Results

The demographics of the pregnant women from the control and PE groups are shown in Table 1. Although no differences were observed in the maternal age, BMI, gestational age at blood drawing and blood pressure at the time of blood drawing between groups, significant differences were noted in birth weight and gestational age at delivery, and these findings were consistent with those reported in the existing literature.

The values of the available markers are reported in Figure 1A–G. The mRNA expression levels of Flt-1, ENG, P-selectin and PLAC1 were higher in the PE group than in the controls, but those of PlGF and HO-1 were lower. TGFβ1, VEGF and SOD did not reach a significant value in the comparison of PE cases versus controls (Kruskal–Wallis test), and were therefore excluded from any further analysis. For all the other markers, a significant difference was found in the comparisons of HELLP cases versus controls and, for some, including Flt-1, ENG and PlGF, a significant difference was found for any generated subgroup (mild PE, severe PE, HELLP) according with the severity (Dunn *post hoc* test). A univariate analysis identified Flt-1 and ENG as markers with the highest sensitivity. Table 2 reports the output of the ROC curve analysis for those markers with a significant difference in PE cases versus controls, together with the actual MoMs for each marker associated with the given sensitivity. The best multivariate model was obtained by combining Flt-1, ENG, PlGF and parity (Table 3). Only the final model is presented, which was obtained by stepwise logistic regression. After adjusting for the incidence of PE (2% or 1:50), the mean quoted odds (obtained by solving the logistic equation having the odds as a dependent variable for each of the subjects in the study) for PE was 1:50 (0.02) for controls and 1:7 (0.14) for women who developed PE. A ROC curve obtained using the estimated score for PE as the test variable yielded a sensitivity of 66% at a 10% 1 – specificity rate with an area under the curve of 0.884 (0.844–0.922, 95% CI; $P < 0.001$; Figure 2), and therefore with a reasonable discrimination to identify cases. Again, sensitivity and the odds for each woman for classification as PE were correlated with the severity (sensitivity of 50% for mild PE, 70% for severe PE and 75% for HELLP; odds of 1:12 for mild PE and 1:5 for both severe PE and HELLP).

Discussion

The present study examined prospectively mRNA expression in the cellular component of maternal blood samples from

Table 1. Demographic characteristics. Data are expressed as the median (minimum–maximum)

	Controls (n = 310)	Mild PE (n = 26)	Severe PE (n = 24)	HELLP (n = 12)	P value*,**
% Nulliparous	59.8	65.4	87.5	91.7	0.008***
Maternal age (years)	28 (15–43)	32 (20–40)	27.5 (19–42)	24.5 (20–35)	0.065
GA at blood test (weeks)	17 (15–20)	18 (15–20)	16 (15–18)	17.5 (15–20)	0.479
BMI at blood test	22.94 (13.79–34.22)	24.93 (19.31–33.33)	21.93 (17.42–29.48)	21.71 (18.39–30.30)	0.150
SBP (mmHg) at blood test	110 (90–130)	110 (90–125)	100 (90–120)	100 (90–130)	0.388
DBP (mmHg) at blood test	70 (60–85)	70 (60–85)	70 (60–80)	70 (60–85)	0.625
Birth weight (g)	3195 (2600–4080)	2775 (1900–3920)	2500 (2100–3900)	2400 (1800–3130)	<0.001***
Birth weight centiles	50 (14–94)	22 (1.5–92)	16 (1.5–55)	10 (1–90)	<0.001
GA at delivery (weeks)	38 (37–41)	37 (33–40)	37 (34–40)	36 (32–40)	<0.001***

*Chi-squared test.
**Kruskall–Wallis and Dunn test.
Significant comparisons: ***control versus severe PE and HELLP.
BMI, body mass index; DBP, diastolic blood pressure; GA, gestational age; HELLP, haemolysis, elevated liver enzymes and low platelets; PE, pre-eclampsia; SBP, systolic blood pressure.

Figure 1. (A–I) Box-and-whisker plots of the distribution of vascular endothelial growth factor receptor-1 (Flt-1) (A), endoglin (B), vascular endothelial growth factor (VEGF) (C), transforming growth factor-β1 (TGFβ1) (D), haem oxygenase-1 (HO-1) (E), placental growth factor (PlGF) (F), superoxide dismutase (SOD) (G), P -selectin (H) and placenta specific-1 (PLAC1) (I) mRNA levels in controls and women with pre-eclampsia, stratified in accordance with the severity of pre-eclampsia [mild and severe pre-eclampsia, and haemolysis, elevated liver enzymes, low platelet (HELLP) syndrome]. The medians are indicated by a line inside each box, and the 75th and 25th percentiles by the box limits; the upper and lower bars represent the 10th and 90th percentiles, respectively. The y-axes represent multiples of the median (MoMs) of each gene expression. Asterisks (*) indicate all specimens above or below the 90th or 10th percentile specimens. CT, control; HELLP, HELLP syndrome; mild, mild pre-eclampsia; severe, severe pre-eclampsia.

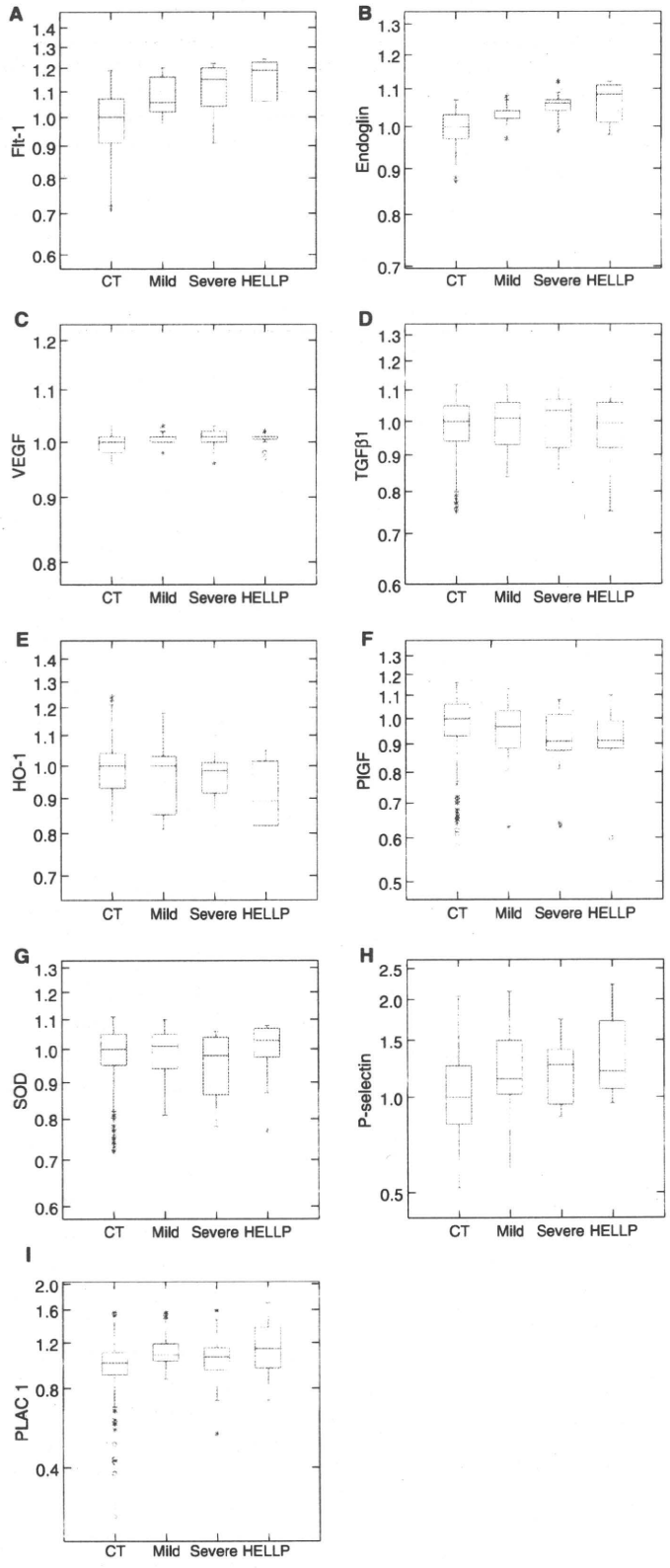

Table 2. Receiver operating characteristic (ROC) curve for each marker

mRNA	AUC	*P* value	Lower 95% bound	Upper 95% bound	Sensitivity (%) at 5% 1 – specificity	Sensitivity (%) at 10% 1 – specificity	MoM cut-off at 5% 1 – specificity	MoM cut-off at 10% 1 – specificity
P-selectin	0.665	0.039	0.588	0.742	18.2	29.1	1.61	1.45
PLAC1	0.631	0.044	0.545	0.717	20	20	1.31	1.26
Flt1	0.806	0.032	0.744	0.868	43.6	52.7	1.11	1.13
Endoglin	0.840	0.029	0.773	0.879	47.3	50.9	1.13	1.15
HO-1	0.588	0.042	0.502	0.675	8.1	14.5	0.84	0.87
PlGF	0.627	0.003	0.547	0.708	24.2	25.8	0.69	0.84

Sensitivity is shown at different cut-off values of 5% and 10% 1 – specificity.
AUC, area under the curve; HO-1, haem oxygenase-1; PLAC1, placenta specific-1; PlGF, placental growth factor.

Table 3. Logistic regression output for plasma cellular RNA levels

Variable	Odds ratio	95% CI		*P* value
		lower	upper	
Flt-1	2.760	1.910	3.988	<0.001
Endoglin	3.214	2.190	4.718	<0.001
PlGF	0.611	0.455	0.821	0.001
Parity (primi or pluri versus nulli)	2.822	1.316	6.052	0.008
Constant (ln)	–7.583			<0.001

The variables were expressed as multiples of the median (MoMs) and categorised at four levels (<25th, 25th–50th, 50th–75th and >75th percentiles).
Flt-1, vascular endothelial growth factor receptor-1; PlGF, placental growth factor.

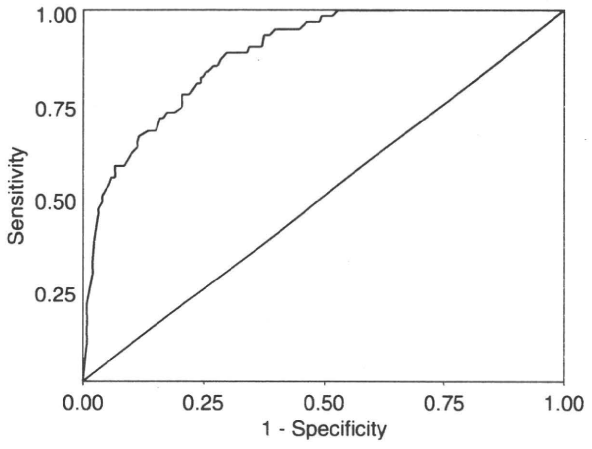

Figure 2. Receiver operating characteristic (ROC) curve obtained by plotting the values of the estimated odds for pre-eclampsia. A multivariate model consisting of endoglin (ENG), vascular endothelial growth factor receptor-1 (Flt-1), placental growth factor (PlGF) and parity was used to generate the ROC curve.

women at gestational weeks 15–20. The study assessed nine species of gene expression associated with angiogenesis and oxidative stress, which coded for factors thought to be important in the pathogenesis of PE. The mRNA levels were compared with the clinical outcomes. As a result, the mRNA expression levels of ENG, Flt-1, P-selectin and PLAC1 were found to be significantly higher in the PE group than in the controls, whereas PlGF and HO-1 levels were lower in the PE group. The blood samples were obtained at an average of 17 weeks, which is almost 20 weeks before the onset of PE. At this gestational age, the mRNA expression of anti-angiogenic factors and anti-oxidants is already altered in pregnant women who subsequently develop PE. These factors play a crucial role in the pathogenesis of PE, and the analysis of cellular components of maternal blood for these transcripts may allow for the prediction of PE.

Although many tests have been proposed for the prediction of PE, the results have been inconsistent and contradictory.[26–28] The present study has demonstrated that a panel of cellular RNA markers quantified long before clinical onset predicts PE occurrence with a degree of accuracy comparable with previous reports, including uterine artery Doppler velocimetry with or without demographic and biochemical parameters.[26] The univariate analysis showed Flt-1 and ENG to be the markers with the highest sensitivity. The best multivariate model was obtained by combining Flt-1, ENG, PlGF and parity. The ROC curve yielded a sensitivity of 66% at a 10% 1 – specificity rate with an area under the curve of 0.884. These results indicate that cellular RNA in maternal blood can be used to assess the pathophysiological alterations which occur in pregnant women who later develop PE.

