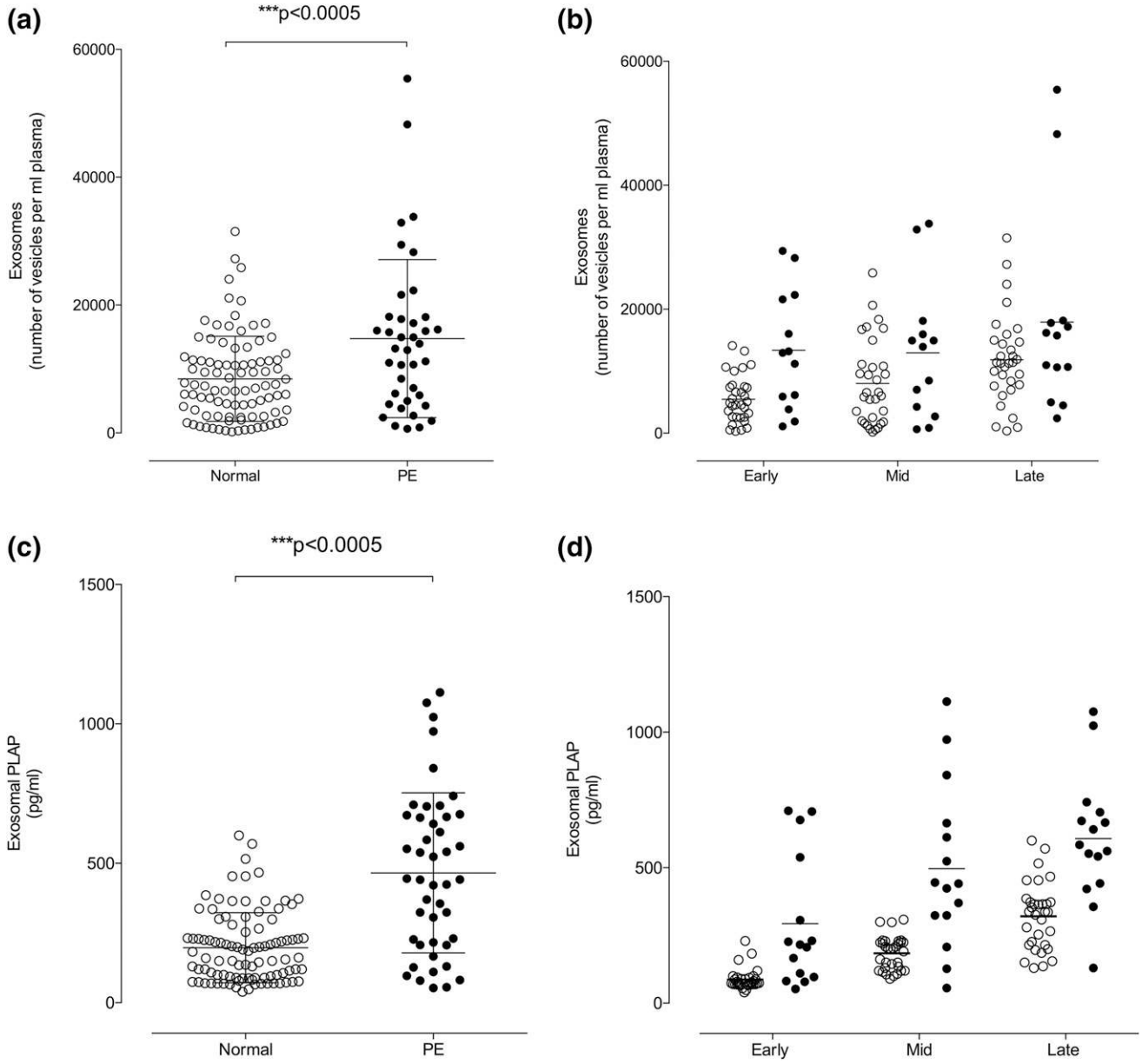

Figure 2. Exosome profiling across PE pregnancies. Enriched exosomes population were quantified in peripheral plasma of women with normal (white circles) and PE (black circles) pregnancies across gestation. (a) Total exosome number presented as the average across early, mid, and late gestation. (b) Gestational age variation in plasma exosome number across pregnancy. (c) Placenta-derived exosome variation is presented as the average across early, mid, and late gestation. (d) Gestational age variation in placenta-derived exosomes across pregnancy. Data are presented as an aligned dot plot, and values are mean ± standard deviation.

