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**Title:** Association of serum angiogenic factors with bronchopulmonary dysplasia. The ANGIODYDYS cohort study.

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**List of abbreviations:**

BPD: bronchopulmonary dysplasia; BW: birth weight; IUGR: intra-uterine growth restriction; PE: preeclampsia; PIGF: placental growth factor; PMA: post-menstrual age; sEng: soluble endogline; sFlt-1: soluble receptor FMS-like tyrosine kinase-1; TGF- $\beta$ : transforming growth factor  $\beta$ ; VEGF: vascular endothelial growth factor; WG: weeks' gestation.

## **Abstract**

**Objectives:** Angiogenic factors may be involved in lung development. To evaluate the relations between maternal and cord blood angiogenic factors (sFlt-1, placental growth factor [PlGF], soluble endogline [sEng], transforming growth factor  $\beta$  [TGF-beta]) and their association with moderate and severe bronchopulmonary dysplasia (BPD) in very preterm growth-restricted infants.

**Study design:** Prospective monocentric cohort study. Twenty-four mother–child dyads featuring antepartum preeclampsia, intra-uterine growth restriction (IUGR) and birth before 30 weeks' gestation were included. This ensured a 80% power to test whether sFlt-1 maternal levels would be twice as high in cases of BPD as in the absence of BPD.

**Main outcome measures:** Four pro/anti-angiogenic factors from two pathways (sFlt-1, PlGF and sEng, TGF-beta) were measured in maternal serum before delivery (at the time of hospitalization or the day of birth) and in neonates' cord blood. Neonatal outcome was moderate to severe BPD, defined as oxygen requirement for at least 28 days and persistent need for oxygen or ventilatory support at 36 weeks' postmenstrual age.

**Results:** sFlt-1 levels were positively correlated in maternal serum and cord blood ( $r_s = .83$ ,  $p < .001$ ) but levels of PlGF and TGF-beta and its receptor sEng were not. Among all the factors studied in cord and maternal blood, none was associated with BPD.

**Conclusions:** In IUGR preterm babies born before 30 weeks' gestation from preeclamptic mothers, serum sFlt-1, PlGF and sEng, TGF- $\beta$  levels were not correlated with BPD. The increased BPD risk in preterm neonates born from preeclamptic mothers cannot be related to high sFlt-1 levels.

## **Introduction**

Bronchopulmonary dysplasia (BPD) is a chronic lung disease in children born premature and characterized by impaired lung growth due to early lung injuries. BPD reveals the effects of persistent abnormalities in distal lung structure including decreased alveolarization and a dysmorphic pulmonary vascular bed [1, 2]. Infants with BPD have prolonged respiratory insufficiency and are at increased risk of lung infections, recurrent hospitalizations during infancy and adverse neurodevelopmental outcomes [1]. Preterm birth, neonatal complications and side effects of respiratory support contribute to the pathogenesis of BPD.

Recently, both clinical and experimental studies revealed the impact of an adverse intra-uterine environment on neonatal lung growth and respiratory outcomes of preterm infants. Among various antenatal factors, pre-eclampsia (PE) has been found associated with a high risk for BPD, but the underlying mechanisms are still unclear [3, 4].

Pre-eclampsia (PE) a major complications of pregnancy and is the consequence of a global maternal endothelial dysfunction resulting in hypertension and proteinuria. PE is an important leading cause of preterm birth and intra-uterine growth restriction (IUGR). We lack a validated treatment that can stop the disease progression apart from delivery. With severe maternal or fetal complications, PE leads to premature delivery of growth-restricted babies.

During pregnancy, the placenta produces and releases to the maternal circulation pro-angiogenic factors such as placental growth factor (PlGF) and transforming growth factor  $\beta$  (TGF- $\beta$ ) and their soluble receptors FMS-like tyrosine kinase-1 (sFlt-1) and soluble endogline (sEng), respectively, which act as anti-angiogenic factors. PlGF is a 45- to 50-kDa dimeric glycoprotein located in the placental trophoblast but also in endothelial and epithelial cells [5]. In normal pregnancies, maternal serum PlGF level follows a bell-shaped curve with advancing gestational age: increased during the first two trimesters, then decreased as