The cell-free RNA in maternal plasma was assessed in a previous study. The expression of seven transcripts was assessed in the plasma of pregnant women at gestational weeks 15–20.[20] The target genes were Flt-1, ENG, VEGF, plasminogen activator inhibitor-1 (PAI-1), tissue-type

plasminogen activator (tPA), PLAC1 and P-selectin. In the statistical univariate analysis, Flt-1 showed the highest degree of discrimination, followed by ENG, PAI-1, P-selectin, tPA, VEGF and PLAC1. The best multivariate model was obtained by the combination of all markers. A ROC curve yielded a sensitivity of 84% (95% CI, 71.8–91.5) at a 5% 1 – specificity rate with an area under the curve of 0.927 ($P < 0.001$).[20] It has been suggested previously that cellular RNA analysis is not as useful as plasma RNA analysis.[17] In our previous study of the hPL gene, the coefficient of variation of our cellular RNA analysis was approximately 20%; it was not calculated in the current study and this is a significant limitation of this study.[17] However, as cellular RNA can be preserved in the PAXgene blood RNA tube, we have confirmed that the RNA is stable below −20°C at least for 1 year. Moreover, the blood processing of cellular RNA is much easier than that of plasma RNA. In addition, the expression levels of hPL and hCG are approximately ten times higher than those in plasma RNA.[17] Therefore, we suggest that the analysis of plasma cellular RNA is a promising method for the evaluation of the pathophysiological alterations occurring in pregnant women who later develop PE. In both the previous cell-free RNA study and the present cellular RNA study, the levels of Flt-1 and ENG were two of the best predictors for PE. This indicates that these anti-angiogenic factors play a crucial role in the pathogenesis of PE.

The origin of the cellular RNA seen in maternal blood has not been resolved. In our previous study, hPL and hCG expression levels in the cellular components of maternal blood correlated with the corresponding protein levels.[17,24] This finding suggests that some trophoblasts or placental debris circulate in maternal blood. The half-times of hPL expression in cellular and plasma RNA were 203.8 and 32.2 minutes, respectively. Therefore, the half-time of cellular RNA is much longer than that of plasma RNA, and cellular RNA is not removed from the maternal circulation rapidly after delivery. The RNA originating from circulating trophoblasts in maternal blood could be detected for several months, as fetal nucleated erythrocytes reportedly circulate in maternal blood for 3 months after delivery.[29] These findings suggest that the levels of trophoblast-derived RNA in the cellular RNA in maternal blood could reflect the pathophysiological alterations of the placenta. However, real-time evaluation of placental function through cellular RNA may be inferior to the evaluation of cell-free RNA.

Another study assessed the mRNA expression of trophoblasts obtained from CVS at week 11. The expression levels of Flt-1, ENG and VEGF in CVS tissue obtained from pregnant women who later developed PE were higher, and those of PlGF and HO-1 were lower, than those of normal pregnancies.[16] These findings indicate that the up-regulation of anti-angiogenic factors and the down-regulation of anti-oxidant factors have already occurred in first-trimester trophoblasts,[16] and that the alterations could be evaluated by the analysis of cellular RNA. All of these findings support the hypothesis that some mRNA expression of Flt-1, ENG, PlGF, VEGF and HO-1 is derived from circulating trophoblasts, and that the alteration of these mRNA levels may reflect mRNA alterations associated with the pathogenesis of PE in the placenta. Therefore, it is suggested that the evaluation of cellular mRNA may allow for the indirect monitoring of placental function.

In this study, PE occurred in 9% of pregnant women, a higher frequency than seen in the published literature. No previous large studies have clarified the prevalence of PE in Indonesian populations, and this higher prevalence could confound the result. Furthermore, because the case number of gestational hypertension or early-onset-type PE was not sufficient for statistical analysis, women with hypertension in pregnancy were excluded and those with early-onset PE were not analysed separately. Further study in more developed countries is needed to confirm the predictive efficiency of cellular RNA in maternal blood.

In conclusion, the current study has demonstrated that Flt-1 and ENG expression increases in the cellular RNA in the blood from pregnant women who develop PE, whereas HO-1 and PlGF expression decreases. These alterations increase with the severity of the clinical symptoms of PE at later gestation. Furthermore, an analysis of the expression of these transcripts allows the accurate detection of high-risk pregnant women who are likely to develop PE in populations at low risk for the development of PE.

Disclosure of interest

None.

Contribution to authorship

AS, TO, YP and AF designed the research and approved the final, submitted version. AS, MN, HS, NW and YP collected, analysed and interpreted the data, and drafted the manuscript. AF and NR performed the statistical analysis.

Details of ethics approval

Approved by the ethics committee of Showa University, #86, and by the University of Indonesia, #92a/PT02.FK/2006.

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References

1 Redman CW, Sargent IL. Latest advances in understanding preeclampsia. *Science* 2005;308:1592–4.
2 Levine RJ, Lam C, Qian C, Yu KF, Maynard SE, Sachs BP, *et al.* Soluble endoglin and other circulating antiangiogenic factors in preeclampsia. *N Engl J Med* 2006;355:992–1005.
3 Levine RJ, Maynard SE, Qian C, Lim KH, England LJ, Yu KF, *et al.* Circulating angiogenic factors and the risk of preeclampsia. *N Engl J Med* 2004;350:672–83.
4 Maynard SE, Min JY, Merchan J, Lim KH, Li J, Mondal S, *et al.* Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. *J Clin Invest* 2003;111:649–58.
5 Venkatesha S, Toporsian M, Lam C, Hanai J, Mammoto T, Kim YM, *et al.* Soluble endoglin contributes to the pathogenesis of preeclampsia. *Nat Med* 2006;12: 642–9.
6 Ferrara N. Role of vascular endothelial growth factor in regulation of physiological angiogenesis. *Am J Physiol Cell Physiol* 2001;280: C1358–66.
7 He H, Venema VJ, Gu X, Venema RC, Marrero MB, Caldwell RB. Vascular endothelial growth factor signals endothelial cell production of nitric oxide and prostacyclin through flk-1/KDR activation of c-Src. *J Biol Chem* 1999;274:25130–5.
8 He Y, Smith SK, Day KA, Clark DE, Licence DR, Charnock-Jones DS. Alternative splicing of vascular endothelial growth factor (VEGF)-R1 (FLT-1) pre-mRNA is important for the regulation of VEGF activity. *Mol Endocrinol* 1999;13:537–45.
9 Toporsian M, Gros R, Kabir MG, Vera S, Govindaraju K, Eidelman DH, *et al.* A role for endoglin in coupling eNOS activity and regulating vascular tone revealed in hereditary hemorrhagic telangiectasia. *Circ Res* 2005;96:684–92.
10 Li H, Gu B, Zhang Y, Lewis DF, Wang Y. Hypoxia-induced increase in soluble Flt-1 production correlates with enhanced oxidative stress in trophoblast cells from the human placenta. *Placenta* 2005;26:210–7.
11 De Marco CS, Caniggia I. Mechanisms of oxygen sensing in human trophoblast cells. *Placenta* 2002;23(Suppl. A):S58–68.
12 Zenclussen AC, Sollwedel A, Bertoja AZ, Gerlof K, Zenclussen ML, Woiciechowsky C, *et al.* Heme oxygenase as a therapeutic target in immunological pregnancy complications. *Int Immunopharmacol* 2005;5:41–51.
13 Zenclussen ML, Anegon I, Bertoja AZ, Chauveau C, Vogt K, Gerlof K, *et al.* Over-expression of heme oxygenase-1 by adenoviral gene transfer improves pregnancy outcome in a murine model of abortion. *J Reprod Immunol* 2006;69:35–52.
14 Ahmed A, Dunk C, Ahmad S, Khaliq A. Regulation of placental vascular endothelial growth factor (VEGF) and placenta growth factor (PlGF) and soluble Flt-1 by oxygen – a review. *Placenta* 2000;21(Suppl. A):S16–24.
15 Cudmore M, Ahmad S, Al-Ani B, Fujisawa T, Coxall H, Chudasama K, *et al.* Negative regulation of soluble Flt-1 and soluble endoglin release by heme oxygenase-1. *Circulation* 2007;115:1789–97.
16 Farina A, Sekizawa A, DeSanctis P, Purwosunu Y, Okai T, Cha DH, *et al.* Gene expression in chorionic villous samples at 11 weeks' gestation from women destined to develop preeclampsia. *Prenat Diagn* 2008;28:956–61.
17 Okazaki S, Sekizawa A, Purwosunu Y, Iwasaki M, Farina A, Okai T. Measurement of mRNA of trophoblast-specific genes in cellular and plasma components of maternal blood. *J Med Genet* 2006;43:e47.
18 Poon LL, Leung TN, Lau TK, Lo YM. Presence of fetal RNA in maternal plasma. *Clin Chem* 2000;46:1832–4.
19 Purwosunu Y, Sekizawa A, Farina A, Wibowo N, Koide K, Okazaki S, *et al.* Evaluation of physiological alterations of the placenta through analysis of cell-free messenger ribonucleic acid concentrations of angiogenic factors. *Am J Obstet Gynecol* 2008;198:124 e1–7.
20 Purwosunu Y, Sekizawa A, Okazaki S, Farina A, Wibowo N, Nakamura M, *et al.* Prediction of preeclampsia by analysis of cell-free messenger RNA in maternal plasma. *Am J Obstet Gynecol* 2009;200:386 e1–7.
21 Purwosunu Y, Sekizawa A, Farina A, Wibowo N, Okazaki S, Nakamura M, *et al.* Cell-free mRNA concentrations of CRH, PLAC1, and selectin-P are increased in the plasma of pregnant women with preeclampsia. *Prenat Diagn* 2007;27:772–7.
22 Purwosunu Y, Sekizawa A, Koide K, Farina A, Wibowo N, Wiknjosastro GH, *et al.* Cell-free mRNA concentrations of plasminogen activator inhibitor-1 and tissue-type plasminogen activator are increased in the plasma of pregnant women with preeclampsia. *Clin Chem* 2007;53:399–404.
23 ACOG Practice Bulletin. Diagnosis and management of preeclampsia and eclampsia. Number 33, January 2002. American College of Obstetricians and Gynecologists. *Int J Gynaecol Obstet* 2002;77:67–75.
24 Okazaki S, Sekizawa A, Purwosunu Y, Farina A, Wibowo N, Okai T. Placenta-derived, cellular messenger RNA expression in the maternal blood of preeclamptic women. *Obstet Gynecol* 2007;110:1130–6.
25 Collett D, editor. *Modelling Binary Data*, 2nd edn. Boca Raton, FL: Chapman & Hall/CRC; 2003.
26 Conde-Agudelo A, Villar J, Lindheimer M. World Health Organization systematic review of screening tests for preeclampsia. *Obstet Gynecol* 2004;104:1367–91.
27 Espinoza J, Romero R, Nien JK, Gomez R, Kusanovic JP, Gontalves LF, *et al.* Identification of patients at risk for early onset and/or severe preeclampsia with the use of uterine artery Doppler velocimetry and placental growth factor. *Am J Obstet Gynecol* 2007;196:326 e1–13.
28 Farag K, Hassan I, Ledger WL. Prediction of preeclampsia: can it be achieved? *Obstet Gynecol Surv* 2004;59:464–82; quiz 485.
29 Sekizawa A, Purwosunu Y, Matsuoka R, Koide K, Okazaki S, Farina A, *et al.* Recent advances in non-invasive prenatal DNA diagnosis through analysis of maternal blood. *J Obstet Gynaecol Res* 2007;33:747–64.

OBSTETRICS

Performance of messenger RNAs circulating in maternal blood in the prediction of preeclampsia at 10-14 weeks

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OBJECTIVE: The purpose of this study was to determine whether the combined distribution of a panel of cellular messenger RNA markers can detect preeclampsia long before onset.

STUDY DESIGN: We compared blood at 10-14 weeks from 11 women who ultimately experienced preeclampsia with 88 matched control subjects. After multiples of the median conversion of all the markers, logistic regression was used to calculate the risk of the development of preeclampsia.

