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Original Article

Detection of angiogenic factors in midtrimester amniotic fluid and the prediction of preterm birth

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ABSTRACT

Objective: We investigated whether the level of vascular endothelial growth factor (VEGF), placental growth factor (PlGF), and soluble VEGF receptor-1 (sFlt-1) in midtrimester amniotic fluid of preterm birth have different values compared with term delivery.
Materials and Methods: Our participants were 86 pregnant women who had undergone amniocentesis from 16 to 19 weeks of gestation. Forty-three cases were women with preterm delivery, and the other 43 cases were matched women with full-term delivery. Stored amniotic fluid was investigated after the delivery. The levels of VEGF, PlGF, and sFlt-1 were measured by enzyme-linked immunosorbent assay and Western blot.
Results: The levels of VEGF and PlGF in the preterm group were significantly higher than in the control group (30.48 ± 8.57 pg/mL vs. 26.06 ± 8.24 pg/mL and 28.83 ± 7.83 pg/mL vs. 25.35 ± 8.26 pg/mL, respectively) ($p = 0.017$ and 0.048, respectively). In terms of sFlt-1, the levels were decreased in the preterm group (10,478.51 ± 4012.56 pg/mL vs. 12,544.05 ± 4140.96 pg/mL) ($p = 0.021$).
Conclusion: This study explains that elevated levels of VEGF and PlGF, suggestive of angiogenesis and tendency of inflammation at midtrimester, are predictive of preterm delivery, and their availability is maximized by downregulation of sFlt-1.

Introduction

Placental angiogenesis and vasculogenesis play an important role for normal development of the fetus [1]. Altered angiogenic marker expression has been studied in obstetric complications, such as preeclampsia [2]. Increased levels of the angiogenic markers like soluble fms-like tyrosine kinase-1 [soluble vascular endothelial growth factor (VEGF) receptor-1, sFlt-1] or soluble endoglin and lower levels of placental growth factor (PlGF) may permit detection of preeclampsia before symptom onset [3,4]. The relation between these angiogenic factors and preeclampsia seems to be very strong and more predictive of risk for preeclampsia [5].

Another important complication during pregnancy is preterm birth, which is defined as delivery that occurs between 24 weeks of gestation and 37 weeks of gestation [6]. Preterm delivery is responsible for a significant percentage of neonatal morbidity and mortality. Based on the known risk factors and pathways of preterm birth, several biomarkers have been tested to see if they can predict spontaneous preterm birth [7]. Relations between angiogenic markers and preterm delivery have become important to characterize the placental aspect associated with preterm labor or the inflammatory role of angiogenic markers in pregnancy [8]. Little is known about angiogenic marker patterns in relation to preterm delivery uncomplicated by preeclampsia. Preeclampsia-like angiogenic marker changes have been reported late in pregnancy among spontaneous preterm labor cases; however, similar changes occur in term pregnancies before labor onset [8].

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Two previous reports suggest that low sFlt-1 levels may mark a subset of spontaneous preterm delivery [8,9]. We measured mid-pregnancy levels of VEGF and PlGF, as well as sFlt-1 in midtrimester amniotic fluid, and assessed their associations with preterm delivery.

Materials and methods

Study design

The research was designed to be a prospective study. Collection of amniotic fluid samples from January 2009 to June 2012 and clinical data were approved by the Institutional Review Board of Kosin Medical Center. All patients gave written informed consent, in accordance with the Helsinki criteria. After excluding fetal aneuploidies, anomalies, and cases who experienced pregnancy loss within 30 days of amniocentesis, we enrolled and stored the samples of amniotic fluid for later analysis. Postdelivery patient obstetric data were reviewed, and the clinical outcomes were obtained. Gestational age was determined based on the last menstrual period and the first trimester obstetric ultrasound evaluation (crown rump length at 7–9 weeks). Preterm delivery was defined as birth before 37 weeks of gestation.

