"Pro-angiogenic vascular endothelial growth factor (VEGF), placental growth factor (PIGF) and anti-angiogenic factor, soluble FMS like tyrosine kinase-1 (sFlt-1) in preeclampsia – A case control study"

Thesis submitted for the award of the degree of

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in

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By

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May 2023

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DECLARATION BY THE CANDIDATE

I declare that the thesis entitled **"Pro-angiogenic vascular endothelial growth factor (VEGF), placental growth factor (PIGF) and anti-angiogenic factor, soluble FMS like tyrosine kinase-1 (sFlt-1) in preeclampsia – A case control study",** submitted by me for the degree of Doctor of Philosophy (PhD) is the record of work carried out by me under the guidance of **Dr.Basavaraj Devaranavadagi,** Professor of Biochemistry, BLDE (Deemed to be University)'s Shri B. M. Patil Medical college, Hospital and Research Centre, Vijayapura, Karnataka and **Dr.Ashalata Mallapur,** Professor and HOD of OBG, S. Nijalingappa Medical College, Bagalkot, Karnataka, and has not formed the basis for the award of any degree, diploma, associateship, fellowship, titles in this university or other similar institution of higher learning.

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This is to certify that the thesis entitled **"Pro-angiogenic vascular endothelial growth factor (VEGF), placental growth factor (PIGF) and anti-angiogenic factor, soluble FMS like tyrosine kinase-1 (sFlt-1) in preeclampsia – A case control study",** submitted for the degree of Doctor of Philosophy (PhD) by **Dr. Sangappa V. Kashinakunti** is the record of research work carried out by him under my supervision and guidance and that this has not formed the basis for the award of any degree, diploma, associateship, fellowship, titles in this university or other similar institution of higher learning.

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CERTIFICATE FROM THE HEAD OF THE INSTITUTION AND THE

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This is to certify that the thesis entitled **"Pro-angiogenic vascular endothelial growth factor (VEGF), placental growth factor (PIGF) and anti-angiogenic factor, soluble FMS like tyrosine kinase-1 (sFlt-1) in preeclampsia – A case control study"**, submitted for the degree of Doctor of Philosophy (PhD) by is the record of research work carried out by Dr. Sangappa V. Kashinakunti under the guidance and supervision of Dr.Basavaraj Devaranavadagi, Professor of Biochemistry, BLDE (Deemed to be University)'s Shri B. M. Patil Medical college, Hospital and Research Centre, Vijayapura, Karnataka and Dr.Ashalata Mallapur, Professor and HOD of OBG, S. Nijalingappa Medical College, Bagalkot, Karnataka,; in partial fulfillment for the award of Doctor of Philosophy in the faculty of Medicine and that this work was carried out by her in the Department of Biochemistry.

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LIST OF ABBREVIATIONS

Abbreviation	Full form
PE	Pre-eclampsia
VEGF	Vascular endothelial growth factor
PIGF	Placental growth factor
sFlt-1	soluble FMS like tyrosine kinase-1
HELLP	Hemolysis, elevated liver enzymes, and low platelets
NO	Nitric oxide
eNOS	Endothelial nitric oxide synthase
MDA	Malondialdehyde
ROC	Receiver operating characteristic
APGAR	Appearance, Pulse, Grimace, Activity, Respiration
PI3K	Phosphoinositide 3-kinase
PKB	Akt/protein kinase B
nNOS	Neuronal Nitric oxide synthase
iNOS	Inducible Nitric oxide synthase
cGMP	Cyclic guanosine 3',5'-cyclic monophosphate
PKA	Protein kinase A
PKG	Protein kinase G
VDCC	Voltage-dependent calcium channels
PMCA	Plasma membrane calcium ATPases
ATP	Adenosine triphosphate
SR	Sarcoplasmic reticulum
SERCA	Sarcoplasmic reticular calcium ATPase
NO_3^-	Nitrite
NO_2^-	Nitrate
ROS	Reactive oxygen species
ACOG	American College of Obstetricians and Gynecologists
LFT	Liver function tests
RFT	Renal function tests
ELISA	Enzyme linked immunosorbent assay

HRP	Horseradish Peroxidase
OD	Optical density
PBS	Phosphate buffered saline
TBS	Tris-buffered saline
ABC	Avidin-Biotin-Peroxidase Complex
TMB	Tetramethylbenzidine
EP	Eppendorf
SBP	Systolic blood pressure
DBP	Diastolic blood pressure
AST	Aspartate transaminase
ALT	Alanine transaminase
LSCS	Lower segment caesarean section
VD	Vaginal delivery
LDH	Lactate dehydrogenase
A/G ratio	Albumin to globulin ratio
RBS	Random blood sugar
P/C ratio	Protein creatinine ratio
HB%	Hemoglobin percentage
RBC	Red blood cell
WBC	White blood cell
BT	Bleeding time
CT	Clotting time
PT	Prothrombin time
Hif	Hypoxia inducible factor

ABSTRACT

ABSTRACT

Background: Pregnancies that are complicated by Preeclampsia (PE) range from three to eight percent. Perinatal and neonatal mortality rate is about ten percent worldwide in developing countries due to PE. This is a multisystem disorder that leads to an increase in maternal and fetal morbidity and mortality. PE is due to impaired placentation that results in placental hypoxia, and endothelial dysfunction, It is characterized by decreased maternal circulating pro-angiogenic proteins like vascular endothelial growth factor (VEGF) and placental growth factor (PIGF), increased anti-angiogenic proteins sFlt-1, before the onset of clinical signs of PE. Oxidative stress also is implicated in the pathogenesis of PE. Studies have demonstrated low VEGF, PlGF, sFlt-1 and sFlt-1: PIGF ratio as a PE predictor but the clinical utility of these parameters is not yet generally established and only few studies have focused on neonatal outcomes.

Aim: The aim of the study was to determine diagnostic criteria for diagnosis of PE using pro-angiogenic VEGF, PIGF and anti-angiogenic sFlt-1 factors.

Objectives: The present study was undertaken to compare maternal VEGF, PIGF, sFlt-1 and sFlt-1:PIGF ratio in PE and normal healthy pregnant women, know the correlation between VEGF, PIGF, sFlt-1 and sFlt-1:PIGF ratio with severity of PE, to find its best cut-off value of these parameters for diagnosis of PE by ROC curve analysis and to study association with neonatal birth weight and APGAR score.

Methods: This was a case control study, conducted at tertiary care hospital, in Karnataka, India. The study was approved by Institutional ethics committee. Informed consent was obtained from all the participants. Forty singleton primigravida pregnant women aged 18-35 years, 20 or more weeks of gestation, diagnosed as PE and

classified as mild/severe PE and 40 healthy pregnant women as controls were selected for the study, based on America college of obstetricians and gynecologists guidelines. VEGF, PIGF and sFlt-1 were estimated by ELISA method. All the participants were visited again to note the neonatal birth weight and APGAR score. Analysis was done using SPSS software version 19.

Results: Maternal serum VEGF and PIGF were significantly (**p=0.0003, p<0.0001respectively**) higher in PE patients than controls and there was significant decrease in mild PE than severe PE (p=0.0465, p=0.0049 respectively) patients. Maternal serum sFlt-1 and sFlt-1:PIGF ratio were significantly higher (p<0.0001) in PE patients than normal healthy pregnant women. Best **cut-off value of VEGF, PIGF, sFlt-1 and sFlt-1:PIGF ratio were 150.0 pg/ml, 105.65 pg/ml, 1843 pg/ml and 28.54 respectively** , and under the curve were 0.734, 0.802, 0.945 and 0.922. Birth weight and APGAR score had significant (p=0.000) correlation with sFlt-1 and sFlt-1:PIGF ratio.

Conclusion: There was a significant decrease in angiogenic factors, significant increase in anti-angiogenic factor and sFlt1:PIGF ratio in PE than healthy pregnant women. There was significant correlation between severity of PE and PIGF, sFlt1 and sFlt-1:PIGF ratio. PIGF, sFlt1 and sFlt-1:PIGF ratio with mentioned cut-off values are better markers and can be used as diagnostic markers in PE. PIGF, sFlt1 and the ratio can be used as prognostic markers with mentioned criterion values for immediate birth outcomes.

Keywords: Preeclampsia, VEGF, PIGF, sFlt-1, sFlt-1:PIGF, Birth weight and APGAR score.

Chapter 1

INTRODUCTION

CHAPTER 1

INTRODUCTION

Preeclampsia (PE) is a multisystem disease, characterized by a de novo hypertension(blood pressure exceeding 140/90 mmHg on two occasions, at least six hours apart), with (or without) proteinuria (>300 mg/dL/24h or a dipstick of >= 2+), and detected after 20 weeks of pregnancy, can occur during labour, even 6 weeks after delivery, in previously normotensive women [1,2]. PE can become severe very quickly or progress slowly. Left untreated, PE can lead to maternal and perinatal morbidity and mortality, such as HELLP (hemolysis, elevated liver enzymes, and low platelets) syndrome and eclampsia [3-5], and it is associated with the development of cardiovascular disease, obesity, renal damage and diabetes in adults [6-9].

This disease affects around 2–8% of pregnancies worldwide, according to World Health Organization, in developing countries, PE is estimated to be responsible for more than 60000 maternal deaths per year and for 16% of all maternal deaths in developed countries. The perinatal and neonatal mortality rate due to PE is about 10% worldwide [10-15], which can be reduced by better understanding of pathogenesis of PE and early diagnosis and treatment of PE [2].

Many factors are being suggested as potential causes for pathogenesis of PE. In PE, invasion of the maternal uterine spiral arteries into the placental trophoblast is inadequate which leads to poor placental perfusion ultimately causing placental and fetal hypoxia. Hypoxia is a potent stimulus for release of the numerous factors into the maternal circulation that may affect widespread endothelial dysfunction of maternal multi organ system, and this can be a reason for hypertension and other signs of the disease [16,17].

The molecular mechanisms such as oxidative stress, genetic, immune and others, which lead to placental dysfunction, resulting in decreased maternal circulating pro-angiogenic placental growth factor (PIGF), vascular endothelial growth factor (VEGF) and also an increase in anti-angiogenic factor like soluble fms like tyrosine kinase (sFlt-1). Hence PE is also called as Anti-angiogenic Placental Syndrome. The exact cellular and molecular mechanism of PE is still elusive [17].
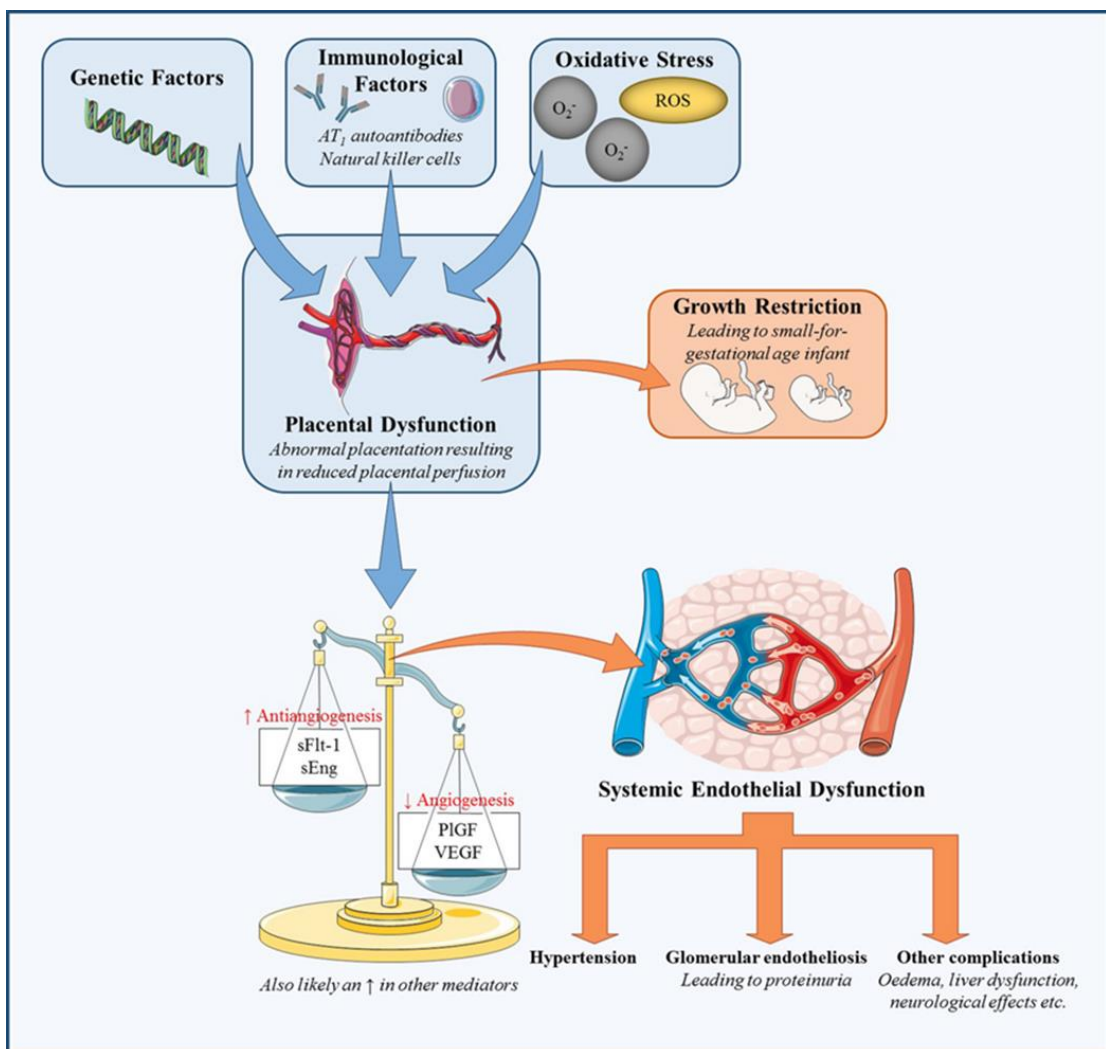
Figure 1.1: Pathogenesis of preeclampsia [17].

The current criteria for diagnosis and to know the severity of PE which is based on blood pressure, proteinuria etc, represent the changes due to systemic inflammation, but the vascular changes like PIGF at 11-12 weeks, VEGF at 17 weeks start decreasing and sFlt1 starts increasing at 16-17 weeks of gestation. These parameters, therefore, can be used in the early diagnosis of PE. VEGF and PlGF bring about their action through nitric oxide (NO) and endothelial derived nitric oxide synthase (eNOS). However, data concerning NO and NOS in pre-eclampsia have been reported lower or unchanged or higher compared to normal controls [2,17,18].

Only few studies have focused on the use of VEGF, PIGF and sFlt1/PlGF ratio as a measure for neonatal outcomes. Hence this study was undertaken.
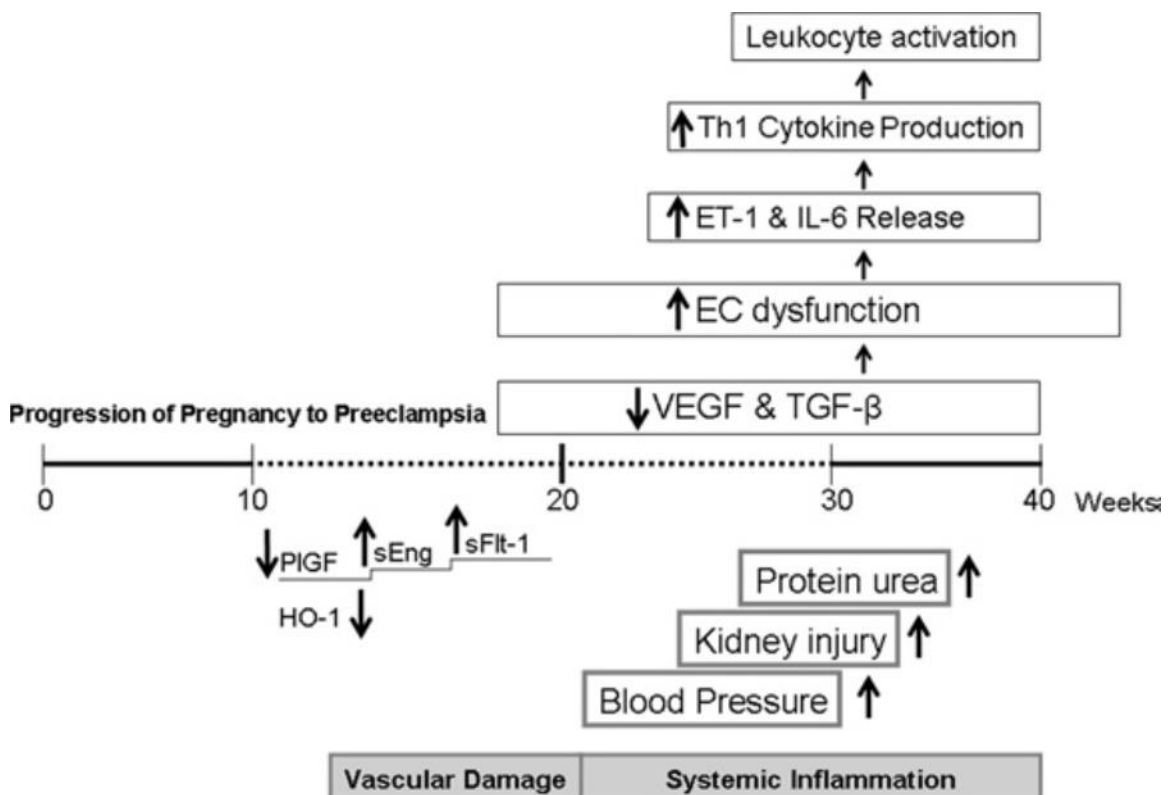
Figure 1.2: Molecular and systemic parameters in preeclampsia[19] (Early diagnosis)

References

1. ACOG practice bulletin. Obstetrics and Gynaecology 2020 Jun;135(6):e237-60.

2. Ives CW, Sinkey R, Rajapreyar I, Tita ATN, Oparil S. Preeclampsia-Pathophysiology and Clinical Presentations: JACC State-of-the-Art Review. J Am Coll Cardiol. 2020 Oct 6;76(14):1690-1702.

3. Duley L. The global impact of pre-eclampsia and eclampsia. Semin Perinatol 2009;33:130-7.

4. Spencer-Jones J. Make every mother and child count. S Afr Med J 2005;95:382,384.

5. Lyons G. Saving mothers'lives: confidential enquiry into maternal and child health 2003-5. Int J ObstetAnesth 2008;17:103-5.

6. Hauth JC, Ewell MG, Levine RJ, Esterlitz JR, Sibai B, Curet LB, et al. Pregnancy outcomes in healthy nulliparas who developed hypertension. Calcium for Preeclampsia Prevention Study Group. Obstet Gynecol 2000;95:24-8.

7. Powe CE, Levine RJ, Karumanchi SA. Preeclampsia, a disease of the maternal endothelium: the role of antiangiogenic factors and implications for later cardiovascular disease. Circulation 2011;123: 2856-69.

8. Vikse BE. Pre-eclampsia and the risk of kidney disease. Lancet 2013;382:104-6.

9. Harder T, Rodekamp E, Schellong K, Dudenhausen JW, Plagemann A. Birth weight and subsequent risk of type 2 diabetes: a meta-analysis. Am J Epidemiol 2007;165:849-57.

10. American College of Obstetricians and Gynecologists. Task Force on Hypertension in Pregnancy. Hypertension in pregnancy. Report of the American College of Obstetricians and Gynecologists' Task Force on Hypertension in Pregnancy. Obstet Gynecol 2013; 122: 1122-31.

11. Bell MJ. A historical overview of preeclampsia-eclampsia. J Obstet Gynecol Neonatal Nurs 2010; 39: 510-18.

12. Silasi M, Cohen B, Karumanchi SA, Rana S. Abnormal placentation, angiogenic factors, and the pathogenesis of preeclampsia. Obstet Gynecol Clin North Am 2010; 37:239-53.

13. Jeyabalan A. Epidemiology of preeclampsia: impact of obesity. Nutr Rev 2013; 71 Suppl 1: S18-25.

14. World Health Organization. World Health report. Make every mother and Child Count. Geneva: World Health Org.; 2005.

15. Altman D, Carroli G, Duley L, Farrell B, Moodley J, Neilson J, et al. Magpie Trial Collaboration Group. Do women with pre-eclampsia, and their babies, benefit from magnesium sulphate? The Magpie Trial: a randomized placebo-controlled trial. Lancet 2002;359:1877-90.

16. Flint EJ, Cerdeira AS, Redman CW, Vatish M. The role of angiogenic factors in the management of preeclampsia. Acta Obstet Gynecol Scand 2019;98:700-707. https://doi.org/10.1111/aogs.13540

17. Tjwa M, Luttun A, Autiero M, Carmeliet P. VEGF and PlGF: two pleiotropic growth factors with distinct roles in development and homeostasis. Cell Tissue Res 2003;314:5-14.

18. Phipps E, Prasanna D, Brima W, Jim B. Preeclampsia: Updates in pathogenesis, definitions, and guidelines. Clin J Am Soc Nephrol 2016 Jun;11:1102–13.

19. Ramma W, Ahmed A. Is inflammation the cause of pre-eclampsia? Biochem Soc Trans 2011;39:1619–27.

Chapter 2

OBJECTIVES

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OBJECTIVES

AIM: To find the diagnostic accuracy of VEGF, PIGF, sFlt-1 levels and sFlt-1:PIGF ratio in preeclampsia

OBJECTIVES OF THESTUDY:

1) To estimate and compare NO, eNOS, VEGF, PIGF, sFlt-1, MDA levels in pre-eclampsia cases and healthy controls.

2) To correlate the NO, eNOS, VEGF, PIGF, sFlt-1 levels and sFlt-1:PIGF with severity of pre-eclampsia.

3) To find the best cut-off of VEGF, PIGF, sFlt-1 levels and sFlt-1:PIGF ratio for the diagnosis of PE by ROC curve analysis.

4) To calculate sensitivity, specificity, positive predictive, negative predictive values and diagnostic accuracy of calculated cut-off values for diagnosis of PE.

5) To study the association and prognostic value of VEGF, PIGF, sFlt-1 levels and sFlt-1: PIGF ratio with pregnancy outcome.

HYPOTHESIS:

VEGF,PIGF, sFlt-1 levels and the ratio between sFlt-1: PIGF act as predictors of pre-eclampsia and also of the neonatal outcome viz birth weight, APGAR score at 1 minute and APGAR score at 5minutes.

Chapter 3

REVIEW OF LITERATURE

CHAPTER 3

REVIEW OF LITERATURE

Pregnancy is a physiological stress in which many changes occur in the milieu interior of the body, more and more stress is being laid on the biochemical changes, to meet increased metabolic requirements of mother and fetus, and also parallel increase in uteroplacental circulation for the growth and development of the fetus, which occur in the blood during normal pregnancy; become exaggerated in complications of pregnancy like pre-eclampsia, where there is insufficient hemodynamic adaptations and can result in maternal and fetal morbidity [1,2]

Preeclampsia: Pre-eclampsia (PE) has been named the "disease of theories" and was described as early as 3000 years ago by the ancient Egyptians. PE is a pregnancy specific disorder, most common, serious multisystem disease and is only cured by delivery [3]

Diagnostic criteria: The criteria for diagnosis of PE have not changed since the past ten years, despite changing context of the PE (proteinuria $\geq$ 300mg/24h; systolic blood pressure (SBP) >140 mmHg; diastolic blood pressure (DBP) $\geq$90 mmHg). PE may however be diagnosed as hypertension in association with impairment of liver functions, insufficiency of kidneys, decreased platelet count, pulmonary oedema, or also cerebral / visual disturbances, in the absence of proteinuria, (Table 3.1). Clinical features of PE along with abnormal laboratory values are used to grade the severity of the disease [4].

Table 3.1. Diagnostic criteria for pre-eclampsia [4].

Blood pressure	Greater than or equal to 140 mm Hg systolic or greater than or equal to 90 mmHg diastolic on two occasions at least 6 hours apart after 20 weeks of gestation in a woman with a previously normal blood pressure Greater than or equal to 160 mm Hg systolic or greater than or equal to 110 mm Hg diastolic hypertension can be confirmed within a short interval (minutes) to facilitate timely antihypertensive therapy
and	
Proteinuria	Greater than or equal to 300 mg per 24-hour urine collection (or this amount extrapolated from a timed collection)) **or** Protein/creatinine ratio greater than or equal to 0.3* Dipstick reading of 1+ (used only if other quantitative methods not available)
Or in the absence of proteinuria, new-onset hypertension with the new onset of any of the following:	
Thrombocytopenia	Platelet count less than100,000/microliter
Renal insufficiency	Serum creatinine concentrations greater than 1.1 mg/dL or a doubling of the serum creatinine concentration in the absence of other renal disease
Impaired liver function	Elevated blood concentrations of liver transaminases to twice normal concentration
Pulmonary edema	
Cerebral or visual symptoms	

* Each measured as mg/dL.

Table3. 2. Severe Features of Preeclampsia (Any of these findings)[4]

Systolic blood pressure of 160 mm Hg or higher, or diastolic blood pressure of 110 mm Hg or higher on two occasions at least 4 hours apart while the patient is on bed rest (unless antihypertensive therapy is initiated before this time)
Thrombocytopenia (platelet count less than100,000/microliter)
Impaired liver function as indicated by abnormally elevated blood concentrations of liver enzymes (to twice normal concentration), severe persistent right upper quadrant or epigastric pain unresponsive to medication and not accounted for by alternative diagnoses, or both
Progressive renal insufficiency (serum creatinine concentration greater than 1.1 mg/dL or a doubling of the serum creatinine concentration in the absence of other renal disease)
Pulmonary edema
New-onset cerebral or visual disturbances

Pro-angiogenic vascular endothelial growth factor (VEGF)

Vascular endothelial growth factor (VEGF), a 45-kDa glycoprotein, is a powerful inducer of angiogenesis, also affecting vascular permeability, endothelial cell survival, and hematopoiesis. VEGFs, of which there are 4 isoforms (VEGFA, VEGFB, VEGFC, and VEGFD), signal through VEGFR. Of the 3 VEGFR subtypes (VEGFR1, VEGFR2, and VEGFR3), VEGFR2 is the primary receptor through which VEGF, especially VEGFA, signals in endothelial cells. VEGFR2-mediated activation of phosphoinositide 3-kinase (PI3K)-Akt/protein kinase B (PKB) pathway leads to

endothelial nitric oxide synthase (eNOS) phosphorylation, increased nitric oxide (NO) generation,and consequent vasodilation (Figure 3.1) [5-7].

NOS consists of three isoforms, including neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). The eNOS isoform is expressed constitutively in the vascular endothelium and maintains vascular tone through the intrinsic synthesis of NO. NO is a potent vasodilator that acts to induce relaxation in vascular smooth muscle cells via guanosine 3',5'-cyclic monophosphate (cGMP) produced by soluble guanylylcyclase. cGMP activates protein kinase A (PKA) and protein kinase G (PKG). Activated PKA and PKG induce smooth muscle relaxation through the attenuation of myosin light chain kinase activity and augmentation of myosin light chain phosphatase activity, which induces dephosphorylation of the 20-kDa, regulatory, myosin light chain (Figure 3.2) [8].

Alteration in the production of VEGF, a biomarker of endothelial dysfunction, has been associated with pre-eclampsia although conflicting results of increased, decrease and normal maternal serum VEGF levels have been reported [9].

Down-regulation of VEGF in pregnancy may result in a suboptimal increase in NO and endothelial dysfunction in pre-eclampsia. However, data concerning NO production in pre-eclampsia have been conflicting. Investigators have reported lower, unchanged, and higher production compared to normal controls, even after controlling for diet, medications and urinary excretion that can affect serum nitrate levels.

Decreased levels of NO and increased levels of arginase (which degrades a precursor molecule in the NOS pathway) have been reported in preeclampsia (figure 3.2). These findings suggest that an intact NOS system is essential for normal spiral artery remodeling and pregnancy [8,10,11].

Pro-angiogenic placental growth factor (PIGF)

The placental growth factor was discovered in 1991 by an Italian scientist Maria GrazellaPersico [12].Placental growth factor (PIGF), a member of the VEGF family, is a proangiogenic protein, and is produced by villous syncytiotrophoblast in the placenta [13]. In normal pregnancies, PlGF is involved in angiogenesis and vasculogenesis, which are important processes in embryogenesis [14] and placentation; hence helps in trophoblastic invasion of the maternal spiral arteries. Median PIGF levels in the serum show a curvilinear relationship with gestational age and increase in the first and second trimesters. This level typically reaches a maximum value at approximately 30 weeks gestation age and then decreases[15].

In preeclampsia, circulating PlGF is decreased, although there are some discrepant findings [16]. In predicting preeclampsia, some studies have demonstrated that low concentrations of PlGF predict the disease from late first trimester [17,18]. Other studies did not find any predictive value early in pregnancy [18]. Most studies have demonstrated low PlGF as a preeclampsia predictor from the second trimester onwards, especially in severe preeclampsia [19,20].The predictive power of a low maternal PlGF blood concentration seems, in general, poorer for predicting preeclampsia [21].

Anti-angiogenic factor soluble fms-like tyrosine kinase-1 [Soluble Flt-1 (sFlt-1)]

Soluble fms-like tyrosine kinase-1 (sFlt-1) is a truncated splice variant of membrane-bound Flt-1. It is believed that sFlt-1 acts as a "decoy protein" in pregnancy. sFlt-1, naturally circulating without any limits, inhibits the angiogenic activity of circulating VEGF and PlGF by binding to them and obstructing the interaction with their membrane receptors, thereby reducing the vasodilation effect on the endothelium,

thus inducing vasoconstriction and contributing to the development of hypertension and proteinuria (Figure 3.1 and Figure 3.4). It appears that a significant role is played by enhanced placental expression and secretion of sFlt-1 in the development of preeclampsia [22].

A study on pregnant rats revealed that sFlt-1 administration can cause hypertension, proteinuria and renal pathologic changes in glomerular endothelium. In an in-vitro study, sFlt-1 was removed from supernatants of preeclamptic tissue, and it was observed that the culture reassumed its normal endothelial function and angiogenesis. On the other hand, the anti-angiogenic status produced by excess of sFlt-1 reversed by exogenous intake of VEGF and PlGF [23].