pregnancy progresses to term [5]. Ranges in cord blood are still not well established in large cohorts. Placental oversynthesis and release of sFlt-1 causes maternal endothelial dysfunction and plays a key role in the pathogenesis of PE. sFlt-1 traps vascular endothelial growth factor (VEGF) and PlGF, thus decreasing their free bioactive form. The magnitude of excess sFlt-1 in cord blood is inversely correlated with birthweight and neonate platelet levels [6]. In animal models, intra-amniotic administration of sFlt-1 can impair lung development and VEGF signaling [7]. Other angiogenic factors, sEng and TGF- $\beta$ , are produced by the placenta and have recently been found involved in the pathogenesis of PE. TGF- $\beta$  regulates lung development from the pseudoglandular phase to the end of the alveolarization program [8]. Knockout mice of TGF- $\beta$  isoforms indicate multiples functions. For example, TGF- $\beta$ 1-deficient mice show in the lungs generalized perivascularitis or interstitial pneumonia, TGF- $\beta$ 2-null mice have postnatal lung defects with collapsed distal airways and TGF- $\beta$ 3 deficiency leads to defective lungs with alveolar hypoplasia and mesenchymal thickening. All of these indicate that TGF- $\beta$  signaling is required for epithelial-mesenchymal interactions to achieve lung branching morphogenesis and alveologensis [9].

In this study, we evaluated the relations between maternal and cord blood angiogenic factors (i.e., sFlt-1, PlGF, sEng and TGF- $\beta$ ) and their association with moderate and severe BPD in very preterm growth-restricted infants enrolled in a prospective birth cohort. To explore mechanisms that may link PE with BPD, we hypothesized that antenatal exposure to excess sFlt-1 in the maternal circulation may disrupt normal fetal lung development, thereby leading to BPD.

## **Materials and methods**

### Study Design and Ethics

We conducted a prospectively cohort study from June 2012 to March 2015 at Cochin–Port Royal hospital, Paris, France, which performs 5500 deliveries a year, and was approved by the CPP Ile de France III (no.: 2958) and by Institutional Review Board (EUDRACT): 2012-A00002-41. Consecutive singleton pregnant women with a diagnosis of antepartum PE and IUGR before 30 WG were included. All patients gave informed consent to participate to the study. Multiple pregnancies, postpartum PE or eclampsia and gestational hypertension were excluded. Babies born after 30 WG were excluded. Maternal blood was also collected immediately after the delivery at inclusion and at the time of delivery. Samples were stored at -80°C (as part of the PERINAT Collection project, Equipex ANR 2010). The patient data and samples were de-identified before analysis.

### Diagnosis of PE

PE was defined as hypertension (blood pressure  $\geq$  140/90 mmHg) and proteinuria ( $\geq$  3 g/l) after 20 WG (10). IUGR was defined by fetal weight  $<10^{\text{th}}$  centile [11] or abdominal circumference  $<10^{\text{th}}$  centile according to Chitty reference curves [12].

### Clinical outcome

The primary outcome was moderate to severe BPD defined as oxygen requirement for at least 28 days and persistent need for oxygen or ventilatory support at 36 weeks' postmenstrual age (PMA) [1]. Because of the competitive risk of death and BPD, we also examined a secondary composite outcome “death or moderate to severe BPD” at 36 weeks' PMA.

### Perinatal data

Maternal and neonatal data were collected from medical records. The antenatal corticosteroid course was considered complete if betamethasone was administered twice at a 24-h interval, incomplete if only one injection was administered, or absent. Gestational age was estimated by the first trimester ultrasound if available and otherwise, the last menstrual date. Main complications of prematurity were recorded: in-hospital deaths, necrotizing enterocolitis, neonatal late-onset infections, severe cerebral lesions, patent ductus arteriosus (PDA), hemodynamic insufficiency requiring vascular filling or inotropic drugs, hyperglycemia requiring insulin treatment, and retinopathy of prematurity. PDA was diagnosed with clinical signs and echocardiography findings. Necrotizing enterocolitis was classified according to the Bell criteria [14]. Neonatal late-onset infections were defined as any positive culture from blood, cerebrospinal fluid, tracheal aspirate or urine sample occurring more than 72 h after birth. Severe cerebral lesions were considered periventricular leukomalacia and grade III and IV intraventricular hemorrhage according to the Papile classification [13].

Ventilation protocols, fluid intake and PDA management were unchanged throughout the study. High-frequency oscillation ventilation was used if hypercarbia or hypoxia persisted despite high pressure with conventional ventilation and with pulmonary hemorrhage or pneumothorax. Inhaled corticosteroids were used only to allow extubation in infants with continuous assisted-ventilation dependency.