Patients

A total of 596 pregnant women with singleton gestations underwent amniocentesis and their samples of amniotic fluid were stored until delivery. Amniocentesis was carried out for proper clinical indications (advanced maternal age, abnormal quad/triple test, family history of chromosomal abnormalities, suspected fetal anomalies or viral infection, and maternal request) at 16–19 weeks of gestation. Among 596 women with available samples of amniotic fluid, the study included 86 women for study objects. Patients were invited to donate amniotic fluid for research purposes. The clinical outcome was obtained by chart review. Inclusion criteria were uneventful pregnancy course before the procedure, absence of congenital fetal malformations, absence of clinical signs of infection, normal volume of amniotic fluid as assessed by ultrasound, and healthy pregnant woman without chronic or medical disease. Any preterm delivery associated with an obstetrical complication, such as hypertensive disorders in pregnancy, obstetrical hemorrhage, fetal growth restriction, or premature rupture of the membrane, was excluded from the amniotic fluid analysis.

We retrieved samples from every case known to have resulted in delivery before 37 weeks of gestation ($n = 43$) and 43 control samples from women who delivered at $\geq$37 weeks of gestation. The control samples were matched with the preterm group at a 1:1 ratio from sampling until testing (storage time). Matches were based on maternal age, gestational age (weeks) at the time of amniocentesis, and the indication for the procedure.

Collection of amniotic fluid and storage

Transabdominal amniocentesis was performed with a 21-gauge needle under ultrasound guidance to evaluate the position of the fetus. Amniotic fluid was first taken for further diagnostic testing, depending on the indication of the invasive procedure. Afterward, 5 mL from a total volume of 20 mL of amniotic fluid was collected for research purposes. Samples were transported immediately to the laboratory in a capped sterile syringe; amniotic fluid samples were then centrifuged for 10 minutes at 400 rpm and stored in aliquots at −70°C until analysis at the completion of follow-up.

Enzyme-linked immunosorbent assay

Invitrogen assay kits (Carlsbad, CA, USA) were used for VEGF, PlGF, and sFlt-1. These kits are based on the solid phase sandwich enzyme-linked immunosorbent assay (ELISA) method. During the first incubation, samples were pipetted into wells coated with antibodies specific for human VEGF, PlGF, and sFlt-1, followed by the addition of a biotinylated secondary antibody. During the first incubation, the human antigen bound simultaneously to the immobilized (capture) antibody on one site and to the solution phase-biotinylated antibody on a second site.

After washing, streptavidin-peroxidase (enzyme) was added, which binds to the biotinylated antibody to complete the four-member sandwich. After a third incubation and wash to remove any unbound enzyme, a substrate solution was added, upon which the bound enzyme acts to produce color. The intensity of this colored product is directly proportional to the concentrations of VEGF, PlGF, and sFlt-1 present in the specimen. The coefficients of variation of intra-assay and interassay precision were 5.1–9.8% for VEGF, 8.5–10.2% for PlGF, and 5.0–5.6% for sFlt-1, respectively. The minimum detectable doses of VEGF, PlGF, and sFlt-1 were <5 pg/mL, 1 pg/mL, and 2 pg/mL, respectively.

Western blot

A total of 1–2 mL of amniotic fluid was prepared by dilution with sodium dodecyl sulfate loading buffer (Fermentas, Waltham, MA, USA), followed by boiling and cooling. The amniotic fluid samples underwent electrophoresis in a 13.5% sodium dodecyl sulfate-polyacrylamide gel (Koma Biotech, Seoul, Korea). Thereafter, proteins were electrotransferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA) at 30 V for 1 hour. Nonspecific binding was blocked for 1 hour in noise-cancelling reagents (Millipore). After washing, membranes were incubated for 2 hours at room temperature with antibodies. The antibodies used (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were rabbit antihuman VEGF antibody (147) (CAT. # sc-507), goat antihuman PlGF antibody (C-20) (CAT. # sc-1880), and mouse antihuman sFlt-1 antibody (C-20) (CAT. # sc-315). A 5-bromo-4-chloro-3-indolyl-phosphate (BCIP)/nitro blue tetrazolium (NBT) tablet (Sigma-Aldrich, St Louis, MO, USA) dissolved in distilled water was used as growth substrate. Chemiluminescence analysis was conducted with Luminata Crescendo Western HRP substrate (Millipore) and autoradiography film (Agfa-Gevaert, Mortsel, Belgium), according to the manufacturer's instructions. The experiment was replicated three times. Bands produced from the Western blot were shown using Gel Doc XR+ with Image Lab software (Bio-Rad, Hercules, CA, USA).

