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An Imbalance between Angiogenic and Anti-angiogenic Factors precedes Fetal Death in a subset of patients: Results of a Longitudinal Study

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Abstract

OBJECTIVE—Fetal death at the time of diagnosis has higher maternal plasma concentrations of the anti-angiogenic factor, soluble vascular endothelial growth factor receptor (sVEGFR)-1, than that of women with normal pregnancy. An important question is whether these changes are the cause or consequence of fetal death. To address this issue, we conducted a longitudinal nested case-control study and measured the maternal plasma concentrations of selective angiogenic and anti-angiogenic factors before the diagnosis of a fetal death. The anti-angiogenic factors studied included sVEGFR-1, soluble endoglin (sEng), and the angiogenic factor, placental growth factor (PlGF).

Methods—A retrospective longitudinal nested case-control study was conducted. It included 143 singleton pregnancies in the following groups: (1) patients with uncomplicated pregnancies who delivered a term infant with an appropriate weight for gestational age (n=124); and (2) patients who had a fetal death (n=19). Samples were collected at each prenatal visit, scheduled at 4-week intervals from the first trimester until delivery. Plasma concentrations of sVEGFR-1, sEng, and PlGF were determined by specific and sensitive ELISA. A linear mixed-effects model was used for analysis.

Results—1) Linear mixed-effects model analyses indicated that the average profiles of analyte concentrations as a function of gestational age for sVEGFR-1, sEng and PlGF were different between women destined to have a fetal death and those with normal pregnancy outcome after adjusting for covariates ($p<0.05$); 2) Plasma sVEGFR-1 concentrations in patients destined to have a fetal death were significantly lower between 7 and 11 weeks of gestation and became significantly higher than those of women with a normal pregnancy between 23 and 35 weeks of gestation ($p<0.05$); 3) Similarly, plasma sEng concentrations of women destined to have a fetal death were lower at 7 weeks of gestation ($p=0.04$) and became significantly higher than those of

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women destined to have a normal pregnancy between 23 and 41 weeks of gestation ($p<0.05$); 4) In contrast, plasma PlGF concentrations were higher among patients destined to develop a fetal death between 7 and 13 weeks of gestation and became significantly lower than those in the control group between 22 and 40 weeks of gestation ($p<0.05$); 5) The ratio of PlGF/sVEGFR-1 x sEng was significantly higher in women destined to have a fetal death between 7 and 13 weeks of gestation (89%–765%) and significantly lower (45%–76%) than those in normal pregnant women between 20 and 41 weeks of gestation ($p<0.05$); 6) Similar results were obtained when patients with a fetal death were stratified into those who were diagnosed before or after 37 weeks.

Conclusions—1) Patient destined to have a fetal death, compared to normal pregnancy, was characterized by higher plasma concentrations of PlGF during the first trimester, and this profile changed into an anti-angiogenic one during the second and third trimesters.

Keywords

soluble VEGF receptor-1 (sVEGFR-1); sFlt-1; soluble endoglin (sEng); placental growth factor (PLGF); mixed- effect model; fetal demise; stillbirth; pregnancy; anti-angiogenic state

Introduction

Fetal death is one of the "great obstetrical syndromes" [1]. As such, it may be expected to have: 1) multiple etiologies; 2) a preclinical stage; and 3) genetic and environmental predisposing factors (which, alone or in combination, may modify the risk of its occurrence) [1]. In addition, fetal death may be considered, in some cases, to be adaptive in nature. Indeed, fetal death of one twin may result in the improvement of preeclampsia [2–5]. Thus far, most research about the causes of fetal death has been based on epidemiologic studies [6–15]. The Tulip classification system suggests that the leading causes are: 1) pathology of the placenta and the placental bed (64.3%); 2) congenital anomalies (5.8%); 3) infection (1.7%); 4) others (4.9%); and 5) unknown causes (23.3%) [16]. It is important to recognize that the term "cause" is used when referring to an association between fetal death and a clinical or pathologic condition, and that the postulates for causation are often not met. Another limitation of the current classifications of the factors associated with fetal death is that subclinical pathologic processes have not been studied. For example, intra-amniotic infection/inflammation is rigorously excluded only in a minority of cases [17–20]. Inadequate fetal growth is often considered a cause of fetal death [7,21–23], but many cases of intrauterine growth restriction result in live birth. Thus, the causal link remains open to question. We propose that progress in understanding the causes of fetal death has been hampered by the cross-sectional nature of the studies. Indeed, most reports examine conditions present at the time of fetal death or after (but not before). Therefore, longitudinal studies are required to gain insight into causality.

Pregnancy is a unique state in which both vasculogenesis and extensive angiogenesis are required for fetal and placental development [24,25]. The balance between angiogenesis and anti-angiogenesis is important for successful reproduction [26–28]. Indeed, increased circulating concentrations of anti-angiogenic factors -soluble vascular endothelial growth factor receptor (sVEGFR)-1 and soluble endoglin (sEng)-and decreased concentrations of an angiogenic factor -placental growth factor (PlGF)-have been reported in patients with preeclampsia [29–64], small for gestational age (SGA) fetus [36,44,47,55,64–69], placental abruption [70], "mirror syndrome" [71–73], and twin-to-twin transfusion syndrome [74].

The overlapping clinical features as well as placental pathology among fetal death, preeclampsia and fetal growth restriction [7,21,75–80] suggest that pregnancies with a fetal death may have an anti-angiogenic state as reflected by abnormal profiles of the maternal

plasma concentrations of angiogenic and anti-angiogenic factors. A cross-sectional study also demonstrated a higher median delta maternal plasma sVEGFR-1 concentration among patients presenting with a fetal death than those with a normal pregnancy [81]. Moreover, spontaneous resolution of early-onset preeclampsia accompanied by an improvement of anti-angiogenic state after fetal demise in a twin pregnancy has recently been reported [82].

A longitudinal study reported by our group demonstrated that patients with SGA neonates and those who developed preeclampsia differed in the maternal plasma concentration of specific angiogenic and anti-angiogenic factors during pregnancy [64]. This observation suggests that each obstetrical syndrome may have a unique angiogenic and anti-angiogenic profile, and that this difference may reflect the underlying mechanisms of disease, timing and magnitude of the insult responsible for the clinical phenotype. To test this hypothesis, we conducted a longitudinal study to determine whether patients who subsequently had a fetal death have a different profile in maternal plasma concentrations of sVEGFR-1, sEng, and PlGF as a function of gestational age from those with normal pregnancy outcome.

Material and Methods

A retrospective, longitudinal nested case-control study included 143 patients with singleton pregnancies with the following diagnoses: 1) uncomplicated pregnancies who delivered appropriate for gestational age neonates (controls; n=124); and 2) patients who had a fetal death (n=19).

Plasma samples were obtained at each prenatal visit, scheduled at 4-week intervals from the first or early second trimester until delivery. In cases in which more than one sample from the same patient in a specific gestational age group was available, the earliest sample was chosen. Fetal death was defined as death of the fetus after 20 weeks of gestation and confirmed by ultrasound examination. Fetuses with known congenital or chromosomal abnormalities, as well as pregnancies complicated by preeclampsia or multiple gestations, were excluded. SGA was diagnosed as a birth weight below the 10th percentile for gestational age [83].

Samples collection

Blood samples were collected into tubes containing EDTA. The samples were centrifuged for 10 minutes at 4°C and stored at −70°C. Laboratory personnel were blinded to the clinical diagnosis.

