Figure 1c

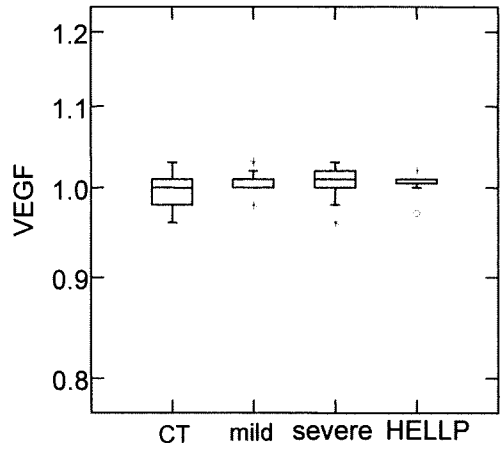


Figure 1d

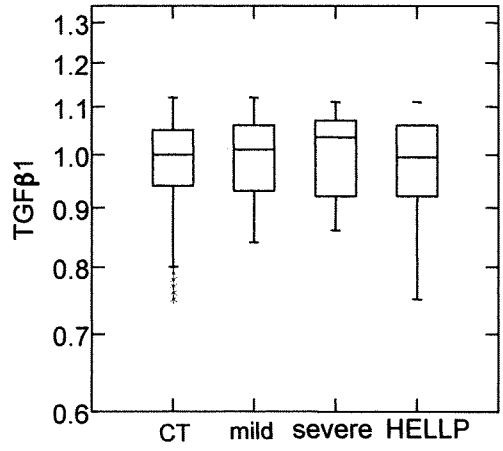


Figure 1e

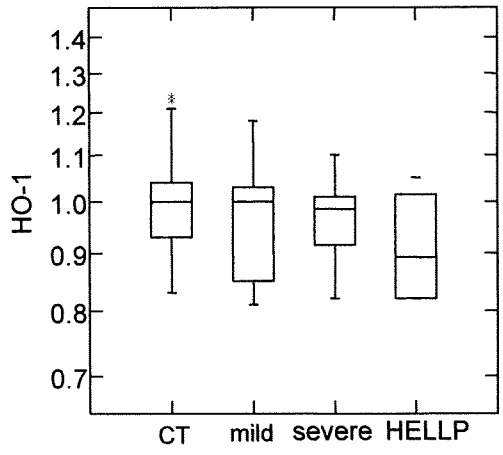


Figure 1f

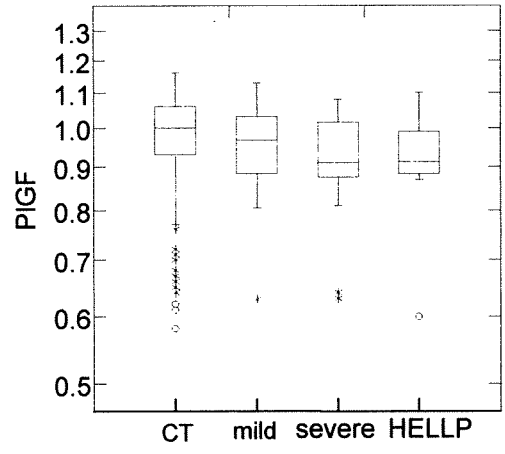


Figure 1g

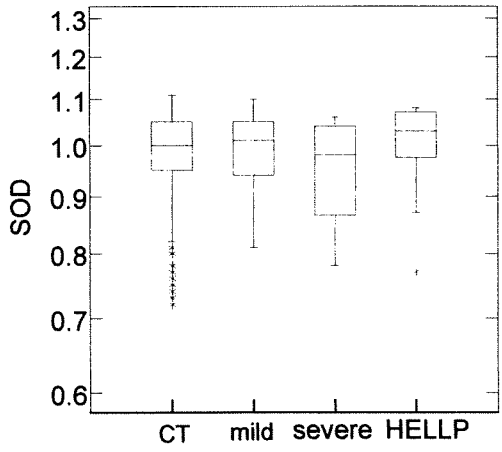


Figure 1h

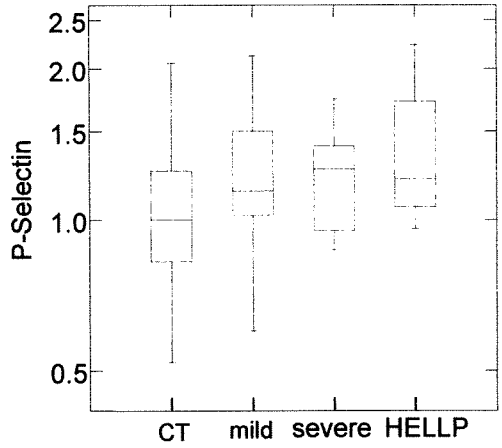


Figure 1i

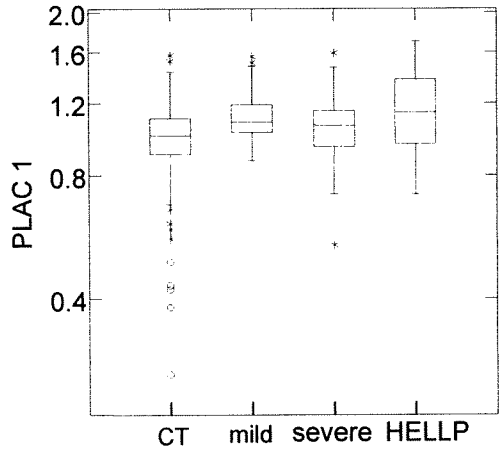
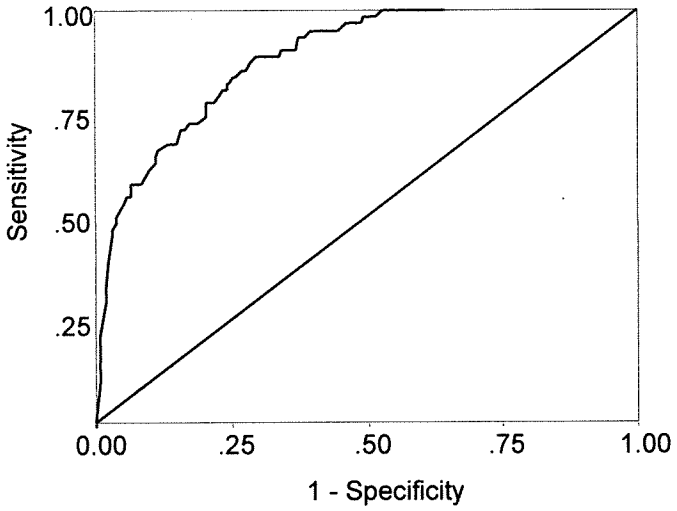


Figure 2



OBSTETRICS

Prediction of preeclampsia by analysis of cell-free messenger RNA in maternal plasma

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OBJECTIVE: The purpose of this study was to predict the occurrence of preeclampsia in a series of patients at gestational week 15-20 weeks, with the use of a panel of messenger RNA markers.

STUDY DESIGN: Data from 62 patients with preeclampsia who were asymptomatic at the time of blood testing and 310 control subjects were analyzed. Multivariable analysis was performed with discriminant analysis.