Statistical analyses

Results are expressed as mean and standard deviation according to the distribution of data. Kolmogorov-Smirnov's test was used to evaluate the normality of the distribution of the continuous data. Comparisons between the two groups were conducted using the Student t test in a normal distribution and X^2 test for univariate analysis in the categorized variables. The receiver operating characteristic (ROC) curve was applied to calculate each factor's predictive value for preterm delivery. SPSS version 18.0 (SPSS Inc., Chicago, IL, USA) was used for statistical calculations. A p value < 0.05 was considered statistically significant.

Results

A total of 43 patients delivered at <37 weeks of gestation; all spontaneous preterm labors included an intact membrane. The

included cases were classified into the preterm group. The matched control group included 43 healthy pregnancies with full term delivery. Table 1 shows the clinical and demographic characteristics of the study samples. There were no significant differences between the preterm delivery group and controls with respect to the mother's age, the gestational age of the fetus at amniocentesis, body mass index, neonatal sex, indication of amniocentesis, or gravidity.

In comparing biomarkers between the preterm and full-term groups, we found the following mean values: VEGF levels in the amniotic fluid were 30.48 pg/mL and 26.06 pg/mL, respectively, and PlGF levels in amniotic fluid were 28.83 pg/mL and 25.35 pg/mL, respectively. Both concentrations were significantly different between the groups. By contrast, the levels of sFlt-1 were 10,478.51 pg/mL in the preterm group and 12,544.05 pg/mL in the full-term group. This represents a significant difference between the groups (Table 2, Figure 1).

The above values were analyzed for their ability to predict preterm birth using a ROC curve (Table 3, Figure 2). ROC analysis was also used to determine the optimal amniotic fluid levels for VEGF, PlGF, and sFlt-1 that would predict preterm delivery. An appropriate cut-off level for VEGF in amniotic fluid was 25.25 pg/mL, with a sensitivity of 76.7% and a specificity of 60.5%. For PlGF in amniotic fluid, the cut-off value was 23.80 pg/mL, with a sensitivity of 76.7% and a specificity of 53.5%. Amniotic sFlt-1 levels had the cut-off value of 11,666.00 pg/mL with the area under the curve of 0.629 in the consideration of the inversely rendered curve. Its sensitivity and specificity were 62.8% and 55.8%, respectively.

Figure 3 shows the representative Western blot for preterm and full-term groups showing levels of VEGF (55 kDa and 40 kDa, respectively), PlGF (45 kDa), and sFlt-1 (110 kDa) in midtrimester amniotic fluid. The samples were randomly selected among the participants—four samples from the preterm delivery group and four samples from the full-term delivery group. Amniotic fluid from the preterm delivery group showed thicker and darker bands of VEGF and PlGF, but not of sFlt-1. The bands of sFlt-1 were more prominent in the full-term delivery group.

Discussion

The data in this study indicate that the detection of angiogenic factors in the midtrimester amniotic fluid can provide a valuable tool for the subsequently developed spontaneous preterm labor. The set of VEGF, PlGF, and sFlt-1 values obtained in midtrimester amniotic fluid was investigated alongside the reference values in the study. High concentrations of VEGF and PlGF were confirmed in the spontaneous preterm group both with ELISA and Western blot analysis. By contrast, the association between spontaneous preterm labor and the lower level of sFlt-1 in midtrimester amniotic fluid was provided for the first time.

Angiogenesis is essential for the development of the fetoplacental vascular network, as well as vasculogenesis [10]. In early placental development, villous cytotrophoblasts produce a variety of angiogenic factors, which induce the differentiation, proliferation, and migration of pluripotent mesenchymal cells via a paracrine manner [11]. Secreted angiogenic factors activate angiogenic cell cords causing further differentiation of endothelial precursor cells. Pregnancy-specific growth factors and hormones, such as human chorionic gonadotropin, α-fetoprotein, and insulin like growth factor, may have a role in the fine regulation of vascular development of the fetoplacental unit [12]. Then, remodeling of primary vessels occurs on extracellular matrix components. In this process, vessel stabilization is supported by angiopoietin, its respective receptors, VEGF, PlGF, nitric oxide (NO), vascular endothelial-cadherin, and low oxygen [13]. Modification of placental vascular assembly may lead to subsequent obstetrical pathologic conditions. Miscarriage, preterm birth, preeclampsia, and intrauterine growth restriction may result from this pathophysiologic cascade [14].

