# Longitudinal changes in maternal soluble endoglin and angiopoietin-2 in women at risk of preeclampsia

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**Short Title:** Soluble endoglin and angiopoietin-2 in preeclampsia

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**ABSTRACT**

**Objective:** To investigate longitudinal changes in maternal plasma levels of soluble endoglin (sEng) and angiopoietin 2 (Ang-2) in pregnancies that develop preeclampsia (PE) and gestational hypertension (GH).

**Methods:** Nested case control study drawn from a larger prospective longitudinal study in singleton pregnancies identified by screening at $11^{+0}$-$13^{+6}$ weeks' gestation as being at high-risk for PE. Blood samples were taken every four weeks until delivery. Values were compared in pregnancies that developed preterm-PE (requiring delivery before 37 weeks), term-PE, GH, and those that remained normotensive.

**Results:** A total of 471 samples were analyzed in 122 women, including 85 that remained normotensive, 12 that developed GH, 13 that developed term-PE and 12 that developed preterm-PE. In the normotensive group, there was an increase of $\log_{10}$sEng levels with gestational age. In the preterm-PE group, compared to the normotensive group, sEng was higher from 18 weeks onwards and the difference increased significantly with gestational age ($p<0.001$). In the GH and term-PE groups, sEng did not differ significantly from the normotensive group ($p=0.583$ and $p=0.890$, respectively). The square root of Ang-2 decreased significantly with gestational age, but did not differ significantly among the different outcome groups ($p=0.571$).

**Conclusion:** Maternal plasma sEng, but not Ang-2, may be a useful mid- and late-gestation biomarker for development of PE.

## Introduction

Pre-eclampsia (PE) remains a leading cause of maternal and perinatal mortality and morbidity in both developed and developing countries[1-3]. Several studies have reported that the maternal plasma concentration of soluble endoglin (sEng) is increased, both at the time of and prior to the onset of the clinical diagnosis of PE[4-14]. These changes have been described as early as the first trimester[15,16], and levels return to normal after pregnancy[17,18]. Most studies were cross-sectional and have focused on specific gestational age windows, commonly 11-13, 20-24 and 30-33 weeks[15,16,19,20]. Fewer studies have investigated the longitudinal changes in sEng levels in women who go on to develop PE[12,21-24]. The results of these studies have proposed that the longitudinal changes are better predictors of PE than measurement at a single time point in pregnancy and that increased sEng levels also appear to be more strongly associated with early-onset disease[22,25]. Most of these longitudinal studies have included mainly low-risk pregnancies.

The evidence around angiopoietin-2 (Ang-2) levels in PE is controversial. While some studies have reported lower levels in pregnancies complicated by PE[26,27], others have reported increased levels in severe PE[28], higher levels before the onset of clinical PE[29,30] and reduced placental tissue levels of Ang-2 mRNA compared to gestational age matched controls[31]. Still, some studies have failed to demonstrate any significant change in PE, either in the first[32,33] or third[34] trimesters Studies of maternal serum Ang-2 levels in PE have been mainly small and cross-sectional[26,27,32] and the investigators have called for larger cohort and longitudinal studies.

The aim of this study was to investigate the longitudinal changes in maternal plasma concentrations of sEng and Ang-2, from the first trimester onwards, in women identified as high-risk following first trimester screening and subsequently developed PE, gestational hypertension (GH) or remained normotensive.

## Methods

### Study Population

At University College London Hospital, the risk for development of PE was routinely assessed at $11^{+0}$-$13^{+6}$ weeks' gestation, using a combination of maternal history, uterine artery Doppler mean pulsatility index, mean arterial pressure and serum pregnancy associated plasma protein A[35]. Those considered to be at high risk for early-onset PE were followed up in a specialist hypertension clinic in which blood samples were collected every four weeks until delivery. The study took place between December 2009 and May 2012. Written informed consent was obtained from all women participating in the study which was approved by the London-Surrey Borders Research Ethics Committee. None of these pregnancies were complicated by aneuploidy or major structural abnormalities.

Maternal plasma sEng and Ang-2 levels were measured in 122 women: 12 that later developed PE and required delivery before 37 weeks' gestation (preterm-PE), 13 that developed PE requiring delivery at or after 37 weeks (term-PE), 12 that developed gestational hypertension (GH) and 85 unaffected controls. The controls had one or more samples within the preselected gestational age windows of 11-14, 15-19, 20-24, 25-29, 30-34, and 35-38 weeks' gestation, delivered phenotypically normal babies of appropriate weight for gestational age at term and did not develop any hypertensive disorder of pregnancy. None of the samples in this nested case-control study were previously thawed and re-frozen.