RESULTS: Univariable analysis identified vascular endothelial growth factor receptor 1 as the marker with the highest detection rate; placenta-specific 1 with the lowest. Mean estimated score for preeclampsia was 9.4 for control subjects and 72.5 for subjects who experienced preeclampsia. A receiver operating characteristic curve that was obtained with the estimated score for preeclampsia as a test variable yielded a detection rate of 84% (95% CI, 71.8-91.5) at a 5% false-positive rate with an area under the curve of 0.927 ($P < .001$). Again, detection rate and score for each patient for classification as preeclamptic correlated with severity.

CONCLUSION: A panel of messenger RNA is able to detect subjects who will experience preeclampsia.

Key words: cell-free mRNA, endoglin, prediction, preeclampsia, plasma RNA, VEGF

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Preeclampsia arises as a complication in 3-7% of pregnancies and remains 1 of the main causes of maternal and fetal death and morbidity. Because preeclampsia is known to have a long preclinical phase before clinically manifesting in later gestation, clinical prediction offers the possibility of redirecting maternal and prenatal care in high-risk pregnancies.[1]

Analogous to the discovery of circulating RNA in the plasma of patients with cancer, the discovery of circulating fetal/placental RNA in maternal plasma has enabled the development of several promising approaches for noninvasive evaluation of placental function.[2,3] We quantified messenger RNA (mRNA) expressions of human chorionic gonadotropin and human placental lactogen in maternal plasma.[4] The mRNA levels of human chorionic gonadotropin and human placental lactogen were correlated with the corresponding protein concentrations.[4] Evaluation of placental mRNA levels in maternal plasma therefore may allow indirect monitoring of placental function. Ng et al[5] recently demonstrated increased plasma concentrations of corticotrophin-releasing hormone (CRH) mRNA among pregnant women with preeclampsia. Farina et al[6] reported that plasma CRH mRNA correlates with clinical severity of preeclampsia.

To identify candidate genes for which mRNA expression in maternal plasma reflects placental gene expressions (including pathophysiologic alterations in preeclampsia), we conducted a microarray analysis of villous trophoblasts.[7] We then selected several target genes that are produced mainly by the placenta and that show increased protein concentrations in patients with preeclampsia. Based on the result, mRNA levels of plasminogen activator inhibitor-1 (SERPINE1), tissue-type plasminogen activator (PLAT), vascular endothelial growth factor (VEGFA), VEGFA receptor 1 (FLT1), endoglin, placenta-specific 1 (PLAC1) and selectin P (SELP) were assessed in the plasma of women with and without preeclampsia.[8-10] Expressions of all 8 genes were found to be increased in the plasma of patients with preeclampsia. All

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TABLE 1
Characteristics of control subjects and women with preeclampsia at enrolment and characteristics of their infants[a]

Characteristic	Control subjects (n = 310)[b]	Women with preeclampsia (n = 62)	*P* value[c]
Women			
Age (y)[d]	27.90 ± 5.37	28.90 ± 5.65	.278
Height (cm)[d]	154 ± 5.89	155 ± 6.42	.328
Weight (kg)[d]	55.58 ± 9.17	56.71 ± 9.58	.557
Body mass index[d,e]	27.37 ± 3.45	23.51 ± 3.75	.180
Systolic blood pressure (mm Hg)[d]	105 ± 9.6	106 ± 10.6	.479
Diastolic blood pressure (mm Hg)[d]	69 ± 7.1	69 ± 7.5	.933
Primigravida (n)	268 (86.4%)	19 (30.6%)	< .0002
Gestational age at enrolment (wk)[d]	17.3 ± 2.3	17.3 ± 2.2	.977
Current smoker (%)	0.9	3.2	.164
Previous hypertension (%)	1.4	3.2	.283
Previous preeclampsia (%)	1.4	0	1
Previous preterm delivery (%)	1.1	0	1
Previous fetal growth restriction (%)	0.9	1.6	.476

[a] Enrolment in this study was at gestational week 15-20. Probability values are given only for significant differences in comparison with control subjects; [b] Women with no preexisting medical diseases or antenatal complications were the control subjects; [c] Student *t* test or χ^2 test; [d] Data are given as mean ± SD; [e] Body mass index: weight in kilograms divided by the square of height in meters.

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expressions correlated positively with disease severity. An additional study of postpartum samples found that all expressions decreased rapidly after delivery, which indicates that most of these transcripts were derived from the placenta/fetus.[8-10] Furthermore, these findings also raised the question of whether those cell-free mRNAs have any significance or role in the development of preeclampsia and indicate pathologic alterations of the placenta in early pregnancy. No previous studies have explored cell-free mRNA concentrations in early gestation of pregnant women who subsequently experienced preeclampsia. In the present study, we quantified these mRNA expressions of placenta-derived genes in maternal plasma from women at gestational weeks 15-20 who were asymptomatic at the time of blood testing and assessed the possibility of predicting preeclampsia.

Materials and Methods

Subjects

The study was designed as a prospective cohort study in early pregnant women (gestational weeks 15-20) who visited the Department of Obstetrics and Gynaecology, University of Indonesia, at Cipto Mangunkusumo National Hospital, from mid 2005-2006. All women provided informed consent to participate in the study that was approved by the Institutional Research Ethics Committee.

Of the 683 women who were enrolled, we excluded 23 women who had incomplete information about outcome, whose pregnancy ended at < 20 weeks, or who experienced stillbirth. Among the remaining 660 women, 62 women experienced preeclampsia. Each case was matched with 5 control subjects for the same gestational age at the time of blood testing, maternal weight, and fetal gender. We therefore enrolled 62 women who experienced preeclampsia and 310 control subjects with a normal course of pregnancy. We did not do any special management or treatment other than antenatal care and before clinical sign of preeclampsia. If abnormalities of blood pressure and/or proteinuria were found, the patients were recommended to be admitted to the hospital.

Mild and severe preeclampsia and hemolysis, elevated liver enzymes, and low platelets (HELLP) syndrome were defined as described in a previous report.[11] In brief, *preeclampsia* was defined as gestational hypertension (systolic pressure > 140 mm Hg or diastolic blood pressure > 90 mm Hg on ≥ 2 occasions after gestational week 20) with proteinuria (> 0.3 g/d). *Severe preeclampsia* was defined by the presence of ≥ 1 of the following occurrences: (1) severe gestational hypertension (systolic pressure > 160 mm Hg or diastolic blood pressure > 110 mm Hg on ≥2 occasions after gestational week 20) or (2) severe proteinuria (≥ 5 g protein in a 24-hour urine specimen or ≥ 3 g in 2 random urine samples that were collected ≥ 4 hours apart). *Fetal growth restriction* was defined as birth weight of ≥ 2.0 SD below the mean expected weight for gestational age. The control group included pregnant women with no preexisting medical diseases or antenatal complications.