Previous studies revealed a relationship between increased sFlt-1 levels and PE. As early as 5 weeks before the onset of PE, sFlt-1 levels increase and remain elevated compared to normal pregnant women [24]. In a study by Powers et al,one subpopulation had elevated maternal sFlt before 35gestational weeks[19], in comparison to the second group of preeclamptic patients with an sFlt1 increase after 35 weeks of gestation [17].The association is stronger in severe preeclampsia [25], however, not all studies agree hereto [26,27].

As for predicting preeclampsia, low sFlt1 concentrations in the first trimester appear to predict early-onset preeclampsia in some studies [28,29], and a high sFlt1 increase from the first to the second trimester augment the risk of preeclampsia [30]. For the second and third trimester, most studies show that elevated maternal sFlt-1 concentrations predict preeclampsia and is a better predictor of severe preeclampsia [31,32].

A nested case control study shows that sFlt-1 values increased during pregnancy in all women but, women who developed PE began this increase earlier in gestation as compared to healthy pregnant women (21- 24 weeks vs 33-36 weeks of gestation) and reached higher values. There was a significant rise in the serum sFlt-1 concentration of PE patients, five weeks prior the clinical onset of the disease, as compared to control group. PlGF and VEGF levels decrease simultaneously with the increase in sFlt-1 [33]. The sFlt-1/PIGF ratio might be of value in the prediction of PE [34,35].
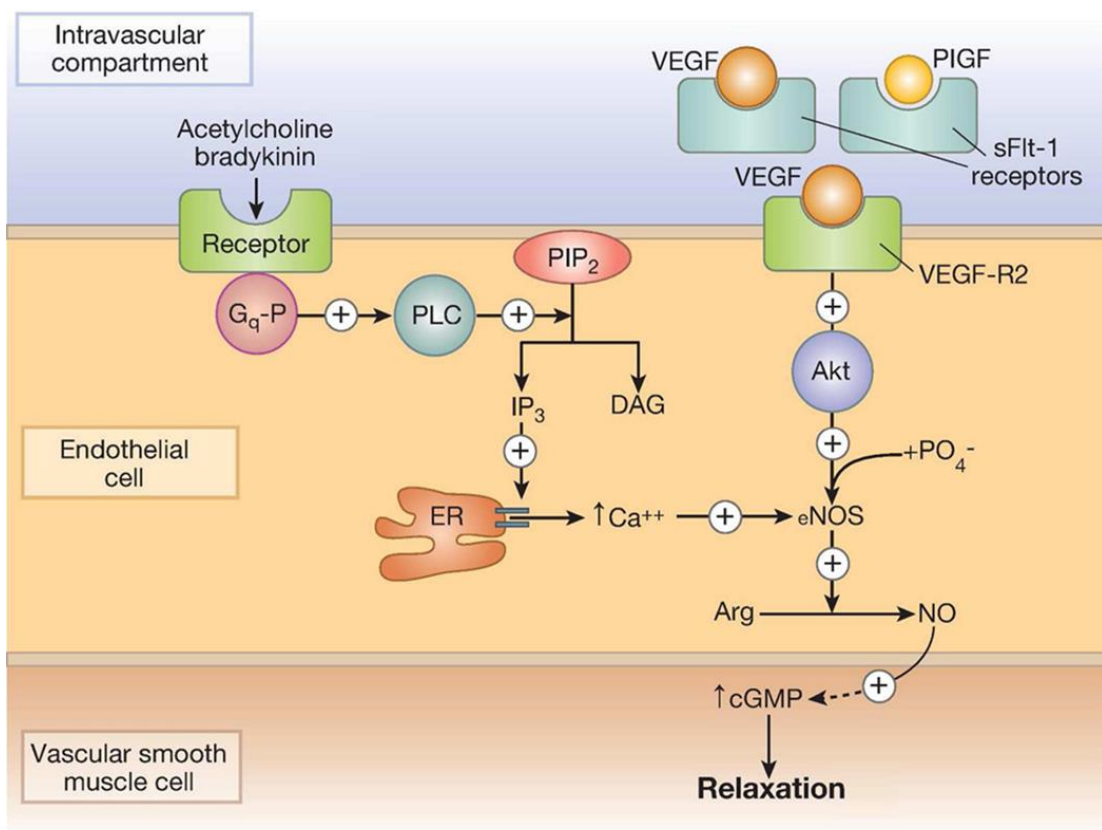
Figure 3.1: Mechanism of action of VEGF and PIGF [11]: Left side: Calcium-dependent, mediated by acetylcholine and bradykinin. Right side: Calcium-independent, activated by vascular endothelial growth factor (VEGF). In PE, increased levels of soluble fms-like tyrosine kinase 1 (sFlt-1) may act as a decoy protein, thus interfering with VEGF-dependent phosphorylation of eNOS. Akt, protein kinase B; Arg, arginine; cGMP, cyclic guanosine monophosphate; DAG, diacylglycerol; ER, endoplasmic reticulum; Gq-P, phosphorylated Gq protein; IP3, inositol triphosphate; PIP2, phosphatidylinositol biphosphate; PLC, phospholipase C.
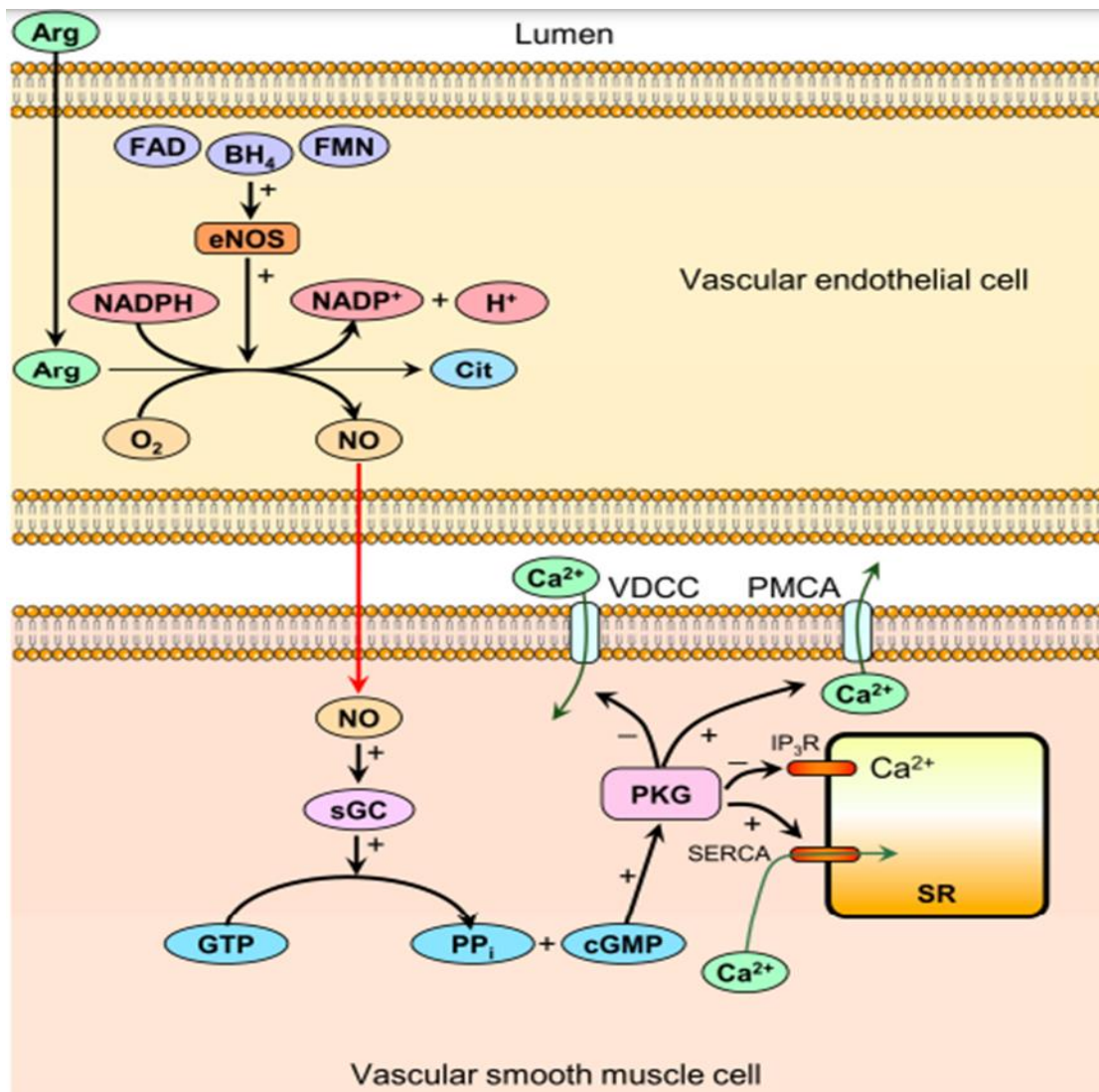
Figure 3.2: Mechanism action of NO [36] :Mechanisms of nitric oxide-mediated vasodilation: Plasma Arginine is the substrate for the synthesis of nitric oxide (NO), endothelial nitric oxide synthase (eNOS) acts as enzyme. The reaction requires O2 and nicotinamide adenine dinucleotide phosphate (NADPH) and the cofactors BH4, flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN). NO diffuses from the endothelial cell in to the smooth muscle cell and activates soluble guanylyl cyclase (sGC), leads to increased cyclic guanosine monophosphate (cGMP) production. cGMP further activates protein kinase G (PKG), results in decreased [Ca2+] through the following mechanisms: 1. Inhibiting the voltage-dependent calcium channels (VDCC), reducing calcium influx. 2. Stimulation of plasma membrane calcium ATPases (PMCA), increasing ATP-dependent calcium efflux. 3. Inhibiting inositol triphosphate receptors (IP3R), decreases the calcium release from the sarcoplasmic reticulum (SR) to the cytoplasm. 4. Activation of sarcoplasmic calcium ATPases (SERCA), increasing the ATP-dependent sequestration of calcium from the cytoplasm to the SR. Decreased [Ca2+] mediates smooth muscle relaxation through activation of myosin light chain kinase and the inhibition of myosin light chain phosphatase, leads to vasodilation.
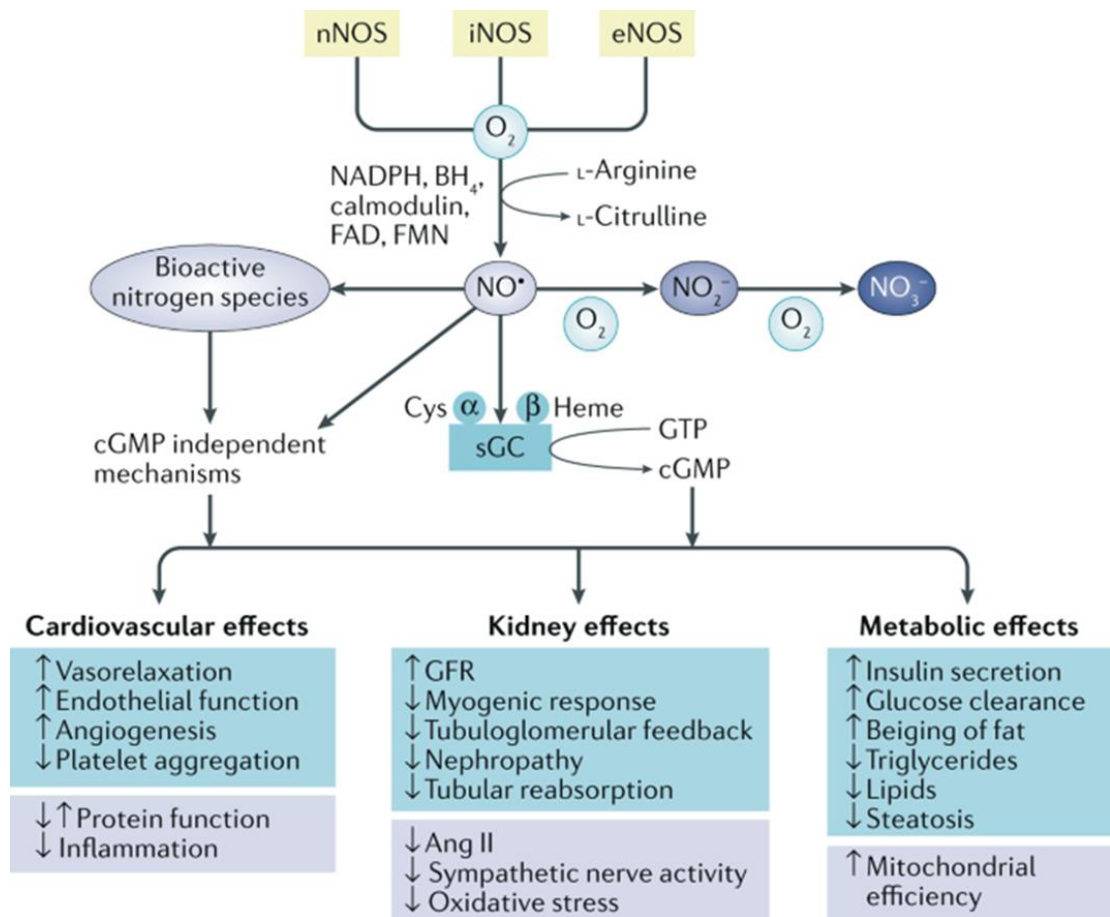
Fig 3.3: Effects of NO on cardiovascular, renal and metabolic functions [37]. The NOS pathway and its effects of NO on cardiovascular, renal and metabolic functions: Nitric oxide (NO) is endogenously synthesized by the reaction catalysed by three different nitric oxide synthase (NOS) isoforms: neuronal NOS (nNOS), inducible (iNOS) and endothelial NOS (eNOS). NO has short-life that is oxidized to form nitrite (NO3 −), nitrate (NO2 −) and other bioactive nitrogen species. NO has been associated with various effects on cardiovascular, renal and metabolic systems, mainly through cGMP-dependent mechanisms.
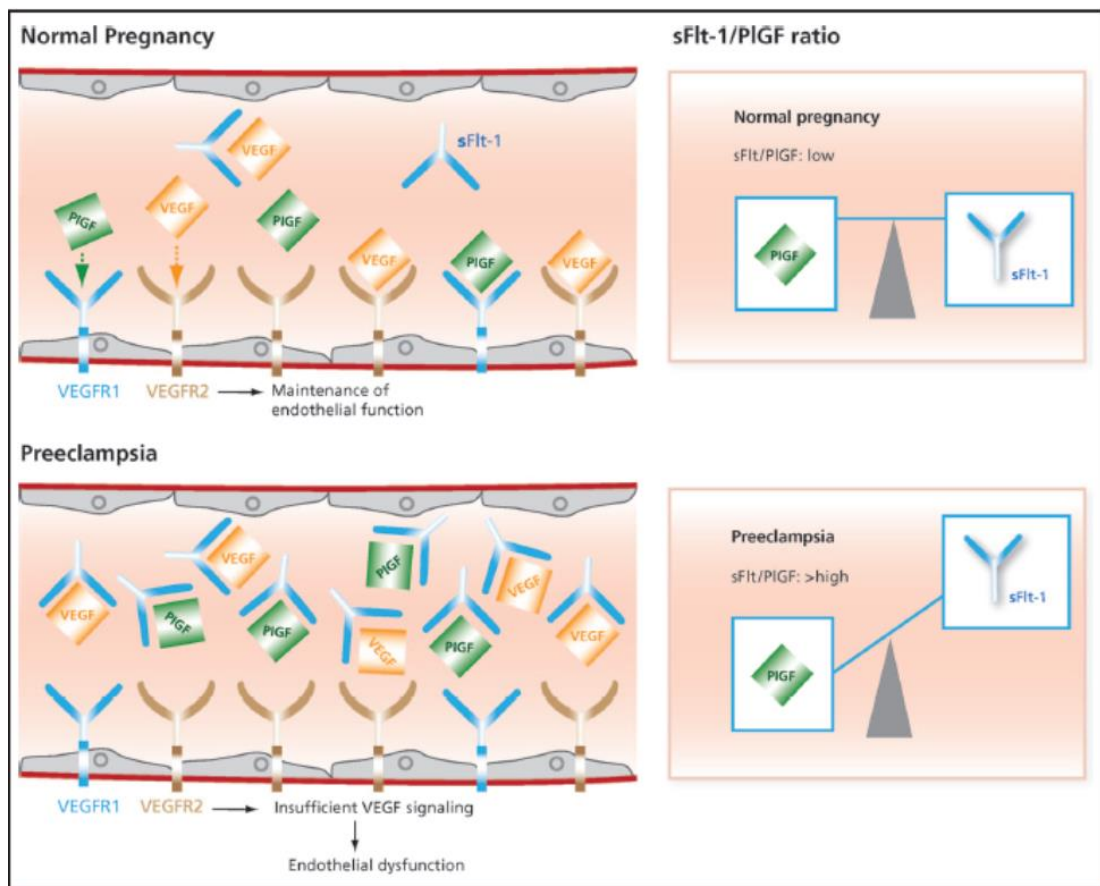
Figure 3.4: Role of VEGF and PIGF in pathogenesis of preeclampsia. In normal pregnancy, low sFlt-1 concentrations allow for proper VEGF and PlGF signaling. The anticoagulant and vasodilatory phenotype of the healthy endothelium is maintained. In preeclampsia, increased production and release of the antiangiogenic factor sFlt-1 from the placenta leads to a decrease in bioavailable VEGF and PlGF. This leads to an impairment of the VEGF/PlGF signaling axis and generalized endothelial dysfunction [38].

sFlt-1/PIGF ratio

In normal pregnancies, the level of sFlt-1 starts to rise after 30–32 weeks of gestation and PlGF level starts to decrease after 30 weeks of gestation. Actually cellular stress in the syncytiotrophoblast, which occurs during the last 8–10 weeks of a pregnancy leads to biochemical changes in levels of sFlt-1 and PlGF in normal pregnancies. Circulating levels of sFlt-1 and PlGF alter in PE patients. These alterations in sFlt-1 and PlGF levels begins prior to the disease onset and stays during the course of the disease. In women with PE, sFlt-1 increases approximately 5 weeks prior to the onset

of the disease, while PIGF decreases before the increase of sFlt-1: Hence, to improve the quality of diagnosis, some studies have suggested sFlt-1/PIGF ratio as a better marker for diagnosis of PE as compared to sFlt-1 and PIGF alone [39].

Recent studies have demonstrated that the sFlt-1/PlGF ratio is a more helpful predictor than either of these parameters alone. In the second trimester, sFlt-1/PlGF ratio might be an ideal parameter to diagnose early-onset preeclampsia (< 34 weeks) or preterm preeclampsia (< 37 weeks) [26]. The ratio between sFlt-1 and PlGF has shown to be elevated in women with diagnosed preeclampsia and markedly elevated before its clinical onset [24,38,40,41].

A recent case-control study defined cut-offs in early-onset and late-onset preeclampsia. In early-onset preeclampsia, between 20 + 0 and 33 + 6 weeks of gestation, a sFlt-1/ PlGF ratio of ≤33 was negative, and an sFlt-1/PlGF ratio of ≥85 was positive for confirmation of preeclampsia or

HELLP syndrome, with a sensitivity/specificity of 95%/ 94% and 88%/99%, respectively. In late-onset preeclampsia developed after 34 + 0 weeks of gestation, a sFlt-1/PlGFratio below the cutoff of ≤33 was negative, and an sFlt-1/ PlGF ratio of ≥110 was positive. The cutoffs in late-onset preeclampsia have lower sensitivity/specificity than the cutoffs for early-onset preeclampsia (90%/73% and 58%/96%, respectively) [42].

European multicenter case control study summarized that sFlt-1/ PlGF ratio, combined with appropriate gestational age-related cutoff values, could better help identify individuals with high risk of developing preeclampsia, compared with the PlGF measurement alone, thus allowing the optimization of their care [43].

Prediction of Short-Term Outcome in Pregnant Women with Suspected Preeclampsia: A prospective observational study conducted in 14 countries showed a cutoff point of 38 for the sFlt-1:PlGF ratio and was useful for predicting the short-term outcome of PE in clinically suspected PE women [44].

The relation between these biomarkers in preeclampsia have suggested that the reduction in circulating maternal PlGF concentration can be detected prior to the sFlt-1elevations, favoring PlGF as an early predictive marker of preeclampsia [45].

PlGF and sFlt-1 in relation to pregnancy outcome

The association of sFlt-1/PlGF ratio with pregnancy outcomes, in pregnant women with fetal IUGR and/or pre-eclampsia was studied by Chang Y et al and they found that women with high SFlt-1/PlGF ratio group had higher rates of intrauterine fetal demise (2/13 vs. 0/12) and early termination (1/13 vs. 0/12). The surviving offspring in this group had a higher incidence of preterm birth (GA: 31.4 ±2.9 weeks vs. 37.3 ± 1.3 weeks, p < 0.001), lower birth weight (1142± 472 g vs. 2311 ± 236 g, p < 0.001), higher incidence of respiratory distress syndrome (6/10 vs. 0/12, p=0.002)and bronchopulmonary dysplasia (4/10 vs. 0/12, p=0.01). They recommended the monitoring of sFlt-1/PlGF ratio in pregnant women with fetal IUGR and timely management for placenta-associated diseases [46].

Masoura et al. found that the increased serum values of sFlt-1 were associated with increased rates of late preterm, early term births and very-low-birth-weight [38]. Voller et al. showed that higher sFlt-1 levels appeared to increase the risk of postnatal growth failure[47].

Role of oxidative stress in PE

Oxidative stress has been shown to play one of the key roles in the pathogenesis of preeclampsia. Pathophysiological mechanisms responsible for PE are still obscure, but it is known that imbalance between the generation of oxidative stress and antioxidant defence system is associated with altered placental growth during the early phase of pregnancy. Oxidative stress is generally characterized by the formation of large amounts of reactive oxygen species (ROS) in cell membranes, endoplasmic reticulum, and mitochondria. ROS generated within the cells further contributes to mitochondrial structural damage. This process often leads to a vicious cycle generating more ROS due to an electron transport chain. There are specifc oxidative stress biomarkers one of them being malondialdehyde (MDA) which is used to estimate the oxidative stress. MDA is a three-carbon aldehyde and produces free radicals, which act on polyunsaturated fatty acids on the lipid bilayer. MDA levels increase beyond normal pregnancy levels by second trimester in PE [48,49].

References

1. Kashinakunti SV, Sunitha H, Shankarprasad DS, Suryaprakash G, Ingin JB. Lipid Peroxidation and Antioxidant Status in Preeclampsia. Al Ameen J Med Sci 2010;3(1):38-41.

2. Rodie VA, Freeman DJ, Sattar N, Greer IA. Pre-eclampsia and cardiovascular disease: metabolic syndrome of pregnancy? Atherosclerosis. 2004 Aug;175(2):189-202.

3. Kashinakunti SV, Sunitha H, Manjula R, Gurupadappa K, Kavita H. Correlation of Spot Urine Protein:Creatinine Ratio with 24-hour Urinary Protein in Preeclampsia: A Cross Sectional Study. BJMMR 2015;5(12):1571-9.

4. ACOG Practice Bulletin No. 202: Gestational Hypertension and Preeclampsia. Obstet Gynecol 2019;133:e1–25.

5. Facemire CS, Nixon AB, Griffiths R, Hurwitz H, Coffman TM. Vascular endothelial growth factor receptor 2 controls blood pressure by regulating nitric oxide synthase expression. Hypertension 2009;54:652-8.

6. Touyz RM, Lang NN, Herrmann J, Van den Meiracker AH, Jan Danser AH. Recent advances in hypertension and cardiovascular toxicities with vascular endothelial growth factor inhibition. Hypertension 2017;70:220-6.

7. Lankhorst S, Jan Danser AH, Van den Meiracker AH. Endothelin-1 and antiangiogenesis. Am J Physiol Regul Integr Comp Physiol 2016;310:R230–4,

8. Phipps E, Prasanna D, Brima W, Jim B. Preeclampsia: Updates in pathogenesis, definitions, and guidelines. Clin J Am Soc Nephrol 2016 Jun;11:1102–13.

9. Adu-Bonsaffoh K, Antwi DA, Gyan B, Amenyi Obed SA. Endothelial dysfunction in the pathogenesis of pre-eclampsia in Ghanaian women. BMC Physiology 2017;17:5. DOI 10.1186/s12899-017-0029-4.

10. Dai B, Liu T, Zhang B, Zhang X, Wang Z: The polymorphism for endothelial nitric oxide synthase gene, the level of nitric oxide and the risk for pre-eclampsia: A meta-analysis. Gene 2013;519:187–93.

11. Craici IM, Wagner SJ, Weissgerber TL, Grande JP, Garovic VD. Advances in the pathophysiology of pre-eclampsia and related podocyte injury. Kidney Int 2014 Aug;86(2):275-85.

12. Roberts JM, Druzin M, August PA, Gaiser RR, Bakris G, Granger JP, et al. ACOG Guidelines: Hypertension in pregnancy. American College of Obstetricians and Gynecologists. 2012.

13. Weed S, Bastek JA, Anton L, Elovitz MA, Parry S, Srinivas SK. Examining the correlation between placental and serum placenta growth factor in preeclampsia. Am J Obstet Gynecol 2012; 207: 140 e141-146.

14. Khalil A, Muttukrishna S, Harrington K, Jauniaux E. Effect of antihypertensive therapy with alpha methyldopa on levels of angiogenic factors in pregnancies with hypertensive disorders. PLoS ONE 2008;3(7):e2766.

15. Tsiakkas A, Duvdevani N, Wright A, Wright D, Nicolaides KH. Serum placental growth factor in the three trimesters of pregnancy: effects of maternal characteristics and medical history. Ultrasound Obstet Gynecol 2015;45:591-8.

16. Masuyama H, Segawa T, Sumida Y, Masumoto A, Inoue S, Akahori Y, et al. Different profiles of circulating angiogenic factors and adipocytokines between early- and late-onset preeclampsia. BJOG 2010 Feb;117(3):314-20.

17. Rasmussen LG, Lykke JA, Staff AC. Angiogenic biomarkers in pregnancy: defining maternal and fetal health. AOGS-14-0445-R2-accepted for publication.

18. Staff A. Circulating predictive biomarkers in preeclampsia. Pregnancy Hypertension: An International Journal of Women's Cardiovascular Health 2010;1:28-42.

19. Powers RW, Roberts JM, Plymire DA, Pucci D, Datwyler SA, Laird DM, et al. Low placental growth factor across pregnancy identifies a subset of women with preterm preeclampsia: type 1 versus type 2 preeclampsia? Hypertension 2012 Jul;60(1):239-46.

20. Audibert F, Boucoiran I, An N, Aleksandrov N, Delvin E, Bujold E, et al. Screening for preeclampsia using first-trimester serum markers and uterine artery Doppler in nulliparous women. Am J Obstet Gynecol 2010 Oct;203(4):383-8.

21. Cowans NJ, Stamatopoulou A, Matwejew E, von Kaisenberg CS, Spencer K. First-trimester placental growth factor as a marker for hypertensive disorders and SGA. Prenat Diagn 2010 Jun;30(6):565-70.

22. Staff AC, Johnsen GM, Dechend R, Redman CW. Preeclampsia and uteroplacental acute atherosis: immune and inflammatory factors. J Reprod Immunol2013; 101-102: 120-126.

23. Steegers EA, Von Dadelszen P, Duvekot JJ, Pijnenborg R. Preeclampsia. Lancet 2010;376: 631-644.

24. Levine RJ, Maynard SE, Qian C, Lim KH, England LJ, Yu KF, et al. Circulating angiogenic factors and the risk of preeclampsia. N Engl J Med 2004;350:672-83.

25. Maynard SE, Min JY, Merchan J, Lim KH, Li J, Mondal S, et al. Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. J Clin Invest 2003 Mar;111(5):649-58.

26. Kim YN, Lee DS, Jeong DH, Sung MS, Kim KT. The relationship of the level of circulating antiangiogenic factors to the clinical manifestations of preeclampsia. Prenat Diagn 2009 May;29(5):464-70.

27. Sezer SD, Kucuk M, Yenisey C, Yuksel H, Odabasi AR, Turkmen MK, et al. Comparison of angiogenic and anti-angiogenic factors in maternal and umbilical cord blood in early- and late-onset pre-eclampsia. Gynecol Endocrinol 2012 Aug;28(8):628-32

28. Garner PR, D'Alton ME, Dudley DK, Huard P, Hardie M. Preeclampsia in diabetic pregnancies. Am J Obstet Gynecol 1990 Aug;163(2):505-8.

29. Vatten LJ, Eskild A, Nilsen TI, Jeansson S, Jenum PA, Staff AC. Changes in circulating level of angiogenic factors from the first to second trimester as predictors of preeclampsia. Am J Obstet Gynecol 2007 Mar;196(3):239-6.

30. Moore AG, Young H, Keller JM, Ojo LR, Yan J, Simas TA, et al. Angiogenic biomarkers for prediction of maternal and neonatal complications in suspected preeclampsia. J Matern Fetal Neonatal Med 2012 Aug 22.

31. Stepan H, Geipel A, Schwarz F, Kramer T, Wessel N, Faber R. Circulatory soluble endoglin and its predictive value for preeclampsia in second-trimester pregnancies with abnormal uterine perfusion. Am J Obstet Gynecol 2008 Feb;198(2):175-6.

32. Roberts JM, Bodnar LM, Patrick TE. The role of obesity in preeclampsia. Pregnancy Hypertens 2011;1: 6–16.

33. American College of Obstetricians and Gynecologists (2013) Hypertension in Pregnancy Induced Practice Guideline WQ 244. Avalilable at: Link: https://goo.gl/M6ANOu : Accessed on 15-09-2018.

34. Perni U, Sison C, Sharma V, Helseth G, Hawfield A, et al. Angiogenic factors in superimposed preeclampsia: a longitudinal study of women with chronic hypertension during pregnancy. Hypertension 2012;59: 740–6.

35. Illanes S, Parra M, Serra R, Pino K, Figueroa-Diesel H, et al. Increased free fetal DNA levels in early pregnancy plasma of women who subsequently develop preeclampsia and IUGR. Prenat Diagn 2009;29: 1118-22.

36. Khalaf D, Krüger M, Wehland M, Infanger M, Grimm D. The Effects of Oral l-Arginine and l-Citrulline Supplementation on Blood Pressure. Nutrients. 2019 Jul 22;11(7):1679. doi: 10.3390/nu11071679. PMID: 31336573; PMCID: PMC6683098.