RESULTS: Higher multiples of the median values than expected were found for endoglin, fms-related tyrosine kinase 1, and transforming growth factor–β1. Lower multiples of the median values were found for placental growth factor and placental protein 13. Endoglin fms-related tyrosine kinase 1 and transforming growth factor–β1 had the best discriminant power. Messenger RNA species provided independent contributions to the prediction of preeclampsia. In fact, 11 women with preeclampsia scored a median risk of 50% of experiencing preeclampsia. Control subjects scored a median risk of preeclampsia of 0.18%. The detection rate at a 5% false positive rate was 72.3%.

CONCLUSION: The messenger RNA dosage in maternal blood would be a useful method for the calculation of the risk of the development of preeclampsia.

Key words: logistic regression, mRNA, maternal blood, preeclampsia, screening

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Hypertensive disorders in pregnancy, including preeclampsia and pregnancy-induced hypertension, are associated with significant morbidity and mortality rates for the mother and the neonate.[1] Preeclampsia is a multisystem disorder that is unique to pregnancy and causes significant morbidity and death worldwide. It affects 2-5% of pregnancies[2]; the clinical features are well recognized and characteristically manifest in the second to third trimester, whereas the underlying disease and major pathogenic changes already occur at earlier stages of pregnancy because of failure of the trophoblast cells to invade, with an increase in the apoptotic and necrotic index. Because several known risk factors are not highly predictive or modifiable, attempts to prevent preeclampsia are confined mostly to those patients who are at increased risk because of their medical history. Given the morbidity that is associated with preeclampsia, an enormous variety of biomolecules have been studied to detect those that show evidence of alteration in the maternal circulation during early pregnancy, before the manifestation of clinical symptoms.[3] The identification of reliable screening markers thus would permit major improvements in obstetric care through better targeting of antepartum surveillance. A great number of possible predictive markers have been tested, which include placental factors,[4] abnormal fibrolytic activity,[5] markers of endothelial dysfunction,[6] markers of oxidative stress,[7] and markers of abnormal trophoblast invasion.[8,9] An aberrant quantitative expression of some circulating placental-specific messenger RNAs (mRNAs) in the maternal blood that was found in preeclampsia cases compared with control subjects seems to be a promising tool for the early detection of the disease.[10] Even if there is no clear evidence that mRNAs offer some advantages over protein markers, molecular analysis has the advantage of enrolling a great number of markers at the same time and can also provide some useful information about the pathophysiologic condition of the disease. The disturbances in the expression of these molecules have led to the proposal that they may be used as early predictive markers of preeclampsia and/or intrauterine growth restriction before the onset of

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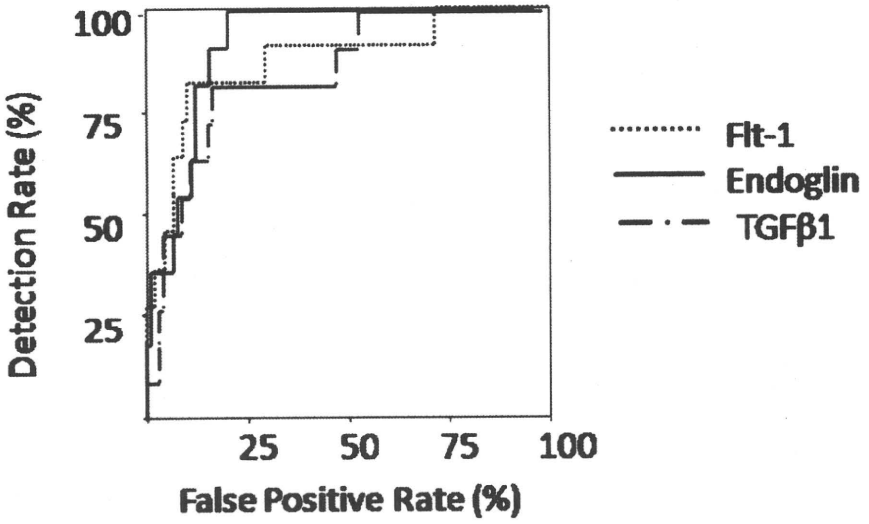
FIGURE 1
Receiver operating characteristic curve for the messenger RNA species that show discriminant power between cases of preeclampsia and control subjects



The X-axis shows 1-specificity or false positive rate; Y-axis shows the detection rate.

FLT1, fms-related tyrosine kinase 1; *TGFβ1*, transforming growth factor beta 1.

Farina. Prediction of preeclampsia by means of circulating placental mRNA at 10-14 weeks of gestation. Am J Obstet Gynecol 2010.

clinical symptoms. However, because it seems that no single test is highly sensitive or predictive, several markers might plausibly be used at the same time.

In this study, we dosed the concentration of a panel of cellular mRNAs that was comprised of fms-related tyrosine kinase 1 (FLT1), endoglin, placental growth factor (PlGF), transforming growth factor–β1 (TGFβ1), and placental protein 13 (PP13) that were circulating in maternal blood of women with preeclampsia who had been matched with appropriate control subjects, with a view to the calculation of a new posterior risk of preeclampsia for each subject in the data series.

Materials and Methods

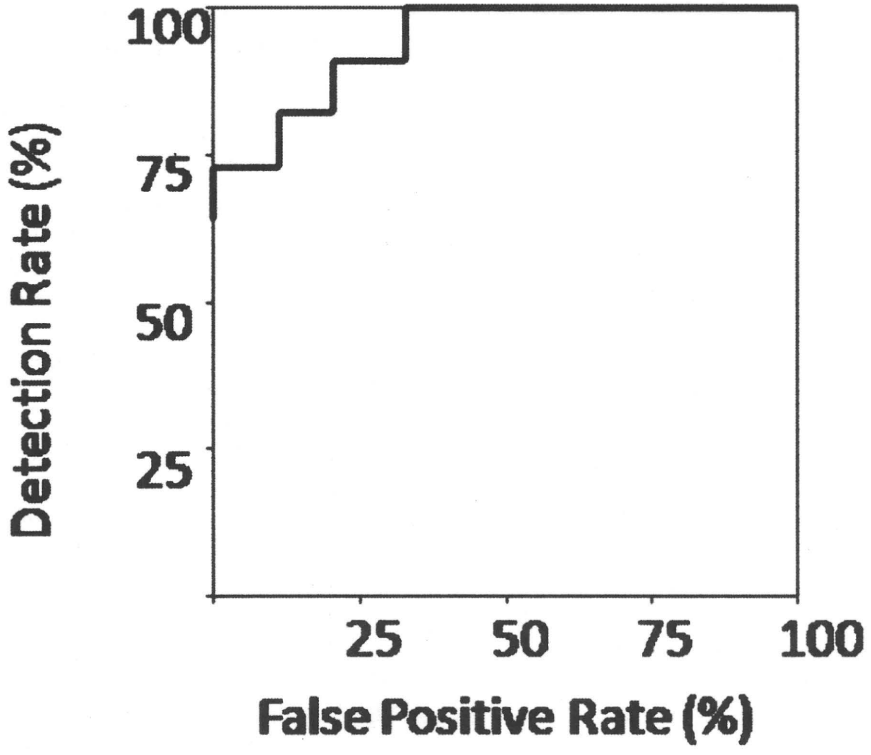
The study population consisted of 99 women who attended the Division of Prenatal Medicine, University of Bologna, Bologna, Italy. Gestational age was calculated by ultrasound measurements at 11-14 weeks' gestation. A second ultrasound examination was performed at 22-24 weeks' gestation for measurement of fetal growth and examination for fetal defects. Those cases in which no major fetal defects were detected were included in the study. Eleven women with preeclampsia but who were asymptomatic at the time of blood drawing were matched with 88 control subjects (1:8 match for fetal sex and gestational age expressed in weeks + days). All women were informed and agreed to participate in the study, which was approved by the local Hospital Ethics Committee.

Preeclampsia was defined as gestational hypertension (systolic pressure of >140 mm Hg or diastolic blood pressure of >90 mm Hg on at least 2 occasions after 20 weeks' gestation) with proteinuria (>0.3 g/d). *Severe preeclampsia* was defined by the presence of 1 or both of the following events: (1) severe gestational hypertension (systolic pressure of >160 mm Hg or diastolic pressure of >110 mm Hg on at least 2 occasions after 20 weeks' gestation) and (2) severe proteinuria (≥5g protein in a 24-hour urine specimen or ≥3 g on 2 random urine samples that were collected at least 4 hours apart). *Intrauterine growth restriction* was defined as the estimated fetal weight being 2.0 standard deviations below the mean expected weight for the gestational age, as determined by ultrasound evaluation.

Blood samples were taken at the time of first examination (11-14 weeks' gestation) from subjects who had been scheduled for chorionic villous sampling or amniocentesis. The blood samples (2.5 mL) were collected in PAXgene blood RNA tubes (PreAnalytic, Hombrechtikon, Switzerland), kept at room temperature for 3 hours, and then stored at –20°C until being transported to Japan. Molecular analysis was performed in the Department of Obstetrics and Gynecology at Showa University School of Medicine, Tokyo, Japan. RNA extraction and real-time polymerase chain reaction (PCR) were performed according to protocols described elsewhere.[11] In brief, cellular component samples were centrifuged twice at 4000 g for 10 minutes at room temperature to remove the entire supernatant and any mRNA that was present in the residual plasma. The pellet was then washed, resuspended, and incubated in optimized buffer solution that contained proteinase K to digest protein. A second round of centrifugation was performed to remove any residual cell debris, and the resulting supernatant was transferred to a fresh microcentrifuge tube. We added 100% ethanol to the supernatant to adjust the binding conditions; the resultant lysate was then applied to a PAXgene spin column (PreAnalytiX; PreAnalytic), which resulted in selective binding of RNA to the silica-gel membrane of the spin column. After the column was washed 3 times, pure RNA was eluted in 80 μL of RNase-free water.

Reverse transcription of the mRNA was performed using an Omniscript RT Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. 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's components in a reaction volume of 20 μL. QuantiTect PCR analyses for vascular endothelial growth factor, FLT1, endoglin, PlGF, and TGFβ1 were performed with predeveloped and com-

FIGURE 2
Receiver operating characteristic curve shows the combined discriminant power of Flt-1, endoglin, TGFβ1, and parity



The X-axis shows 1-specificity or false positive rate; Y-axis shows the detection rate.

FLT1, fms-related tyrosine kinase 1; *TGFβ1*, transforming growth factor beta 1.

Farina. Prediction of preeclampsia by means of circulating placental mRNA at 10-14 weeks of gestation. Am J Obstet Gynecol 2010.

mercially available primers and probe sets (catalog no. Hs00900054_m1 for vascular endothelial growth factor; catalog no. Hs01052936_m1 for FLT1; catalog no. Hs00923997_g1 for endoglin; catalog no. Hs00182176_m1 for PlGF; and catalog no. Hs0000171257_m1 for TGFβ1; Applied Biosystems, Foster City, CA). The following thermal cycling protocol was used for PCR: initial denaturation at 95°C for 15 minutes, 40 cycles of denaturation at 94°C for 15 seconds, and annealing at 60°C for 1 minute. 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. Quantification of gene expression was performed with investigators blinded to the outcome of pregnancy. Amounts of mRNA samples were expressed in term of copies per milliliter. To quantify mRNA concentrations, we prepared plasmid DNA for calibration curves as previously described.[11]

Data analysis was performed by nonparametric statistics because of the small sample size. A 1:8 match (1 case with 8 control subjects) for gestational age expressed in days, and fetal sex was determined. Such a match would guarantee a proper comparison, even in the presence of a relatively low number of cases. The median mRNA concentration of each available marker (FLT1, endoglin, PlGF, TGFβ, and PP13) as a function of increasing gestational age was measured initially and calculated by weighted log10–linear regression. All data were expressed as multiples of the median. We used a logistic regression to calculate the posterior risk of preeclampsia in both control subjects and affected cases, taking the panel of available mRNA values that were expressed in multiples of the median and parity as predictors of the disease. The detection and false-positive rates were calculated for each available marker with the use of a univariable receiver operating characteristic (ROC) curve. Multivariable analysis was performed with logistic regression to calculate the risk for each patient for classification as a control or preeclampsia case. The logistic output was adjusted for the incidence of preeclampsia in the general population (2%) by the calculation of the sampling fraction as described by Collett.[12] Again, quantitative data were transformed into an ordinal scale (in 4 categories <25th percentile, 25-50th percentile, 50-75th percentile, and >75th percentile).

