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Serum and plasma determination of angiogenic and anti-angiogenic factors yield different results: the need for standardization in clinical practice

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Abstract

Objective—The importance of an anti-angiogenic state as a mechanism of disease in preeclampsia is now recognized. Assays for the determination of concentrations of soluble vascular endothelial growth factor receptor (sVEGFR)-1, sVEGFR-2, placental growth factor (PlGF) and soluble endoglin (sEng) have been developed for research and clinical laboratories. A key question is whether these factors should be measured in plasma or serum. The purpose of this study was to determine if there are differences in the concentrations of these analytes between plasma and serum in normal pregnancy and in preeclampsia.

Methods—Samples of maternal blood were obtained by venipuncture and collected in EDTA (lavender top) and serum collection tubes (red top). A standard laboratory procedure was implemented for the centrifugation, aliquoting and storage of samples. Plasma and serum from 70 women with normal pregnancies and 34 patients with preeclampsia, were assayed for sVEGFR-1, sVEGFR-2, PlGF and sEng by ELISA. Non-parametric paired tests were used for analyses.

Results—A significant difference between plasma and serum concentration was observed for sVEGFR-1 and sVEGFR-2 in normal pregnancy, and for sVEGFR-1, sVEGFR-2, PlGF and sEng in women with preeclampsia.

Conclusion—The concentrations of sVEGFR-1, sVEGFR-2, PlGF and sEng when measured in maternal plasma and in serum are different. Therefore, the matrix used for the assay (serum versus plasma) needs to be considered when selecting thresholds for predictive studies and interpreting the growing body of literature on this subject.

Keywords

preeclampsia; anti-angiogenic state; sFlt-1; sVEGFR-1; sVEGFR-2; PlGF; placental growth factor; sEng; soluble Endoglin; vascular endothelial growth factor; pregnancy

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INTRODUCTION

An imbalance between angiogenic and anti-angiogenic factors has been proposed to play a central role in the pathogenesis of preeclampsia. Indeed, patients with preeclampsia have lower plasma/serum concentrations of angiogenic factors such as vascular endothelial growth factor (VEGF)[1–4] and placental growth factor (PlGF) [2,4–20] and higher concentrations of anti-angiogenic factors including soluble VEGF receptor-1 (sVEGFR-1), also referred to as sFlt-1,[4,10,12–14,16,18,20–31] and soluble endoglin (sEng).[17,18,31–37] Recently, sVEGFR-2 has also been implicated in the pathophysiology of preeclampsia.[38–40] Since these differences can be observed before the clinical diagnosis of the disease,[6–11,15,26,35,41–47] it has been proposed that the measurement of plasma/serum concentrations of angiogenic and anti-angiogenic factors in the first and/or second trimester of pregnancy,[7,11,14,16–18,48–53] alone or in combination with the results of Doppler velocimetry of the uterine arteries,[20,29,37,54–60] may serve as an assessment tool to identify women at risk to develop preeclampsia.

Among men and non-pregnant women, the concentrations of VEGF and sVEGFR-1 have been reported to be significantly higher in serum than in plasma.[61–67] However, it is unknown if there is a difference in the concentrations of these analytes when measured in maternal serum or plasma during pregnancy. This information is important to avoid inaccurate risk assessment. The objective of this study was to determine if there are differences in the concentrations of sVEGFR-1, sVEGFR-2, PlGF and sEng in the serum and plasma, both in women with a normal pregnancy and in patients with preeclampsia.

METHODS

Study design

This cross-sectional study included 34 patients with preeclampsia and 70 women with a normal pregnancy, identified in our clinical database and bank of biological samples. Women with multiple pregnancies and/or those with fetuses with chromosomal or congenital anomalies were excluded. All patients were enrolled at the Sotero del Rio Hospital, Santiago, Chile and provided written informed consent prior to the collection of blood samples. The utilization of samples for research purposes was approved by the Institutional Review Boards of the Sotero del Rio Hospital, Santiago, Chile (an affiliate of the Pontificia Catholic University of Santiago, Chile), and the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, NIH, DHHS. Many of these samples have been previously employed to study the biology of inflammation, hemostasis, angiogenesis regulation, and growth factor concentrations.