Patient Characteristics

Patient characteristics that were recorded included maternal age, racial origin (Caucasian, Afro-Caribbean, South Asian, East Asian or mixed), method of conception (spontaneous or assisted requiring the use of ovulation inducing drugs), cigarette smoking during pregnancy, history of chronic hypertension or pre-existing diabetes mellitus, history of PE in the mother of the patient, and obstetric history including parity (parous or nulliparous if no previous pregnancies at or after 24 weeks) and previous pregnancy with PE. Maternal weight and height were also measured, and body mass index (BMI) was calculated.

Sample Analysis

The scientist performing the assays was not aware of the clinical data of the patients and was blinded to the pregnancy outcome. Plasma sEng was measured by enzyme-linked immunoassay (ELISA) technique (Quantikine Endoglin ELISA kit; R&D Systems Europe Ltd, Abingdon, UK) and the lower limit of detection of the assays was 0.007μg/l. Plasma Ang-2 was measured by ELISA technique (Quantikine Angiopoietin-2 ELISA kit; R&D Systems Europe Ltd, Abingdon, UK) and the lower limit of detection of the assays was 8.29ng/l. The samples were analysed in duplicate and the tests performed according to the manufacturer's recommendations

Outcome Measures

Data on pregnancy outcomes were collected from the women's hospital maternity records. The obstetric records of women with pre-existing or pregnancy associated hypertension were examined to determine if the condition was chronic hypertension, PE or GH. The diagnosis of PE and GH was made according to the guidelines of the International Society for the Study of Hypertension in Pregnancy[36]. In GH the systolic BP should be 140 mm Hg or more and/or the diastolic BP should be 90 mmHg or more on at least two occasions four hours apart developing after 20 weeks of gestation in a previously normotensive woman in the absence of significant proteinuria. In PE there should be GH with proteinuria of 300 mg or more in 24 hours or two readings of at least ++ on dipstick analysis of a midstream or catheter urine specimen, if no 24-hour collection is available. In PE superimposed on chronic hypertension, significant proteinuria (as defined above) should develop after 20 weeks of gestation in a woman with known chronic hypertension (history of hypertension before conception or the presence of hypertension at the booking visit before 20 weeks of gestation in the absence of trophoblastic disease).

Statistical analysis

Maternal baseline characteristics were compared by $X^2$-test or Fisher's exact test for categorical variables and Kruskal-Wallis test for continuous variables, and comparisons between different outcome groups was by Mann-Whitney test with post-hoc Bonferroni correction for multiple comparisons. Data are presented as median and interquartile ranges (IQR) for continuous data and as n (%) for categorical variables.

The distribution of maternal plasma sEng was $\log_{10}$ transformed, while the distribution of Ang-2 was confirmed to be Gaussian after square root transformation, and it was tested using the Kolmogorov-Smirnov test. Analysis of repeated measures with multilevel mixed-effects linear model (fixed effects and random effects) was performed. The fixed effect component included up to third order polynomial terms of gestational age, hypertensive disorders (PE or GH) and first order interaction between gestational

age and each hypertensive disorder. The random effect component included the intercept, and linear effects of gestational age. Repeated measurements at different weeks of gestation in the same woman constituted level 1 and each individual constituted level 2. The multilevel model was compared to one-level model by the likelihood radio (LR) test. Prior to performing the regression analysis, continuous variables were centered by subtracting the mean from each measured value (70 from maternal weight in kg, 164 from maternal height in centimeters and 32 from maternal age in years). Logistic regression analysis was performed for the prediction of preterm preeclampsia in the first and second trimester. The performance of screening by log10 sEng at 11-13 weeks and at 19-22 weeks was determined by receiver operating characteristic (ROC) curves.

The software programmes MLwiN 2.28 (Centre for Multilevel Modelling, University of Bristol, Bristol, UK) and IBM SPSS Statistics 20 (SPSS Inc., Chicago, IL, USA) were used for the statistical analysis.

