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Urinary Placental Growth Factor: A Promising Marker for Screening Preeclampsia

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Abstract- The present study is aimed to ascertain whether preeclampsia is associated with changes in serum levels of vascular endothelial growth factor (VEGF), placental growth factor (PlGF), soluble fms-like tyrosine kinase -1 (sFlt-1) and urine excretion levels of PlGF in Indian patients. Serum and urine samples were obtained from 40 women with preeclampsia and 40 normotensive, non-proteinuric pregnant women as control and the levels of these factors were analyzed using enzyme-linked immunosorbent assay (ELISA). The serum levels of VEGF (mean ± SD 170.53 ± 36.56 Vs 254.61 ± 47.394 pg/ml, $p<0.0001$), PlGF (mean ± SD 236.77 ± 93.70 Vs 744.98 ± 168.55 pg/ml, $p<0.0001$) and the urinary levels of PlGF (mean ± SD 30.08 ± 9.42 Vs 77.70 ± 24.70 pg/ml, $p<0.0001$) were significantly lower where as serum levels of sFlt-1 were significantly higher (median (range) 11295.25 (2936.2 – 37818) Vs 2893.20 (1180.43- 6706.6) pg/ml, $p<0.0001$) in preeclamptic patients as compared to the control. In conclusion, preeclampsia is associated with alterations in serum and urinary angiogenic factors. The longitudinal measurement of these angiogenic factors in serum might be ideal for ascertaining the risk of preeclampsia, but measuring the levels of PlGF in the urine during routine antenatal care could be a simpler and non-invasive factor, if it can be used as definite, informative screening marker for preeclampsia.

Index Terms- Preeclampsia, Placental growth factor, Soluble fms-like tyrosine kinase-1, Vascular endothelial growth factor, Vascular endothelial growth factor receptor-1

I. Introduction

Preeclampsia is a hypertensive disorder of pregnancy (1), in which the normal hemodynamic response to pregnancy is compromised. It remains as a leading cause of maternal and perinatal morbidity and mortality and is diagnosed primarily by the onset of hypertension and proteinuria after the twentieth week of gestation (2). Preeclampsia is characterized by diffuse endothelial dysfunction possibly secondary to impaired trophoblast invasion of the spiral arteries during implantation (3). In preeclampsia, the reduced invasion of trophoblast cells and incomplete/inadequate transformation of the maternal spiral arteries, may possibly result in insufficient uteroplacental circulation and may cause local placental ischemia which may further release the angiogenic factor(s) into the maternal circulation such as vascular endothelial growth factor (VEGF), placental growth factor (PlGF) and its receptor, soluble vascular endothelial growth factor receptor-1(sVEGFR-1)/soluble fms-like tyrosine kinase1 (sFlt-1) (4).

Evidence from the previous study suggests that preeclampsia may be caused by an imbalance of angiogenic factors (5). Soluble fms-like tyrosine kinase 1 (sFlt1) may play a role in preeclampsia by antagonizing vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) in maternal vasculature (5). Exogenous administration of sFlt-1 to pregnant rats found to induce hypertension, proteinuria and glomerular endotheliosis similar to that of human preeclampsia (6). A study on "*Calcium for Preeclampsia Prevention (CPEP)* trial" demonstrated that serum sFlt-1 levels are elevated approximately 5 weeks before the onset of clinical symptoms of preeclampsia where as the low serum PlGF, begins at 13 to 16 weeks of gestation (7). This study also suggested that the reduced free VEGF antedated the clinical signs of preeclampsia (7).