RNA extraction and real-time quantitative reverse transcription–polymerase chain reaction (PCR)

Processing of blood samples has been described previously.[4] In brief, 7-mL peripheral blood samples were collected in EDTA-containing tubes and centrifuged at 1600*g* for 10 minutes at 4°C twice. Molecular analysis was performed in the Department of Obstetrics and Gynecology at Showa University School of Medicine, Tokyo, Japan. Total RNA was extracted from 1.6 mL of harvested plasma. The plasma was mixed with 2 mL of Trizol LS reagent (Invitrogen, Carlsbad, CA) and 0.4 mL of chloroform. This mixture was centrifuged at 12,000*g* for 15 minutes at 4°C, then the aqueous layer was transferred to new tubes. After 1 volume of 700 mL/L ethanol was added to 1 volume of aqueous layer, the mixture was applied to a QIAamp MinElute Virus col-

TABLE 2
Characteristics of patients with preeclampsia (n = 62) at later gestation and characteristics of their infants

Characteristic[a]	Mild preeclampsia (n = 26)	Severe preeclampsia (n = 24)	HELLP syndrome (n = 12)
Women			
Age (y)[b]	31.5 (20-40)	27.5 (19-42)	24.5 (20-35)
Body mass index[b,c]	24.6 (19.3-33.3)	21.9 (17.4-29.4)	21.7 (18.4-30.3)
Systolic blood pressure (mm Hg)[b]	150 (130-160)	175 (160-195)	185 (160-180)
Diastolic blood pressure (mm Hg)[b]	90 (90-100)	115 (110-195)	110 (100-140)
Proteinuria (g/24h)[b]	0.75 (0.3-6.4)	6 (5.0-8.2)	5.55 (3.8-9.5)
Primigravida (%)	8 (30.7%)	7 (29.1%)	5 (41.6%)
Infants			
Birthweight (g)[b]	2775 (1500-3290)	2475 (1740-3900)	2400 (1600-3130)
Gestational age at delivery (wk)[b]	37.45 (33-40)	37.2 (34-40)	36.4 (34-40)
Small for gestational age, < 10th percentile (n)	8 (30.7%)	11 (45.8%)	7 (58.3%)

[a] Measurement on admission to the hospital; [b] Values represent median (minimum-maximum); [c] Body mass index: weight in kilograms divided by the square of height in meters.

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umn (Qiagen, Hilden, Germany) and processed according to the recommendations of the manufacturer. Total RNA was eluted with 20 μL of RNase-free water and directly reverse-transcripted with an Omniscript RT kit (Qiagen) in accordance with the instructions of the manufacturer. After this, complementary DNA products were amplified by real-time quantitative PCR according to the manufacturer's instructions (QuantiTect Probe PCR kit; Qiagen) with a 2-μL aliquot of complementary DNA and the kit components in a reaction volume of 20 μL. TaqMan PCR analyses for SERPINE1, PLAT, VEGFA, FLT1, endoglin, PLAC1, and SELP were performed with predeveloped and commercially available primers and probe sets (Cat # Hs00167155_m1 for SERPINE1, Cat # Hs00263492_m1 for PLAT, Cat # Hs00900054_m1 for VEGFA, Cat # Hs01052936_m1 for FLT1, Cat # Hs00923997_g1 for endoglin, and Cat # Hs00174583_m1 for SELP; Applied Biosystems, Foster City, CA). Primers and TaqMan-probes for PLAC1 gene have been described previously.[12] As an initial step, we verified that each PCR assay was specific to mRNA and not to genomic DNA. Amplification data were collected and analyzed with an ABI Prism 7900T Sequence Detector (Applied Biosystems). Each sample was analyzed in duplicate, and multiple negative water blanks were included in every analysis. The following thermal profile was used: 15 minutes of denaturation at 95°C, followed by 15 seconds of annealing at 94°C and 1 minute of extension at 60°C. Quantification of gene expression was performed with investigators who were blinded to the outcome of pregnancy. Amounts of mRNA samples were expressed in terms of copies per milliliter. To quantify mRNA concentrations, we prepared plasmid DNA for calibration curves as previously described.[10]

TABLE 3
Cases and control subjects: mean (SD)

MRNA species (Log10 scale)[a]	Control subjects (n = 310)	Women with preeclampsia (n = 62)	*P* value[b]
PLAT	1.17 (0.42)	2.17 (0.52)	< .001
SERPINE1	2.27 (0.39)	2.66 (0.57)	< .001
FLT1	1.90 (0.32)	2.39 (0.32)	< .001
VEGFA	3.25 (0.27)	3.77 (0.38)	.030
Endoglin	3.66 (0.31)	3.99 (0.47)	< .001
SELP	2.42 (0.69)	2.93 (0.85)	.029
PLAC1	3.52 (0.67)	3.94 (0.86)	.040

Power ranged between 0.94 and 1 at a given type I error of 0.05.

[a] Expression levels of SERPINE1, PLAT, VEGFA, FLT1, endoglin, PLAC1, and SELP are expressed as copies per milliliter; [b] Student *t* test.

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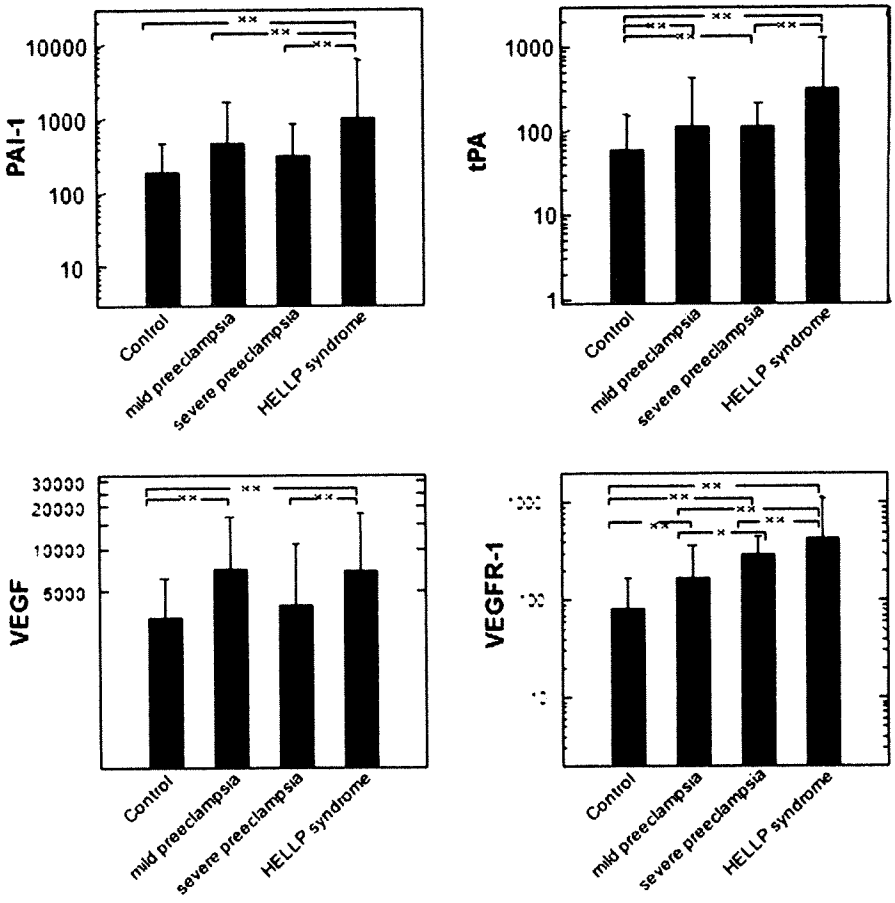
Statistical analysis