37. Carlström, M. Nitric oxide signalling in kidney regulation and cardiometabolic health. Nat Rev Nephrol 2021;17:575–90.

38. Verlohren S, Herraiz I, Lapaire O, Schlembach D, Moertl M, Zeisler H, et al. The sFlt-1/PlGF ratio in different types of hypertensive pregnancy disorders and its prognostic potential in preeclamptic patients. Am J Obstet Gynecol 2012;206:58.e1–58.e8.

39. Nikuei P, Rajaei M, Roozbeh N, Mohseni F, Poordarvishi F, Azad M et al. Diagnostic accuracy of sFlt1/PlGF ratio as a marker for preeclampsia. BMC Pregnancy Childbirth 2020;20:80. https://doi.org/10.1186/s12884-020-2744-2

40. Villa PM, Hämäläinen E, Mäki A, Räikkönen K, Pesonen AK, Taipale P, Kajantie E, Laivuori H: Vasoactive agents for the prediction of early- and late-onset preeclampsia in a high-risk cohort. BMC Pregnancy Childbirth 2013;13:110.

41. Verlohren S, Herraiz I, Lapaire O, Schlembach D, Zeisler H, Calda P, et al. New gestational phase specific cutoff values for the use of the soluble fms-like tyrosine kinase-1/placental growth factor ratio as a diagnostic test for preeclampsia. Hypertension 2014;63:346–52.

42. Tarasevičienė V, Grybauskienė R, Mačiulevičienė R. sFlt-1, PlGF, sFlt-1/PlGF ratio and uterine artery Doppler for preeclampsia diagnostics. MEDICINA 2016;52:34 9-53.

43. Zeisler H, Llurba E, Chantraine F, Vatish M, Staff AC, Sennström M, et al. Predictive value of the sFlt-1:PlGF ratio in women with suspected preeclampsia. N Engl J Med 2016;374:13-22.

44. Thadhani R, Mutter WP, Wolf M, Levine RJ, Taylor RN, Sukhatme VP, et al. First trimester placental growth factor and soluble fms-like tyrosine kinase 1 and risk for preeclampsia. J Clin Endocrinol Metab 2004 Feb;89(2):770-5.

45. Chang YS, Chen CN, Jeng SF, Su YN, Chen CY, Chou HC, et al. The sFlt-1/PlGF ratio as a predictor for poor pregnancy and neonatal outcomes. Pediatrics and Neonatology 2017;58:529-33.

46. Masoura S, Kalogiannidis I, Makedou K, Theodoridis T, Koiou K, Gerou S, et al. Biomarkers of endothelial dysfunction in preeclampsia and neonatal morbidity: a case-control study. Eur J Obstet Gynecol Reprod Biol 2014;175:119-23.

47. Voller SB, Chock S, Ernst LM, Su E, Liu X, Farrow KN, et al. Cord blood biomarkers of vascular endothelial growth (VEGF and sFlt-1) and postnatal growth: a preterm birth cohort study. Early Hum Dev 2014;90:195-200.

48. Guerby P, Tasta O, Swiader A, Pont F, Bujold E, Parant O, et al. Role of oxidative stress in the dysfunction of the placental endothelial nitric oxide synthase in preeclampsia. Redox Biol. 2021 Apr;40:101861. doi: 10.1016/j.redox.2021.101861. Epub 2021 Jan 19. PMID: 33548859; PMCID: PMC7873691.

49. Aouache R, Biquard L, Vaiman D, Miralles F. Oxidative Stress in Preeclampsia and Placental Diseases. Int J Mol Sci. 2018 May 17;19(5):1496. doi: 10.3390/ijms19051496. PMID: 29772777; PMCID: PMC5983711.

Chapter 4

MATERIAL AND METHODS

CHAPTER 4

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Study Design: Case-control study

Study Duration: Two years, from March 2019 to February 2021.

Source of Data: Pre-eclamptic and normal pregnant women from the department of obstetrics and gynecology, Hanagal Sri Kumareshwar Hospital, Bagalkot, Karnataka

Sample Size: Sample size calculation was done using Open Epi software version 2.3:1. At 95% confidence level, 80% power of the study. According to Caillon H et al. [1] proportion of preeclampsia patients with sFlt-1 > 95th percentile- 62%(P1);proportion of healthy controls with sFlt-1 > 95th percentile – 29% (P2). Sample size calculated is 34~ 40. Hence 40 preeclampsia cases and 40 normal gestation controls were included in the study.

Sample size formula

Sample size: $n = (Z\alpha/2 + Z\beta)^2 * (p1(1-p1) + p2(1-p2)) / (p1-p2)^2$,

α (two-tailed) = 0.050 and at 95% confidence level.

β = 0.200 and 80% of power of the study

The standard normal deviate for $\alpha = Z\alpha = 1.960$

The standard normal deviate for $\beta = Z\beta = 0.84$

Ethical Clearance: Institutional Ethics committee clearance was obtained from Shri B. M. Patil Medical College, BLDE (Deemed to be University) (BLDE(DU) /IEC/323/2018-189, dated 21-12-2018) and S. Nijalingappa Medical College, Bagalkot (SNMC/IECHSR/2018-189/A-84/1.1, dated 11-02-2019).

Consent: Informed written consent was obtained from all the participants.

Inclusion criteria:

Cases: Primigravida (singleton) pregnant women aged 18-35 years, 20 or more weeks of gestation, diagnosed and classified as mild and severe pre-eclampsia, according to American College of Obstetricians and Gynecologists (ACOG) [2] guidelines, were included in the study as cases.

Controls: Pregnant healthy primigravida women, aged 18-35 years with singleton pregnancies, matched for age and gestational weeks with the cases, were included in the study as controls.

Exclusion criteria:

Patients with pre-mature rupture of membranes, chorioamnionitis, multiple gestation, Rh isoimmunization, fetal anomalies, intra uterine fetal death, chronic inflammatory diseases, history of DM, past history of systemic HT, cardiovascular, renal diseases and chronic inflammatory diseases were excluded from the study.

Methodology: Detailed history was taken and clinical examination was done at the diagnosis. The required demographic information, clinical findings were noted from the participants according to a pre-designed proforma. Under aseptic measures, blood sample was collected from the antecubital vein. Whole blood (2.0mL) was transferred to EDTA tube and rest 3 mL was collected in plane tube and serum was separated, for estimation of biochemical parameter, liver function tests (LFT), renal function tests (RFT). All biochemical parameters were estimated by Biosystems BA 400 fully automated analyser and kit supplied Biosystems. External quality control is with Biorad. Serum for estimation of NO, eNOS, VEGF, PIGF, sFlt-1 and MDA was collected and stored at -20^{0} C and were estimated in batches by enzyme linked

immunosorbent assay (ELISA) method. **Chromate, Statfax 4300 ELISA reader was used.** Spot urine sample was collected and centrifuged for 5 minutes at 3000 rpm/min. Urine protein and urine creatinine were estimated in semi-automated analyser statfax 3300. Urine protein/creatinine ratio was calculated from the values obtained.

All the participants were followed-up at delivery to collect the placental tissue for histopathology and immunohistochemistry and also to note the birth weight and APGAR score of the neonates.

Statistical Analysis: Analysis was done using SPSS software version 19. Unpaired 't' test and Mann Whitney U test for quantitative data and Pearson's correlation test were applied. $p < 0.05$ was considered as statistically significant. ROC curve analysis was done for cut-off values, to calculate sensitivity, specificity, positive predictive value, negative predictive value and diagnostic accuracy for diagnosis of PE.

Estimation of Nitric Oxide (NO) *(ELISA kit method, Bioassay technology)*

Assay Principle

This kit is an Enzyme-Linked Immunosorbent Assay (ELISA). The plate has been pre-coated with human NO antibody. NO present in the sample is added and binds to antibodies coated on the wells. And then biotinylated human NO Antibody is added and binds to NO in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated NO antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and color develops in proportion to the amount of human NO. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm.

Reagent Preparation

● All reagents should be brought to room temperature before use.

● **Standard:** Reconstitute the 120μl of the standard (640μmol/L) with 120μl of standard diluent to generate a 320μmol/L standard stock solution. Allow the standard to sit for 15 mins with gentle agitation prior to making dilutions. Prepare duplicate standard points by serially diluting the standard stock solution (320μmol/L) 1:2 with standard diluent to produce 160μmol/L, 80μmol/L, 40μmol/L and 20μmol/L solutions. Standard diluent serves as the zero standard(0 μmol/L). Any remaining solution should be frozen at -20°C and used within one month. Dilution of standard solutions suggested are as follows:

320μmol/L	Standard No.5	120μl Original Standard + 120μl Standard
160μmol/L	Standard No.4	120μl Standard No.5 + 120μl Standard Diluent
80μmol/L	Standard No.3	120μl Standard No.4 + 120μl Standard Diluent
40μmol/L	Standard No.2	120μl Standard No.3 + 120μl Standard Diluent
20μmol/L	Standard No.1	120μl Standard No.2 + 120μl Standard Diluent
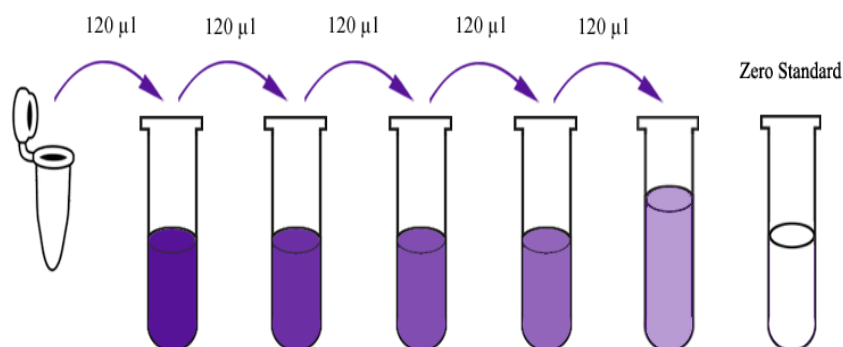
Figure 4.1: Preparation of NO standards

- **Wash Buffer** Dilute 20ml of Wash Buffer Concentrate 30x into deionized or distilled water to yield 500 ml of 1x Wash Buffer. If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved.

Conc µmol/L	20	40	80	160	320	640
OD	0.025	0.0441	0.084	0.178	0.335	0.62
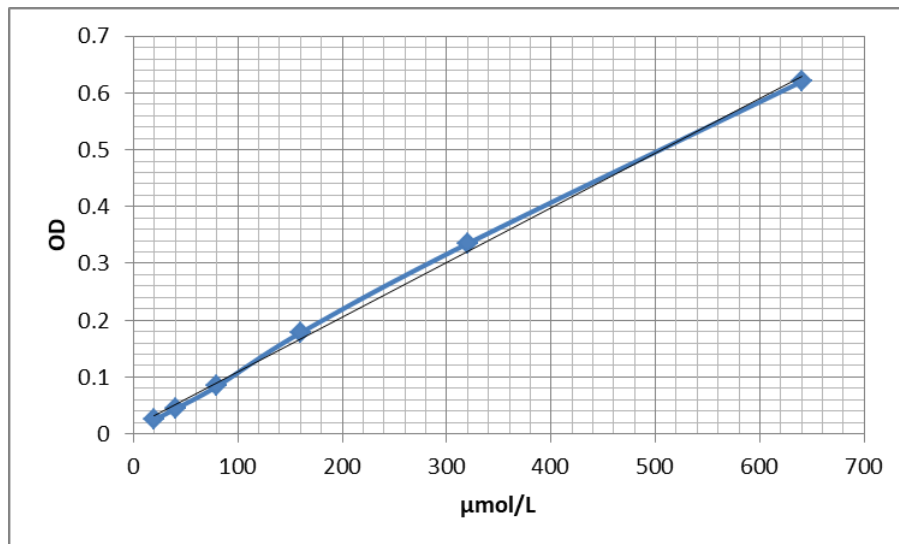
Figure 4.2: NO standard curve

Assay Procedure

1. Prepare all reagents, standard solutions and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature.

2. Determine the number of strips required for the assay. Insert the strips in the frames for use. The unused strips should be stored at 2-8°C.

3. Add 50µl standard to standard well. **Note**: Don't add antibody to standard well because the standard solution contains biotinylated antibody.

4. Add 40μl sample to sample wells and then add 10μl anti-NO antibody to sample wells, then add 50μl streptavidin-HRP to sample wells and standard wells (Not blank control well). Mix well. Cover the plate with a sealer. Incubate 60 minutes at 37°C.

5. Remove the sealer and wash the plate 5 times with wash buffer. Soak wells with at least 0.35 ml wash buffer for 30 seconds to 1 minute for each wash. For automated washing, aspirate all wells and wash 5 times with wash buffer, overfilling wells with wash buffer. Blot the plate onto paper towels or other absorbent material.

6. Add 50μl substrate solution A to each well and then add 50μl substrate solution B to each well. Incubate plate covered with a new sealer for 10 minutes at 37°C in the dark.

7. Add 50μl Stop Solution to each well, the blue color will change into yellow immediately.

8. Determine the optical density (OD value) of each well immediately using a microplate reader set to 450 nm within 10 minuets after adding the stop solution.

Estimation Endothelia derived Nitric Oxide Synthase (eNOS) ELISA (BOSTER BIOLOGICAL TECHNOLOGY)

Assay Principle

Human NOS3 Pre-Coated ELISA (Enzyme-Linked Immunosorbent Assay) is a solid-phase immunoassay, well strip plate that is pre-coated with antibody specific for NOS3. The detection antibody is a biotinylated antibody specific for NOS3. The capture antibody is a monoclonal antibody from mouse and the detection antibody is a biotinylated polyclonal antibody from goat.

To measure Human NOS3, add standards and samples to the wells, then add the biotinylated detection antibody. Wash the wells with PBS or TBS buffer, and add Avidin-Biotin-Peroxidase Complex (ABC-HRP). Wash away the unbounded ABC-HRP with PBS or TBS buffer and add TMB. TMB is an HRP substrate and will be catalyzed to produce a blue color product, which changes into yellow after adding the acidic stop solution. The absorbance of the yellow product at 450nm is linearly proportional to Human NOS3 in the sample. Read the absorbance of the yellow product in each well using a plate reader, and benchmark the sample wells' readings against the standard curve to determine the concentration of Human NOS3 in the sample.

Standard Curve

Conc pg/ml	0	78	156	312	625	1,250	2,500	5,000
O.D.	0.022	0.089	0.161	0.278	0.492	1.121	1.431	2.091
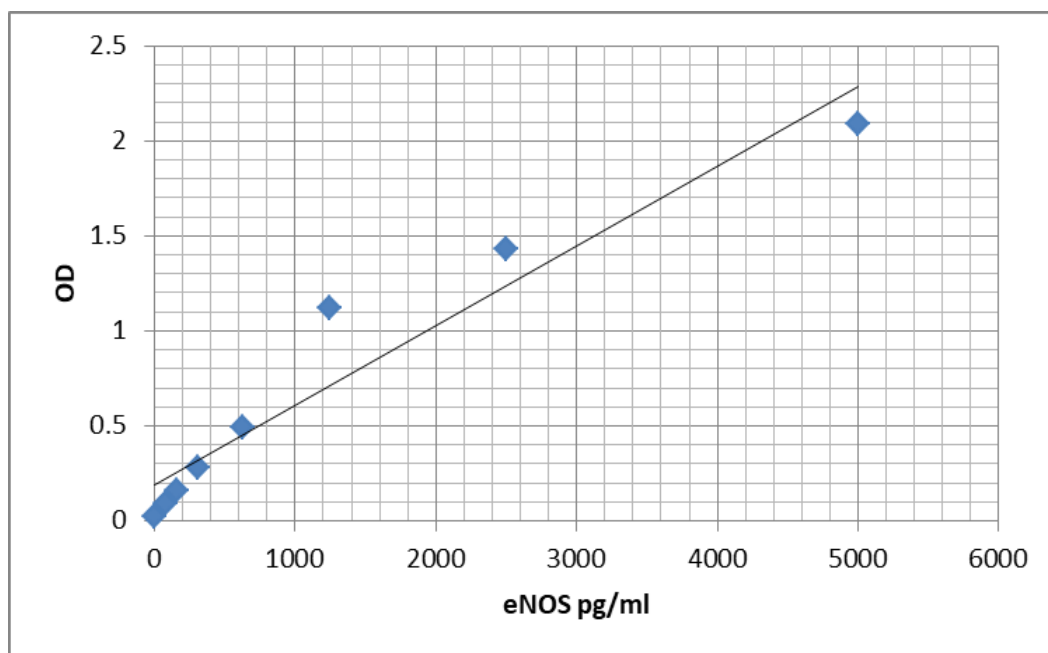
Figure 4.3: Standard Curve for eNOS

Assay Protocol

It is recommended that all reagents and materials be equilibrated to room temperature (18-25°C) prior to the experiment (see Preparation Before The Experiment, if you have missed this information).

1) Prepare all reagents and working standards as directed previously.

2) Remove excess microplate strips from the plate frame and seal and store them in the original packaging.

3) Add 100 µl of the standard, samples, or control per well. Add 100 µl of the **Sample Diluent** into the zero well. At least two replicates of each standard, sample, or control is recommended.

4) Cover with the plate sealer provided and incubate for 120 minutes at room temperature (or 90 min. at 37 °C).

5) Remove the cover and discard the liquid in the wells into an appropriate waste receptacle. Invert the plate on the benchtop onto a paper towel and tap the plate to gently blot any remaining liquid. It is recommended that the wells are not allowed to completely dry at any time.

6) Add 100 µl of the prepared **1x Biotinylated Anti-Human NOS3 antibody** to each well.

7) Cover with a plate sealer and incubate for 90 minutes at room temperature (or 60 min at 37°C).

8) Wash the plate 3 times with the **1x wash buffer**:

   a) Discard the liquid in the wells into an appropriate waste receptacle. Then, invert the plate on the benchtop onto a paper towel and tap the plate to gently blot any remaining liquid. It is recommended that the wells are not allowed to completely dry at any time.

b) Add 300 µl of the **1x wash buffer** to each assay well. (For cleaner background incubate for 60 seconds between each wash).

c) Repeat steps a-b 2 additional times.

d) Discard the wash buffer in the wells into an appropriate waste receptacle. Then, invert the plate on the benchtop onto a paper towel and tap the plate to gently blot any remaining liquid.

9) Add 100 µl of the prepared **1x Avidin-Biotin-Peroxidase Complex** into each well. Cover with the plate sealer provided and incubate for 40 minutes at RT (or 30 min at 37°C).

10) Wash the plate 5 times with the **1x wash buffer**:

a) Discard the liquid in the wells into an appropriate waste receptacle. Then, invert the plate on the benchtop onto a paper towel and tap the plate to gently blot any remaining liquid. It is recommended that the wells are not allowed to completely dry at any time.

b) Add 300 µl of the **1x wash buffer** to each assay well. (For cleaner background incubate for 60 seconds between each wash)

c) Repeat steps a-b 4 additional times.

d) Discard the wash buffer in the wells into an appropriate waste receptacle. Then, invert the plate on the benchtop onto a paper towel and tap the plate to gently blot any remaining liquid.

11) Add 90 µl of **Color Developing Reagent** to each well. Cover with the plate sealer provided and incubate in the dark for 30 minutes at RT (or 15-25 minutes at 37°C).

12) Add 100 µl of **Stop Solution** to each well. The color should immediately change to yellow.

13) Within 30 minutes of stopping the reaction, the O.D. absorbance should be read with a microplate reader at 450nm.

Estimation VEGF (Vascular Endothelial Cell Growth Factor) ELISA Kit (BOSTER BIOLOGICAL TECHNOLOGY)

Assay Principle

VEGFA Pre-Coated ELISA (Enzyme-Linked Immunosorbent Assay) kit is a solid-phase immunoassay specially designed to measure Human VEGFA with a 96-well strip plate that is pre-coated with antibody specific for VEGFA. The detection antibody is a biotinylated antibody specific for VEGFA. The capture antibody is monoclonal antibody from mouse and the detection antibody is polyclonal antibody from goat. The kit includes Human VEGFA protein as standards.

To measure Human VEGFA, add standards and samples to the wells, then add the biotinylated detection antibody. Wash the wells with PBS or TBS buffer, and add Avidin-Biotin-Peroxidase Complex (ABC-HRP). Wash away the unbounded ABC-HRP with PBS or TBS buffer and add TMB. TMB is an HRP substrate and will be catalyzed to produce a blue color product, which changes into yellow after adding the acidic stop solution. The absorbance of the yellow product at 450nm is linearly proportional to Human VEGFA in the sample. Read the absorbance of the yellow product in each well using a plate reader, and benchmark the sample wells' readings against the standard curve to determine the concentration of Human VEGFA in the sample. For more information on assay principle, protocols, and troubleshooting tips, see Boster's ELISA Resource Center at https://www.bosterbio.com/elisa-technical-resource-center.

VEGF Standard

1. Number tubes 1-8. Final Concentrations to be Tube # 1: 2,000.00 pg/ml, # 2: 1,000.00 pg/ml, # 3: 500.00 pg/ml, # 4: 250.00 pg/ml, # 5: 125.00 pg/ml, # 6: 62.50 pg/ml, # 7: 31.25 pg/ml, # 8: Sample Diluent serves as the zero standard (0 pg/ml).

2. To generate standard #1, add 200 µl of the reconstituted standard stock solution of 10 ng/ml and 800 µl of sample diluent to tube #1 for a final volume of 1000 µl. Mix thoroughly.

3. Add 300 µl of sample diluent to tubes # 2-7.

4. To generate standard # 2, add 300 µl of standard # 1 from tube # 1 to tube # 2 for a final volume of 600 µl. Mix thoroughly.

5. To generate standard # 3, add 300 µl of standard # 2 from tube # 2 to tube # 3 for a final volume of 600 µl. Mix thoroughly.

6. Continue the serial dilution for tube # 4-7.

Conc pg/ml	0	31.2	62.5	125	250	500	1000	2000
O.D.	0.020	0.055	0.113	0.189	0.345	0.589	1.106	1.913
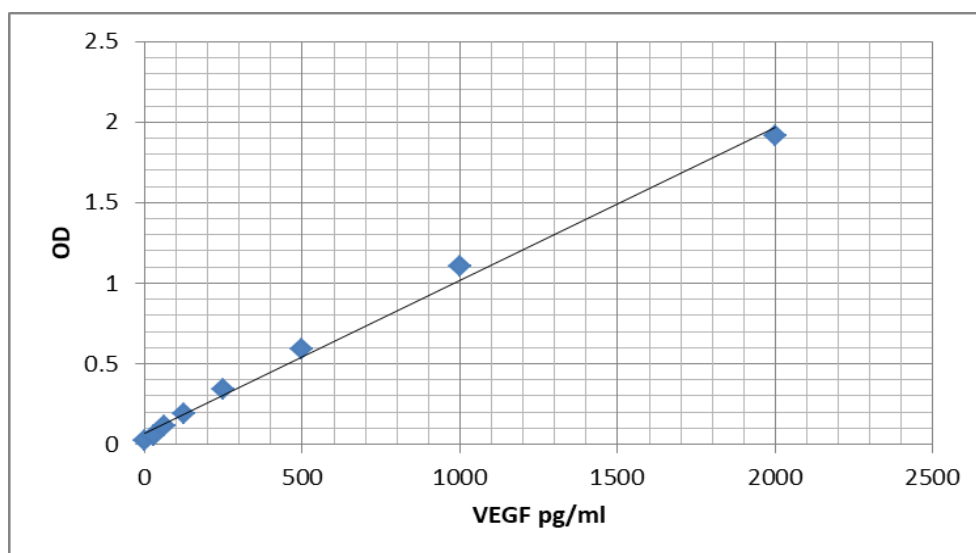
Figure 4.4: Standard Curve for VEGF

Assay Protocol

It is recommended that all reagents and materials be equilibrated to room temperature (18-25°C) prior to the experiment (see Preparation Before The Experiment, if you have missed this information).

1) Prepare all reagents and working standards as directed previously.

2) Remove excess microplate strips from the plate frame and seal and store them in the original packaging.

3) Add 100 µl of the standard, samples, or control per well. Add 100 µl of the Sample Diluent into the zero well. At least two replicates of each standard, sample, or control is recommended.

4) Cover with the plate sealer provided and incubate for 120 minutes at room temperature (or 90 min. at 37 °C).

5) Remove the cover and discard the liquid in the wells into an appropriate waste receptacle. Invert the plate on the benchtop onto a paper towel and tap the plate to gently blot any remaining liquid. It is recommended that the wells are not allowed to completely dry at any time.

6) Add 100 µl of the prepared 1x Biotinylated Anti-Human NOS3 antibody to each well.

7) Cover with a plate sealer and incubate for 90 minutes at room temperature (or 60 min at 37°C).

8) Wash the plate 3 times with the 1x wash buffer:

   a. Discard the liquid in the wells into an appropriate waste receptacle. Then, invert the plate on the benchtop onto a paper towel and tap the plate to gently blot any remaining liquid. It is recommended that the wells are not allowed to completely dry at any time.

b. Add 300 µl of the 1x wash buffer to each assay well. (For cleaner background incubate for 60 seconds between each wash).

c. Repeat steps a-b 2 additional times.

d. Discard the wash buffer in the wells into an appropriate waste receptacle. Then, invert the plate on the benchtop onto a paper towel and tap the plate to gently blot any remaining liquid.

9. Add 100 µl of the prepared 1x Avidin-Biotin-Peroxidase Complex into each well. Cover with the plate sealer provided and incubate for 40 minutes at RT (or 30 min at 37°C).

10. Wash the plate 5 times with the 1x wash buffer:

a) Discard the liquid in the wells into an appropriate waste receptacle. Then, invert the plate on the benchtop onto a paper towel and tap the plate to gently blot any remaining liquid. It is recommended that the wells are not allowed to completely dry at any time.

b) Add 300 µl of the 1x wash buffer to each assay well. (For cleaner background incubate for 60 seconds between each wash)

c) Repeat steps a-b 4 additional times.

d) Discard the wash buffer in the wells into an appropriate waste receptacle. Then, invert the plate on the bench top onto a paper towel and tap the plate to gently blot any remaining liquid.

11. Add 90 µl of Color Developing Reagent to each well. Cover with the plate sealer provided and incubate in the dark for 30 minutes at RT (or15-25 minutes at 37°C).

12. Add 100 µl of Stop Solution to each well. The color should immediately change to yellow.

13. Within 30 minutes of stopping the reaction, the O.D. absorbance should be read with a microplate reader at 450nm.

Estimation placental growth factor (PIGF)- ELISA method (BOSTER BIOLOGICAL TECHNOLOGY)

Assay Principle

Human PIGF Pre-Coated ELISA (Enzyme-Linked Immunosorbent Assay) kit is a solid-phase immunoassay specially designed to measure Human PGF with a 96-well strip plate that is pre-coated with antibody specific for PIGF. The detection antibody is a biotinylated antibody specific for PIGF. The capture antibody is monoclonal antibody from mouse and the detection antibody is polyclonal antibody from goat.

To measure Human PIGF, add standards and samples to the wells, then add the biotinylated detection antibody. Wash the wells with PBS or TBS buffer, and add Avidin-Biotin-Peroxidase Complex (ABC-HRP). Wash away the unbounded ABC-HRP with PBS or TBS buffer and add TMB. TMB is an HRP substrate and will be catalyzed to produce a blue color product, which changes into yellow after adding the acidic stop solution. The absorbance of the yellow product at 450nm is linearly proportional to Human PIGF in the sample. Read the absorbance of the yellow product in each well using a plate reader, and benchmark the sample wells' readings against the standard curve to determine the concentration of PIGF in the sample.

Standard

1. Number tubes 1-8. Final Concentrations to be Tube # 1: 1,000.00 pg/ml, # 2: 500.00 pg/ml, # 3: 250.00 pg/ml, # 4: 125.00 pg/ml, # 5: 62.50 pg/ml, # 6: 31.25 pg/ml, # 7: 15.63 pg/ml, # 8: Sample Diluent serves as the zero standard (0 pg/ml).

2. To generate standard #1, add 100 µl of the reconstituted standard stock solution of 10 ng/ml and 900 µl of sample diluent to tube #1 for a final volume of 1000 µl. Mix thoroughly.

3. Add 300 µl of sample diluent to tubes # 2-7.

4. To generate standard # 2, add 300 µl of standard # 1 from tube # 1 to tube # 2 for a final volume of 600 µl. Mix thoroughly.

5. To generate standard # 3, add 300 µl of standard # 2 from tube # 2 to tube # 3 for a final volume of 600 µl. Mix thoroughly.

6. Continue the serial dilution for tube # 4-7.

Standard Curve

Conc (pg/ml)	0	15.6	31.2	62.5	125	250	500	1000
O.D.	0.021	0.074	0.131	0.226	0.344	0.798	1.388	2.055
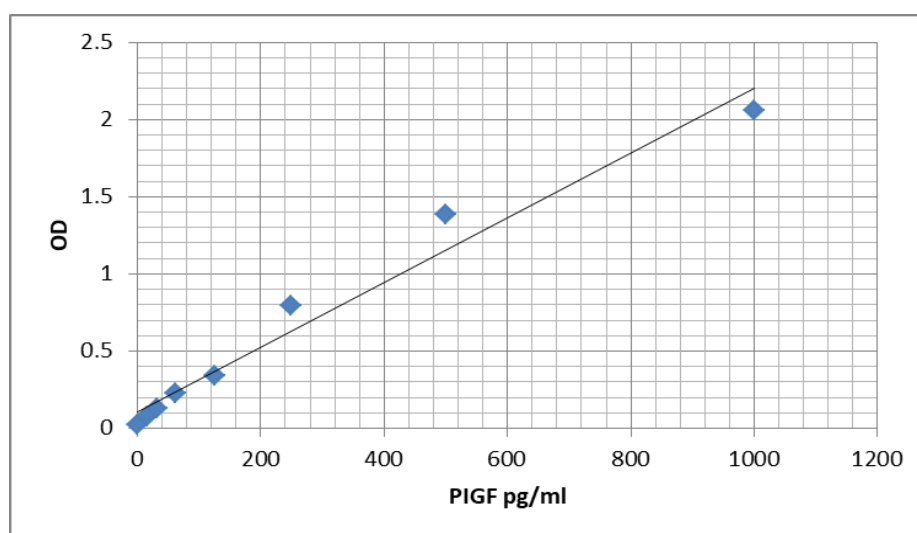
Figure 4.5: Standard curve for PIGF

Assay Protocol

It is recommended that all reagents and materials be equilibrated to room temperature (18-25°C) prior to the experiment (see Preparation Before the Experiment, if you have missed this information).