To obtain a more robust risk estimation, a multivariable ROC curve for the calculation of multivariable detection rate was built with the use of the calculated risk for preeclampsia by logistic regression analysis as the test variable, for each patient in the series.

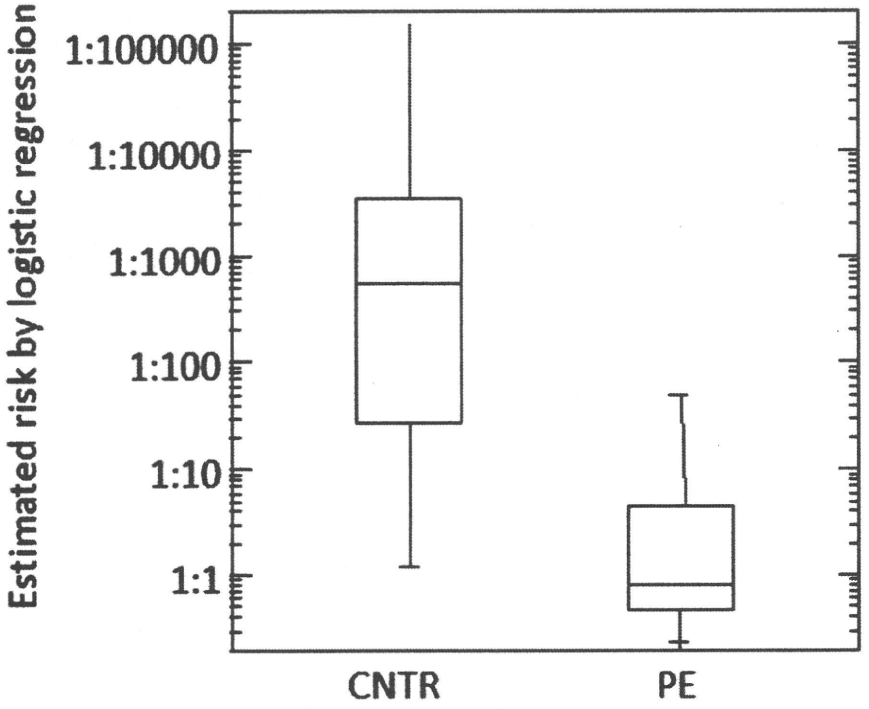
RESULTS

The findings of this study demonstrate that at 11-14 weeks of gestation, women who subsequently experience preeclampsia have an increased maternal concentration of mRNA for FLT1, endoglin, and TGFβ1 and reduced levels of mRNA for PP13 and PlGF. These findings are compatible with previous studies that reported reduced levels of PP13 and PlGF and increased FLT1, endoglin, and TGFβ1 in early second-trimester pregnancy in women destined to experience preeclampsia.[10,13,14]

Tables 1 and 2 show the demographic characteristics of the patients and the log multiples of the median of the mRNA species that were considered in the study, stratified according to the 2 subgroups that were generated. The median gestational age at the time of blood test was 12 + 3 weeks (range, 11–14 weeks) and 12 + 3 weeks (range, 11–14 weeks) for control subjects and cases, respectively.

ROC curve analysis showed that FLT1 has the highest detection rate at a 5%

FIGURE 3
Box and whiskers plot shows the risk distribution for preeclampsia in cases and controls



CNTR, controls; *PE*, preeclampsia.

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false-positive rate, followed by endoglin and TGFβ1 (Figure 1). Table 3 shows the output of the ROC curves for those mRNA species, with a significant detection rate for preeclampsia. As shown, only FLT1, endoglin, and TGFβ1 were included in the analysis.

After adjustment for the incidence of the disease (2%), we found that the median risk of preeclampsia was reduced by 10 times in control subjects and 20 times higher in our cases. Figure 2 expresses the risk (in a box and whiskers plot) for both cases and control subjects. The detection rate of the combined marker panel at 5% false positive was 72.3%, as reported in Figure 3. Table 4 shows the actual risk of preeclampsia for each of the preeclamptic cases found in our series along with the available clinical information.

TABLE 1
Demographic and clinical characteristics of pregnant women who provided CVS

Variable	Controls, n = 88	Preeclampsia, n = 11	*P* value[a]
Gestational age at the time of blood test, d	84 (72-96)	84 (71-96)	ns
Maternal age, y	36 (35–38)	36 (35–38)	ns
Percentage of primiparae	52.3	81.8	ns
Week at delivery	39 (36–41)	36 (34–41)	ns
Neonatal weight, g	3150 (2560–4180)	2930 (2000–3510)	ns

Data are expressed as a median (min-max) or percentage.
CVS, chorionic villous samples; *ns*, not significant.

[a] Mann-Whitney U-test or Fisher's exact test.

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Comment

The onset of preeclampsia is associated with maternal and neonatal morbidity and death. Medical treatment is often started too late when there is the presence of hypertension and proteinuria, which are probably end-stage manifestations of this multisystem disorder. Currently, Doppler ultrasound analysis of the uteroplacental circulation is the most widely used test to detect poor placental perfusion and represents a routine antenatal screening tool to detect women who are at a higher risk of preeclampsia. Many studies are available in the literature; unfortunately, they are difficult to compare because of differences in the Doppler sampling techniques, definitions of abnormal flow velocity waveform, populations, gestational age at examination, and the criteria for a diagnosis of preeclampsia. A review of 43 studies that included >42,000 women and evaluated uterine artery Doppler images reports that the positive predictive value of an abnormal Doppler image is low in both high- and low-risk populations.[9,15,16] In low-risk women, for example, the risk seems to increase from 2.5% to 8-15%, although a negative test reduces the probability of disease to 1.5-2%. In high-risk women, a rise from 14% to 29-32% would be quoted for those women who are screened as positive, and a reduction as low as 6-9% is expected for those women who are screened negative. Thus, Doppler imaging seems more effective in the assignment of a higher risk ratio (compared with baseline) in low-risk patients than in high-risk patients. Again, the predictive ability of Doppler imaging differs significantly according to gestation at delivery. In addition to first trimester Doppler images of the uterine arteries with maternal factor and/or biochemical markers for calculating a posterior risk of preeclampsia,

TABLE 2
Log MoM values in cases and controls

mRNA	PE cases, n = 11	Controls, n = 88	*P* value[a]
FLT1	0.34 (−0.16 to 0.74)	0 (−0.92 to 0.45)	< .001
Endoglin	0.32 (0.25 to 0.51)	0 (−1.05 to 0.84)	< .001
PlGF	−0.35 (−0.57 to 0.31)	0 (−1.39 to 0.97)	.772
TGFβ1	0.28 (−0.04 to 0.61)	0 (−1.19 to 0.49)	< .001
PP13	−0.15 (−1.99 to 0.29)	0 (−1.95 to 0.42)	< .001

Data are expressed in median and SD.
FLT1, fms-related tyrosine kinase 1; *MoM*, multiple of median; *mRNA*, messenger RNA; *PE*, preeclampsia; *PlGF*, placental growth factor; *PP13*, placental protein 3; *TGFβ1*, transforming growth factor β1.

[a] Mann-Whitney U test

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TABLE 3
Univariable and multivariable ROC curves analysis

Variable	Area	SE	*P* value	95% CI	DR% at 5% FPR
FLT1	0.872	0.064	< .001	0.747–0.997	45.5
ENG	0.884	0.034	< .001	0.817–0.952	36.4
TGFβ1	0.835	0.058	< .001	0.720–0.949	27.3
All + Parity	0.946	0.024	< .001	0.899–0.994	72.3

CI, confidence interval; *DR*, detection rate; *ENG*, endoglin; *FLT1*, fms-related tyrosine kinase 1; *FPR*, false positive rate; *MoM*, multiple of median; *ROC*, receiver operating characteristic; *TGFβ1*, transforming growth factor β1.

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more recent papers have used a statistical algorithm that is quite similar to that used in the present study.[17-20]

FLT1 and endoglin recently have been reported as playing important roles in the pathophysiologic condition of preeclampsia.[21] Protein concentrations of FLT1 and endoglin in plasma are increased before the onset of preeclampsia and correlate with disease severity.[21-23] These reports have indicated that FLT1 and endoglin from the placenta induce severe maternal endothelial dysfunction, which 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. Although the precise relationship of endoglin to FLT1 is unknown, it appears that both endoglin and FLT1 contribute to the pathogenesis of the maternal syndrome through separate mechanisms. Several lines of evidence support this hypothesis.[21-23] Endoglin level is elevated in the sera of preeclamptic women 2-3 months before the onset of clinical signs of preeclampsia, correlates with disease severity, and falls off after delivery. Endoglin is a coreceptor for TGFβ1 and is highly expressed on cell membranes of vascular endothelium and syncytiotrophoblasts.[23] Endoglin inhibits TGFβ1 signaling in endothelial cells and blocks TGFβ1-mediated activation of endothe-

TABLE 4
Estimated risk for preeclampsia cases asymptomatic at the time of blood test

Risk	Case ID	Gestational age at the time of PE onset	IUGR	Type of PE	Parity	MoM FLT1	MoM Endoglin	MoM TGFβ1
1:2	IB	25+3	Yes	Severe	0000	2.61	2.58	4.12
1:2	CV	30+2	Yes	Severe	0000	1.97	2.09	2.21
1:2	AB	31+5	No	Mild	0000	3.43	1.95	2.18
1:2	FD	30+3	No	Severe	1001	5.47	2.17	1.91
1:2	ER	37+5	No	Mild	0010	4.29	3.23	2.62
1:2	EB	36+0	No	Mild	2002	2.08	2.04	1.66
1:3	JC	35+2	Yes	Severe	0010	2.48	3.22	2.13
1:3	GD	37+3	Yes	Severe	0000	2.18	2.02	1.61
1:7	JP	35+2	No	Mild	0010	2.19	2.43	0.91
1:21	SE	32+6	No	Mild	0020	0.69	1.91	1.82
1:51	CB	33+5	No	Mild	0000	1.36	1.78	1.04

FLT1, fms-related tyrosine kinase 1; *IUGR*, intrauterine growth restriction; *TGFβ1*, transforming growth factor β1.

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lial nitric oxide synthase and vasodilation, which suggests that dysregulated TGFβ1 signaling may be involved in the pathogenesis of preeclampsia. TGFβ1 has also been associated with the prediction of preeclampsia, but with discordant results, being reported both higher or lower in preeclampsia cases.[24,25]

In previous studies on chorionic villous sampling, we proved for the first time that the pathophysiologic alterations that are involved in preeclampsia that have been identified as mRNA species aberrations start from the trophoblasts long before the clinical onset.[14]

Only 2 previous studies have reported so far the detection rate of mRNA for FLT1 and endoglin at 15-20 weeks' gestation.[13,26] In maternal blood, the detection rate with a 5% false-positive rate was a bit higher than in cellular components (PAX method) for FLT1 (58% vs 43%) but were quite similar for endoglin (43% vs 47%). Even if there is no evidence to demonstrate which source of samples is better for the screening of preeclampsia, we have chosen mononuclear fraction because, based on a previous result, it seems a bit better than whole blood in the detection of preeclamptic patients. The plasma method, instead, so far has been reported more extensively for the diagnosis and screening of fetal genetic diseases because it is more specific in the detection of fetal- and or placental-specific mRNAs sequences. Again, the plasma method needs a more difficult and sophisticated approach, which is not properly available in our laboratories.

Several other mRNA species (eg, plasminogen activator inhibitor-1 selectin P tissue plasminogen activator vascular endothelial growth factor placenta-specific protein 1 heme oxygenase 1 PlGF) have been investigated at 15-20 weeks' gestation, but the detection rate of all of them was <30%. All of them, however, contributed to discriminating preeclampsia cases from control subjects.

In this study, we moved the screening from 15-20 to 11-14 weeks' gestation by using FLT1, endoglin, TGFβ1, and parity. This is a novel result that allows a direct and clear comparison with the risk estimation at the first trimester that has been described by other groups by means of Doppler imaging, biochemical markers, and maternal history.[17-20] The combination of factors that we described yielded a good detection rate and a risk estimation that is realistic. In fact, the risk modification (10-fold reduction and 20-fold increase in preeclampsia risk in control subjects and in cases, respectively) is to be expected in a screening protocol. Again, the enrollment of a population with intrauterine growth restriction, without preeclampsia and/or gestational hypertension, would better define the detection rate and the false-positive rate of the mRNA markers and would also make the risk estimation even more realistic.