Power analysis was performed by means of PASS statistical software (PASS, Rotterdam, The Netherlands). Distributions of demographic characteristics and mRNA concentrations were analyzed by the Student *t* test and $\chi 2$ test. Mean values of the variables of interest were strat-

FIGURE 1
Messenger RNA levels



Mean (±SD mRNA levels of **A,** SERPINE1; **B,** PLAT); **C,** VEGFA; **D,** FLT1; **E,** endoglin; **F,** PLAC1, and **G,** SELP in control subjects and women with preeclampsia; the levels have been stratified in accordance with the severity of preeclampsia (mild and severe preeclampsia and HELLP syndrome). The *double asterisks* denote $P < .01$ by Scheffe post hoc test; the *single asterisk* denotes $P < .05$.

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ified retrospectively according to severity of preeclampsia and development of HELLP syndrome. Analysis of variance and relative Scheffe test were used for comparisons. Detection rate and false-positive rate were calculated for each available marker with a univariable receiver operating characteristic (ROC) curve. Discriminant analysis was used as a multivariable tool with the purpose of pulling together the detection rate of the whole set of markers. Discriminant analysis is useful to build a predictive model of group membership that was based on observed characteristics for each case. The procedure generates a discriminant function that provides the best discrimination between groups (affected vs controls) and is able to assign to each group membership a mutually exclusive score from 0-100 for belonging to the control or affected group. Because discriminant analysis needs a parametric distribution of the markers, the data were converted into Log10 scale and analyzed by Kolmogorov-Smirnov and Shapiro tests. Finally, an ROC curve for the calculation of multivariable detection rate was built with the use of the calculated score of preeclampsia for each patient in the series.

RESULTS

Table 1 shows the clinical characteristics of the pregnant women from control and preeclampsia groups. Preeclampsia occurred in 62 of 683 patients (9%), which is a higher frequency than that seen in the published literature. Although no differences were observed in age, body mass index, smoking status, gestational age at blood drawing, and blood pressure and proteinuria at the time of blood drawing between groups, significant differences were noted in birthweight, frequency of fetal growth restriction, and gestational age at delivery, which was consistent with the existing literature.[13,14] Table 2 shows detailed background of preeclampsia groups. Table 3 shows mean values and relative comparisons for each mRNA. All 7 kinds of mRNA expressions were increased significantly in the preeclampsia group. In the preeclampsia group, 26 (41.9%), 24 (38.7%) and 12 (19.4%) cases showed mild preeclampsia, severe preeclampsia, and HELLP syndrome, respectively. Figure 1 shows comparisons among control subjects, mild and severe preeclampsia, and HELLP syndrome. Almost all comparisons among groups revealed significant differences at the probability value of $< .01$. All mRNAs showed a tendency to increase according to preeclampsia severity. Only VEGFA and PLAC1 did not show any clear correlation with the severity, although concentrations were lower in control subjects than in subjects with HELLP syndrome.

We evaluated the matrix of correlation for patients with preeclampsia and identified SELP, PLAT, and SERPINE1 as the cluster of markers with the strongest associations ($P < .01$), followed by the cluster of endoglin, VEGFA, and PLAC1 ($P < .05$). Table 4 shows ROC outputs for each marker. Univariable ROC curves show FLT1 as the mRNA with the highest degree of discrimination, followed by endoglin, SERPINE1, SELP, PLAT, VEGFA, and PLAC1. All markers displayed very significant probability values. Detection rate at 5% false-positive rate ranged between 17.7% and 58.0% (Table 4). When stratified according to severity, the highest detection rate was found for HELLP syndrome (Table 5). The power of the ROC curve was 83% at a given type I error of 0.05.

TABLE 4
Output of univariable ROC curve for each available marker and multivariable ROC curve with the use of the discriminant score of the development of preeclampsia as a test variable

Variable	Area under the curve	SEM	*P* value	95% CI for area under the curve		Detection rate at 5% false-positive rate (%)	95% CI for detection rate (%)	
				Lower	Upper		Lower	Upper
FLT1	0.846	0.032	< .001	0.783	0.909	58.0	44.8	70.2
Endoglin	0.756	0.038	< .001	0.683	0.830	43.5	31.2	56.6
SERPINE1	0.732	0.037	< .001	0.660	0.805	29.0	18.5	42.1
SELP	0.727	0.033	< .001	0.662	.0792	24.2	14.5	37.0
PLAT	0.686	0.042	< .001	0.604	0.768	33.9	22.6	47.1
VEGFA	0.651	0.042	< .001	0.568	0.734	29.0	18.5	42.1
PLAC1	0.645	0.040	< .001	0.567	0.723	17.7	9.6	29.9
Score	0.927	0.025	< .001	0.877	0.976	84.0	71.8	91.5

Under nonparametric assumption.
Null hypothesis: true area = 0.5.

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In the multivariable model, degree of correlation among markers was taken into account. Mean estimated score for preeclampsia (range, 0-100) was 9.4 for control subjects and 72.5 for preeclampsia cases, which demonstrates that this multimarker model was able to assign a very different score for preeclampsia in those subjects who were destined to have preeclampsia, compared with those who remained control subjects for the entire pregnancy. An ROC curve that was generated by the actual score for preeclampsia that was calculated for each subject yielded a global detection rate of 83.9% and 88.7% at false-positive rates of 5% and 10%, respectively, with an area under the curve of 0.927 ($P < .001$; Figure 2). Finally, the score of each patient for classification of preeclampsia was correlated with severity. In fact, mean scores were 56.3 for mild preeclampsia, 79.3 for severe preeclampsia, and 93.7 for HELLP syndrome.

Comment

The present study, as an extension of the earlier study, prospectively examined maternal blood samples from women at gestational weeks 15-20. Because our preliminary study showed that the mRNA level of CRH was too low to quantify in the early mid-trimester samples, we eliminated CRH quantification in this study. We then assessed 7 kinds of mRNA expressions and compared them with clinical outcomes. Although standard deviations were large for each gene, all of these mRNA expressions were increased in the plasma from pregnant women who experienced preeclampsia later. The mRNA expression levels of PLAT and SERPINE1 were increased 8.9- and 8.0-fold in the preeclampsia group. The results revealed that these gene expressions are increased not only in the third trimester but also in the early second trimester. Because these cell-free mRNA expressions in plasma were cleared rapidly after delivery,[8-10] cell-

TABLE 5
Output of multivariable ROC curve with the use of the discriminant score of the development of preeclampsia as a test variable, according to the severity of the preeclampsia

Variable	Area under the curve	SEM	*P* value	95% CI for area under the curve		Detection rate at 5% false-positive rate (%)	95% CI for detection rate (%)	
				Lower	Upper		Lower	Upper
Mild preeclampsia (n = 26)	0.837	0.055	< .001	0.728	0.945	65.4	44.3	82.0
Severe preeclampsia (n = 24)	0.989	0.005	< .001	0.980	0.998	95.8	76.8	99.7
HELLP syndrome (n = 12)	0.997	0.004	< .001	0.991	1.000	100	69.8	100