## Results

### Study population

Both sEng and Ang-2 were analyzed in a total of 471 samples from 122 women, including 85 that remained normotensive and 12, 13 and 12 that developed GH, term-PE and preterm-PE, respectively. The maternal characteristics of each outcome group are summarized in Table 1. The median maternal weight was greater in women that developed term-PE (p=0.036). There were no significant differences between the different outcome groups in maternal age (p=0.164), height (p=0.095), ethnicity (p=0.079), parity (p=0.970), smoking status (p=0.638), mode of conception (p=0.117) or chronic hypertension (p=0.067).

### Soluble Endoglin

$Log_{10}$ sEng decreased significantly with maternal weight, but was not significantly affected by ethnic origin (p=0.157), maternal age (p=0.968), height (p=0.142), smoking status (p=0.513), mode of conception (p=0.487), previous history of PE (p=0.242) or chronic hypertension (p=0.413), (Table 2). The multilevel model provided a significantly better fit to the data than the single-level model (LR=152, degrees of freedom=3, p<0.001).

In the normotensive group, there was a quadratic increase in $log_{10}$sEng with gestational age (Table 2, Figure 1). In the preterm-PE group, compared to the normotensive group, sEng was higher from 18 weeks onwards and the difference increased significantly with gestational age. In the GH and term-PE groups, sEng did not differ significantly from the normotensive group (p=0.583 and p=0.890, respectively).

### Angiopoietin-2

The square root of Ang-2 decreased significantly with gestational age (Table 2, Figure 2), but did not differ significantly among the different outcome groups (p=0.571), or with maternal age (p=0.115), height (p=0.083), weight (p=0.293), ethnic origin (p=0.345), history of PE (p=0.091), smoking status (p=0.658), mode of conception (p=0.897) or chronic hypertension (p=0.983). The multilevel model provided a significantly better fit to the data than the single-level model (LR=289, degrees of freedom=3, p<0.001).

Logistic regression analysis showed that $\log_{10}$ sEng was a significant predictor for preterm preeclampsia at 19-22 weeks ($p<0.001$), but not at 11-13 weeks ($p=0.155$). The area under the ROC curve (AUC) for the prediction of preterm-PE was 0.58 (95% CI, 0.40-0.75) at 11-13 weeks, and 0.81 (95%CI, 0.62-0.99) at 19-22 weeks.

## Discussion

### Main findings of the study

The study has demonstrated that in normotensive pregnancies there is a quadratic increase in sEng with gestational age with a trough at 20-22 weeks, whereas there is a decrease in Ang-2 levels with gestation. In preterm-PE, compared to the normotensive controls, sEng is significantly higher from an early stage in pregnancy, and the difference is increasing with gestation. Maternal plasma Ang-2 levels are similar in the pregnancies complicated by preterm-PE and those which remained normotensive. In term-PE and GH, neither sEng nor Ang-2 is significantly different from the normotensive group.

### Strengths and limitations of the study

The major strengths of this study are its prospective longitudinal design, the use of a well-defined methodology, and the application of a robust statistical approach that takes into account not only the difference in marker levels in the outcome groups but also the change with gestational age. This approach is different from the calculation of trends with gestation based on large numbers of cross-sectional and unrelated measurements. A limitation of the study is the relatively small number of cases.

### Interpretation of results, comparison with previous studies and future implications

Endoglin (Eng) is a trans-membrane glycoprotein that acts as co-receptor for transforming growth factor (TGFβ) and is highly expressed on cellular membranes of the vascular endothelium and on the syncytiotrophoblast[37-40]. Eng is involved in angiogenesis and has a role in maintaining vascular tone[41,42]. Its soluble circulating form (sEng), produced through the proteocleavage of the placental membrane-bound form, is an anti-angiogenic factor, which was first implicated in the pathogenesis of PE and hemolysis elevated liver enzymes and low platelet (HELLP) syndrome in 2006[4,5]. Placental tissue expression of sEng is upregulated in patients with PE and administration of sEng in mice induces a PE-like syndrome with hypertension and proteinuria[4,8,43]. The discovery that the heme oxygenase (Hmox1)/carbon monoxide (CO) pathway inhibits sFlt-1 and sEng and that statins induce Hmox1 and suppress the release of sFlt-1 and sEng, has led researchers to propose statins and Hmox1 activators as potential novel therapeutic agents for treating PE[44-46]. This also explains the protective effect of smoking against placental cytotoxic damage associated with PE[44-46].

