Original Article

Establishing Reference Values for Both Total Soluble Fms-Like Tyrosine Kinase 1 and Free Placental Growth Factor in Pregnant Women

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It has been reported that the concentration of free placental growth factor (PlGF) is decreased and that of soluble fms-like tyrosine kinase 1 (sFlt-1) is increased before the onset of preeclampsia. However, no study has determined the reference values for sFlt-1 and free PlGF during pregnancy using a commercially available enzyme-linked immunosorbent assay (ELISA) kit. This longitudinal cohort study was undertaken to address this issue. Serum samples were collected from 148 women at 10, 18, 28, and 37 weeks of gestation. Preeclampsia occurred in 6 women: 4 women who delivered at <37 weeks of gestation, and 2 women who delivered at ≥37 weeks. The average and 90% confidence interval (90% CI) of the serum concentration of both sFlt-1 and free PlGF were determined in a total of 433 specimens from 148 subjects with 1 to 4 collections at 7 to 39 weeks of gestation, and were represented as quadric curves. The mean values (90% CI) of sFlt-1 (pg/ml) at 10, 18, 28, and 37 weeks of gestation were 413 (174–981), 296 (125–704), 413 (174–982), and 1,130 (477–2,690), respectively. The mean values (90% CI) of free PlGF (pg/ml) were 36 (14–89), 206 (83–515), 518 (207–1,290), and 354 (142–884), respectively. We also established the reference values for the ratio of sFlt-1/PlGF. These values may be useful for predicting the subsequent occurrence of preeclampsia. (*Hypertens Res* 2005; 28: 727–732)

***Key Words*: placental growth factor, preeclampsia, pregnancy, reference values, soluble fms-like tyrosine kinase 1**

Introduction

Preeclampsia is associated with maternal and infantile morbidity and mortality (*1*, *2*). The early detection of high risk women may be clinically important. Advice could be given on what symptoms the patient should look for and how she might change her lifestyle; and preventive treatment with, for example, aspirin or antioxidants could be applied (*3*). Preeclampsia is associated with a failure of trophoblasts to invade the spiral arteries (*4*), which may be associated with increased vascular resistance of the uterine artery, leading to decreased perfusion of the placenta (*5*).

Recently, it was shown that concentrations of soluble fms-like tyrosine kinase 1 (sFlt-1), a circulating anti-angiogenic protein, are increased in the placenta and serum of women with preeclampsia (*6*, *7*). This protein acts by adhering to the receptor-binding domains of placental growth factor (PlGF) and vascular endothelial growth factor (VEGF), preventing their interaction with endothelial receptors on the cell surface.

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Table 1. Clinical Profiles of the Pregnant Cohort by the Presence or Absence of Preeclampsia

	Total (n=148)	Normal pregnancy (n=142)	Preeclampsia, <37 weeks at delivery (n=4)	Preeclampsia, ≥37 weeks at delivery (n=2)
Age (years)	28.8±4.8	28.7±4.8	32.5±5.8	28.0±2.1
Nullipara	74 (50%)	72 (50%)	0 (0%)	2 (100%)
Height (m)	1.58±0.06	1.58±0.06	1.61±0.06	1.48±0.04
Weight (kg)	53.3±9.0	53.4±9.1	54.5±6.6	42.5±0.7
BMI (kg/m^2)	21.4±3.3	21.4±3.4	21.1±2.6	19.4±0.8
Gestational week at delivery	38.5±2.0	38.7±1.3	29.3±1.0	39.5±2.1
Birth weight (g)	2,932±480	2,993±360	1,048±198	2,390±382
Cesarean section	40 (27%)	35 (25%)	4 (100%)	1 (50%)
Preterm delivery	5 (3.4%)	1 (0.7%)	4 (100%)	0 (0%)
Overterm delivery	0 (0%)	0 (0%)	0 (0%)	0 (0%)
LBWI	16 (11%)	11 (8%)	4 (100%)	1 (50%)
Giant baby	1 (0.7%)	1 (0.7%)	0 (0%)	0 (0%)
FGR infants	15 (10%)	11 (7.7%)	2 (50%)	2 (100%)

Data are mean±SD or number (%). BMI, body mass index; LBWI, low birth weight infant; FGR, fetal growth restriction.

