Sequential Changes in Antiangiogenic Factors in Early Pregnancy and Risk of Developing Preeclampsia

Sarosh Rana, S. Ananth Karumanchi, Richard J. Levine, Shivalingappa Venkatesha, Jose Alejandro Rauh-Hain, Hector Tamez, Ravi Thadhani

Abstract—Concentrations of soluble fms-like tyrosine kinase 1 (sFlt1) and soluble endoglin (sEng) increase in maternal blood with the approach of clinical preeclampsia. Although alterations in these circulating antiangiogenic factors herald the signs and symptoms of preeclampsia, in vitro studies suggest they may also play a role in regulating early placental cytotrophoblast functions. Early pregnancy changes in sFlt1 and sEng may thus identify women destined to develop preeclampsia. We performed a nested case-control study of 39 women who developed preeclampsia and 147 contemporaneous normotensive controls each with serum collected in the first (11 to 13 weeks of gestation) and second (17 to 20 weeks) trimesters. Whereas levels of sFlt1 and sEng at 11 to 13 weeks were similar between cases and controls (sFlt1: 3.5±0.3 ng/mL versus 3.0±0.1, P=0.14; sEng 6.9±0.3 ng/mL versus 6.6±0.2, P=0.37, respectively), at 17 to 20 weeks both were elevated in the women destined to develop preeclampsia (sFlt1: 4.1±0.5 ng/mL versus 3.1±0.1, P<0.05; sEng, 6.4±0.4 ng/mL versus 5.2±0.1, P<0.01). Women who developed preterm (<37 weeks) preeclampsia demonstrated even greater sequential changes: difference [\(\Delta d\)] between second and first trimester levels: dsFlt1, 0.63±0.91 ng/mL in preterm PE versus 0.05±0.15 in controls; dsEng, 0.73±0.77 ng/mL versus −1.32±0.18, P<0.01. Similar findings were noted in a cross-sectional analysis of specimens collected from the Calcium for Preeclampsia Prevention Study. In conclusion, sequential changes in antiangiogenic factors during early pregnancy may be useful for predicting preterm preeclampsia. (Hypertension. 2007;50:137-142.)

Key Words: antiangiogenic factors • sFlt1 • soluble endoglin • preeclampsia • predictive test

Preeclampsia affects 3% to 5% of pregnancies and causes substantial maternal and neonatal morbidity and mortality. Predicting the development of preeclampsia is important both for prevention (when useful preventive measures become available) and for early referral of high risk pregnancies. Measures to predict preeclampsia, however, are not standardized, and currently there are no reliable biomarkers that are routinely measured in the clinic.

Recently, our improved understanding of the pathogenesis of preeclampsia has raised the possibility that blood levels of antiangiogenic proteins might eventually be used to predict this devastating condition. Circulating soluble fms-like tyrosine kinase 1 (sFlt1) and soluble endoglin (sEng) have been hypothesized to play a pathogenic role in preeclampsia. Adenoviral overexpression of sFlt1 and sEng induce development of preeclampsia-like illness in rats. Furthermore, alterations in circulating sFlt1 and sEng are noted several weeks preceding clinical signs. Although there is evidence that angiogenic factor alterations may be strongly associated with the development of the clinical preeclampsia, it is surprising that their levels are not dramatically altered during early pregnancy (11 to 18 weeks of gestation) when the abnormal placentation associated with severe premature preeclampsia develops. This led us to hypothesize that sequential angiogenic factor changes from the first to the second trimesters of pregnancy might differ in women destined to develop preeclampsia, explaining the apparent paradox and that these changes could have predictive value. The aim of the present study, therefore, was to measure concentrations of sFlt1 and sEng in paired serum specimens collected in first and second trimesters from women with well-characterized pregnancy outcomes followed prospectively during pregnancy.