Definitions

Patients were considered to have a normal pregnancy if they did not have any medical, obstetrical, or surgical complications, and if they delivered a term (≥37 weeks), singleton neonate of appropriate birth weight for gestational age [68] without complications. Preeclampsia was diagnosed in the presence of hypertension (systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg) and proteinuria (≥300 mg in a 24-hour urine collection, two dipstick measurement of 1+ or one dipstick measurement ≥2+) according to ACOG [69] and the National High Blood Pressure Education Program.[70] Small-for-gestational age (SGA) neonate was defined as a birthweight <10th percentile for the gestational age at birth according to the Chilean birth weight distribution of a Hispanic population.[68]

Sample collection and immunoassays

Maternal blood samples were obtained by venipuncture and collected in serum collection tubes (red top) and EDTA containing tubes (lavender top). The samples were centrifuged shortly after collection, and stored at −70°C until assay. The serum and plasma concentrations of sVEGFR-1, sVEGFR-2, PlGF, and sEng were determined by sensitive and specific immunoassays obtained from R&D Systems (Minneapolis, MN). All four immunoassays utilized the quantitative sandwich enzyme immunoassay technique. The concentrations of sVEGFR-1, sVEGFR-2, PlGF, and sEng were determined by interpolation from the standard curve. The inter- and intra-assay coefficients of variation obtained in our laboratory were: sVEGFR-1: 1.4% and 3.9%, respectively; sVEGFR-2: 2% and 4%, respectively; PlGF: 6% and 4.8%, respectively; and sEng: 2.3% and 4.6% respectively. The sensitivity of the assays was: sVEGFR-1: 16.97 pg/ml; sVEGFR-2: 19.07 pg/ml; PlGF: 9.52 pg/ml; and sEng: 0.08 ng/ml. The laboratory personnel performing the assays were blinded to the clinical information of each subject.

Statistical analysis

The normality of the data was tested using the Kolmogorov-Smirnov test. Since the concentrations of sVEGFR-1, sVEGFR-2, PlGF and sEng were not normally distributed, non-parametric tests were used for analyses. Paired-Wilcoxon ranks tests were used for comparison of the concentrations of sVEGFR-1, sVEGFR-2, PlGF and sEng in plasma and serum samples from the same patients. The median percentage of difference between paired plasma and serum concentrations for each analyte was calculated (plasma was considered as the reference sample). The statistical package used was SPSS v.15.0 (SPSS Inc., Chicago, IL, USA). A p-value of <0.05 was considered significant.

RESULTS

Table I displays the demographic and clinical characteristics of the study population. In the normal pregnancy group, the median serum concentrations of sVEGFR-1 and sVEGFR-2 were significantly higher than those of plasma, while those of PlGF and sEng did not change significantly (Table II). The median percentage of difference of the maternal serum over plasma concentrations for sVEGFR-1, sVEGFR-2, PlGF and sEng were 14.2%, 6.5%, 4%, and −1.1%, respectively. Serum concentrations of sVEGFR-1 and sVEGFR-2 were significantly higher than those of plasma through all trimesters (Table III). No significant differences were observed in the median percentage of difference of the maternal serum over plasma concentrations of any analyte among trimesters.

Among patients with preeclampsia, the median concentrations of sVEGFR-1, sVEGFR-2, PlGF and sEng were significantly higher in serum than in plasma (Table IV and Figure 1). The median percentages of difference of the maternal serum over plasma concentrations were 6.5%, 8.3%, 14.6% and 9.2%, respectively.

DISCUSSION

Principal findings of the study

The concentration of angiogenic and anti-angiogenic factors in plasma and serum of pregnant women is different. In normal pregnancy, the concentrations of sVEGFR-1 and sVEGFR-2, but not those of PlGF or sEng, were significantly higher in serum than in plasma. In contrast, among patients with preeclampsia, a significant difference was observed for sVEGFR-1, sVEGFR-2, PlGF and sEng.

