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Plasma soluble vascular endothelial growth factor receptor-1 concentration is elevated prior to the clinical diagnosis of pre-eclampsia

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Abstract

Objective: Accumulating evidence suggests that the balance between vascular endothelial growth factor (VEGF), placental growth factor (PlGF), and their receptors is important for effective vasculogenesis, angiogenesis, and placental development. Recently, the soluble form of VEGFR-1 (sVEGFR-1), an antagonist to VEGF and PlGF, has been implicated in the pathophysiology of pre-eclampsia. Plasma sVEGFR-1 concentration is elevated in pre-eclampsia at the time of clinical diagnosis and correlates with the severity of the disease. The purpose of this study was to determine whether the concentrations of sVEGFR-1 in plasma of pre-eclamptic patients change prior to the clinical manifestations of the disease.

Methods: A longitudinal case-control study was conducted in normal pregnant women ($n = 44$) and patients with pre-eclampsia ($n = 44$). Blood sampling was performed at six intervals: (1) 7–16 weeks; (2) 16–24 weeks; (3) 24–28 weeks; (4) 28–32 weeks; (5) 32–36 weeks; and (6) more than 37 weeks of gestation. To examine the relationship between plasma sVEGFR-1 concentration and interval to clinical diagnosis of pre-eclampsia, plasma samples of pre-eclamptic patients at different gestational ages were stratified according to the interval from blood sampling to clinical development of the disease into five groups: (1) at clinical manifestation; (2) 2–5 weeks; (3) 6–10 weeks; (4) 11–16 weeks; and (5) 17–25 weeks before clinical manifestations. Plasma concentrations of sVEGFR-1 were determined by enzyme-linked immunoassay. Parametric statistics and repeated measure procedures were used for the analysis.

Results: The mean plasma sVEGFR-1 concentration in pre-eclamptic patients before the clinical manifestation of the disease was significantly higher than in normal pregnant women at 24–28, 28–32, and 32–37 weeks of gestation ($p = 0.02$, $p < 0.001$, and $p < 0.001$, respectively). In contrast, no significant differences in the mean plasma sVEGFR-1 concentration between patients with pre-eclampsia and normal pregnant women were observed both at 7–16 weeks and 16–24 weeks of gestation ($p = 0.1$ and $p = 0.9$). Similarly, the mean plasma sVEGFR-1 concentration was significantly higher in pre-eclamptic patients than in normal pregnant women at clinical manifestation, at 2–5 weeks (mean 3.8 weeks), and at 6–10 weeks (mean 8.2 weeks) prior to the development of clinical pre-eclampsia ($p < 0.001$, $p < 0.001$, and $p = 0.002$, respectively). Among patients with early-onset pre-eclampsia (defined as gestational age of 34 weeks or less), the mean plasma sVEGFR-1 concentration was significantly higher in pre-eclampsia (before clinical diagnosis) than in normal pregnant women at 24–28 (mean 26.4) weeks of gestation ($p = 0.008$). In contrast, among patients with the late-onset disease (defined as gestational age of more than 34 weeks), plasma sVEGFR-1 concentration in pre-clinical pre-eclampsia was significantly higher than in normal pregnant women at 28–32 (mean 30.2) weeks of gestation ($p < 0.001$).

Conclusions: Plasma sVEGFR-1 concentration is elevated in pre-eclampsia prior to the clinical diagnosis of the disease. This elevation began 6–10 weeks prior to the clinical manifestations, and the increase was more pronounced at 2–5 weeks before the diagnosis, as well as at clinical presentation. Furthermore, in early-onset pre-eclampsia, plasma concentration of sVEGFR-1 is elevated earlier than the late-onset disease.

Keywords: *Pre-eclampsia, plasma-soluble vascular endothelial growth factor 1, longitudinal study, early onset, late onset, VEGF, PIGF*

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Introduction

Pre-eclampsia, a syndrome diagnosed by the presence of hypertension and proteinuria [1–10], has been proposed to be a two-stage disease [4] in which abnormal placentation [8,11–14] is followed by generalized endothelial cell dysfunction [15]. For decades, the term ''toxemia of pregnancy'' was used to refer to the disease, implying that an unknown ''toxic factor(s)'' produced by the placenta gains access into maternal circulation, causing systemic endothelial dysfunction, hypertension, and multiorgan involvement/damage [16–24].

Two angiogenic factors, vascular endothelial growth factor (VEGF) and placental growth factor (PlGF), are important for effective function of endothelial cells and placental development [25–30]. VEGF can promote endothelial cell proliferation, migration [31], and survival [32], and exerts its biologic effects through two high-affinity tyrosine kinase receptors: VEGFR-1 (VEGF receptor-1 or flt-1 or fms-like tyrosine kinase-1) and VEGFR-2 (VEGF receptor-2 or KDR/Flk-1 or kinase domain receptor). VEGFR-1 has two isoforms: a transmembranous form and a soluble form (sVEGFR-1). The latter is generated by a splice variant of the VEGFR-1 gene and contains the extracellular ligand-binding domain, while lacking the signaling tyrosine kinase domain. Thus, this isoform binds VEGF and inhibits its biological activities [31]. Another ligand for VEGFR-1 is PlGF, whose function is to potentiate the angiogenic effects of VEGF. Whereas VEGFR-2 is the major mediator of the mitogenic, angiogenic, permeability-enhancing, and endothelial survival effects of VEGF, the precise function of VEGFR-1 is still a subject of debate [31]. Most investigators believe that VEGFR-1 may not primarily be a receptor transmitting a mitogenic signal, but rather a ''decoy'' receptor able to inhibit the activity of VEGF on vascular endothelium by preventing binding of VEGF to VEGFR-2. However, there is evidence suggesting that VEGFR-1 signaling can be functional, especially in pathologic conditions such as ischemic limb, retina, or heart [33–35].

A role for the balance between angiogenic/anti-angiogenic factors in the genesis of pre-eclampsia has been proposed by several investigators [25,27–30,36–56]. However, a major set of recent observations have been made by the group of Maynard and Karumanchi [57], who proposed that a blockage of VEGF plays a major role in the genesis of the disease. Evidence in support of this includes: (1) the placenta of patients with pre-eclampsia over-expresses s-VEGFR-1 mRNA and protein [55,57]; (2) the median plasma/serum concentration of sVEGFR-1 is higher in pre-eclampsia at the time of diagnosis than in patients with normal pregnancies [55,57,58] and the plasma concentration correlates with the severity of the disease [59]; (3) pre-eclampsia is associated with decreased plasma/serum concentrations of VEGF and PlGF [47,53,57,60]; (4) serum of pregnant women with pre-eclampsia have anti-angiogenic effects in the endothelial cell tube formation bioassay and these effects can be restored by the addition of VEGF and PlGF to serum [57]; and (5) administration of sVEGFR-1 to pregnant animals can induce the clinical manifestations of pre-eclampsia, including hypertension and proteinuria [57]. Moreover, these animals develop glomerular endotheliosis (a pathologic change observed) [61–63], but not pathognomonic of pre-eclampsia [64].

A critical question is, however, whether the increase in plasma sVEGFR-1 occurs before or only at the time of the diagnosis of the disease. The purpose of this study was to determine whether the plasma concentrations of the sVEGFR-1 increase prior to the diagnosis of pre-eclampsia.