Human sVEGFR-1, sEng, and PlGF immunoassays

Maternal plasma concentrations of sVEGFR-1, sEng and PlGF were determined by sensitive and specific immunoassays (R&D Systems. Minneapolis, MN, USA). All immunoassays utilized a sandwich enzyme immunoassay technique and had been validated for plasma determinations of the analytes. The inter- and intra-assay coefficients of variation (CV) were: sVEGFR-1: 1.4% and 3.9%, sEng: 2.3% and 4.6% respectively; and PlGF: 6.02% and 4.8%, respectively. The sensitivity of the assays was: sVEGFR-1: 16.97 pg/ml, sEng: 0.08 ng/ml and PlGF: 9.52 pg/ml.

Statistical Analysis

Cross-sectional analysis of demographic and clinical characteristic data

The Kolmogrov-Smirnov and the Shapiro-Wilk tests were used to assess the distribution of the data. Since the data were not normally distributed, we used the Kruskal-Wallis test for comparisons among groups, and the Mann-Whitney test for comparisons between groups for

continuous variables. Chi-square or Fisher's exact tests were used for comparisons of categorical variables.

Longitudinal analysis of plasma sVEGFR-1, sEng and PlGF concentrations

Changes in the plasma concentrations of angiogenic-related factors over time and between groups were tested using a linear mixed-effects model [fixed effects + random effects]. The fixed effects were the diagnosis (a factor with two levels: normal pregnancy and fetal death), the linear and quadratic effects of gestational age on the concentration of the analytes, and several covariates including maternal age, body mass index (BMI), smoking, nulliparity and duration of sample storage. The interaction terms of the linear and quadratic effects of gestational age with the diagnosis were included in the model. This allowed testing the difference in analyte concentrations between the fetal death and control groups at specific gestational ages. The random effect used in the mixed-effects model was the intercept of each individual patient (i.e. the baseline concentration at 7 weeks of gestation). The model was fitted to the natural log ($\log_e$) transformed plasma concentration after replacement of zero concentration (below the detection limit) with 99% of the smallest non-zero concentration observed in the entire dataset for a given analyte (there were 16 zero values for PLGF in the control group and they were replaced with 6.33 pg/ml, and there was one zero value in the control group for sVEGFR-1 which was replaced with 384.6 pg/ml).

A natural logarithmic transformation was employed to stabilize the variance across the entire gestational age range. Statistical significance of fixed effects model was assessed using t-tests, and a p value < 0.05 was considered significant. The R statistical environment (www.r-project.org) and the specialized *nlme* package [Jose Pinheiro, Douglas Bates, Saikat DebRoy, Deepayan Sarkar and the R Core team (2008). nlme: Linear and Nonlinear Mixed Effects Models. R package version 3.1–90] were used for all longitudinal analyses.

Results

The demographic and clinical characteristics of the study population

The demographic and clinical characteristics of the study groups are displayed in Table I. There was no significant difference in the frequency of nulliparity, smoking, the median maternal age and the median gestational age at enrollment between patients destined to have a fetal death and those who had a normal pregnancy outcome.

The median gestational age at delivery and birthweight were lower in patients with a fetal death than that of those in the control group ($p<0.001$; Table I). Two patients (10.5%) with a fetal demise had clinical placental abruption, and 6 patients (33%) delivered neonates whose birthweight were less than the 10^{th} percentile for gestational age. Fetal death was diagnosed before 24 weeks in 3 patients (15.7%) and at term gestation (37 weeks or more) in 8 patients (42%).

This study included a total of 973 samples with 867 samples from the control group and 106 samples from patients with a fetal death. All patients except 1 in the control group had 7 blood samples, while patients in the fetal death group had a median number of blood samples of 6, with a range of 2–10.

Plasma concentrations of sVEGFR-1, sEng and PlGF

Plasma concentrations of sVEGFR-1, sEng, and PlGF in normal pregnant women and those of patients with fetal death for every 4 week gestational interval are displayed in Table II. The plasma concentration of PlGF in the first trimester (6–13 weeks) was above the detection limit of the assay in 83% (72/87) of the control group, and all samples (n=12)

obtained in the first trimester (6–13 weeks) from women destined to have a fetal death had PlGF concentrations above the sensitivity of the assay. This result was unexpected.

The changes of plasma concentrations of sVEGFR-1, sEng and PlGF in patients destined to have a fetal death and those who had a normal pregnancy across all gestational ages are displayed in Figures 1, 2 and 3 respectively. The curves in the figures represent a quadratic fit of the analyte concentration based on the gestational age (without adjustment for covariates). By examining these curves, an overview of the relationship of plasma concentrations of sVEGFR-1, sEng, PlGF and gestational age in patients who subsequently had a fetal death and those with a normal pregnancy outcome can be surmised. The mean maternal plasma concentration of sVEGFR-1 in the fetal death group was lower during the first trimester, and became higher than that of the control group during the second and third trimesters (Figure 1). Similar changes were observed for sEng (Figure 2). In contrast, the mean maternal plasma PlGF concentration in patients destined to have a fetal death was higher in the first trimester, but lower than that of the control group until term (Figure 3).

Longitudinal analysis of plasma sVEGFR-1, sEng and PlGF concentrations

A linear mixed-effects model was used to assess the relationship between fetal death and angiogenic/anti-angiogenic factor plasma concentrations while adjusting for gestational age at venipuncture, maternal age, body mass index (BMI), smoking, nulliparity and duration of sample storage. Overall, the average profiles of analyte concentrations as a function of gestational age for sVEGFR-1, sEng and PlGF were different between the two groups as determined by the p-values ($p<0.05$) for the “Fetal Death x GA” and “Fetal death x GA2” coefficients in Table III (see also Fig 1–3). The inclusion of the interaction terms “Fetal Death x GA” and “Fetal death x GA2” in the mixed effects model allowed evaluation of the significance and magnitude of the differences in plasma angiogenic /anti-angiogenic factor concentrations between groups at specific gestational ages (i.e. GA=0). Since a gestational age at 0 does not have a meaningful interpretation, the test was performed at 7 weeks. Table III demonstrates that the difference in concentrations of each analyte at 7 weeks was significant (sVEGFR-1: $p=0.0002$; sEng: $p=0.0464$ and PlGF: $p<0.0001$).

By preserving the same model structure, the differences in plasma angiogenic/anti-angiogenic factor concentrations between the two groups while adjusting for all covariates were evaluated from 7 to 40 weeks of gestation (Table IV). The magnitude of differences in plasma concentrations of sVEGFR-1, sEng and PlGF between the two groups was a function of gestational age. The maternal plasma sVEGFR-1 concentrations were significantly lower (26%–48%) among patients destined to have a fetal death than those of the control group from 7 to 11 weeks of gestation (each $p<0.05$; Table IV). Subsequently, maternal plasma sVEGFR-1 concentrations in the cases increased until term, while those of normal pregnancy trended down in the second trimester and rose again in the third trimester (see Figure 1). Maternal plasma sVEGFR-1 concentrations in women destined to have a fetal death were significantly higher (26%–51%) than in those with a normal pregnancy from 20 to 37 weeks of gestation ($p<0.05$; Table IV).

