Research Article

Prediction of preeclampsia with novel biomarkers At second trimester of pregnancy

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Abstract
Objective: To evaluate the ability of the soluble form of vascular endothelial growth factor receptor (sFlt-١), neutrophil-flt-١, monocyte-flt-١, pentraxin٣ (PTX٣), nitric oxide (NO) and alpha fetoprotein (AFP) measurements at gestational weeks ١٤-١٨ to predict preeclampsia. **Subjects:** ٢٥ preeclamptic females, ٢٥ normotensive pregnant females and ٢٥ healthy non-pregnant females served as control. **Methods:** Maternal samples were collected at ١٤-١٨ gestational weeks. EDTA samples were investigated for both neutrophil- and monocyte-flt-١. Serum samples were frozen and stored at -٤٠ºC. Levels of sFlt-١, PTX٣, NO and AFP were measured in the stored serum samples. **Results:** Alpha fetoprotein, sflt-١ and pentraxin ٣ were found to be statistically significantly increased in group III when compared with group II (P-value = ٠.٠٢٤, P-value = < ٠.٠٠١ & P-value = ٠.٠٠٦ respectively). However, there was statistically significant decrease in neutrophil-flt-١ and nitric oxide in group III when compared with group II (P-value = < ٠.٠٠١ & P-value = ٠.٠١٦). Soluble flt-١ was found to have the highest predictive value for predicting preeclampsia (AUC = & P-value = < ٠.٠٠١) **Conclusion:** Soluble flt-١ was the best single biomarker to predict preeclampsia at second trimester of pregnancy.
Keywords: Preeclampsia, Biomarkers, alpha fetoprotein, sflt-١, neutrophil-flt-١, monocyte-flt-١, pentraxin ٣ and nitric oxide.

Introduction

Preeclampsia (PE) is a major contributor to the maternal and neonatal mortality and morbidity. It is the ٢nd largest cause of maternal mortality affecting ٣٪ to ٨٪ of pregnant women worldwide(١).

Preeclampsia is a multisystemic disorder of unknown etiology. The symptoms of PE manifest themselves in the late second or third trimester of pregnancy. It can be manifested as a maternal syndrome (hypertension, proteinuria with or without other multisystemic alterations), or as a fetal syndrome (fetal growth restriction, diminished amniotic fluid, and altered oxygenation). A rare complication of the condition is eclampsia involving encephalopathy and seizures, sometimes with a fatal result. Fetal complications include low birth weight, prematurity or perinatal death(٢).

Therefore we are in need of a widely applicable and affordable test that could permit presymptomatic diagnosis in order to identify and monitor the patients at risk for developing preeclampsia and thus provide the best prenatal care for these women and their child(٣).

Preeclampsia has been associated with an elevation of alpha fetoprotein (AFP) in maternal serum. Subsequent investigations have clarified that in a significant proportion of women, elevated maternal AFP is associated with a higher frequency of adverse pregnancy outcomes at later gestations. Such adverse outcomes included preeclampsia, intrauterine growth restriction (IUGR), antepartum bleeding, and preterm labor(٤).

Previous studies have shown that placenta of preeclamptic women secretes multiple proteins in response to impaired placental perfusion and hypoxia. One of the secreted placental factors is vascular endothelial growth factor (VEGF) which is a multifunctional cytokine that plays a pivotal role in angiogenesis in vivo. It exerts its biological effects through VEGF receptor-١ [Fms-like tyrosine kinase ١ (Flt-١)] and VEGF receptor-٢. Flt-١ has recently gained attention because of its overall implication in pathological angiogenesis and inflammation.

Placental cells also secrete a soluble isoform of flt-١(sflt-١) that acts as an anti-angiogenic factor by interacting with, and thereby neutralizing, VEGF. Also, the increased level of sflt-١ during PE inhibits the upregulation and decreases neutrophil expression of flt-١ by neutralizing VEGF[٥].

Long pentraxins such as long pentraxin ٣ (PTX٣) are produced in several tissues. Pentraxin ٣ has been proposed as a marker of endothelial dysfunction and inflammation in PE due to continuous shedding of placental debris into maternal circulation. Pentraxin ٣ levels have been found to be elevated in normal pregnancy and also shown to be significantly higher at the time of diagnosis of PE when compared with normal pregnancy. Additional longitudinal studies throughout pregnancy may be warranted to determine if PTX٣ will be a useful early marker for PE[٦].

Nitric oxide (NO) production is significantly elevated in normal pregnancy. Because NO is an important physiological vasodilator in normal pregnancy, it follows that NO deficiency during PE has been implicated in the disease process. It is unclear whether NO deficiency occurs in women with PE. Nitric oxide deficiency can be attributed to either increased oxidative stress during PE or the effect of placental factors inhibiting synthesis of NO. Much of the uncertainty in this area of research originates from the difficulty in directly assessing the activity of the NO system in the clinical setting[٧].