Our finding in normotensive pregnancies, of an initial decrease in maternal plasma sEng levels between 11 weeks and 22 weeks and subsequent increase with advancing gestational age, is compatible with the results of previous longitudinal studies[12,21-23]. Similarly, our finding of increased sEng in preterm-PE, prior to the clinical onset of the disease, is consistent with both cross-sectional and longitudinal studies[9,12,14,18,20,21-24]. In term-PE we found no significant differences in plasma sEng from normotensive controls. Previous longitudinal studies reported that in term-PE sEng sequential change was either higher[21] or not significantly different from controls[22].

The onset of significant increase in sEng levels in preterm-PE in our study was 18 weeks' gestation and this is similar to the gestation of 17 weeks reported in a previous longitudinal study[12]. The difference in sEng levels in the pregnancies complicated by preterm-PE and those that remained normotensive increased with gestation. This finding could have implications for the prediction of PE; measuring maternal plasma sEng levels during the second and third trimesters could potentially improve its predictive accuracy for PE. Studies have repeatedly demonstrated that, when measured longitudinally, changes in protein level are better predictors of disease than measurement at a single time point in pregnancy[25]. Compared with pregnancies where sEng levels decreased, those where the levels increased between the first and second trimesters had an odds ratio (OR) for preterm-PE of 14.9[22]. This association was stronger than that seen for soluble fms-like tyrosine kinase 1 (sFlt-1) or placental growth factor (PlGF). An increase in sFlt-1 alone had an OR of 3.9 and an increase in PlGF concentration less than the median had an OR of 4.3. When combined in a ratio, the change in PlGF/(sEng x sFlt-1) had an OR of 3.7 while the change in sEng/sFlt-1 had an OR of 10.4 for preterm-PE[22].

Angiopoietins are angiogenic growth factors released by the villous trophoblast and are thought to play an integral role in placental vascular development[47-49]. Angiopoietin-1 (Ang-1) promotes vascular maturation by recruiting and stabilizing pericytes, while Ang-2 is an inhibitor of Ang-1, loosening the attachment of pericytes[50-52]. The angiopoietins act via tyrosine kinase receptors expressed mainly in the endothelial cells (Tie-1 and Tie-2)[53,54].

We noted a significant decrease with gestational age, which was not reported in the study by Akolekar et al; this could be simply due to the narrow gestational age window between 11 and 13 weeks in that study[32]. Interestingly, our study was also consistent with Akolekar et al in reporting similar Ang-2 levels in pregnancies developing hypertension and those remaining normotensive[32]. Studies examining maternal serum Ang-2 levels in PE have reported contradictory results. This could be explained by the different study populations in different studies or sometimes failure to perform the correct statistical approach, such as adjusting for potential confounding variables or taking into account the effect of gestational age.

In the only previous longitudinal study, maternal plasma Ang-1 and Ang-2 levels were measured in 19 women with PE and 43 controls and reported that the ratio Ang1/Ang2 was significantly lower in PE at 25 and 28 weeks but not thereafter[29]. However, the performance of screening for PE of this test was poor with a detection rate (DR) of 47% and false positive rate (FPR) of 13% at 25 weeks and respective values of 50% and 20% at 28 weeks[29].

Conclusion

This study describes the longitudinal changes in maternal plasma sEng and Ang-2 levels in high-risk pregnancies which remained normotensive and those that developed GH or PE. These results are likely to be useful for the continuing efforts to improve the accuracy of predicting PE. In screening for PE, measuring maternal plasma sEng levels during the second and third trimesters is likely to improve its predictive accuracy and reduce the false positive rate. In contrast, Ang-2 is not a useful biomarker for PE.

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## Figure Legends

**Figure 1.** Maternal plasma soluble endoglin (sEng) levels in pregnancies with normal outcome. The solid line represents the mean values, while the interrupted lines represent the 5$^{th}$ and 95$^{th}$ centiles. The dotted lines show the 95% confidence intervals of the mean values.

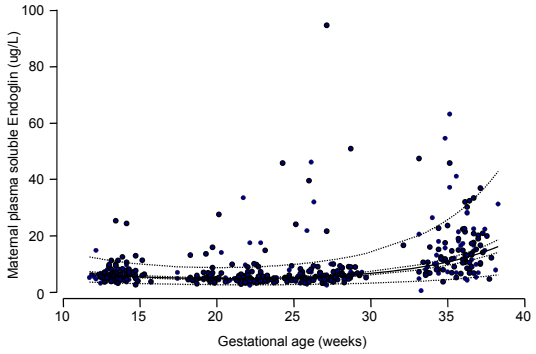


Figure 1

**Figure 2.** Maternal plasma soluble endoglin (sEng) levels in pregnancies with normal outcome (black) and those complicated (red) with preterm preeclampsia (PE), term PE and gestational hypertension (GH) for a woman of 70 kg of weight. The solid lines represent the mean values, while the interrupted lines show the 95% confidence intervals (CI) in each outcome group.