Methods

Study Population

We performed a nested case-control study of women who enrolled in the Massachusetts General Hospital Obstetric Maternal Study (MOMS), whose methods have been described previously. In brief, the MOMS cohort was established in 1998 for the prospective study of early gestational risk factors for adverse outcomes that occur...
later in pregnancy. Women who received prenatal care at Massachusetts General Hospital and affiliated health centers were eligible for inclusion in the cohort. For the current study, consecutive women with singleton gestations between June 1, 2001, and May 1, 2003, who enrolled in the MOMS cohort at or before 12 weeks of gestation and who delivered after 20 weeks were eligible for inclusion. Cases (n=39) were defined as those with blood collections in the first and second trimester who subsequently developed preeclampsia, and controls (n=147) were consecutive contemporaneous women enrolled in the same cohort who delivered at term (>37 weeks) and remained normotensive, normoglycemic, and without evidence of proteinuria throughout pregnancy. Cases and controls were matched by age (±2 years) and body mass index (≥1 kg/m²) because of the potential for confounding by these exposures. Sixteen of the 39 cases in this study had preterm preeclampsia. All subjects provided written informed consent, and this study was approved by the Institutional Review Board of the Massachusetts General Hospital.

Measurements of sFlt1 and sEng

Blood samples were collected from all women at the first prenatal visit (11 to 13 weeks) and again in the second trimester (17 to 20 weeks). After collection, samples were stored at −80°C until analysis. Serum sFlt1 and sEng were measured using commercial ELISA kits (R&D Systems). The intraassay precision coefficients of variation for sFlt1 and sEng in our laboratory were 3.5% and 3.2%, respectively. The interassay precision coefficients of variation for sFlt1 and sEng were 8.1% and 9.5%, respectively. All samples were run in duplicate, and if more than 25% variation existed between duplicates, the assay was repeated, and averages were reported. One author (S.R.) performed all assays and was blinded to case status. Samples were randomly ordered for analysis.

Covariates and Confounders

The electronic medical record, which is the medical record used at the Massachusetts General Hospital, provides clinical and demographic data that details the events of pregnancy through the early postpartum period. Specific information obtained from the electronic medical record collected at baseline (first prenatal visit) and at all subsequent prenatal visits included age, race, height, weight, smoking status, gestational age estimated from the last menstrual period and verified by ultrasound, blood pressure, and the results of routine urine analysis and fetal gestational age estimation. All pregnancy outcome information is also entered in the electronic medical record, including results of glucose tolerance tests, other routinely measured laboratory values, and delivery characteristics such as birth weight, route of delivery, and diagnosis of preeclampsia.

Primary Outcomes

All outcomes were verified by detailed examination of medical records, including prenatal flow sheets and laboratory measurements. Blood pressure (K1 and K5) was measured at each prenatal visit using standard mercury sphygmomanometers. Exclusion criteria included histories of hypertension, renal disease, and diabetes. All subjects had received prenatal care within the MOMS network, delivered a live infant, and had no evidence of hypertension 6 weeks after delivery.

Preeclampsia was defined as new onset hypertension and proteinuria after gestational week 20; hypertension defined as blood pressure elevation to systolic ≥140 mm Hg or diastolic ≥90 mm Hg, respectively, and proteinuria, as ≥2+ by dipstick or ≥300 mg/24 hour in the absence of urinary tract infection. Preeclampsia was designated and analyzed as “term preeclampsia” (≥37 weeks) and “preterm preeclampsia” (<37 weeks).

Statistical Analysis

Demographic and clinical characteristics were compared using χ² tests or Student t test, as appropriate. Log transformation was needed for the primary exposures given their skewed distributions. sFlt1 and sEng were examined as continuous variables, and with cut points and tertile analyses based on the distribution of the controls and simplified for clinical interpretation. Multiple regression analysis was performed using logistic regression techniques. A sample size of 35 cases and 105 controls (1:3) was estimated to provide at least 80% power to detect differences of ~20% between cases and controls. All probability values were 2-tailed, and a probability value <0.05 was considered statistically significant.