Patients and methods

Study design

A retrospective longitudinal case-control study was conducted by searching our clinical database and bank of biologic samples. This study included patients with pre-eclampsia ($n = 44$) and normal pregnant women ($n = 44$). All women were enrolled in the prenatal clinic at the Sotero del Rio Hospital, Santiago, Chile, and followed until delivery. Prenatal visits were scheduled at 4-week intervals in the first and second trimester, and every two weeks in the third trimester until delivery. Blood sampling was performed at enrollment and during every visit, whenever the patient consented. Pre-eclampsia was defined as hypertension (systolic blood pressure $\geqslant 140$ mmHg or diastolic blood pressure $\geqslant 90$ mmHg on at least two occasions, 4 h to 1 week apart) and proteinuria ($\geqslant 300$ mg in a 24-h urine collection or one dipstick measurement $\geqslant 2+$) [65]. Severe pre-eclampsia was defined as either severe hypertension (diastolic blood pressure $\geqslant 110$ mmHg) plus mild proteinuria or mild hypertension plus severe proteinuria (a 24-h urine sample containing 3.5 g protein or urine specimen $\geqslant 3+$ protein by dipstick measurement) [65]. Patients with an abnormal liver function test (aspartate aminotransferase > 70 IU/L) plus thrombocytopenia (platelet count $< 100{,}000/\text{cm}^3$), as well as those with eclampsia, were also classified as having severe pre-eclampsia [65]. Pregnant women were considered normal if they had no medical, obstetrical or surgical complications, and delivered a normal term ($\geqslant 37$ weeks) infant whose birthweight was appropriate for gestational age (10th–90th percentile). The

exclusion criteria comprised: (1) known major fetal anomaly or fetal demise; (2) active vaginal bleeding; (3) multifetal pregnancy; (4) serious medical illness (renal insufficiency, congestive heart disease, chronic respiratory insufficiency, etc.); (5) chronic hypertension, asthma requiring medication; and (6) patients requiring anti-platelet or non-steroidal anti-inflammatory drugs. For this study, subjects were selected only if they had plasma samples available at least once before 24 weeks of gestation, and at least another sample after this gestational age with a total of at least three samples during pregnancy. Plasma samples were selected from each patient only once for each of the following six intervals: (1) 7–16 weeks; (2) 16–24 weeks; (3) 24–28 weeks; (4) 28–32 weeks; (5) 32–37 weeks; and (6) more than 37 weeks of gestation. For each pre-eclamptic case, one control was selected by matching for gestational age ($\pm$ 2 weeks) at the time of clinical diagnosis of pre-eclampsia. Since the clinical presentation of pre-eclampsia developed at different gestational ages and all control cases delivered at term, more plasma samples from normal pregnant women were included in the study. In an effort to reduce the unequal number of plasma samples between cases and controls, samples from normal pregnant women obtained at gestational ages beyond the time the diagnosis of pre-eclampsia was made in the matched case were not used for this study. The collection and utilization of the samples was approved by both the Human Investigation Committee of the Sotero del Rio Hospital, Santiago, Chile (a major affiliate of the Catholic University of Santiago) and the IRB of the National Institute of Child Health and Human Development. Many of these samples were used in previous studies.

Sample collection and human sVEGFR-1 immunoassay

Venipuncture was performed and blood collected into tubes containing EDTA. The samples were centrifuged and stored at -70°C. The concentrations of sVEGFR-1 were measured using an enzyme-linked immunoassay (ELISA; R&D Systems, Minneapolis, MN). This assay employs the quantitative sandwich immunoassay technique. Briefly, recombinant human VEGFR-1 standards and maternal plasma specimens were incubated in duplicate wells of the microtiter plates pre-coated with monoclonal antibodies specific for VEGFR-1. During this incubation, the immobilized antibodies in the microtiter plate bound the VEGFR-1 in both the standards and samples. After washing unbound substances, polyclonal antibodies to human VEGFR-1 conjugated to an enzyme (horseradish peroxidase) were added to the assay wells. After an incubation period, the assay plates were washed to remove unbound antibody–enzyme reagents. Upon addition of a substrate solution (tetramethylbenzidine), color developed in the assay plates proportionally to the amount of VEGFR-1 bound in the initial step. The microtiter plates were read with a programmable spectrophotometer (Ceres 900 Microplate Workstation, Bio-Tek Instruments, Winooski, VT). The inter- and intra-assay coefficients of variation (CVs) were 4.8% and 6.9%, respectively. The sensitivity was 17.8 pg/ml.

Statistical analysis

Kolmogorov–Smirnov tests were used to test for normal distribution of the data. After logarithmic transformation (Log sVEGFR-1), unpaired Student's *t*-tests or analysis of variance (ANOVA) and post hoc tests with Bonferroni or Dunnett's T3 correction for multiple comparisons were utilized to determine the differences of the mean between and among groups, respectively. Analysis of covariance (ANCOVA) was used to assess the difference in plasma concentrations of sVEGFR-1 between patients destined to develop pre-eclampsia and in normal pregnancy after adjusting for gestational age at blood sampling and intervals of sample storage. Chi-square or Fisher's Exact tests were employed for comparisons of proportions. Repeated-measure analysis was used to examine the difference of the changes in plasma sVEGFR-1 concentrations in relationship to various intervals of blood sampling between normal pregnant women and patients with pre-eclampsia. The statistics package used was SPSS V.12 (SPSS Inc., Chicago, IL). Significance was assumed for a p value of less than 0.05.

Results

Clinical characteristics of the study population are displayed in Table I. Patients with pre-eclampsia were slightly younger than normal pregnant women and had a history of smoking less frequently than

Table I. Clinical characteristics of the study population.

	Normal pregnancy ($n=44$)	Pre-eclampsia ($n=44$)	p
Age (years)	29 $\pm$ 6	26 $\pm$ 6	0.04*
Nulliparity	11 (25%)	30 (68.2%)	$<$0.001*
Smoking	10 (22.7%)	1 (2.3%)	0.007*
GA at delivery (weeks)	39.7 $\pm$ 1.1	36.9 $\pm$ 2.7	$<$0.001*
Birthweight (g)	3372 $\pm$ 383	2710 $\pm$ 766	$<$0.001*
Birthweight $<$10th percentile	0	16 (36.4 %)	$<$0.001*

Values are expressed as mean $\pm$ SD or number (percent). GA, gestational age.

patients in the control group. The group with pre-eclampsia included more nulliparous women than the control group. As expected, patients with pre-eclampsia delivered earlier and had a lower mean birthweight than normal pregnant women. Sixteen (36%) patients with pre-eclampsia delivered neonates whose birthweights were less than the 10th percentile for gestational age. The clinical characteristics of patients with pre-eclampsia are displayed in Table II. Thirty-two (72%) patients had severe pre-eclampsia, while ten (22%) had early-onset pre-eclampsia (defined as gestational age at 34 weeks or less).

Table II. Clinical characteristics of patients with pre-eclampsia.