Table 2. The Level of the Logarithm of Serum Total sFlt-1, Free PlGF, and the Ratio of sFlt-1/PlGF in Women Who Were Serially Examined in the Four Periods

	Weeks of gestation				Significant pairs among periods
	8–12 w (period 1)	16–20 w (period 2)	26–30 w (period 3)	35–39 w (period 4)	
$\log_{10}$ sFlt-1	2.68±0.25	2.56±0.26	2.65±0.25	3.10±0.21	all pairs
$\log_{10}$ PlGF	1.53±0.27	2.39±0.20	2.88±0.23	2.60±0.26	all pairs
$\log_{10}$ (sFlt-1/PlGF)	1.16±0.26	0.15±0.26	−0.24±0.27	0.51±0.30	all pairs

w, weeks; sFlt-1, soluble fms-like tyrosine kinase 1; PlGF, free placental growth factor.

Whereas free VEGF concentrations are low throughout pregnancy (*7*), free PlGF concentrations increase during pregnancy (*7*). Therefore, free PlGF, not free VEGF (*8–11*), is considered pivotal for maintaining vascular endothelial cell homeostasis during pregnancy. In a nested case-control study as part of the Calcium for Preeclampsia Prevention Trial, it was disclosed that increased levels of sFlt-1 and reduced levels of free PlGF are potentially useful for predicting the subsequent development of preeclampsia (*7*).

Torry *et al*. (*12*) reported reference values for PlGF during pregnancy. However, they used an enzyme-linked immunosorbent assay (ELISA) originally developed by themselves (*12*). The determination of reference values for sFlt-1 and PlGF using commercially available kits may have more clinical importance. In this study, we tried to construct reference curves representing the 90% confidence interval (90% CI) for sFlt-1, free PlGF, and the ratio of sFlt-1/PlGF throughout pregnancy using a commercially available ELISA kit (R&D Systems, Minneapolis, USA).

Methods

Participants and Specimens

With the informed consent of all the participants of this study and the approval of the Ethics Committee of our institute, peripheral blood samples were collected throughout the pregnancy period. Study subjects were 157 healthy Japanese women with singleton pregnancies attending antenatal clinics within 23 weeks of gestation. A total of 9 women were excluded from the analysis because they delivered at another hospital and details of the clinical outcome were unavailable.

Of the 148 women included in the study, 1 woman (0.7%) contributed one serum specimen (a case of preeclampsia), 49 (33.1%) contributed two specimens, 58 (39.2%) contributed three, and 40 (27.0%) contributed four.

For 33 of the 40 women from whom four specimens were collected, serum samples were collected at 8 to 12 weeks, 16 to 20 weeks, 26 to 30 weeks, and 35 to 39 weeks of gestation. In those women, we analyzed the data in a longitudinal man-