exosomes present in the maternal circulation increased 2.2-fold in the control group over gestation ($P = 0.0001$) [Fig. 2(b)]. No effect of gestational age on the total number of exosomes in maternal plasma was identified in the PE group. At 11 to 14 weeks, exosome concentration was 2.4-fold higher in the PE group than in the control group [Fig. 2(b)]. Statistically significant independent effects of gestational age ($P = 0.0052$) and clinical status ($P < 0.0001$) on the concentration of exosomes in maternal plasma were also confirmed by two-way ANOVA.

Similarly, the concentration of exosomal PLAP in maternal plasma was significantly greater than that observed in controls throughout pregnancy [Fig. 2(c)]. The concentration of placenta-derived exosomes in maternal plasma increased 3.7-fold and 2.1-fold in the control and PE groups, respectively, over gestation ($P = 0.0001$) [Fig. 2(d)]. At 11 to 14 weeks, the concentration exosomal PLAP was 3.4-fold higher in the PE group than in the control group [Fig. 2(d)]. Statistically significant independent effects of gestational age ($P < 0.0001$) and clinical status ($P < 0.0001$) on the concentration of exosomal PLAP in maternal plasma were confirmed by two-way ANOVA with repeat measures and with *post hoc* pairwise comparisons using Bonferroni multiple

comparisons tests (*i.e.*, normal compared with PE). As the maternal BMI was higher in PE compared with normal pregnancies, we did an analysis of covariance using maternal BMI and total or placental exosomes as continuous variables. A significant effect of gestational age (early, mid-, or late gestation) and condition (*i.e.*, normal or PE) on the exosome concentration was identified; however, there was no significant effect of maternal BMI ($P = 0.12$).

Exosomes and physiological correlates

To establish whether changes in the uteroplacental circulation are associated with exosome concentrations across gestation, the association between PI and exosome concentration and PLAP was determined. Linear regression analysis identified a negative association between PI and total exosomes for the normal group ($P = 0.0415$), whereas no significant association was observed for the PE group ($P = 0.6991$) [Supplemental Fig. 3(a)]. Furthermore, linear regression analysis identified a negative association between PI and exosomal PLAP concentration for both normal ($P < 0.0001$) and PE ($P = 0.001$) groups, where approximately 31% and approximately 26% of the variance in exosomal PLAP is accounted for by variation in PI for normal pregnancies and PE pregnancies, respectively [Supplemental Fig. 3(b)]. Additionally, the number of placental exosomes was significantly lower in female newborns compared with male newborns from PE pregnancies (Supplemental Fig. 4).

To estimate changes in the relative contribution of placental exosomes (*i.e.*, $PLAP^+$) to total exosomes present in maternal plasma, PLAP content per exosome (PLAP ratio) was determined. No significant difference in the PLAP ratio between normal and PE pregnancies was identified (Supplemental Fig. 5).

Classification efficiency of maternal plasma concentration of total exosome number and exosomal PLAP

The classification efficiency (*i.e.*, the proportion of cases correctly identified) by measuring total exosome number and exosomal PLAP concentration in maternal plasma at 11 to 14 weeks of pregnancy was assessed by ROC curve analysis (Fig. 3). The area under the ROC curves for total exosome number and exosomal PLAP concentration was 0.745 ± 0.094 (standard error; $P = 0.0106$) and 0.829 ± 0.077 ($P = 0.0006$), respectively. The classification efficiencies for total and placenta-derived exosome concentration were 78.0% and 85.1%, respectively (Fig. 3). Using total exosomes at early gestation (*i.e.*, 11 to 14 weeks), the proportion of positive tests in patients with PE (*i.e.*, PPV) was 69% and the proportion of negative tests in control patients (without PE) (*i.e.*, negative predictive value) was 63% [Fig. 3(a) and 3(b)]. However, the PPV and negative predictive value for the quantification of placental exosomes at early gestation to identify women who developed PE later during gestation were 75% and 76%, respectively [Fig. 3(c) and 3(d)].