Blood pressure (mmHg)	
Systolic	155 ± 15
Diastolic	100 ± 8
Mean arterial pressure	118 ± 9
Proteinuria (dipstick)	3 ± 0.8
Aspartate aminotransferase[a] (SGOT) (U/L)	29 ± 31
Platelet count[b] ($\times 10^3$) (μ/L)	206 ± 59
Severe pre-eclampsia	32 (72.7%)
GA at pre-eclampsia diagnosed $\leqslant$ 34 weeks	10 (22.7%)
GA at pre-eclampsia diagnosed $\geqslant$ 37 weeks	27 (61.4%)

Values are expressed as mean ± SD or number (percent). GA, gestational age. [a]$n = 26$. [b]$n = 42$.

The gestational age at which pre-eclampsia was diagnosed varied. Four patients had hypertension and proteinuria at 28–32 weeks, 13 at 32–37 weeks, and 27 at term ($\geqslant$ 37 weeks). There were no significant differences in the mean gestational age at which venipuncture was performed by the interval window (for sVEGFR-1 plasma determination, see above) between the group of patients who eventually developed pre-eclampsia and the control group. No significant differences in the mean plasma sVEGFR-1 concentration between patients with pre-eclampsia and normal pregnant women were observed both at 7–16 weeks and 16–24 weeks of gestation ($p = 0.1$ and $p = 0.9$; see Table III and Figure 1a,b). However, at 24–28, 28–32, and 32–37 weeks of gestation, the mean plasma sVEGFR-1 concentrations in women who subsequently developed pre-eclampsia were

Table III. Plasma sVEGFR-1 concentrations in normal pregnancy and pre-eclampsia.

	Normal pregnancy	*P*	Pre-clinical samples pre-eclampsia	*p*	Clinical samples pre-eclampsia	p^{β}
1st blood sampling (7–16 weeks)						
sVEGFR-1 (pg/ml)	464 ± 260	0.1	546 ± 271			
Gestational age (weeks)	12.3 ± 2.2	0.2	11.6 ± 2.4			
Range	8.4–15.9		7.7–15.1			
	$n = 37$		$n = 34$			
2nd blood sampling (16.1–24 weeks)						
sVEGFR-1 (pg/ml)	560 ± 351	0.9	585 ± 369			
Gestational age (weeks)	19.4 ± 1.7	0.06	20.2 ± 2.1			
Range	16.3–23.4		16.7–24.0			
	$n = 44$		$n = 36$			
3rd blood sampling (24.1–28 weeks)						
sVEGFR-1 (pg/ml)	708 ± 426	0.02*	931 ± 436			
Gestational age (weeks)	25.9 ± 1.3	0.2	26.4 ± 1.1			
Range	24.1–28.0		24.6–28.0			
	$n = 38$		$n = 29$			
4th blood sampling (28.1–32 weeks)						
sVEGFR-1 (pg/ml)	823 ± 376	< 0.001*	1621 ± 1212	0.2	15716 ± 11290	0.2
Gestational age (weeks)	29.9 ± 1.1	1.0	30.2 ± 1.0	1.0	30.4 ± 1.4	1.0
Range	28.3–32.0		28.7–32.0		29.4–31.4	
	$n = 42$		$n = 33$		$n = 2$[a]	
5th blood sampling (32.1–37 weeks)						
sVEGFR-1 (pg/ml)	1183 ± 482	< 0.001*	2585 ± 1172	< 0.001*	8426 ± 6603	< 0.001*
Gestational age (weeks)	34.7 ± 1.3	1.0	34.8 ± 1.5	1.0	34.5 ± 1.2	1.0
Range	32.4–36.6		32.6–36.7		32.6–36.6	
	$n = 37$		$n = 20$		$n = 13$	
6th blood sampling ($\geq$37weeks)						
sVEGFR-1 (pg/ml)	2193 ± 1374		–		4995 ± 3145	< 0.001*
Gestational age (weeks)	39.4 ± 1.0				38.8 ± 1.1	0.05
Range	37.0–40.7				37.6–41.4	
	$n = 27$				$n = 27$	

Values are expressed as mean ± SD. p^{β}: compared between samples at clinical manifestation of pre-eclampsia and normal pregnancy.
[a]Two pre-eclamptic patients had no blood samples available at clinical manifestation.

