Original Articles

Prediction of pre-eclampsia during early pregnancy in primiparas with soluble fms-like tyrosine kinase-1 and placental growth factor


ABSTRACT

Background. We hypothesized that pre-eclampsia (PE) can be predicted early in primiparas by measuring serum levels of soluble fms-like tyrosine kinase-1 (sFlt-1) and placental growth factor (PIGF).

Methods. All normotensive primiparas attending the antenatal clinics of Aga Khan University Hospital and Aga Khan Hospital for Women, Karachi, Pakistan without any known risk factor for PE were invited to participate in the study. They were divided into two groups based on the development of PE. Their blood samples were collected at 8–15, 16–22, 23–28, 29–34 weeks of pregnancy and once within 1 week of delivery. All samples were analysed for sFlt-1 and PIGF.

Results. Six hundred and eleven (46.7%) of 1307 recruited primiparas completed the study according to the protocol. Of these, 39 (6.4%) women developed PE. The difference in serum sFlt-1 was evident as early as 15 weeks of gestation. Higher levels of serum sFlt-1 were present in women who later developed PE. Relatively higher levels of PIGF were observed in non-PE women compared to PE women up to 22 weeks of gestation. However, after 23 weeks of pregnancy, PIGF levels increased in both the groups, but less so in the PE group. Receiver operator characteristics (ROC) curve analysis showed that even in early pregnancy (<15 weeks of gestation), serum sFlt-1 alone has the potential to predict PE with area under the curve (AUC), sensitivity and specificity of 0.81, 75.9 and 72.4, respectively.

Conclusion. PE can be predicted in primiparas in the early part of second trimester with serum levels of sFlt-1 and in the later part of second trimester with serum levels of PIGF.

INTRODUCTION

Pre-eclampsia (PE) is a pregnancy-specific syndrome of elevated blood pressure and proteinuria after 20 weeks of gestation. Five to ten per cent of pregnancies get complicated with this condition. In addition to acute maternal and foetal morbidity and mortality, adverse long-term effects such as hypertension and other metabolic derangements have also been reported in children born to women with PE.

The pathophysiology of PE is not fully understood. However, it is agreed that widespread endothelial dysfunction is the hallmark feature of PE. Poor placentation has been proposed as a major contributory factor underlying the endothelial dysfunction in PE. Incomplete or lack of trophoblastic invasion of the spiral arteries results in placental ischaemia leading to the release of factor(s), which are responsible for damage to the maternal vascular endothelium. It has been shown that sera of PE patients injected into mice mimicked the clinical features of PE. Various circulating factors were suggested to be implicated in this endothelial dysfunction underlying PE but were subsequently excluded. However, Maynard et al. convincingly showed the association of PE with increased serum levels of soluble fms-like tyrosine kinase (sFlt-1) and corresponding decrease in levels of free vascular endothelial growth factor (VEGF) and placental growth factor (PIGF). Subsequent studies confirmed these findings and showed dysregulation of sFlt-1 and angiogenic factors.

VEGF and PIGF are growth factors which are key molecules in angiogenesis and vasculogenesis, in particular during embryogenesis, sFlt-1, also known as soluble vascular endothelial growth factor receptor (sVEGFR-1), is a soluble protein and splice variant of VEGFR or Flt-1. It lacks transmembrane and cytoplasmic domains whereas Flt-1 is a membrane-bound protein. Flt-1 binds to VEGF and PIGF and produces the biological responses of VEGF and PIGF. On the other hand, sFlt-1 acts as a decoy receptor and regulates the levels of free VEGF and PIGF available to signal via membrane-bound Flt-1.

The overall maternal mortality rate in Pakistan is 276/100 000 live-births and hypertensive disorders are the second leading cause of maternal mortality. By the time PE is diagnosed, it already has caused marked endothelial dysfunction in maternal vessels leading to placental hypoxia and compromised foetal growth. Clinically, it has been observed that the time between the first detection of hypertension and proteinuria and the subsequent development of serious complications such as seizures can be
of + or more on dipstick analysis of a midstream urine specimen). Currently, there is no specific treatment for PE. In a majority of cases, the only option available to a physician is termination of pregnancy. Placental hypoxia leading to foetal growth restriction increases foetal mortality when delivered before reaching the age of viability. Therefore, the information that a primipara with normal ongoing pregnancy is likely to develop PE during the later part of pregnancy would be important to both mothers and physicians.