### Measurement of angiogenic factors

Serum sFlt-1, PlGF, sEng and TGF- $\beta$  levels were assessed by the automated immunoassays PlGF Cobas (REF: 05144671) and sFlt-1 Cobas (REF: 05109523) (Roche Immunodiagnostic, Meylan, France) or the manual immunoassays sEng Quantikine and TGF- $\beta$ 1 Quantikine, R&D (Minneapolis, MN, USA).

The treating physicians were unaware of the test results. All de-identified samples were thawed once for analysis, the technician being blinded to the patient's diagnosis.

### Statistical analysis

Two different maternal serum samplings were planned with the study protocol: at the time of inclusion and at delivery. However, hospitalization length before delivery ranged from 0 to 28 days; moreover, 8 mothers had only one blood sample collected because cesarean section occurred just after their admission. Therefore, for each mother, we analyzed the sample collected closest to delivery. We first analyzed the correlation between maternal and cord blood levels of sFlt-1, PlGF, sEng and TGF- $\beta$  as well as ratios of sFlt-1 to PlGF and sEng to TGF- $\beta$  by using non-parametric Spearman tests.

The main analysis was the association between maternal sFlt-1 level and neonatal respiratory outcome (i.e., moderate/severe BPD at 36 weeks' PMA). Median levels of maternal sFlt-1 (and interquartile range) were compared between infants with moderate/severe BPD and those with no/mild BPD. Multiple logistic regression was then performed with adjustment for gestational age and birthweight z-score [14], because both these variables are the main predictors of neonatal outcomes [14]. The same analysis was repeated with the secondary outcome "death or moderate to severe BPD" at 36 weeks' PMA. Significance was set at  $p < .05$ .

According to preliminary analyses, 50% of the IUGR preterm babies born before 30 WG from pre-eclamptic mothers were affected by BPD at 36 weeks' PMA. Our hypothesis was that the mean sFlt-1 level in maternal blood would be approximately 3000 pg/ml in the absence of BPD in the neonate and twice higher with BPD. Therefore, with an alpha risk of .05, 80%



power (bilateral test) and standard deviation of 2500 pg/ml, we needed to recruit 24 "mother-child dyads" (12 children in each group).

Furthermore, we analyzed the association between each angiogenic factor (in maternal blood or in cord blood) and moderate/severe BPD by the Wilcoxon rank-sum test. To account for multiple analyses (12 exposure factors), the Dunn-Sidak correction was applied and significance was set at  $p < .005$ . Statistical analyses involved use of SAS v9.4 (SAS Institute, Inc, Cary, NC).

## Results

Thirty-four mother–child dyads met the inclusion criteria; 33 mothers gave their consent and 26 dyads were included in the study (Supplemental data S1). All births were cesarean section mainly due to fetal indications (76.9%).

Demographic and clinical characteristics of the study population are in Table 1 and outcomes are in Tables 2 and 3. All mothers except one received antenatal corticosteroids. Most of the pregnancies were monitored for pathological Doppler findings. None of the demographic and obstetrical factors listed in Table 1 were significantly associated with moderate to severe BPD at 36 WG.

About half of the newborns required high-frequency oscillation ventilation during their neonatal hospitalization. The median duration of invasive ventilation was < 10 days. Most of the babies did not receive postnatal corticosteroids. Two babies (7.7%) died before 36 weeks' PMA and 83.3% had BPD (any severity). More than half of the children had risk factors for BPD such as patent ductus arteriosus or late-onset sepsis.

### Association between maternal sFlt-1 level and criteria for BPD severity at 36 weeks' PMA

Serum sFlt-1 level was 40-fold higher in maternal blood than cord blood (Table 4). Maternal sFlt-1 level was not significantly associated with the respiratory outcome at 36 weeks' PMA. We found no significant relation between maternal sFlt-1 blood level and moderate/severe BPD after adjustment on birthweight or gestational age at birth (Table 5). Because of the competitive risk of death or BPD, we examined the association between serum sFlt-1 level and the combined outcome death or moderate/severe BPD but found no correlation with the combined outcome in maternal blood levels ( $p=.73$ ) or cord blood levels ( $p=.48$ ).