Evaluation of alterations in placental developments which can threaten the obstetrical progress is not easy because of the difficulty in obtaining living tissues. However, amniocentesis is preformed easily with a very low risk of miscarriage in the early period of gestation. Obtained amniocentesis samples may have information of the angiogenic markers, which would be used to predict subsequent obstetric complications. Even though the amniotic membrane would only allow minimal transportation of high molecular angiogenic factors, the concentration of those molecules in the amniotic fluid may reflect the distribution in fetoplacental tissues and maternal serum [15]. The levels of the molecules in amniotic fluid are consistent with their site of production and local tissue conditions. One other important source of high molecular materials in amniotic fluid is materno-embryonic transportation or fetal production. The transportation to the amniotic cavity depends on the molecular size, and as the amniotic membrane is almost impermeable to large molecules, the fetal and maternal effects on amniotic fluid composition have been also considered [16].

For these reasons, we evaluated the levels of VEGF, PlGF, and sFlt-1 as remarkable angiogenic factors in midtrimester amniotic fluid for the prediction of subsequent preterm birth. The angiogenic markers VEGF and PlGF are abundantly expressed by the placenta and several fetal tissues, and play key roles in the regulation of effective vasculogenesis, angiogenesis, and placental development [17,18]. Both VEGF and PlGF induce proliferation, migration, and activation of endothelial cells, which they contribute to induction of vascular permeability and maintenance of integrity of newly formed blood capillaries. Failure to get the above adaptation may result in reduced fetoplacental perfusion, associated obstetrical complications like angiopoietin, fetal death, preeclampsia, and preterm labor [19].

Table 1
Clinical and demographic characterizations of the participants.

Clinical characteristics	Preterm delivery ($n = 43$)	Term delivery ($n = 43$)	p
Maternal age (y)	34.8 ± 4.3	34.2 ± 4.8	0.786
Gestational age at delivery (wk)	33.5 ± 1.8	39.5 ± 0.8	0.021
Gestational age at sampling (wk)	18.3 ± 0.2	18.0 ± 0.2	0.573
BMI (kg/m^2)	21.7 ± 2.3	21.2 ± 1.5	0.562
Sex (male/female)	21/22	20/23	0.829
Gravidity	1.19 ± 0.45	0.98 ± 0.28	0.056
Indication for amniocentesis			
Abnormal maternal serum markers	32 (74.4)	34 (79.1)	
Increased nuchal translucency	2 (4.7)	1 (2.3)	
Abnormal findings in ultrasound	0 (0)	1 (2.3)	
Anomaly of previous baby	1 (2.3)	2 (4.7)	
Pregnancy by IVF	4 (9.3)	3 (6.9)	
Advanced maternal age	4 (9.3)	2 (4.7)	

Results are expressed as mean ± standard deviation and n (%).
BMI = body mass index; IVF = *in vitro* fertilization.

Table 2
Concentrations of VEGF, PlGF, and sFlt-1 measured by enzyme-linked immunosorbent assay in midtrimester amniotic fluid of preterm and term groups.

	Preterm delivery ($n = 43$)	Term delivery ($n = 43$)	p
VEGF (pg/mL)	30.48 ± 8.57	26.06 ± 8.24	0.017
PlGF (pg/mL)	28.83 ± 7.83	25.35 ± 8.26	0.048
sFlt-1 (pg/mL)	10,478.51 ± 4,012.56	12,544.05 ± 4,140.96	0.021

Results are expressed as mean ± standard deviation.
PlGF = placental growth factor; sFlt-1 = soluble VEGF receptor-1; VEGF = vascular endothelial growth factor.