1) Prepare all reagents and working standards as directed previously.

2) Remove excess microplate strips from the plate frame and seal and store them in the original packaging.

3) Add 100 µl of the standard, samples, or control per well. Add 100 µl of the Sample Diluent into the zero well. At least two replicates of each standard, sample, or control is recommended.

4) Cover with the plate sealer provided and incubate for 120 minutes at room temperature (or 90 min. at 37 °C).

5) Remove the cover and discard the liquid in the wells into an appropriate waste receptacle. Invert the plate on the benchtop onto a paper towel and tap the plate to gently blot any remaining liquid. It is recommended that the wells are not allowed to completely dry at any time.

6) Add 100 µl of the prepared 1x Biotinylated Anti-Human NOS3 antibody to each well.

7) Cover with a plate sealer and incubate for 90 minutes at room temperature (or 60 min at 37°C).

8) Wash the plate 3 times with the 1x wash buffer:

   a. Discard the liquid in the wells into an appropriate waste receptacle. Then, invert the plate on the benchtop onto a paper towel and tap the plate to gently blot any remaining liquid. It is recommended that the wells are not allowed to completely dry at any time.

b. Add 300 µl of the 1x wash buffer to each assay well. (For cleaner background incubate for 60 seconds between each wash).

c. Repeat steps a-b 2 additional times.

d. Discard the wash buffer in the wells into an appropriate waste receptacle. Then, invert the plate on the benchtop onto a paper towel and tap the plate to gently blot any remaining liquid.

9. Add 100 µl of the prepared 1x Avidin-Biotin-Peroxidase Complex into each well. Cover with the plate sealer provided and incubate for 40 minutes at RT (or 30 min at 37°C).

10. Wash the plate 5 times with the 1x wash buffer:

a. Discard the liquid in the wells into an appropriate waste receptacle. Then, invert the plate on the benchtop onto a paper towel and tap the plate to gently blot any remaining liquid. It is recommended that the wells are not allowed to completely dry at any time.

b. Add 300 µl of the 1x wash buffer to each assay well. (For cleaner background incubate for 60 seconds between each wash)

c. Repeat steps a-b 4 additional times.

d. Discard the wash buffer in the wells into an appropriate waste receptacle. Then, invert the plate on the benchtop onto a paper towel and tap the plate to gently blot any remaining liquid.

11. Add 90 µl of Color Developing Reagent to each well. Cover with the plate sealer provided and incubate in the dark for 30 minutes at RT (or15-25 minutes at 37°C).

12. Add 100 µl of Stop Solution to each well. The color should immediately change to yellow.

13. Within 30 minutes of stopping the reaction, the O.D. absorbance should be read with a microplate reader at 450nm.

Estimation of sFLT1 (soluble fms-like tyrosine kinase-1) ELISA method (BOSTER BIOLOGICAL TECHNOLOGY)

Assay Principle

FLT1 Pre-Coated ELISA (Enzyme-Linked Immunosorbent Assay) kit is a solid-phase immunoassay specially designed to measure Human FLT1 with a 96-well strip plate that is pre-coated with antibody specific for FLT1. The detection antibody is a biotinylated antibody specific for FLT1. The capture antibody is polyclonal antibody from goat and the detection antibody is polyclonal antibody from goat. The kit includes Human FLT1 protein as standards.

To measure Human sFLT1, add standards and samples to the wells, then add the biotinylated detection antibody. Wash the wells with PBS or TBS buffer, and add Avidin-Biotin-Peroxidase Complex (ABC-HRP). Wash away the unbounded ABC-HRP with PBS or TBS buffer and add TMB. TMB is an HRP substrate and will be catalyzed to produce a blue color product, which changes into yellow after adding the acidic stop solution. The absorbance of the yellow product at 450nm is linearly proportional to Human FLT1 in the sample. Read the absorbance of the yellow product in each well using a plate reader, and benchmark the sample wells' readings against the standard curve to determine the concentration of Human FLT1 in the sample.

sFlt-1 Standard

1) Number tubes 1-8. Final Concentrations to be Tube # 1: 10,000.00 pg/ml, # 2: 5,000.00 pg/ml, # 3: 2,500.00 pg/ml, # 4: 1,250.00 pg/ml, # 5: 625.00 pg/ml, # 6: 312.50 pg/ml, # 7: 156.25 pg/ml, # 8: Sample Diluent serves as the zero standard (0 pg/ml).

2) For standard #1, add 1000 µl of undiluted standard stock solution to tube #1.

3) Add 300 µl of sample diluent to tubes # 2-7.

4) To generate standard # 2, add 300 µl of standard # 1 from tube # 1 to tube # 2 for a final volume of 600 µl. Mix thoroughly.

5) To generate standard # 3, add 300 µl of standard # 2 from tube # 2 to tube # 3 for a final volume of 600 µl. Mix thoroughly.

6) Continue the serial dilution for tube # 4-7.

Conc (pg/ml)	0	156	312	625	1250	2500	5000	10000
O.D.	0.031	0.069	0.124	0.164	0.366	0.681	1.178	1.920
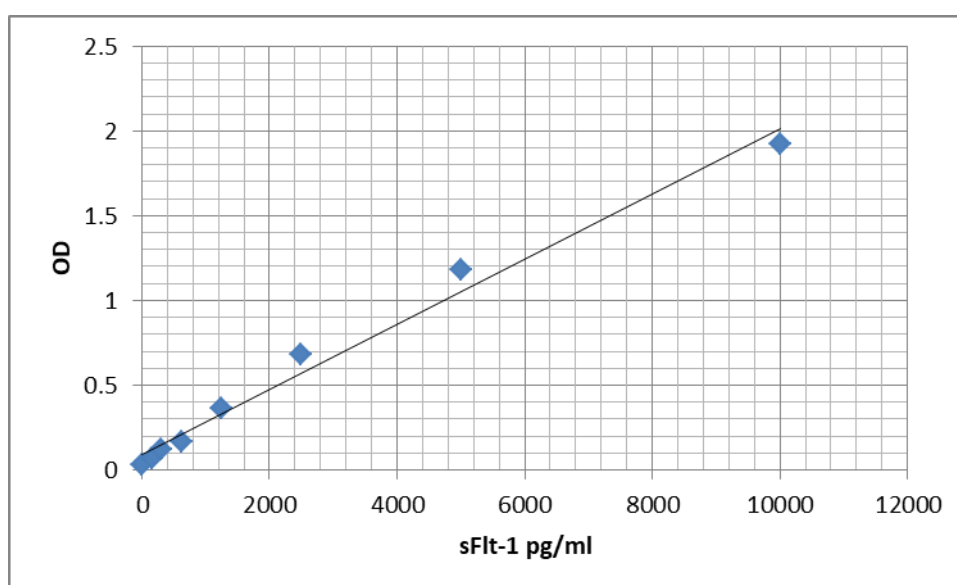
Figure 4.6 : Standard Curve for sFlt-1

Assay Protocol

It is recommended that all reagents and materials be equilibrated to room temperature (18-25°C) prior to the experiment (see Preparation Before The Experiment, if you have missed this information).

1. Prepare all reagents and working standards as directed previously.

2. Remove excess microplate strips from the plate frame and seal and store them in the original packaging.

3. Add 100 µl of the standard, samples, or control per well. Add 100 µl of the Sample Diluent into the zero well. At least two replicates of each standard, sample, or control is recommended.

4. Cover with the plate sealer provided and incubate for 120 minutes at room temperature (or 90 min. at 37 °C).

5. Remove the cover and discard the liquid in the wells into an appropriate waste receptacle. Invert the plate on the benchtop onto a paper towel and tap the plate to gently blot any remaining liquid. It is recommended that the wells are not allowed to completely dry at any time.

6. Add 100 µl of the prepared 1x Biotinylated Anti-Human NOS3 antibody to each well.

7. Cover with a plate sealer and incubate for 90 minutes at room temperature (or 60 min at 37°C).

8. Wash the plate 3 times with the 1x wash buffer:

    a) Discard the liquid in the wells into an appropriate waste receptacle. Then, invert the plate on the benchtop onto a paper towel and tap the plate to gently blot any remaining liquid. It is recommended that the wells are not allowed to completely dry at any time.

b) Add 300 µl of the 1x wash buffer to each assay well. (For cleaner background incubate for 60 seconds between each wash).

c) Repeat steps a-b 2 additional times.

d) Discard the wash buffer in the wells into an appropriate waste receptacle. Then, invert the plate on the benchtop onto a paper towel and tap the plate to gently blot any remaining liquid.

9. Add 100 µl of the prepared 1x Avidin-Biotin-Peroxidase Complex into each well. Cover with the plate sealer provided and incubate for 40 minutes at RT (or 30 min at 37°C).

10. Wash the plate 5 times with the 1x wash buffer:

a) Discard the liquid in the wells into an appropriate waste receptacle. Then, invert the plate on the benchtop onto a paper towel and tap the plate to gently blot any remaining liquid. It is recommended that the wells are not allowed to completely dry at any time.

b) Add 300 µl of the 1x wash buffer to each assay well. (For cleaner background incubate for 60 seconds between each wash)

c) Repeat steps a-b 4 additional times.

d) Discard the wash buffer in the wells into an appropriate waste receptacle. Then, invert the plate on the benchtop onto a paper towel and tap the plate to gently blot any remaining liquid.

11. Add 90 µl of Color Developing Reagent to each well. Cover with the plate sealer provided and incubate in the dark for 30 minutes at RT (or15-25 minutes at 37°C).

12. Add 100 µl of Stop Solution to each well. The color should immediately change to yellow.

13. Within 30 minutes of stopping the reaction, the O.D. absorbance should be read with a microplate reader at 450nm

Estimation of MDA (Malondialdehyde) ELISA Kit (Wuhan Fine Biotech Co., Ltd.)

Principle of the Assay This kit was based on Competitive-ELISA detection method. The microtiter plate provided in this kit has been pre-coated with target. During the reaction, target in the sample or standard competes with a fixed amount of target on the solid phase supporter for sites on the Biotinylated Detection Antibody specific to target. Excess conjugate and unbound sample or standard are washed from the plate, and HRP-Streptavidin (SABC) is added to each microplate well and incubated. Then TMB substrate solution is added to each well. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450nm. The concentration of target in the samples is then determined by comparing the OD of the samples to the standard curve.

2, Standards: 1). Add 1 ml Sample Dilution Buffer into one Standard tube (labeled as zero tube), keep the tube at room temperature for 10 minutes and mix them thoroughly.

2). Label 7 eppendorf (EP) tubes with 1/2, 1/4, 1/8, 1/16, 1/32, 1/64 and blank respectively. Add 0.3ml of the Sample Dilution Buffer into each tube. Add 0.3ml of the above Standard solution (from zero tube) into 1st tube and mix them thoroughly. Transfer 0.3ml from 1st tube to 2nd tube and mix them thoroughly. Transfer 0.3ml

from 2nd tube to 3rd tube and mix them thoroughly, and so on. Sample Dilution Buffer was used for the blank control.
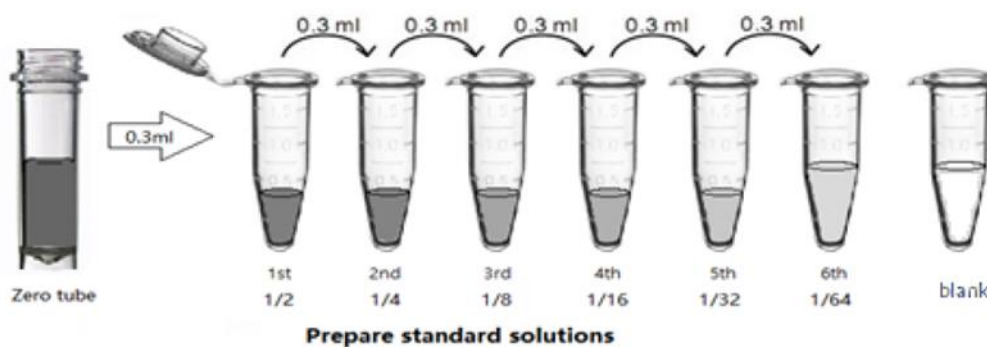
Figure 4.7: Preparation of standards for MDA

ng/ml	0	7.812	15.625	31.25	62.5	125	250	500
OD	2.22	1.535	0.893	0.613	0.373	0.217	0.111	0.071
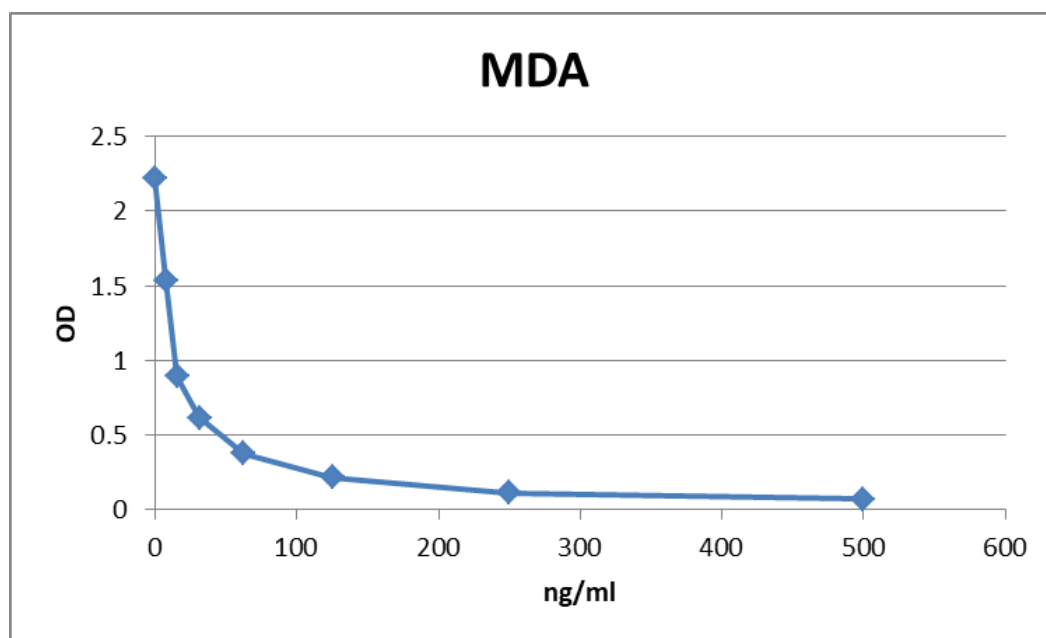
Figure 4.8: Standard curve for MDA

Assay Procedure

When diluting samples and reagents, they must be mixed completely and evenly. Before adding TMB into wells, equilibrate

TMB Substrate for 30 minutes at 37°C. It is recommended to plot a standard curve for each test.

1) Set standard, test samples, control (blank) wells on the pre-coated plate respectively, and then, records their positions. It is recommended to measure each standard and sample in duplicate. Wash plate 2 times before adding standard, sample and control (blank) wells!

2) Add Sample and Biotin-labeled Antibody: Add 50ul of Standard, Blank, or Sample per well. The blank well is added with Sample/Standard Dilution Buffer. Immediately add 50ul Biotin-labeled Antibody Working Solution into each well. Cover with the Plate sealer we provided. Gently tap the plate to ensure thorough mixing. Incubate for 45 minutes at 37°C. (Solutions are added to the bottom of microplate well, avoiding inside wall touching and foaming as much as you can.)

3) Wash: Remove the cover, and wash plate 3 times with Wash Buffer, and let the wash buffer stay in the wells for 1 minute each time. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. HRP-Streptavidin Conjugate (SABC): Add 100ul SABC Working Solution into each well. Cover it with a new Plate sealer. Incubate for 30 minutes at 37°C.

4) Wash: Remove the cover and wash plate 5 times with Wash Buffer, and let the wash buffer stay in the wells for 1 -2 minutes each time.

5) TMB Substrate: Add 90ul TMB Substrate into each well, cover the plate and incubate at 37°C in dark within 10-20 minutes. (Before adding TMB into wells,

equilibrate TMB Substrate for 30 minutes at 37°C. It is recommended to plot a standard curve for each test.) (Note: The reaction time can be shortened or extended according to the actual color change, but not more than 30 minutes. You can terminate the reaction when apparent gradient appeared in standard wells.)

6) Stop: Add 50ul Stop Solution into each well. The color will turn yellow immediately. The adding order of Stop Solution should be as the same as the TMB Substrate Solution.

7) OD Measurement: Read the O.D. absorbance at 450nm in Microplate Reader immediately after adding the stop solution. Regarding calculation, the standard curve can be plotted as the O.D.450 of each standard solution (Y) vs. the respective concentration of the standard solution (X). The target concentration of the samples can be interpolated from the standard curve

References

1. Caillon H, Tardif C, Dumontet E, Winer N, Masson D. Evaluation of sFlt-1/PlGF Ratio for Predicting and Improving Clinical Management of Pre-eclampsia: Experience in a Specialized Perinatal Care Center. Ann Lab Med. 2018 Mar;38(2):95-101. doi: 10.3343/alm.2018.38.2.95. PMID: 29214752; PMCID: PMC5736685.

2. American College of Obstetricians and Gynecologists; Task Force on Hypertension in Pregnancy. Hypertension in pregnancy. Report of the American College of Obstetricians and Gynecologists' Task Force on Hypertension in Pregnancy. Obstet Gynecol 2013; 122: 1122-31.

Chapter 5

RESULTS

CHAPTER 5

RESULTS:

Forty normal healthy pregnant women as controls and forty PE patients as cases were selected for the present study. Women aged between 18 to 35 years of age were included in the study. Only two(4.9%) were between 30-35 years of age in cases, all others were between 18 to 29 years of age in both cases and controls (Table 5.1).

Table 5.1: Age distribution in controls and cases

Age in Years	Controls	Cases
18-20	13(32.5%)	19 (47.5%)
21-30	27 (67.5%)	19(47.5%)
31-35	0	2(4.9%)
Total	40	40

Among the PE cases, 8 had mild PE, 32 had severe PE (Table 5.2).

Table 5.2: Mild and severe cases preeclampsia

	Cases
Mild	8
Severe	32

In severe cases of PE, 22 Patients had systolic blood pressure (SBP) >160 mm Hg and 17 had diastolic blood pressure (DBP)> 110 mm Hg, Thrombocytopenia was found in 4 patients, 3 patients had epigastric pain, 3 patients had raised aspartate transaminase (AST) and alanine transaminase (ALT), renal insufficiency (S. Creatinine>1.1 mg/dl), abdominal oedema, pulmonary oedema and venous thrombosis was present in one patient each. New onset of Cerebral or visual disturbance; i.e, headache was found in 14 patients and 3 patients had visual disturbance. Two patients presented with HELLP syndrome (Table 5.3).

Table 5.3: Severity criteria as per ACOG guidelines and number of PE cases

Severity criteria	No of cases
SBP >160 mm Hg	22 (14 DBP>110)
DBP> 110 mm Hg	17 (10 SBP>160)
Thrombocytopenia	4
Epigastric pain	3
Abdominal oedma	1
Raised AST and ALT	3
Renal insufficiency (S. Creatinine >1.1 mg/dl)	1
Pulmonary oedma	1
New onset of Cerebral or visual disturbance	Headache 14 Visual disturbance 3
Venous thrombosis	1
HELLP syndrome	2

30 normal pregnant women underwent LSCS, 28 PE patients underwent LSCS, 6 PE women had pre-term vaginal delivery (VD). 10 control women had full term vaginal delivery and 6 PE women had full term vaginal delivery (Table 5.4).

Table 5.4: Mode of delivery in controls and cases

	Controls	Cases
LSCS	30	28
Pre-term VD	-	6
Full term VD	10	6

There were 5 neonatal deaths in PE patients. 39and 30 neonates of control women and PE women cried immediately after birth, one neonate of PE patient had birth asphyxia and 2 responded to tactile stimulation. One neonate of control woman and 2 of PE women required bag and mask ventilation respectively (Table 5.5 and 5.6).

Table 5.5: Gender Distribution of Neonates in controls and cases

	Controls	Cases
Female	23	19(2 Dead)
Male	17	21(3 Dead)
Total	40	40

Table 5.6: Neonatal outcome in both controls and cases

	Controls	Cases
Cried Immediately	39	30
Birth asphyxia	-	1
Responded to tactile stimulation	-	2
Bag and mask	1	2
Death	0	5
Total	40	40

Comparison of general characteristics between controls and cases: Mean gestational age was 37.78±2.97 weeks in controls and 35.63±4.37 weeks in PE cases, which was not statistically significant (p value = 0.084). There was significant increase in pulse rate (p=0.003), systolic blood pressure (p=0.000), diastolic blood pressure (p=0.000), body mass index (p=0.004) in PE group than control group (Table 5.7) .

Table 5.7: Comparison of general characteristics between controls and cases

	Controls	Cases	t	p
Gestational weeks	37.78±2.97	35.63±4.37	2.511	0.084
Pulse (Beats/min)	86.54±6.98	92.28±9.27	-3.044	0.003
SBP (mmHg)	117.41±10.75	157.55±17.75	-11.878	0.000
DBP (mmHg)	74.70±9.45	102.40±9.10	-13.093	0.000
BMI (kg/m^2)	24.82±3.65	28.99±5.41	-3.008	0.004

Comparison of hematological parameters between controls and cases: Eosinophil count was significantly (p=0.001) higher in normal pregnant women than PE patients, other haematological parameters did not show significant change between cases and controls (Table 5.8).

Table 5.8: Comparison of hematological parameters between controls and cases

Parameter	Controls	Cases	t	p
HB gm%	11.57±1.48	11.06±2.12	1.19	0.23
RBC millions/µL	4.10±0.50	4.05±0.94	0.25	0.80
WBC	12370.27±3126.48	12933.33±4473.21	-0.63	0.52
P%	70.92±8.42	71.51±12.52	-0.241	0.81
L%	23.84±8.05	24.36±12.01	-0.221	0.82
E%	3.32±1.08	2.67±1.19	2.50	0.01
M%	1.92±1.27	1.46±1.44	1.45	0.14
B%	0.00±0.00	0.00±0.00	1.87	0.06
Platelet	228891.89±49402.42	199743.59±81379.78	-0.67	0.50
BT, min	2.92±0.37	2.80±0.35	0.01	0.98
CT, min	5.72±0.38	5.71±0.38	1.19	0.23
PT, Sec	12.72±1.26	13.49±2.23	0.67	0.50

Comparison of Blood Sugar and Renal Functions of controls and cases: There was no significant difference in random blood glucose level and serum creatinine (p= 0.575, 0.590 respectively) in controls and cases. The renal function tests in tearms of serum urea, serum uric acid, urine protein, urine P/C ratio were significantly higher in preeclampsia cases than healthy pregnant women (p value was 0.025, 0.000, 0.010, 0.001respectively) (Table 5.9).

Table 5.9: Blood Sugar and Renal Functions of controls and cases

	Controls	Cases	t	p
RBS mg/dl	88.52±21.46	85.87±20.37	0.563	0.575
Urea mg/dl	21.30±6.48	24.28±4.91	-2.291	0.025
Creatinine mg/dl	0.73±0.13	0.75±0.15	-0.542	0.590
Uric acid mg/dl	3.73±1.00	5.02±1.49	-4.434	0.000
Urine Protein mg/l	120.26±158.84	2464.68±3185.03	-2.73	0.010
Urine P/C ratio	0.23±0.28	4.47±4.14	-3.801	0.001

Comparison of liver function tests in PE controls and cases: There was significant decrease (p=0.003) in serum albumin in preeclampsia patients (**3.27±0.51gm/dl)** than control (**3.60±0.44gm/dl)** group. AST was significantly higher (p=0.04) in cases(**43.43±61.28IU/L)** than the control group (**23.37±10.40 IU/L)**, ALT was also more in preeclampsia cases than normal pregnant women but it was not statistically significant (p=**0.07)**. The other parameters of liver function tests did not show significant change (Table 5.10) .

Table 5.10: Comparison of liver function tests in PE controls and cases

Parameter	Controls	Cases	t	p
Total bilirubin mg/dl	0.57±0.20	0.67±0.45	-1.23	0.22
Direct bilirubin mg/dl	0.27±0.12	0.31±0.21	-1.02	0.30
Indirect bilirubin mg/dl	0.30±0.13	0.36±0.27	-1.18	0.24
Total proteins gm/dl	6.26±0.70	5.99±0.69	1.694	0.094
Albumin gm/dl	3.60±0.44	3.27±0.51	3.065	**0.003**
Globulin gm/dl	2.66±0.63	2.72±0.69	-0.421	0.675
A/G ratio	1.46±0.54	1.34±0.78	0.746	0.458
AST IU/L	23.37±10.40	43.43±61.28	-2.015	**0.04**
ALT IU/L	15.82±10.17	35.36±67.37	-1.791	0.07
ALP IU/L	198.69±80.58	195.815±90.81	0.148	0.88
LDH IU/L	232.13±67.78	380.54±262.16	-0.96	0.34

Comparison of Birth weight, APGAR between neonates of controls and cases:
Birth weight, APGAR at 1 minute and APGAR score at 5 min were significantly (**p=** 0.000, 0.015, 0.023) lower in neonates born to preeclampsia women (2.17±0.83 kg, 6.59±2.66,7.68±2.951) as compared to neonates of healthy pregnant women (2.85±0.57 kg, 7.81±0.78, 8.91±0.39) (Table 5.11).

Table 5.11: Comparison of Birth weight, APGAR between neonates of controls and cases

	Controls	**Cases**	**t**	**p**
Birth Weight (Kg)	2.85±0.57	2.17±0.83	3.888	0.000
APGAR 1min	7.81±0.78	6.59±2.66	2.499	0.015
APGAR 5min	8.91±0.39	7.68±2.951	2.337	0.023

Comparison of general characteristics between mild and severe cases of PE: In the present study the comparison between mild and severe cases of PE was done, systolic blood pressure (161.56±17.24 mm Hg), diastolic blood pressure (104.06±8.87 mm Hg) were significantly (p= 0.003 and 0.019 respectively) higher in severe cases of PE as compared to mild cases of PE. Birth weight of neonates born to severe PE (2.01±0.83kg) was significantly (p= 0.025) lower as compared to mild cases (2.79±0.51 kg) of PE (Table 5.12) .

Table 5.12: Comparison of general characteristics between mild and severe cases of PE

	Mild	Severe	p
SBP mmHg	141.50±8.46	161.56±17.24	**0.003**
DBP mmHg	95.75±7.04	104.06±8.87	**0.019**
AST IU/L	31.62±19.35	46.38±67.79	0.549
ALT IU/L	15.33±12.43	40.36±74.47	0.354
Creatinine mg/dl	0.71±0.13	0.76±0.16	0.376
Platelet	249500.00±41582.44	191656.25±85353.00	0.116
Total proteins gm/dl	6.11±0.37	6.01±0.77	0.727
Albumin gm/dl	3.43±0.43	3.23±0.52	0.314
Globulin gm/dl	2.67±0.50	2.78±0.76	0.712
A/G ratio	1.35±0.45	1.33±0.86	0.940
B. Wt in Kg	2.79±0.51	2.01±0.83	**0.025**
APGAR 1min	8.00±0.000	6.29±2.85	0.156
APGAR 5min	9.00±0.00	7.39±3.19	0.232

Comparison of NO, eNOS, VEGF, PIGF, sFlt-1, Sflt1:PIGF and MDA between controls and PE: There was significant decrease in NO (Median cases=56.56 µmol/L, controls=77.77 µmol/L; inter quartile range, cases=28.74-78.53, controls=61.93-111.34), eNOS (Median cases =81.63pg/ml, controls=124.00pg/ml; inter quartile range cases=54.87-132.22, controls=62.15 to 226.00),VEGF(Median cases=90.12pg/ml, controls=180.40pg/ml; inter quartile range cases=52.50-105.93, controls=86.25 to 255.11) and PIGF (Median cases=48.98pg/ml, controls=124.11pg/ml; inter quartile range cases=31.84 to 72.71, controls=73.04 to 182.35) in PE cases as compared to normal pregnant women. sFlt1 (Median cases=3984.00pg/ml, controls=787.00pg/ml; inter quartile range cases=2313.00-5117.00, controls=404.25-1011.00) and sFlt1:PIGF(Median cases=75.25, controls=5.30; interquartile range cases=37.18-138.71,

controls=2.09 to 10.68) ratio were significantly higher (p<0.0001) in PE cases than control group (Significance p values between PE and control group for **NO, eNOS, VEGF, PIGF, sFlti and Sflt1:PIGF were 0.0033, 0.0473, 0.0003, < 0.0001, < 0.0001 and < 0.0001** respectively) (Table 5.13, Figure 5.1-5.6).