Association between serum levels of angiogenic/anti-angiogenic factors (except sFlt-1) and moderate/severe BPD at 36 weeks' PMA

Except for TGF- $\beta$  level, levels of PlGF and sEng were approximately 10-fold higher in maternal blood than in cord blood (Table 4). Moderate or severe BPD was not associated with levels of circulating angiogenic factors or their ratios in maternal blood (Table 4). Because of the competitive risk of death or BPD, we examined the association between PlGF, sEng and TGF- $\beta$  levels and the combined outcome death or moderate/severe BPD but found no correlation (data not shown).

Correlation between angiogenic /anti-angiogenic factors levels in maternal and cord blood

sFlt-1 levels were significantly correlated in maternal blood and in cord blood ( $r_s = .83$ ,  $p < .001$ ), but sFlt-1/PlGF and sEng/TGF- $\beta$  ratios were not (Figure 1). We found no correlation between the two angiogenic pathways we investigated (supplemental data S2).

## Discussion

To our knowledge, this is among the first cohort studies to describe the association between sFlt-1 and sEng levels in maternal blood or cord blood and BPD in very preterm babies. None of the angiogenic factors we investigated was associated with BPD, either in maternal blood or cord blood.

Pre-eclampsia is characterized by an altered angiogenic state with increased levels of anti-angiogenic factors, especially sFlt-1 and sEng. The sFlt-1 protein binds to VEGF and PlGF, thus reducing their active fraction in blood [5]. sEng is another anti-angiogenic protein that traps circulating TGF- $\beta$ , thereby decreasing its binding to its receptors and impairing the downstream signaling, which includes activation of endothelial nitric oxide synthase and vasodilatation [15]. Few studies have measured angiogenic factor levels in the mother–infant dyad. As compared with maternal sera, in cord blood, levels of sFlt-1 and sEng were very low, as reported previously [16, 17].

The sources of sFlt-1 and sEng in the fetal circulation are still unclear and the relation between maternal and cord blood levels is not known [18]. Faupel-Badger et al. studied 95 pregnant women, 48 with preeclampsia and found increased maternal levels of sFlt-1 and sEng in pre-eclamptic mothers but not increased sFlt-1 level in cord blood [19]. Another study of 36 women comparing growth-restricted fetuses to controls also showed higher maternal levels of VEGF and sFlt-1 in the IUGR than control group. Cord blood levels of VEGF and sFlt-1 were very reduced and were not significantly different between IUGR fetuses and controls. However, the pre-eclampsia status was unknown [20]. In another study, 38 dyads of pre-eclamptic mothers and infants revealed a positive association between fetal and maternal serum levels of sFlt-1. However, the range of gestation was wide (from 24 to 38 WG) [16]. To our knowledge, no study has reported maternal serum levels of sEng and TGF- $\beta$  or the

ratio of these levels in maternal or cord blood. We showed that among these angiogenic factors, only sFlt-1 level was significantly correlated in the mother–infant dyads.

For more than a decade, epidemiological studies have shown an increased risk of BPD in preterm infants from pre-eclamptic mothers, but the underlying mechanisms are unknown [3, 4]. Experimental studies have shown that excess sFlt-1 in amniotic fluid during the late canalicular and early saccular stage of lung development reduces VEGF signaling and increases apoptosis in the newborn rat lung. This increase is followed by sustained reductions in alveolarization and pulmonary vascular growth [7]. IUGR induced by a low protein diet mimicking pre-eclampsia in a rat model disturbed alveolarization and angiogenesis [21]. The role of PlGF in the developing lung has been studied only once in postnatal experimental models. Its overexpression in transgenic mice resulted in an enlarged air space and enhanced pulmonary compliance, but the pattern is not exactly the same as in BPD [22].

Epidemiological data concerning angiogenic factors and BPD at 36 weeks' PMA are scarce. In a cohort of 190 preterm infants, PlGF cord blood levels were significantly decreased with maternal vascular underperfusion diagnosed on histological placental examination. The pre-eclampsia status was not described. As in our study, BPD was not correlated with any angiogenic factors measured in cord blood [23]. The only significant association described was a decrease in PlGF cord blood levels in BPD with pulmonary hypertension. Yang et al. found increased cord-blood PlGF level in BPD infants [24] and Procianoy et al. reported an increased VEGF/PlGF ratio in postnatal blood samples (within 72 h after birth) in infants with BPD at 28 days of life [25]. However, both studies concerned preterm infants of a much wider range of gestational age (<34 and <35 WG, respectively). In our study, we examined homogeneous mother–child pre-eclamptic dyads with very preterm babies (<30 WG) and found no correlation between biological levels of angiogenic factors and clinical BPD at 36 weeks' PMA.