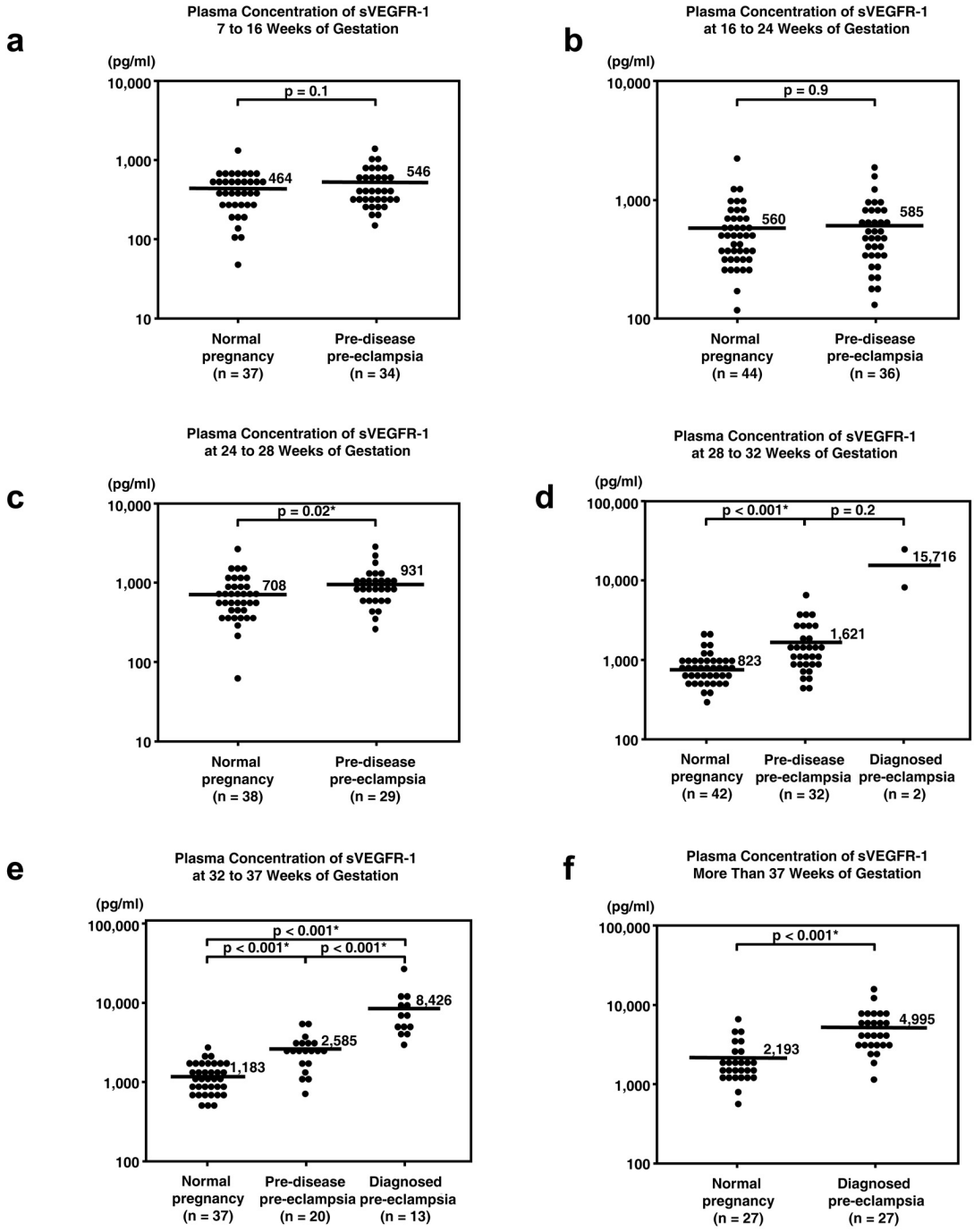

Figure 1. Mean plasma sVEGFR-1 concentration of normal pregnant women and patients with pre-eclampsia according to the interval of gestational age at blood samplings. No significant differences in the mean plasma sVEGFR-1 concentration between patients with pre-eclampsia and normal pregnant women were observed both at 7–16 weeks (a) and 16–24 weeks of gestation (b) ($p = 0.1$ and $p = 0.9$; see Table III). However, at 24–28 (c), 28–32 (d) and 32–37 weeks of gestation (e), the mean plasma sVEGFR-1 concentrations in pre-eclamptic patients before the clinical manifestations of the disease were higher than in normal pregnant women ($p = 0.02$, $p < 0.001$ and $p < 0.001$, respectively; see Table III). Pre-eclamptic patients at the time of clinical diagnosis had a mean plasma sVEGFR-1 concentration significantly higher than those before clinical manifestations at 32–37 weeks of gestation (e; $p < 0.001$), and higher than normal pregnant women both at 32–37 weeks and at term ($p < 0.001$ for both comparisons; e and f). The statistical tests used were unpaired Student's *t*-test or ANOVA with Bonferroni correction for multiple comparisons. The vertical axis in the figure is in the logarithmic scale.

higher than in normal pregnant women ($p = 0.02$, $p < 0.001$ and $p < 0.001$, respectively; see Table III and Figure 1c,d,f). Patients with pre-eclampsia at the time of clinical diagnosis had a significantly higher mean plasma sVEGFR-1 concentration than those before clinical manifestations at 32–37 weeks of gestation ($p < 0.001$; see Figure 1e) and higher than normal pregnant women both at 32–37 weeks and at

term ($p < 0.001$ for both comparisons; see Figure 1e, f).

To examine the relationship between plasma sVEGFR-1 concentration and the interval to clinical diagnosis of pre-eclampsia, plasma samples of pre-eclamptic patients at different gestational ages were stratified according to the interval from blood sampling to clinical diagnosis into five groups: (1) at clinical diagnosis; (2) 2–5 weeks before diagnosis; (3) 6–10 weeks before diagnosis; (4) 11–16 weeks before diagnosis; and (5) 17–25 weeks before diagnosis. The plasma samples from normal pregnant women (who were matched with patients who developed pre-eclampsia; see *Patients and methods*) were matched for gestational age with the plasma samples of pre-eclamptic patients at different gestational ages according to the intervals pre-specified above (e.g. 2–5 weeks before diagnosis, etc.). The mean plasma sVEGFR-1 concentration was significantly higher in patients who developed pre-eclampsia than in normal pregnant women at clinical diagnosis, at 2–5 weeks (mean 3.8 weeks), and at 6–10 weeks (mean 8.2 weeks) before the clinical diagnosis ($p < 0.001$, $p < 0.001$ and $p = 0.002$, respectively; see Table IV and Figure 2a–c). The results remained significantly different even after adjusting for gestational age at blood sampling and intervals of sample storage (all $p < 0.05$). No significant differences in the mean plasma sVEGFR-1 concentration were observed between patients who eventually developed pre-eclampsia and the normal control group, both between 11 and 16 weeks before the diagnosis and between 17 and 25 weeks before clinical manifestations of pre-eclampsia ($p = 0.6$ and $p = 0.5$, respectively, see Table IV and Figure 2d,e).

Since clinical pre-eclampsia developed at various gestational ages, performing repeated-measure analysis with samples stratified by gestational age alone would make the results difficult to interpret. Therefore, repeated measure analysis was conducted including samples from the following intervals: (1) 17–25 weeks before the diagnosis – the mean interval from blood sampling to the clinical diagnosis was 20.6 weeks and the mean gestational age at sampling was 16.5 weeks; (2) 11–16 weeks before the diagnosis – the mean interval from blood sampling to clinical diagnosis was 13.2 weeks and the mean gestational age at sampling was 24.2 weeks; (3) 6–10 weeks before the diagnosis – the mean interval from blood sampling to clinical diagnosis of 8.2 weeks and mean gestational age at diagnosis was 28.5 weeks; (4)

Table IV. Plasma sVEGFR-1 concentrations in normal pregnant women and pre-eclampsia.

Blood sampling	Normal pregnancy	Pre-eclampsia	*p*
At clinical manifestation			
sVEGFR-1 (pg/ml)	1820 ± 1249	6568 ± 5380	< 0.001*
Gestational age (weeks)	37.2 ± 3.0	37.1 ± 2.7	0.9
Range	28.9–40.7	29.4–41.4	
	$n = 42$	$n = 42$[a]	
2–5 weeks before clinical manifestation			
sVEGFR-1 (pg/ml)	1004 ± 486	2623 ± 1312	< 0.001*
Gestational age (weeks)	31.6 ± 3.8	32.8 ± 2.8	0.2
Range	24.1–36.3	27.1–36.7	
Interval before clinical manifestation (weeks)	$n = 27$	3.8 ± 1.1	
		$n = 27$	
6–10 weeks before clinical manifestation			
sVEGFR-1 (pg/ml)	794 ± 411	1093 ± 481	0.002*
Gestational age (weeks)	28.5 ± 2.9	28.5 ± 2.9	0.9
Range	19.7–32.6	19.6–34.4	
Interval before clinical manifestation (weeks)	$n = 37$	8.3 ± 1.4	
		$n = 37$	
11–16 weeks before clinical manifestation			
sVEGFR-1 (pg/ml)	804 ± 509	703 ± 294	0.8
Gestational age (weeks)	24.5 ± 3.1	24.2 ± 3.3	0.8
Range	17.6–27.9	17.7–28.0	
Interval before clinical manifestation (weeks)	$n = 19$	13.2 ± 1.3	
		$n = 19$	
17–25 weeks before clinical manifestation			
sVEGFR-1 (pg/ml)	523 ± 359	558 ± 348	0.5
Gestational age (weeks)	17.6 ± 3.5	16.5 ± 4.5	0.2
Range	9.1–23.4	8.0–22.7	
Interval before clinical manifestation (weeks)	$n = 42$	20.6 ± 3.6	
		$n = 42$	

Values are expressed as mean ± SD. [a]Two pre-eclamptic patients had no blood samples available at clinical manifestation.