Several lines of evidence indicate a variation in the levels of angiogenic factors in different population (8-11). The threshold values of these factors have not been reported in Indian population. Thus, it is important to analyze the baseline values of angiogenic factors in Indian patients so as to determine the threshold value of these factors for predicting and early diagnosis of preeclampsia. Although longitudinal measurement of these angiogenic factors in serum might be ideal for ascertaining the risk of preeclampsia, but measuring the levels of angiogenic factors in urine of preeclamptic patients during routine antenatal check up may be easier and cost effective when proved to be diagnostic. sFlt-1 is a large molecule to be filtered into urine (12) and VEGF is derived from glomerular podocytes and tubular cells of the kidney, thus it is unlikely to reflect the circulating angiogenic state (13). The urinary PlGF acts as a more clinically feasible alternative. Therefore the study was aimed to evaluate the alterations of angiogenic factors (sFlt-1, VEGF, PlGF) in serum and PlGF in urine of preeclamptic and normotensive, non-proteinuric pregnant women in Indian population.

II. Materials and Methods

Study Design

Forty each preeclamptic patients and normotensive non-proteinuric pregnant women who served as control were selected from the antenatal clinic and the inpatient ward of the Department of Obstetrics and Gynecology, All India Institute of

Medical Sciences, New Delhi, India. The preeclamptic women immediately after the clinical diagnosis were included whereas the cases with chorioamnionitis, chronic hypertension, pre-gestational hypertension, renal disease, cardiac disease, active asthma, diabetes, thyroid disease and epilepsy were excluded from the study. Preeclampsia was defined according to the research definition criteria of the International Society for the Study of Hypertension in Pregnancy (ISSHP) (14); systolic and diastolic blood pressures above 140 and 90 mmHg respectively, in at least two consecutive measurements and at least 4 hours apart occurring after twentieth week of gestation and accompanied by proteinuria (>300 mg per liter in a 24 h urine collection/>1+ on a urine dipstick). The normotensive, non-proteinuric pregnant women without any other medical complications were taken as the control group. The preeclamptic patients and the control women were matched for maternal age and gestational age. Gestational age was established based on menstrual date and/or ultrasonographic examination before 20 weeks of gestation. This study was approved by the institute ethics committee and written informed consent was obtained from all the women recruited for the study.

III. Immunoassay Procedures

5 ml of venous blood was collected and centrifuged at 3000 rpm for 20 minutes and the serum was then separated and stored in aliquots at -20ºC. 5 ml of urine sample was also collected by "clean catch" technique from the same patient and centrifuged at 3000 rpm for 20 minutes, aliquoted and immediately stored at -20ºC. VEGF, PlGF, sFlt-1in serum and PlGF in urine were measured by a sandwich-type enzyme linked immunosorbent assay (ELISA; Quantikine ® human VEGF, Quantikine ® human PlGF and Quantikine ® human sVEGFR1/sFlt-1, R&D Systems Inc., Minneapolis, MN, U.S.A). According to the kit, the minimum detectable level for VEGF was 7 ρg/ml, 9.0 ρg/ml for PlGF and 1.5 – 13.3 ρg/ml for sFlt-1. The intra assay variation and inter assay variation were 4.5% and 7.0% for VEGF 3.6% and 11.0% for PlGF and 3.8% and 7.0% for sFlt-1, respectively.

IV. Statistical Analysis

Between the preeclamptic patients and the control group, quantitative variables like systolic blood pressure, diastolic blood pressure, body mass index, serum VEGF, serum PlGF, serum sFlt-1 and urinary PlGF were compared using paired t test/ Wilcoxon sign rank test. The result was considered to be statistically significant at 5% level of significance (i.e., $p<0.05$). Statistical analysis was performed using the statistical package Stata 9.

V. Results

The clinical characteristics such as systolic blood pressure, diastolic blood pressure and body mass index are shown in table 1. The systolic and diastolic blood pressures and body mass index were significantly higher in women with preeclampsia than in controls.

Patients with preeclampsia showed reduced serum levels of VEGF than controls (mean ± SD 170.53 ± 36.55 Vs 254.61 ± 47.39 pg/ml, $p< 0.0001$) (Fig.1). The serum PlGF levels were also significantly lower in women with preeclampsia than in controls (mean ± SD 236.77 ± 93.70 Vs 744.98 ± 168.55 pg/ml, $p< 0.0001$) (Fig.2). The serum sFlt-1 levels were significantly higher in women with preeclampsia than in controls (median (range) 11295.25 (2936.2 – 37818) Vs 2893.20 (1180.43-6706.6) pg/ml, $p<0.0001$) (Fig.3).The levels of urinary PlGF were also significantly lower in women with preeclampsia than in controls (mean ± SD 30.08 ± 9.42 Vs 77.70 ± 24.70 pg/ml, $p< 0.0001$) (Fig.4).