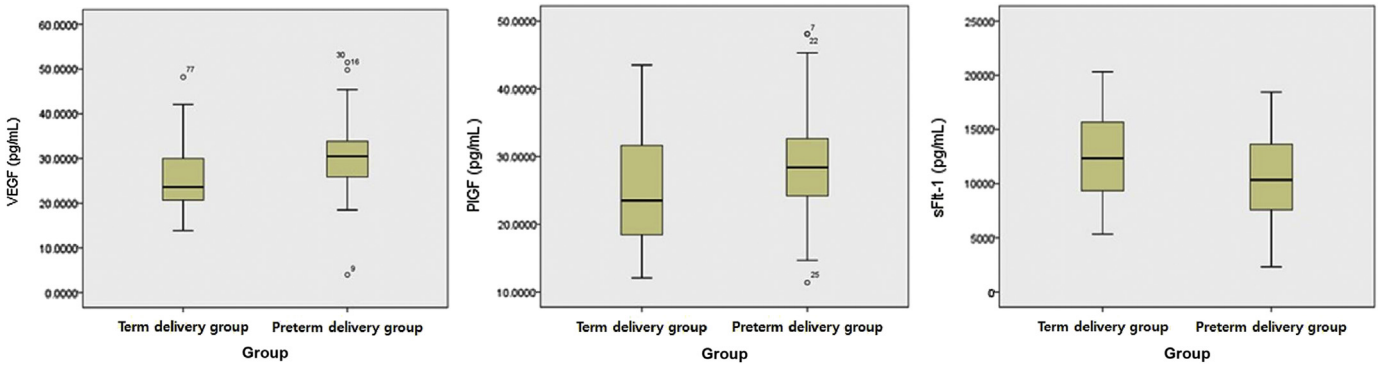

Figure 1. Concentrations of VEGF, PlGF, and sFlt-1 as measured by enzyme-linked immunosorbent assay in midtrimester amniotic fluid in preterm and term delivery groups. PlGF = placental growth factor; sFlt-1 = soluble VEGF receptor-1; VEGF = vascular endothelial growth factor. group 0, preterm delivery; group 1, term delivery.

In terms of sFlt-1, a splice variant of VEGF receptor is expressed and secreted from several different tissues, including human endometrium, endothelial cells, and placental villous tissues [9,20]. Secreted sFlt-1 binds to VEGF and PlGF with high affinity, thereby decreasing their availability and acting as inhibitor of VEGF and PlGF. Hypoxic condition is believed to induce the secretion of sFlt-1, but the physiological significance of the variable levels detected in conception tissue is not yet fully understood [15]. During normal pregnancy, sFlt-1 serum levels increase with advancing gestation, but are markedly increased in both serum and placental tissue from preeclamptic pregnancies [21]. Increased serum levels of sFlt-1 in preeclampsia have been argued as a potential direct cause of several manifestations of the disease, and it has been postulated that in preeclampsia, the abnormal placentation following placental hypoxia may result in increased sFlt-1 levels, thus contributing to the pathogenesis of this disease [22]. Increases in VEGF in maternal serum may be a trigger for elevation of placental sFLT1 expression, which leads to preeclampsia. In addition, placental sFLT1 has been regarded as having a role in placental functions. Higher and earlier levels of sFlt-1 and PlGF were suggested to have a decreased risk of adverse perinatal outcomes [23].

The association between the concentrations of sFlt-1 in amniotic fluid and the development of following preterm labor has not been studied. As an antagonist of both VEGF and PlGF, it may play an important role as antiangiogenic factor, and the prominent shift of the levels in amniotic fluid may also reflect the alteration of implantation, placentation, pregnancy maintenance, and/or pregnancy termination. Mijal et al [5] reported that low sFlt-1 levels were associated with preterm delivery unexplained by preeclampsia and small for gestational age [5]. Unlike preeclampsia, representative of the hypoxic condition, relatively reduced levels in amniotic fluid for preterm birth were shown in this study. It is not explained that the nonhypoxic condition of intraamniotic cavity is related to the development of preterm labor. The molecular role of sFlt-1 must be focused for its secondary play as a receptor for VEGF and PlGF. sFlt-1 cannot act effectively in living tissues without some molecules which bind with it, and decreased levels of sFlt-1 mean the amplified action of VEGF and PlGF for the particular pathway. The action may stand not only for angiogenesis or vasculogenesis, but also for subclinical inflammation [24]. Thus, the detection of sFlt-1 levels in amniotic fluid can give information for preterm birth, indirectly.

Table 3
Receiver operating characteristic (ROC) analysis with the concentrations of VEGF, PlGF, and sFlt-1 measured by enzyme-linked immunosorbent assay in midtrimester amniotic fluid.

	Preterm delivery (n = 43) vs. Term delivery (n = 43)					
	Area under curve	95% CI	p	Cut-off value	Sensitivity	Specificity
VEGF (pg/mL)	0.681	0.565–0.798	0.004	25.25	76.7	60.5
PlGF (pg/mL)	0.635	0.514–0.756	0.032	23.80	76.7	53.5
sFlt-1 (pg/mL)	0.371	0.254–0.488	0.039	11,666.00	62.8	55.8

CI = confidence interval; PlGF = placental growth factor; sFlt-1 = soluble VEGF receptor-1; VEGF = vascular endothelial growth factor.