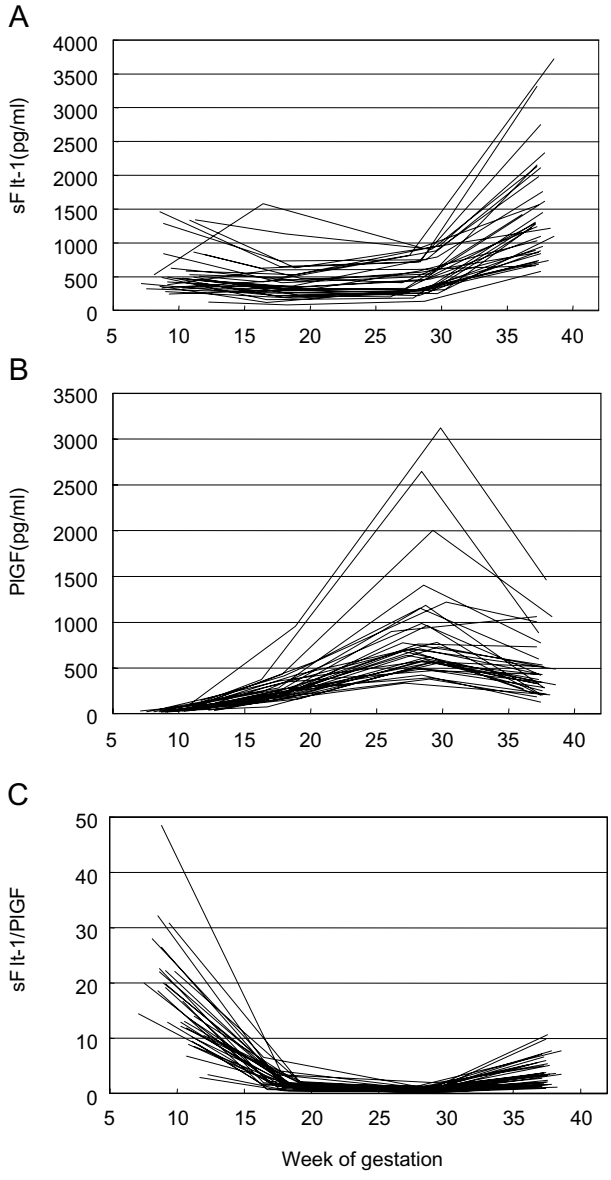

Fig. 1. *Individual changes in the serum concentration of soluble fms-like tyrosine kinase 1 (sFlt-1) (A), placental growth factor (PlGF) (B), and the ratio of sFlt-1/PlGF (C) at 8–12 weeks, 16–20 weeks, 26–30 weeks, and 35–39 weeks of gestation.*

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Preeclampsia was defined as a systolic blood pressure (SBP) ≥140 mmHg or a diastolic blood pressure (DBP) ≥90 mmHg, respectively, on 2 occasions recorded 24-h apart in association with the onset of proteinuria in a patient who had been normotensive during the first 20 weeks of pregnancy.

Procedure

Blood samples were collected and then centrifuged at room temperature at 2,500 rpm for 15 min. Serum aliquots were stored at −70°C until assayed. ELISAs for human sFlt-1 (also called soluble VEGF receptor 1) and free PlGF were performed in duplicate according to the manufacturer's instructions (R&D Systems). Briefly, the samples for ELISA measurements were diluted in 0.1% bovine serum albumin (BSA)/Tris-buffered saline and incubated in a 96-well plate precoated with a capture mouse monoclonal antibody directed against sFlt-1 or free PlGF for 2 h. The wells were then washed four times in 0.05% Tween 20/phosphate-buffered saline (PBS) and incubated with a secondary antibody against sFlt-1 and free PlGF conjugated to horseradish peroxidase for an additional 2 h. The plates were washed again four times, a substrate solution containing H_2O_2 and tetramethylbenzidine was added, and optical density was determined at 450 nm. A standard curve was generated for each sample plate, and sample concentrations of sFlt-1 and free PlGF were calculated with SoftMax 4-parameter logistic curve fit software (Molecular Devices Corp., Sunnyvale, USA). The minimal detectable doses in the assays for sFlt-1 and free PlGF were 5 and 7 pg/ml, respectively. The inter-assay and intra-assay coefficients of variation were 7.6% and 3.3% for sFlt-1, and 10.9% and 5.6% for free PlGF, respectively.

Statistical Analysis

The results are presented as the mean±SD. For sFlt-1 and free PlGF, statistical testing was conducted after logarithmic transformation. Fisher's exact tests were used for the comparison of categorical variables, and unpaired *t*-tests or paired *t*-tests were used for the comparison of two continuous variables if appropriate. All *p* values are two-tailed. All analyses were performed with the SPSS software package (version 13.0J for Windows; SPSS Inc., Chicago, USA). A level of $p<0.05$ was considered statistically significant.

Results

Among the 148 women, preeclampsia occurred in 6—*i.e.*, 4 women who delivered at <37 weeks of gestation, and 2 who delivered at ≥37 weeks of gestation (Table 1). In 33 women for whom serial serum data were examined throughout the pregnancy, there was no preeclampsia (Table 2, Fig. 1). The average of $\log_{10}$ sFlt-1 decreased from 8–12 weeks to 16–20 weeks, gradually increased at 26–30 weeks, and was rapidly increased at 35–39 weeks of gestation. The average of $\log_{10}$ PlGF increased from 8–12 weeks to 26–30 weeks, then decreased at 35–39 weeks of gestation. The average of the ratio of sFlt-1/PlGF decreased from 8–12 weeks to 26–30 weeks, then increased at 35–39 weeks of gestation.