VI. Discussion

Despite many years of intensive research, preeclampsia still remains as an elusive disorder of pregnancy complicated with high maternal and fetal morbidity and mortality. Prompt diagnosis and interventions are of vital importance in reducing the complications of this disorder which re-emphasize the need of developing a proper screening marker for preeclampsia. Numerous studies are available where the role of serum angiogenic factors in preeclampsia has been investigated (5,15,16)' but very few are available on urinary placental growth factor although it is a very simple and cost effective method (17-19). Thus in the present study, a combined analysis of serum as well as urine angiogenic factors was performed. Moreover, the levels of these angiogenic factors vary across the world which may be due to geographical, social, economic and racial differences (20). Variation in the levels of angiogenic factors among different populations has been reported by number of workers (8-11). Thus it is important to analyze the baseline values of angiogenic factors in a given population so as to determine the threshold value for predicting and early diagnosis of preeclampsia.

In the present study, a significant decrease in the levels of free VEGF was found in the sera of preeclamptic pregnant women compared to the sera of control women (Fig.1). VEGF is a key survival factor for the vascular endothelium and important for maintaining vascular endothelial cell homeostasis. It is a disulphide-linked homodimeric, heparin-binding glycoprotein with a molecular weight of 34–42 kDa (21). It is a potent angiogenic, mitogenic factor which enhances the vascular permeability activities related to endothelial cells. It also promotes neovascularization, reduces blood pressure and is important for the maintenance of the normal glomerular filtration (22-24). Therefore, its deficiency or low levels could cause the disturbances or alterations in the vasculature of placenta in preeclampsia leading to increased blood pressure, defective glomerular filtration leading to proteinuria and oedema. VEGF also exerts its biological effect through 2 high affinity tyrosine kinase receptors: VEGF receptor-1 (VEGFR-1 or fms-like tyrosine kinase-1/Flt-1) and VEGF receptor-2 (VEGFR- 2 or kinase domain receptor/KDR) (25,26). VEGFR-1 has two isoforms: a transmembranous and a soluble isoform (27). The soluble isoform of VEGFR-1 / Flt-1 binds with free VEGF in the circulation thereby preventing their interaction with the endothelial receptors, thus antagonizing their action (28). An adequate and organized interaction of VEGF with its receptors is

essential for normal placental development and function, as well as maintenance of the maternal vasculature. Also optimal levels of VEGF are required in the circulation for stimulation of endothelial cells to survive for prolonged periods and to function properly throughout pregnancy (29). Thus a decrease in the concentration of free VEGF in the serum of women with preeclampsia may lead to endothelial cell dysfunction of maternal blood vessels. Therefore, when serum levels of sFlt-1 rise, their binding with VEGF may reduce the circulating (or free) VEGF levels below a critical threshold required for vasculogenesis and the maintenance of the placental circulation. Apart from that, the anti-VEGF antibodies and sFlt-1 cause rapid glomerular endothelial cell detachment and hypertrophy (30). The studies on anti-angiogenic trials showed that down-regulation or neutralization of circulating VEGF have resulted in proteinuria and hypertension, which supports the view that low levels of serum VEGF may play an important role in the induction of proteinuria in preeclampsia (30). However, controversial reports are available where elevated levels of total VEGF in the serum of women with preeclampsia have been reported. This discrepancy could be because of the fact that total VEGF protein (bound and unbound) is undetectable by the Sandwich type ELISA (31, 32). The earlier studies which reported a decreased concentration of VEGF have used an ELISA kit, which measures only free (unbound) VEGF (32) whereas the studies which reported an increased concentration of VEGF in preeclampsia have used either a radioimmunoassay or an ELISA system measuring both bound and unbound VEGF (33-35).