Table 5.13: Comparison of maternal serum NO, eNOS, VEGF, PIGF, sFlt-1, sFlt1: PIGF between controls and cases

	Median		Interquartile range		Average rank		MWU	Z	P
	Controls	Cases	Controls	Cases	Control	cases			
NO#	77.77	56.56	61.93-111.34	28.74 to 78.53	47.67	32.51	480.50	2.93	0.0033
eNOS*	124.00	81.63	62.15 to 226.00	54.87-135.22	45.52	32.50	630.00	2.71	0.0473
VEGF*	180.40	90.12	86.25 to 255.11	52.50-105.93	50.58	31.64	436.50	3.62	0.0003
PIGF*	124.11	48.98	73.04 to 182.35	31.84 to 72.71	52.38	28.52	308.50	4.61	< 0.0001
sFLT1*	787.00	3984.0	404.25-011.00	313.0-117.0	23.24	59.75	92.00	6.94	< 0.0001
Ratio	5.30	75.25	2.09 to 10.68	37.18-138.71	22.21	55.09	119.00	6.39	< 0.0001

#: µmol/L, * :pg/ml
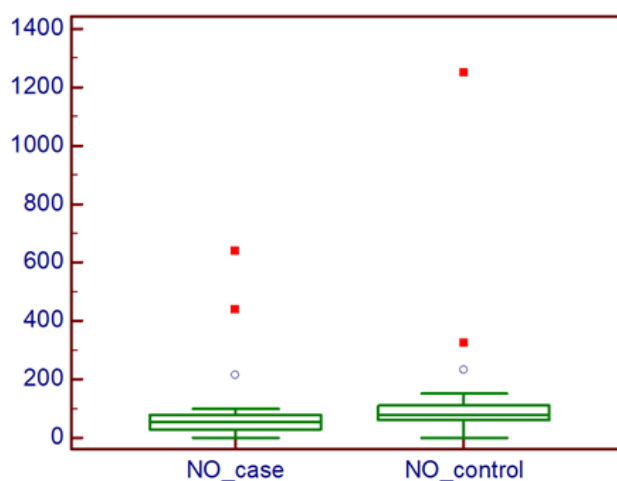
Figure 5.1 Comparison of maternal serum NO between cases and controls
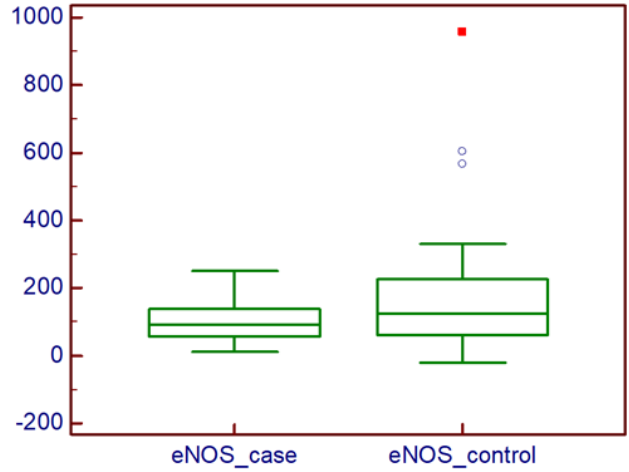
Figure 5.2: Comparison of maternal serum eNOS between cases and controls
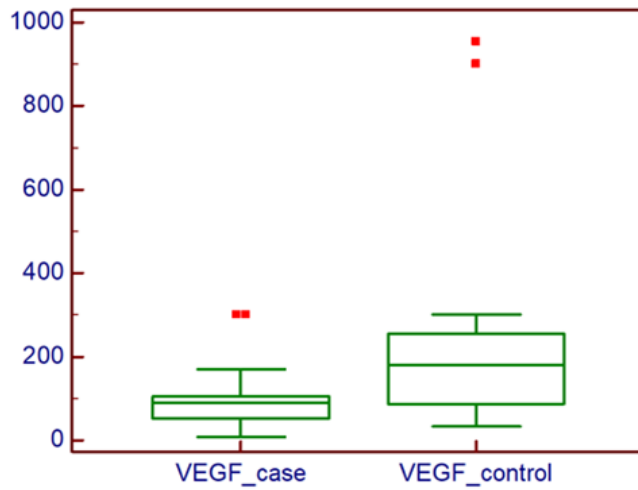
Figure 5.3 : Comparison of maternal serum VEGF between cases and controls
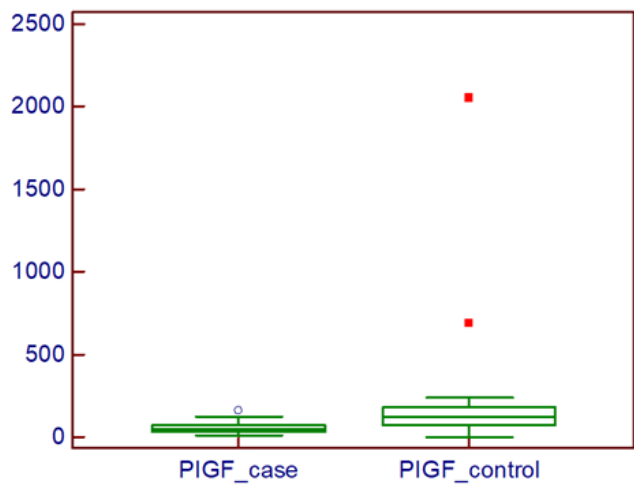
Figure 5.4: Comparison of maternal serum PIGF between cases and controls
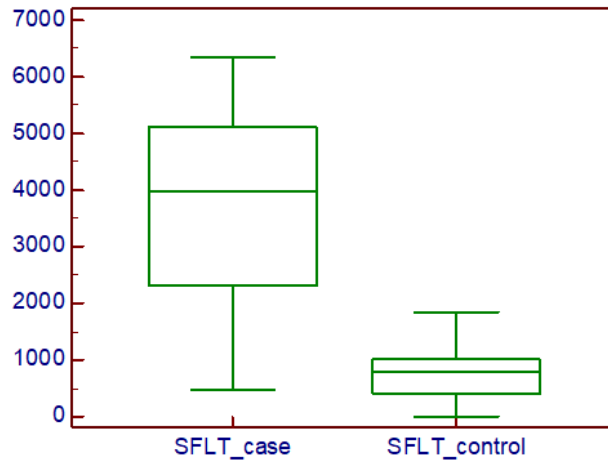
Figure 5.5: Comparison of maternal serum sFlt-1 between cases and controls
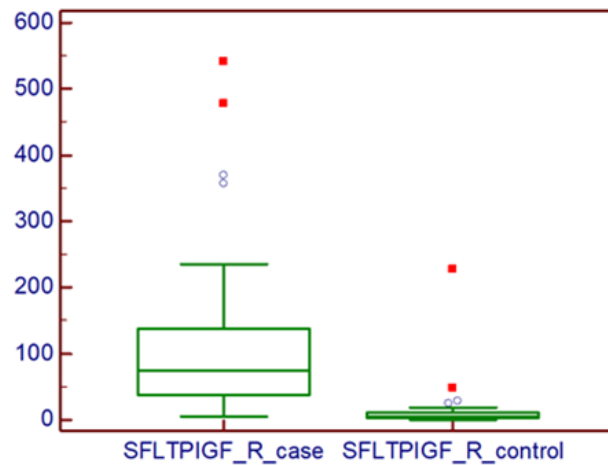
Figure 5.6 : Comparison of maternal serum sFlt-1:PIGF ratio between cases and controls

The oxidative stress marker serum malondialdehyde (MDA) was increased significantly (p=0.001) in PE patients (445.13±150.64 ng/ml) than control group (209.08±36.05 ng/ml) (Table 5.14, Figure 5.7).

Table 5.14: Comparison maternal serum malondialdehyde in Controls and PE patients

	Controls	**Cases**	**p**
Serum malondialdehyde (ng/ml) (MDA)	**209.08±36.05**	**445.13±150.64**	**0.001**
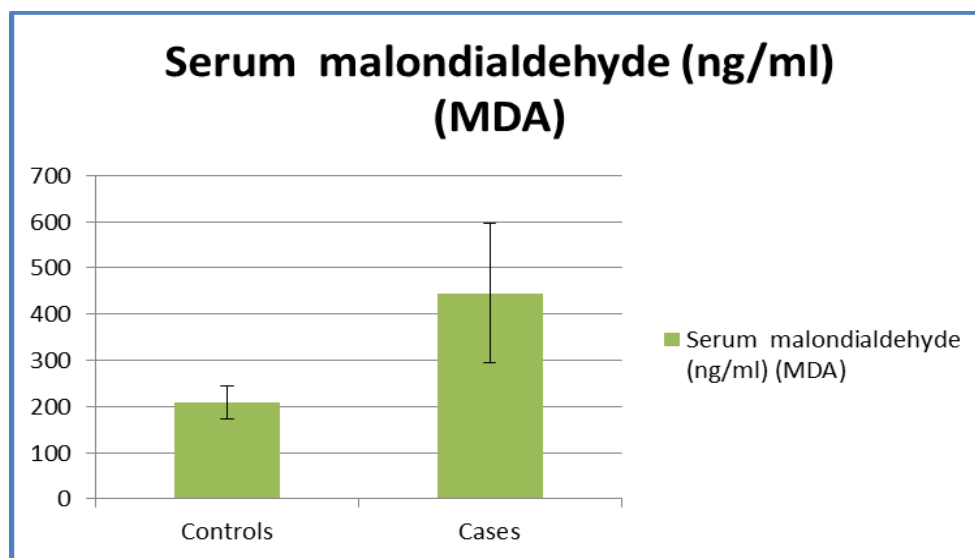
Figure 5.7: Comparison of maternal serum malondialdehyde between cases and controls

Comparison of NO, eNOS, VEGF, PIGF, sFlt-1 and Sflt1:PIGF between mild PE and severe cases of PE: There was significant decrease in VEGF (Median mild=91.40pg/ml, severe=72.81pg/ml, inter-quartile range mild=73.12-124.68, severe=22.50 to 85.62) and PIGF (Median mild=72.53pg/ml, severe=44.08pg/ml, inter-quartile range mild=61.95-95.87, severe=27.85-61.31) in severe PE cases as compared to mild cases of PE, p values were 0.0465,0.0049respectively. sFlt1 (Median mild=1618.0pg/ml, severe=4448.0pg/ml, inter-quartile range mild=958.0-2996.0, severe=3239.0-5331.00) and sFlt1: PIGF ratio (Median mild=25.40, severe=84.94, inter-quartile range mild=8.16-54.32, severe=48.66-183.04)were significantly higher in severe PE cases than mild cases of PE(p=0.0040, 0.0014 respectively).Whereas NO and eNOS did not show difference between the mild (p= 0.1073)and severe (p= 0.9345) cases of PE (Table 5.15, Figure 5.8-5.13).

Table 5.15: Comparison of maternal serum NO, eNOS, VEGE, PIGF, sFLT1 and sFLT1: PIGF in mild and severe preeclampsia

	Median		Interquartile range		Average rank		MWU	Z	P
	Mild	Severe	Mild	Severe	Mild	Severe			
NO$_{\#}$	85.76	62.40	44.03-76.77	64.24-122.64	13.71	21.37	68.00	1.61	0.1073
eNOS*	92.85	91.53	63.25-123.21	52.42-141.25	20.68	21.07	129.50	0.0822	0.9345
VEGF*	91.40	72.81	73.12-124.68	22.50 to 85.62	22.83	13.43	71.50	1.99	0.0465
PIGF*	72.53	44.08	61.95-95.87	27.85-61.31	31.68	18.40	46.50	2.813	0.0049
sFLT1*	1618.0	4448.0	958.0-2996.0	3239.0-5331.00	10.06	23.65	44.50	2.879	0.0040
Ratio	25.40	84.94	8.16-54.32	48.66-183.04	8.87	23.93	35.00	3.191	0.0014

#: μmol/L, * :pg/ml
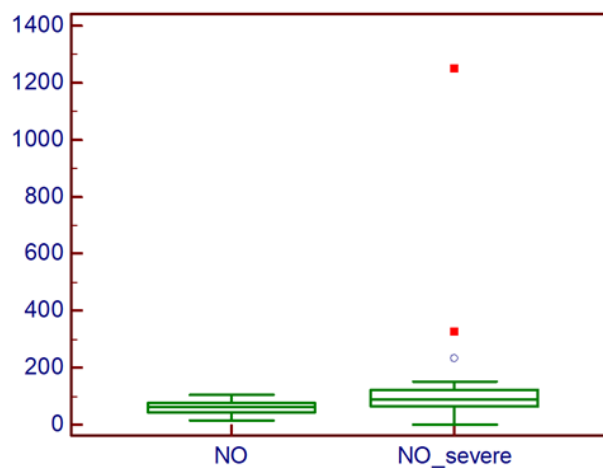
Figure 5.8: Comparison of maternal serum NO between mild and severe cases of PE
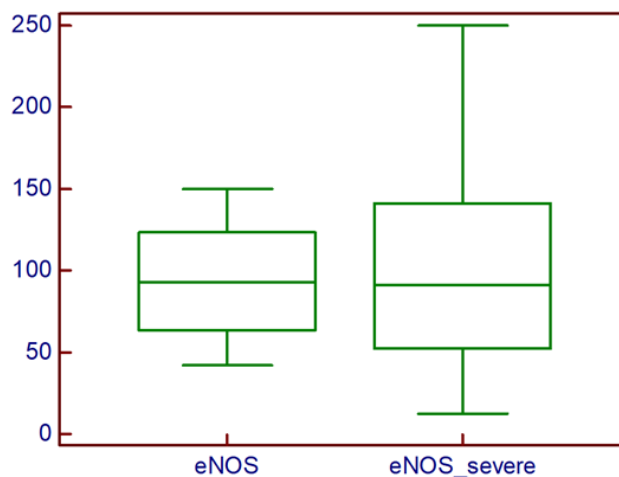
Figure 5.9 : Comparison of maternal serum eNO between mild and severe cases of PE
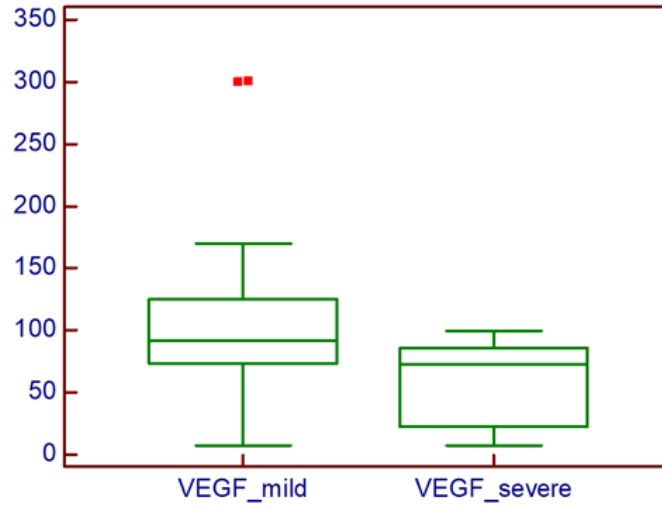
Figure 5.10: Comparison of maternal serum VEGF between mild and severe cases of PE
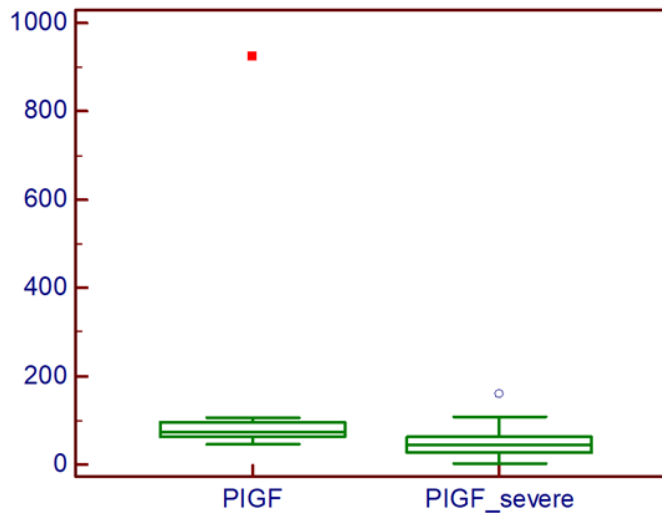
Figure 5.11 : Comparison of maternal serum PIGF between mild and severe cases of PE
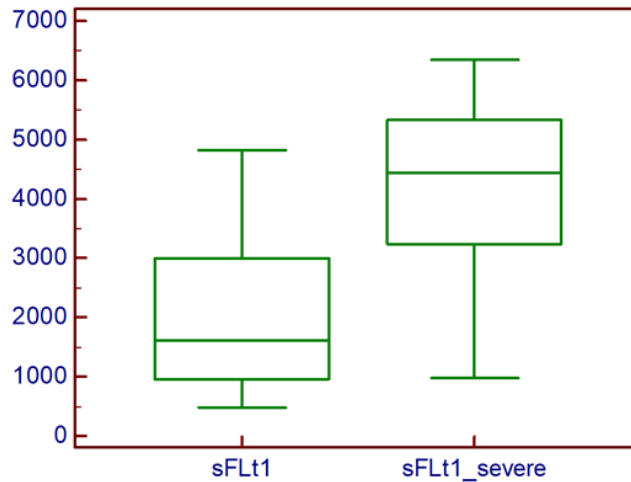
Figure 5.12 : Comparison of maternal serum sFlt-1 between mild and severe cases of PE
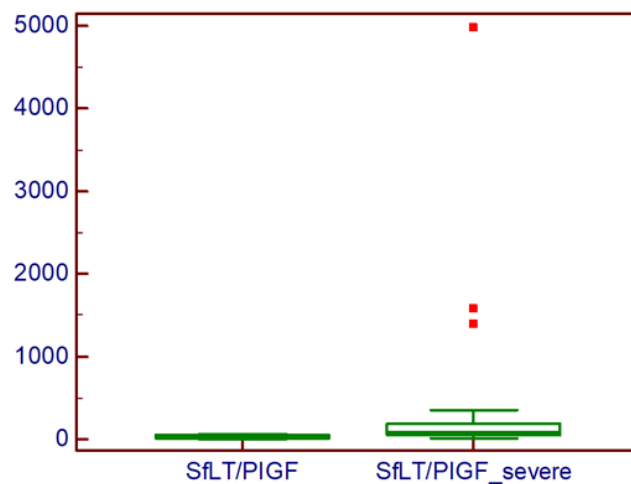
Figure 5.13: Comparison of maternal serum sFlt-1:PlGF between mild and severe cases of PE

Correlation of NO, eNOS, VEGF, PIGF, sFlt-1, Sflt1:PIGF ratio with severity of PE: The correlation between the severity of PE and PIGF showed significant (p=0.005) negative correlation (r=-0.436), sFlt1 and sFlt1: PIGF ratio showed significant (p= 0.003, 0.001 respectively) positive correlation (r=0.452 and 0.498 respectively) with severity of PE (Table 5.16).

Table 5.16: Correlation of maternal serum NO, eNOS, VEGF, PIGF, sFlt-1 levels and sFlt-1:PIGF ratio with severity of PE cases.

		NO	eNOS	VEGF	sFLT1	PIGF	sFLT1/PIGF
Severity	r	0.251	0.014	0.306	0.452**	-0.436**	0.498**
	p	0.129	0.934	*0.055*	0.003	0.005	0.001

Diagnostic validity of VEGF, PIGF, sFlt1, sFlt1:PIGF ratio: The diagnostic validity of all parameters was calculated using ROC curve analysis, the results were as follows: area under the curve for VEGF, PIGF, sFlt-1 and sFlt-1:PIGF ratio were 0.734, 0.802, 0.945 and   0.922 respectively, the sensitivity 90.2, 92.7, 80.5 and 82.9, the specificity 55.0, 65.8, 100.0 and 94.6% respectively. The optimum cutoff value for the diagnosis of PE, for VEGF, PIGF, sFlt1 and sFlt1:PIGF were 150.0 pg/ml, 105.65pg/ml, 1843. 0 pg/ml and 28.54 respectively, positive predictive values were 67.27%, 74.51%, 80.49% and 91.89% respectively, negative predictive values were 84.62%, 89.29%, 100% and 82.93% respectively. Diagnostic accuracy was 72.84% for VEGF, 79.75% for PIGF, 90.12%for sFlt1 and 87.18%   for   sFlt1:PIGF ratio (Table 5.17, Figure 5.14).

Table 5.17: Compilation of Diagnostic Validity of VEGF, PIGF, sFlt1, sFlt1:PIGF ratio

	VEGF	PIGF	sFlt1	sFlt1:PIGF
Area under the ROC curve (AUC)	0.734	0.802	0.945	0.922
95% Confidence interval [b]	0.624 to 0.826	0.697 to 0.883	0.871to 0.983	0.838 to 0.970
z statistic	4.072	5.435	18.279	12.998
Significance level P (Area=0.5)	<0.0001	<0.0001	<0.0001	<0.0001
Sensitivity	90.2	92.7	80.5	82.9
Specificity	55.0	65.8	100.0	94.6
Best cut off (pg/ml)	150	105.65	1843	28.54
+ve Predictive Value	67.27%	74.51%	80.49%	91.89%
-Ve Predictive Value	84.62%	89.29%	100%	82.93%
Diagnostic Accuracy	72.84%	79.75%	90.12%	87.18%
Odds Ratio	11.31	24.36	165	55.05
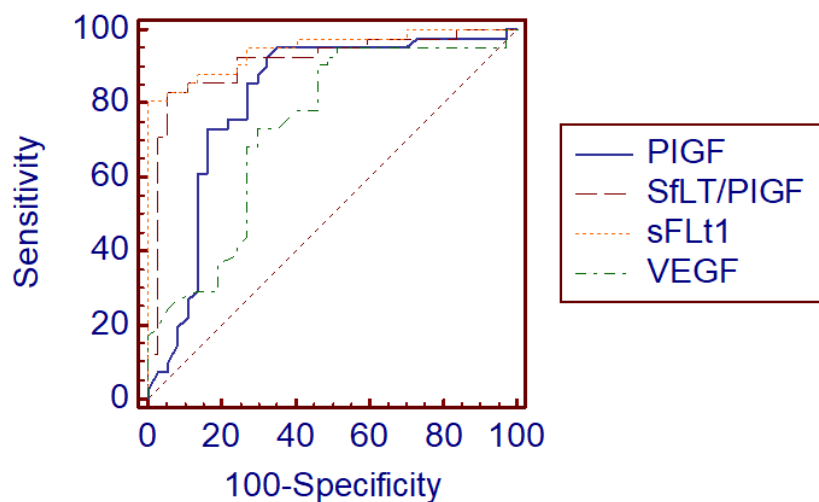
Figure 5.14: ROC curve analysis of VEGF, PIGF, sFlt1, sFlt1:PIGF ratio

Correlation of birth weight and APGAR score with maternal serum VEGF, PIGF, sFlt1and sFlt1:PIGF ratio: Correlation of birth weight and APGAR score with maternal serum angiogenic and anti-angiogenic factors showed that sFLt1and sFlt1/PIGF ratio had significant (p=0.000 for all parameters) negative correlation

(r= -0.545,-0.386,-0.362 respectively) with the neonatal birth weight , APGAR score at 1 minute and APGAR score at 5 minutes, VEGF and PIGF did not show significant correlation with these three birth outcomes (Table 5.18).

Table 5.18: Correlation of birth weight and APGAR score with maternal serum angiogenic and anti-angiogenic factors

		VEGF	sFLt1	PIGF	sFlt1/PIGF
Birth weight In Kg	r	0.081	-0.545[**]	0.095	-0.700[**]
	p	0.472	0.000	0.405	0.000
APGAR 1 min	r	0.163	-0.386[**]	0.137	-0.687[**]
	p	0.146	0.000	0.230	0.000
APGAR 5 min	r	0.129	-0.362[**]	0.117	-0.704[**]
	p	0.253	0.001	0.306	0.000
**. Correlation is significant at 0.01 level (2-tailed).					
*. Correlation is significant at 0.05 level (2-tailed).					

Prognostic value of maternal serum VEGF, PIGF, sFlt1and sFlt1:PIGF ratio for birth weight and APGAR score: With VEGF cutoff value of <150 pg/ml, 90.9% of PE and 16.6% of controls had low birth weight babies. With PIGF cutoff value of <105.65 pg/ml, 100% of PE and 33.3% of controls had low birth weight neonates. With sFlt-1 cutoff value of >1843 pg/ml, 95% of PE and none from control had low birth weight babies. With sFlt-1:PIGF ratio >28.5, 100% of PE and 8.3% of normal pregnant women gave birth to low birth weight babies. These differences for VEGF, PIGF, sFlt-1 and sFlt-1:PIGF ratio were found to be statistically significant (p< 0.001). APGAR score at 1 minute and APGAR score at 5 minutes were depressed in more number of cases as compared to controls at diagnostic cutoff of proangiogenic and antiangiogenic factors. But only at VEGF cutoff <150 pg/ml, the APGAR score at 1 minute was depressed in 37.0% of PE cases compared to 2.5% of controls, which was statistically significant (p< 0.001) (Table 5.19).

Table 5.19: Prognostic value of maternal serum pro-angiogenic and anti-angiogenic factors for birth out come

	Controls						Cases					
	LBW		APGAR 1		APGAR 5		LBW		APGAR 1		APGAR 5	
			Depressed		Depressed				Depressed		Depressed	
	No	%	No	%	No	%	No	%	No	%	No	%
VEGF<150 pg/ml	2	16.6*	1	2.5	0	0	20	90.9*	10	37.0*	7	17.5
PIGF 105.65 pg/ml	4	33.3*	0	0	0	0	22	100*	10	37.0	7	17.5
sFlt1>1843pg/ml	0	0	0	0	0	0	21	95.4	9	22.5	6	15
sFlt1: PIGF >28.5	1	8.3*	0	0	0	0	22	100*	10	37.0	7	17.5

Histopathology features of normal and PE placenta: Histopathology of PE placentae showed syncytial knots was the most common finding which was seen in all examined placentae with pre eclampsia followed by morphologically thin slender villi and fibrinoid necrosis of vessesls, Hypovascularisation of placental villi was the other more observed finding. Intervillous haemorrhage and calcification was less common histopathological finding (Figure 5.15, 5.16, 5.17, 5.18, Table 5.20). Whereas normal placentae showed better vascularised villi and less syntitial knots.

Table 5.20: Histopathological features of PE placenta

Particulars	**No of Placenta**
Fibrinoid necrosis of vessels	87.5% (35)
Syncytial knots in the villi	100% (40)
Calcifications	15% (06)
Hypovascularisation of villi	75% (30)
Intervillous hemorrhage	35% (14)
Slender villi with thickened basement membrane	80% (32)
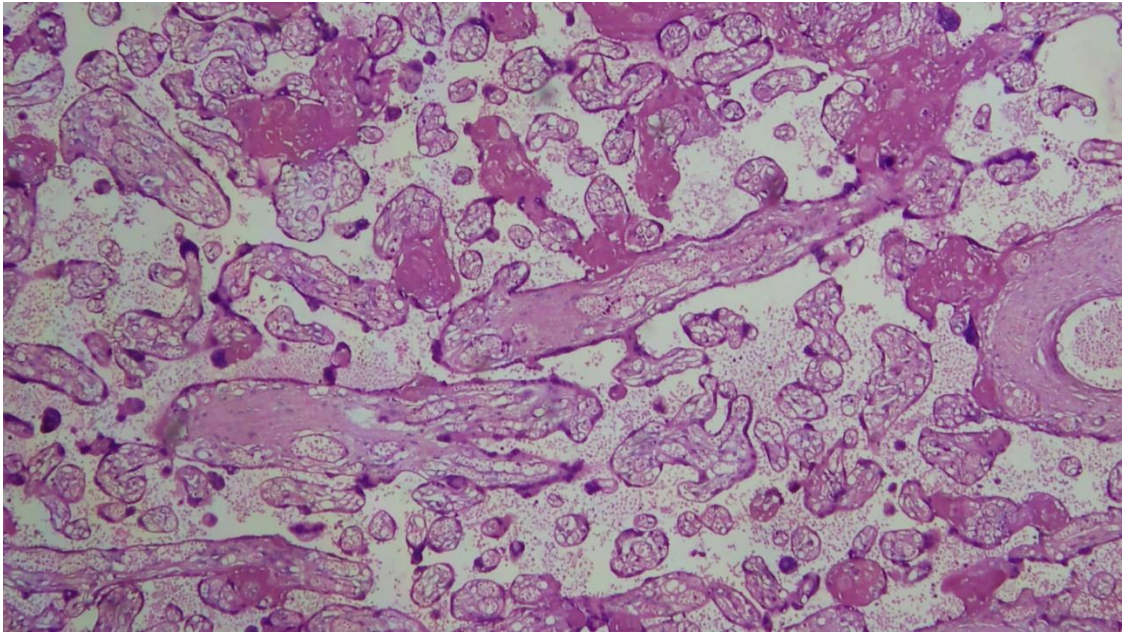
Figure 5.15: **Small slender villi with intervillous fibrinoid material deposition (H&E x40).**
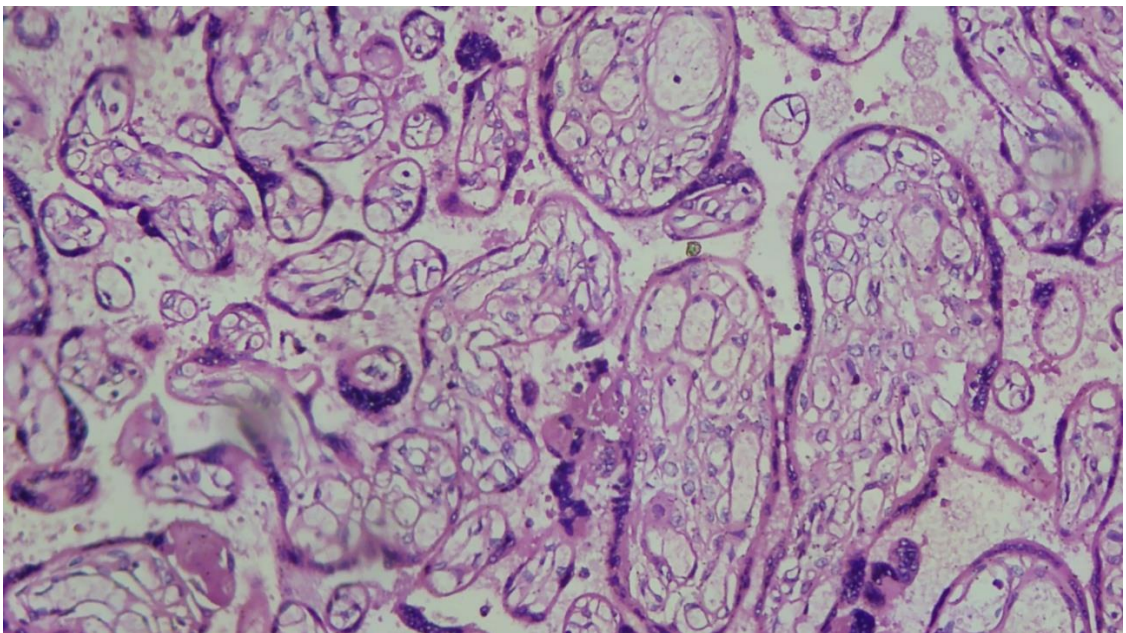
Figure 5.16: **Thickening of trophoblastic basement membrane of villi with presence of many syncytial knots (H&E x100).**
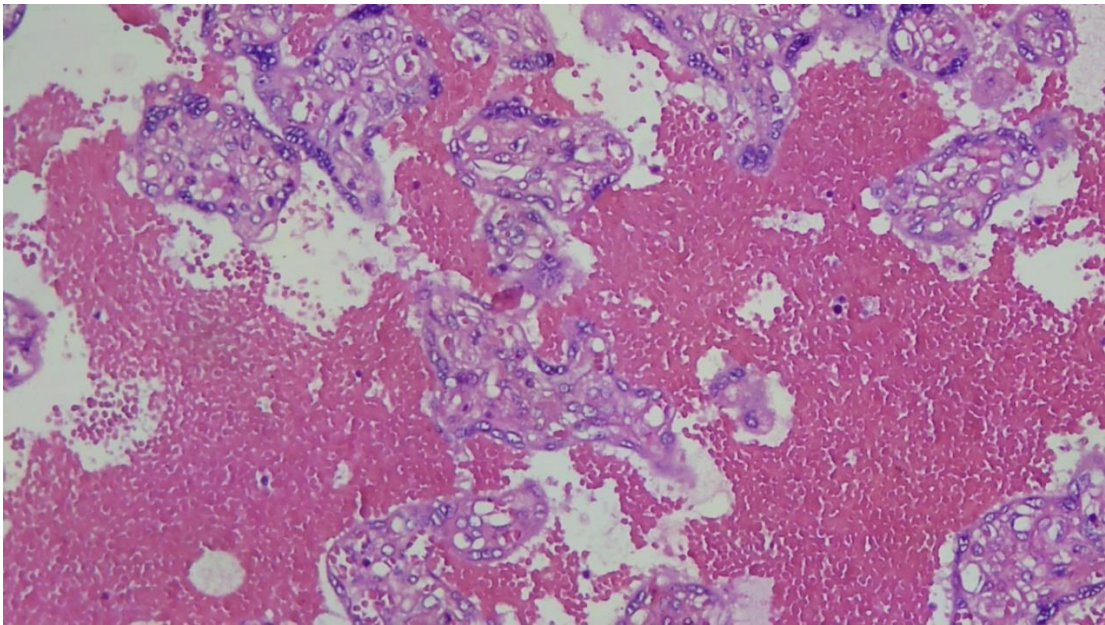
Figure 5.17: Intervillous haemorrhage with syncytial knots (H&E x100).
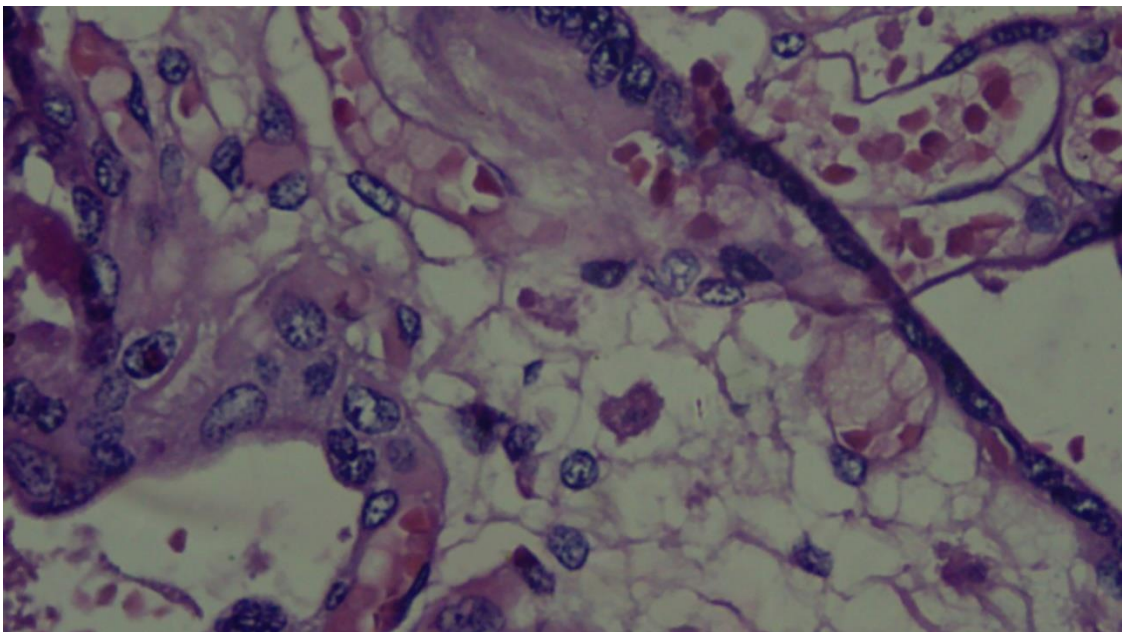
Figure 5.18: Cytotrophoblastic proliferation within the villi (H&E x400).