Only a few studies have described sEng patterns. Mestan et al. did not find any association between sEng level and maternal vascular underperfusion at placental examination but did not study the association between sEng level and BPD. Kim et al. measured amniotic fluid levels of sEng in women without pre-eclampsia and found a significant positive correlation with BPD and with a composite outcome of neonatal morbidity. However, BPD was not clearly defined and the inclusion criteria covered a wide range of gestational age (<37 WG) [26].

The strength of our study is the prospective analysis of four angiogenic factors (pro- or anti-angiogenic) to search for a biomarker of BPD in a very homogeneous population of IUGR preterm babies born from pre-eclamptic mothers. However, our study also has some limitations. First, this was not a case–control study and the sample size was limited. Second, as a strength and limitation, this was a single-center cohort. Subsequently, the protocols are the same for all babies for the main factors of BPD (such as PDA or respiratory management, use of postnatal corticosteroids). Third, we studied a selected number of markers previously described to be involved in lung development impairment in animal models.

The current literature supports a potential role of angiogenic factors in the pathogenesis and diagnosis of pre-eclampsia. The identification of specific biomarkers that predict the risk of BPD offers hope for developing pre- or postnatal interventions. However, despite a correlation between maternal and fetal blood levels of sFlt-1, our findings could not demonstrate biological evidence that links PE with alveolarization disorders in preterm infants.

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## Tables and figures

**Table 1:** Maternal and neonatal characteristics

<b>Maternal age</b> (median, IQR)	32 [27;36]
<b>Number of pregnancies</b> (median, IQR)	2 [1;4]
<b>Aspirin use during pregnancy</b> (% , n/N)	19 (5/26)
<b>Abnormal uterine Dopplers</b> (% , n/N)	88.5 (23/26)
<b>Antenatal corticosteroids (≥1 dose)</b> (% , n/N)	96.2 (25/26)
<b>HELLP syndrome</b> (% , n/N)	11.5 (3/26)
<b>Mode of delivery : Cesarean-section</b> (% , n/N)	100 (26/26)
<b>Gestational age at delivery (WG)</b> (median, IQR)	28.4 [27.3 - 29.0]
<b>Birthweight</b> (g) (median, IQR)	787 [650 - 840]
<b>Boys</b> (% , n/N)	30.8 (8/26)

IQR, interquartile range. WG : weeks' gestation

**Table 2:** Respiratory outcome and death

<b>Severe respiratory disease requiring high-frequency oscillation ventilation (% , n/N)</b>		46.1 (12/26)
<b>Duration of invasive ventilation * (d) (median, IQR)</b>		3.2 [0.1-8]
<b>Postnatal corticosteroids (% , n/N)</b>		11.5 (3/26)
<b>BPD at 36 weeks' PMA*</b>		
	<b>None</b>	16.7 (4/24)
	<b>Mild</b>	25.0 (6/24)
	<b>Moderate/severe</b>	58.3 (14/24)
<b>Death before 36 weeks' PMA</b>		7.7 (2/26)

IQR, interquartile range; PMA, postmenstrual age; WG, weeks' gestation

\*: Among the 24 infants alive at 36 week's PMA

**Table 3:** Neonatal outcome

<b>Patent ductus arteriosus</b> (% <i>, n/N</i> )	61.5 (16/26)
<b>Late-onset sepsis</b> (% <i>, n/N</i> )	65.4 (17/26)
<b>Platelet count at day 0</b> (/mm <sup>3</sup> , IQR)	152 000 [135 000 ; 183 000]
<b>Necrotizing enterocolitis or spontaneous ileal perforation</b> (% <i>, n/N</i> )	0
<b>Intraventricular hemorrhage III–IV</b> (% <i>, n/N</i> )	7.7 (2/26)

**Table 4:** Association between serum and cord blood levels of sFlt-1, PlGF, sEng and TGF- $\beta$  and moderate/severe BPD