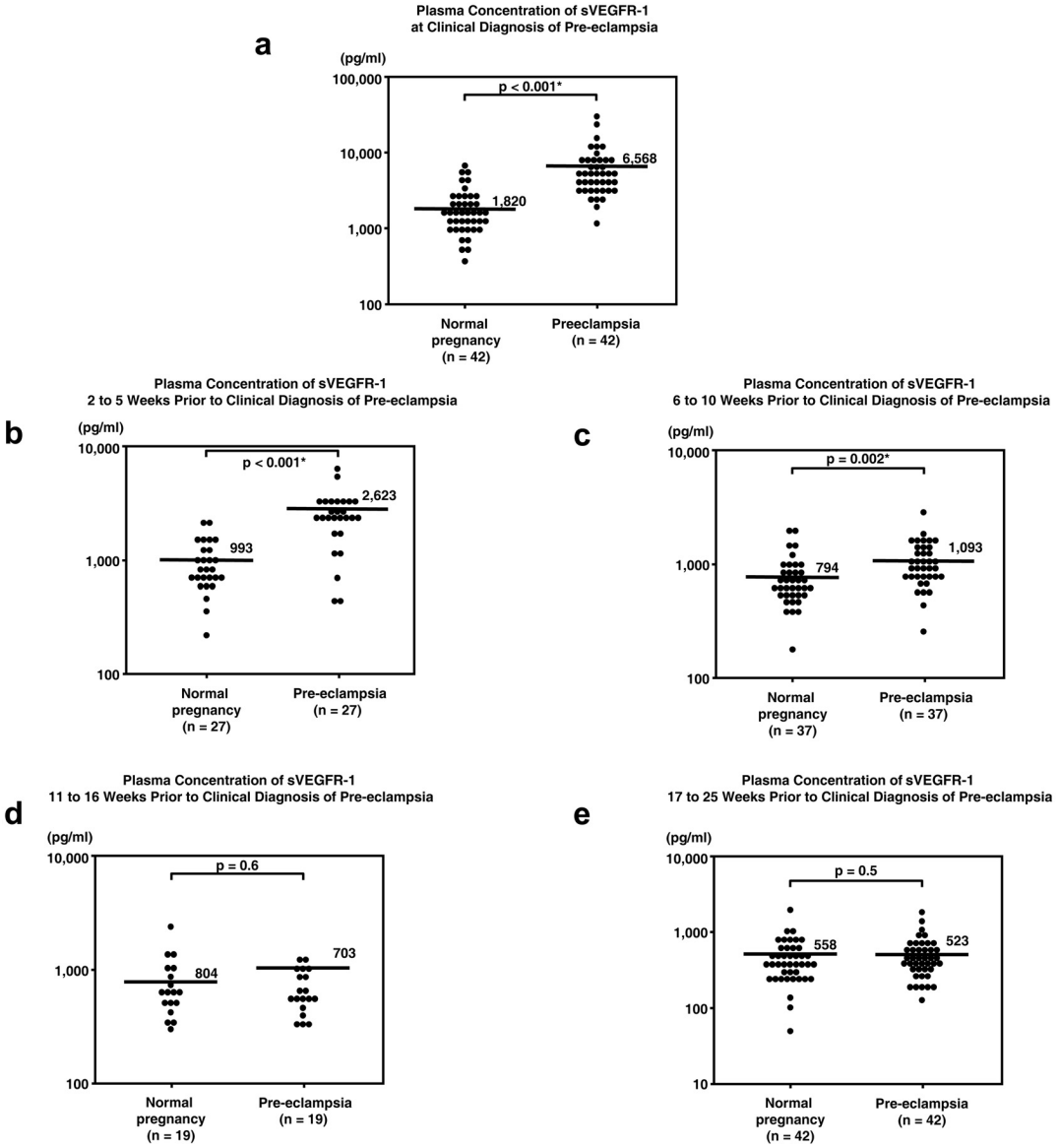

Figure 2. Mean plasma sVEGFR-1 concentration of normal pregnant women and patients with pre-eclampsia stratified according to the interval from blood sampling to clinical diagnosis of pre-eclampsia. The mean plasma sVEGFR-1 concentration was significantly higher in patients who developed pre-eclampsia than in normal pregnant women at clinical diagnosis (a), at 2–5 weeks (b), and at 6–10 weeks (c) before the clinical diagnosis ($p < 0.001$, $p < 0.001$ and $p = 0.002$, respectively; see Table IV). No significant differences in the mean plasma sVEGFR-1 concentration were observed between patients who eventually developed pre-eclampsia and the normal control group both between 11 and 16 weeks (e) before the diagnosis and between 17 and 25 weeks (f) before clinical manifestations of pre-eclampsia ($p = 0.6$ and $p = 0.5$, respectively, see Table IV). The statistical test used was unpaired Student's *t*-test. The vertical axis in the figure is in the logarithmic scale.

2–5 weeks before the clinical diagnosis – the mean interval from blood sampling to the clinical diagnosis was 3.8 weeks and the mean gestational age was 32.8 weeks; and (5) at clinical diagnosis of pre-eclampsia. Only those patients who donated a blood sample in these intervals were included in the repeated measure analysis.

The results demonstrated that patients who subsequently developed pre-eclampsia ($n = 10$) had a significantly different profile (plasma concentration over time) of plasma sVEGFR-1 concentration than patients with normal pregnancies ($n = 10$) as a function of the interval of blood sampling to the diagnosis of the disease ($p = 0.01$; repeated measure analysis; see Figure 3). The mean plasma sVEGFR-1 concentration was higher in pre-eclamptic patients than in normal pregnant women at 6–10 weeks before clinical manifestations, and the increase was more pronounced at 2–5 weeks before as well as at the time pre-eclampsia was diagnosed, but not before 11–25 weeks prior to clinical manifestations (see Figure 3).

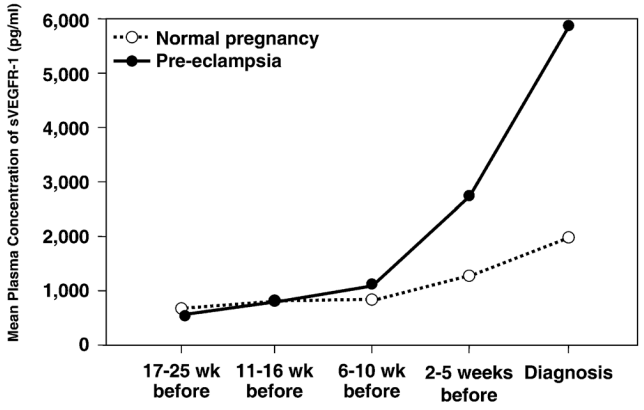

Figure 3. Mean plasma sVEGFR-1 concentration of normal pregnant women and patients with pre-eclampsia stratified according to the interval from blood sampling to clinical diagnosis of pre-eclampsia derived from repeated measure analysis. Patients who subsequently developed pre-eclampsia ($n = 10$) had a significantly different profile of plasma sVEGFR-1 concentration than patients with normal pregnancies ($n = 10$) as a function of the interval of blood sampling to the diagnosis of the disease ($p = 0.01$). The mean plasma sVEGFR-1 concentration in pre-eclamptic patients was higher than normal pregnant women at 6–10 weeks before clinical manifestation, and the increase was more pronounced at 2–5 weeks before as well as when pre-eclampsia was diagnosed, but not before (11–25 weeks prior to clinical manifestations). The analysis was performed after logarithmic transformation.