Similarly, plasma sEng concentrations of women destined to have a fetal death were lower (21%) at 7 weeks of gestation ($p=0.04$) and became significantly higher (18%-62%) between 20 and 40 weeks of gestation than those of women destined to have a normal pregnancy ($p<0.05$; Table IV). In contrast to the anti-angiogenic analytes, the maternal plasma PlGF concentrations were higher (45%–262%) among patients destined to have a fetal death than in women who had a normal pregnancy outcome between 7 and 14 weeks of gestation ($p<0.05$; Table IV). After the first trimester, plasma concentrations of PlGF in patients with fetal death increased at a slower rate than those with a normal pregnancy until the early third trimester (see Figure 3). Between 22 and 39 weeks of gestation, the maternal plasma PlGF

concentrations became significantly lower (26%–45%) among patients destined to have a fetal death than in those in the control group ($p<0.05$; Table IV). Note that the assessment of the magnitude and significance of between group differences shown in Table IV included an adjustment for all covariates explored in this study.

Collectively, patients destined to have a fetal death compared to those who had a normal pregnancy were characterized by higher plasma concentrations of PlGF in the first trimester, and lower plasma concentrations of sVEGFR-1 and sEng. This profile changed to favor higher concentrations of sVEGFR-1 and sEng, but lower PlGF in the second and third trimesters.

Longitudinal analysis of the ratio between angiogenic factor (PlGF) and anti-angiogenic factor (sVEGFR-1 and/or sEng) concentrations

A similar longitudinal analysis was conducted on the ratios of analytes [PlGF/sVEGFR-1, PlGF/sEng, and PlGF/ (sVEGFR-1 x sEng)] instead of individual concentrations (see Table VI). The ratio of PlGF/sVEGFR-1 x sEng was significantly higher in women destined to have a fetal death between 7 and 14 weeks of gestation (94%–780%) and significantly lower (43%–75%) between 20 and 40 weeks of gestation than those in normal pregnant women (each $p<0.05$; Table V). The ratio of PlGF/ (sVEGFR-1 x sEng), PlGF/sEng or PlGF/ sVEGFR-1 differed significantly ($p <0.05$) between patients with fetal death and those with normal pregnancy in 28 out of 34 (82%) gestational weeks evaluated (Table V and Figure 4). However, among the three ratios evaluated, the ratio PlGF/ (sVEGFR-1 x sEng) provided the best discrimination between women destined to have a fetal death and those destined to have a normal pregnancy outcome [as determined by the number of weeks from 7 to 40 when the differences were statistically significant and also based on the magnitude of the differences expressed in percentages, Table V]. When the patients with placental abruption and those who were SGA were excluded, The ratios of PlGF/ (sVEGFR-1 x sEng) was significantly higher in women destined to have a fetal death between 7 and 12 weeks of gestation (103%–585%) and significantly lower (47%–61%) between 21 and 34 weeks of gestation than those in normal pregnant women (each $p<0.05$).

Longitudinal analysis of the ratio of PlGF/ (sVEGFR-1 x sEng) in fetal death in preterm and term gestations

We next subdivided patients with fetal death into those who were diagnosed before or after 37 weeks and compared the results with those of normal pregnancy (Table VI). The ratio of PlGF/ (sVEGFR-1 x sEng) in patients with preterm fetal death was significantly higher between 7 and 11 weeks of gestation (111%–327%) and significantly lower (49%–93%) between 20 and 37 weeks of gestation than those in normal pregnant women (each $p<0.05$; Table V and Figure 5). In patients with term fetal death, the ratio of PlGF/ (sVEGFR-1 x sEng) was significantly higher between 7 and 14 weeks of gestation (119%–1146%) and significantly lower (47%–54%) between 25 and 34 weeks of gestation than those in normal pregnant women (each $p<0.05$; Table V and Figure 6). The ratio PlGF/ (sVEGFR-1 x sEng) in patients with fetal death in preterm and term gestation was significantly different from that of normal pregnant women in 74% (23/31) and in 53% (18/34) of the number of weeks evaluated.

Comments

Principal findings of the study

1) this is the first longitudinal study that we are aware of reporting a change in biological markers before fetal death. In this case, the analytes measured included angiogenic and anti-angiogenic factors implicated in the genesis of pregnancy complications; 2) patients destined

to have a fetal death had higher maternal plasma concentrations of PlGF and lower plasma concentrations of sVEGFR-1 and sEng in the first trimester than women destined to have a normal pregnancy; 3) In contrast, during the second and third trimesters, patients destined to have a fetal death had higher plasma concentrations of sVEGFR-1 and sEng, but lower plasma PlGF concentrations than that of women destined to have a normal pregnancy; and 4) The ratio of PlGF / (sVEGFR-1 x sEng) in patients destined to have a fetal death is higher in the first trimester, and lower in the second and third trimester than that in normal pregnant women. The association between the fetal death and the profile of the ratio of PlGF / (sEng x sVEGFR-1) is present in both preterm and term fetal deaths.

Fetal death has a unique anti-angiogenic profile

We have previously proposed that fetal death is one of the "great obstetrical syndromes" [1]. The observations herein demonstrate that mothers destined to have a fetal death have a different angiogenic and anti-angiogenic profile in their blood than the one previously described in other obstetrical syndromes in the context of longitudinal studies (e.g. preeclampsia and SGA) [64]. The most obvious difference between fetal death, preeclampsia and SGA is that a subset of patients destined to have fetal death had increased concentrations of PlGF in the first trimester of pregnancy and lower sVEGFR-1. The opposite is the case for preeclampsia, in which PlGF is lower in the first trimester while there is no difference in the maternal plasma concentration of sVEGFR-1 [35,64,84]. In SGA, the maternal plasma concentrations of sEng are higher in the first trimester, while PlGF is lower than in normal pregnancy [64]. Of interest is that in a subset of patients destined to develop preterm labor with intact membranes, changes in PlGF, sVEGFR-1 and sEng in maternal plasma were not detected in the first trimester in a longitudinal study reported by our group [85]. Collectively, these observations suggest that the behavior of maternal concentrations of angiogenic and anti-angiogenic factors among different complications of pregnancy differ according to the specific phenotype. Thus, it is not simply a matter of whether there is an angiogenic or anti-angiogenic profile, but also when such profile exists and what specific growth factor is involved.

Changes in maternal plasma sVEGFR-1

Patients destined to have a fetal death, compared to those destined to have a normal pregnancy, had lower maternal plasma sVEGFR-1 concentrations at the beginning of the first trimester (between 7 and 11 weeks), which then became higher between 20 and 37 weeks of gestation. This observation is consistent with a previous cross-sectional study which demonstrated that high maternal serum concentrations of sVEGFR-1 between 10 and 14 weeks were associated with a reduced risk of stillbirth [55]. Previous studies indicate that patients destined to develop early-onset preeclampsia and late-onset preeclampsia have a significantly higher plasma sVEGFR-1 concentration than those destined to have a normal pregnancy only after 26 (range 24–28) and 30 (range 28–32) weeks of gestation, respectively [35,39,64]. Moreover, the magnitude of the changes is much higher in patients destined to develop preeclampsia than those destined to have a fetal death.

The changes in sVEGFR-1 concentrations in patients who delivered an SGA neonate were less dramatic than those observed in patients who developed preeclampsia [64]. Indeed, significantly higher maternal plasma concentrations of sVEGFR-1 were reported among patients with an SGA neonate at the time of diagnosis, in particular, among those with Doppler abnormalities in the uterine and umbilical arteries [69]. However, in a previous longitudinal study reported by our group, there was no significant difference in the changes of maternal plasma sVEGFR-1 concentrations between patients who were destined to deliver an SGA neonate and those with a normal pregnancy [64]. This suggests that

sVEGFR-1 may be more important in determining the phenotype of preeclampsia and fetal death than that of SGA without Doppler abnormalities.