In the present study, a significant decrease in the concentrations of free PlGF was also found in the sera of women with preeclampsia compared to the sera of control women (Fig.2). PlGF also belongs to the VEGF family and shares 53% homology with VEGF (36). At physiological concentrations, it has been shown to be a very weak stimulator of endothelial cell proliferation (37). On the other hand, PlGF potentiates the action of low doses of VEGF on microvascular endothelial cells. PlGF also acts by binding with VEGFR- 1/Flt-1 and results in non-branching or least branching of blood vessels (38, 39). Thus PlGF and VEGF, together enhance the growth of new blood vessels (angiogenesis), and maintain the normal function of endothelial cells lining the blood vessels (39). Physiologically, the two growth factors, together with the oxygen diffusive placental tissue conductance, may promote remodeling of the materno-fetal interface. The decreased concentration of free PlGF and VEGF in the serum of preeclamptic patients may lead to endothelial cell dysfunction in maternal circulation (29).

A significantly increased concentration of sFlt-1 in the sera of preeclamptic women was also observed in the present study when compared to control (Fig.3). One possible reason for this is the effect of inadequate perfusion on the growth of the feto-placental unit, which may lead to an increase in serum sFlt-1 values (40). sFlt-1 is a soluble receptor of VEGF (23,27) which is generated by a splice variant of the *Flt-1* gene and contains the extracellular ligand-binding domain, and lacks the transmembrane and cytoplasmic domains (27). sFlt-1 may not primarily be a receptor transmitting a mitogenic signal, but may inhibit the activity of VEGF on the vascular endothelium by preventing the binding of VEGF to VEGFR-2 (41). Previous studies indicate that the source of the serum sFlt-1 is the placenta and both the transmembranous and soluble forms were detected in it (4). However, sFlt-1 protein was also found in the supernatant from explants of placental villi, suggesting that sFlt-1 could be released in to the intervillous space directly (4). The increased sFlt-1 in the placentas of preeclamptic women may thus impair the placental vascularization by antagonizing the angiogenic activities of VEGF on endothelial cells of placental blood vessels, leading to reduced placental perfusion. When sFlt-1 was administered to pregnant rats, it resulted in hypertension, proteinuria and glomerular endotheliosis (6). These data suggest that the elevated sFlt-1 may induce preeclampsia but preeclampsia may not necessarily increase the levels of sFlt-1. The authors also suggested that excess placental production of sFlt-1 contributes to these maternal symptoms in patients with preeclampsia (6).Thus, carefully regulated balance of VEGF, PlGF, and sFlt-1 is essential to maintain optimal/high level of angiogenesis which is necessary for proper placental vasculature and thereby successful placental circulation required for maintenance of normal pregnancy. Any deviation in the normal values of these angiogenic factors will lead to disturbance in the normal flow of blood in the placenta leading to conditions like preeclampsia as has been observed in the present study.

In the present study, significantly reduced levels of urinary PlGF were observed in preeclamptic women compared to control women (Fig.4). This finding is concomitant with the previous findings which showed the reduced levels of PlGF in the urine of preeclamptic women (12, 17-19). The reduced levels of urinary PlGF may be due to the reduced circulating PlGF which in turn attributed to the increased binding of sFlt-1 to the free PlGF in the circulation (42). Optimal levels of free PlGF along with VEGF is necessary to promote the growth and proliferation of glomerular and peritubular endothelial cells (12, 17,18). In contrast, the excess circulating levels of sFlt-1in preeclampsia promotes glomerular endothelial hypertrophy, apoptosis and cell detachment from the glomerular basement membrane which leads to proteinuria (12, 17,18). sFlt-1 is a large molecule of 100 KDa which can't be filtered into urine in the absence of renal damage whereas PlGF and VEGF are much smaller proteins of 30 KDa and 45 KDa respectively and are readily filtered (12). During glomerular development, the podocytes express numerous vascular growth factors such as VEGF-A, while the glomerular endothelial cells express VEGF receptors fetal liver kinase 1 (Flk-1) and fms-like tyrosine kinase-1 (Flt-1) (43, 44). In addition, the podocytes are geographically situated at the developing vascular cleft adjacent to the incoming endothelial cells. The location and gene expression profile of podocytes suggests that they are required to provide migratory cues to glomerular endothelial cells to establish the renal filtration barrier (44). Furthermore, similar to other fenestrated vascular beds in the body, podocytes continue to express VEGF-A in the mature glomerulus. This suggests that VEGF plays a role in maintaining the filtration barrier either through survival, proliferation, and/or differentiation cues to the adjacent specialized endothelia (44). Since the expression of mRNA for VEGF and its receptors were found predominantly in glomerular podocytes, distal tubules and collecting ducts (13, 45), the urinary VEGF acts as a poor marker to reflect the circulating angiogenic state. Therefore in the present study, urinary PlGF