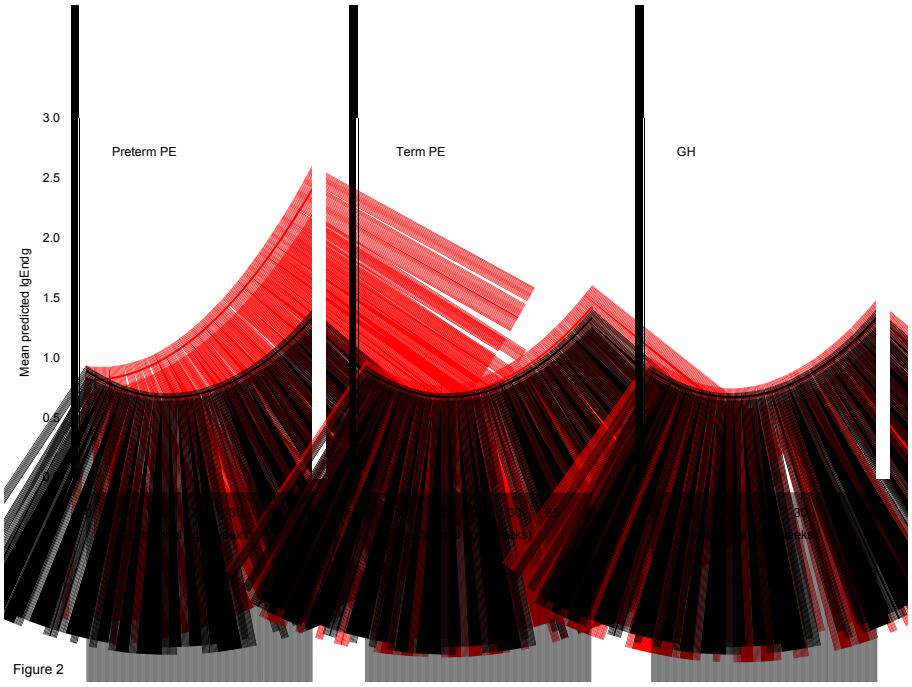


Figure 2

**Figure 3.** Individual maternal plasma soluble endoglin (sEng) levels in pregnancies with normal outcome (grey) and those complicated with preterm preeclampsia (PE) (red), term PE (pink) and gestational hypertension (GH) (blue).

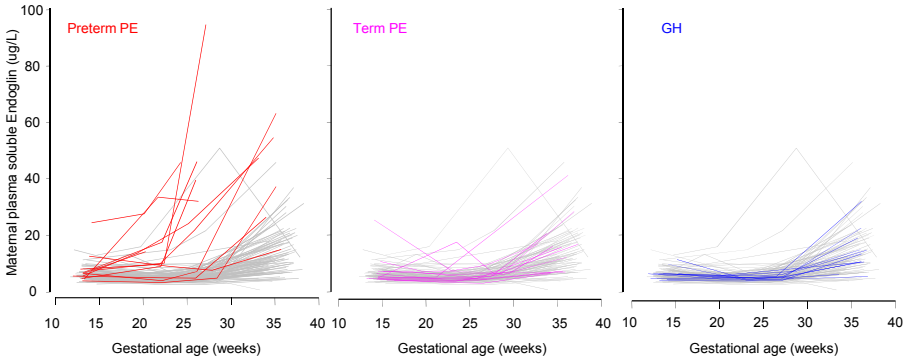


Figure 3

**Figure 4.** Maternal plasma angiopoietin-2 (Ang-2) levels in pregnancies with normal outcome. The solid line represents the mean values, while the interrupted lines represent the 5$^{th}$ and 95$^{th}$ centiles. The dotted lines show the 95% confidence intervals of the mean values.