Angiogenic and anti-angiogenic factors in plasma and in serum

Despite the accumulating body of evidence about the changes in the maternal concentrations of circulating angiogenic and anti-angiogenic factors in preeclampsia, the comparability between plasma and serum values has received little attention. In contrast, this has been more extensively addressed in non-pregnant patients for VEGF, a molecule which has been studied for a long time for its role in pathological vascularization, such as inflammatory diseases and malignancies. The concentration of VEGF in the serum is significantly higher than in the plasma of patients with cancer[61,65–67] and also in healthy individuals.[61–67] This difference is mainly attributed to the release of VEGF by platelets during the clotting process in serum preparation,[61–65,67] and it varies according to the technique of plasma preparation (e.g. EDTA plasma, citrate plasma, CTAD plasma, platelet poor plasma, platelet rich plasma) [65] as well as the duration of clotting and temperature.[63] Because the procedures for serum preparation and temperature are not standardized among laboratories, it has been suggested that the use of plasma should be preferred[62,67] when studying VEGF as a marker of tumor angiogenesis. Of note, the concentration of free VEGF in maternal plasma and serum is close to the limit of detection in both normal pregnancy and preeclampsia, and changes in its concentration are difficult to detect [10]. For this reason, VEGF has not been proposed as a biomarker for risk assessment in preeclampsia, and it was not included in this study.

In agreement with our findings, an increase in sVEGFR-1 concentration in serum compared to plasma has previously been demonstrated in healthy males and non-pregnant female volunteers [71] and in neonatal cord blood obtained from the umbilical vein at cesarean section.[28] Similarly, the concentration of sEng in umbilical vein blood has been previously found to be significantly higher in serum than in plasma.[36]

Differences between plasma and serum concentration of angiogenic and anti-angiogenic factors in preeclampsia

To our knowledge, this is the first study comparing the concentration of angiogenic and anti-angiogenic factors in plasma and serum in normal pregnancy and in pregnancies complicated with preeclampsia. Our findings indicate that the concentrations of sVEGFR-1, sVEGFR-2, PlGF and sEng in patients with preeclampsia, and those of sVEGFR-1 and sVEGFR-2 in normal pregnant women, vary significantly between plasma and serum and that values measured in serum are higher than those from plasma. Such difference should be considered when comparing the results of studies which have used different biological samples, but it becomes even more meaningful if the measurement of these angiogenic and anti-angiogenic factors is introduced in clinical practice as a screening or diagnostic test. In that case, standardization of the method is crucial because thresholds established in serum may not have the same predictive value in plasma and vice versa.

The cause of the observed differences is not known. It can be speculated that, similar to what has been observed for VEGF in non-pregnant individuals, the clotting process can influence the production or release of angiogenic and anti-angiogenic factors by several cell types so that each factor measured in plasma and serum is derived by different cellular sources. Indeed, sVEGFR-1 is known to be produced by endothelial cells, monocytes-macrophages, and neutrophils[71,72] as well as the placenta.[73,74] Endothelial cells are probably the main source of circulating sVEGFR-2,[75] but the contribution of other cells, such as circulating endothelial progenitor cells[75,76] and megakaryocytes,[75] is also possible. sEng is released by vascular endothelial cells,[77,78] macrophages,[79] immature erythroid cells[80] and syncytiotrophoblast.[78] Finally, while PlGF was first isolated in the placenta,[81,82] it is also expressed by other tissues such as activated endothelial cells,[83]

inflammatory cells,[83] erytroblasts,[83] neurons,[84] and keratinocytes during wound healing.[85]

In conclusion, the concentration of sVEGFR-1 and sVEGFR-2 in normal pregnancy is significantly different when measured in plasma and in serum. Interestingly, this difference is significant for sVEGFR-1, sVEGFR-2, PlGF and sEng in patients with preeclampsia. Therefore, the matrix used for each angiogenic and anti-angiogenic factor must be taken into account when reviewing the growing body of evidence that links these factors with the pathophysiology of preeclampsia, as well as in the clinical implementation of the determination of these analytes for the risk assessment for preeclampsia and other obstetrical syndromes.