We used all 433 specimens from the 148 subjects, and calculated the average and 90% CI of the serum concentrations of sFlt-1 and free PlGF, and the ratio of sFlt-1/PlGF from 7 to 39 weeks. The averages at each week could be represented by a quadric curve (Fig. 2, Table 3). As the Levine tests for both

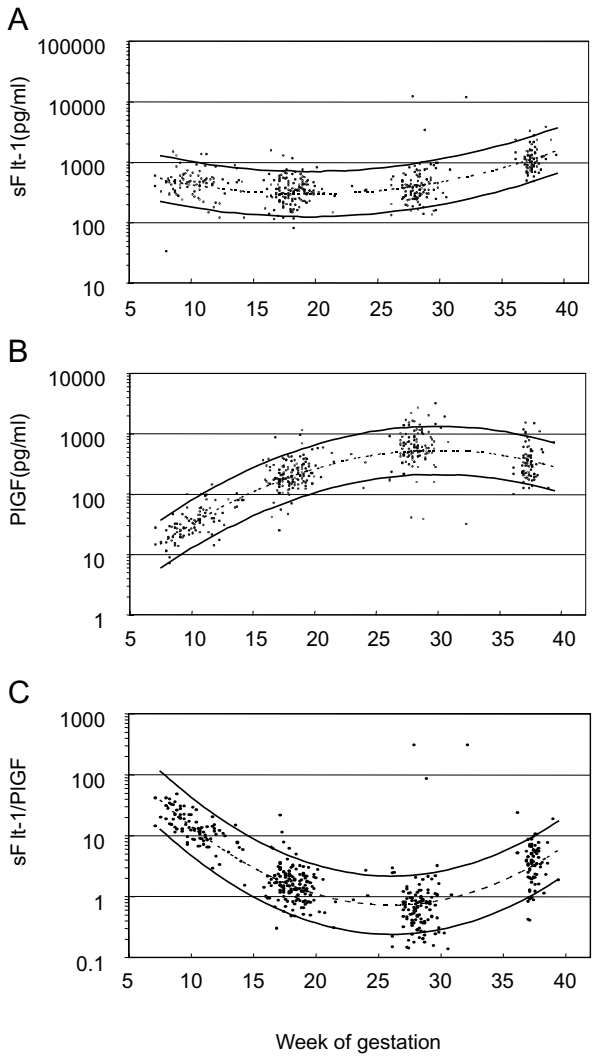

Fig. 2. *Quadric curves representing the mean and 5th and 95th percentile for the serum level of soluble fms-like tyrosine kinase 1 (sFlt-1) (A), placental growth factor (PlGF) (B), and the ratio of sFlt-1/PlGF (C) from 7 to 39 weeks of gestation. Closed circles indicate the 148 women examined in the study. One woman (0.7%) contributed one serum specimen (a case of preeclampsia), 49 (33.1%) contributed two specimens, 58 (39.2%) contributed three, and 40 (27.0%) contributed four, including 6 cases of the subsequent onset of preeclampsia.*

$\log_{10}$ sFlt-1 and $\log_{10}$ PlGF among weeks with the cases of $n>15$ (at 8 to 11, 16 to 19, 27 to 29, and 37 weeks of gestation) were not significant ($p=0.391$ and $p=0.479$, respectively), we assumed that the SDs for both $\log_{10}$ sFlt-1 and $\log_{10}$ PlGF at each week were almost the same. However, the Levine tests for $\log_{10}$ sFlt-1/PlGF among weeks were slightly significant ($p=0.047$), but the ranges of SD for $\log_{10}$ sFlt-1/PlGF at each week showed no regular tendency of increase or decrease. Therefore, we presumed that the SD for $\log_{10}$ sFlt-1/PlGF at each week was also almost the same. We estimated the SDs of the fitted curves by averaging the SDs at each week. The SDs for $\log_{10}$ sFlt-1, $\log_{10}$ PlGF, and the ratio of $\log_{10}$ sFlt-1/PlGF were 0.228, 0.242, and 0.291, respectively. Thus, the 5th and 95th percentile of $\log_{10}$ sFlt-1, $\log_{10}$ PlGF, and $\log_{10}$ sFlt-1/PlGF at each week were calculated by the equation, mean $-$ 1.645 $\times$ SD and mean + 1.645 $\times$ SD, respectively (Fig. 2, Table 3). For example, the mean values (90% CI) of total sFlt-1 (pg/ml) at 10, 18, 28, and 37 weeks of gestation were 413 (174–981), 296 (125–704), 413 (174–982), and 1,130 (477–2,690), respectively. The mean values (90% CI) of free PlGF (pg/ml) were 36 (14–89), 206 (83–515), 518 (207–1,290), and 354 (142–884), respectively. The mean values (90% CI) of the ratio of sFlt-1/PlGF were 11.8 (3.93–35.4), 1.39 (0.46–4.17), 0.77 (0.26–2.32), and 3.29 (1.10–9.90), respectively.