Now, it must be of concern why the proangiogenic profiles in midtrimester amniotic fluid are related to the subsequent spontaneous preterm labor without preeclampsia. In previous studies, the relation between preterm birth and higher angiogenic markers has become apparent in the cases of preeclampsia, small for gestational age, or hypertensive conditions which may overlap or affect the prevalence the preterm delivery [5,8,9]. However, in spontaneous preterm birth, the association is not considered obvious without obstetric complications. Brou et al [16] stated that maternally and fetally derived intra-amniotic compartments showed cell-to-cell interaction for both preponderant proinflammatory conditions and angiogenesis, which are mutually dependent, in response to a hostile environment. This may indicate the altered fetal/placental growth and an innate immune response in reaction to a hostile environment and preterm birth yet to happen [16,24]. Balanced activity between these markers, which are detectable or undetectable in amniotic fluid, may be nonexistent or active only to a lesser degree, thereby inducing promotion of an adverse environment for maintaining conception. The availability of biologically active molecules and their balance with regulatory molecules can determine the final outcome [25]. Therefore, understanding such environments and associated markers can provide the key for prediction for preterm labor with risk assessment and management.

An authentic predictor of spontaneous preterm delivery is clinically useful because it would allow for the identification of women at high risk of preterm birth, in whom a specific intervention could be tested. The possibility to predict which women are likely to have a preterm birth is a prerequisite for the effective use of most interventions aimed at preventing preterm birth. Also, studies that identify predictors of spontaneous preterm birth may be helpful to understand the mechanisms of biological pathways and possibly lead to better interventions. Furthermore, they can indicate predictive markers with noninvasive methods, unlike amniocentesis. Finally, another reason for predicting preterm birth is that being able to identify women with low risk would avoid