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Figure 1 A

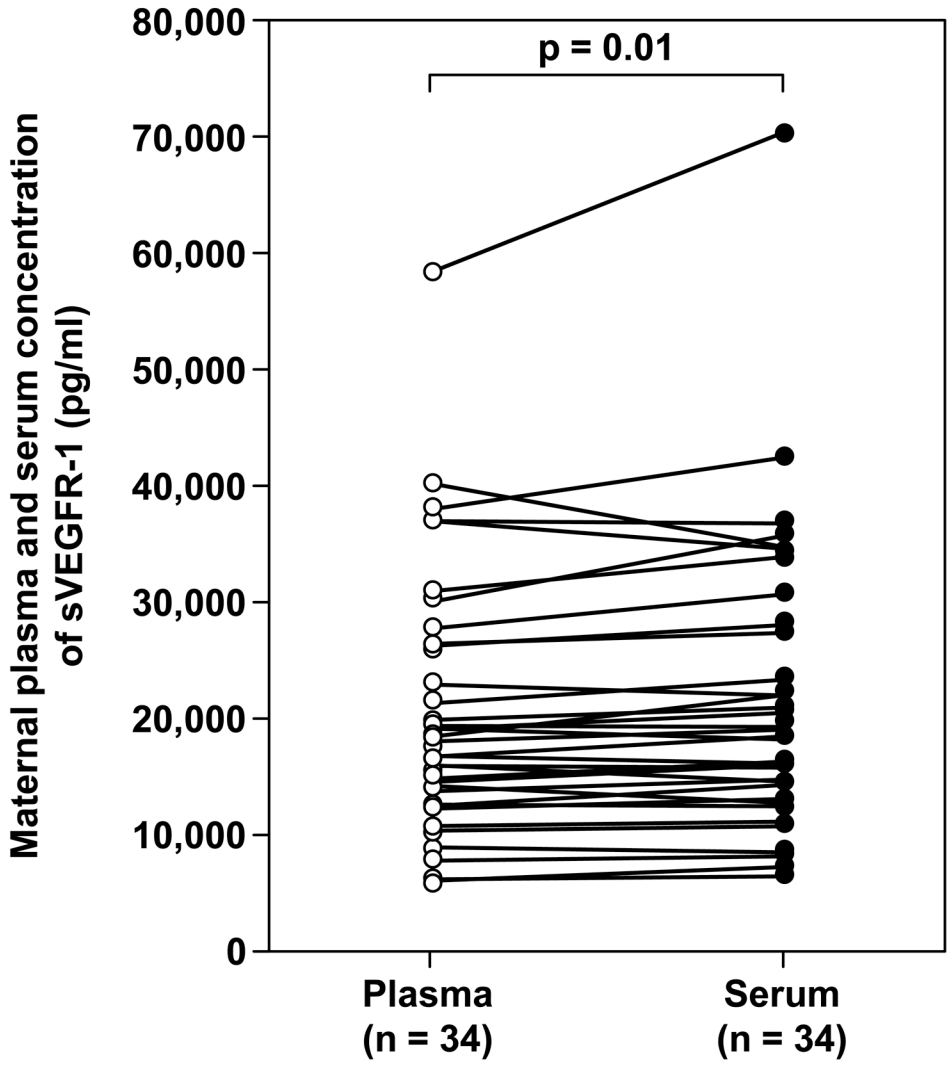
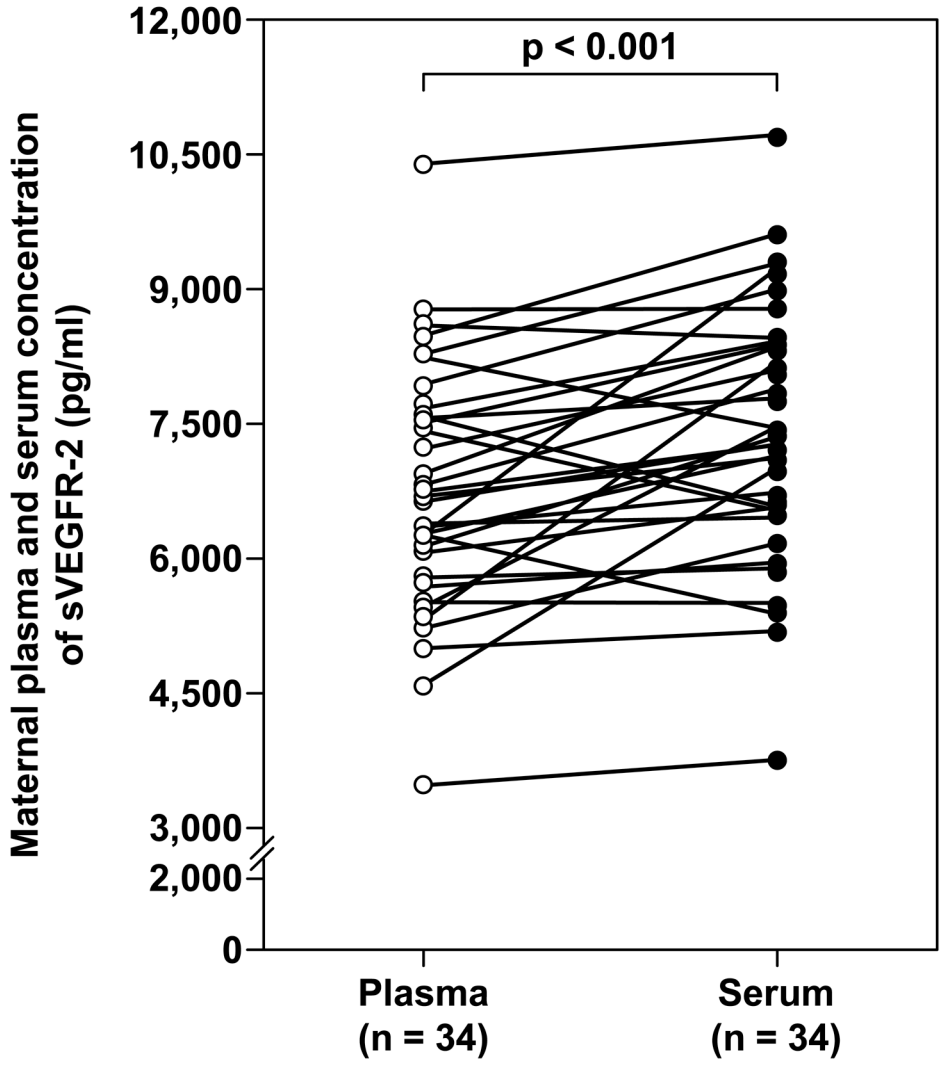