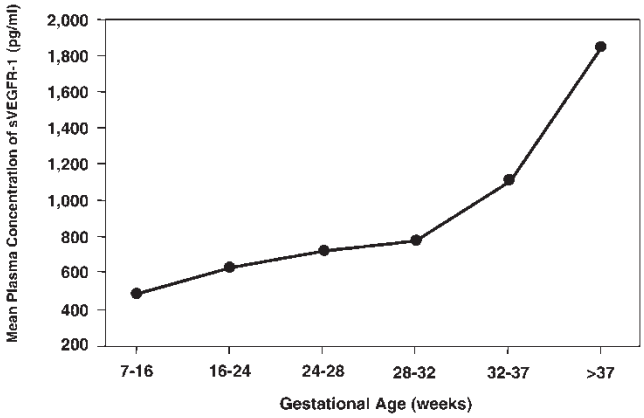

Figure 4. Mean plasma sVEGFR-1 concentration of normal pregnant women stratified according to the interval of gestational age at blood samplings derived from repeated measure analysis. The mean plasma sVEGFR-1 concentration in normal pregnancy ($n = 19$) increased with advancing gestational age with a small increase at 24–28 weeks (1st interval vs 3rd interval; $p = 0.035$) and a higher rise after 28–32 weeks interval (4th interval vs 5th interval; $p = 0.001$, repeated measure analysis). The analysis was performed after logarithmic transformation.

To examine the relationship between the changes of plasma sVEGFR-1 concentration as a function of gestational age in normal pregnant women, those patients who donated a blood sample in the six following intervals were included in repeated measure analysis ($n = 19$): (1) 7–16 weeks; (2) 16–24 weeks; (3) 24–28 weeks; (4) 28–32 weeks; (5) 32–37 weeks; and (6) more than 37 weeks of gestation. In normal pregnant women, the mean plasma sVEGFR-1 concentration increased with advancing gestational age with a small increase at 24–28 weeks (1st interval vs 3rd interval; $p = 0.035$) and a higher rise after 28–32 weeks interval (4th interval vs 5th interval; $p = 0.001$; repeated-measure analysis; see Figure 4).

To examine the diagnostic potential of plasma sVEGFR-1 concentrations to identify those destined to develop pre-eclampsia, patients were stratified into early-onset pre-eclampsia (defined as gestational age of 34 weeks or less) and late-onset pre-eclampsia (defined as gestational age of more than 34 weeks). The gestational ages at which the plasma sVEGFR-1 concentration in pre-eclampsia began to rise above those of normal pregnant women varied. For patients with early-onset pre-eclampsia, the mean plasma sVEGFR-1 concentration was significantly higher in pre-eclampsia (before clinical diagnosis) than in normal pregnancy starting around 24–28 (mean 26.8) weeks of gestation ($p = 0.008$; see Table V). In contrast, for patients with late-onset pre-eclamp-

Table V. Plasma sVEGFR-1 concentrations in normal pregnant women and patients who developed clinical pre-eclampsia at 34 weeks of gestation or less.

	Normal pregnancy	p	Pre-clinical samples pre-eclampsia	p	Clinical samples pre-eclampsia	p^{β}
1st blood sampling (7–16 weeks)						
sVEGFR-1 (pg/ml)	464 ± 260	0.5	522 ± 231			
Gestational age (weeks)	12.3 ± 2.2	0.4	11.6 ± 2.6			
Range	8.4–15.9		8.0–15.1			
	$n = 37$		$n = 8$			
2nd blood sampling (16.1–24 weeks)						
sVEGFR-1 (pg/ml)	560 ± 351	0.7	548 ± 215			
Gestational age (weeks)	19.4 ± 1.7	0.7	19.8 ± 2.9			
Range	16.3–23.4		17.3–23.9			
	$n = 44$		$n = 7$			
3rd blood sampling (24.1–28 weeks)						
sVEGFR-1 (pg/ml)	708 ± 426	0.008*	1324 ± 580			
Gestational age (weeks)	25.9 ± 1.3	0.03*	26.8 ± 0.6			
Range	24.1–28.0		26.0–27.3			
	$n = 38$		$n = 6$			
4th blood sampling (28.1–32 weeks)						
sVEGFR-1 (pg/ml)	823 ± 376	0.05	2516 ± 1088	0.2	15716 ± 11290	0.2
Gestational age (weeks)	29.9 ± 1.1	1.0	29.7 ± 1.1	1.0	30.4 ± 1.4	1.0
Range	28.3–32.0		28.7–31.3		29.4–31.4	
	$n = 42$		$n = 6$		$n = 2$[a]	
5th blood sampling (32.1–37 weeks)						
sVEGFR-1 (pg/ml)	1183 ± 482				11221 ± 9100	< 0.001*
Gestational age (weeks)	34.7 ± 1.3				33.5 ± 0.5	< 0.001*
Range	32.4–36.6				32.6–34.0	
	$n = 37$				$n = 6$	

Values are expressed as mean ± SD. p^{β}: compared between samples at clinical manifestation of pre-eclampsia and normal pregnancy.
[a]Two pre-eclamptic patients had no blood samples available at clinical manifestation.

sia, the plasma sVEGFR-1 concentration in pre-clinical pre-eclampsia was significantly higher than in normal pregnancy at 28–32 (mean 30.3) weeks of gestation ($p < 0.001$; see Table VI).

Using a cut-off of 1560 pg/ml (which represents 2 SD above the mean for pregnant women at the same gestational age range) for gestational age 24–28 weeks, a high plasma sVEGFR-1 concentration had a sensitivity and specificity of 16.7% (1/6) and 97.4% (37/38), respectively, to identify early-onset pre-eclampsia (at 29–34 weeks). In contrast, using the cut-off of 1575 pg/ml for gestational ages of 28–32 weeks, the sensitivity and specificity were 83% (5/6) and 95% (40/42), respectively, to identify early-onset pre-eclampsia (at 32–34 weeks).

Similarly, using the cut-off of 1575 pg/ml for gestational ages of 28–32 weeks, high plasma sVEGFR-1 concentration had a sensitivity and specificity of 18.5% (5/27) and 95% (40/42), respectively, for the identification of late-onset pre-eclampsia (at more than 34 weeks). In contrast, using the cut-off of 2164 pg/ml for gestational ages ranging from 32–36 weeks (mean 34 weeks), the sensitivity and specificity were 70% (14/20) and 97% (36/37), respectively, to predict late-onset pre-eclampsia (at 37 weeks or more).

Discussion

Our results demonstrate that: 1) women destined to develop pre-eclampsia have a higher plasma sVEGFR-1 concentration than those who will have a normal pregnancy; and 2) the increase is detectable several weeks prior to the clinical recognition of the disease. Such change could be detected about 6–10 weeks prior to the clinical diagnosis, and was more pronounced 2–5 weeks before diagnosis and at the time of clinical presentation. Moreover, both early-onset ($\leqslant 34$ weeks) and late-onset (> 34 weeks) pre-eclampsia were associated with an elevation of plasma sVEGFR-1 concentration prior to the diagnosis.

The observation that plasma sVEGFR-1 concentration in pre-eclamptic patients was elevated prior to the clinical diagnosis is consistent with the study of Levine et al., who reported that serum concentration of sVEGFR-1 increased approximately five weeks before the onset of the disorder [66]. Indeed, the view that pre-eclampsia is a chronic process with a long sub-clinical phase was first recognized by Gant et al. in 1974, who demonstrated that patients destined to develop pre-eclampsia had an abnormal angiotensin II sensitivity test as early as the 22nd

Table VI. Plasma sVEGFR-1 concentrations in normal pregnant women and patients who developed clinical pre-eclampsia at more than 34 weeks of gestation.