Secondary Analysis of Angiogenic Markers in the Calcium for Preeclampsia Prevention Study

We also performed a secondary analysis of angiogenic factor changes during early pregnancy, using data obtained from the published nested case controlled study within the trial of Calcium for Preeclampsia Prevention (CPEP) cohort. CPEP was a randomized double-blind clinical trial conducted from 1992 to 1995 to evaluate the effects of calcium supplementation on the incidence and severity of preeclampsia. Healthy nulliparous women with singleton pregnancies were enrolled within 13 to 21 completed weeks of gestation at 5 participating U.S. medical centers and followed until 24 hours after delivery. We examined data from samples of normotensive women, who developed preeclampsia before 37 weeks, and women who developed preeclampsia after 37 weeks at 3 different time intervals—10 to 12 weeks, 13 to 16 weeks, and 17 to 20 weeks.

Results

Demographic and Clinical Characteristics

Baseline and delivery characteristics of the MOMS study population are displayed in Table 1. There were no significant differences in age and body mass index between the 2 groups. Women who subsequently developed preeclampsia had higher systolic and diastolic blood pressures at the first prenatal visit, and as would be expected blood pressure levels were considerably higher when overt disease occurred.

First and Second Trimester Levels of sFlt1 and sEng in Normal and Preeclamptic Pregnancy

The mean first trimester serum sFlt1 levels were nonsignificantly higher in women who later developed preeclampsia compared with women who had normal pregnancies, but in the second trimester the levels were significantly higher in preeclampic group (2nd trimester sFlt1: 4.1±0.5 ng/mL compared with 3.1±0.1 ng/mL in preeclampsia versus controls respectively, P=0.01; Table 2). Serum levels of sEng showed a similar pattern, not significantly different in the first trimester, but greater in the second (2nd trimester sEng: 6.4±0.4 ng/mL versus 5.2±0.1 ng/mL in preeclampsia versus controls respectively, P=0.004; Table 2).

Sequential Changes in Angiogenic Factors

Figure 1 graphically displays the delta or d (difference between second and first trimester values of sFlt1 and sEng) in women with normal pregnancies, in all women who later developed preeclampsia, and in those who developed preterm preeclampsia (<37 weeks). In normal pregnancy, there was little change in sFlt1 between first and second trimesters (dFlt1=−0.05±0.15 ng/mL). dFlt1 was relatively higher in all women who developed preeclampsia 0.71±0.47 ng/mL (P=0.08 compared with controls) and also higher in a subset of women destined to develop preterm preeclampsia 0.63±0.91 ng/mL, although not statistically significant (P=0.2 compared with controls).

sEng decreased between first and second trimesters in normal pregnancy (−1.32±0.18 ng/mL), but this decrement
Predictive Algorithms
Second trimester levels of both sFlt1 and sEng were higher in women who developed preterm preeclampsia. To determine whether these alterations could be used as a predictive test for preterm preeclampsia, we analyzed the data using the product of sFlt1×sEng: (product-1 in first trimester and product-2 in second trimester). Both product-1 and product-2 were elevated in patients with preeclampsia and in particular significantly elevated in preterm preeclampsia (Figure 2). Importantly, the delta of the product (dproduct) was strikingly positive in contrast with a negative value in normal controls (Figure 2). The dproduct was especially amplified in patients with preterm preeclampsia as compared with normal controls (P=0.004; Figure 2).

To assess the relationship between altered levels of angiogenic factors and risk of preterm preeclampsia, we computed adjusted odds ratios (aOR) and 95% confidence intervals (95% CI) for preterm preeclampsia in the highest tertile of the control distribution of dproduct concentrations with respect to the lower two tertiles after adjustment for race/ethnicity, body-mass index, and gestational age at specimen collection (Figure 3). Substantial increases in risk of preterm preeclampsia were observed in the group whose delta product levels were greater than +1 [aOR 5.5, 95% CI 1.4 to 22.4], compared with women whose delta product was less than −1.

Secondary Analysis of the CPEP Nested Case-Control Study
To evaluate whether sequential alterations of angiogenic factors are also observed in a different population of women who developed preterm preeclampsia, we performed a secondary analysis of angiogenic factor alterations from the recently published nested case control study from the CPEP trial cohort.5 Among women in this study who had remained normotensive, the mean values of sFlt1 increased from 3.7 ng/mL to 4.9 ng/mL between 10 to 12 weeks and 13 to 16 weeks and decreased to 4.3 ng/mL at 17 to 20 weeks, whereas in women with preterm preeclampsia, the mean values of sFlt1 increased from 3.4 ng/mL to 4.2 ng/mL and further increased to 5.4 ng/mL at 10 to 12, 13 to 16 and 17 to 20 weeks of gestation. This pattern was not obvious in women with term preeclampsia (Table 3).