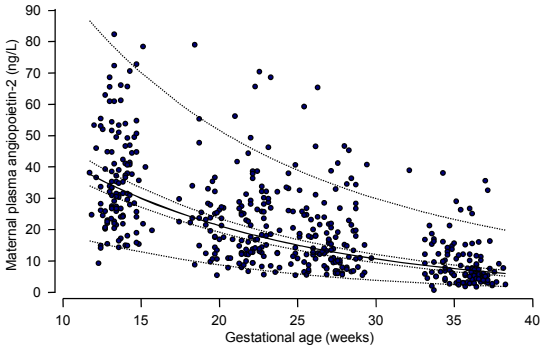


Figure 4

**Figure 5.** Maternal plasma angiopoietin-2 (Ang-2) levels in pregnancies with normal outcome (black) and those complicated (red) with preterm preeclampsia (PE), term PE and gestational hypertension (GH). The solid lines represent the mean values, while the interrupted lines show the 95% confidence intervals (CI) in each outcome group.

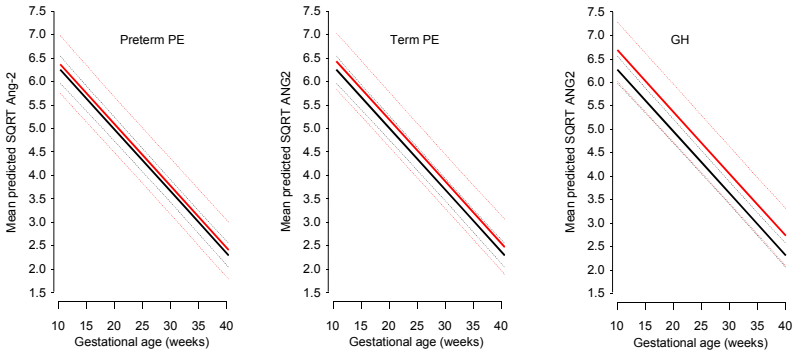


Figure 5

**Figure 6.** Individual maternal plasma angiopoietin-2 (Ang-2) levels in pregnancies with normal outcome (grey) and those complicated with preterm preeclampsia (PE) (red), term PE (pink) and gestational hypertension (GH) (blue).

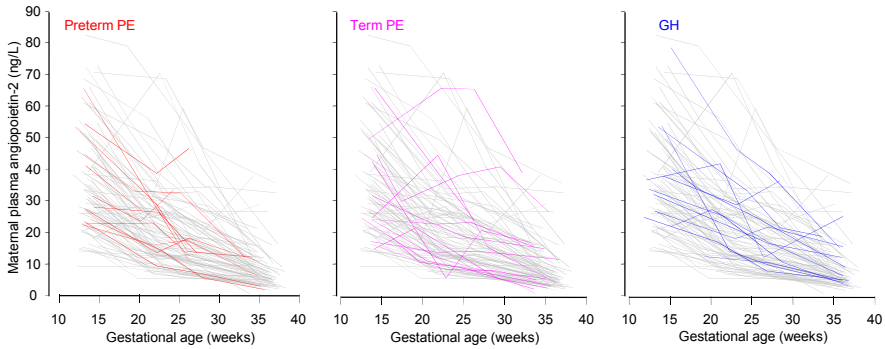


Figure 6

**Figure 7.** Receiver operating characteristic (ROC) curve for the prediction of preterm pre-eclampsia using maternal plasma soluble endoglin (sEng) levels at 11-13 weeks (solid line) and at 19-22 weeks (dashed line).