Figure 1 B



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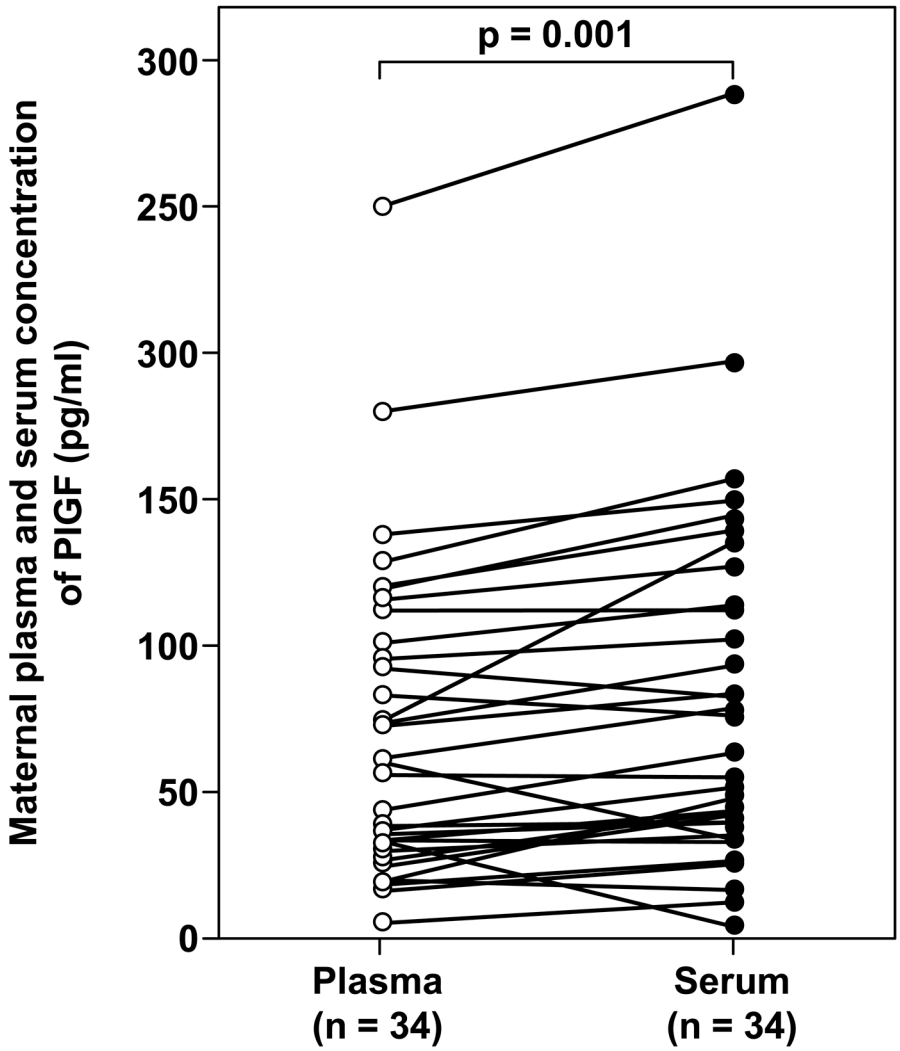