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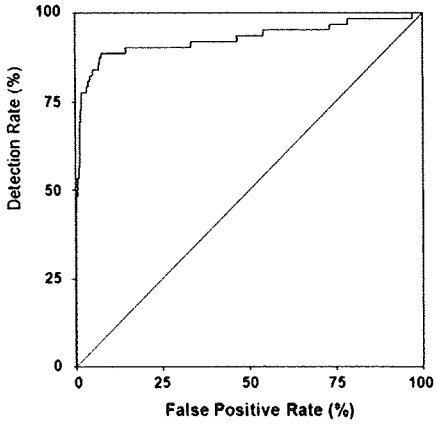
FIGURE 2
Multivariable ROC curve



Multivariable receiver operating characteristic (ROC) curve that was obtained with the discriminant score as a test variable for the prediction of preeclampsia.

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free mRNA analyses allow the evaluation of placental pathophysiologic alterations. This approach thus could have significant clinical value and should lead to the development of real-time monitoring of placental function.

Furthermore, we assessed the possibility of prediction by analyzing these transcripts in plasma. Although many tests have been proposed for the prediction of preeclampsia, results have been inconsistent and contradictory.[15-17] However, in the present study, a panel of mRNAs were dosed long before clinical onset properly predicted preeclampsia occurrence with a degree of accuracy comparable or substantially higher than that reported for Doppler ultrasonography with or without demographic and biochemical parameters.[15] Univariable analysis showed FLT1 as the marker with the highest detection rate and PLAC1 with the lowest detection rate. The best multivariable model was obtained by the combination of all markers. An ROC curve yielded a detection rate of 84% at 5% false-positive rate with an area under the curve of 0.927 ($P < .001$). To the best of our knowledge, this result offers the best prediction of preeclampsia in low-risk populations so far. Furthermore, this study also observed that the score, as generated by discriminant analysis of the development of any form of preeclampsia (mild, severe, or HELLP syndrome) that was analyzed long before clinical onset, is proportional to the degree of severity that was observed later in pregnancy. In fact, patients who had mild preeclampsia had a mean score for preeclampsia of 53, compared with 97.3 for those who had HELLP syndrome.

In the previous study of plasma mRNA from patients with preeclampsia, SERPINE1 and PLAT expressions were the most closely correlated with disease severity. However, the present study revealed that the area under the curve was highest for FLT1, followed by endoglin. These findings suggest that antiangiogenic factors such as FLT1 and endoglin play more critical roles in the earlier steps of preeclampsia development than do SERPINE1 and PLAT, whereas SERPINE1 and PLAT play important roles in the final steps of clinical manifestations of proteinuria and hypertension. FLT1 and endoglin recently have been reported to play important roles in the pathophysiologic condition of preeclampsia.[18] Protein concentrations of FLT1 and endoglin in plasma are increased before the onset of preeclampsia and correlate with disease severity.[18,19] Overexpression of FLT1 in pregnant rats results in a preeclampsia-like phenotype. Overexpression of soluble endoglin in rodents by means of adenoviral vectors also leads to increased vascular permeability and induction of modest hypertension without significant proteinuria.[18,19] Furthermore, adenoviral-mediated overexpression of both FLT1 and endoglin causes severe vascular damage, nephrotic-range proteinuria, severe hypertension, and a syndrome similar to HELLP syndrome.[20] These reports have indicated that FLT1 and endoglin from the placenta induce severe maternal endothelial dysfunction. This is concordant with the present finding that FLT1 and endoglin are likely to offer the best predictors among genes that are derived from the placenta. FLT1 and endoglin are suggested as the main factors that cause preeclampsia at gestational weeks 15-20; expressions in the placenta can be evaluated through the analysis of cell-free mRNA in plasma from pregnant women.

Although the reason that those mRNA expressions increased cannot be explained in this article, the increased mRNA expression may reflected mRNA alterations that are associated with the pathogenesis of preeclampsia in the placenta. Evaluation of cell-free mRNA may allow indirect monitoring of placental function.[4] Further research of other placental mRNA expression to resolve the mechanism that regulates the trophoblasts during early gestation may elucidate the pathogenesis of preeclampsia. Several other hypotheses that are related to increased plasma RNA have been reported,[5] but the exact mechanism that produces the increase of those mRNA expressions in maternal plasma requires further investigation.

In view of stability of mRNA in maternal plasma, Ng et al[2] have shown that placental mRNAs are very stable in maternal plasma. This stability may suggest the practicality of the mRNA marker in maternal plasma for clinical use. In this study, preeclampsia occurred in 9% of patients, which is a higher frequency than seen in the published literature. No previous large studies have clarified the prevalence of preeclampsia in Indonesia populations; this higher prevalence could confound the result. Furthermore, because the case number of gestational hypertension or early onset type preeclampsia is not enough to analyze statistically, we did not include patients of with hypertension in pregnancy and did not analyze those patients with early onset of preeclampsia separately.

In conclusion, we demonstrated that mRNA expression levels of FLT1, VEGFA, endoglin, PLAT, SERPINE1, PLAC1, and SELP are increased in plasma from pregnant women at gestational weeks 15-20 who subsequently experience preeclampsia and that alterations in placental function can be evaluated through analyses of plasma mRNA in pregnant women at early gestation. Furthermore, in populations that are at low risk of preeclampsia, a panel of these mRNA expressions allows accurate detection of high-risk pregnant women who are likely to experience preeclampsia. ■

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21年度　分担研究報告書

「わが国における新しい妊婦健診体制構築のための研究」
分担研究：母体血による疾患の早期診断確立に向けて

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研究要旨

【目的】現在、妊娠高血圧腎症（preeclampsia, PE）の発症を高感度、高特異度でスクリーニングする方法が確立されていない。我々は、子宮動脈血流速度波形計測と血管新生因子関連物質(soluble fms-like tyrosine kinase 1 [sFlt1], placental growth factor [PlGF], soluble endoglin [sEng])の測定を組み合わせると、早産となる妊娠高血圧腎症（preterm PE）の発症を高率に予知できる可能性を示してきた。本研究では、妊娠中期にPE発症のハイリスク妊婦を一次スクリーニングし、さらに、血清 sFlt1:PlGF 比及びsEngを4週毎に反復測定する二次スクリーニングによってpreterm PE発症を早期診断する方法の確立を目指す。

【方法】平成20年度～22年度にかけて、以下5つの研究を行う。研究1.　血清sFlt1:PlGF比、sEngの妊娠20～38週の正常域の決定。研究2.　妊娠中期の子宮動脈血流速度波形で両側notchesがみられる妊婦における、妊娠20～23週、および妊娠27～30週のsFlt1:PlGF比、sEngによるpreterm PE発症予知精度の検討。研究3.　PEハイリスク妊婦における、sFlt1:PlGF比、sEng反復測定によるpreterm PE早期診断法の開発。研究4.　子宮動脈血流速度波形 mean resistance index (mRI), mean pulsatility index (mPI), mean notch depth index (mNDI)の妊娠16～24週の正常域の設定。研究5.　妊娠中期の妊婦コホートにおける、妊娠中期の平均血圧（mean blood pressure, MBP）、mNDI, PlGFを用いたpreterm PEの発症予測式の作成。