Aim of the study

To study the clinical utility of AFP, sflt-١, neutrophil-flt-١, monocyte-flt-١, PTX٣ and NO as predictive biomarkers of preeclampsia in first half of the second trimester pregnant women before the appearance of clinical symptoms of the disease.

Material and Methods

Fifty pregnant women who attended the Minia University Hospital for routine antenatal care, over a period of one year, between ١٤-١٨ weeks of gestation and ٢٥ age matched apparently healthy females (served as control) were included in the study after obtaining informed consent. Women with history of chronic hypertension, diabetes and renal disease were excluded from the study. Women who had proteinuria by dipstick method at booking, as well as those with a baseline blood pressure of more than or equal to ١٤٠/٩٠mmHg at booking were excluded from the study. All women were clinically evaluated at booking to rule out exclusion criteria. All these women were followed up till delivery.

Blood pressure was measured in the semi recumbent posture with a left lateral tilt, in the right arm and proteinuria was excluded by testing the spot sample for albumin by dipstick method.

A spot urine sample was collected for estimation of calcium, creatinine and albumin.

Six ml of venous blood were withdrawn by sterile venipuncture without prior fasting. Two ml of venous blood were divided equally into ٢ EDTA-containing tube and mixed well. The first tube was used for estimation of complete blood count (CBC) and flowcytometric analysis of CD١٣, CD١٤ and Flt-١. The other tube was centrifuged at ٣٠٠٠ rpm for ١٥ minutes, plasma separated, aliquoted and kept frozen at -٤٠°C until analysis of pentraxin٣. Four ml of venous blood were evacuated into plain tube, allowed to clot for ٣٠ minutes after collection then centrifuged at ٣٠٠٠ rpm for ١٥ minutes to separate serum. Half ml of serum was used for instant analysis of random blood glucose, blood urea, serum creatinine, uric acid, total bilirubin, ALT, AST and albumin. The remaining serum was aliquoted and kept frozen at -٤٠°C until analysis of AFP, sFlt-١ and nitric oxide.

Complete blood count: determined by automated cell counter Sysmex K-٢١n ***(TAO Medical Incorporation, Japan)***.

Glucose, blood urea, serum and urine creatinine, uric acid, total bilirubin, ALT, AST and serum albumin: determined by fully automated clinical chemistry analyzer Konelab ٢٠i ***(Thermo Scientific, Finland)***.

Serum and urine calcium: determined by Flexor EL٢٠٠ ***(Elitech Clinical Systems, Puteaux France)***.

Albumin/creatinine ratio: urinary albumin was determined in a random urine sample by turbidimetric method using sulfosalicilic acid ٣٪ to precipitate albumin in urine then the absorbance was measured using spectrophotometer at wave length ٥٧٨ nm. the ratio is then calculated by dividing urinary albumin (mg/dl) on urinary creatinine (gm/dl). Levels were considered normal if less than ٣٠, microalbumin if values were ٣٠ to ٣٠٠ and albuminuria if greater than ٣٠٠ [٨].

Calcium/creatinine ratio: was calculated by dividing urinary calcium (mg/dl) on urinary creatinine (gm/dl) in a random urine sample. Normal values were considered normal if less than ٢٢٠ [٩].

The AFP was based on the principle of a solid phase enzyme-linked immunosorbent assay ***(Kits were supplied by R&D systems)***.

Soluble flt-١ assay employed the quantitative sandwich enzyme immunoassay technique. ***(Kits were supplied by R&D systems)***.

Maternal neutrophils and monocytes were identified using monoclonal mouse anti human antibodies against neutrophil surface markers CD١٣ conjugated with fluorescent isothiocyanate (FITC) ***(Clone WM-٤٧, Dako, Denmark)*** and against monocyte surface markers CD١٤ conjugated with FITC for CD١٤ ***(Clone TUK٤, Dako, Denmark)***.

Washed cells from whole blood were incubated with FITC- conjugated antibodies against neutronphils CD١٣ along with the phycoerythrin (PE)-labeled monoclonal antibody, which bound to cells expressing VEGF R١ in one tube. FITC-conjugated antibodies against monocyte CD١٤ were incubated along with PE-labeled monoclonal antibody against VEGF R١ in another tube. Unbound PE-conjugated antibody was then washed from the cells. Cells expressing VEGF R١ were fluorescently stained, with the intensity of staining directly proportional to the density of VEGF R١. Cell surface expression of VEGF R١ was determined by flow cytometric analysis using ٤٨٨ nm wavelength laser excitation ***(BD FAC SCalibur, BD Biosciences, USA)***.

PTX٣ assay employed the quantitative sandwich enzyme immunoassay technique ***(Kits were supplied by R&D systems)***.