In conclusion, we have demonstrated that a panel of mRNA levels in the cellular components of blood from first-trimester pregnant women can be used to predict preeclampsia. Among the genes that were analyzed, antiangiogenic genes (such as FLT1 and endoglin) were the best markers to predict the subsequent onset of preeclampsia. These findings indicate that these factors play a crucial role in the pathogenesis of preeclampsia and that the alteration has started and can be assessed by the first trimester. ■

REFERENCES

1. Walker JJ. Pre-eclampsia. Lancet 2000;356:1260-5.

2. De Groot CJ, Bloemenkamp KW, Duvekot EJ, et al. Preeclampsia and genetic risk factors for thrombosis: a case-control study. Am J Obstet Gynecol 1999;181:975-80.

3. Myatt L, Miodovnik M. Prediction of preeclampsia. Semin Perinatol 1999;23:45-57.

4. Lambert-Messerlian GM, Silver HM, Petraglia F, et al. Second-trimester levels of maternal serum human chorionic gonadotropin and inhibin a as predictors of preeclampsia in the third trimester of pregnancy. J Soc Gynecol Investig 2000;7:170-4.

5. Halligan A, Bonnar J, Sheppard B, Darling M, Walshe J. Haemostatic, fibrinolytic and endothelial variables in normal pregnancies and pre-eclampsia. BJOG 1994;101:488-92.

6. Ballegeer V, Spitz B, Kieckens L, Moreau H, Van Assche A, Collen D. Predictive value of increased plasma levels of fibronectin in gestational hypertension. Am J Obstet Gynecol 1989;161:432-6.

7. Cotter AM, Molloy AM, Scott JM, Daly SF. Elevated plasma homocysteine in early pregnancy: a risk factor for the development of severe preeclampsia. Am J Obstet Gynecol 2001;185:781-5.

8. Clausen T, Djurovic S, Brosstad FR, Berg K, Henriksen T. Altered circulating levels of adhesion molecules at 18 weeks' gestation among women with eventual preeclampsia: indicators of disturbed placentation in absence of evidence of endothelial dysfunction? Am J Obstet Gynecol 2000;182:321-5.

9. Chien PF, Arnott N, Gordon A, Owen P, Khan KS. How useful is uterine artery Doppler flow velocimetry in the prediction of pre-eclampsia, intrauterine growth retardation and perinatal death? An overview. BJOG 2000;107:196-208.

10. Purwosunu Y, Sekizawa A, Okazaki S, et al. Prediction of preeclampsia by analysis of cell-free messenger RNA in maternal plasma. Am J Obstet Gynecol 2009;200:386.e1-7.

11. Okazaki S, Sekizawa A, Purwosunu Y, Iwasaki M, Farina A, Okai T. Measurement of mRNA of trophoblast-specific genes in cellular and plasma components of maternal blood. J Med Genet 2006;43:e47

12. Collett D. Modelling binary data. New York, NY: Chapman & Hall; 1999.

13. Shimizu H, Sekizawa A, Purwosunu Y, et al. PP13 mRNA expression in the cellular component of maternal blood as a marker for pre-eclampsia. Prenat Diagn 2009;29:1231-6.

14. Farina A, Sekizawa A, De Sanctis P, et al. Gene expression in chorionic villous samples at 11 weeks' gestation from women destined to develop preeclampsia. Prenat Diagn 2008;28:956-61.

15. Papageorghiou AT, Yu CK, Nicolaides KH. The role of uterine artery Doppler in predicting adverse pregnancy outcome. Best Pract Res Clin Obstet Gynaecol 2004;18:383-96.

16. Conde-Agudelo A, Villar J, Lindheimer M. World Health Organization systematic review of screening tests for preeclampsia. Obstet Gynecol 2004;104:1367-91.

17. Akolekar R, Etchegaray A, Zhou Y, Maiz N, Nicolaides KH. Maternal serum activin at 11-13 weeks of gestation in hypertensive disorders of pregnancy. Fetal Diagn Ther 2009;25:320-7.

18. Poon LC, Staboulidou I, Maiz N, Plasencia W, Nicolaides KH. Hypertensive disorders in pregnancy: screening by uterine artery Doppler at 11-13 weeks. Ultrasound Obstet Gynecol 2009;34:142-8.

19. Poon LC, Kametas NA, Maiz N, Akolekar R, Nicolaides KH. First-trimester prediction of hypertensive disorders in pregnancy. Hypertension 2009;53:812-8.

20. Onwudiwe N, Yu CK, Poon LC, Spiliopoulos I, Nicolaides KH. Prediction of pre-eclampsia by a combination of maternal history, uterine artery Doppler and mean arterial pressure. Ultrasound Obstet Gynecol 2008;32:877-83.

21. Levine RJ, Lam C, Qian C, et al. Soluble endoglin and other circulating antiangiogenic factors in preeclampsia. N Engl J Med 2006;355:992-1005.

22. Levine RJ, Maynard SE, Qian C, et al. Circulating angiogenic factors and the risk of preeclampsia. N Engl J Med 2004;350:672-83.

23. Venkatesha S, Toporsian M, Lam C, et al. Soluble endoglin contributes to the patho-

genesis of preeclampsia. Nat Med 2006;12: 642-9.

24. Enquobahrie DA, Williams MA, Qiu C, Woelk GB, Mahomed K. Maternal plasma transforming growth factor-beta1 concentrations in preeclamptic and normotensive pregnant Zimbabwean women. J Matern Fetal Neonatal Med 2005;17:343-8.

25. Lim JH, Kim SY, Park SY, Yang JH, Kim MY, Ryu HM. Effective prediction of preeclampsia by a combined ratio of angiogenesis-related factors. Obstet Gynecol 2008;111:1403-9.

26. Sekizawa A, Purwosunu Y, Farina A, et al. Prediction of preeclampsia by an analysis of placenta-derived cellular mRNA in the blood of pregnant women at 15-20 weeks of gestation. BJOG 2010;117:557-64.

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Gene expression in chorionic villous samples at 11 weeks of gestation in women who develop pre-eclampsia later in pregnancy: implications for screening

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Objectives To determine the gene expression profile in chorionic villous samples (CVSs) of women destined to develop pre-eclampsia (PE).

Method Case-control study encompassing five women destined to develop PE [cases matched for gestational age with 30 controls]. We quantified mRNA expression on tissue samples from CVS of normal and PE patients. We then assessed mRNA expressions of cathepsin (CTSD), angiopoietin 2 (ANGPT2), interleukin 8, chemokine (C-X-C motif) ligand 10, neurokinin B (NKB), matrix metallopeptidase 9, major histocompatibility complex, class I, C (HLA-C)and human leukocyte antigen-G (HLA-G). Data were analyzed by nonparametric rank analysis.

Results For all the mRNA species considered in this study, except CTSD and ANGPT2, all the mean observed ranks in the PE group were significantly altered compared with the rank expectation among controls. mRNA for NKB and HLA-C were the markers with the highest degree of aberration in PE, compared with those in controls.

Conclusion Our study has directly showed that gene expressions relating to trophoblastic cell invasion or utero-placental hemodynamic adaptation are altered in the first trimester trophoblasts that go on to develop PE later. These results posit the use of residual CVS as a possible screening method for PE. Copyright © 2011 John Wiley & Sons, Ltd.

KEY WORDS: mRNA; screening for pre-eclampsia; real-time PCR; chorionic villous samples

INTRODUCTION

Pre-eclampsia (PE) is a serious complication of pregnancy affecting the mortality and the morbidity of both mothers and infants, with a prevalence of approximately 2–7% (De Groot *et al.*, 1999). Although the primary mechanism of PE is still unknown, it is characterized by impaired placental function, abnormal trophoblast invasion, deficient physiologic maternal spiral artery modification, increased apoptosis of trophoblastic cells and placental ischemia. Failure of normal invasion by trophoblast cells leads to inappropriate development of the maternal spiral artery (Walker *et al.*, 2000) which can interfere with normal villous development and reduce placental perfusion. PE is unique to human pregnancy and its clinical features are well recognized, characteristically manifesting in the second to third trimester. However, the underlying pathologic changes, related to early abnormal trophoblast invasion into the maternal vascular tissue associated with increased apoptotic and necrotic indices, occur much earlier. The earlier the gestational age (GA) at diagnosis, the higher the risk of maternal death (MacKay *et al.*, 2001). In fact, the increased risk of maternal death is fourfold if PE is diagnosed before gestational week 32 as compared with the risk after that GA. Given the morbidity associated with PE and the long preclinical phase before it manifests clinically, several studies have attempted to identify early-pregnancy proteins that might be predictive of PE and intrauterine growth restriction, offering the possibility of reducing maternal and fetal risks by administration of prophylactic low-dose aspirin started early during the gestation in high-risk pregnancies. PE can be classified as early- and late-onset PE, defined by the development of symptoms before or after 32 weeks of pregnancy, respectively. The early-onset form is more severe, frequently leading to the delivery of growth-retarded premature babies or poor outcome for the mother.

This study belongs to a series of already published studies tending to demonstrate that direct alterations in gene expression among trophoblasts will later develop into PE. We prospectively collected tissue samples of villous trophoblasts at the time of fetal karyotype analysis through chorionic villous sampling (CVS). mRNA expressions of factors that are reported to play

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roles in the development of PE were assessed and compared with the clinical outcomes.

METHODS

Thirty-five pregnant women in care at the Division of Prenatal Medicine at the University of Bologna, Bologna, Italy were enrolled in the present prospective case-control study. All the women were older than 35 years at delivery and bearing a single male fetus. Pregnancies with major fetal defects (like congenital heart diseases and aneuploidies) were excluded. CVS was performed for assessment of fetal karyotype. Residual CVS from five women with a diagnosis of PE (performed at the third trimester of pregnancy) was matched with 30 controls (1 : 6 ratio) for GA and fetal gender. GA was calculated by ultrasound measurements at 11 weeks of gestation. All women gave informed consent to participate in the study, which was approved by the local Institutional Review Board.

PE was defined as gestational hypertension (systolic pressure >140 mmHg or diastolic blood pressure >90 mmHg on ≥2 occasions after gestational week 20, with proteinuria (>0.3 g/day). Severe PE was defined by the presence of ≥1 of the following: (1) severe gestational hypertension (systolic pressure >160 mmHg or diastolic blood pressure >110 mmHg on ≥2 occasions after gestational week 20 or (2) severe proteinuria (≥5 g protein in a 24-h urine specimen). (ACOG practice bulletin, 2002)

Tissues and RNA preparation

Villous samples were centrifuged at 1500 rpm for 5 min at 4 °C, resuspended in 1 mL of phosphate buffered solution (PBS) and then centrifuged at 1500 rpm for 5 min at 4 °C. Each villous sample was mixed with 0.8 mL of Trizol Reagent (Invitrogen, Carlsbad, CA, USA) and lysated by repetitive pipetting. For RNA extraction, 0.16 mL of chloroform was added to the sample; after vigorous shaking, the sample was incubated at 4 °C for 15 min and centrifuged at 12 000 rpm for 15 min at 4 °C; following centrifugation, the aqueous phase was transferred to a fresh microtube. Precipitation of total RNA from the aqueous phase was obtained by mixing with 0.4 mL of isopropyl alcohol, incubation at 4 °C for 15 min and centrifugation at 12 000 rpm for 15 min at 4 °C. The RNA pellet was washed once in 0.8 mL of 75% ethanol and briefly air-dried. Finally, the total RNA was dissolved in 20 μL of RNase-free water and stored at −80 °C.

Real-time quantitative reverse transcription-PCR

RNA samples were transferred to Japan at under −20 °C. Molecular analysis was performed in the Department of Obstetrics and Gynecology at Showa University School of Medicine, Tokyo. RNA was reverse-transcripted by an Omniscript RT kit (Qiagen, Hilden, Germany). cDNA products were amplified by real-time quantitative PCR according to the manufacturer's instructions (Quanti-Tect Probe PCR kit; Qiagen) using a 2-μL aliquot of cDNA and the kit components in a reaction volume of 20 μL. TaqMan PCR analyses for cathepsin D (CTSD), angiopoietin 2 (ANGPT2), interleukin 8 (IL8), chemokine (C-X-C motif) ligand 10 (IP-10), neurokinin B (NKB), matrix metallopeptidase 9 (MMP9), major histocompatibility complex, class I, C (HLA-C) and human leukocyte antigen G (HLA-G) mRNAs; (Applied Biosystems, Foster City, CA, USA). 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 thermal profile used was as follows: 15 min of denaturation at 95 °C, followed by 15 s of annealing at 94 °C and 1 min of extension at 60 °C. Quantification of gene expression was performed by investigators blinded to the outcome of pregnancy. Amounts of mRNA samples were expressed in terms of copies per millilitre. To quantify these mRNA concentrations, we prepared plasmid DNA for calibration curves as previously described (Purwosunu *et al.*, 2007). As initial volumes of tissues could not be measured, the level of each gene expression was expressed as a ratio to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression.