Figure 1 D

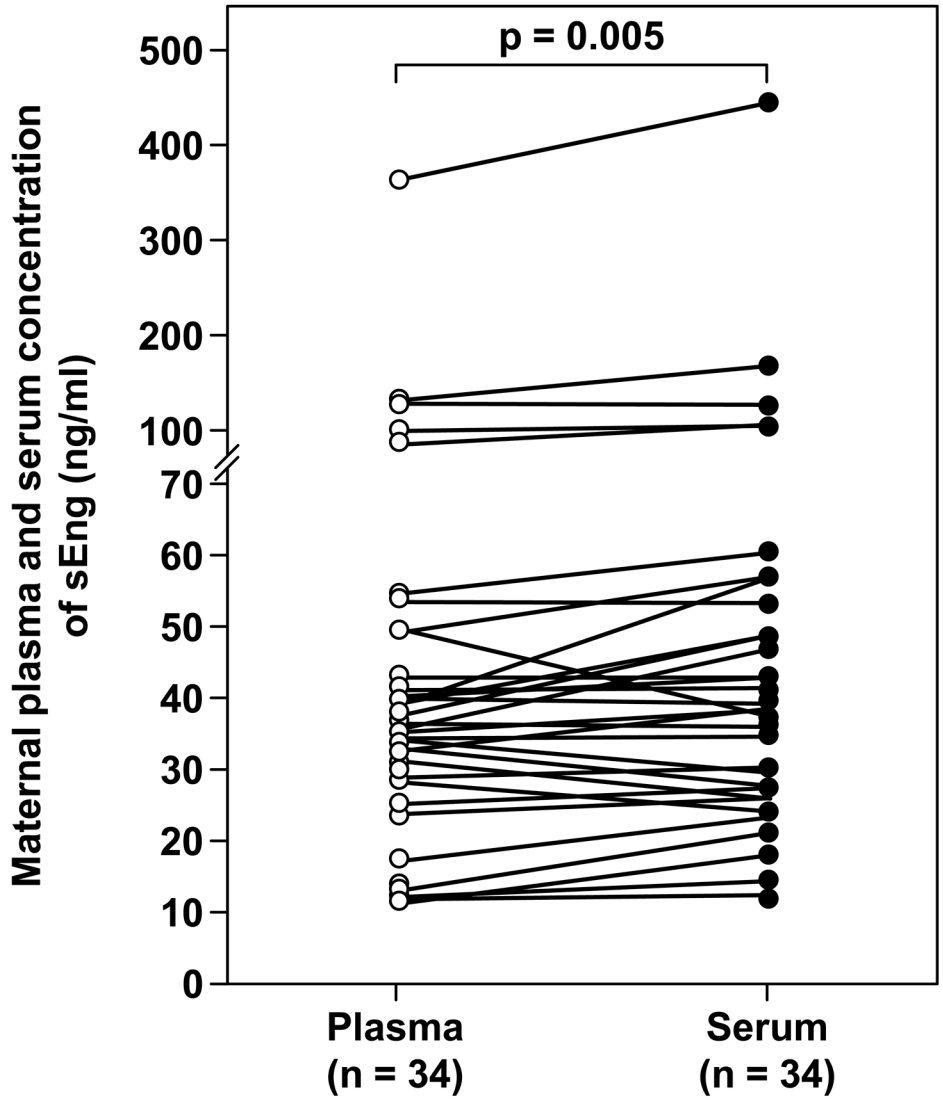

Figure 1.
Paired comparisons of the maternal plasma and serum concentrations of: **A.** sVEGFR-1, **B.** sVEGFR-2, **C.** PlGF, and **D.** sEng in patients with preeclampsia.