Changes in maternal plasma sEng

The pattern of maternal plasma sEng concentrations among patients destined to have a fetal death throughout pregnancy is unique. During the first trimester, the maternal plasma concentration is lower than that of women destined to have a normal pregnancy only at 7 weeks of gestation, and the change over time (advancing gestational age) is subtle in comparison to the magnitude of the change of sVEGFR-1. After the first trimester, the sEng concentrations increased in women destined to have a fetal death in comparison to those who went on to have a normal pregnancy; these became statistically significant between 20 and 40 weeks of gestation.

Our group reported that sEng was elevated from the 10^{th} week of gestation among patients who were destined to deliver an SGA neonate [64]. In contrast, patients who developed preterm preeclampsia had a significant elevation of the maternal plasma concentrations of this analyte starting at 23 weeks of gestation, while those who subsequently developed term preeclampsia had a significant increase in the maternal plasma sEng concentration only after 30 weeks gestation [64]. Similarly, in the study conducted by Levine et al, sEng was increased among patients who developed preterm preeclampsia or delivered an SGA neonate between 17–20 weeks of gestation, and between 25–28 weeks among those destined to develop term preeclampsia [44].

Changes in maternal plasma PlGF

The changes in the maternal plasma PlGF concentrations among patients destined to have a fetal death demonstrated a different pattern than those previously reported in patients destined to develop preeclampsia or to deliver an SGA neonate [64]. While our previous longitudinal study demonstrated that the plasma PlGF concentration in patients destined to develop preeclampsia or deliver an SGA neonate was significantly lower than those with a normal pregnancy in the first trimester from 10–11 weeks [64], the plasma PlGF concentration in patients with a fetal death reported herein was higher than that in normal pregnant women between 7–14 weeks. Moreover, the gestational age at which maternal plasma PlGF concentration peaked among patients destined to have a fetal death was slightly earlier (approximately 26 weeks) than that of normal pregnant women (approximately 28–30 weeks), and that of patients with SGA neonate or term preeclampsia (approximately 27 weeks) as reported in a previous longitudinal study [64]. In contrast, patients with preterm preeclampsia had an earlier peak in their maternal plasma PlGF concentrations (before 25 weeks of gestation) [64] and substantially lower concentrations during the third trimester than normal pregnant women [35,64]

Possible mechanisms to explain the changes in angiogenic and anti-angiogenic factors in fetal death

Recent studies using hysteroscopy [86], hysterectomy specimens [86–88], Doppler velocimetry [89–92] and an advanced oxygen sensing probe [93–95] suggest that the circulation in the intervillous space is not established until 10–12 weeks [96]. Before this time, extravillous trophoblast plugs the tips of the spiral arteries; therefore, the conceptus is in a state of hypoxia. Upon dislodging of the trophoblast plugs between 10 and 14 weeks, maternal blood enters the intervillous space and the oxygen tension increases. These developmental stages are designed to limit exposure of the trophoblast to oxygen [93,97–99]. The latter can induce the production of reactive oxygen radicals and oxidative stress and damage the placenta [100–104]. Untimely and premature opening of the spiral arteries leads

to a state of hyperoxia in the intervillous space, and this can damage the placenta and lead to a spontaneous abortion [105–107].

A recent study of pregnancies undergoing termination for psychosocial reasons between 6–12 weeks of gestation demonstrated an inverse relationship between the partial pressure of oxygen and the concentrations of sVEGFR-1 in blood from the placental bed, suggesting that the changes in oxygen tension can modulate the expression of specific placental proteins including anti-angiogenic factors in early pregnancy [108]. Moreover, high oxygen tension (40%) has been shown to up-regulate PlGF and down-regulate sVEGFR-1 protein expression in term villous explants [109,110]. Thus, it is possible that the observed lower plasma concentrations of sVEGFR-1 and higher plasma concentrations of PlGF in patients destined to have a fetal death in early first trimester than in those with a normal pregnancy result from abnormal high oxygen tension in the intervillous space.

Although the profile of angiogenic and anti-angiogenic factors in the second and third trimesters of patients destined to have a fetal death is somewhat similar to that of those destined to develop preeclampsia, the magnitude of the changes is much higher in those with preeclampsia especially for the early-onset disease. It is noteworthy that, in contrast to patients destined to have a fetal death; those who subsequently developed preeclampsia have lower plasma concentrations of PlGF than normal pregnant women, while there is no difference in sVEGFR-1 or sEng concentrations in the first trimester.

Strengths and limitations of this study

The strengths of this study are its longitudinal nature and that several analytes have been measured. This represents the first longitudinal study of angiogenic and anti-angiogenic factors in women destined to have a fetal death. Another strength of this study is the analytical approach. Many longitudinal studies (including those published in prestigious journals) have been analyzed using a cross-sectional approach. We have previously discussed the limitations of such an approach [64].

It is now accepted that testing hypotheses about the association between the maternal plasma concentration of anti-angiogenic/angiogenic factors or other analytes and important covariates [such as pregnancy outcome (normal pregnancy or fetal death), gestational age, BMI, etc] requires a different set of analytical tools. The classical use of generalized linear models or repeated measure analysis of variance has limitations. Generalized linear models could overestimate the significance of the covariates, while the use of repeated measure ANOVA has limitations when there is missing data. These problems are overcome by using the linear mixed-effects model analyses. Other statistical approaches may also be appropriate. It is important to stress that whatever analytical approach is employed, it must take into account the correlated nature of the observations and missing values. A mixed-effects model addresses this issue by allowing each individual to have its own “random effect” on the baseline analyte concentration. Therefore, these models have the capability to fit the observed data much better than generalized linear models.

We have incorporated all the available data in the figures to enable the reader to visualize the main trends in the raw data on a logarithmic scale in each study group, while the individual profiles are presented in the supplementary Figure 1. The curves in the figures presented in the manuscript represent a quadratic fit of the analyte concentration based on the gestational age alone. The purpose of these overall curves is to provide the reader with a description of the behavior of the group’s average concentration for a particular analyte at a given gestational age.

Limitations of this study are that the change of plasma angiogenic/anti-angiogenic factor concentrations in fetal demise was based on the assumption that the early or the late fetal demise group had similar profiles of angiogenic/anti-angiogenic factor concentrations. We tried to address this potential problem by comparing plasma angiogenic/anti-angiogenic concentrations between cases of fetal death that occurred before 24 or 28 weeks of gestation and controls and could not demonstrate a statistically significant difference between the two groups. However, we acknowledge that we have a small sample size (n=3 and 4 respectively), and that the small number of repeated measurements for each patient obtained before 24 or 28 weeks of gestation. It is possible that we do not have the statistical power to detect a difference. However, we have demonstrated that patients destined to have a fetal death (preterm and term) had a different profile of maternal angiogenic/anti-angiogenic factors from that of women with normal pregnancies. Since the mechanisms leading to a fetal death in the second and third trimester may be different [6,111], one would expect that the gestational age at which the fetal death was diagnosed might influence the maternal profile of angiogenic and anti-angiogenic factors. A larger study that is specifically designed to address this question is necessary; yet the low prevalence of fetal death in the general obstetric population makes this a challenge. It could also be argued that the observed higher PlGF concentrations in women destined to have a fetal death than those in the control group could result from undetectable concentrations of PlGF in the controls in the first trimester. We have replaced these values with 99% of the smallest non-zero concentration observed in the entire dataset. This was done to reduce the chance of type I error for this observation. The development of more sensitive assays for PlGF should address this problem, and we will be in a position to employ such assays in the near future.