We hypothesized that the pathophysiological process for the development of PE begins in the first trimester. The normal process of trophoblastic invasion is complete before 20 weeks of gestation. If raised level of sFlt-1 and corresponding decreased level of PIGF is the cause of endothelial dysfunction and hence PE, there will be altered levels of these proteins in women during early pregnancy, when PE is clinically unrecognizable. Thus, this study was conducted with the primary objective to determine if PE can be predicted in primiparas by measuring levels of sFlt-1 and PIGF in maternal serum. The secondary objective was to investigate and compare the pattern of changes in the levels of serum sFlt-1 and PIGF in primiparas who develop PE and those who do not.

METHODS
Study design and participants
This was a nested case–control study. The study participants were recruited from the antenatal clinics of Aga Khan University Hospital and Aga Khan Hospital for Women, Karachi, Pakistan. All primiparas attending antenatal clinics were screened for identification of potential study participants. The inclusion criteria were normotensive primiparas without any known risk factors such as small vessel disease (autoimmune diseases such as diabetes mellitus, systemic lupus erythematosus, etc.) and family history of hypertensive disorders of pregnancy. Normotensive primiparas with any of the above-mentioned risk factors, women with history of smoking, essential hypertension and evidence of renal disease were excluded from the study. Participants who developed hypertension before 20 weeks of pregnancy were also excluded. A primipara was defined as a woman with first pregnancy as well as those women who had a history of foetal loss in the previous pregnancy before 20 weeks of gestation. Gestational age was calculated from the last menstrual period.

Study protocol
Potential study participants were identified by trained research medical officers and recruited into the study after obtaining written informed consent. A semi-structured questionnaire was administered to collect the demographic information and contact details of the study participants. A flow chart of all the participants was maintained in which pulse, blood pressure, body temperature, weight and proteinuria status was recorded during all antenatal visits. Five ml of blood was drawn from all recruited participants during pregnancy at 8–15, 16–22, 23–28, 29–34 weeks and once within 1 week of delivery.

Each sample was timed with other routine antenatal investigations to avoid an extra phlebotomy in most patients. The participants were divided into two groups: those who developed PE (PE) and those who did not develop PE (non-PE). PE was defined according to the guidelines of the International Society for the Study of Hypertension in Pregnancy, which considers PE as (i) systolic blood pressure of ≥140 mmHg and diastolic blood pressure ≥90 mmHg after 20 weeks of gestation in a previously normotensive patient, and (ii) new onset proteinuria (two readings of + or more on dipstick analysis of a midstream urine specimen).

From the pool of non-PE patients, serum samples of a similar number of women (in a ratio of 1:1 with PE patients) were taken for analysis who were comparable with the PE patients in terms of age, gestational age and body mass index (BMI) at the time of each blood sampling. The recruited participants were excluded when they developed other risks factors of PE such as gestational diabetes, twin pregnancy and essential hypertension. The participants were considered to have completed the study when no more than one sample was missed from them.

Sample collection and measurement of serum sFlt-1 and PIGF
Venepuncture was done and blood collected into serum tubes. The samples were centrifuged and serum was separated. Aliquots were prepared and stored at −70°C till subsequent use. The concentrations of sFlt-1 and PIGF were measured using enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, Minn, USA). Briefly, the microtitre plates were coated with monoclonal antibodies specific for either sFlt-1 or PIGF and standards and samples were added to the wells. During the incubation period, sFlt-1 or PIGF present in the standards and samples became bound to the immobilized antibody. After a thorough wash, an enzyme-linked polyclonal antibody specific for each protein was pipetted into the wells, and after a second incubation and wash step, a substrate solution was added and colour developed in proportion to the amount of bounded protein. The colour development was subsequently stopped and the absorbance at 540 nm with wavelength correction at 450 nm was measured using an ELISA Plate Reader (Dynatech MR 5000). The results were calculated according to the manufacturer’s recommendations. The inter- and intra-assay coefficients of variation were 3.8% and 7% for sFlt-1 and 3.6% and 11% for PIGF, respectively.

Sample size
A minimum of 30 participants in each group (PE and non-PE) were required to achieve 80% power and 5% level of significance, assuming a minimum change of 20% in mean sFlt-1 and PIGF readings and 30% change in coefficient of variation of sFlt-1 and PIGF readings.