Table I

Demographic and clinical characteristics of the study population

	Normal pregnancy (n= 70)	Preeclampsia (n=34)
Maternal age (years)	25.5 (15–39)	22.5 (18–38)
Pre-pregnancy BMI (Kg/m^2)	23.5 (17.1–37.4)	25.7 (18.6–36.3)
Nulliparity (%)	25.7 (18/70)	64.7 (22/34)
Systolic blood pressure (mmHg)	120 (110–137)	160 (140–220)
Diastolic blood pressure (mmHg)	80 (65–88)	100 (90–150)
Gestational age at delivery (weeks)	39.4 (37–41.4)	36.4 (28–41)
Birth weight (g)	3,400 (2,770–4,090)	2,400 (830–4,580)
Gestational age at maternal plasma/serum collection (weeks)	28.9 (6.1–40)	36.1 (27.6–40.9)
Early-onset preeclampsia (%)	-	38.2 (13/34)
SGA (%)	-	26.5 (9/34)

The results are expressed as percentage (proportion) or median (range)

BMI: body mass index.

Early-onset preeclampsia: preeclampsia diagnosed <34 weeks of gestation

SGA: small for gestational age

Table II

Comparison of the maternal plasma and serum concentrations of sVEGFR-1, sVEGFR-2, PlGF and sEng in patients with a normal pregnancy

Analyte	Plasma (n=70)	Serum (n=70)	p*
sVEGFR-1 (pg/mL)	2,081.8 (264.8–19,210.9)	2,288.6 (277.7–15,861.8)	<0.001
sVEGFR-2 (pg/mL)	9,161.7 (6,201.1–13,817.8)	10,231.4 (6,379.4–16,168)	<0.001
PlGF (pg/mL)	189.2 (6.8–1,896.3)	197.5 (6.8–1,841.8)	0.1
sEng (ng/mL)	8.6 (4.5–38.4)	8.4 (4.7–44.9)	0.7

The results are expressed as median (range)

*Wilcoxon ranks test

Table III

Comparison of the maternal plasma and serum concentrations of sVEGFR-1, sVEGFR-2, PlGF and sEng in patients with a normal pregnancy in the first, second and third trimester

Analyte	Plasma (n=70)	Serum (n=70)	p*
First trimester	*n=10*	*n=10*	
sVEGFR-1 (pg/mL)	1,403.7 (264.8–3,259.1)	1,699.6 (277.7–3,568.9)	0.005
sVEGFR-2 (pg/mL)	10,178.8 (6,916.3–11,100.4)	10,708.6 (7,183.5–11,804.7)	0.04
PlGF (pg/mL)	27 (6.8–58.9)	29.2 (6.8–50.5)	0.5
sEng (ng/mL)	7.3 (6.4–12)	7.3 (5.8–11.3)	0.1
Second trimester	*n=22*	*n=22*	
sVEGFR-1 (pg/mL)	1,542.9 (386.0–4,133.6)	1,698.7 (512.2–4,686.2)	<0.001
sVEGFR-2 (pg/mL)	8,970.9 (6,673.4–13,787.5)	10,192.9 (6,469.5–13,573.9)	<0.001
PlGF (pg/mL)	145.3 (26.1–1,896.3)	157.3 (27.3–1,841.8)	0.5
sEng (ng/mL)	6.5 (4.5–38.4)	6.6 (4.7–43.6)	0.6
Third trimester	*n=38*	*n=38*	
sVEGFR-1 (pg/mL)	2,793.3 (827.1–19,210.9)	3,083.3 (885–15,861.8)	<0.001
sVEGFR-2 (pg/mL)	8,996.4 (6,201.1–13,817.8)	9,947.8 (3,379.4–16,168)	0.003
PlGF (pg/mL)	304.4 (39.9–1,606.3)	292.1 (41.8–1,540)	0.2
sEng (ng/mL)	10 (5.6–34.9)	10.1 (5.6–44.9)	1.0

The results are expressed as median (range)

*Wilcoxon ranks test

Table IV

Comparison of the maternal plasma and serum concentrations of sVEGFR-1, sVEGFR-2, PlGF and sEng in patients with preeclampsia

Analyte	Plasma (n=34)	Serum (n=34)	p*
sVEGFR-1 (pg/mL)	17,747.7 (5,557.8 – 58,140.6)	18,284.5 (5,955.4 – 70,199.1)	0.01
sVEGFR-2 (pg/mL)	6,668 (3,487.8 – 10,399.1)	7,298 (3,746.4 – 10,725.4)	<0.001
PlGF (pg/mL)	60.5 (6.8 – 250.5)	61.4 (6.8 – 289.9)	0.001
sEng (ng/mL)	35.9 (11.2 – 353.2)	39.1 (12 – 437.5)	0.005

The results are expressed as median (range)

*Wilcoxon ranks test