Conclusions

1) Patients destined to have a fetal death have higher maternal plasma concentrations of PlGF and lower plasma concentrations of sVEGFR-1 and sEng in the first trimester than women destined to have a normal pregnancy; 2) In contrast, during the second and third trimesters, patients destined to have a fetal death have higher plasma concentrations of sVEGFR-1 and sEng, but lower plasma PlGF concentrations than that of women destined to have a normal pregnancy; 3) These changes in maternal plasma concentrations of these angiogenic/anti-angiogenic factors, especially in the first trimester, are different from those we have previously reported in patients destined to develop preeclampsia or SGA. The mechanisms responsible for the occurrence of a fetal death may be operational in the first trimester of pregnancy. This has important implications because it requires a major emphasis on the organization of prenatal care so that we can focus on the study of biological markers of disease and interventions in early pregnancy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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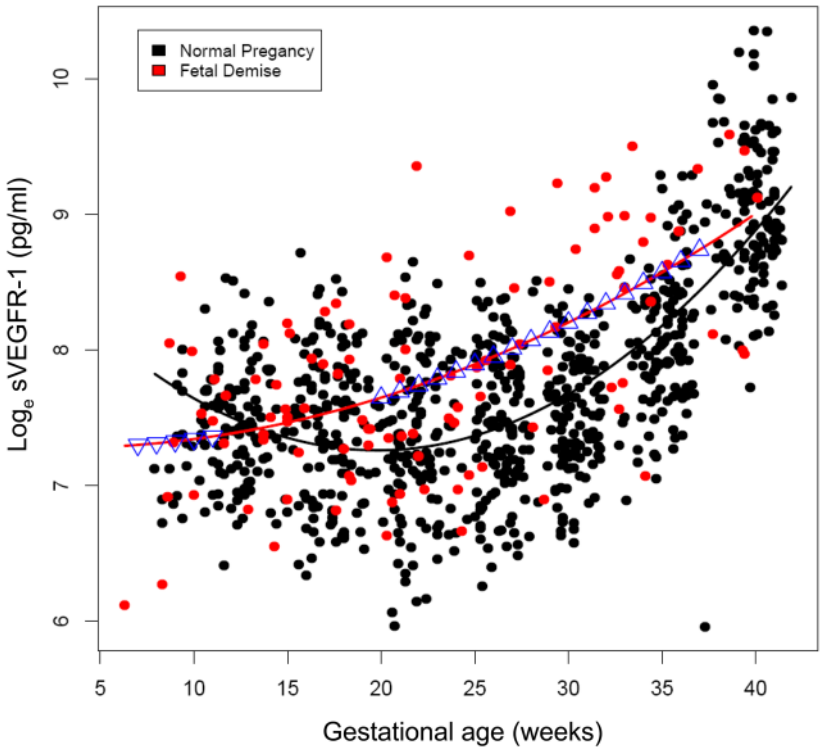

Figure 1.
Mean maternal plasma concentrations (on a $\log_e$ scale) of soluble vascular endothelial growth factor receptor-1 (sVEGFR-1) as a function of gestational age in women with normal pregnancies and patients destined to have a fetal death without adjustment for covariates. Triangles denote statistical significance between the two groups at each gestational age according to a linear mixed-effects model adjusting for covariates. For the individual plots of the patients, please see supplementary figure 1.

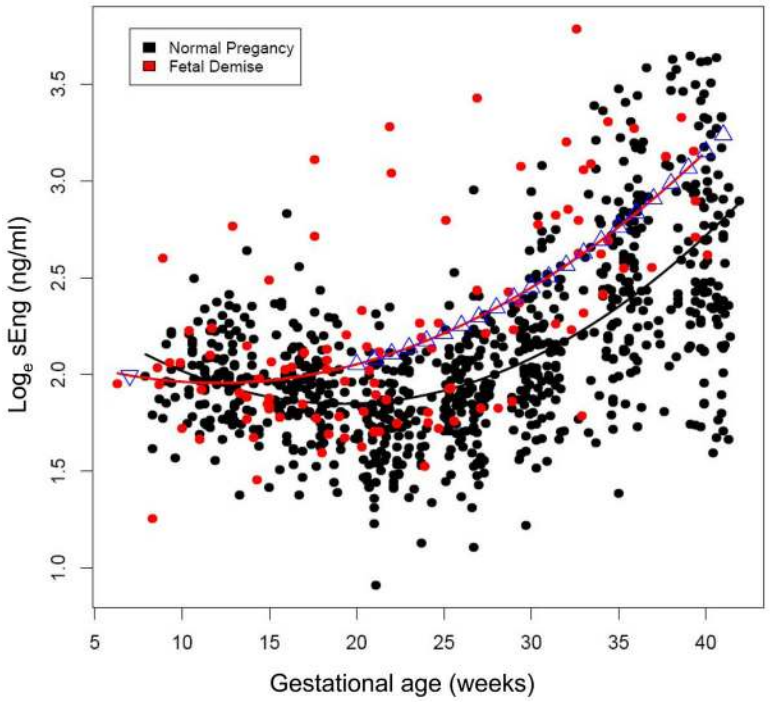

Figure 2.

Mean maternal plasma concentrations (on a $\log_e$ scale) of soluble endoglin (sEng) as a function of gestational age in women with normal pregnancies and patients destined to have a fetal death without adjustment for covariates. Triangles denote statistical significance between the two groups at each gestational age according to a linear mixed-effects model adjusting for covariates. For the individual plots of the patients, please see supplementary figure 1.

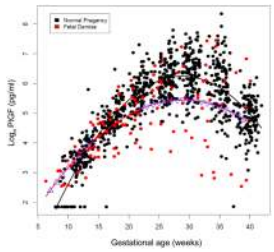

Figure 3.
Mean maternal plasma concentrations (on a $\log_e$ scale) of placental growth factor (PlGF) as a function of gestational age in women with normal pregnancies and patients destined to have a fetal death without adjustment for covariates. Triangles denote statistical significance between the two groups at each gestational age according to a linear mixed-effects model adjusting for covariates. For the individual plots of the patients, please see supplementary figure 1.

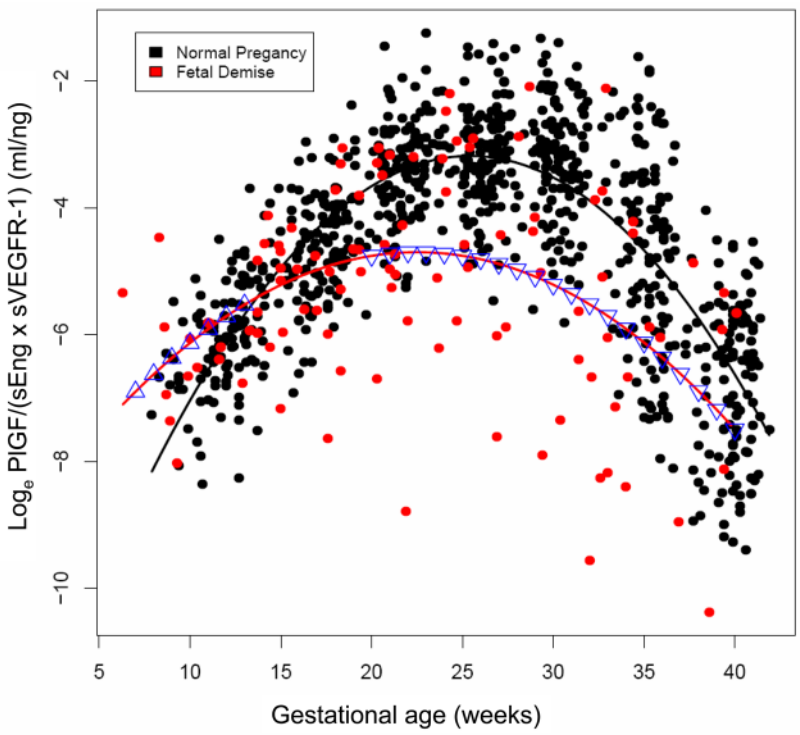

Figure 4.