Similar results were observed in the levels of sEng. In normotensive women the levels of sEng increased from 6.8

| TABLE 1. Demographics and Clinical Characteristics of Subjects Enrolled in MOMS Study |
|-----------------------------------------------|-----------------|-----------------|-----------------|
| Variable                                      | Normal (n=147)  | Preeclampsia (n=39) | P Value |
| Baseline characteristics                      |                 |                 |       |
| Age, y                                        | 31.4±5.4        | 32.8±5.4        | 0.06  |
| BMI, kg/m²                                     | 28.5±6.3        | 29.8±9.1        | 0.40  |
| Smoker, % never                               | 47%             | 60%             | 0.21  |
| Parity                                        | 0.7±0.9         | 0.8±1.0         | 0.47  |
| DBP, mm Hg                                    | 70±7            | 75±8            | <0.01 |
| SBP, mm Hg                                    | 113±7           | 120±13          | <0.01 |
| Delivery Characteristics                      |                 |                 |       |
| Gestational age at delivery in weeks          | 39.3±1.9        | 37.2±2.3        | <0.001|
| Birth weight in grams                         | 3444±532        | 3300±809        | 0.35  |
| Characteristics at presentation              |                 |                 |       |
| Maximum DBP, mm Hg                            | 78±5            | 93±7            | <0.01 |
| Maximum SBP, mm Hg                            | 122±7           | 147±10          | <0.01 |
| Maximum SPOT, g/g                             | Negative*       | 1.4±0.9         |       |
| BMI indicates body mass index; DBP, diastolic blood pressure; SBP, systolic blood pressure; SPOT, urine protein/creatinine ratio. |
*By dipstick analysis; values are mean±SD.

| TABLE 2. Angiogenic Factors in Normal and Preeclamptic Pregnancy |
|---------------------------------------------------------------|----------------|----------------|----------------|
| Angiogenic Factor                                             | Normal Pregnancy | Preeclampsia | P Value |
| sFlt1, ng/mL, first trimester                                 | 3.0±0.1         | 3.5±0.3       | 0.14  |
| sFlt1, ng/mL, second trimester                                | 3.1±0.1         | 4.1±0.5       | 0.01* |
| sEng, ng/mL, first trimester                                  | 6.6±0.2         | 6.9±0.3       | 0.37  |
| sEng, ng/mL, second trimester                                 | 5.2±0.1         | 6.4±0.4       | 0.004*|

Values are presented as mean±SE.
*Statistical significance (P<0.05).
ng/mL to 7.1 ng/mL at 10 to 12 weeks and 13 to 16 weeks and then decreased to 5.8 ng/mL at 17 to 20 weeks, whereas in women with preterm preeclampsia the levels of endoglin increased from 7.1 ng/mL to 7.9 ng/mL and further increased to 10.2 ng/mL between 10 to 12 weeks, 13 to 16 weeks, and 17 to 20 weeks. The same trend was noted in women with term preeclampsia (although not as pronounced) where the mean levels of sEng increased from 7.4 ng/mL to 7.8 ng/mL to 8.3 ng/mL at 10 to 12 weeks, 13 to 16 weeks, and 17 to 20 weeks, respectively (see Table 3).

Discussion

Both sFlt1 and sEng are elevated during second trimester in patients destined to develop preeclampsia. Normal pregnancy is characterized by a fall in sEng levels from first to second trimester without significant change in sFlt1. However, in patients who develop preeclampsia, particularly preterm preeclampsia, both sFlt1 and sEng concentrations continue to rise from first to second trimester. The changes in sFlt1 and sEng during first and second trimesters may be potentially useful for screening patients at high risk for subsequent development of preterm preeclampsia.