		Moderate/severe BPD at 36 weeks' PMA*		No/mild BPD at 36 weeks' PMA*		
		N=14		N=10		
		Median	IQR	Median	IQR	p
<b>sFlt-1 (pg/ml)</b>						
	Maternal blood	7718	[5162-11876]	7236	[5953-12743]	.99
	Cord blood	200	[116-344]	186	[140-227]	.86
<b>PlGF (pg/ml)</b>						
	Maternal blood	26.3	[12.1-36.2]	9.6	[4.1-37.4]	.40
	Cord blood	3	[3-9.2]	3	[3-3]	.06
<b>sEng (ng/ml)</b>						
	Maternal blood	35.8	[15.6-58.9]	23.7	[13.8-27.2]	.08
	Cord blood	4.3	[3.0-6.1]	6.7	[2.1-7.5]	.25
<b>TGF-<math>\beta</math> (ng/ml)</b>						
	Maternal blood	1034	[682-1870]	1771	[462-2992]	.32
	Cord blood	1320	[682-3432]	660	[308-1342]	.14

<b>sFlt-1/PlGF</b>						
	Maternal blood	449.5	[197.5-710.4]	627.9	[252.5-1655.2]	.40
	Cord blood	38.3	[21.7-105.6]	62.1	[46.7-75.6]	.86
<b>sEng/TGF-<math>\beta</math></b>						
	Maternal blood	0.034	[0.017-0.043]	0.009	[0.003-0.19]	.08
	Cord blood	0.003	[0.001-0.005]	0.007	[0.002-0.025]	.30
*: among the 24 infants alive at 36 weeks' PMA (post-menstrual age).						
Significance set at $p < .005$ to account for multiple analyses						