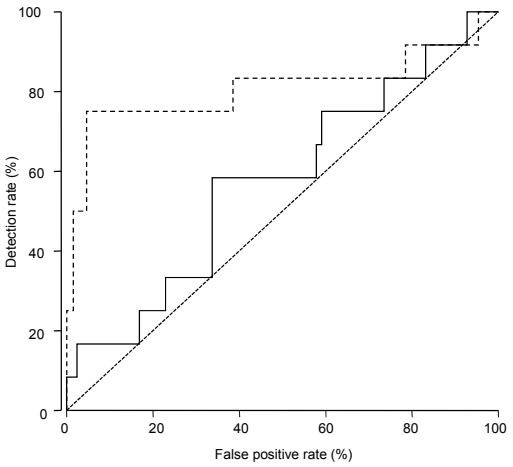


Figure 7

**Table 1.** Maternal characteristics in the outcome groups.

| Maternal Characteristics | Normal (n=85) | Gestational hypertension (n=12) | Term preeclampsia (n=13) | Preterm preeclampsia (n=12) |
|---|---|---|---|---|
| Maternal age in years, median (IQR) | 32.0 (28.5-35.5) | 34 (30.3-38.8) | 28.0 (25.5-33.5) | 28.0 (26.3-36.5) |
| Weight in kilograms , median (IQR) | 65.0 (56.5-75.0) | 72.5 (65.0-83.9) | 78.0 (64.5-93.3)* | 62.5 (56.8-65.3) |
| Height in cm , median (IQR) | 164.0 (160.3-167.8) | 165.0 (162.7-169.0) | 162.0 (157.0-167.6) | 157.0 (152.3-167.3) |
| Racial origin | | | | |
| Caucasian, n (%) | 66 (77.6) | 8 (66.7) | 5 (38.5) | 6 (50.0) |
| Afro-Caribbean, n (%) | 8 (9.4) | 3 (25.0) | 3 (23.1) | 3 (25.0) |
| South Asian, n (%) | 7 (8.2) | 1 (8.3) | 3 (23.1) | 3 (25.0) |
| East Asian, n (%) | 2 (2.4) | 0 | 2 (15.4) | 0 |
| Mixed, n (%) | 2 (2.4) | 0 | 0 | 0 |
| multiparous, n (%) | 24 (28.2) | 3 (25.0) | 3 (23.0) | 4 (33.3) |
| Cigarette smoker, n (%) | 4 (4.7) | 1 (8.3) | 0 | 0 |
| Mode of conception | | | | |
| Spontaneous, n (%) | 80 (94.1) | 10 (83.3) | 12 (92.3) | 12 (100) |
| Ovulation induction, n (%) | 3 (3.5) | 2 (16.7) | 1 (7.7) | 0 |
| In vitro fertilization, n (%) | 2 (2.4) | 0 | 0 | 0 |
| Chronic hypertension, n (%) | 1 (1.2) | 0 | 2 (15.4) | 1 (8.3) |
| Gestational age at delivery, median (IQR) | 39.7 (38.9-40.6) | 40.2 (38.0-41.4) | 39.3 (39.4-40.0) | 33.0 (29.3-36.5)** |

IQR=interquartile range

Comparisons between outcome groups (Chi-square test for categorical variables and Kruskall-Wallis for continuous variables). Comparison of each outcome group with normal outcome by Mann-Whitney Test with post-hoc Bonferroni correction. *p<0.005, **p<0.001

**Table 2.** Application of Multilevel Linear Mixed-effects model on $Log_{10}$ Endoglin and Square root Angiopoietin-2.

| | Soluble Endoglin | | | Square root Angiopoietin-2 | | |
|---|---|---|---|---|---|---|
| **FIXED PART** | | | | | | |
| | **Estimate** | **Std Error** | **P value** | **Estimate** | **Std** | **P value** |
| Intercept | 1.445100 | 0.061509 | <0.001 | 7.562358 | 0.164446 | <0.001 |
| Gestational hypertension | 0.050212 | 0.091158 | 0.583 | | | |
| Term preeclampsia | -0.012157 | 0.088104 | 0.890 | | | |
| Preterm preeclampsia | -0.467573 | 0.096719 | <0.001 | | | |
| Gestational age (weeks) | -0.074952 | 0.005000 | <0.001 | -0.132031 | 0.004696 | <0.001 |
| Gestational age (weeks)$^2$ | 0.001826 | 0.000097 | <0.001 | | | |
| Gestational age (weeks)$^3$ | | | | | | |
| Interaction Gestational hypertension with gestational age (weeks) | -0.002191 | 0.004164 | 0.600 | | | |
| Interaction Term preeclampsia with gestational age (weeks) | 0.003681 | 0.004060 | 0.366 | | | |
| Interaction Preterm preeclampsia with gestational age (weeks) | 0.038663 | 0.004628 | <0.001 | | | |
| Weight-70 | -0.003202 | 0.000816 | <0.001 | | | |
| | | | | | | |
| **RANDOM PART** | | | | | | |
| **Level 2:** | | | | | | |
| Variance (constant) | 0.047362 | 0.011781 | | 2.227997 | 0.434765 | |
| Variance (Gestational age) | 0.000122 | 0.000025 | | 0.001014 | 0.000385 | |
| Covariance (constant, gestational age) | -0.002046 | 0.000511 | | -0.035857 | 0.011638 | |
| **Level 1:** | | | | | | |
| Residual | 0.013761 | 0.001313 | | 0.402102 | 0.038064 | |