Our findings could have implications for the prediction and the pathogenesis of preterm preeclampsia. Although advances in obstetric and neonatal care have led to a considerable reduction in morbidity and mortality from preeclampsia, there have been no substantial advances in the prediction, prevention, and diagnosis of preeclampsia. The pursuit of a safe and reliable screening test for preeclampsia has been a goal of researchers for many years. Previous efforts have focused on detecting early manifestations of disease such as microalbuminuria, weight gain and plasma volume changes. In a large metaanalysis, Conde-Agudelo et al analyzed 87 of 7191 (211 369 women) potentially relevant articles to assess the usefulness of clinical, biophysical, and biochemical tests in the prediction of preeclampsia. They concluded that as of 2004, there was no clinically useful screening test for predicting the development of preeclampsia. That article only noted the just-then appearing literature on angiogenic proteins as too early to analyze nor did it discuss combinations of tests, but suggested that both areas might prove more fruitful. Indeed a series of subsequent reports have indicated that serum concentrations of angiogenic proteins may prove to be powerful predictors of preeclampsia. The present report extends these observations by suggesting that changes in sFlt1 and sEng levels between first and second trimesters may prove to be early predictors of preeclampsia, especially...
preterm disease. In a recent study by Stepan et al, second trimester levels of sFlt1 were higher and levels of placental growth factor were lower in pregnancies with adverse pregnancy outcome. Combining this information with uterine artery Doppler studies significantly improved the sensitivity and specificity of Doppler alone for the prediction of preeclampsia.\textsuperscript{15}

An imbalance in angiogenic factors is thought to play an intimate role in the pathogenesis of clinical preeclampsia. Placentas of women with severe preeclampsia are characterized by shallow implantation and abnormal vascular remodeling including impaired pseudo-vasculogenesis.\textsuperscript{16,17} These placental changes are thought to occur between 12 to 18 weeks of pregnancy and are particularly important for the pathogenesis of the vast majority of severe preterm pre eclampsia. It is believed that placental abnormalities may lead to the elaboration of systemic factors that induce the maternal syndrome of preeclampsia. Both sFlt1 and sEng, two antiangiogenic proteins, have been found to be elevated in preeclampsia not only during clinical disease but also several weeks before onset of symptoms.\textsuperscript{5} Importantly, both factors have been implicated in inducing a preeclampsia-like syndrome in rats.\textsuperscript{6,10} However, because alterations in concentrations of angiogenic factors in the maternal circulation occur relatively late in pregnancy, increased production of these antiangiogenic factors may be a secondary phenomenon in response to abnormal placentation. In vitro data using placental villous explants and primary cytotrophoblast cultures suggest that in addition to its role in inducing maternal endothelial dysfunction, antiangiogenic factors may be involved in cytotrophoblast migration and differentiation.\textsuperscript{18} Our findings that pregnancies in which preterm preeclampsia later develops exhibit a more pronounced rise in serum sFlt1 concentration and a less pronounced fall or even an increase in serum sEng from first to second trimester than in normal pregnancies suggests that abnormalities of circulating angiogenic factors may occur at the same time as abnormalities in placental differentiation. Our findings are similar to those reported by Wathen et al, who observed a fall of 15% in the serum concentration of sFlt1 from 14 to 19 weeks during normal pregnancy in contrast to increased levels of sFlt1 from 16 to 20 weeks in women with subsequent preeclampsia.\textsuperscript{18} Of further interest is the similarity of the measurements from the CPEP trial performed more than 12 years ago and the present MOMS protocol, supporting the use of these assays in samples appropriately stored over long periods of time.

The etiology of the increased concentrations of circulating sFlt1 and sEng in preeclamptic patients is unknown. Hypoxia, genetic factors, or immunologic factors may be involved. Expression of both sFlt1 and sEng is elevated in response to hypoxia where increased expression is mediated by hypoxia inducible factor-1.\textsuperscript{19,20} Furthermore, it is believed that during normal pregnancy the placenta is relatively hypoxic early in pregnancy and that hypoxia disappears with increased blood flow to the placenta during the second trimester. Although never formally documented in preeclamptic pregnancies, it is believed that hypoxia is central to most preeclamptic pregnancies based on surrogate evidence of increased hypoxia induced transcription factor expression (hypoxia inducible factor-1\textalpha and hypoxia inducible factor-2\textalpha) and impaired Doppler blood flow to the placenta.\textsuperscript{21} Our findings that both sFlt1 and sEng remain elevated from first to second trimester in women with preterm preeclampsia in contrast to normal pregnancies where these increase little or decrease suggest that placental ischemia may in fact play a role in the increased production of these antiangiogenic proteins in women who develop preterm preeclampsia.