Statistical analysis
The data were entered twice by two different data entry operators in EPIDATA (version 3.1). The final data were converted into SPSS (version 16.0) for analysis. Age and height of the participants were compared for PE and non-PE groups using independent samples t-test. Gestational ages, weights and BMI at the time of each blood sampling for PE and non-PE groups were also compared using independent samples t-test. Repeated measures ANOVA was used to investigate the pattern of changes in the levels of sFlt-1 and PIGF for the two groups at different gestational ages. Graphs of sFlt-1 and PIGF levels at different gestational ages are reported for the two groups. Receiver operator characteristics (ROC) curve analysis was done and locally derived cut-off values for sFlt-1 were obtained for each range of gestational age (weeks). Area under receiver operator curve (AUC), sensitivity, specificity, negative predictive value and positive predictive value with 95% CI were reported for each gestational age (weeks). An AUC of 0.5 showed no discrimination and 1 showed complete discrimination.

RESULTS
A total of 1665 primipara women were identified as potential study subjects based on the inclusion criteria of the research study.
Of these, 1307 (78.5%) gave consent to participate in the study and 611 (46.7%) completed the study as per protocol (Fig. 1). Of these 611 primiparas, 39 (6.4%) developed PE. The first blood sample was missing in 5 study participants and the second, third and fourth blood samples were missing in 2, 3 and 2 participants, respectively. Among the 39 PE women included, the first, second, third and fourth blood sample was missing in 5, 2, 3 and 3 women, respectively. As a result, the corresponding samples from the non-PE group were also not analysed. A comparison of the characteristics of the PE and non-PE groups is shown in Table I.

**Gestational changes in serum sFlt-1 levels**

Figure 2 shows a comparison of changes in the levels of sFlt-1 according to gestational age in PE and non-PE groups. A statistically significant difference in the serum sFlt-1 level was evident as early as in the first sample (up to 15 week of gestation; mean gestational age 11.6 weeks; range 7.3–15 weeks). The difference in the protein levels widened as pregnancy progressed from 22 weeks onwards and peaked after 29 weeks of gestation. Exceptionally higher levels of serum sFlt-1 were found to be present in women who developed PE. The difference in levels of sFlt-1 between PE and non-PE groups was not seen after termination of pregnancy (Fig. 2).

**Gestational changes in serum PIGF levels**

Higher levels of serum PIGF were observed in the non-PE group compared with those in the PE group up to 22 weeks of gestation, but were not statistically significant. However, with progression of pregnancy beyond 23 weeks, serum PIGF levels increased in both the groups but less in the PE group compared with the non-PE group (Fig. 3). In samples obtained after 29 weeks of pregnancy, PIGF continued to increase in non-PE women but decreased in PE women, thus widening the difference between the two groups (Fig. 3). Similar to sFlt-1, the difference in the serum levels of PIGF between the two groups was no longer significantly after termination of pregnancy (Fig. 3).

**Predictive potential of serum sFlt-1 for PE**

Figure 4 shows the raw data of serum sFlt-1 values of PE and non-PE women. The pattern of changes in sFlt-1 levels at different gestational ages is different between the two groups. To determine the predictive value of sFlt-1, ROC curves for sFlt-1 and PIGF at different gestational ages were built and analysed (Fig. 5). The predictive accuracy as measured by AUC was much better for sFlt-1 as compared with PIGF. The cut-off values, AUC, sensitivity, specificity, positive predictive value and negative predictive values for sFlt-1 at various gestational ages are shown in Table II. It is obvious from this data that even in early pregnancy (<15 weeks of gestation), sFlt-1 alone has the potential to predict PE with AUC, sensitivity and specificity of 0.81, 75.9 and 72.4, respectively. The cut-off value at this gestational age was found to be 2106.84 pg/ml.

**DISCUSSION**

We observed changes in the levels of serum sFlt-1 and PIGF at various gestational ages and compared them with PE and non-PE.

![Fig 1. Study outcomes of women after counselling (for definitions, see Methods)](image)

![Fig 2. Patterns of changes in serum levels of soluble fms-like tyrosine kinase-1 (sFlt-1) in non-pre-eclamptic and pre-eclamptic women. The mean (SD) of sFlt-1 concentration have been presented at four times during pregnancy and once within 1 week of delivery. The p value represents the statistical difference between the two groups at each gestational age.)](image)
primiparas to determine the predictive potential of these proteins for PE (Fig. 5). We showed that PE can be predicted by measuring serum levels of sFlt-1 and PlGF at a stage when the syndrome is clinically unrecognizable. All PE women had significantly higher levels of sFlt-1 and correspondingly lower levels of PlGF at gestational ages where their blood pressure was within normal limits. To the best of our knowledge, this is the first study to show that PE can be effectively predicted in the early part of second trimester in primiparas with no other known risk factors for developing PE.