Discussion

In this study, we established the normal range of the serum concentration of both sFlt-1 and free PlGF throughout pregnancy. The concentration of sFlt-1 decreased from 8–12 weeks to 16–20 weeks, gradually increased at 26–30 weeks, and rapidly increased at 35–39 weeks of gestation. The concentration of free PlGF increased from 8–12 weeks to 26–30 weeks, and then decreased at 35–39 weeks of gestation. Thus, the cutoff value for abnormality should be changed according to the gestational period. In short, from 16 to 19 weeks of gestation, a level of free PlGF of <58 pg/ml is abnormal, while after 20 weeks, a level <100 pg/ml is abnormal. For sFlt-1, from 10 to 28 weeks of gestation, a level $>1{,}000$ pg/ml is abnormal. However, at 35–39 weeks of gestation, 2,000 pg/ml of sFlt-1 is within the normal range.

Our reference value for free PlGF was slightly different from the value previously reported by Torry *et al*. (*12*). The pattern of change during pregnancy was almost the same, but the levels at 28 to 30 weeks, and at term in Torry's report were slightly less than those in our report. These differences may have been due to the difference in anti-PlGF antibodies between the two ELISA kits. The ELISA kit used in this study was coated with a mouse monoclonal antibody against human PlGF, whereas the ELISA kit in the study by Torry *et al*. (*12*) was coated with rabbit anti-human PlGF. Torry *et al*. (*12*) used an ELISA originally developed by themselves. Because the ELISA kits that we used for this study were commercially available (R&D Systems), our reference value for free PlGF may be more useful for clinical studies.

We also established the reference values for the ratio of sFlt-1/PlGF. Because Maynard *et al*. (*6*) reported that angiogenesis was restricted by both the lower level of serum free PlGF in preeclamptic women and addition of exogenous sFlt-1 to the serum of non-pregnant women, the ratio of sFlt-1/PlGF may represent the anti-angiogenic status in pregnant women.

Table 3. The Mean, 5th Centile, and 95th Centile of the Serum Total sFlt-1, Free PlGF, and the Ratio of sFlt-1/PlGF Defined by the Estimated Curves in Pregnant Women at 7 to 39 Weeks of Gestation

		Weeks of gestation																
		7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Total sFlt-1 (pg/ml)	5th centile	226	205	188	174	162	152	144	138	133	129	126	125	124	125	126	129	133
	Mean	536	487	447	413	385	362	343	327	315	306	300	296	295	297	300	307	316
	95th centile	1,270	1,160	1,060	981	914	859	814	778	749	728	713	704	702	705	713	728	750
Free PlGF (pg/ml)	5th centile	6	8	11	14	19	24	31	38	48	58	70	83	96	111	126	141	156
	Mean	15	20	27	36	47	60	77	96	119	145	174	206	241	278	315	353	390
	95th centile	37	50	67	89	117	151	191	240	297	362	435	515	602	694	788	883	975
Ratio of sFlt-1/PlGF	5th centile	12.8	8.45	5.69	3.93	2.77	2.00	1.48	1.12	0.87	0.69	0.56	0.46	0.39	0.34	0.30	0.28	0.26
	Mean	38.5	25.4	17.1	11.8	8.32	6.01	4.45	3.36	2.60	2.06	1.67	1.39	1.18	1.03	0.91	0.83	0.78
	95th centile	116	76.2	51.4	35.4	25.0	18.1	13.4	10.1	7.82	6.20	5.03	4.17	3.55	3.08	2.74	2.50	2.33