was estimated which acts as a better alternative factor for the measurement. The urinary PlGF possible be a non invasive screening marker for preeclampsia.

VII. Conclusion

Data from the present study showed the significant variations in the levels of angiogenic factors not only in the serum but also in the urine of preeclamptic patients as compared to the control women; which opened the possibility of urinary PlGF as a screening marker for preeclampsia. Further prospective, longitudinal studies in which urinary concentrations of PlGF are measured throughout pregnancy is needed to better assess the relevance of this factor for the early diagnosis of preeclampsia and the prediction of its severity. If a reliable and valid urinary dipstick assay could be developed for screening all pregnant women for urine PlGF during their regular ANC check up, there will be a hope for early diagnosis of preeclampsia which will reduce not only the expense of the serum analysis but will minimize the complications of preeclampsia.

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Table:1 Clinical characteristics of pregnant women with preeclampsia and normotensive, non-proteinuric pregnant women at the enrolment of the study

	Preeclamptic women (n=40)	Normotensive, non-proteinuric pregnant women (n=40)	Statistical significance (p-value)
Systolic blood pressure (mmHg) Mean±SD	153.8 ± 10.30	113.15 ± 5.43	0.0001**
Diastolic blood pressure (mmHg) Mean±SD	100.75 ± 8.27	75.15 ± 5.08	0.0001**
Body Mass Index Mean±SD	24.91 ± 4.75	22.85 ± 3.50	0.01*