Immunohistochemistry features of normal and PE placenta: Strong immunoreactivity was seen to VEGF antibody in normal placentae whereas placenta with PE showed weak expression of VEGF antibody (Figure 5.19, 5.20,5.21, 5.22).
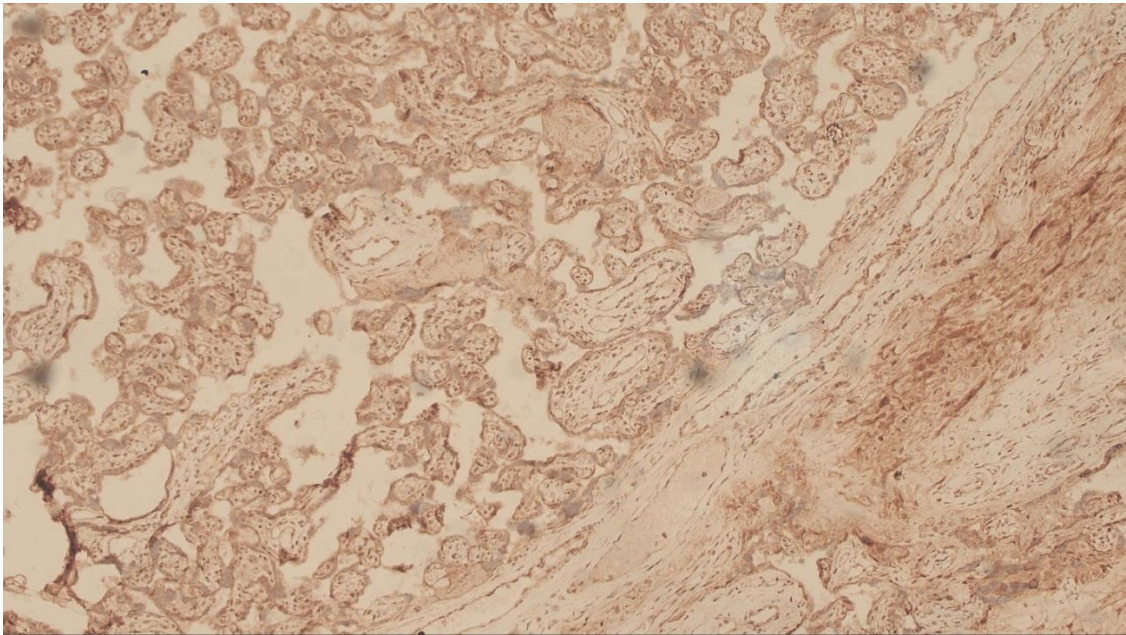
Figure 5.19: **Strong immunoreactivity of VEGF expression by vascular endothelial cells of capillaries within the villi in normal placenta (x100).**
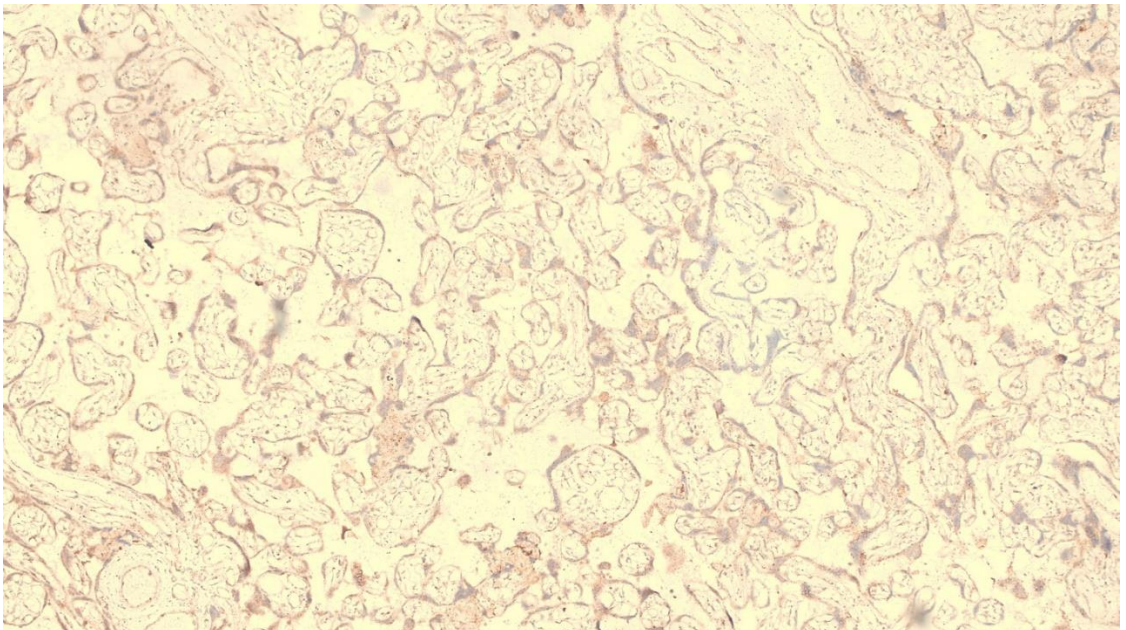
Figure 5.20: **Weak immunoreactivity of VEGF expression by vascular endothelial cells of capillaries within the villi in placenta with pre eclampsia (x100).**
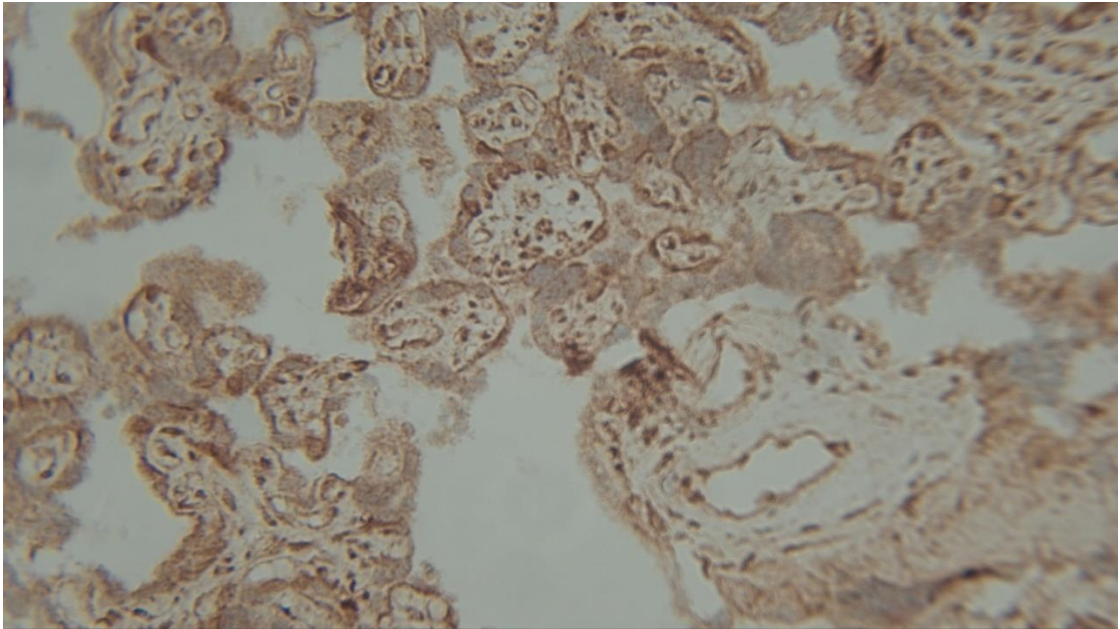
Figure 5.21: Strong immunoreactivity of VEGF expression by vascular endothelial cells of capillaries within the villi in normal placenta (x200).
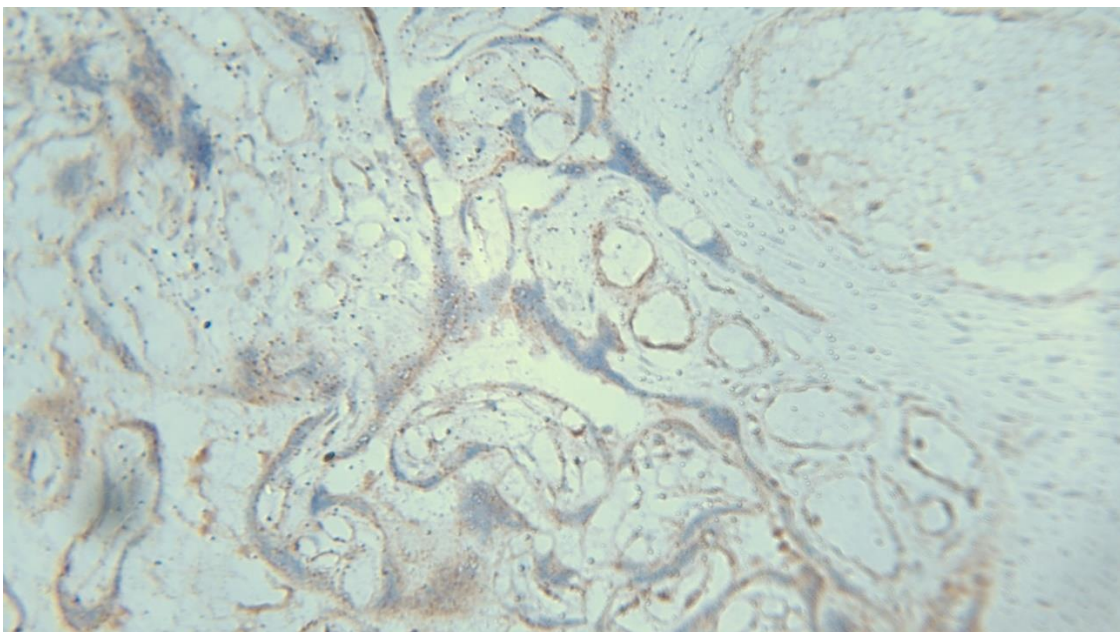
Figure 5.22:   Weak immunoreactivity of VEGF expression by vascular endothelial cells of capillaries within the villi in placenta with pre eclampsia (x200).

Chapter 6

DISCUSSION

CHAPTER 6

DISCUSSION:

Forty cases of PE and forty controls of normal healthy pregnant women were selected for study. These two groups were comparable with respect to maternal age and gestational age; there was statistically insignificant difference in these parameters. This is important to know whether these confounding factors are equally distributed among cases and controls, and thus, cases and controls are matched.

Nitric oxide, eNOs, VEGF, PIGF and sFlt-1: In the present study, we found a significant (p=0.0033) decrease in NO. Similar results were shown by Sahu S. et al [1] (p=0.000); Darkwa et al [2] showed decrease in NO but it was not statistically significant (p=0.16), but Adu-Bonsaffoh et al [3] found significant (<p=001) increase in NO. Studies not only have shown that there was significant decrease in VEGF and PIGF in PE cases as compared to normal pregnant women but also found sFlt1 and sFlt1:PIGF ratio were significantly higher in PE cases than control group; In the current study also we found similar results, but Caillon H et al found no significant change in PIGF and sFlt1 but got significant (p=0.01) decrease in sFlt-1/PIGF ratio in PE than control group.

Studies done by Nikuei P et al [4] and Pant V et al [5] did not find significant rise in sFlt-1/PIGF ration in severe cases of PE cases than mild cases of PE. Pant V et al [5] also showed there was no significant decrease in PIGF in severe cases of PE than mild cases of PE. Studies have shown significant rise in sFlt-1 in severe PE cases than mild cases of PE [6-8]. Shaker OG et al [7] and Basuni M et al [9] showed significant decrease in PIGF in severe cases of PE than mild cases of PE.

Probable mechanisms of lowered serum concentration of VEGF in PE: The etiology of PE is still poorly understood, pathogenesis may be because of defective decidualization, impaired cytotrophoblast invasion, endothelial dysfunction, and inappropriate immune responses to the allogenic fetus. The fetal trophoblast cells invade the maternal decidua during implantation, resulting in reduced placental perfusion, failure of spiral artery remodeling, and abnormal placentation with resulting placental ischemia and placental hypoxia (Figure 6.1)[10].
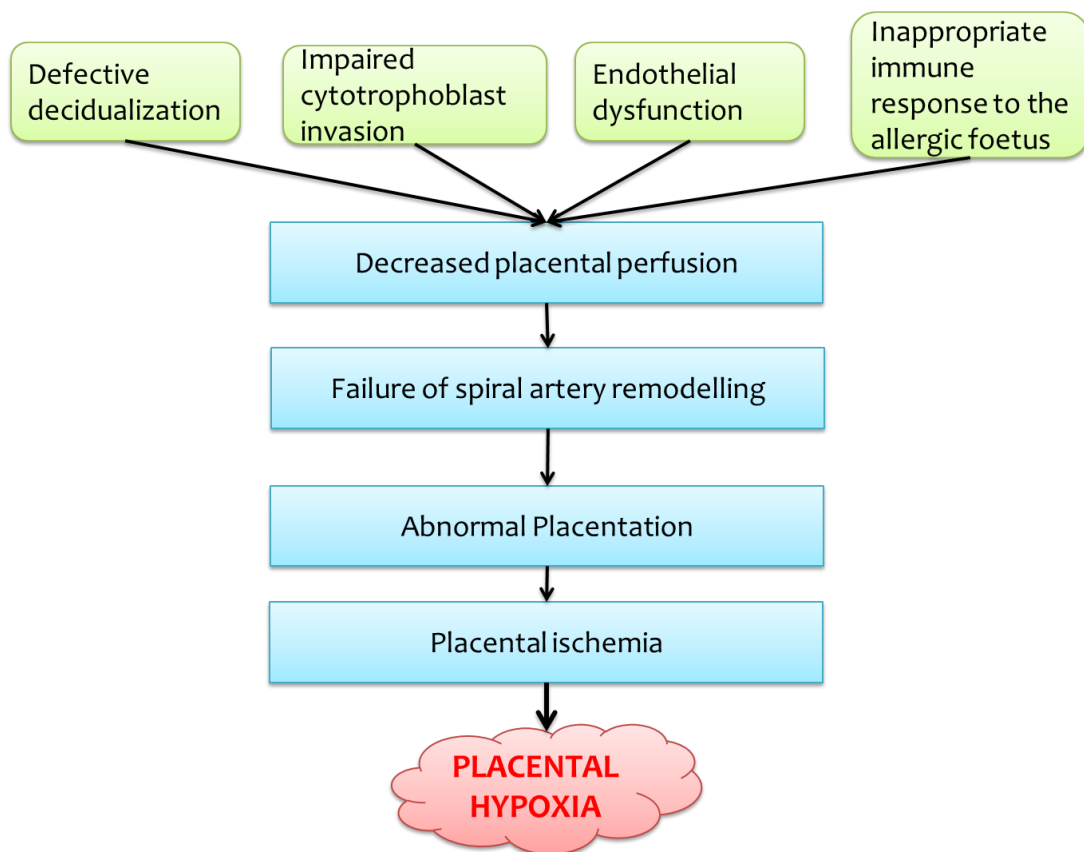
Figure 6.1: Etiology and pathogenesis of PE

In hypoxia VEGFR-1 commonly referred to as soluble fms-like tyrosine kinase receptor-1 (sFlt-1), is transactivated by transcription factor Hif-2α, increasing angiogenesis, while Hif-1 also mediates enhanced sFlt-1 production. The serum levels of VEGF in PE cases were significantly higher compared to healthy pregnancies. In

turn, sFlt-1 sequesters the free form of VEGF and acts as an anti-angiogenic factor thus limiting the availability of free VEGF for fetal and placental angiogenesis (Figure 6.2). The sFlt1 interfering with VEGF-dependent phosphorylation/stimulation of endothelial NOS (eNOS), which catalyses the synthesis of NO from L-Arginine, Nitric oxide (NO), a potent vasodilator that mediates endothelium-dependent relaxation, has been linked to endothelial dysfunction in PE [11-14].
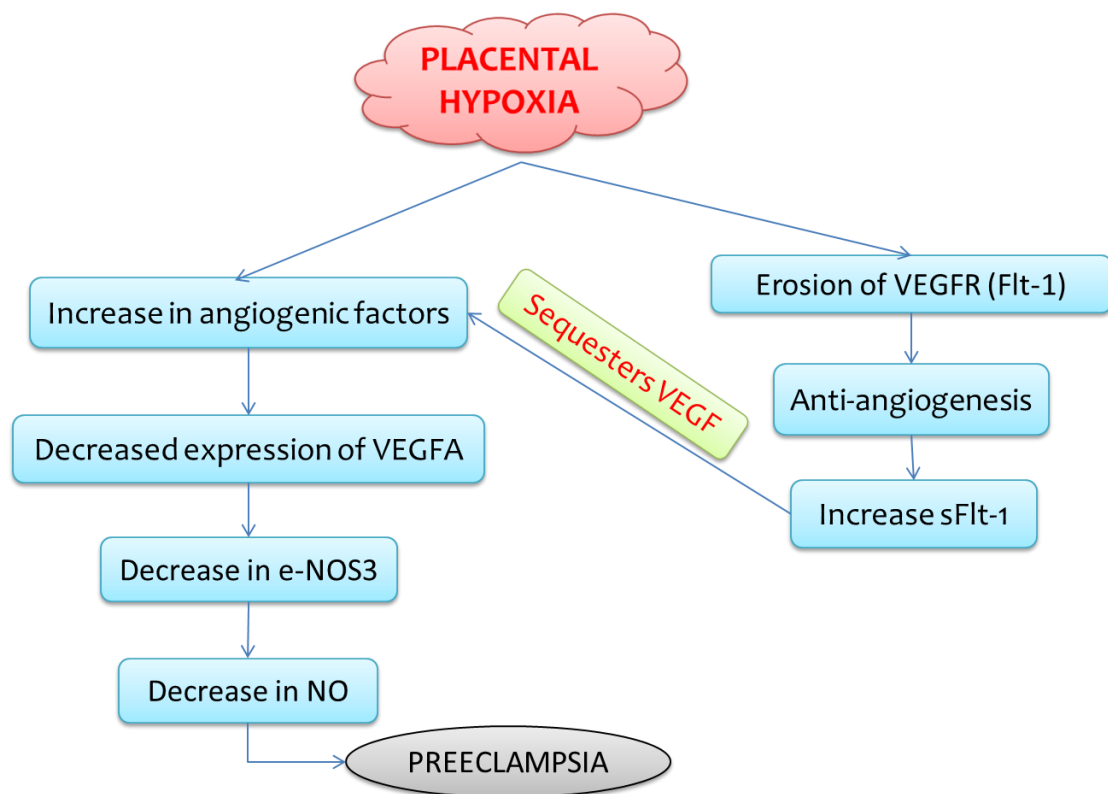
Figure 6.2 : Probable mechanisms of lowered serum concentration of VEGF in PE

Probable mechanisms of lowered serum concentration of PIGF in PE: Decrease in PlGF may be because of combined effect of decreased expression of PlGF and reduced free PlGF, due to sequestration by sFLT-1, which is increased in PE women. Persistent placental hypoxia resulting from an underdeveloped utero-placental circulation leads to decreased expression of PlGF. Regulation of PlGF expression is explained by many mechanisms like endoplasmic reticular stress and epigenetic

changes, and changing the effect of the transcription factor hypoxia-inducible factor-1α (HlF1-α). Inflammation also lowers the expression of PlGF (Figure 6.3) [15-17].
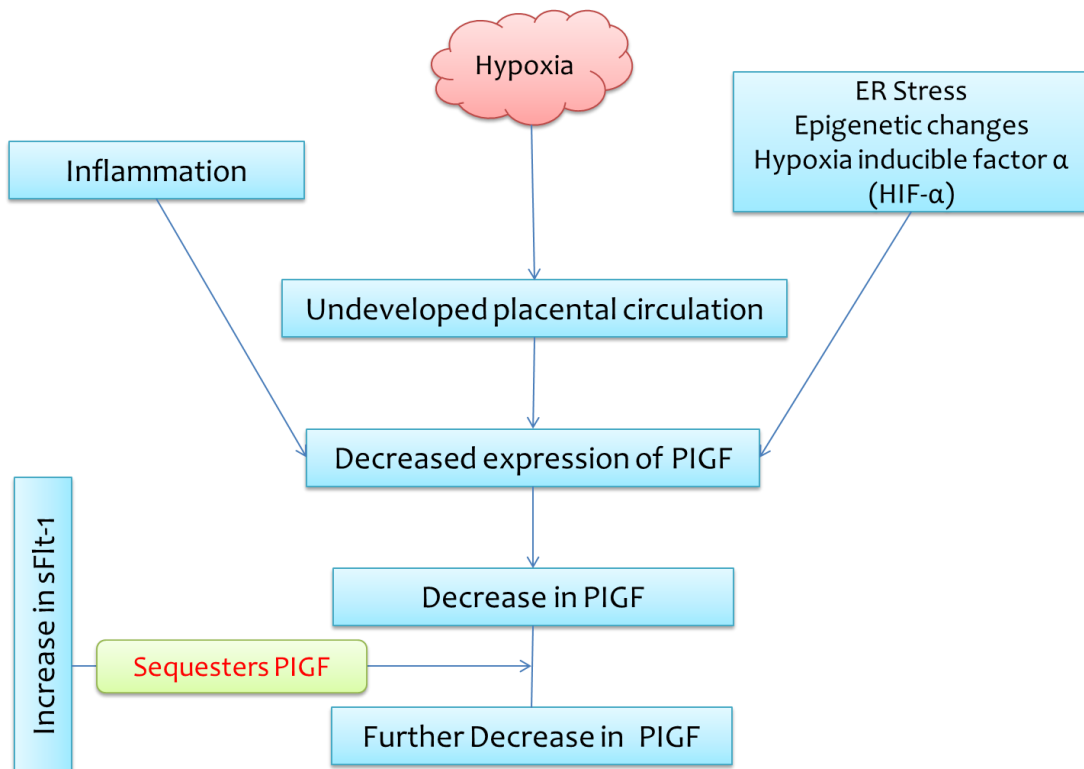
Figure 6.3: Probable mechanisms of lowered serum concentration of PIGF in PE

Mild and severe PE: Adu-Bonsaffoh et al. [3]showed markedly elevated serum levels of NO in early-onset PE (Severe form) compared to late-onset PE that is considered as a mild disease. This may represent a compensatory mechanism to offset the ongoing pathological process and indicating the generalized endothelial dysfunction. Erdemoğlu et al. [18], concluded that there was no difference between means of NO in healthy, mild, and severe preeclamptic patients. Shaker et al., in their study showed that the no significant difference was found between the mild and severe groups (p > 0.05).

In the present study, PIGF was significantly decreased in severe PE than mild PE. sFlt-1 and sFlt-1/PIGF ratio were significantly increased in severe PE than mild PE.