The finding that there are altered levels of angiogenic and anti-angiogenic factors in women who are likely to become pre-eclamptic has already been observed. However, our study focuses only on primiparas, who were selected for this study as nulliparity itself is a risk factor for the development of PE. Previous studies have shown that nulliparity increases the risk of developing PE up to three-fold. Women who had any other known risk factors for developing PE were excluded from the study. Smokers were also not included in the study because this has been shown to alter the sFlt-1 levels. BMI was matched between PE and non-PE women because obesity has been reported to increase the risk of PE. This approach effectively minimized the influence of confounding factors and we were able to determine the predictability of PE only in a selected population of high-risk group, i.e. primiparas. Of importance is the finding that PE in primiparas can be predicted as early as 15 weeks of gestation with the help of increased levels of serum sFlt-1, whereas previous studies have shown the predictive value of this and other proteins in later parts of pregnancy especially during the late second or early third trimester. This shows that raised level of serum sFlt-1 can predict PE much earlier in primiparas who do not have any other known risk or confounding factor. The ratio of sFlt-1 and PlGF has been reported to be a more sensitive parameter for the prediction of PE. However, in this study the sensitivity and specificity of this ratio was not better than that for sFlt-1 alone (data not shown).

We have found higher levels of serum sFlt-1 in PE women even before placentation is complete, which is about 16–18 weeks of gestation. Our study does not answer whether a defect in placentation culminates in the imbalance between angiogenic and anti-angiogenic factors or vice versa. Generally, the placenta is considered to be the source of increased production of sFlt in PE. However, this question about whether placental hypoxia is responsible for increased levels of sFlt-1 or higher levels of sFlt-1 cause placental hypoxia in PE, has been discussed by Karumanchi and Bdlolah. They suggest that increased production of sFlt-1 and placental hypoxia may complement each other and create a vicious cycle resulting in PE. In fact, the role of sFlt-1 in angiogenesis has been

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<th>Table II. Cut-off values of soluble fms-like tyrosine kinase 1 (sFlt-1) at different gestational weeks for the prediction of pre-eclampsia</th>
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<td>Gestational age (weeks)</td>
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AUC area under ROC curve  PPV positive predictive value  NPV negative predictive value

Fig 3. Patterns of changes in serum levels of placental growth factor (PlGF) in non-pre-eclamptic and pre-eclamptic women. The mean (SD) of PlGF concentration have been presented at four times during pregnancy and once within 1 week of delivery. The p value represents the statistical difference between the two groups at each gestational age.

Fig 4. Raw data of serum sFlt-1 levels of pre-eclamptic and non-pre-eclamptic women at various gestational ages.
explored and it has been found that raised levels of sFlt-1 inhibit angiogenesis and produce endothelial dysfunction. From these studies and our findings, it is reasonable to suggest that dysregulation of sFlt-1 may be an important causal event leading to poor placentation and subsequent PE.

The pattern of changes in the levels of sFlt-1 and PlGF as shown in this study is consistent with the findings of Levine et al. who have shown that levels of sFlt-1 remain stable in normotensive pregnancies till 29 weeks of gestation with a steep rise after that, and more so in women who develop PE.11

Corresponding to the increase in levels of serum sFlt-1, a decline in the level of PIGF was observed in samples of PE women. Contrary to the finding in PE women, PIGF continued to increase even in the last part (29 weeks and onwards) of the pregnancy in normotensive women despite a modest rise in sFlt-1 levels during that time.

Our study is the first to show that PE can be effectively predicted in primiparas who are at high risk of developing PE in the early part of second trimester with circulating levels of serum sFlt-1 and in the later part of second trimester with serum PIGF.

It is important to know as early as possible that a particular woman is likely to develop PE since much of the infant mortality in PE is attributable to prematurity. We have shown that altered levels of serum sFlt-1 and PIGF can safely predict PE much earlier than the clinical onset of this syndrome. Effective prediction of PE will open the arena for developing compounds aimed at enhancing pro-angiogenic molecules and/or antagonizing the anti-angiogenic factors. This approach might allow delivery to be safely postponed to a stage when there are better chances of survival of the foetus.

There are some limitations of our study. We do not have data on the age of onset and severity of PE. Therefore, we could not analyse the correlation between levels of angiogenic factors and these attributes. Another limitation is that the age of delivery, foetal outcome and infant data have not been analysed between the two groups.

In conclusion, PE can be predicted in primiparas in the early part of second trimester by measuring serum sFlt-1 and in the later part of second trimester with serum PIGF.
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REFERENCES