Statistical analysis

Data were matched for GA in a 1 : 6 case-control study design. Median mRNA concentrations of each available marker CTSD, ANGPT2, IL8, IP-10, NKB, MMP9, HLA-C and HLA-G in cases and controls were calculated. Rank analysis and nonparametric analysis (Mann–Whitney U test or Fisher exact test) were used to detect differences between cases and controls. Differences were considered significant for a p-value <0.05.

RESULTS

There were two cases of severe PE that were treated according to the guideline for 'expectant management' (Sibai *et al.*, 2007). Tables 1 and 2 report the clinical information available for the data set. The ranks of the mean mRNA levels from each PE sample were found to be significantly higher or lower than the expected rank for six controls. Table 3 reports the whole data set for PE cases and the medians [intraquartile range (IQR)] for controls and the mean observed ranks for PE cases and 30 controls generated by a 1 : 6 match showing a statistically different distribution between cases and controls. CTSD, ANGPT2, MMP9, HLA-C and HLA-G showed lower values than controls. IP-10, IL8 and NKB by contrast showed higher values. NKB and HLA-C were the markers with the highest difference, followed by MMP9.

Table 1—Demographic and clinical characteristics of patients. Data are expressed as medians (minimum–maximum) or percentages

Variable	PE $n = 5$	Controls $n = 30$	p Value[a]
Gestational age (days) at time of CVS	82 (81–84)	82 (81–84)	ns
Maternal age	36 (35–38)	36 (35–38)	ns
Percentage of primiparae	36	38	ns
Week at delivery	38 (35–41)	39 (36–41)	ns
Neonatal weight (g)	2850 (2000–3510)	3100 (2560–4180)	ns

[a] Mann–Whitney U test or Fisher exact test.
CVS, chorionic villous samples; PE, pre-eclampsia.

Table 2—Clinical data available for pre-eclampsia (PE) cases

Case ID	Week of PE insurgence	PE degree	Second-trimester Doppler of uterine arteries (mRI[a])	Type of delivery
SB	29	Severe	0.68, no incisura	Cesarean section
IR	31	Severe	0.50, monolateral incisura	Cesarean section
DM	33	Mild	0.38, no incisura	Cesarean section
AF	32	Mild	0.44, no incisura	Vaginal
MC	36	Mild	0.46, no incisura	Vaginal

[a] mRI = medium resistance index.

Table 3—Median value (×1000) for mRNA species in pre-eclampsia (PE) cases and controls (numbers in parentheses are the nonmedian raw values and the intraquartile range, respectively)

mRNA species	PE $n = 5$	Controls $n = 30$	Mean rank in PE	Mean rank in controls	p Value*	Expression in PE cases versus controls
CTSD	142 (104,136,1 871 947)	275 (149–483)	2.00	4.00	0.170	↓
ANGPT2	1621 (1134,1514,1945,2395)	4480 (1188–6943)	3.00	4.00	0.369	↓
IL8	2389 (2058,2123,2496,2566)	21322013–2259)	7.00	3.50	0.016	↑
IP-10	632 (309,418,731,989)	307 (84–420)	6.00	3.50	0.016	↑
NKB	6618 (2742,4369,8245,14 816)	2431 (1103–5426)	7.00	3.50	<0.001	↑
MMP9	1566 (1430,1451,2006,7742)	8720 (5810–10474)	1.00	4.50	0.007	↓
HLA-C	43 (11,19,44,60)	120 (70–257)	1.00	4.50	<0.001	↓
HLA-G	1634 (1173,1308,1698,1198)	2476 (1677–3047)	2.00	4.50	0.016	↓

Unit is copies per mL. The gene expression is normalized for GAPDH.
CTSD, cathepsin; ANGPT2, angiopoietin; IL8, interleukin; IP-10, ligand 10; NKB, neurokinin; MMP9, matrix metallopeptidase 9; HLA-C, major histocompatibility complex, class I, C; HLA-G, major histocompatibility complex, class I, G.
* Rank sum test.

NKB and HLA-C proved to be the markers with the lowest level of aberration in PE. CTSD and ANGPT2 did not reach the level of statistical significance.

DISCUSSION

This study, as an extension of the earlier study, prospectively examined CVSs from women at gestational week 11. We assessed eight kinds of mRNA expressions and compared them with clinical outcomes.

All of them had been previously evaluated in molecular, biochemical and immunohistochemistry studies and were associated with PE. They belong to different categories including trophoblast invasion, villous angiogenesis, remodelling of extracellular matrix, maternal immunity and hemodynamic adaptation. As is widely known, all of these are biological functions associated with PE development.

Matrix metalloproteinases (MMPs) play a crucial role in restructuring the extracellular matrix by activating the secretion of gelatinases, collegenases and proteolytic enzymes. The balance between MMPs and tissue inhibitors of matrix metalloproteinase (TIMPs); is likely to play an important role in remodeling uterine arteries in pregnancy and maintaining vasodilatation in later pregnancy (Kelly *et al.*, 2003). The maternal plasma concentration of MMP9 is about 15 times higher than in nonpregnant women. MMP9 has been associated with a prediction of PE but with discordant results. For example, trophoblasts and placentas from pre-eclamptic pregnancies were recently found to express lower levels of MMP9 (Kolben *et al.*, 1996; Campbell *et al.*, 2004; Montagnana *et al.*, 2009), but some investigators have found higher expression levels of MMP9 in placental sections from PE tissue (Wang *et al.*, 2009). Again, Poon *et al.* (2009), in disagreement with our results, demonstrate that the maternal serum concentration of MMP9 at 11 ± to 13+ weeks of gestation is increased in pregnancies that subsequently develop PE, suggesting an association with an underlying inflammatory process.

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Natural Killer (NK) cells express an array of receptors, some of which are known to bind HLA-C molecules expressed by extravillous trophoblastic cells. Several recent studies have suggested that NK cell and trophoblast interaction are required to attract trophoblasts to the decidua and to promote placental vascular remodeling and differentiation (Hanna *et al.*, 2006; Le Bouteiller and Tabiasco, 2006). In PE, it has been found that some killer cell immunoglobulin-like receptor/HLA-C combinations appear unfavorable to trophoblastic cell invasion (Hiby *et al.*, 2004), suggesting a role for this gene in the pathogenesis of PE. Thus, in normal pregnancies NK cell activation through interaction with HLA-C in extravillous trophoblasts may promote placental development and maternal decidual spiral artery modifications. Insufficient NK cell activation would halt this process prematurely, resulting in poor decidual artery remodeling and increasing the risk of PE (Hiby *et al.*, 2004; Parham *et al.*, 2004). It is therefore also possible that a lower HLA-C expression, as shown by our results, could reduce and make less efficient such an interaction.

HLA-G is a nonclassic class I HLA molecule that is expressed in extravillous trophoblast cells. The molecular properties of HLA-G have recently been reviewed (Apps *et al.*, 2008). A number of studies have reported significantly lower concentrations of soluble HLA-G in maternal blood in cases of PE than in control pregnancies when measured at the end of the third trimester, (Yie *et al.*, 2004; Hackmon *et al.*, 2007), in the second trimester and in the first trimester (Yie *et al.*, 2005). Decreased HLA-G RNA (Hara *et al.*, 1996; Lim *et al.*, 1997; O'Brien *et al.*, 2000) and protein (Hara *et al.*, 1996; Lim *et al.*, 1997; Goldman-Wohl *et al.*, 2000a,b) as found in placental tissue of patients affected by PE suggests that lower levels of HLA-G do not protect the invading trophoblast from decidual NK cells. Thus, trophoblast invasion is defective, leaving a high degree of resistance in the uterine spiral arteries. Goldman-Wohl *et al.* (2000b) demonstrated HLA-G expression in anchoring extravillous trophoblasts with an increasing gradient of expression in more invasive cells. The authors suggested that HLA-G expression correlates with increased invasiveness and that HLA-G may be a necessary precondition for invasion. This indicates that, in PE, clusters of trophoblasts that do not express HLA-G may be unable to invade into maternal spiral arteries. Our results are in accord with these previous observations.

IP-10 is a chemokine of the CXC family (Neville *et al.*, 1997). Its principal biological activity is regulation and control of the basal homeostatic and inflammatory leukocyte movement (Rosenkilde *et al.*, 2004). It has potent angiogenic properties (Belperio *et al.*, 2000; Bernardini *et al.*, 2003; Rosenkilde *et al.*, 2004; Strieter *et al.*, 2005) and promotes adhesion, migration and invasion of trophoblast cells. PE is associated with a higher maternal serum concentration of IP-10 than normal pregnancy (Gotsch *et al.*, 2007). This result is consistent with the view that PE may reflect an anti-angiogenic state as well as an enhanced systemic inflammatory response.

IL8, a member of the CXC family of chemokines known as CXCL-8, is one of the main chemo-attractants for neutrophils and can activate the neutrophils (Goldsby *et al.*, 2003). It is also involved in angiogenesis (Baggiolini *et al.*, 1989). IL-8 and IP-10, produced by decidual NK cells, have been shown to attract trophoblasts expressing specific chemokine receptors. A block in trophoblast migration has been demonstrated by the addition of anti-IL-8 and anti-IP-10 monoclonal antibodies in culture (Hanna *et al.*, 2006). Scott Kauma *et al.* (2002) found that circulating plasma levels of IL8 were elevated in pre-eclamptic than in normal pregnant women (Sharma *et al.*, 2007). Although activated vascular endothelial cells are probably responsible, in part, for increased circulating IL-8 levels in pre-eclamptic women, other potential sources for these chemokines during pregnancy are the placenta, maternal decidua and circulating leukocytes (Denison *et al.*, 1997). Hence, higher mRNA expression in CVS, as shown in our results, is consistent with previous studies.

NKB belongs to a family of neuropeptides called the tachykinins. Traditionally, these peptides have been classified as neurotransmitters, being found in discrete neurons and immune cells (Page, 2004, 2005). Recently, this dogma was challenged when the placenta, a tissue devoid of nerves, was found to be a source of *TAC3* gene expression (Page *et al.*, 2000, 2001).

A role for the tachykinins in the placenta has remained as yet undefined. Nevertheless, recent and consistent evidence suggest that they may play a role in uteroplacental hemodynamic adaptation by inducing uterine and placental vasodilatation, thereby increasing placental blood flow (Page *et al.*, 2000, 2001; Brownbill *et al.*, 2003; D'Anna *et al.*, 2004; Laliberte *et al.*, 2004). Elevated circulating levels of the tachykinin NKB have been observed in women with PE during the third trimester of pregnancy and may also explain the sequelae of PE, including hypertension.

Currently, Doppler ultrasound analysis of the uteroplacental circulation combined with clinical history and, sometimes, with biochemical markers like pregnancy-associated plasma protein-A (PAPP-A), forms the most common screening tool in routine prenatal settings to detect women at increased risk for PE. Molecular screening tests (alone or combined with Doppler analysis) seem to have a sufficient predictive power and could be extensively used although the costs are still very high. For these reasons, it seemed promising to search for the best mRNAs with the highest predictive power, in order to reduce costs and optimize the screening results. This study presents eight new mRNAs that could be evaluated in further studies for a possible use in PE screening. However, screening PE by CVS analysis affords a slightly different point of view representing an extra opportunity for early screening. In fact, given that women who undergo CVS for fetal karyotype analysis are at higher risk for PE because of advanced maternal age, the mRNA dosage may be a possible way of detecting high-risk patients. Again, the CVS itself is considered, even if controversial, a possible extra risk for PE occurrence (Adusumalli *et al.*, 2007).