Exosome microRNA profiles can be used to distinguish between normal and PE pregnancies

The overall microRNA profile for each sample was subjected to principal component analysis (PCA), and the microRNAs contributing to the differences between samples were shown as eigenvectors [Fig. 4(a)]. Using these microRNA profiles, the samples were separated into two distinct groups, namely, normal and PE pregnancies. The microRNA contributing the most to the separation between normal and PE pregnancy groups is hsa-miR-486-1-5p and hsa-miR-486-2-5p, as shown by the eigenvector. Subsequently, statistical analysis between normal and PE pregnancies was performed, and statistically significant microRNAs (adjusted $P < 0.05$) were displayed as a heatmap [Fig. 4(b)]. This analysis demonstrated that normal and PE pregnancy groups can be separated from each other, based on 12 microRNAs. Interestingly, hsa-miR-486-1-5p and hsa-miR-486-2-5p are statistically different between normal and PE pregnancy groups, which correlates with the PCA and eigenvector analysis. Both hsa-miR-486-1-5p and hsa-miR-486-2-5p are upregulated in PE pregnancy compared with normal pregnancy.

Gene target and gene ontology analysis of microRNAs differentially expressed and statistically significant between normal and PE pregnancies

The 12 statistically significant microRNAs (containing 9 unique microRNA species) differentially expressed between normal and PE pregnancies were analyzed to identify their gene targets using the Cytoscape application CyTargetLinker. A total of 824 gene targets were identified to be targeted by our candidate microRNAs [Fig. 5(a)]. Further analysis of these 824 gene targets using the Cytoscape application BiNGO revealed that a total of 1038 gene ontology terms were regulated by these genes [Fig. 5(b)]. Within this gene ontology network, the gene ontology pathways involved with migration, placenta development, and angiogenesis were enriched and statistically significant (adjusted P value of <0.05). This statistical significance can be seen for each gene ontology node, where the increasing color of the gene ontology node (from yellow to orange) is proportional to increasing statistical significance, whereas white nodes are not statistically significant.

Discussion

To have a meaningful impact on the maternal and fetal morbidity and mortality resulting from PE, two strategies