Paired t-test ; * Statistical significance, $p<0.05$

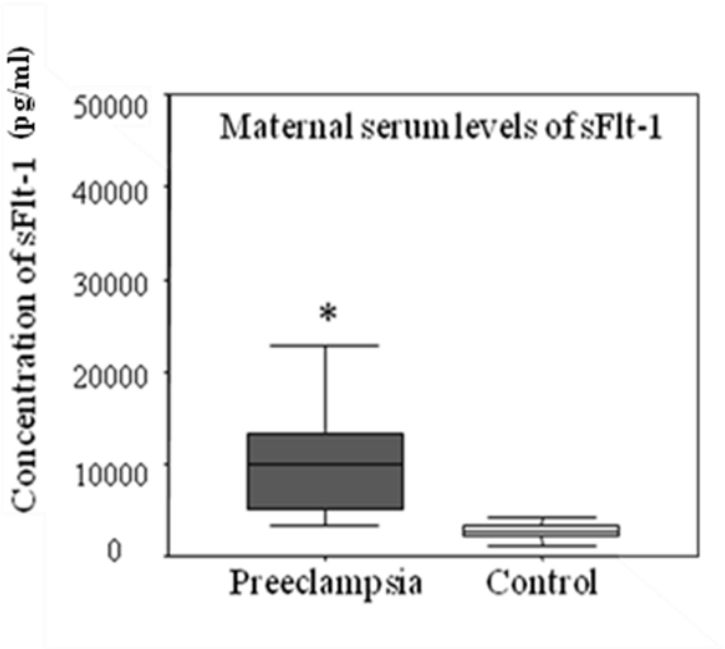

FIG.1

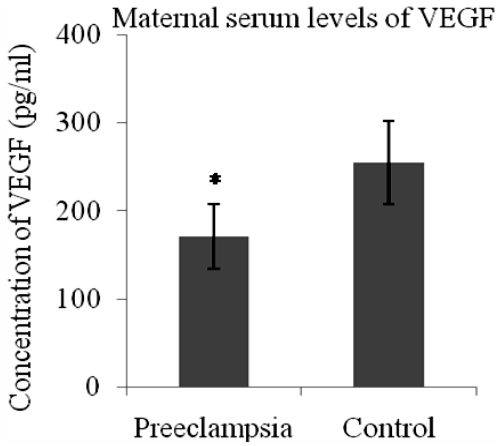

FIG.2

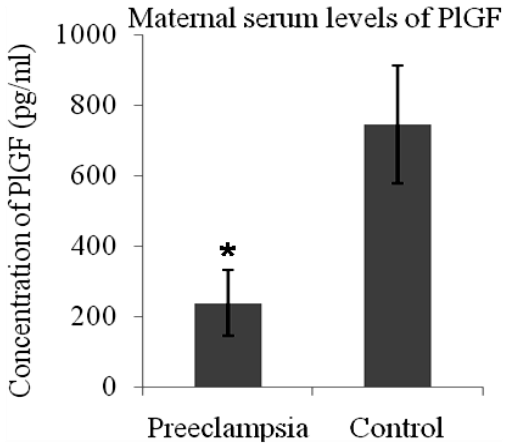

FIG.3

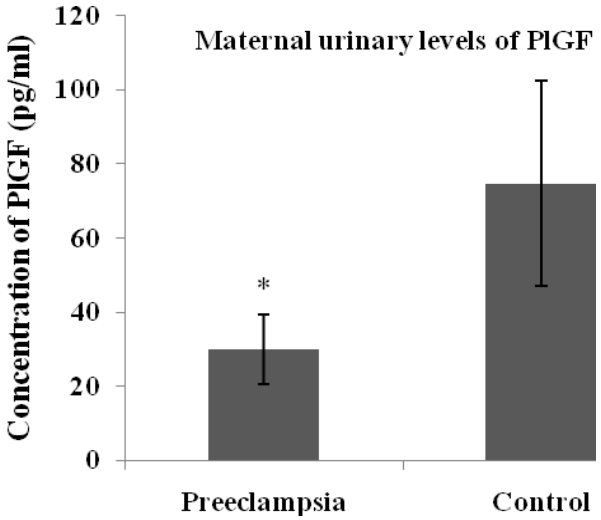

FIG.4

Legends to figures

FIG.1. Box plots indicate the serum levels of Soluble fms-like tyrosine kinase-1 (sFlt-1) in preeclamptic and normotensive, non-proteinuric pregnant women. Boxes denote the interquartile range with the upper and lower horizontal edges representing the 75^{th} and 25^{th} percentiles, respectively. The central horizontal lines represent the medians.
* represents the statistical significance, $p<0.05$.

FIG.2. Bar diagram represents the serum levels of Vascular Endothelial Growth Factor (VEGF) in preeclamptic and normotensive, non-proteinuric pregnant women. Values are given as mean$\pm$SD. Error bars on the bar diagram represents the standard deviation.
* represents the statistical significance, $p<0.05$.

FIG.3. Bar diagram represents the serum levels of Placental Growth Factor (PlGF) in preeclamptic and normotensive, non-proteinuric pregnant women. Values are given as mean$\pm$SD. Error bars on the bar diagram represents the standard deviation.
* represents the statistical significance, $p<0.05$.

Fig.4 Bar diagram represents the urinary levels of Placental Growth Factor (PlGF) in preeclamptic and normotensive, non-proteinuric pregnant women. Values are given as mean$\pm$SD. Error bars on the bar diagram represents the standard deviation.
* represents the statistical significance, $p<0.05$.