In conclusion, in this report we evaluated a new list of genes possibly involved in PE development and screening. They belong to categories widely associated with

PE onset including trophoblast invasion, villous angiogenesis, remodelling of extracellular matrix, maternal immunity and hemodynamic adaptation. All of them potentially open up new opportunities for screening the disease long before clinical onset; however, because of the small sample size only those genes that showed a higher degree of aberration have a higher chance to predict PE in prospective studies.

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REFERENCES

Adusumalli J, Han CS, Beckham S, Bartholomew ML, Williams J III. 2007. Chorionic villus sampling and risk for hypertensive disorders of pregnancy. *Am J Obstet Gynecol* **196**: 591.e1–e7. discussion 591.e7.

Apps R, Gardner L, Moffett A. 2008. A critical look at HLA-G. *Trends Immunol* **29**: 313–321.

Baggiolini M, Walz A, Kunkel SL. 1989. Neutrophil-activating peptide-1/interleukin 8,a novel cytokine that activates neutrophils. *J Clin Invest* **84**: 1045–1049.

Belperio JA, Keane MP, Arenberg DA, *et al.* 2000. CXC chemokines in angiogenesis. *J Leukoc Biol* **68**: 1–8.

Bernardini G, Ribatti D, Spinetti G, *et al.* 2003. Analysis of the role of chemokines in angiogenesis. *J Immunol Methods* **273**: 83–101.

Brownbill P, Bell NJ, Woods RJ, Lowry PJ, Page NM, Sibley CP. 2003. Neurokinin B is a paracrine vasodilator in the human fetal placental circulation. *J Clin Endocrinol Metab* **88**: 2164–2170.

Campbell S, Rowe J, Jackson CJ, Gallery ED. 2004. Interaction of cocultured decidual endothelial cells and cytotrophoblasts in preeclampsia. *Biol Reprod* **71**: 244–252.

D'Anna R, Baviera G, Corrado F, *et al.* 2004. Neurokinin B and nitric oxide plasma levels in pre-eclampsia and isolated intrauterine growth restriction. *BJOG* **111**: 1046–1050.

De Groot CJ, Bloemenkamp KW, Duvekot EJ, *et al.* 1999. Pre-eclampsia and genetic risk factors for thrombosis: a case-control study. *Am J Obstet Gynecol* **181**: 975–980.

Denison FC, Kelly RW, Calder AA. 1997. Differential secretion of chemokines from peripheral blood in pregnant compared with non-pregnant women. *J Reprod Immunol* **34**: 225–240.

Goldman-Wohl DS, Ariel I, Greenfield C, Hanoch J, Yagel S. 2000a. HLA-G expression in extravillous trophoblasts is an intrinsic property of cell differentiation: a lesson learned from ectopic pregnancies. *Mol Hum Reprod* **6**: 535–540.

Goldman-Wohl DS, Ariel I, Greenfield C, *et al.* 2000b. Lack of human leukocyte antigen-G expression in extravillous trophoblasts is associated with pre-eclampsia. *Mol Hum Reprod* **6**: 88–95.

Goldsby RA, Kindt TJ, Osborne BA, Kuby J. 2003. Leukocyte migration and inflammation. *Immunology*, W. H. Freeman and Company: New York; 338–360.

Gotsch F, Romero R, Friel L, *et al.* 2007. CXCL10/IP-10: a missing link between inflammation and anti-angiogenesis in preeclampsia? *J Matern Fetal Neonatal Med* **20**: 777–792.

Hackmon R, Koifman A, Hyodo H, Glickman H, Sheiner E, Geraghty DE. 2007. Reduced third-trimester levels of soluble human leukocyte antigen G protein in severe preeclampsia. *Am J Obstet Gynecol* **97**: 255.e1–5.

Hanna J, Goldman-Wohl D, Hamani Y, *et al.* 2006. Decidual NK cells regulate key developmental processes at the human fetal-maternal interface. *Nat Med* **12**: 1065–1074.

Hara N, Fujii T, Yamashita T, Kozuma S, Okai T, Taketani Y. 1996. Altered expression of human leukocyte antigen G (HLA-G)on extravillous trophoblasts in preeclampsia: immunohistological demonstration with anti-HLA-G specific antibody "87G" and anticytokeratin antibody "CAM5 2". *Am J Reprod Immunol* **36**: 349–358.

Hiby SE, Walker JJ, O'Shaughnessy KM, *et al.* 2004. Combinations of maternal KIR and fetal HLA-C genes influence the risk of preeclampsia andreproductive success. *J Exp Med* **200**: 957–965.

Kauma S, Takacs P, Scordalakes C, Walsh S, Green K, Peng T. 2002. Increased endothelial monocyte chemoattractant protein-1 and interleukin-8 in preeclampsia. *Obstet Gynecol* **100**: 706–714.

Kelly BA, Bond BC, Poston L. 2003. Gestational profile of matrix metalloproteinases in rat uterine artery. *Mol Hum Reprod* **9**: 351–358.

Kolben M, Lopens A, Blaser J, *et al.* 1996. Proteases and their inhibitors are indicative in gestational disease. *Eur J Obstet Gynecol Reprod Biol* **68**: 59–65.

Laliberte C, DiMarzo L, Morrish DW, Kaufman S. 2004. Neurokinin B causes concentration-dependent relaxation of isolated human placental resistance vessels. *Regul Pept* **117**: 123–126.

Le Bouteiller P, Tabiasco J. 2006. Killers become builders during pregnancy. *Nat Med* **12**: 991–992.

Lim KH, Zhou Y, Janatpour M, *et al.* 1997. Human cytotrophoblast differentiation/invasion is abnormal in pre-eclampsia. *Am J Pathol* **151**: 1809–1918.

MacKay AP, Berg CJ, Atrash HK. 2001. Pregnancy-related mortality from preeclampsia and eclampsia. *Obstet Gynecol* **97**: 533–538.

Montagnana M, Lippi G, Albiero A, *et al.* 2009. Evaluation of metalloproteinases 2 and 9 and their inhibitors in physiologic and pre-eclamptic pregnancy. *J Clin Lab Anal* **23**: 88–92.

Neville LF, Mathiak G, Bagasra O. 1997. The immunobiology of interferon-gamma inducible protein 10 kD (IP-10): a novel, pleiotropic member of the C-X-C chemokine superfamily. *Cytokine Growth Factor Rev* **8**: 207–219.

O'Brien M, Dausset M, Carosella E, Moreau P. 2000. Analysis of the role of HLA-G in preeclampsia. *Human Immunol* **61**: 1126–1131.

Page NM. 2004. Hemokinins and endokinins. *Cell Mol Life Sci* **61**: 1652–1663.

Page NM. 2005. New challenges in the study of the mammalian tachykinins. *Peptides* **26**: 1356–1368.

Page NM, Woods RJ, Gardiner SM, *et al.* 2000. Excessive placental secretion of neurokinin B during the third trimester causes pre-eclampsia. *Nature* **405**: 797–800.

Page NM, Woods RJ, Lowry PJ. 2001. A regulatory role for neurokinin B in placental physiology and pre-eclampsia. *Regul Pept* **98**: 97–104.

Parham P. 2004. NK cells and trophoblasts: partners in pregnancy. *J Exp Med* **200**: 951–955.

Poon LC, Nekrasova E, Anastassopoulos P, Livanos P, Nicolaides KH. 2009. First-trimester maternal serum matrix metalloproteinase-9 (MMP-9) and adverse pregnancy outcome. *Prenat Diagn* **29**: 553–559.

Purwosunu Y, Sekizawa A, Koide K, *et al.* 2007. Cell-free mRNA concentrations of plasminogen activator inhibitor-1 and tissue-type plasminogen activator are increased in the plasma of pregnant women with preeclampsia. *Clin Chem* **53**: 399–404.

Rosenkilde MM, Schwartz TW. 2004. The chemokine system—a major regulator of angiogenesis in health and disease. *APMIS* **112**: 481–495.

Sharma A, Satyam A, Sharma JB. 2007. Leptin, IL-10 and inflammatory markers (TNF-alpha, IL-6 and IL-8) in pre-eclamptic, normotensive pregnant and healthy non-pregnant women. *Am J Reprod Immunol* **58**: 21–30.

Sibai BM, Barton JR. 2007. Expectant management of severe preeclampsia remote from term: patient selection, treatment, and delivery indications. *Am J Obstet Gynecol* **196**: 514.e1–e9.

Strieter RM, Burdick MD, Gomperts BN, *et al.* 2005. CXC chemokines in angiogenesis. *Cytokine Growth Factor Rev* **16**: 593–609.

Wang Z, Lu S, Liu C, *et al.* 2009. Expressional and epigenetic alterations of placental matrix metalloproteinase 9 in preeclampsia. *Gynecol Endocrinol* **31**: 1–7.

Walker JJ. 2000. Pre-eclampsia. *Lancet* **356**: 1260–1265.

Yie SM, Li LH, Li YM, Librach C. 2004. HLA-G protein concentrations in maternal serum and placental tissue are decreased in preeclampsia. *Am J Obstet Gynecol* **191**: 525–529.

Yie SM, Taylor RN, Librach C. 2005. Low plasma HLA-G protein concentrations in early gestation indicate the development of preeclampsia later in pregnancy. *Am J Obstet Gynecol* **193**: 204–208.

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REVIEW SERIES

Diurnal blood pressure variation and cardiovascular prognosis in a community-based study of Ohasama, Japan

Diurnal variations in blood pressure: clinical implications and pathogenesis

Hirohito Metoki[1,2], Takayoshi Ohkubo[3] and Yutaka Imai[1]

The introduction of 24-h ambulatory blood pressure (BP) monitoring has enabled BP evaluations at specific times of the day. Associations between diurnal BP variation and cardiovascular prognosis have been investigated in the Ohasama study, which is an epidemiological survey of hypertension using ambulatory and home BP monitoring that has been ongoing since 1985 in the general population of Ohasama, a town located in northern Japan. A diminished nocturnal decline in systolic BP was associated with a greater common carotid intima-media thickness as well as a higher risk of cardiovascular morbidity and mortality, especially the risk for cerebral infarction. The consumption of large amounts of alcohol was associated with a higher morning pressor surge. A large nocturnal decline in BP and a large morning pressor surge were both associated with a risk of cerebral hemorrhage. Ambulatory BP monitoring provides not only static, but also dynamic information about BP that should be considered to ensure effective management of hypertension and cardiovascular diseases.
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Keywords: ambulatory blood pressure monitoring; morning pressor surge; nocturnal decline in blood pressure

INTRODUCTION

High blood pressure (BP) is associated with target-organ damage and a poor cardiovascular prognosis. The introduction of 24-h ambulatory BP monitoring has enabled BP to be evaluated at specific times of day. A single BP value obtained using an ambulatory device on rising in the morning is a better discriminator of future cardiovascular events than the mean of three measurements taken under standardized conditions in a hospital or clinic.[1] Several recently proposed indices of circadian BP variation might be relevant to the diagnosis and management of hypertension with special reference to target-organ damage and prognosis. The present review describes diurnal BP variation and cardiovascular prognosis from the results of the Ohasama study, which is an epidemiological survey of hypertension based on ambulatory BP monitoring that was started in 1985 among the general population of Ohasama, a town located in northern Japan.

STUDY POPULATION

Ohasama had a population of 9400 in 1985. We have obtained ambulatory BP data over the past 20 years by monitoring over 3000 inhabitants aged $\geqslant$20 years, as well as outcomes and information about risk factors and predictors. To prospectively investigate the association between BP levels and subsequent risk of outcomes, we excluded individuals aged $<$40 years at the time of ambulatory BP monitoring because death or stroke occurrence was less frequent among younger persons. Thus, several indexes of BP obtained by ambulatory monitoring were prospectively analyzed among 1542 inhabitants of Ohasama aged $\geqslant$40 years.

NOCTURNAL DECLINE IN BP

BP generally increases on awakening in the morning and falls while asleep during the nighttime. This circadian variation in BP is regulated by the autonomic nervous and endocrine systems, and modified by several factors such as physical and mental activities as well as environmental stressors. Nocturnal BP usually falls 10–20% from the diurnal value and is referred to as nocturnal dipping. However, nocturnal dipping is attenuated or disappears under several patho-physiological conditions and persons with this phenomenon are referred to as 'non-dippers.' Those with higher nocturnal BP than the diurnal value are referred to as 'inverted dippers' or 'risers.' A person with a large nocturnal decline in BP is defined as an 'extreme

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dipper.' However, nocturnal BP remains normal or high among hypertensive patients. Therefore, 'extreme dippers' among hypertensive patients are equivalent to 'diurnal risers.'[2] Such disordered circadian BP variations are associated with a poor cardiovascular prognosis[3–6] and are regarded as targets for antihypertensive therapy.