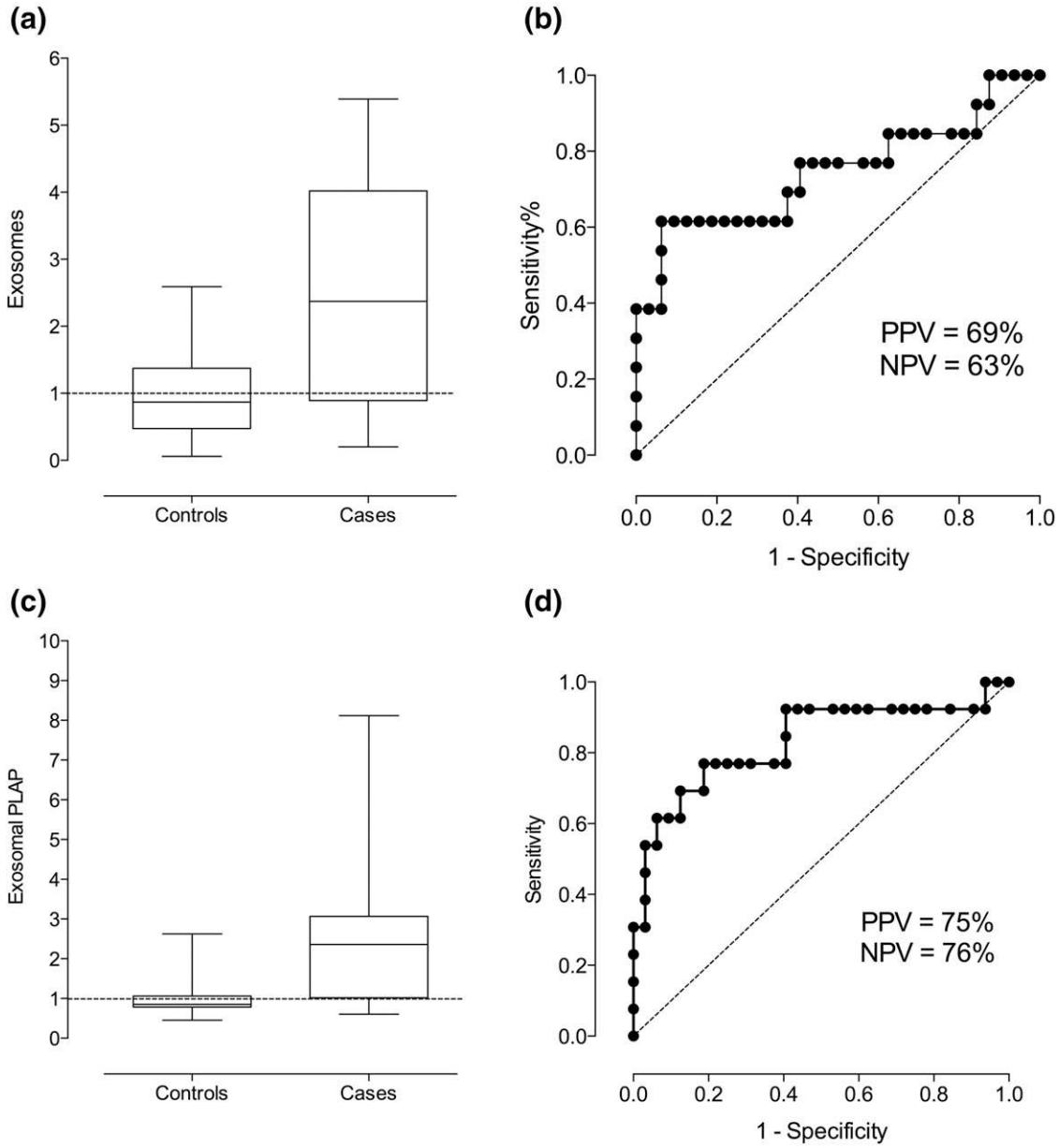

Figure 3. ROC analysis. The classification model was based on both quantification of exosomes and clinical data and developed using LogitBoost regression analysis. (a and b) The predicted likelihood and ROC curve of total exosomes for predicting PE, respectively. (c and d) The predicted likelihood and ROC curve of placental exosomes for predicting PE, respectively. NPV, negative predictive value.

must be implemented: (1) early pregnancy screening to identify women at risk for developing PE and (2) the development, evaluation, and implementation of intervention strategies to reduce the incidence of poor pregnancy outcomes. The aim of this study was to characterize changes in the exosome concentration and microRNA cargo derived from maternal peripheral plasma in PE pregnancies and to determine whether these changes can be used for a early pregnancy biomarker for PE.

The data obtained in this longitudinal study established that PE pregnancies are associated with a 1.74-fold increase in the concentration of exosomes in maternal plasma when compared with normal pregnancies. We established that the concentration of total exosomes and placental exosomes (defined and exosome-associated immunoreactive PLAP) present in maternal circulation increased during pregnancy, for both normal and PE pregnancies. Interestingly, the concentration of exosomes was higher in PE when compared with normal pregnancies matched by gestational age. These results suggest that, in early pregnancy (*i.e.*, 11 to 14 weeks), presymptomatic women who subsequently develop PE can be identified by the concentration of exosomes within their plasma. Identifying women during early pregnancy who are at risk for developing PE would allow their