Nitric oxide assay ***(Kits were supplied by R&D systems)*** was based on the enzymatic conversion of nitrate to nitrite by nitrate reductase followed by colorimetric detection of nitrite as an azo dye product of the Griess Reaction. The Griess Reaction was based on the two-step diazotization reaction in which acidified NO_2^- produced a nitrosating agent, which reacted with sulfanilic acid to produce the diazonium ion. This ion was then coupled to N-(١-naphthyl) ethylenediamine to form the chromophoric azo-derivative which absorbed light at ٥٤٠ - ٥٧٠ nm ***(Alderton, WK et al. ٢٠٠١)***.

Statistical analysis

Data were analyzed by using SPSS (***Statistical Package for Social Science)*** version ١٦. Results were expressed as mean ± standard deviation (SD) unless otherwise specified. Student t-test of Mann Whitney was used to compare variables between studied groups. Pearson correlation was used to perform correlation between different variables. Results were considered significant if P-value was < ٠.٠٥ and highly significant if P-value < ٠.٠٠١. Receiver Operating Characteristic (ROC) curve was used to estimate area under the curve (AUC) of different variables where ٠.٩ - ١.٠ indicating an excellent test, ٠.٨ - ٠.٩ a good test, ٠.٧ - ٠.٨ a fair test and ٠.٦-٠.٧ a poor test. ROC curve was used to estimate the optimal cut-off value for best sensitivity and specificity of coordinate points of the curve. The optimal cut-off value was used to estimate both positive predictive value (PPV) and negative predictive value (NPV) of the studied variable.

Results

Group III had statistically significant increase in age (P-value = ٠.٠٣) when compared to group II. Group III had the statistically highest value (P-value = < ٠.٠٠١) when compared to both group I and group II., BMI (P-value = and parity when compared to both group. Group III had statistically significant increase in parity when compared with group II (P-value = ٠.٠١٨).

There was statistically significant increase in both systolic and diastolic blood pressure in

group III when compared with group II (P-value = < ٠.٠٠١). Baby birth weight was statistically significantly decreased in group III when compared with group II (P-value = < ٠.٠٠١).

Both hemoglobin and hematocrit were significantly decreased in group II and group III when compared with group I (P-value = < ٠.٠٠١). Group III had the statistically significant highest total leukocytic count when compared with group I and group II (P- value = ٠.٠٤٨ & P-value = ٠.٠٤٧ respectively). Platelet count was significantly decreased in group II and group III when compared with group I (P-value = ٠.٠٠٩ & P-value = ٠.٠٠٥ respectively).

There was statistically significant increase in random blood glucose (P-value = ٠.٠٤٩), ALT (P-value = ٠.٠١٥), total bilirubin (P-value = ٠.٠٠١) and A/C ratio (P-value = < ٠.٠٠١) when compared with group II. However, there was statistically significant decrease in albumin (P-value = ٠.٠٣٧) and calcium/creatinine ratio (P-value = ٠.٠٠٤) in group III when compared with group II.

Alpha fetoprotein, sflt-١ and pentraxin ٣ were found to be statistically significantly increased in group III when compared with group II (P-value = ٠.٠٢٤, P-value = < ٠.٠٠١ & P-value = ٠.٠٠٦ respectively). However, there was statistically significant decrease in neutrophil-flt-١ and nitric oxide in group III when compared with group II (P-value = < ٠.٠٠١ & P-value = ٠.٠١٦).

There was significant positive correlation between sflt-١ and pentraxin ٣ (P-value = ٠.٠٤٩), neutrophil-flt-١ and monocyte-flt-١ (P-value = ٠.٠٠٤), neutrophil-flt-١ and uric acid (P-value = ٠.٠٢٢) in group III. However, there was significant negative correlation between sflt-١ and monocyte-flt-١ (P-value = ٠.٠٠٩), sflt-١ and neutrophil-flt-١ (P-value = < ٠.٠٠١), neutrophil-flt-١ and pentraxin ٣ (P-value = ٠.٠٠٧), pentr-axin ٣ and nitric oxide (P-value = ٠.٠١٢).