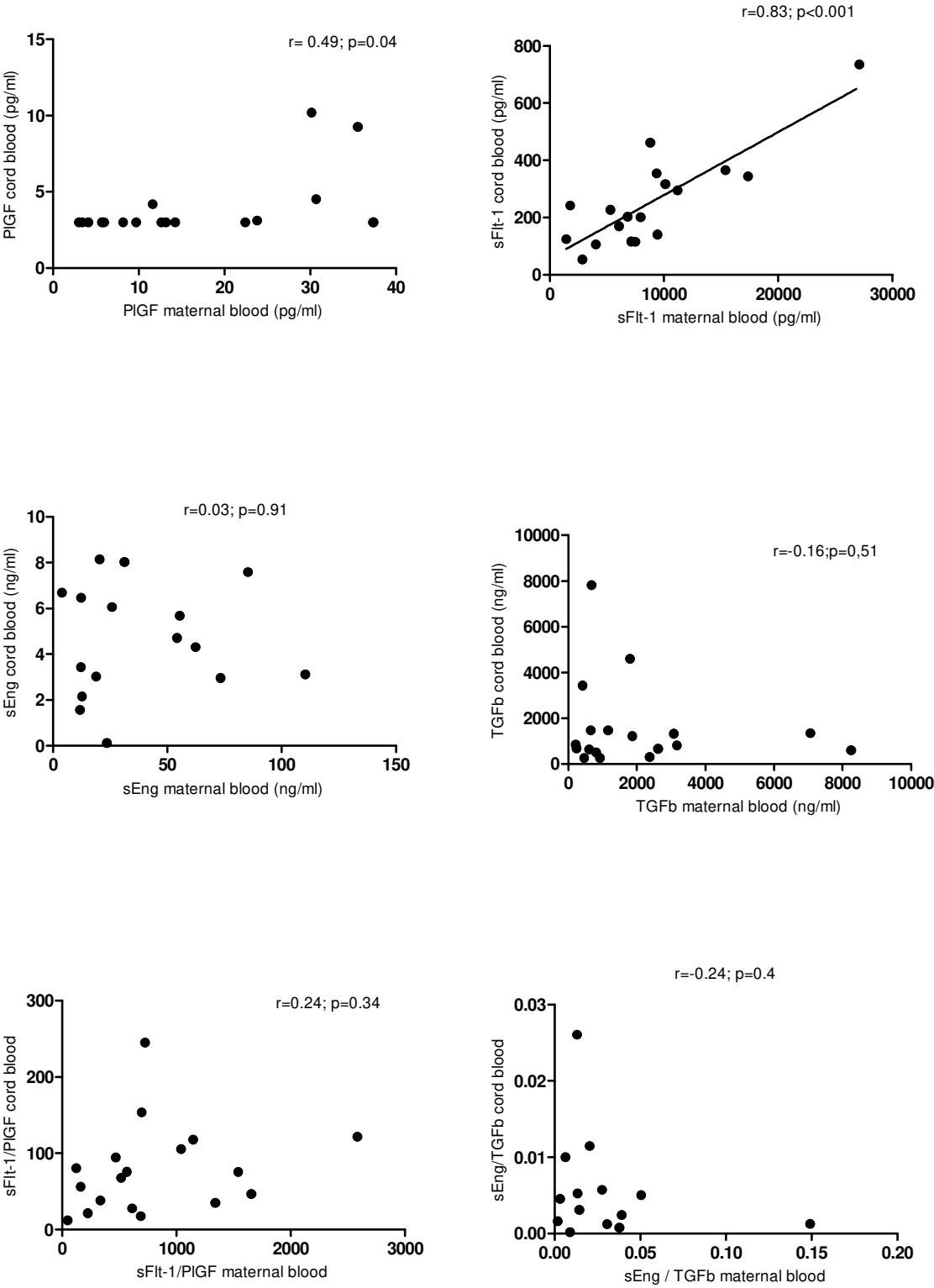


**Table 5:** Multivariate analysis: association of sFlt-1 maternal levels with moderate/severe BPD

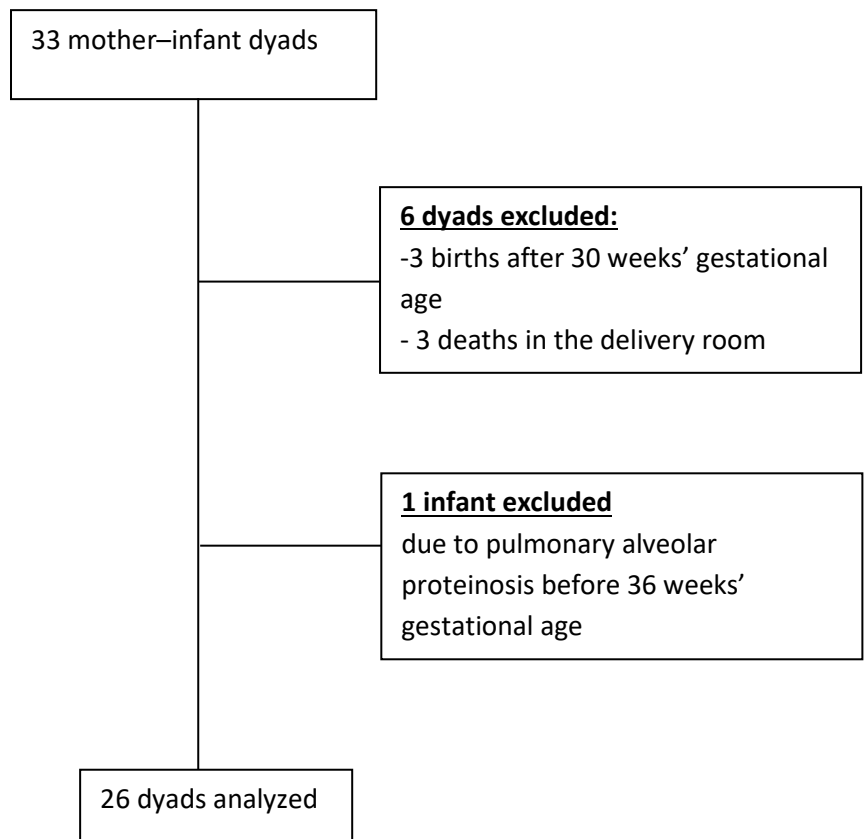
	<b>Moderate/severe BPD</b>					
	OR	95% CI	p	OR	95% CI	P
<b>sFlt-1 in maternal blood</b> (per 1000 pg/mL)	1.00	[0.99-1.01]	.71	1.00	[0.99-1.01]	.88
<b>GA</b> (continuous variable)				0.79	[0.29-2.10]	.63
<b>BW</b> (z-score)				0.27	[0.07-0.99]	.05

OR, odds ratio; 95% CI, 95% confidence interval; GA, gestational age, BW, birth weight

**Figure 1:** Correlation between maternal and cord blood levels of angiogenic and anti-angiogenic factors. Among pro- and anti-angiogenic factors involved in pre-eclampsia, only sFlt-1 was positively correlated inside the fetal maternal dyad.



**Supplemental data S1:** Flow chart of dyads in the study.



**Supplemental data S2:** correlation between soluble receptor and angiogenic factor in maternal and cord blood.

	Correlation in maternal blood		Correlation in cord blood	
	Coefficient	p	Coefficient	p
<b>sFlt-1 – sEng</b>	0.23	.31	0.30	.24
<b>PlGF – TGF<math>\beta</math></b>	0.11	.59	0.05	.83