【成績】

（研究1）平成20年度は、正常妊婦85例の保存血清を用いて、sFlt1, PlGF, sFlt1:PlGF、比、およびsEngの4指標について、妊娠20～38週の95%値および5%値を決定した。

（研究2）平成21年度は、2003～2008年に、妊娠中期に子宮動脈血流速度波形を計測し、かつ、妊娠20～30週で採血を行った妊婦1346例中、妊娠16～24週に両側notchesを呈した妊婦323例（24.0%）を対象に、妊娠20～23週の血清322検体、妊娠27～30週の血清211検体について、sFlt-1、PlGF、sEng濃度の測定を開始した。

（研究3）平成20年度から、妊娠16～23週の子宮動脈血流速度波形のNDI値が≧90値以上、あるいは、妊娠16～23週の血圧が130/85mmHg以上、あるいは、既往PIHの妊婦を対象に、妊娠20週以降4週毎に妊娠35週まで反復して採血を行った。平成21年12

月までに27例が登録され、現在も症例を追加中である。

（研究４）平成21年度は、妊娠高血圧症候群を発症しなかった正常妊婦1530例の子宮動脈血流速度波形を用いて、mRI, mPI, およびmNDIの3指標について、妊娠16～24週の80%値、90%値、95%値および97.5%値を決定した。mNDIは、mRI、mPIと比較して、すべてのPE、早発型PEのスクリーニング特性がほぼ同様であった。

（研究５）平成21年度は、妊婦コホート1252例を対象に、妊娠中期のMBP、mNDI、PlGFとpreterm PE発症との関連を調べた。ROC曲線解析の結果、MBP, AUCは各々0.878、0.842、0.686であった。最適なカットオフ値は、MBPは98 mmHg、mNDIは90%値、そしてPlGFは10%値であった。多重ロジスティック分析の結果、この3指標は独立してpreterm PEの発症に関与していたため、この3指標を組み合わせてpreterm PEの発症を予知する予測式を構築した。3指標を用いた予測式とその後の早産となるPE発症との関連を、ROC曲線解析したところ、AUCは0.961と非常に高くなった。妊娠中期のMBP、mNDI、およびPlGFの3つを組み合わせた予測式を用いることで、大部分のpreterm PE妊婦（93%）を、10%の擬陽性率で、妊娠中期に一次スクリーニング可能であった。

【結論】平成20年、21年度の研究によって、sFlt1, PlGF, sFlt1:PlGF比、およびsEngの4指標の妊娠20～38週の正常域、子宮動脈血流速度波形のmRI, mPI, および,mNDIの3指標の妊娠16～24週の正常域を決定した。また、妊娠中期のMBP、mNDIおよびPlGFの3つを組み合わせた予測式を用いることで、妊娠20～23週の時点で、preterm PE発症リスクの高い妊婦を高感度で一次選別できることを明らかにした。最終年度は、(1) 妊娠中期の子宮動脈血流速度波形の両側notchesを用いて選別されたPEハイリスク妊婦を対象とした、妊娠20～23週および妊娠27～30週でのsFlt1:PlGF比、sEng濃度を用いたpreterm PE発症予知法（研究2)、(2)妊娠中期のNDI高値、血圧130／85mmHg以上あるいは既往PIHを用いて選別されたPEハイリスク妊婦を対象とした、妊娠20週以降4週毎のsFlt1:PlGF比、sEng濃度の反復測定法によるpreterm PE早期診断法（研究3）を評価する。

A．緒言

Papageorghiou らは 8000 人以上の妊婦を対象に、妊娠 22-24 週で子宮動脈血流速度波形を計測した。その結果、子宮動脈血流速度波形異常は、結果的に早産となった妊娠高血圧腎症 (preterm preeclampsia:preterm PE)の発症予知能が高いことを ROC 分析により明らかにした(1)。一方、Levine らは 4500 人以上の妊婦コホートを対象に、妊娠 21-32 週で soluble fms-like tyrosine kinase 1 : placental growth factor 比（sFlt1:PlGF 比）及び soluble endoglin (sEng)濃度をそれぞれ計測・測定した(2)。その結果、正常妊婦における sFlt1:PlGF 比及び sEng 濃度の上位 25%を高値（異常）と定義した場合に、両者が異常を示した場合には、preterm PE の発症リスクは約 32 倍と高かった(2)。以上の知見は、子宮動脈血流速度波形計測と血管新生因子関連物質(sFlt1, PlGF, sEng)の測定を組み合わせれば、preterm PE の発症を高率に予知できる可能性を示唆している。現在の妊婦健診では、一部の PE ハイリスク妊婦（高血圧合併妊婦、糖尿病合併妊婦、腎炎合併妊婦、SLE 合併妊婦、抗リン脂質抗体陽性妊婦、既往妊娠高血圧症候群[pregnancy-induced hypertension, PIH]妊婦など）は PE ハイリスク妊婦として最初から高次施設へ紹介され管理される場合が多いが、大部分の PE 発症妊婦は合併症もなく、初産婦での発症率が高いこともあって、一次施設で妊婦健診が行われている。そして、突然 PE と診断され、慌てて高次施設へ紹介・搬送されることが多い。そのため、高次施設に紹介・搬送された時には、すでに重症合併症（子癇、HELLP 症候群、DIC、常位胎盤早期剥離、高度の胎児発育不全［intrauterine fetal restriction, FGR］、あるいは胎児機能不全など）を合併していることが多い。中には、重症合併症の発見、治療が遅れ、母体死亡や周産期死亡の原因になることもある。もしも、preterm PE の発症を高い精度で予知できる方法が開発され、一次施設での妊婦健診にこの方法が取り入れられれば、preterm PE を発症する危険性の高い妊婦を早期診断できるようになるであろう。そうなれば、PE 発症前に高次施設へ紹介・搬送が可能となり、PE に伴う重症合併症への対応が早まるので、例え PE を発症しても母児予後の改善を期待できるであろう。

我々は、これまでの研究から、以下の成果を得ている。

1. preterm PE を発症した妊婦の 73%は、妊娠 16～23 週でmNDI 高値（≧90%値）を示した。
2. preterm PE を発症した妊婦の 60%は、妊娠 16～23 週で平均血圧(mean blood pressure, MBP)≧98mmHg（収縮期血圧 128mmHg、拡張期血圧 84mmHg に相当）を示した。
3. preterm PE を発症した妊婦の 98%において、sFlt1:PlGF 比及び sEng の両者異常高値（≧95 パーセント値）を認めた(3-5)。
4. preterm PE となった大部分の妊婦では、PE 発症前に sFlt1:PlGF 比及び sEng 両者の上昇が見られた(3-5)。