ROC curve analysis **(Table ١)** revealed that BMI at > ٢٥ had an AUC of ٠.٨٢, sensitivity, specificity, PPV and NPV of ٨٤, ٥٢, ٦٣.٦ and ٧٦.٥ % respectively with P-value = < ٠.٠٠١. Uric acid at > ٦.٠ had an AUC of ٠.٦٤٦, sensitivity, specificity, PPV and NPV of ٨, ٨٨, ٤٠ and ٤٨.٩ % respectively with P-value = ٠.٠٧٧. A/C ratio at > ٣٠, had an AUC of ٠.٧٨٤, sensitivity, specificity, PPV and NPV of ١٦, ٩٢, ٦٦.٧ and ٥٢.٣ % respectively with P-value = ٠.٠٠١. CCR at ≤ ٤٠, had an AUC of ٠.٧٥٤, sensitivity, specificity, PPV and NPV of ٢٠, ٨٤, ٥٥.٦ and ٥١.٢ % respectively with P-value = ٠.٠٠٢. AFP at > ٨.٥ ng/ml had an AUC of ٠.٦٧٤, sensitivity, specificity, PPV and NPV of ٦٨, ٦٠, ٦٣ and ٦٥.٢ % respectively with P-value = ٠.٠٣٥. Soluble flt-١ at > ١٩٨٦ pg/ml had an AUC of ٠.٩٤١, sensitivity, specificity, PPV and NPV of ٨٤ % P-value = < ٠.٠٠١. Monocyte-flt-١ at < ٤٢.٣ % had an AUC of ٠.٧١, sensitivity, specificity, PPV and NPV of ٧٦, ٦٨, ٧٠.٤ and ٧٣.٩ % respectively with P-value = ٠.٠١١. Neutrophil-flt-١ < ٤١.٣ % had an AUC of ٠.٨٧٤, sensitivity, specificity, PPV and NPV of ٨٠, ٨٤, ٥٥.٦ and ٥٣.١ % respectively with P-value = < ٠.٠٠١. Pentraxin ٣ at > ٥.٩ ng/ml had an AUC of ٠.٨٢, sensitivity, specificity, PPV and NPV of ٦٨, ٧٢, ٦٨ and ٦٨ % respectively with P-value = ٠.٠٠١. Nitric oxide at < ٧٣.٥ µmol/l had an AUC of ٠.٧٠٢, sensitivity, specificity, PPV and NPV of ٦٤, ٦٨, ٦٦.٧ and ٦٥.٤ % respectively with P-value = ٠.٠١٤.

Table (١): Data of ROC curve analysis including AUC, sensitivity, specificity, PPV, NPV and P-value of BMI, uric acid, A/C ratio, CCR, AFP, CD١٤+Flt-١+ monocytes, CD١٣+Flt-١+ neutrophils, PTX٣ and NO.

Test	AUC	Cutoff value	Sensitivity	Specificity	PPV	NPV	P-value
BMI	٠.٨٢٠	> ٢٥	٨٤ %	٥٢ %	٦٣.٦ %	٧٦.٥ %	< ٠.٠٠١
Uric acid	٠.٦٤٦	> ٦.٠ mg/dl	٨٪	٨٨٪	٤٠٪	٤٨.٩٪	٠.٠٧٧
A/C ratio	٠.٧٨٤	> ٣٠	١٦٪	٩٢٪	٦٦.٧٪	٥٢.٣٪	٠.٠٠١
CCR	٠.٧٥٤	≤ ٤٠	٢٠٪	٨٤٪	٥٥.٦٪	٥١.٢٪	٠.٠٠٢
AFP	٠.٦٧٤	> ٨.٥ ng/ml	٦٨٪	٦٠٪	٦٣٪	٦٥.٢٪	٠.٠٣٥
sFlt-١	٠.٩٤١	> ١٩٨٦ pg/ml	٨٤٪	٨٤٪	٨٤٪	٨٤٪	< ٠.٠٠١
CD١٤+Flt-١+ monocytes	٠.٧١٠	< ٤٢.٣ %	٧٦٪	٦٨٪	٧٠.٤٪	٧٣.٩٪	٠.٠١١
CD١٣+Flt-١+ neutrophils	٠.٨٧٤	< ٤١.٣ %	٨٠٪	٨٤٪	٥٥.٦٪	٥٣.١٪	< ٠.٠٠١
Pentraxin ٣	٠.٧٧٤	> ٥.٩ ng/ml	٦٨٪	٧٢٪	٦٨٪	٦٨٪	٠.٠٠١
NO	٠.٧٠٢	< ٧٣.٥ µmol/l	٦٤٪	٦٨٪	٦٦.٧٪	٦٥.٤	٠.٠١٤

Conclusion

Among all studied biomarkers, soluble flt-١ was found to be the best single biomarker in predicting preeclampsia at ١٤-١٨ weeks of gestation with the best diagnostic sensitivity and specificity.

Soluble flt-١ was significantly negatively correlated with neutrophil-flt-١ and monocyte-flt-١. So, the predictive utility of sflt-١ could be strengthened when combined with both neutrophil- and monocyte-flt-١. This may be an approach to develop a novel predictive model for preeclampsia combining the three biomarkers with better sensitivity and specificity.

Also, combining nitric oxide "which decrease" and pentraxin ٣ "which increase" could be another novel predictive model used as an early screening tool to predict preeclampsia at second trimester of pregnancy.

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