Our study has limitations. Only a small number of women developed preeclampsia before 34 weeks. The study lacks additional controls such as women with gestational hypertension and those with normotensive pregnancies who deliver small-for-gestational age infants, thus not allowing us to determine whether the angiogenic factor changes we observed in preterm preeclampsia are unique to this condition. Furthermore, because placental bed histology was not available, we cannot conclude that there is a correlation between placental changes and the changes in antiangiogenic factors. Finally, we do not know whether sequential measurements of placental growth factor would enhance the predictive potential of antiangiogenic factors for preeclampsia.

**Perspectives**

Although there is abundant evidence for the role of circulating antiangiogenic factors as mediators of the clinical signs and symptoms of preeclampsia, there are no definitive data linking early abnormalities in angiogenic factors with the impaired placentation of preeclampsia. This work—which relies on using sequential samples obtained during early pregnancy—suggests that the abnormal rise in antiangiogenic factors that occurs in patients who develop preterm preeclampsia may be antedated by early trimester alterations that reflect placentation abnormalities. Moreover, it suggests that

### TABLE 3. Analysis of Angiogenic Factors in the CPEP Database

<table>
<thead>
<tr>
<th>Pregnancy Outcome</th>
<th>sFlt1 (ng/mL)</th>
<th>sEng (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10–12 wks</td>
<td>13–16 wks</td>
</tr>
<tr>
<td>Normal</td>
<td>3.7±0.3</td>
<td>4.9±0.3</td>
</tr>
<tr>
<td>n</td>
<td>6</td>
<td>62</td>
</tr>
<tr>
<td>PE &lt;37 wks</td>
<td>3.4±0.3</td>
<td>4.2±0.4</td>
</tr>
<tr>
<td>n</td>
<td>13</td>
<td>28</td>
</tr>
<tr>
<td>PE ≥37 wks</td>
<td>4.1±0.3</td>
<td>4.5±0.4</td>
</tr>
<tr>
<td>n</td>
<td>17</td>
<td>50</td>
</tr>
</tbody>
</table>

PE indicates preeclampsia. Values are presented as mean±SE.
sequential measurement of antiangiogenic factors may be useful as a screening test for premature preeclampsia, possibly when combined with other early predictive tests such as Doppler ultrasound. This will be important for timely referral of patients at high risk of developing early preeclampsia and for indicating the need for therapeutic measures when they become available. Large prospective studies with longitudinal specimen collection from early pregnancy are required to assess definitively whether sequential changes in antiangiogenic proteins can be used to predict preterm preeclampsia.

Sources of Funding
S.A.K. is funded by R01 grants from the National Institute of Diabetes, Digestive and Kidney Diseases (DK 065997) and the National Heart, Lung, and Blood Institute (HL079594). R.T. is funded by R01 grants from the National Institute of Child Health and Human Development (HD 39223) and National Institute of Diabetes, Digestive and Kidney Diseases (DK 67397). R.J.L. receives salary support from the intramural program of the National Institute of Child Health and Human Development, National Institutes of Health.

Disclosures
S.A.K. is coinventor on patents held by the Beth Israel Deaconess Medical Center that have been licensed to multiple companies. R.T. is a coinventor of patents held by the Massachusetts General Hospital that have been licensed to multiple companies for the prediction of preeclampsia. S.A.K. and R.T. are consultants to Beckman Coulter, Abbott Diagnostics, and Johnson & Johnson. The remaining authors report no conflicts.

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Hypertension. 2007;50:137-142; originally published online May 21, 2007;
doi: 10.1161/HYPERTENSIONAHA.107.087700

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