以上の結果は、妊娠中期に、子宮動脈血流速度波形異常、血圧レベルで一次スクリーニングを行い、続いて、 sFlt1:PlGF 比及び sEng 高値を反復測定すること（二次

スクリーニング）で、preterm PE の発症リスクの高い妊婦を早期診断できる可能性を強く示唆している。現在、この sFlt1:PlGF 比や sEng は研究レベルであり、実用化段階に至っていないが、欧米では、PE の診断に用いるための sFlt1:PlGF 比測定キットが開発され、すでに臨床で使用できるようになっており(6, 7)、本研究の成果は直ちに臨床応用可能と考えられる。

本研究の目的は、(1)妊娠中期における preterm PE ハイリスク妊婦の高感度一次スクリーニング法を開発すること、(2)4 週間隔での sFlt1:PlGF 比、sEng 反復測定法が、preterm PE の早期診断法として有用かどうかを検証することである。そして、本研究の目標は、母体血による preterm PE の早期診断法を確立し、それを新しい妊婦健診体制に組み込むことである。その結果、preterm PE のハイリスク妊婦が疾患発症前に高次医療機関に紹介されるようになり、preterm PE の重症合併症による母体死亡、周産期死亡が更に減少することが期待される。これが、本研究の意義である。

B．研究方法

以下 5 つの研究を行う。

1. 【sFlt1:PlGF 比の妊娠中の正常域の決定】

2003 年～2008 年において前方視的に行われた「大学院整備重点化経費－研究科特別経費、期間：平成 15-17 年度、研究課題名：妊娠中期の子宮動脈血流速度波形異常妊婦における血清 sFlt1 の経時的変化と preeclampsia 発症に関する追跡研究」および「文部科学省科学研究費 研究番号：18591809、平成 18－19 年度、研究課題名：血清 P l GF を含む早発型 PE 発症に関与するリスク因子の前向き研究」（以下先行研究と略）において、妊婦 1500 例の血清が保存されている。平成 20 年度は、同意を得て凍結保存した検体のうち、妊娠 20-38 週の間に 3 回採血し得た正常妊婦血清 255 検体の sFlt1、PlGF を測定し、sFlt1:PlGF 比の正常域を決定した。

2. 【子宮動脈血流速度波形異常妊婦における、sFlt1:PlGF 比、sEng の preterm PE 発症予知精度の検討】

2003 年～2008 年の先行研究において、妊娠中期に子宮動脈血流速度波形計測を行った妊婦 1500 例の妊婦血清が保存されている。平成 21 年度は、妊娠 16～24 週の子宮動脈血流速度波形で両側 notches を認めた妊婦 323 例を対象に、同意を得て凍結保存した検体を用いて、妊娠 16-23 週および妊娠 27-30 週での sFlt1:PlGF 比、sEng を測定し、PE ことに preterm PE 発症の positive predictive value を明らかにする。

3. 【PE ハイリスク妊婦における、sFlt1:PlGF 比、sEng 反復測定による preterm PE 発症早期診断法の開発】

20 年度 10 月より研究を開始した。子宮動脈血流速度波形異常、外来血圧 130/85 以上、及びその他の PE ハイリスク因子を保持する妊婦において、4 週毎に血液を採取保存し、sFlt1:PlGF 比及び sEng 濃度を適宜測定する。最終年度に、sFlt1:PlGF 比、sEng 反復測定が preterm PE 発症の早期診断法として有用か否かを検証する。

4. 【子宮動脈血流速度波形 RI, PI, NDI の正常域の設定】

平成 21 年度は、2003 年～2008 年先行研

究において、妊娠中期に子宮動脈血流速度波形計測を行った妊婦 1606 例について、PIH を発症しなかった正常妊婦 1530 例の子宮動脈血流速度波形を用いて、RI, PI, および NDI の 3 指標について、妊娠 16～24 週の 80%値、90%値、95%値および 97.5%値を決定する。各指標は、両側子宮動脈血流速度波形の指標の平均値を用い、各々 mean RI (mRI), mean PI (mPI), mean NDI (mNDI)と表現した。

5. 【妊娠中期の MBP、NDI, PlGF 濃度を用いた preterm PE の発症予測式の作成】

2003～2008 年の先行研究において、妊娠中期の MBP、NDI、PlGF 濃度についてデータが得られた妊婦 1252 例について、これらのリスク因子を組み合わせることで、preterm PE の発症予知精度が向上するかどうかを検討する。

研究 2 については、20 年度報告において、本学臨床研究倫理審査委員会へ提出した研究計画書の概要を記載した。

C．研究結果

1.【sFlt1:PlGF 比の妊娠中の正常域の決定】

平成 20 年度報告に研究結果を記載した。

2. 【子宮動脈血流異常妊婦における、sFlt1:PlGF 比、sEng の preterm PE 発症予知精度の検討】

平成 21 年度は、2003～2008 年に、妊娠中期に子宮動脈血流速度波形を計測し、かつ、妊娠 20～30 週で採血を行った妊婦 1346 例中、妊娠 16～24 週に両側 notches を呈した妊婦 323 例（24.0%）について、妊娠 20～23 週の血清 322 個、妊娠 27～30 週の血清 211 個について、sFlt-1、PlGF、sEng 濃度の測定を開始した。

3. 【PE ハイリスク妊婦における、sFlt1:PlGF 比、sEng 反復測定による preterm PE 発症予知法の開発】

平成 20 年 10 月より、妊娠 16～23 週の子宮動脈血流速度波形の mNDI 値が≧90%値以上、あるいは、妊娠 16～23 週の血圧が 130/85mmHg 以上、あるいは、既往 PIH の妊婦を対象に、妊娠 20 週以降 4 週毎に妊娠 35 週まで反復して採血を行った。平成 21 年度の 12 月までに 27 例が登録された。

4.【子宮動脈血流速度波形 RI, PI, NDI の正常域の設定】

平成 21 年度は、妊娠中期に子宮動脈血流速度波形計測を行った妊婦 1606 例中、妊娠高血圧症候群を発症しなかった正常妊婦 1530 例の子宮動脈血流速度波形を用いて、mRI, mPI, および mNDI の 3 指標について、妊娠 16～24 週の 80%値、90%値、95%値および 97.5%値を決定した（図 1：mRI の正常域；図 2：mPI の正常域；図 3：mNDI の正常域）。各々の指標を用いて、すべての PE および早発型 PE 発症との関連について ROC 曲線解析を行った（図 4）。その結果、mRI、mPI および mNDI は、すべての PE および早発型 PE の発症予知について、そのスクリーニング特性がほぼ同様であることを明らかにした（表 1）。

本研究の結果は、3rd SGI International Summit 2009, "Preeclampsia" in Sendai, Japan において発表した。