	Normal pregnancy	p	Pre-clinical samples pre-eclampsia	p	Clinical samples pre-eclampsia	p^{β}
1st blood sampling (7–16 weeks)						
sVEGFR-1 (pg/ml)	464 ± 260	0.1	553 ± 286			
Gestational age (weeks)	12.3 ± 2.2	0.2	11.6 ± 2.4			
Range	8.4–15.9		7.7–15.1			
	$n = 37$		$n = 26$			
2nd blood sampling (16.1–24 weeks)						
sVEGFR-1 (pg/ml)	560 ± 351	0.9	594 ± 400			
Gestational age (weeks)	19.4 ± 1.7	0.04*	20.3 ± 1.9			
Range	16.3–23.4		16.7–24.0			
	$n = 44$		$n = 29$			
3rd blood sampling (24.1–28 weeks)						
sVEGFR-1 (pg/ml)	708 ± 426	0.1	828 ± 336			
Gestational age (weeks)	25.9 ± 1.3	0.4	26.3 ± 1.1			
Range	24.1–28.0		24.6–28.0			
	$n = 38$		$n = 23$			
4th blood sampling (28.1–32 weeks)						
sVEGFR-1 (pg/ml)	823 ± 376	< 0.001*	1422 ± 1164			
Gestational age (weeks)	29.9 ± 1.1	0.2	30.3 ± 1.0			
Range	28.3–32.0		28.7–32.0			
	$n = 42$		$n = 27$			
5th blood sampling (32.1–37 weeks)						
sVEGFR-1 (pg/ml)	1183 ± 482	< 0.001*	2585 ± 1172	< 0.001*	6030 ± 1922	< 0.001*
Gestational age (weeks)	34.7 ± 1.3	1.0	34.8 ± 1.5	0.9	35.4 ± 0.9	0.7
Range	32.4–36.6		32.6–36.7		34.3–36.6	
	$n = 37$		$n = 20$		$n = 7$	
6th blood sampling ($\geq$ 37weeks)						
sVEGFR-1 (pg/ml)	2193 ± 1374		–		4995 ± 3145	< 0.001*
Gestational age (weeks)	39.4 ± 1.0				38.8 ± 1.1	0.05
Range	37.0–40.7				37.6–41.4	
	$n = 27$				$n = 27$	

Values are expressed as mean ± SD. p^{β}: compared between samples at clinical manifestation of pre-eclampsia and normal pregnancy.

week of gestation [67,68]. Similarly, abnormal uterine artery Doppler velocimetry [69] (defined as the mean resistance index above 95th percentile for gestational age [70] or the presence of bilateral notching [71]), an index of the increased impedance to blood flow in the uterine artery [72–77], is present weeks before the onset of clinical hypertension and proteinuria in a subset of patients with pre-eclampsia [78–86].

The elevation of plasma sVEGFR-1 in early-onset pre-eclampsia was first observed a few weeks (24–28 weeks) after the reported persistence of abnormal uterine artery Doppler in pre-eclamptic patients, suggesting the existence of a temporal relationship between failure to increase utero-placental perfusion and elevated plasma sVEGFR-1 concentration. It is possible that inadequate perfusion on the growth of feto-placental unit may lead to an increase in plasma sVEGFR-1 concentration. Consistent with this hypothesis is that plasma sVEGFR-1 concentration in pre-eclampsia at the time of clinical diagnosis is associated with the abnormality of uterine artery Doppler velocimetry, and that there is an inverse relationship between plasma sVEGFR-1 concentration and birthweight, as well as adjusted birthweight for gestational age (multiple of median), suggesting that an elevated plasma sVEGFR-1 concentration is indicative of a problem with the utero-placental supply line (Chaiworapongsa et al., to be submitted). The findings that plasma sVEGFR-1 concentration is elevated both in early-onset and late-onset pre-eclampsia suggest that these two conditions have a common final pathophysiological pathway. Perhaps an anti-angiogenic state is part of the common pathway of pre-eclampsia.

Several lines of evidence indicate that the source of the plasma sVEGFR-1 is likely to be the placenta. Immunohistochemistry and in situ hybridization studies localized VEGFR-1 protein and mRNA to extravillous trophoblast and villous cytotrophoblast, and more intense staining has been observed in syncytiotrophoblast in the latter half of gestation [87–89]. Clark et al. demonstrated that both membranous and soluble forms of VEGFR-1 were detectable

in the placenta, and that sVEGFR-1 protein could be found in the supernatant from explants of placental villi [38], suggesting that sVEGFR-1 could be released into the intervillous space. Indeed, the sVEGFR-1 concentrations obtained from cytotrophoblast-conditioned medium were higher in tissues derived from pre-eclamptic patients than from those of normal pregnancies [25]. The expressions of both membranous and soluble forms of VEGFR-1 mRNA and protein are also up-regulated in the placenta of pre-eclamptic patients [57].

The mechanisms responsible for the elevation of plasma sVEGFR-1 concentration in patients with pre-eclampsia remain to be determined. Since experimental evidence suggests that hypoxia could stimulate VEGFR-1 expression in trophoblast [90–92], it is possible that an ischemic placenta could induce expression and release sVEGFR-1 from villous trophoblast, which is in direct contact with maternal blood in the intervillous space. Evidence in support of this includes: (1) increased expression of VEGFR-1 protein in villous trophoblast of patients with pre-eclampsia and some patients with SGA [93]; (2) increased mRNA expression for both membrane and soluble forms of VEGFR-1 in villous trophoblast of placenta obtained from patients with ischemic villi [94]; and (3) increased expression of VEGFR-1 mRNA in the placenta of pre-eclamptic patients, but not from the placenta of neonates born with severe hypoxic ischemic encephalopathy has been reported [95]. This evidence also suggests that a chronic process, rather than acute severe hypoxia, is associated with an up-regulation of VEGFR-1 expression.

Alternatively, since the function of VEGFR-1 and its ligands (VEGF and PlGF) are involved in angiogenesis and trophoblast proliferation/differentiation [36,39,44,96,97], it is possible that the increased expression of VEGFR-1 in trophoblast is associated with the structural remodeling process of the villous tree and blood vessels [98–102] in response to chronic depletion of utero-placental nutrients (e.g., oxygen, but also amino acid, glucose, etc.). Indeed, previous morphological studies of the villous tree indicate that pre-eclampsia is associated with excessive branching capillary network (branching angiogenesis) in peripheral placental villi [98,99], a process thought to be an adaptive response of the fetus to increase gas-nutrient exchange [103]. Moreover, plasma concentrations of sVEGFR-1 in patients with pre-eclampsia and those with SGA with abnormal uterine artery Doppler are related inversely to gestational age at clinical diagnosis (Chaiworapongsa et al., to be submitted), probably due to the need for more extensive remodeling of the villous tree in earlier gestation. Furthermore, our previous observations in patients with pre-eclampsia and those with SGA with abnormal uterine artery Doppler at the time of clinical diagnosis indicate that the increased impedance to blood flow in the villous circulation as measured by abnormal umbilical artery Doppler velocimetry is associated with an increased plasma sVEGFR-1 concentration (delta value). There is evidence suggesting that impedance to flow in the umbilical arteries is a function of the number of tertiary stem villi and their morphology [100,104–106].