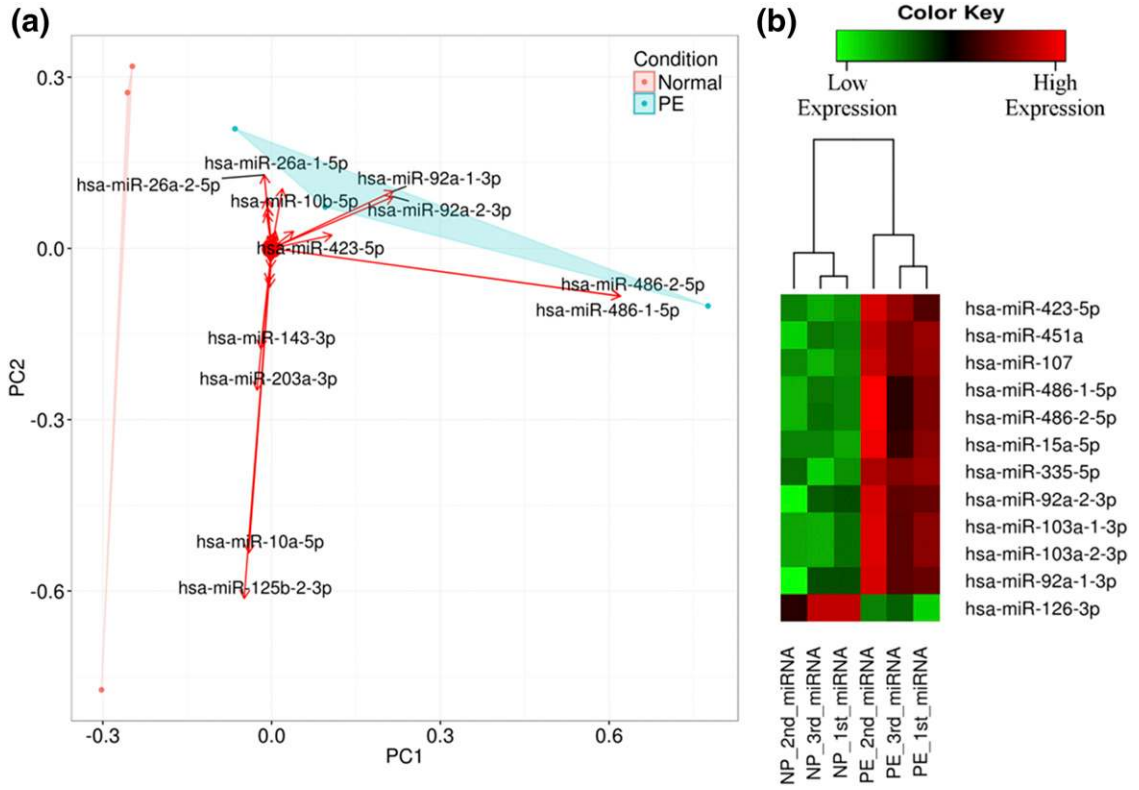

Figure 4. PCA and statistical analysis reveal a set of candidate microRNA biomarkers to differentiate between normal and PE pregnancies. Analysis was performed on exosome microRNA profiles generated using next-generation sequencing. (a) PCA and eigenvector analysis of microRNAs between normal and PE pregnancies was performed, where red and blue represent normal and PE pregnancy samples, respectively. Statistical analysis of reads was performed by DESeq2 using a Wald test and corrected for multiple testing using the Benjamini and Hochberg procedure. (b) Heatmap of statistically significant microRNAs (adjusted *P* value of <0.01), where red and green are high and low expression, respectively. PC1, principal component 1; PC2, principal component 2.

referral for more intensive surveillance or treatment (*e.g.*, low-dose aspirin (38) to prevent or reduce the risk of severe disease. Preventing or reducing the severity of PE will improve pregnancy outcomes for both mother and baby. The primary strength of our study was our repeated measures of total exosomes and placental exosomes across gestation. However, we did not consider the treatment of PE (*e.g.*, corticosteroids or heparin) on the exosome concentration. Additionally, the metabolic state of the patient should be considered for the analysis of exosome concentration in biological fluids. This is because the secretion of exosomes from placental cells has been shown to be regulated by the microenvironment milieu (*e.g.* hypoxia and glucose) (27, 39) and maternal BMI (40).

Several studies have been conducted to identify blood-borne biomarkers of abnormal placental function in asymptomatic pregnant women during the first trimester. Commonly detected blood-borne biomarkers include, Pregnancy Associated Plasma Protein-A, Placental Protein 13, soluble Fms-like tyrosine kinase-1, pentraxin, inhibin-A, placental growth factor, and vascular endothelial growth factor (41–43). Some microRNAs, lipids, and enzymes have also been found to be dysregulated in PE, such as miR-455 (44), miR-1233 (18), glycerophosphoserines (GP01, GP02, and GP03), flavanoids (PK12) (45), and matrix metalloproteinases (2 and 9) (46). However, many of these biomarkers cannot be detected at significant concentrations in the early first trimester. Therefore, it is important to identify a panel of biomarkers with greater predictive value that warrant further clinical evaluation.