5.【妊娠中期の平均血圧、mNDI, PlGF 濃度を用いた preterm PE の発症予測

式の作成】

平成21年度は、妊婦コホート1252例において、妊娠中期のMBP、mNDI、血清PlGF濃度（以下単にPlGFと略）とその後の早産となるPE発症との関連を検討した。このコホートよりPEは35例（2.8%）、pretermPEは15例(1.2%)、早発型PEは9例（0.7%）に発症した。ROC解析したところ、MBP、mNDIおよびPlGFのAUCは各々0.878、0.842、0.686であった(図5)。最適なカットオフ値は、MBPは98 mmHg、mNDIは90%値、そしてPlGFは10%値であった。多重ロジスティック分析の結果、この3指標は独立して早産となるPEの発症に関与していたため、この3指標を同時に用いて早産となるPEを予知する予測式を構築した。3指標を用いた予測式とその後の早産となるPE発症との関連を、ROC解析したところ、AUCは0.961と非常に高くなった（図5)。このことから、妊娠中期のMBP、mNDI、およびPlGFの3つを組み合わせることで、大部分の早産となるPE妊婦（93%）を、90%の特異度で、妊娠中期にスクリーニングできることがわかった（表2)。

本研究の結果は、3rd SGI International Summit 2009, "Preeclampsia" in Sendai, Japanにおいて発表した。

D．考察

平成20年度において、妊娠20～38週における血清sFlt1, PlGF, sFlt1:PlGF比、およびsEngの4指標の正常域を決定し、本年度は、妊娠16～24週における子宮動脈血流速度波形のRI、PIおよびNDIの正常域を決定した。これにより、妊娠16～24週の子宮動脈血流速度波形異常でPEハイリスク妊婦を選別し、妊娠16週以降、4週間毎にsFlt1:PlGF比、sEng値を測定し、異常値の出現を追跡していくことが可能となった。

これまでの血管新生関連因子とPEとの関連を調べた研究において、血管新生関連因子の変化は、早発型PEにおいて、遅発型PEよりもより顕著であることが示されてきている(2, 3-5)。また、遅発型PEにおいては、必ずしも疾患発症後に血管新生関連因子の変化が見られるとは限らないこともわかってきた(3-5)。従って、血管新生関連因子を用いてPEの発症予知を行う場合、その対象をpreterm PEに限定することは妥当な選択と考えられる。

これまで、妊娠中期の子宮動脈血流速度波形と血管新生関連因子の組み合わせによって、PEの発症予知精度が上昇することが、1000例以上を対象とした（または30例以上のPE発症例を持つ）前方視的研究によって明らかにされている(8-11)。また、これまでの子宮動脈血流速度波形を用いたPE発症予知研究より、子宮動脈血流速度波形はpreterm PEの発症においてその感度が高いことが明らかにされている(1, 12)。従って、子宮動脈血流速度波形によるスクリーニングにおいて、すべてのPEを予知することをoutcomeとした場合、感度の低下が起こる。我々の前方視的研究でも、preterm PEをoutcomeとすれば、RIとNDIを組み合わせることによりその80%を捉えることができたが、term PEの発症予知感度は5%と極めて低率であった（未発表データ)。臨床的にその発症を予知して

我々が積極的に介入すべき対象はpreterm PEであることを考慮すると、PEの発症予知の対象をpreterm PEに限定することは妥当な選択と考えられる。

我々は、1518例の妊婦後ろ向きコホート研究において、妊娠中期の血圧レベル120/80mmHg未満の至適血圧群に比較して、正常血圧（120-129/80-85mmHg)、正常血圧高値(130-139/85-89mmHg)、及び高血圧(140/90mmHg以上)では、PEの発症率が各々5.6倍、9.5倍及び20倍高いことを明らかにした(13)。我々の検討で、早発型PEの中に、子宮動脈血流速度波形の異常を呈さず、血圧高値あるいは高血圧を背景に発症する一群が存在し、これらの妊婦では疾患発症時にsFlt1:PlGF比及びsEng値が上昇していることを確認している（未発表データ)。従って、preterm PEをより多くスクリーニングするためには、子宮動脈血流速度波形異常妊婦のみならず、妊娠中期の血圧レベル上昇例までを対象に含める必要があると考えられる。また、既往PE妊婦では、血管新生関連因子の上昇がその後のPE発症と関連すること(14, 15)、preterm PEの既往妊婦はpreterm PEを反復しやすいことが知られていることから(16)、既往PE発症妊婦、糖尿病、慢性腎炎、SLE、抗リン脂質抗体症候群などを合併している妊婦といったPEハイリスク妊婦も対象に含める必要があると考えられる。

本年度の研究5において、妊娠中期のMBP、mNDIおよびPlGFは各々独立してpreterm PEの発症に関連していること、そして、これらの3指標を組み合わせた予測式を用いれば、10%の擬陽性率で93%のpreterm PEをスクリーニングできることを明らかにした。最近、妊娠初期においても、MBP、mPIおよびPlGFを組み合わせることで、早発型PEの発症予知精度を改善できることが報告された(17)。PIHを発症した妊婦209例、正常コントロール408例において、BMI、初産経産の有無、既往PEの有無、妊娠初期のMBP、mPI、PlGFは早発型PEの発症に関与し、5%の擬陽性率で93%の早発型PEの発症を予知した(15)。我々の結果とあわせて考えると、妊娠初期から中期にかけての、MBP、子宮動脈血流速度指標およびPlGF値を組み合わせると、単独の指標を用いた場合と比較して、PEの発症予知精度が改善すると結論できる。そして、妊娠初期から妊娠中期において、重症合併症を併発しやすい早発型PEあるいはpreterm PEのハイリスク妊婦を、高い感度でスクリーニングできると結論できる。

本年度は、研究2、研究3の測定が終了せず、血清sFlt1/PlGF比およびsEngの反復測定法がpreterm PEの早期診断法として有用かどうかまで結論を出すことができなかった。また、研究3はハイリスクの選別が厳しすぎるためか、まだ症例が27例しか集まっていない。少なくとも50例は必要と思われ、症例数の追加が当面の課題である。

E．結論

これまでの妊婦健診では、PEは疾患発症前に高感度、高特異度で予知する方法がなかった。このため、PEとして搬送されてくるときには、すでに、母体が重篤な高血圧、肺水腫、常位胎盤早期剥離、子癇、脳出血、あるいはDICといった母体死亡に直結する

重症合併症を併発している場合が少なくない。また、早発型PEでは、約半数以上でFGRを合併するため、すでに子宮内胎児死亡であったり、あるいは重症の胎児機能不全によって神経学的後遺症を合併したりすることも少なくない。母児の予後を少しでも改善するためには、PE発症のハイリスク妊婦を高感度で選別し、適切な診断法によって早期診断できる方法を確立し、そして、重症化する以前に医学的介入を行う新しい妊婦健診方法の開発が喫緊の課題である。本研究により、生体情報（血圧レベル、超音波検査所見、採血データ）を用いて、高い感度と特異度を持った一次スクリーニング法を開発できた。来年度には、sFlt1:PlGF比、sEngの反復測定法による二次スクリーニングがpreterm PEの早期診断に有用かどうかがはっきりしてくるであろう。これらの、PEスクリーニング法をわが国における新しい妊婦健診体制に組み入れることができれば、疾患発症前にPEが早期診断されるようになり、妊娠高血圧腎症に伴う母児の予後は大きく改善されると期待される。また、本研究で用いる正常情報は何れも臨床レベルで実施可能なものであることから、直ちに妊婦健診に反映できると考えられる。

F. 文献

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