Mean maternal plasma concentrations (on a $\log_e$ scale) of the ratio PlGF / (sEng x sVEGFR-1) as a function of gestational age in women with normal pregnancies and patients destined to have a fetal death without adjustment for covariates. Triangles denote statistical significance between the two groups at each gestational age according to a linear mixed-effects model adjusting for covariates.

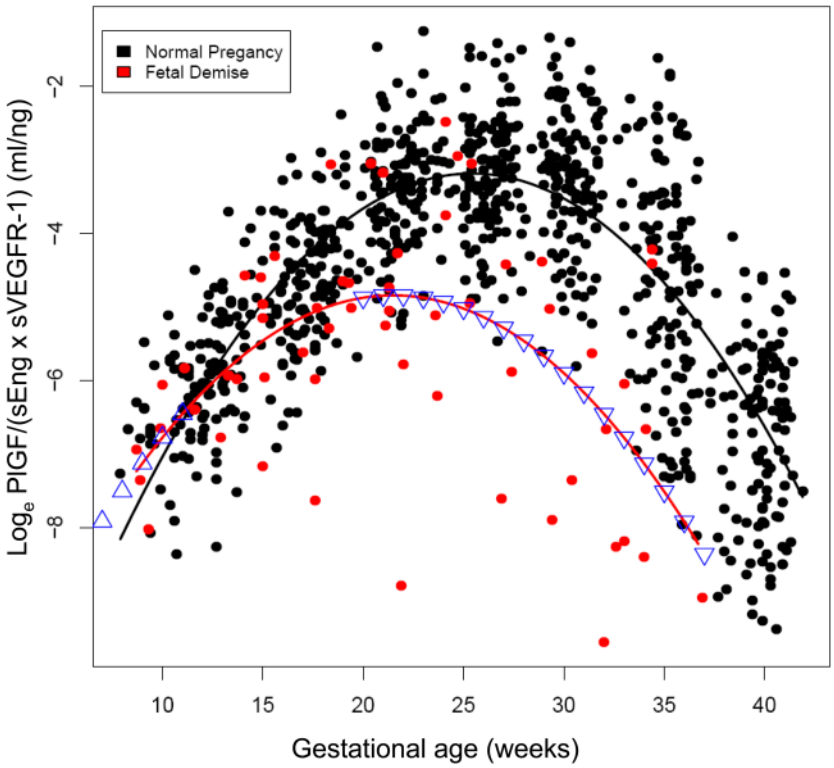

Figure 5.
Mean maternal plasma concentrations (on a $\log_e$ scale) of the ratio PlGF / (sEng x sVEGFR-1) as a function of gestational age in women with normal pregnancies and patients destined to have a fetal death before 37 weeks of gestation without adjustment for covariates. Triangles denote statistical significance between the two groups at each gestational age according to a linear mixed-effects model adjusting for covariates.

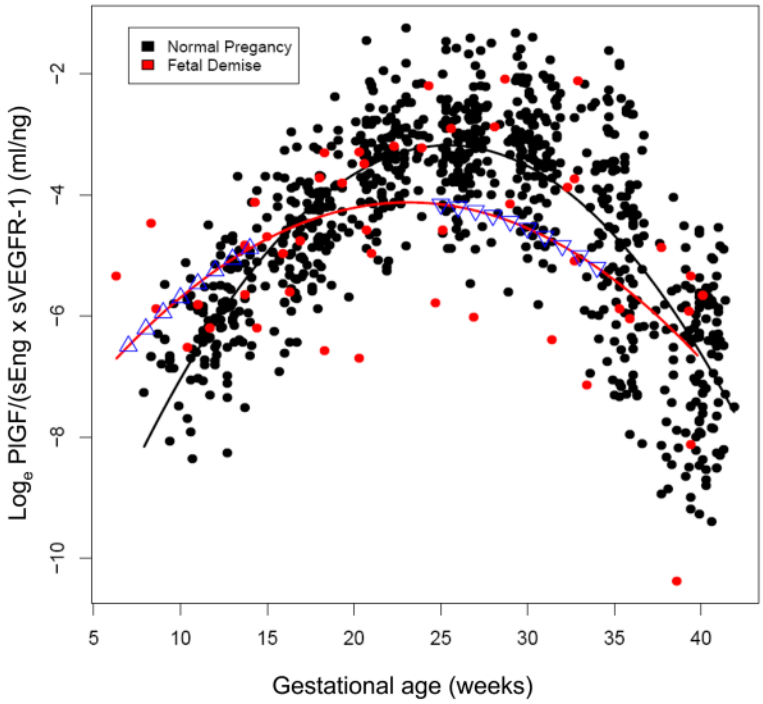

Figure 6.
Mean maternal plasma concentrations (on a log$_e$ scale) of the ratio PlGF / (sEng x sVEGFR-1) as a function of gestational age in women with normal pregnancies and patients destined to have a fetal death at 37 weeks or more without adjustment for covariates. Triangles denote statistical significance between the two groups at each gestational age according to a linear mixed-effects model adjusting for covariates.

Table I

Demographic and clinical characteristics of the study groups

	Normal Pregnancy (n=124)	Fetal demise (n=19)	p
Maternal Age (years)	24 (16–47)	24 (16–44)	0.4
Body mass index (BMI) (Kg/m^2)	23.5 (16–37)	23.7 (19–44)	0.4
Smoking	14 (11.3%)	1 (5.3%)	0.7
Nulliparity	58 (46.8%)	11 (57.9%)	0.4
GA at enrollment (weeks)	12.2 (7.9–14.9)	11.1 (6.3–19.4)	0.3
GA at delivery (weeks)	40.0 (37.3–41.9)	36.0 (20.6–40.3)	<0.001*
Birthweight (grams)$^{\alpha}$	3390 (2580–4090)	2280 (420–3650)	<0.001*
Birthweight <10th percentile	0	6 (33.3%)	<0.001*
Delivery < 37 weeks	0	11 (57.9%)	<0.001*

Data expressed as median (range) and number (percentage).