Investigation of the literature reveals that placenta-specific biomarkers (such as PLAP) can be used to isolate a specific population of exosomes from the plasma of pregnant women (47). These placenta-specific PLAP exosomes have also been shown to modulate expression of CD3-zeta, JAK3, and SOCS-2 in T cells. Another study has shown that the concentration of exosomal proteins in plasma increased proportionally to disease severity and/or progression (48). Interestingly, impaired placental function in PE-complicated pregnancies results in placental apoptosis and necrosis, which further cause an increased release of microvesicles and nanovesicles (*i.e.*, exosomes) (49, 50). Consistent with previous research, our study confirms that the exosomal PLAP concentration in maternal blood increases during gestation and is elevated in pregnancies complicated by PE compared with normal pregnancies. These extracellular vesicles

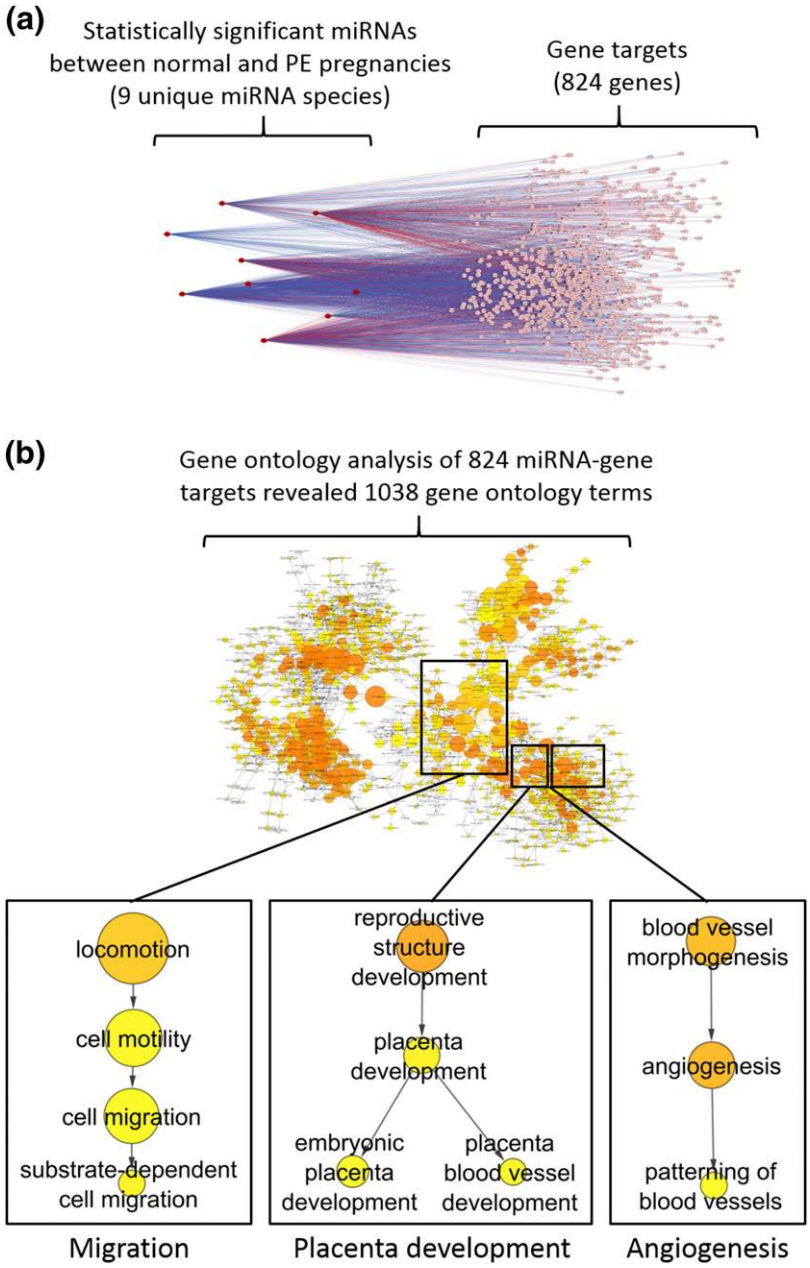

Figure 5. MicroRNA gene target and gene ontology pathway analysis. (a) Gene target identification using CyTargetLinker was performed in the nine microRNAs that were statistically significant between normal and PE pregnancies. A total of 824 genes were identified to be regulated by at least two of our candidate microRNAs and are detected within at least two microRNA-gene target databases. (b) Gene ontology analysis using BiNGO was performed on all 824 genes and displayed as a network. The increase in circle size is proportional to the number of genes involved in the gene ontology term. Furthermore, increasing significance is represented by the gradient of yellow (least significant) to orange (most significant). All colored circles indicate a *P* value (adjusted) of <0.05. Analysis shows that statistically significant microRNAs between normal and PE pregnancies regulate placenta development, migration, and angiogenesis.

may contain biomarkers (*e.g.*, proteins and microRNAs) that can be used to differentiate between PE and normal pregnancies (51).