Nikuei P. et al. [4] found that there was significant (p= 0.389) change in sFlt-1:PIGF ratio between mild and severe cases of PE. Pant V. et al. [5] showed there was no significant difference in PIGF (p=0.143) and sFlt-1:PIGF (p=0.945) ratio between mild and severe cases of PE, but they found significant increase in sFlt-1 (p=0.010) in severe PE than mild PE, Shaker OG et al. [19] and Basuni M et al. [9] found significant increase in sFlt-1 (p<0.05 and <0.001 respectively) and significant decrease in PIGF(p<0.001) in severe PE than mild PE patients. The present study also showed similar results. The probable mechanism may be the more increase in hypoxia results in further increase in sFlt-1 and decrease in VEGF and PIGF leads to decrease in NO and eNOS causes more increase in ROS , hence results in severe PE (Figure 6.4).
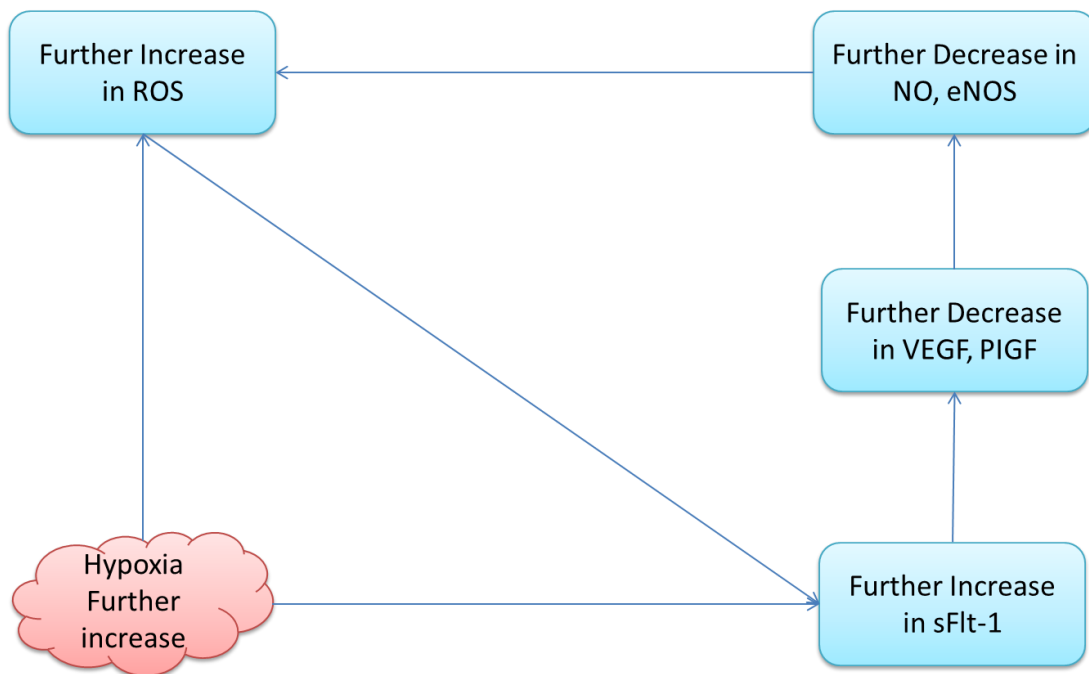
Figure 6.4: Mechanism in severe PE

Role of reactive oxygen species (ROS) in PE: Defective spiral artery remodeling leads to the placental ischemia and alters the biological activities of mitochondria, and hence increases the production of reactive oxygen species (ROS). ROS stabilizes Hif-1α, and induces the production sFlt-1. Thus, dysfunction of mitochondria (increased oxidative stress) plays an important role in increased production of sFlt-1 [20].
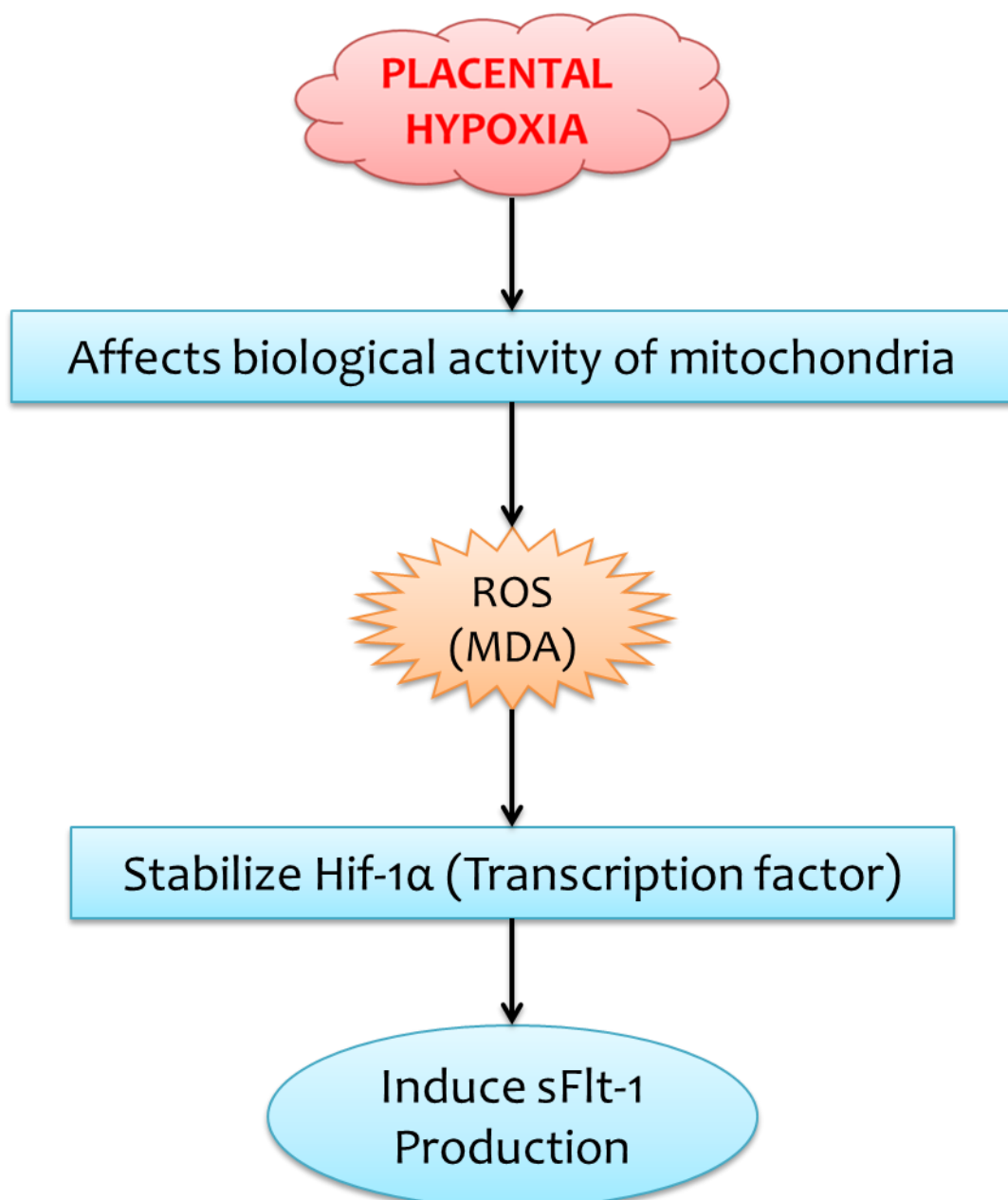
Figure 6.5: Role of ROS in production of sFlt-1 in PE [20].

The mechanisms mentioned above enhance the production of reactive oxygen species (ROS), triggers oxidative stress. Elevated levels of reactive oxygen species (ROS) are a common finding in preeclampsia, and likely contributed by superoxide, hydrogen peroxide, hydroxyl radical, and peroxynitrite. In the current study also we found significant increase (p=0.001) in MDA, lipid peroxidation product, oxidative stress marker, in PE patients than healthy pregnant women. An imbalance between pro-oxidant: NADPH oxidase , xanthine oxidase, cyclooxygenases, and complexes I, II, and III of the mitochondrial electron transport chain; and anti-oxidant enzymes viz, superoxide dismutase, catalase, and hemeoxygenase are all hypothesized to contribute to elevated ROS in preeclampsia; and thereby contributing to impaired NO bioavailability and signaling [11,21].

It is observed that in endothelial cells upon hypoxia-reoxygenation conditions, eNOS undergoes S-glutathionylation, leading to eNOS uncoupling, decreased NOS activity, increased superoxide generation, and impaired endothelium-dependent vasodilation. The decrease of tetrahydrobiopterin under conditions of oxidative stress seems to be a main cause of eNOS uncoupling [21].
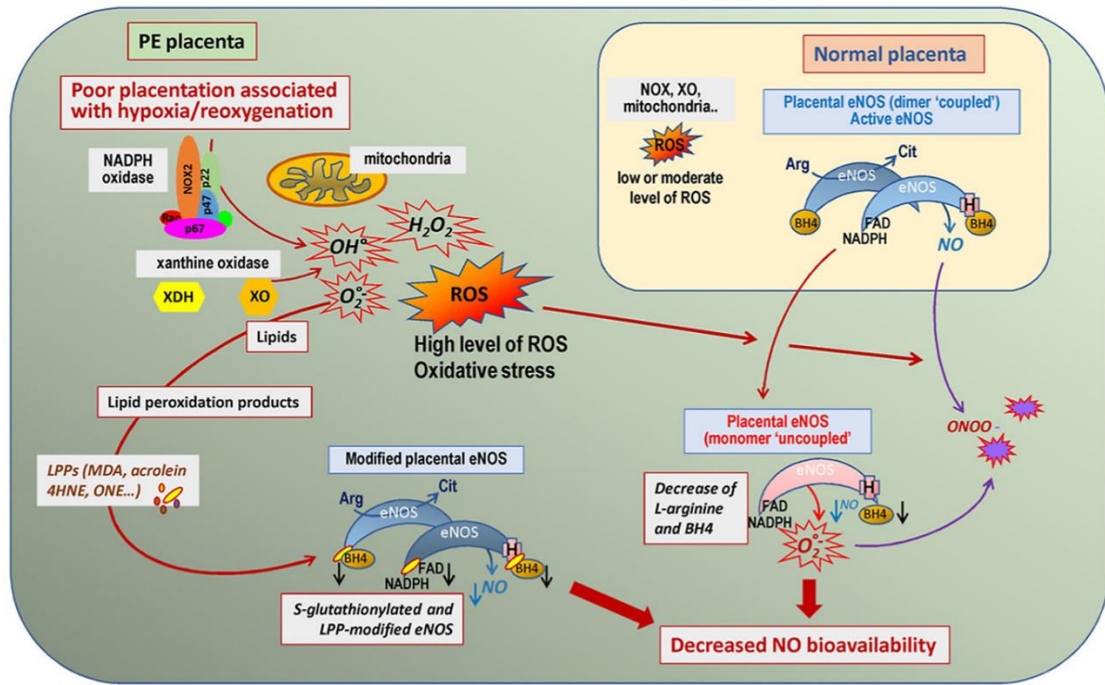
Figure 6.6: Mechanism of ROS production in PE[21].

Diagnostic and prognostic validity of VEGF, PIGF, sFlt-1 and sFlt-1:PIGF ratio:

In the present study we also calculated the diagnostic and prognostic parameters, Tandon V et al [19] showed AUC for VEGF was 0.738 and in the current study it was 0.734 and it was statistically significant, they also found sensitivity and specificity of VEGF as 68.3% and 96.6% respectively where as in the present study it was 90.24% and 55% respectively.

Sharabi Nov et al [22] found AUC for PIGF and sFlt-1:PIGF ratio as 0.82 and 0.85 respectively; in the present study it was, 0.802 and 0.922 respectively; they got best cut-off value for sFlt-1/PIGF ratio as 38, where as in the current study it was 28.5. Sá CPN et al [23] found best cut off value for PIGF at 104 pg/ml and specificity at 60%, where as in the present study best cut-off value was 105.65 pg/ml, specificity was 65.79%. Andersen LB et al [24] showed specificity and NPV for PIGF as 68.80% and 96.40% respectively; in the current study it was 65.79% and 89.29% respectively. Andersen LB et al [24] also found NPV for sFlt-1/PIGF as 85% in the present study, it

was 82.93%. Phupong V et al [25] showed best cut-off value for PIGF and sFlt-1 was 113.8 pg/ml and 1724.5 pg/ml respectively; similar results were found in the present study which were 105.65 pg/ml and 1843 pg/ml respectively. Radulescu C et al [26], showed sensitivity of 100% for PIGF, where as in the present study it was 92.68%. Basuni M et al [9] found AUC and NPV for PIGF as 0.872 and 90.7% respectively; similarly in the present study these were 0.802 and 89.29% respectively. They also showed best cut-off value, AUC and PPV for sFlt-1 as 2075 pg/ml, 0.883 and 94.4% respectively, in the present study found 1843 pg/ml, 0.945 and 80.49% respectively. Most of the findings of present study coincides with Taraseviciene V et al [27] mainly AUC of PIGF (0.977), sFlt-1 (0.954) and sFlt-1/PIGF(0.983) ratio, sensitivity of PIGF (95.8%) and sFlt-1 (84.7%) and specificity of sFlt-1 (65.8%) and sFlt-1/PIGF ratio (96.2%). The results of the present study for correlation of birth weight (r=-0.545, p=0.000) are in accordance with Shaker OG et al [7], who found significant correlation of birth weight (r=0.39,p<0.001) with sFlt-1.

Conclusion: There was a significant decrease in NO, eNOS, angiogenic factors, significant increase in anti-angiogenic factor, oxidative stress and sFlt1:PIGF ratio in PE than healthy pregnant women. There was significant correlation between severity of PE and PIGF, sFlt1 and sFlt-1:PIGF ratio. PIGF, sFlt1 and sFlt-1:PIGF ratio with mentioned cut-off values are better markers and can be used as diagnostic markers in PE. PIGF, sFlt1 and the ratio can be used as prognostic markers with mentioned criterion values for immediate birth outcomes.

References

1. Sahu S, Daniel M, Abraham R, Vedavalli R, Senthilvel V. Study of uric acid and nitric oxide concentrations in preeclampsia and normal pregnancy. Int J Biol Med Res. 2011; 2(1): 390-393.

2. Darkwa EO, Djagbletey R, Essuman R, Sottie D, Dankwah GB, Aryee G. Nitric Oxide and Pre-Eclampsia: A Comparative Study in Ghana. Open Access Maced J Med Sci. 2018 Jun 20; 6(6): 1023-1027.

3. Adu-Bonsaffoh K, Antwi DA, Obed SA, Gyann B. Nitric oxide dysregulation in the pathogenesis of preeclampsia among Ghanaian women. Integrated Blood Pressure Control 2015;8:1-6.

4. Nikuei P, Rajaei M, Roozbeh N, Mohseni F, Poordarvishi F, Azad M, et al. Diagnostic accuracy of sFlt1/PlGF ratio as a marker for preeclampsia. BMC Pregnancy and Childbirth 2020;20:80. https://doi.org/10.1186/s12884-020-2744-2

5. Pant V, Yadav B, Sharma J. A cross sectional study to assess the sFlt1:PlGF ratio in pregnant women with and without preeclampsia. BMC Pregnancy and Childbirth 2019;19:266. https://doi.org/10.1186/s12884-019-2399-z

6. ACOG practice bulletin. Obstetrics and Gynaecology 2020 Jun;135(6): e237-60.

7. Shaker OG, Shehata H. Early prediction of preeclampsia in high-risk women. J Womens Health (Larchmt). 2011 Apr;20(4):539-44. doi: 10.1089/jwh.2010.2378. Epub 2011 Mar 17. PMID: 21413896

8. Verlohren S, Herraiz I, Lapaire O, Schlembach D, Moertl M, Zeisler H, et al. The sFlt-1/PlGF ratio in different types of hypertensive pregnancy disorders and its prognostic potential in preeclamptic patients. Am J Obstet Gynecol 2012;206:58.e1–58.e8.

9. Basuni M, Fathy WM, Gaber W. Assessment of placental growth factor and soluble vascular endothelial growth factor receptor 1 in the prediction of pre-eclampsia. Egypt J Haematol 2012;37:281-6.

10. Flint EJ, Cerdeira AS, Redman CW, Vatish M. The role of angiogenic factors in the management of preeclampsia. Acta Obstet Gynecol Scand 2019;98:700-707. https://doi.org/10.1111/aogs.13540

11. Phipps E, Prasanna D, Brima W, Jim B. Preeclampsia: Updates in pathogenesis, definitions, and guidelines. Clin J Am Soc Nephrol 2016 Jun;11:1102–13.

12. Touyz RM, Lang NN, Herrmann J, Van den Meiracker AH, Jan Danser AH. Recent advances in hypertension and cardiovascular toxicities with vascular endothelial growth factor inhibition. Hypertension 2017;70:220-6.

13. Lankhorst S, Jan Danser AH, Van den Meiracker AH. Endothelin-1 and antiangiogenesis. Am J Physiol Regul Integr Comp Physiol 2016;310:R230–4.

14. Khalaf D, Krüger M, Wehland M, Infanger M, Grimm D. The Effects of Oral l-Arginine and l-Citrulline Supplementation on Blood Pressure. Nutrients. 2019 Jul 22;11(7):1679. doi: 10.3390/nu11071679. PMID: 31336573; PMCID: PMC6683098.

15. Ives CW, Sinkey R, Rajapreyar I, Tita ATN, Oparil S. Preeclampsia-Pathophysiology and Clinical Presentations: JACC State-of-the-Art Review. J Am Coll Cardiol. 2020 Oct 6;76(14):1690-1702.

16. Phupong V, Areeruk W, Tantbirojn P, Lertkhachonsuk P. Soluble fms-like tyrosine kinase 1 and placental growth factor ratio for predicting preeclampsia in elderly gravida. Hypertens Pregnancy 2020 May;39(2):139-144. doi: 10.1080/10641955.2020.1745227. Epub 2020 Mar 22

17. Rsdulescu C, Bacârea A, Hut,anu A, Gabor R, Dobreanu M. Placental Growth Factor, Soluble fms-Like Tyrosine Kinase 1, Soluble Endoglin, IL-6, and IL-16 as Biomarkers in Preeclampsia. Mediators of Inflammation 2016. Article ID 3027363, 8 pages. http://dx.doi.org/10.1155/2016/3027363.

18. Erdemoğlu, E., Uğur, M., Erdemoğlu, E. Plasma homocysteine and nitric oxide levels in preeclampsia. J Turkish-German Gynecol Assoc. 2009; 10: 26-29.

19. Shaker OG, Shehata H. Early prediction of preeclampsia in high-risk women. J Womens Health (Larchmt). 2011 Apr;20(4):539-44. doi: 10.1089/jwh.2010.2378. Epub 2011 Mar 17. PMID: 21413896

20. Jena MK, Sharma NR, Petitt M, Maulik D, Nayak NR. Pathogenesis of Preeclampsia and Therapeutic Approaches Targeting the Placenta. Biomolecules. 2020 Jun 24;10(6):953. doi: 10.3390/biom10060953. PMID: 32559856; PMCID: PMC7357118.

21. Guerby P, Tasta O, Swiader A, Pont F, Bujold E, Parant O, et al. Role of oxidative stress in the dysfunction of the placental endothelial nitric oxide

synthase in preeclampsia. Redox Biol. 2021 Apr;40:101861. doi: 10.1016/j.redox.2021.101861. Epub 2021 Jan 19. PMID: 33548859; PMCID: PMC7873691.

22. Sharabi-Nov A, Sršen TP, Kumer K, Vodušek VF, Fabjan T, Hamutal Meiri TN. Maternal Serum Inhibin-A Augments the Value of Maternal Serum PlGF and of sFlt-1/PlGF Ratio in the Prediction of Preeclampsia and/or FGR Near Delivery- A Secondary Analysis. Reprod Med 2021;(2):35–49. https://doi.org/10.3390/reprodmed2010005

23. Sá CPN, Jiménez MF, Rosa MW, Arlindo EM, Ayub ACK, Cardoso RB, et al. Evaluation of Angiogenic Factors (PlGF and sFlt-1) in Pre-eclampsia Diagnosis. Rev Bras Ginecol Obstet. 2020 Nov;42(11):697-704. English. doi: 10.1055/s-0040-1713916. Epub 2020 Nov 30. PMID: 33254263.

24. Andersen LB, Dechend R, Jørgensen JS, Luef BM, Nielsen J, Barington T, Christesen HT. Prediction of preeclampsia with angiogenic biomarkers. Results from the prospective Odense Child Cohort. Hypertens Pregnancy. 2016 Aug;35(3):405-19. doi: 10.3109/10641955.2016.1167219. Epub 2016 May 9. PMID: 27159383.

25. Phupong V, Areeruk W, Tantbirojn P, Lertkhachonsuk P. Soluble fms-like tyrosine kinase 1 and placental growth factor ratio for predicting preeclampsia in elderly gravida. Hypertens Pregnancy 2020 May;39(2):139-144. doi: 10.1080/10641955.2020.1745227. Epub 2020 Mar 22

26. Rsdulescu C, Bacârea A, Hut,anu A, Gabor R, Dobreanu M. Placental Growth Factor, Soluble fms-Like Tyrosine Kinase 1, Soluble Endoglin, IL-6, and IL-

16 as Biomarkers in Preeclampsia. Mediators of Inflammation 2016. Article ID 3027363, 8 pages. http://dx.doi.org/10.1155/2016/3027363.

27. Tarasevičienė V, Grybauskienė R, Mačiulevičienė R. sFlt-1, PlGF, sFlt-1/PlGF ratio and uterine artery Doppler for preeclampsia diagnostics. Medicina (Kaunas). 2016;52(6):349-353. doi: 10.1016/j.medici.2016.11.008. Epub 2016 Nov 29. PMID: 27940029.

Chapter 7

SUMMARY

SUMMARY

In the present study, there was a significant decrease in NO, eNOS, angiogenic factors (VEGF, PIGF), significant increase in anti-angiogenic factor (sFlt-1), oxidative stress (MDA) and sFlt1:PIGF ratio in PE than healthy pregnant women. There was significant correlation between severity of PE and PIGF, sFlt1 and sFlt-1/PIGF ratio. PIGF (105.65 pg/ml), sFlt1(1843 pg/ml) and ratio (28.54) with mentioned cut-off values are better markers and can be used as diagnostic markers in PE. PIGF, sFlt1 and sFlt-1/PIGF ratio can be used as prognostic markers with mentioned criterion values for immediate birth out comes.

Research hypothesis that VEGF,PIGF, sFlt-1 levels and sFlt-1: PIGF ratio act as predictors of pre-eclampsia and also of the neonatal outcome viz birth weight, APGAR score at 1 minute and APGAR score at 5minute, has been **proved**.

Clinical implications:

Adding of maternal serum VEGF, PlGF, sFlt-1 levels and sFlt-1:PIGF ratio to the current clinical assessment could achieve an earlier diagnosis.

There may be double benefit: targeting of resources to those at highest risk, while minimizing excessive assessment and intervention in women at lower risk.

Research implications:

- *The results of the present study have helped in understanding the pathophysiology of PE.*
- *Transition of the present research may be done clinically for early diagnosis and hence better clinical outcomes for mother and neonate.*

Limitations of the study:

- Genetic expression of eNOS, VEGF, PIGF, sFlt-1 and estimation of L-Arginine, BH4, ADMA was not done, as it was not feasible with the available resources. It would have given better understanding of pathophysiology of PE in this ethnic group.

Future perspective:

- Follow up study with serial VEGF, PIGF and sFlt-1 estimations will help with emphasis on early diagnosis of PE.

- Estimation of L-Arginine, BH4, ADMA and genetic expression of eNOS, VEGF, PIGF and sFlt-1 will help for better understanding of pathophysiology of PE.

Chapter 8

ANNEXURES

CHAPTER 8

Annexures

CONSENT FORM I

INFORMATION FOR PARTICIPANTS OF THE STUDY

01	**Title of the project**	**Pro-angiogenic vascular endothelial growth factor (VEGF), placental growth factor (PIGF) and anti-angiogenic factor, soluble FMS like tyrosine kinase-1 (sFlt-1) in preeclampsia – A case control study**
02	**Name of the PI/Ph.D Student and Department**	**Dr. SANGAPPA V. KASHINAKUNTI** Professor, Department of Biochemistry, S.Nijalingappa Medical College, Navanagar, Bagalkot-587102, Karnataka, India. **E-mail: drsvkashinakunti@yahoo.co.in** **Cell No.: 9481981701**
03	**Name, Designation, Address, Phone No. and Email ID of the Guide**	**Dr. BASAVARAJ DEVARANAVADAGI** Professor and HOD, Department of Biochemistry, Shri. B. M. Patil Medical College, BLDE (Deemed to be University), **VIJAYAPURA**, Karnataka Cell No: 9448745957 Email: rohit123@gmail.com
04	**Name of Co-guide/Co-investigator with designation, Department, Phone No. and Email ID**	**DR. ASHALATA MALLAPUR** Professor and HOD Department of OBG S. Nijalingappa Medical College, Bagalkot. 587102 **Phone:** 9945699986 **Email:** drashalatamallapur@gmail.com

05	**Purpose/ Objectives of this project /study**	1) To estimate and compare VEGF, PIGF, nitric oxide, nitric oxide synthase, sFlt-1 and MDA levels in pre-eclempsia cases and healthy controls.
		2) To correlate the VEGF, PIGF, nitric oxide, nitric oxide synthase levels and sFlt-1 levels with severity of pre-eclampsia cases.
		3) To find out the best cut-off of VEGF, PIGF, sFlt-1 levels and sFlt-1: PIGF ratio for the diagnosis of pre-eclempsia by ROC curve analysis.
		4) To calculate sensitivity, specificity, positive predictive value, negative predictive value and diagnostic accuracy of calculated cut-off of VEGF, PIGF, sFlt-1 levels and sFlt-1: PIGF ratio for diagnosis of pre-eclampsia.
		5) To do the follow-up of study subjects for the outcome of pregnancy and to study its association with the VEGF, PIGF, sFlt-1 levels and sFlt-1: PIGF ratio.
		6) To study the prognostic value of VEGF, PIGF, sFlt-1 levels and sFlt-1: PIGF ratio on the outcome of pregnancy among study subjects.
06	**Procedure/Methods of the study**	**Group 1 (Preeclampsia group):** Singleton pregnancies, 40 primigravida patients will be examined and tested. All patients will be diagnosed with preeclampsia according to American College of Obstetrics and Gynecology (Table 1).
		Group 2 (Control group): Singleton

pregnancies, 40 healthy primigravida patients.

All patients will be subjected to the following:

Detailed history taking: History of present pregnancy of persistent headache, visual disturbances or epigastric pain in the present pregnancy. Past history of chronic hypertension, diabetes mellitus or autoimmune diseases. Family history of similar conditions.

Physical examination: General: Blood pressure: It will be measured to obtain hypertension as it was defined as the repeated measurement of systolic blood pressure of 140mmHg or greater and diastolic blood pressure of 90mmHg or greater. It will be repeated 6 hours apart to confirm the condition.Examination for pallor, jaundice, chest auscultation and lower limb edema will be done.

Abdominal examination: Fundal level, gestational age, lie and presentation of fetus.

Lab investigations: Serum samples will be collected by venipuncture in two tubes, 2ml with anticoagulant and 2 ml without anticoagulant. Complete blood count, blood grouping and rhesus factor. After clotting, the samples were centrifuged and serum will be pipetted and the following will be measured: blood glucose, Alanine aminotransferase, Asspartate transaminase, Uric acid, Serum albumin, Serum creatinine, Nitric oxide,

Nitric oxide synthase, VEGF, PlGF, sFlt-1 and MDA.

Sample for immunoassays for measurement of Nitric oxide, Nitric oxide synthase, VEGF, PlGF, sFlt-1 and MDA will be stored at -20C till estimation. Urine analysis for proteinuria will be done.

USG will be done for gestational age, fetal heart sounds.

All the participants will be visited again at the time of delivery to collect the placental tissue for histopathology and immunohistochemistry and the outcome findings will be noted.

07	**Expected duration of the subject participation**	1 Hour
08	**Expected benefits from the research to the participant**	Results of the present study will help in the further management of preeclampsia
09	**Any risks expected from the study to the participants**	Minimal risk
10	**Maintenance of confidentiality of records**	Confidentiality will be maintained
11	**Provision of free treatment for research related injury**	Free treatment will be given by the Institution
12	**Compensation of the participants for disability or death resulting from such injury**	Compensation for any unforeseeable research related injury or death resulting from such injury will be duly given to you through hospital insurance policy number 68040236180200000009
13	**Freedom to withdraw from the study at any time during the study period without the loss of benefits that the participant would otherwise be entitled**	Yes, participants can withdraw from the study whenever they wish
14	**Possible current and future uses of the biological material and of the data to be**	Not Applicable

	generated from the research and if the material is likely to be used for secondary purposes or would be shared with others, this should be mentioned	
15	**Contact details of Chairman of the IEC for appeal against violation of rights**	Dr. S.L. Hoti, Director Grade Scientist (Scientist G), ICMR-National Institute of Traditional Medicine (Formerly RMRC), Belagavi- 590010 Phone No. 0831-2477477 Fax. 0831-2475479

CONSENT FORM-II
PARTICIPANT CONSENT FORM

Participant's name:

Address:

Phone No.

Email ID:

Title of the project:	**Pro-angiogenic vascular endothelial growth factor (VEGF), placental growth factor (PIGF) and anti-angiogenic factor, soluble FMS like tyrosine kinase-1 (sFlt-1) in preeclampsia – A case control study**

The details of the study have been provided to me in writing and explained to me in my own language. I confirm that I have understood the above study and had the opportunity to ask questions. I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason, without the medical care that will normally be provided by the hospital being affected. I agree not to restrict the use of any data or results that arise from this study provided. Such a use is only for scientific purpose(s).

I have been given an information sheet giving details of the study. I fully consent to participate in the above study.

Signature of the Participant: Date:

Signature of the Witness: Date:

ಒಪ್ಪಿಗೆ ಪತ್ರ

ಭಾಗವಹಿಸುವವರ ಹೆಸರು:

ವಿಳಾಸ:

ದೂರವಾಣಿ ಸಂಖ್ಯೆ:

ಇಮೇಲ್ ಐಡಿ:

ಯೋಜನೆಯ ಶೀರ್ಷಿಕೆ:   ಪ್ರಿ-ಎಂಕ್ಲಾಂಶಿಯಾ ರೋಗಿಗಳಲ್ಲಿನ ಪ್ರೊಯಿಂಜಿಯೋಜೆನಿಕ್ ಮತ್ತು ಯಂಟ-
                          ಯಿಂಜಿಯೋನೆನಿಕ್ ಕುರಿತು ಒಂದು ಅಧ್ಯಯನ.
ಅಧ್ಯಯನದ ವಿವರಗಳನ್ನು ನನಗೆ ಬರೆಯಲು ಮತ್ತು ನನ್ನ ಸ್ವಂತ ಭಾಷೆಯಲ್ಲಿ ನನಗೆ ವಿವರಿಸಲಾಗಿದೆ. ನಾನು ಮೇಲಿನ
ಅಧ್ಯಯನವನ್ನು ಅರ್ಥಮಾಡಿಕೊಂಡಿದ್ದೇನೆ ಮತ್ತು ಪ್ರಶ್ನೆಗಳನ್ನು ಕೇಳುವ ಅವಕಾಶವನ್ನು ಹೊಂದಿದ್ದೇನೆ ಎಂದು ನಾನು
ದೃಢೀಕರಿಸುತ್ತೇನೆ. ಅಧ್ಯಯನದ ನನ್ನ ಪಾಲ್ಗೊಳ್ಳುವಿಕೆ ಸ್ವಯಂಪ್ರೇರಿತವಾಗಿರುವುದನ್ನು ನಾನು ಅರ್ಥ ಮಾಡಿಕೊಂಡಿದ್ದೇನೆ
ಮತ್ತು ಆಸ್ಪತ್ರೆಯಿಂದ ಪರಿಣಾಮಕಾರಿಯಾದ ವೈದ್ಯಕೀಯ ಆರೈಕೆಯಿಲ್ಲದೆ ಯಾವುದೇ ಕಾರಣವನ್ನು ನೀಡದೇ ನಾನು
ಯಾವುದೇ ಸಮಯದಲ್ಲಿ ಹಿಂತೆಗೆದುಕೊಳ್ಳಲು ಮುಕ್ತನಾಗಿರುತ್ತೇನೆ. ಒದಗಿಸಿದ ಈ ಅಧ್ಯಯನದಿಂದ ಉದ್ಭವಿಸುವ
ಯಾವುದೇ ಡೇಟಾ ಅಥವಾ ಫಲಿತಾಂಶಗಳ

ಬಳಕೆಯನ್ನು ನಿರ್ಬಂಧಿಸಲು ನಾನು ಸಮ್ಮತಿಸುತ್ತೇನೆ. ಇಂತಹ ಬಳಕೆ ವೈಜ್ಞಾನಿಕ ಉದ್ದೇಶಕ್ಕಾಗಿ ಮಾತ್ರ, ಅಧ್ಯಯನದ
ವಿವರಗಳನ್ನು ನೀಡುವ ಮಾಹಿತಿ ಶೀಟ್ ನನಗೆ ನೀಡಲಾಗಿದೆ. ಮೇಲಿನ ಅಧ್ಯಯದಲ್ಲಿ ಪಾಲ್ಗೊಳ್ಳಲು ನಾನು
ಸಂಪೂರ್ಣವಾಗಿ ಸಮ್ಮತಿಸುತ್ತೇನೆ.
ಪಾಲ್ಗೊಳ್ಳುವವರ ಸಹಿ:

                                        ದಿನಾಂಕ:

ಸಾಕ್ಷಿ ಸಹಿ

Proforma for collection of data

Patient Id:			
Name:	Age:	Ht:	Wt:
Occupation:			
Address		Mobile no	
Clinical features:			
Obstetric history:			
Past history:			
Physical examination	BP, pulse rate, pallor, jaundice, chest and heart auscultation and lower limb edema.		
Abdominal examination	Fundal level± gestational age, lie and presentation of fetus		
Laboratory investigations	Complete blood count, blood grouping and rhesus factor, blood glucose, ALT, AST, uric acid, serum albumin, serum creatinine, VEGF, PlGF, sFlt-1. sFlt-1/PlGF		
Placental tissue			
USG report			
Pregnancy outcome (IUGR, intrauterine demise, early termination, preterm birth, lower birth weight, bronchopulmonary dysplasia etc.)			