GA- gestational age

$^{\alpha}$n of fetal demise group = 18

Table II

Median (interquartile range) plasma concentrations of sVEGFR-1, sEng, and PlGF in each gestational age intervals

		Controls	Fetal death
1st Interval(6–10 weeks)	GA	9.3 (8.9–9.4)	8.8 (8.5–9.5)
	sVEGFR-1 (pg/ml)	1363 (1006–1746)	1258 (886–2999)
	sEng (ng/ml)	7.1 (6.3–7.6)	7.3 (6.6–7.9)
	PlGF (pg/ml)	9.8 (5.8–24.4)	18.2 (13.3–21.3)
	n	16	8
2nd Interval (10.1–14 weeks)	GA	12.3 (11.3–13.0)	12.3 (11.2–13.6)
	sVEGFR-1 (pg/ml)	1732 (1287–2238)	1810 (1549–2333)
	sEng (ng/ml)	7.3 (6.3–8.6)	7.5 (6.6–9.1)
	PlGF (pg/ml)	34.3 (24.5–48.9)	38.0 (26.1–45.1)
	n	98	10
3rd Interval (14.1–18 weeks)	GA	16.7 (16.0–17.5)	15.4 (15.0–17.0)
	sVEGFR-1 (pg/ml)	1628 (991–2378)	1931 (1514–2772)
	sEng (ng/ml)	7.0 (6.0–7.7)	6.4 (6.0–7.8)
	PlGF (pg/ml)	99.1 (79.1–121.7)	88.9 (45.8–108.9)
	n	99	17
4th Interval (18.1–22 weeks)	GA	20.7 (19.3–21.4)	20.4 (19.3–21.1)
	sVEGFR-1 (pg/ml)	1606 (1192–2357)	1658 (1362–2986)
	sEng (ng/ml)	6.0 (5.2–7.0)	7.1 (6.0–8.4)
	PlGF (pg/ml)	229.2 (154.5–323.4)	142.6 (99.0–235.8)
	n	95	17
5th Interval (22.1–26 weeks)	GA	24.0 (22.7–25.4)	24.3 (23.9–25.1)
	sVEGFR-1 (pg/ml)	1697 (1124–2695)	1802 (1184–2460)
	sEng (ng/ml)	6.1 (5.1–7.4)	6.8 (5.8–8.9)
	PlGF (pg/ml)	399.9 (275.3–565.0)	318.1 (175.6–442.6)
	n	108	12
6th Interval (26.1–30 weeks)	GA	27.6 (26.8–29.6)	28.4 (27.2–29.0)
	sVEGFR-1 (pg/ml)	1672 (1236–2390)	3320 (2592–4873)
	sEng (ng/ml)	7.2 (6.1–8.8)	10.0 (7.1–11.4)
	PlGF (pg/ml)	565.2 (362.4–853.1)	238.2 (113.6–525.6)
	n	108	10
7th Interval (30.1–34 weeks)	GA	31.1 (30.6–31.7)	32.7 (32.0–33.0)
	sVEGFR-1 (pg/ml)	2069 (1473–2712)	6435 (4830–7998)
	sEng (ng/ml)	8.6 (6.9–11.9)	16.2 (11.0–20.3)
	PlGF (pg/ml)	535.1 (368.3–891.4)	203.7 (60.7–394.8)
	n	78	11

		Controls	Fetal death
8th Interval (34.1–38 weeks)	GA	35.6 (34.9–36.1)	35.3 (34.4–36.4)
	sVEGFR-1 (pg/ml)	3686 (2621–4977)	5580 (3803–7535)
	sEng (ng/ml)	11.9 (9.1–17.1)	14.8 (12.8–24.6)
	PlGF (pg/ml)	312.9 (185.8–654.5)	443.1 (109.1–1001.5)
	n	108	7
9th Interval (38.1–42 weeks)	GA	39.9 (39.4–40.7)	39.4 (39.3–39.4)
	sVEGFR-1 (pg/ml)	7571 (5855–10965)	9166 (2958–12936)
	sEng (ng/ml)	13.1 (9.8–19.3)	18.1 (15.0–23.4)
	PlGF (pg/ml)	128.2 (86.8–194.7)	184.1 (15.6–438.8)
	n	112	5

Table III

Longitudinal analysis of the association between anti-angiogenic (sVEGFR-1 and sEng) or angiogenic factor (PLGF) and fetal demise after adjusting for confounding factors

	sVEGFR-1					s-Eng					PlGF				
	Value	**Std. Error**	**DF**	**t-value**	**p-value**	**Value**	**Std. Error**	**DF**	**t-value**	**p-value**	**Value**	**Std. Error**	**DF**	**t-value**	**p-value**
(Intercept)	8.1391	0.2522	825	32.28	0.0000	2.3068	0.1687	825	13.67	0.0000	2.2029	0.3295	825	6.69	0.0000
Fetal death	−0.6585	0.1722	137	−3.82	0.0002	−0.2350	0.1169	137	−2.01	0.0464	1.2877	0.2278	137	5.65	0.0000
GA	−0.1015	0.0064	825	−15.78	0.0000	−0.0507	0.0044	825	−11.49	0.0000	0.4430	0.0086	825	51.63	0.0000
GA^2	0.0039	0.0002	825	22.19	0.0000	0.0020	0.0001	825	17.06	0.0000	−0.0100	0.0002	825	−43.02	0.0000
mage	−0.0022	0.0058	137	−0.39	0.7007	0.0058	0.0039	137	1.49	0.1380	−0.0097	0.0076	137	−1.28	0.2026
BMI	−0.0172	0.0079	137	−2.19	0.0300	−0.0150	0.0053	137	−2.85	0.0050	−0.0112	0.0103	137	−1.09	0.2774
smoking	−0.0730	0.1082	137	−0.68	0.5007	0.0132	0.0722	137	0.18	0.8550	0.2394	0.1411	137	1.70	0.0920
Nulliparity	0.2112	0.0749	137	2.82	0.0055	0.0768	0.0500	137	1.54	0.1271	−0.1544	0.0977	137	−1.58	0.1164
Duration of storage	0.0002	0.0001	825	3.45	0.0006	0.0000	0.0000	825	0.97	0.3342	−0.0003	0.0001	825	−4.15	0.0000
Fetal Death x GA	0.0975	0.0188	825	5.19	0.0000	0.0368	0.0129	825	2.86	0.0043	−0.1514	0.0251	825	−6.04	0.0000
Fetal death x GA^2	−0.0022	0.0005	825	−4.05	0.0001	−0.0005	0.0004	825	−1.23	0.2203	0.0030	0.0007	825	4.14	0.0000

GA- gestational age; Mage- maternal age; BMI- body mass index

Fetal Death x GA – interaction between diagnosis and GA

Fetal death x GA^2 - interaction between diagnosis and GA^2

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Table IV

The statistical differences (p value) and the percentage changes [(fetal demise - control/control) *100] in the maternal plasma concentrations of angiogenic and antiangiogenic factors between patients with a fetal demise and normal pregnant women according to gestational age

	sVEGFR-1		sEng		PlGF	
Gestational Age	P value	Percentagchange	P value	Percentage change	P value	Percentage change
7	0.0002	−48.24	0.0464	−20.94	0.0000	262.43
8	0.0006	−43.06	0.0681	−18.02	0.0000	212.46
9	0.0018	−37.64	0.1062	−15.06	0.0000	171.00
10	0.0062	−32.02	0.1724	−12.07	0.0000	136.47
11	0.0219	−26.21	0.2843	−9.07	0.0000	107.59
12	0.0722	−20.25	0.4601	−6.04	0.0003	83.34
13	0.2050	−14.20	0.7063	−3.01	0.0024	62.91
14	0.4715	−8.10	0.9969	0.03	0.0156	45.63
15	0.8605	−2.00	0.6955	3.07	0.0753	30.97
16	0.7280	4.04	0.4384	6.11	0.2563	18.50
17	0.4024	9.97	0.2517	9.13	0.6103	7.86
18	0.1989	15.72	0.1334	12.14	0.9340	−1.22
19	0.0916	21.23	0.0662	15.12	0.5266	−8.99
20	0.0409	26.44	0.0311	18.08	0.2554	−15.64
21	0.0183	31.29	0.0140	21.00	0.1106	−21.34
22	0.0084	35.73	0.0060	23.88	0.0445	−26.20
23	0.0041	39.69	0.0025	26.71	0.0172	−30.34
24	0.0021	43.14	0.0010	29.48	0.0066	−33.85
25	0.0012	46.02	0.0004	32.20	0.0026	−36.81
26	0.0007	48.30	0.0001	34.85	0.0010	−39.27
27	0.0005	49.94	0.0000	37.42	0.0005	−41.27
28	0.0004	50.94	0.0000	39.92	0.0002	−42.87
29	0.0003	51.27	0.0000	42.33	0.0001	−44.09
30	0.0003	50.93	0.0000	44.65	0.0001	−44.95
31	0.0004	49.92	0.0000	46.87	0.0001	−45.47