The decline in nocturnal BP was calculated in the Ohasama study as follows: nocturnal decline in BP (%)=(daytime BP−nighttime BP)×100/daytime BP. We classified the subtypes of nocturnal decline in BP as follows: extreme dipper (20% nocturnal decline in BP from diurnal value), dipper (10–19% nocturnal decline in BP), non-dipper (0–9% nocturnal decline in BP) and riser (0% nocturnal decline in BP or nocturnal elevation).

NOCTURNAL DECLINE IN BP AND CARDIOVASCULAR MORTALITY

We reported the association between ambulatory BP and cardiovascular prognosis in 1997. That report describes that ambulatory BP predicted mortality more effectively than casual screening of BP during a mean follow-up period of 5.1 years.[7] During the same observation period, the mortality risk was highest among risers, followed by non-dippers. Mortality rates did not differ between extreme dippers and dippers. This relationship in both treated and untreated individuals was more remarkable for cardiovascular, than for non-cardiovascular mortality, and was not changed after adjustment for 24-h, daytime and nighttime BP levels.[8] Follow-up for a mean of 9.2 years showed that a diminished nocturnal decline in BP was associated with a risk for cardiovascular mortality, which was independent of the overall BP load during a 24-h period.[9]

NOCTURNAL DECLINE IN BP AND RISK OF STROKE

Analysis of data over a mean follow-up period of 10.4 years revealed no consistent association between dipping profile and the risk of total stroke. The data did not fit a linear model; the relative hazard per 1 s.d. increase of nocturnal decline in BP was 1.1 (95% CI, 0.8–1.4, P=0.7). The risk for cerebral infarction was significantly higher among individuals with a diminished nocturnal decline (risers and non-dippers) than among those with a decline of $\geqslant$10% (dippers and extreme dippers). The relative hazard among individuals with a diminished nocturnal decline was 1.6 (95% CI, 1.0–2.5, P=0.04). Extreme dippers had 2.7-fold higher risk (95% CI, 1.1–6.4, P=0.02) of cerebral hemorrhage than those with a nocturnal decline of <20% (dippers, non-dippers and risers).

MORNING BP SURGE

BP that abruptly increases around awakening in the morning is called the 'morning pressor surge.'[10,11] The association between morning BP surge and cardiovascular disease has received focus because cardiovascular events occur more frequently in the morning[12–14] and a mean follow-up of 20 months has revealed that elderly patients with a large morning pressor surge have a threefold higher risk of stroke.[15]

The amplitude of the morning pressor surge in the Ohasama study was defined based on earlier findings[11] as follows: morning pressor surge in systolic blood pressure (SBP)=2-h mean SBP after waking−2-h mean SBP before waking.

The morning pressor surge can also be calculated by a method that generates the 'sleep-trough' morning pressor surge,[15] which is calculated as follows: sleep-trough morning pressor surge in SBP=2-h mean SBP after waking−lowest SBP defined as mean BP of three readings centered on the lowest nighttime reading.

An association between morning pressor surge and the incidence of total stroke (n=128) and of cerebral infarction (n=86) was not identified during a 10.4-year follow-up. However, the risk for cerebral hemorrhage (n=27) was significantly high in the fifth quintile group with a morning pressor surge amplitude of 25 mm Hg (RH, 4.0; 95% CI, 1.1–14.6, P=0.04), when the second quintile of the morning pressor surge (amplitude 3–11 mm Hg) was set as the reference category.[16] The predictive value of the sleep-trough morning surge was similar to that of the sleeping-to-waking morning surge in the same population.[16]

The International Database of Ambulatory Blood Pressure in relation to Cardiovascular Outcome has recently been established.[17,18] A morning surge in BP exceeding the 90th percentile in this database was a significant and independent predictor of mortality and cardiovascular events even after correcting the night-to-day BP ratio, the 24-h BP level and other covariables.[19] Moreover, consistent with our earlier findings,[16] Asians with a morning surge in the top decile were at a significantly higher risk for hemorrhagic stroke (n=51; HR [95% CI], 2.28 [1.09–4.26], P=0.03), but not for ischemic stroke (n=127; HR, 1.41 [0.67–2.98], P=0.37), than those with a lower morning surge.

BP AT A SPECIFIC TIME OF DAY

Although the predictive value of BP increases with increasing numbers of measurements,[20,21] BP values obtained at different times of the day (nighttime, morning and daytime) have not been compared with values obtained by the same number of measurements. A simple calculated mean of the BP values recorded every 30 min during the nighttime (8 h) generates 16 values; similarly, a simple mean of the daytime (16 h) BP values recorded every 30 min yields 28 values. Therefore, if the predictive power of BP obtained during the daytime was more powerful than taken during the nighttime, it could reflect the larger number of measurements taken during the daytime.

The means of four BP readings obtained every 30 min for two consecutive hours in a day (moving averages) during the Ohasama study were defined as '2h-BP' (see Figure 1).[22] When readings were omitted because of missed and/or artifactual measurements, calculations were based on the remaining readings (minimum of one) obtained during the 2-h period. The 2h-BP allows a comparison of

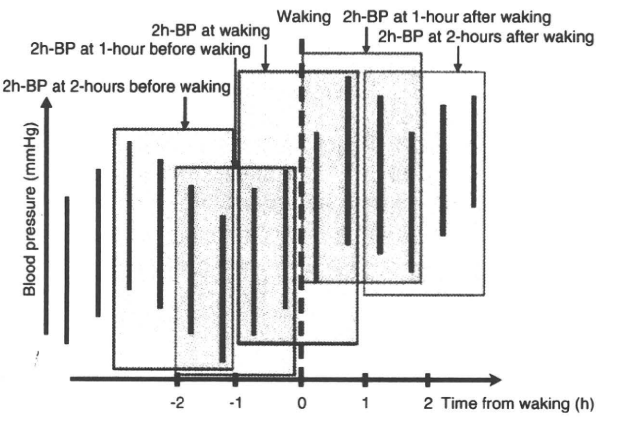

Figure 1 Definition of moving average of 2-h SBP/2-h DBP. Means of four SBP/DBP readings obtained over two consecutive daytime hours (moving averages) are defined as 2h-SBP/2h-DBP. Horizontal line indicates time (hours) from waking. Vertical line shows BP (mm Hg). Reproduced from our earlier article22 with permission from the Lippincott Williams & Wilkins.

the predictive value of BP taken at different times using the same number of BP measurements.

BP AT A SPECIFIC TIME OF DAY AND CARDIOVASCULAR MORTALITY

When nighttime and daytime SBP values were simultaneously included in the same Cox model, only nighttime BP significantly predicted cardiovascular mortality risk from the 10.8-year follow-up data. We concluded that the relationship between ambulatory SBP and cardiovascular mortality is not U- or J-shaped, and that the prognostic value of BP during the nighttime is better than that during the daytime.[23] We applied 2h-BP to evaluate the relevance of BP at a particular time of day to the risk of stroke mortality. Total cerebrovascular and cardiovascular mortality risk was significantly associated with elevated 2h-BP recorded during the night and early morning. Hemorrhagic stroke mortality was significantly associated with elevated daytime 2h-BP. The mortality of cerebral infarction and heart disease was significantly associated with elevated nighttime 2h-BP.[22]

BP AT A SPECIFIC TIME OF DAY AND RISK OF STROKE

The risk of stroke incidence in the Ohasama study was more closely associated with daytime, than with nighttime BP over a mean follow-up of 6.4 years.[24]

Using 2h-BP to evaluate the relevance of BP taken at a specific time of day to determine the risk of stroke morbidity showed that risk for total stroke incidence was significantly associated with systolic 2h-BP values (2h-SBPs) throughout the day (Figure 2). Risk for incidence of intracerebral hemorrhage was significantly associated with elevated daytime 2h-SBPs, but less so with nighttime 2h-SBPs (Figure 3a). Risk for the incidence of cerebral infarction was significantly associated with nighttime 2h-SBPs, but less so with daytime 2h-SBPs (Figure 3b).

DIURNAL BP VARIATION AND TARGET-ORGAN DAMAGE

Cross-sectional analyses regarding target-organ damage were performed during the Ohasama study. Nighttime BP was most closely associated with carotid artery alterations among values for daytime, nighttime and casual BP. Although a morning pressor surge was not associated with carotid artery alterations, a diminished nocturnal decline in SBP was associated with common carotid intima-media thickness after adjustment for confounding factors.[25] Daytime and nighttime BP values were both associated with silent cerebrovascular lesions, whereas casual BP in the same population was not.[26]

FACTORS ASSOCIATED WITH DIURNAL BP VARIATION

Alcohol consumption and diurnal BP variation

We found, using 2h-BP, that BP rapidly increased before awakening and that morning BP was higher among Ohasama inhabitants who consumed alcohol. The morning pressor surge was significantly higher among those who consumed large amounts of alcohol than in those who consumed none, whereas alcohol consumption status was not significantly associated with the magnitude of the nocturnal decline in BP.[27]

Genetic polymorphisms and diurnal BP variation

Daytime SBP and diastolic blood pressure (DBP) values were higher in individuals with the C allele in the angiotensin II type 1 receptor gene A/C^{1166} polymorphism (130.5 ± 14.0/77.4 ± 8.3 mm Hg) than in those with the AA genotype (127.7 ± 13.6/75.8 ± 8.3 mm Hg, P=0.03/0.04), although the difference was not statistically significant after adjusting for age, gender, body mass index and smoking status.[28] Nighttime BP values were significantly lower among individuals with the MM genotype in the angiotensinogen M235T polymorphism than in those with the T allele (105.2 ± 13.0/60.1 ± 6.9 *vs.* 110.6 ± 13.3/63.6 ± 7.8 mm Hg, P=0.04/0.02). The nocturnal decline in BP was significantly larger among those with the MM genotype than with the T allele (17.4/19.8 *vs.* 13.5/16.2 mm Hg, P=0.004/0.01).[29] The nocturnal decline in BP was significantly greater among individuals with a homozygous CC aldosterone synthase gene (CYP11B2) C-334T polymorphism than in others (15.4%/17.7% *vs.* 13.3%/16.1%, P=0.007/0.03), although 24-h ambulatory BP levels did not significantly differ among the genotypes.[30]

We recently focused on the (pro)renin receptor gene. Although casual BP was not associated, 24-h, daytime and nighttime SBP and DBP values were significantly higher among male carriers of the IVS5+169T rather than the C allele of the (pro)renin receptor gene. BP values did not significantly differ among the three genotypes of female IVS5+169C>T carriers.[31]

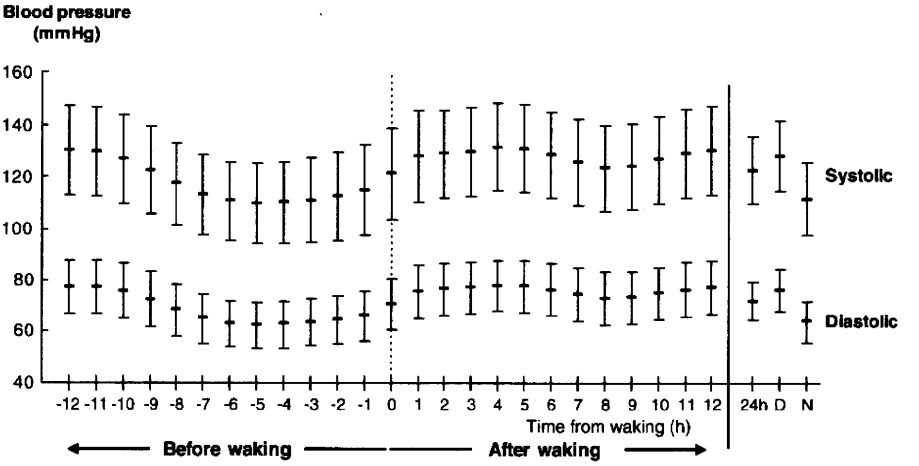

Figure 2 Circadian BP variation of SBP and DBP using 2-h SBP/2-h DBP. Left panel: 2-h moving averages of SBP and DBP over 24-h period based on time (hours) from waking. Right panel: 24-h, daytime and nighttime mean BP values are shown as 24 h, D and N, respectively.