		Weeks of gestation															
		24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39
Total sFlt-1 (pg/ml)	5th centile	138	144	152	162	174	188	205	226	251	280	316	360	412	477	556	654
	Mean	328	343	362	385	413	447	488	537	596	666	751	855	980	1,130	1,320	1,550
	95th centile	779	815	860	915	982	1,060	1,160	1,280	1,420	1,580	1,790	2,030	2,330	2,690	3,140	3,690
Free PlGF (pg/ml)	5th centile	170	182	193	201	207	210	210	207	202	193	183	170	156	142	126	111
	Mean	425	456	482	503	518	525	525	518	504	483	456	425	390	354	316	278
	95th centile	1,060	1,140	1,210	1,260	1,290	1,310	1,310	1,290	1,260	1,210	1,140	1,060	976	884	789	695
Ratio of sFlt-1/PlGF	5th centile	0.25	0.24	0.24	0.25	0.26	0.28	0.30	0.34	0.39	0.46	0.55	0.68	0.85	1.10	1.45	1.95
	Mean	0.74	0.72	0.72	0.74	0.77	0.83	0.91	1.02	1.17	1.37	1.65	2.03	2.56	3.29	4.35	5.87
	95th centile	2.22	2.17	2.17	2.22	2.32	2.48	2.72	3.05	3.51	4.12	4.95	6.10	7.68	9.90	13.1	17.6

sFlt-1, soluble fms-like tyrosine kinase 1; PlGF, placental growth factor.

In our preliminary longitudinal study, the levels of $\log_{10}$ PlGF were all lower than the 5th percentile at 16–20 weeks in 4 women with both the subsequent onset of preeclampsia and preterm delivery (24 pg/ml at 17 weeks, 41 pg/ml at 17 weeks, 46 pg/ml at 18 weeks, and 54 pg/ml at 17 weeks; unpublished data). Our finding of a decrease in the concentration of free PlGF in the second trimester is consistent with previous reports which examined free PlGF levels at 13 to 28 weeks (*7*), 15 to 25 weeks (*13*), 16 to 20 weeks (*14*), and 18 to 24 weeks (*15*), except for one study (*16*). The levels of $\log_{10}$ PlGF at 8 to 12 weeks in 3 women with subsequent onset of preeclampsia and preterm delivery were all lower than the 5th percentile (7 pg/ml at 8 weeks, 17 pg/ml at 12 weeks, and 14 pg/ml at 12 weeks; unpublished data). This finding of a decrease in the concentration of PlGF in the first trimester is also in agreement with two previous reports which examined free PlGF levels at 5 to 15 weeks (*14*), and 7 to 13 weeks (*17*). Thus, data obtained by our group and other researchers (*7*, *13–15*, *17*) indicate that the level of free PlGF in both the first and the second trimester is decreased before the onset of preeclampsia.

In our preliminary longitudinal study, the level of $\log_{10}$ sFlt-1 at 16 to 20 weeks in 4 women with both the subsequent onset of preeclampsia and preterm delivery (535 pg/ml at 17 weeks, 256 pg/ml at 17 weeks, 282 pg/ml at 18 weeks, and 613 pg/ml at 17 weeks; unpublished data) did not differ from that in normal pregnant women. This agrees well with the observation made by Levine *et al*. (*7*) that there were no differences in the serum sFlt-1 level at 17 to 20 weeks of gestation between normal and subsequent preeclampsia groups. However, Stepan *et al*. reported that the level of sFlt-1 was increased at 20 weeks of gestation in some women with findings of abnormal uterine perfusion on Doppler ultrasonography (*18*). Therefore, a small fraction of preeclamptic patients may show an early increase in the concentration of sFlt-1 at around 20 weeks of gestation.

Levine *et al*. (*7*) reported that serum sFlt-1 levels in the highest quartile at 21 to 32 weeks and 33 to 41 weeks of gestation predicted the occurrence of preterm preeclampsia and term preeclampsia, respectively. In 2 cases of preeclampsia in our preliminary study, the levels of $\log_{10}$ sFlt-1 at around 28 weeks increased over the 95th percentile before the onset of preeclampsia (unpublished data). Thus, a higher sFlt-1 level after 21 weeks of gestation, but not before 21 weeks, is probably associated with an increased risk of preeclampsia.

In conclusion, we constructed quadric curves representing the 90% CI of sFlt-1, free PlGF, and the ratio of sFlt-1/PlGF throughout pregnancy. We also established cutoff values of sFlt-1, free PlGF, and the ratio of sFlt-1/PlGF for identifying pregnant women at risk for the subsequent onset of preeclampsia. In our preliminary cohort study, the levels of free PlGF in the second trimester in 4 women with the subsequent

onset of preeclampsia with preterm delivery were all below the 5th percentile. However, we studied only a small number of women. The present findings need to be confirmed in larger studies.

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