Gestational Age	sVEGFR-1		sEng		PlGF	
	P value	Percentagchange	P value	Percentage change	P value	Percentage change
32	0.0006	48.27	0.0000	48.98	0.0001	−45.65
33	0.0012	45.98	0.0000	50.99	0.0001	−45.51
34	0.0028	43.09	0.0000	52.89	0.0002	−45.04
35	0.0070	39.64	0.0000	54.67	0.0004	−44.22
36	0.0182	35.67	0.0000	56.33	0.0010	−43.05
37	0.0462	31.23	0.0000	57.85	0.0031	−41.51
38	0.1071	26.37	0.0000	59.25	0.0091	−39.56
39	0.2183	21.15	0.0000	60.51	0.0250	−37.16
40	0.3880	15.64	0.0000	61.63	0.0606	−34.28

Table V

The statistical differences (p value) and the percentage changes [(fetal demise - control/control) *100] in the ratio of maternal plasma concentrations of angiogenic and antiangiogenic factors between patients with a fetal demise and normal pregnant women according to gestational age

Gestational Age	PlGF/ (sVEGFR-1 x sEng)		PlGF/sEng		PlGF/sVEGFR-1	
	P value	Percentage change	P value	Percentage change	P value	Percentage change
7	0.0000	780.73	0.0000	362.08	0.0000	592.52
8	0.0000	565.41	0.0000	284.21	0.0000	442.38
9	0.0000	408.51	0.0000	221.68	0.0000	329.30
10	0.0000	293.07	0.0000	171.20	0.0000	243.40
11	0.0001	207.33	0.0002	130.22	0.0000	177.60
12	0.0008	143.06	0.0012	96.80	0.0002	126.79
13	0.0082	94.43	0.0088	69.39	0.0027	87.24
14	0.0620	57.33	0.0489	46.81	0.0265	56.23
15	0.2864	28.76	0.1942	28.13	0.1594	31.73
16	0.7849	6.60	0.5292	12.60	0.5491	12.26
17	0.6256	−10.74	0.9846	−0.36	0.8602	−3.32
18	0.2306	−24.40	0.5261	−11.22	0.3687	−15.86
19	0.0642	−35.23	0.2274	−20.34	0.1191	−25.99
20	0.0147	−43.87	0.0826	−28.04	0.0314	−34.22
21	0.0030	−50.80	0.0264	−34.53	0.0074	−40.91
22	0.0006	−56.38	0.0077	−40.03	0.0016	−46.35
23	0.0001	−60.88	0.0021	−44.68	0.0004	−50.78
24	0.0000	−64.52	0.0006	−48.62	0.0001	−54.37
25	0.0000	−67.44	0.0002	−51.95	0.0000	−57.24
26	0.0000	−69.79	0.0000	−54.75	0.0000	−59.51
27	0.0000	−71.64	0.0000	−57.09	0.0000	−61.25
28	0.0000	−73.07	0.0000	−59.03	0.0000	−62.52
29	0.0000	−74.13	0.0000	−60.61	0.0000	−63.37
30	0.0000	−74.87	0.0000	−61.86	0.0000	−63.82
31	0.0000	−75.31	0.0000	−62.82	0.0000	−63.88

Gestational Age	PlGF/ (sVEGFR-1 x sEng)		PlGF/sEng		PlGF/sVEGFR-1	
	P value	Percentage change	P value	Percentage change	P value	Percentage change
32	0.0000	−75.46	0.0000	−63.50	0.0000	−63.57
33	0.0000	−75.33	0.0000	−63.93	0.0000	−62.85
34	0.0000	−74.91	0.0000	−64.09	0.0000	−61.73
35	0.0000	−74.19	0.0000	−64.02	0.0000	−60.15
36	0.0000	−73.15	0.0000	−63.68	0.0001	−58.07
37	0.0000	−71.74	0.0000	−63.10	0.0007	−55.41
38	0.0001	−69.92	0.0001	−62.24	0.0038	−52.07
39	0.0007	−67.62	0.0003	−61.09	0.0168	−47.95
40	0.0035	−64.73	0.0011	−59.64	0.0579	−42.86

Table VI

The statistical differences (p value) and the percentage changes [(fetal demise - control/control) *100] in the ratio of maternal plasma concentrations of angiogenic and antiangiogenic factors [PlGF/(sVEGFR-1 x sEng)] between patients with a fetal demise and normal pregnant women stratified by gestational age at which fetal death was diagnosed

	<37 weeks		≥ 37 weeks	
Gestational Age	P value	Percentage change	P value	Percentage change
7	0.0033	327.67	0.0000	1146.16
8	0.0046	257.20	0.0000	835.28
9	0.0076	199.14	0.0000	611.15
10	0.0148	151.19	0.0000	447.81
11	0.0329	111.48	0.0001	327.52
12	0.0793	78.52	0.0005	238.01
13	0.1901	51.10	0.0029	170.74
14	0.4147	28.23	0.0147	119.70
15	0.7706	9.11	0.0607	80.62
16	0.8090	−6.91	0.1888	50.43
17	0.4421	−20.37	0.4400	26.93
18	0.2006	−31.70	0.7915	8.51
19	0.0764	−41.27	0.8411	−6.03
20	0.0248	−49.36	0.5364	−17.55
21	0.0069	−56.22	0.3230	−26.71
22	0.0016	−62.05	0.1894	−34.01
23	0.0003	−67.01	0.1112	−39.79
24	0.0001	−71.25	0.0669	−44.35
25	0.0000	−74.88	0.0420	−47.89
26	0.0000	−77.99	0.0280	−50.57
27	0.0000	−80.67	0.0200	−52.50
28	0.0000	−82.97	0.0156	−53.75
29	0.0000	−84.96	0.0135	−54.38
30	0.0000	−86.69	0.0130	−54.41

Gestational Age	<37 weeks		≥ 37 weeks	
	P value	Percentage change	P value	Percentage change
31	0.0000	−88.18	0.0142	−53.84
32	0.0000	−89.48	0.0176	−52.66
33	0.0000	−90.61	0.0249	−50.81
34	0.0000	−91.60	0.0394	−48.21
35	0.0000	−92.46	0.0680	−44.77
36	0.0000	−93.22	0.1226	−40.32
37	0.0000	−93.88	0.2200	−34.68
38			0.3759	−27.56
39			0.5932	−18.61
40			0.8525	−7.37