Why does trophoblast release sVEGFR-1 during utero-placental insufficiency? In mammals, growth of the fetus is exponential during the last half of gestation, whereas growth of the placenta is slower [48]. Unless the efficiency of placental transport of nutrients is increasing to keep pace with the demand of the fetus, fetal growth could be compromised. Placental transport capacity could increase as gestational age advances by two mechanisms [48]: (1) increased uterine and umbilical blood flow, and (2) increased rate of nutrient extraction. Evidence from animal experiments suggests that the former seems to be a preferable method [107] and, thus, failure to increase utero-placental perfusion during mid-gestation could compromise fetal growth. Villous ''maturation'' and ''differentiation'' could be considered as established responses to increase the rate of nutrient extraction [98,103]. The normal villous maturation involves differential growth of capillaries by endothelial cell proliferation followed by remodeling, as well as morphological changes from stem villi and immature intermediate villi (branching angiogenesis) to mature intermediate villi and terminal villi (non-branching angiogenesis) [98,103]. This mechanism would be more important in cases of chronic limitations to the utero-placental blood flow (e.g., pre-eclampsia). The period between mid-gestation and the start of the third trimester is an important transitional phase in villous development (angiogenic switch). The growth of capillaries rises dramatically at 20–24 weeks of gestation, and remodeling or enlargement occurs thereafter. Similarly, the preferential growth of terminal villi (the most efficient villi for nutrient exchange) occurs by 26 weeks of gestation [98,101]. Our observation that plasma sVEGFR-1 concentration of pre-eclamptic patients began to rise at 24–28 weeks in early-onset pre-eclampsia and at 28–32 weeks for the late-onset disease suggests that a problem in the supply line (e.g., inadequate utero-placental perfusion) may occur at different stages of angiogenesis and structural development of the villi. This hypothesis could explain, at least in part, the various villous morphologic findings between the early-onset and the late-onset disease. Early-onset disease is associated with poorly developed and non-branching villous capillaries, while late-onset pre-

eclampsia is associated with abundant villous branching [27,98,99,101,106]. As a result, the prevalence of abnormal umbilical artery Doppler is higher in early-onset than that in the late-onset disease [108–110].

VEGFR-1 has been localized to villous trophoblast and endothelial cells of villous blood vessels, suggesting that this protein may play an autocrine role in trophoblast function and a paracrine role in vascular growth at feto-maternal interface. Clark et al. observed that the expression of VEGFR-1 mRNA in villous trophoblast varied considerably even in a single small villus [38,89]. This indicated that VEGFR-1 production is regulated in a specific manner and may play an important role in villus development. However, the precise function of the membrane and the soluble form of VEGFR-1 in the development of human villous tree remains unclear.

Roberts and Lain proposed that the pathophysiology of pre-eclampsia consists of two stages: uteroplacental insufficiency, followed by generalized endothelial cell dysfunction [4]. The soluble form of VEGFR-1 could be one of several factors linking these two pathological processes in pre-eclampsia.

The clinical consequence of elevated plasma sVEGFR-1 concentration could be the alteration of endothelial cell function. Normal nonpregnant women have low levels of serum VEGF [46], which is thought to be required for the maintenance of endothelial cell function and survival [32,111,112]. The high plasma sVEGFR-1 concentration observed herein may interfere with this physiological process. Plasma VEGF and PlGF, two important pro-angiogenic factors, increased during normal pregnancy, but decreased in pre-eclampsia weeks before the clinical diagnosis [51,52,113–115]. Since sVEGFR-1 binds to VEGF and PlGF with a higher affinity than VEGFR-2 [31,116], a functional receptor of endothelium, the increased plasma sVEGFR-1 observed in pre-eclampsia would result in a net effect of anti-angiogenesis [57].

Moreover, the increased availability of sVEGFR-1 in pre-eclampsia may counteract the nitric oxide [117–120]/prostacyclin-induced [120,121] vasodilatation effect of VEGF and result in the elevation of maternal blood pressure. Consistent with this hypothesis, several recent studies in both animals and humans have suggested a role for the blockade of VEGF action that could induce clinical hypertension and proteinuria [57,122,123,124]. However, the effect of chronic elevation of sVEGFR-1 to human endothelial cells during pregnancy requires further investigation.

It is likely that factors other than sVEGFR-1 participate in the pathophysiology of pre-eclampsia [3,4]. The alterations of endothelial cell function depend not only on anti-angiogenic factors, but on others as well. Examples of factors detrimental to endothelial cells include lipid peroxides [125–128] and oxidative stress [129–132], while examples of protective factors are growth factors [32,111] and anti-oxidant agents [133–135]. The balance between the detrimental and protective factors of endothelial cells could determine the susceptibility of the patients and clinical presentation; for example, whether the patients will develop hypertension and proteinuria or not. An extensive longitudinal study [136] of biochemical markers in serum of pre-eclamptic patients indicated that the concentration of triglyceride is higher, but high-density lipoprotein cholesterol, ascorbic acid, and PIGF are lower in serum of patients destined to develop pre-eclampsia compared to those in normal pregnant women at 20 weeks of gestation. This finding may indicate that pre-eclamptic patients are more susceptible to generalized endothelial cell dysfunction beginning in early pregnancy. The increased plasma sVEGFR-1 concentration in the early third trimester would be a second-hit phenomenon. Consistent with this hypothesis is that non-pregnant women with a history of pre-eclampsia had higher plasma concentrations of low-density lipoprotein cholesterol and triglyceride and a higher susceptibility to lipoprotein oxidation when compared with women who had a normal pregnancy at 1–3 years after delivery [137]. Similarly, non-pregnant women who had a history of pre-eclampsia had a significantly decreased total anti-oxidant status than those who never had the condition [138].

While examining the diagnostic potential for plasma sVEGFR-1 concentration, we used the cut-off derived from plasma sVEGFR-1 concentration which is two standard deviations above the mean of normal pregnant women, instead of opting for the concentration derived from receiver operating curve to limit the false-positive rate in normal pregnant women. Although plasma sVEGFR-1 concentration is higher in pre-eclampsia than in the control group, approximately 2 months prior to the clinical manifestations the diagnostic indices are very poor. The optimal time to determine plasma sVEGFR-1 concentrations from a diagnostic/prognostic point of view is 28–32 weeks of gestation (mean 30 weeks) for early-onset pre-eclampsia, and 30–34 weeks of gestation (mean 32 weeks) for the late-onset disease, or approximately one month before the clinical diagnosis.

In conclusion, our study demonstrates that plasma sVEGFR-1 concentration is elevated in pre-eclampsia approximately 8 weeks before, and the increase is more pronounced at 2–5 weeks prior to the clinical manifestations of the disease. This observation may

have clinical and therapeutic implications. Preventive strategies such as neutralization against sVEGFR-1 [139] or administration of pro-angiogenic factors (i.e., PlGF and VEGF) could be considered. However, it is noteworthy to emphasize that although these measures could prevent further endothelial cell damage and probably delay clinical hypertension and proteinuria, they may not correct utero-placental insufficiency. Thus, fetal death could still occur. Furthermore, it is crucial to maintain the balance between plasma sVEGFR-1 and other pro-angiogenic factors, since excess VEGF could, in turn, induce exaggerated vasodilatation and increased vessel permeability.

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