Exosomes contain various proteins, microRNAs, messenger RNAs, and bioactive lipids that play essential roles in reproduction through to the adaptation of the maternal body system for pregnancy (52). For example, microRNAs such as miR-376c (53) and miR-520g (54) have been identified to be differentially expressed between normal and PE pregnancies in serum. Thus these microRNAs could potentially be encapsulated within exosomes. Furthermore, miR-520g and miR-376c were shown to regulate trophoblast migration and proliferation (53, 54). In corroboration, our study has revealed a range of statistically significant and differentially expressed microRNAs in exosomes between normal and PE pregnancies. Furthermore, our gene ontology analysis demonstrated that these microRNAs regulate biological processes such as migration, placenta development, and angiogenesis. Interestingly, the dysregulation of these biological processes has been linked to PE (55).

Additionally, our PCA and statistical analysis identified hsa-miR-486-1-5p and hsa-miR-486-2-5p as key candidate biomarkers that can be used to differentiate between normal and PE pregnancies. Specifically, both these microRNAs are upregulated within exosomes derived from PE pregnancies, which is independent of gestational age. Previous studies have demonstrated that hsa-miR-486 is suppressed in many cancers such as lung cancer, hepatocellular carcinoma, and thyroid carcinoma (56–58). Furthermore, transfection of this microRNA into cells suppressed migration, proliferation, and invasion, key processes involved in cancer (56–58). To date, studies that identified or functionally investigated this microRNA in relation to its role in PE have not been reported.

Recently, a study has demonstrated that the incidence of PE could be reduced, using a combination of early screening (*i.e.*, between 11 and 14 weeks) and treatment with a low dose of aspirin (38). In a separate study using 7797 singleton pregnancies, a combination of ultrasonography and clinical markers was used for early screening of PE (59). Using these methods, the aforementioned study reported a 93% detection rate for early

onset of PE, but only had a positive predictive value of 7%. In our study, we have identified hsa-miR-486-1-5p, hsa-miR-486-2-5p, and the concentration of exosomes to be differentially expressed between normal and PE pregnancies, which was independent of gestation age. Based upon data obtained to date, we suggest that the combined detection of both the concentration of exosomes as well as the expression of hsa-miR-486-1-5p and hsa-miR-486-2-5p could outperform existing predictive modalities in identifying women at risk for developing PE.

In summary, the data obtained in this study demonstrate that exosome concentration, hsa-miR-486-1-5p, and hsa-miR-486-2-5p are elevated in PE compared with normal pregnancies. We propose that the combined measurement of exosome concentration, hsa-miR-486-1-5p, and hsa-miR-486-2-5p might allow for the early identification of women at risk for developing PE; however, a larger trial is required to further validate utility of this approach for population screening. Early detection of women at risk for developing PE would allow referral of these women for more intensive surveillance or application of preventative therapy to reduce PE complications and to improve pregnancy outcomes for both mother and baby.

Conclusions

Current management for PE offers no effective antenatal treatment other than steroid administration to induce fetal lung maturity and timely delivery. Therefore, there is a need for strategies for early identification of patients who will develop PE to establish preventive strategies to reduce the prevalence and severity of PE and associated complications. Recently, it has been demonstrated that the human placenta can communicate with the maternal physiology via small nanovesicles called exosomes. These exosomes are of interest because it is believed that they contain a biomolecular "fingerprint" of the cell origin and may give information regarding their metabolic status. The availability of a screening test with appropriate prognostic performance would improve patient management, allow for a triage of intervention therapies (such as low-dose aspirin), and improve health outcomes for mother and baby and would be economically cost effective. However, as the pathogenesis of PE is not always the same, patient heterogeneity (*e.g.*, BMI) should be considered to implement strategies to improve management, treatment, and clinical outcomes. In this study, we suggest that the quantification of placenta-derived exosomes present in maternal blood and the measurement of hsa-miR-486-1-5p and hsa-miR-486-2-5p expression may improve our ability to identify asymptomatic women at risk for developing PE.

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