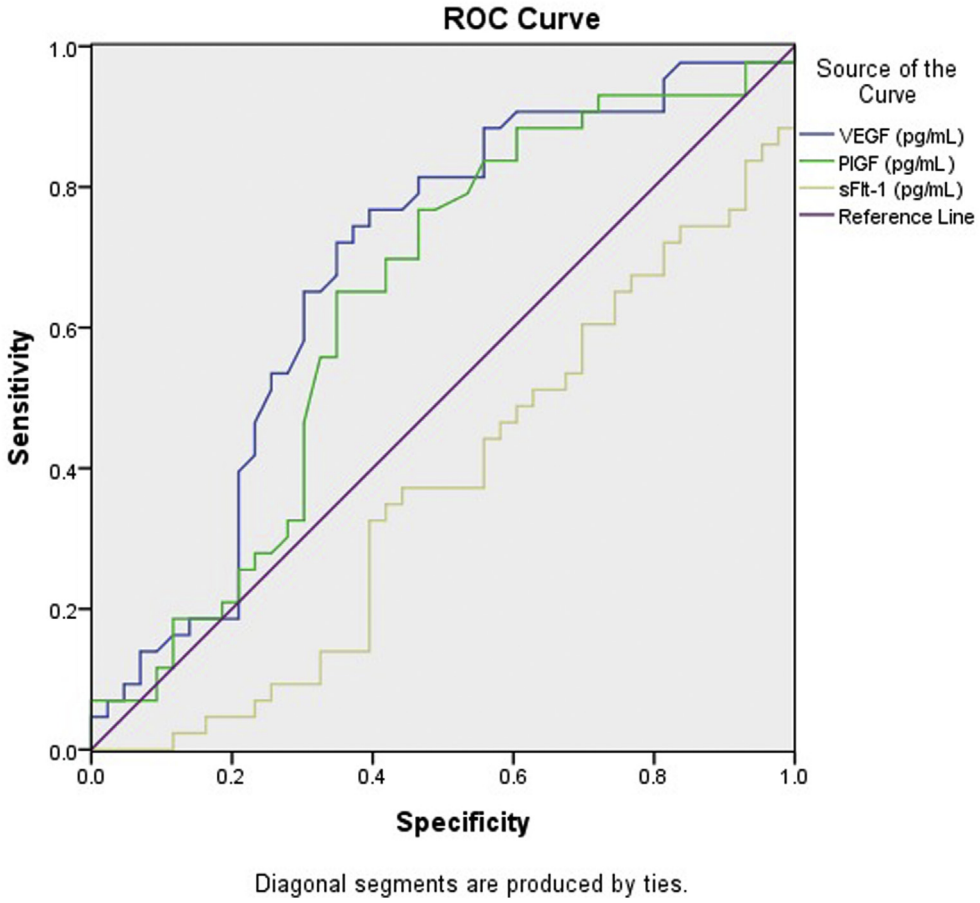

Figure 2. Receiver operating characteristic (ROC) curves that compare the values of VEGF, PlGF, and sFlt-1 for preterm delivery. PlGF = placental growth factor; sFlt-1 = soluble VEGF receptor-1; VEGF = vascular endothelial growth factor.

unnecessary and costly interventions [7]. This strategy may prevent both the initiation of preterm labor and the fetal complications associated with prematurity by monitoring with technologies such as cervical sonography and treating with interventions to reduce the rate of preterm delivery.

This study provides an early demonstration of a possible preterm labor instigating association or interaction between angiogenic biomarkers in amniotic fluid. The concentrations measured through ELISA were confirmed by adjunctive Western blotting, which is a widely accepted analytical technique used to detect specific proteins in this type of tissue homogenate or extract (Figure 3). We evaluated the cut-off values for useful biomarkers to predict preterm birth in midtrimester amniotic fluid. The values were 25.25 pg/mL, 23.80 pg/mL, and 11,666.00 pg/mL for VEGF, PlGF, and sFlt-1, respectively. These levels cannot yet be applied in clinical work and this is one of the limitations of this work; further studies are needed to replicate and corroborate these results. There seems to be variability in biomarker levels over time or between individuals and races, making it difficult to determine the threshold level for risk of spontaneous preterm birth [25]. Indeed, the combined use of markers has been shown to have better predictive accuracy than individual markers alone because there are several heterogeneous pathways that lead to spontaneous preterm birth including inflammatory and angiogenic responses. The other limitation is the lack of repeated angiogenic marker measurements during pregnancy. Recent study has demonstrated the utility of longitudinal measurements, showing stronger associations between changes in biomarker concentrations and preterm labor risk [8].

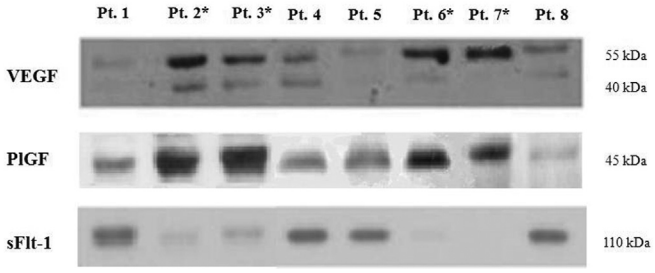

Figure 3. Representative Western blot for preterm (Pt2*, Pt3*, Pt6*, and Pt7*) and full-term (Pt1, Pt4, Pt5, and Pt8) groups showing levels of VEGF (55 kDa and 40 kDa, respectively), PlGF (45 kDa), and sFlt-1 (110 kDa) in midtrimester amniotic fluid. PlGF = placental growth factor; Pt = patient; sFlt-1 = soluble VEGF receptor-1; VEGF = vascular endothelial growth factor.

This study has several strengths. The first is that we did not know or compare the levels of target molecules in the amniotic fluid until the termination of pregnancy. Another strength is the homogeneity of the participant population, since it is a complex phenotype that is related to multiple mechanisms, including infection/inflammation, uteroplacental ischemia or hemorrhage, uterine overdistension, stress, and other immunologically-mediated processes [26]. We homogenized the preterm delivery group by including only patients with spontaneous preterm delivery <37 weeks of gestation in singleton pregnancy.

This report concluded that it is feasible to measure the VEGF, PlGF, and sFlt-1 concentrations in midtrimester amniotic fluid for the prediction of spontaneous preterm birth for asymptomatic women. These angiogenic parameters can be useful and strong biomarkers to distinguish high-risk patients and to discriminate the expected preterm birth and to provide the understandable key for the complex mechanism of preterm labor.

Conflicts of interest

The authors have no conflicts of interest relevant to this article.

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