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Annexure -I

<u>PLAGIARISM VERIFICATION CERTIFICATE</u>

1. Name of the Student: **Dr. Sangappa V. Kashinakunti** Reg No**: 17PhD006**
2. Title of the Thesis: **"Pro-angiogenic vascular endothelial growth factor (VEGF), placental growth factor (PIGF) and anti-angiogenic factor, soluble FMS like tyrosine kinase-1 (sFlt-1) in preeclampsia – A case control study"**
3. Department: **Biochemistry**
4. Name of the Guide & Designation: **Dr. Basavaraj Devaranavadagi,** Professor and HOD of Biochemistry, Shri. B. M. Patil Medical College, BLDE (Deemed to be University), Vijayapura
5. Name of the Co Guide & Designation: **Dr. Ashalata A. Mallapur,** Professor and HOD, Department of OBG, S. Nijalingappa Medical College, Navanagar, Bagalkot.

The above thesis was verified for similarity detection. The report is as follows:

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Signature of the Guide   Signature of Co-Guide   Signature of Student   Verified by (Signature)

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Ethical clearance certificates

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SHRI. B. M. PATIL MEDICAL COLLEGE, HOSPITAL & RESEARCH CENTRE, VIJAYAPURA

BLDE (DU)/IEC/323/2018-19

21-12-2018

INSTITUTIONAL ETHICAL CLEARANCE CERTIFICATE

The ethical Committee of this University met on 21st December 2018 at 11 a.m.to scrutinizes the Synopsis/ Research Projects of Post Graduate Student / Under Graduate Student Faculty members of this University / College from ethical clearance point of view. After scrutiny, the following original/ corrected and revised version Synopsys of the thesis/ research projects has been accorded ethical clearance.

Title. Pro-angiogenic vascular endothelial growth factor (VEGF), placental growth factor (PIGF) and anti-angiogenic factor, soluble FMS like tyrosine kinase-1(sFlt-1) in preeclampsia – A case control study

Name of the Faculty member /Ph.D/PG/UG student. Dr.Sangappa V. Kashinakunti

Name of the Guide; DrBASAVARAJ DEVARANAVADAGI.Professor&HOD Dept of.Biochemistry

Dr. Sharada Metgud

Chair person
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Dr.G.V.Kulkarni

Member Secretary
IEC, BLDE (DU),
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MEMBER SECRETARY
Institutional Ethics Committee
BLDE (Deemed to be University)
Vijayapura-586103. Karnataka

Note:-Kindly send Quarterly progress report to the Member Secretary

Following documents were placed before ethical committee for Scrutinization.

- Copy of Synopsis/Research Projects
- Copy of inform consent form
  Any other relevant documents

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S. Nijalingappa Medical College & Hanagal Shri Kumareshwar Hospital & Research Centre
Navanagar, Bagalkot-587102, Karnataka State, India.

(Recognized by Medical Council of India and Affiliated to Rajiv Gandhi University of Health Sciences, Karnataka)

SNMC-INSTITUTIONAL ETHICS COMMITTEE ON HUMAN SUBJECTS RESEARCH

☎08354-235340 Fax: 08354-235360 Website: www.snmcbgk.in Email: iechsrsnmcbgk@gmail.com

Office of the Institutional Ethice Committee

Ref. No. :
File No: SNMC/IECHSR/2018-19/A-84/1.1

Date:
Date: 11/02/2019

To:

Dr Sangappa Kashinakunti
Professor of Biochemistry
SNMC, Bagalkot

Topic of Protocol: Pro-angiogenic vascular endothelial growth factor (VEGF), placental growth factor (PIGF) and anti-angiogenic factor, soluble FMS like tyrosine kinase-1 (sFlt-1) in preeclampsia – A case control study submitted by Dr. Sangappa V. Kashinakunti Professor, Department of Biochemistry, S.Nijalingappa Medical College, Navanagar, Bagalkot-587102, Karnataka, India

Subject: Approval for conducting the above mentioned study & related documents by IEC.

Dear Dr Sangappa Kashinakunti

The Ethics Committee (EC) meeting of SNMC was held on 19-01-2019 from 09.30 AM onwards in the Hall of Medical Education Department of S. Nijalingappa Medical College & Hanagal Shri Kumareshwar Hospital & Research Centre, Bagalkot.

Following members of the committee were present:

1. Dr. S. L Hoti, Scientist-G,Director grade scientist ICMR-NITN, Belgaum.	Chairman
2. Dr.Yasmeen Maniyar , Professor & HOD of Pharmacology , SNMC, Bagalkot.	Member
3. Dr Anita Herur Professor of Physiology,SNMC,Bagalkot	Member
4. Dr Ashalata Mallapur Prof &HoD OBG, SNMC,Bagalkot	Member
5. Dr Chandrashekar V M Professor of Pharmacology HSK Pharmacy college	Member
6. Dr. Chandrashekharayya S. Hiremath, Professor of ENT, SNMC, Bagalkot	Member
7. Dr Manjula R Associate professor of Community Medicine	Member
8. Mr. Vittal Kamble , Near Vallabhbai chowk, Bagalkot.	Member
9 Mr.Jagdeesh, Budihal, advocate Navanagar , Bagalkot.	Member
10. Mr. D. G. Bannur, Holebasaveshwar Nilaya, 10th Cross, Vidyagiri, Bagalkot.	Member
11. Dr.Vijayamahantesh SN Professor of Forensic Medicine, SNMC, Bagalkot.	Member Secretary

Office of the Institutional Ethice Committee

Ref. No. :   Date:

The Ethical Committee of SNMC reviewed the following documents:

1. Research Protocol entitled) Pro-angiogenic vascular endothelial growth factor (VEGF), placental growth factor (PlGF) and anti-angiogenic factor, soluble FMS like tyrosine kinase-1 (sFlt-1) in preeclampsia – A case control study

2. Information sheet for participants of the study (Consent Form –I) and (Consent Form –II) of) Pro-angiogenic vascular endothelial growth factor (VEGF), placental growth factor (PlGF) and anti-angiogenic factor, soluble FMS like tyrosine kinase-1 (sFlt-1) in preeclampsia – A case control study

NOTE: It is to be noted that neither PI nor any of the proposed study team members were present during the decision-making procedures of the Ethics Committee, and members who are independent of the Investigator, have voted/ provided opinion on the trial.

Discussion points:
After reviewing the documents submitted by the Principal Investigator, the Committee has decided to grant approval for conducting the above mentioned study.

You are requested to report to the Ethics Committee the Following:
1. Progress of the study at the end of 4 months.
2. Provide a report to the Ethics Committee on completion of the study.

The Ethics Committee of SNMC follows procedures that are in compliance with the requirements of ICH (International Conference on Harmonization) related to GCP (Good Clinical Practice), schedule Y and all other applicable Indian regulations.

If you have any Questions concerning the above, please feel free to contact the undersigned.

Thanks & Regards,

(Dr. Vijayamahantesh SN)

Member Secretary
Member Secretary,
IEC
S.N. Medical College
BAGALKOT

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Paper presentation certificates

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August 6-7, 2020

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Certificate of Merit

This is to certify that, **Dr. Sangappa Kashinakunti** of Department of Biochemistry, S. Nijalingappa Medical College, Bagalkot, Karnataka, India has been awarded **'CERTIFICATE OF MERIT'** in PhD category for his/her presentation entitled **"Diagnostic accuracy of placental growth factor (PIGF) in pre-eclampsia- A case control study"** during UNESCO/UNITWIN Network Web Seminar during August 6 & 7,2020.

PROF. DR. ARAVIND V. PATIL
The Dean, FoM & Principal
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PROF. R. B. KOTNAL
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41 Annual Conference of Indian Association of Biomedical Scientists

International Virtual Conference on

RECENT TRENDS IN BIOMEDICAL RESEARCH

12- 14th March, 2021

CERTIFICATE

This is to certify that ___Sangappa Virupaxappa Kashinakunti___

has participated as and presented ___Oral Presentations___ entitled

___Diagnostic accuracy of maternal serum endothelial derived nitric oxide synthase (eNOS) and its correlation with birth out comes, in preeclampsia - A case control study___

and Won ___1st Prize___ in the "International Virtual Conference on "RECENT TRENDS IN BIOMEDICAL RESEARCH" Organized by the Indian Association of Biomedical Scientists held during 12-14 March 2021.

Dr. G. K. Pal
President
IABMS

Dr. E. Padmini
General Secretary, IABMS
Organizing Secretary

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27th, 28th, 29th February 2020

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DEPARTMENT OF BIOCHEMISTRY
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CERTIFICATE

Type of Conference-Multi speciality

(CPD Code–MMC/MAC/2020/F–014720)

This is to certify that

Dr/Mr/Ms ___Sangappa Y. Kashinakunti___

has Participated as Delegate / Delivered an Oral / ~~Poster~~ presentation in the 9th Annual International Conference of Indian Academy of Biomedical Sciences.

He / She is granted 4 CPD credit points by Maharashtra Medical Council.

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President, IABS

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(MMC/MAO-01898/2018)

Dr.Shailesh Kore
MMC Observer
(MMC/MAO-00603/2015)

PUBLICATIONS

Research article (Award paper)

Diagnostic accuracy of maternal serum endothelial-derived nitric oxide synthase (eNOS) and its correlation with birth outcomes, in preeclampsia - a case-control study

Sangappa Virupaxappa Kashinakunti[1,2], Basavaraj Devaranavadagi[3], Manjula Rangappa[4], Ashalata Mallapur[5]

[1]PhD Scholar, [3]Professor and Head, Department of Biochemistry, Shri B M Patil Medical College, BLDE University, Vijayapur-586101, Karnataka, India
[2]Professor, Department of Biochemistry, [4]Professor, Department of Community Medicine, [5]Professor and Head Department of Obstetrics and Gynaecology, S. Nijalingappa Medical College, Navanagar, Bagalkot-587102 Karnataka, India

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Research article

Comparison of serum nitric oxide levels with case severity in pre-eclampsia – A case control study

Sangappa Virupaxappa Kashinakunti[1,2], Basavaraj Devaranavadagi[3], Manjula Rangappa[4], Ashalata Mallapur[5]

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Research article

Comparison of serum nitric oxide levels with case severity in pre-eclampsia – A case control study
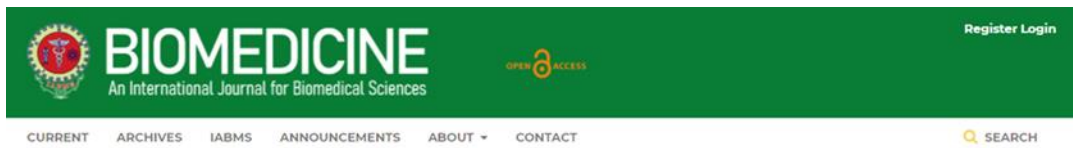
Sangappa Virupaxappa Kashinakunti[1,2], Basavaraj Devaranavadagi[3], Manjula Rangappa[4], Ashalata Mallapur[5]

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ABSTRACT

Introduction and Aim: In preeclampsia (PE) due to abnormal trophoblastic invasion of the maternal spiral arteries leads to decreased uteroplacental circulation. Nitric oxide (NO) is involved in regulation of feto-placental vascular permeability and resistance. It also affects maturation, development of the placenta and foetal survival. Maternal serum NO levels are controversial in PE and only few studies have focused on birth outcomes. Hence, the current study was undertaken to estimate and compare the maternal serum NO levels in normal pregnant and PE women and also see the correlation between maternal serum NO level with severity of PE and to study its association with birth outcome.

Materials and Methods: The study was conducted at a tertiary care hospital in North Karnataka, India. Forty singleton primigravida PE patients diagnosed and classified as mild/severe PE, as per ACOG guidelines and 40 healthy pregnant were selected for the study. NO was estimated by ELISA method.

Results: NO was high in PE cases than controls but it was not statistically significant. But NO was significantly (p=0.03) higher in mild PE than severe PE patients. The NO level negatively correlated significantly (p=0.031) with case severity. Maternal serum NO did not show any significant correlation with birth weight and APGAR score of the new born baby.

Conclusion: In mild PE rise in maternal serum NO, may be to due compensatory mechanism to normalise persistently elevated blood pressure and decreased level of serum NO in severe PE may be due to down-regulation of the nitric oxide synthase.

Keywords: Nitric oxide; pre-eclampsia; birth weight; APGAR score.

INTRODUCTION

Preeclampsia (PE) is a hypertensive multisystem disorder in previously normotensive women, complicating 6-10% of all pregnancies over 20 weeks of gestation. World Health Organisation estimates that frequency of PE is seven times higher in developing countries than developed countries. PE is one of the leading causes of foetal growth disorders, foetal morbidity and mortality. There is a five-fold increase in perinatal deaths from intrauterine growth restriction and prematurity as a result of PE. Maternal complications of PE include eclampsia, stroke, liver and renal failure, disseminated intravascular coagulation, premature labour and maternal death (1-4).

Evidence suggests that general vascular endothelial dysfunction, which occurs during PE can explain mechanism responsible for its pathogenesis. Primary disturbance appears to result from reduced uteroplacental circulation due to abnormal trophoblastic invasion of the maternal spiral arteries resulting in poor placentation. These spiral arteries retain their endothelial lining and the underlying smooth muscles, which make them reactive to

vasoactive agents expressed by the endothelial cells. Endothelial cell dysfunction can cause hypertension with its increased production of vasoconstrictor agents such as plasma endothelin or reduced release of vasodilator agents such as Nitric oxide (NO) and prostacyclin (5-7).

NO is a physiological agent involved in regulation of feto-placental vascular permeability and resistance and platelet aggregation in the placenta. Maturation and development of the placenta and foetal survival is significantly affected by nitric oxide. Endothelial nitric oxide produces its action through cyclic guanosine monophosphate dependent pathway. Serum NO level is considered as a valuable biomarker of endothelial function. NO biosynthesis data are also controversial in PE (7-9).

The outcome of current study may give more sight to clinicians as to whether the pathogenesis of PE is linked to maternal serum NO levels and how such knowledge can be utilized in effective prevention of PE. Some authors depicted different results and they showed that the biosynthesis of NO in preeclampsia was decreased, unchanged or raised and hence, this issue is still debatable (10-12). Hence the current

study was undertaken to estimate and compare maternal serum levels of NO in normal pregnant and PE women; and also to find the correlation between serum nitric oxide level with severity of PE and to study its association of NO levels with birth outcome.

MATERIALS AND METHODS

This is a case control study, conducted in department of obstetrics and gynecology and department of biochemistry at Hanagal Shri Kumareshwara Hospital, Bagalkot, Karnataka India, from Jan 2019 to Jun 2019. The study was approved by Institutional ethics committee. Informed consent was obtained from all the participants.

Primigravida (singleton) pregnant women aged 18-35 years, 20 or more weeks of gestation, diagnosed as PE and classified as mild/severe PE based on America college of obstetricians and gynecologists guidelines (13) were selected for the study. Patients with pre-mature rupture of membranes, chorioamnionitis, multiple gestation, Rh isoimmunization, fetal anomalies, intra uterine fetal death, chronic inflammatory diseases, history of diabetes mellitus, past history of systemic hypertension, cardiovascular or renal diseases and chronic inflammatory diseases were excluded from the study. Healthy pregnant women, matched for age, gravida and gestational weeks with the cases were selected as controls.

All participants were subjected for history taking and followed by general physical examination.

Abdominal examination was performed for fundal level, lie and presentation of fetus. Blood samples were collected by venipuncture in two tubes, 2ml with anticoagulant and 2 ml without anticoagulant. Complete blood count, blood grouping and rhesus factor were done. After clotting, the samples were centrifuged and serum was pipetted and the following parameters were measured blood glucose, liver function tests, urea. serum creatinine, uric acid and nitric oxide. Sample for NO measurement was stored at -20º C till estimation. Urine analysis for proteinuria was done. Abdominal ultrasonography was done for gestational age, fetal heart sound. All the participants were visited again at the outcome of the pregnancy and the outcome findings was noted.

Sample size calculation was done using Open Epi software version 2.3:1, retrospectively with 83.47% power of the study, using case severity in to consideration, sample size calculated was 34~ 40. Hence, 40 preeclampsia cases and 40 normal gestation controls were included in the study.

Analysis was done using SPSS software version 19. Unpaired 't' test for quantitative data and Pearson's correlation tests were applied. P<0.05 will be considered as statistically significant.

RESULTS

Seven women were mild PE and 33 were severe PE patients.

Table 1: Diagnostic and severity features of healthy pregnant women and preeclampsia group

	Controls	Cases	t	p
Gestational weeks	37.78±2.97	35.63±4.37	2.511	0.014
Pulse Beats/min	86.54±6.98	92.28±9.27	-3.044	0.003
SBP mmHg	117.41±10.75	157.55±17.75	-11.878	0.000
DBP mmHg	74.70±9.45	102.40±9.10	-13.093	0.000
BMI	24.82±3.65	28.99±5.41	-3.008	0.004
Haemoglobin g%	11.57±1.48	11.06±2.12	1.19	0.23
Platelet	228891.89±49402.42	199743.59±81379.78	-0.67	0.50
RBG mg/dl	88.52±21.46	85.87±20.37	0.563	0.575
Serum Urea mg/dl	21.30±6.48	24.28±4.91	-2.291	0.025
Serum Creatinine mg/dl	0.73±0.13	0.75±0.15	-0.542	0.590
AST IU/L	23.37±10.40	43.43±61.28	-2.015	0.04
ALT IU/L	15.82±10.17	35.36±67.37	-1.791	0.07
Urine Protein mg/l	120.26±158.84	2464.68±3185.03	-2.73	0.010

SBP - Systolic blood pressure; **DBP**- Diastolic blood pressure; **BMI**- Body mass index; **RBG**- Random blood glucose; **Urine P/C ratio-** Urine protein creatinine ratio; **AST**- Aspartate transaminase; **ALT**- Alanine transaminase.

There was significant decrease in gestational weeks in preeclampsia. Pulse, systolic blood pressure, diastolic blood pressure and body mass index were significantly higher in preeclampsia women than normal pregnant women. Haemoglobin and Platelet count were less in preeclampsia cases than the control group but it was not statistically significant. All renal function tests were significantly higher in

preeclampsia cases than healthy pregnant women except serum creatinine which also more was in cases than control group but it was not statistically significant. Aspartate transaminase was significantly higher in cases than the control group, alanine transaminase was also more in preeclampsia cases than normal pregnant women, but it was not statically significant.

Table 2: Nitric oxide (NO) in controls and cases

	Controls	Cases	t	p
NO µmol/l	76.38±117.02	115.77±195.32	-1.090	0.279

NO was also high in cases than controls, but it was not statistically significant.

Table 3: Nitric oxide (NO) in mild and severe cases of PE

	Mild	Severe	t	p
NO µmol/l	165.27±217.77	58.39±77.86	2.24	0.03

NO was significantly higher in mild PE patients than severe PE patients.

Table 4: Co-relation of Nitric oxide (NO) with case severity

	Pearson Correlation	p
NO	-0.350	0.031

Significant negative correlation was between NO and case severity of PE, which meant as the severity of PE increases the NO levels, would decrease.

Table 5: Correlation between maternal serum Nitric oxide (NO) level with birth outcome

	Birth weight	APGAR 1 min	APGAR 5 min
r	-0.020	0.079	0.055
p	0.909	0.663	0.762

NO did not show any significant correlation with birth weight and APGAR score at 1 minute and 5 minutes.

DISCUSSION

Many studies showed that the biosynthesis of NO was increased in normal pregnancy, especially in the second trimester, with its peak in the third trimester of pregnancy. Study done by Adu-Bonsaffoh *et al.,* (14) found that PE women had a significantly higher maternal serum nitrite concentration (p<0.001) while women with chronic hypertension had a significantly lower nitrite concentration than the control group (P=0.04).Women with gestational hypertension had a similar nitrite level to the control group (15-17).

In the current study, there was increase in maternal serum NO levels of PE women compared to normal healthy pregnant women, but it was not statistically significant (p=0.279). Literature has reported inconsistent results as far as serum nitric oxide levels in PE compared to healthy pregnant women.

They attributed the marked increase in NO levels to compensatory mechanism to the pathological effect of PE. It is possible that the amount of bioavailable NO needed to restore the persistently elevated BP was insufficient (14).

Conversely, Darkwa *et al.,* (18) showed a statistically non-significant reduction in plasma NO levels in PE compared to healthy pregnant women (P = 0.160) agreeing with the observations of other studies. The studies have indicated that maternal serum NO levels are reduced in PE. Sahu *et al.,* (19) reported a reduction in NO production in PE women compared with normotensive pregnant women. The reduction in NO as a result of down-regulation of the nitric oxide synthase enzyme, leading to a relative deficiency of NO and an excess of ONOO- and/or occurrence of endothelial damage in the development of the disorder, the production of O2- by NOS is central to the pathophysiology eventually leading to clinical syndrome and NO donors have been hypothesized to prevent PE. Another reason for reduced NO serum levels of PE women could be due to increased binding and reduced releasing of nitrates from red blood cells (4, 20, 21).

The determination of nitric oxide levels is confounded by several internal factors including a source of sample (plasma, serum, urine), external factors like method of assaying, diet rich in nitrates such as cured meats or vegetables, alcohol consumption, atmospheric pollution, exercise and cigarette smoking. It may be difficult to find a significant change over the uncontrolled external factors and eliminate the inter-subject variations (18, 23).

In the present study, the serum NO levels were compared between the mild and severe PE women, the results showed significantly higher level of NO in mild PE patients compared to severe PE women. There was a significant negative correlation between maternal serum NO and severity of PE.

However, a study of Adu-Bonsaffoh *et al.,* (14) showed markedly elevated serum levels of NO in early-onset PE (Severe form) compared to late-onset PE that is considered as a mild disease. This may represent a compensatory mechanism to offset the ongoing pathological process and indicating the generalized endothelial dysfunction. On the other hand, other researchers have reported that circulating nitrate and nitrite levels are not reduced in patients with severe PE compared with normotensive controls (11, 14, 24).

Erdemoğlu *et al.,* concluded that there was no difference between means of NO in healthy, mild, and severe preeclamptic patients, neither severity of preeclampsia nor eclampsia was correlated with NO values (24). Shaker *et al.,* in their study showed that the, mean levels of serum NO products were significantly decreased in both the mild and severe groups compared with the normotensive group (p1, p2 < 0.001), but no significant difference was found between the mild and severe groups (p3 > 0.05). They also did not get any correlation between maternal serum NO and birth weight. In the current study also there was no correlation between maternal serum NO and birth outcomes viz birth weight and APGAR score (25).

The limitation of present study was that the diet history was not considered and history of alcohol consumption and smoking were not included because these are culturally sensitive issues in this part of India. Further longitudinal nitric oxide synthase estimation and genetic expression are required to find the facts of pathogenesis of PE.

CONCLUSION

In conclusion, in mild PE, significant rise in maternal serum NO may be due the compensatory mechanism to normalise persistently elevated blood pressure where the regulatory mechanism works at its zenith; and decreased level of serum NO in severe PE may be due to down-regulation of the nitric oxide synthase, which ultimately leads to endothelial dysfunction, presenting as a syndrome.

CONFLICT OF INTEREST

Authors declare no conflicts of interest.

REFERENCES

1. Bernardi, F. C., Vuolo, F., Petronilho, F., Michels, M., Ritter, C., Dal-Pizzol, F. C. Plasma nitric oxide, endothelin-1, arginase and superoxide dismutase in the plasma and placentae from preeclamptic patients. An Acad Bras Cienc. 2015; 87(2): 713-719.

2. Carty, D. M., Delles, C., Dominiczak, A. F. Preeclampsia and future maternal health. J Hypertens 2010; 28: 1349-1355.

3. World Health Organization. Make every mother and child count. The world health report 2005. Geneva, Switzerland: World Health Organization, 2005. Available at: http://www.who.int/whr/2005/whr2005_en.pdf (Accessed on 30 June 2017)

4. Gilbert, J. S., Ryan, M. J., La Marca, B. B., Sedeek, M., Murphy, S. R., Granger, J. P. Pathophysiology of hypertension during preeclampsia: linking placental ischemia with endothelial dysfunction. Am J Physiol Heart Circ Physiol 2008; 294(2): 541-550.

5. George, E. M., Granger, J. P. Recent insights into the pathophysiology of preeclampsia. Expert Rev Obstet Gynecol 2012; 5(5): 557-556.

6. Su, J. B. Vascular endothelial dysfunction and pharmacological treatment. World J Cardiol 2015; 26: 719-741.

7. Myatt, L. Placental adaptive responses and fetal programming. J Physiol 2006; 572: 25-30.

8. Krause, B. J., Hanson, M. A., Casanello, P. Role of nitric oxide in placental vascular development and function. Placenta 2011; 32: 797-805.

9. Suzuki, T., Mori, C., Yoshikawa, H., Miyazaki, Y., Kansaku, N., Tanaka, K., *et al.,* Changes in nitric oxide production levels and expression of nitric oxide synthase isoforms in the rat uterus during pregnancy. Biosci Biotechnol Biochem. 2009; 73: 2163-2166.

10. Matsubara, K., Matsubara, Y., Hyodo, S., Katayama, T., Ito, M. Role of nitric oxide and reactive oxygen species in the pathogenesis of preeclampsia. J Obstet Gynaecol Res. 2010; 36: 239-247.

11. Zeng, Y., Li, M., Chen, Y., Wang, S. Homocysteine, endothelin-1 and nitric oxide in patients with hypertensive disorders complicating pregnancy. Int J Clin Exp Pathol. 2015; 8: 15275-15279.

12. Acauan Filho, B. J., Pinheiro da Costa, B. E., Ogando, P. B., Vieira, M. C., Antonello, I. C., Poli-de Figueiredo, C. E. Serum nitrate and NOx levels in preeclampsia are higher than in normal pregnancy. Hypertens Pregnancy. 2016; 35: 226-233.

13. American College of Obstetricians and Gynecologists; Task Force on Hypertension in Pregnancy. Hypertension in pregnancy. Report of the American College of Obstetricians and Gynecologists' Task Force on Hypertension in Pregnancy. Obstet Gynecol. 2013; 122: 1122-1131.

14. Adu-Bonsaffoh, K., Antwi, D. A., Obed, S. A., Gyann, B. Nitric oxide dysregulation in the pathogenesis of preeclampsia among Ghanaian women. Integrated Blood Pressure Control 2015; 8: 1-6.

15. Osol, G., Ko1, N. L., Mandalà, M. Altered Endothelial Nitric Oxide Signaling as a Paradigm for Maternal Vascular Maladaptation in Preeclampsia. Curr Hypertens Rep 2017; 19: 82.

16. Pimentel, A.M.L., Pereira, N. R., Costa, C. A., Mann, G. E., Cordeiro, V.S.C., de Moura, R. S., *et al.,* L-arginine-nitric oxide pathway and oxidative stress in plasma and platelets of patients with pre-eclampsia. Hypertens Res. 2013; 36(9): 783–788.

17. Eleuterio, N. M., Palei, A.C.T., Machado, J.S.R., Tanus-Santos, J. E., Cavalli, R. C., Sandrim, V. C., *et al.* Relationship between adiponectin and nitrite in healthy and preeclampsia pregnancies. Clin Chim Acta. 2013; 423: 112-115.

18. Darkwa, E. O., Djagbletey, R., Essuman, R., Sottie, D., Dankwah, G. B., Aryee, G. Nitric Oxide and Pre-Eclampsia: A Comparative Study in Ghana. Open Access Maced J Med Sci. 2018 Jun 20; 6(6): 1023-1027.

19. Sahu, S., Daniel, M., Abraham, R., Vedavalli, R., Senthilvel, V. Study of uric acid and nitric oxide concentrations in preeclampsia and normal pregnancy. Int J Biol Med Res. 2011; 2(1): 390-393.

20. Diejomaoh, F., Omu, A., Al-Busiri, N., Taher, S., Al-Othman, S., Fatinikun, T., *et al.,* Nitric oxide production is not altered in preeclampsia. Arch Gynecol Obstet. 2004; 269(4): 237-243.

21. Poorolajal, J., Jenabi, E. The association between body mass index and preeclampsia: a meta-analysis. J Matern Neonatal Med 2016; 29(22): 3670-3676.

22. Owusu, B. Y., Stapley, R., Honavar, J., Patel, R. P. Effects of erythrocyte aging on nitric oxide and nitrite metabolism. Antioxid Redox Signal 2013; 19: 1198-1208.

23. Hodžić, J., Izetbegović, S., Muračević, B., Iriškić, R., Jović, H. S. Nitric oxide biosynthesis during normal pregnancy and pregnancy complicated by preeclampsia. Med Glas (Zenica). 2017; 14(2): 211-217.

24. Erdemoğlu, E., Uğur, M., Erdemoğlu, E. Plasma homocysteine and nitric oxide levels in preeclampsia. J Turkish-German Gynecol Assoc. 2009; 10: 26-29.

25. Shaker, O. G., Hany Shehata, H. Early Prediction of Preeclampsia in High-Risk Women. Journal of women's health 2011; 20(4): 539-544.

Research article (Award paper)

Diagnostic accuracy of maternal serum endothelial-derived nitric oxide synthase (eNOS) and its correlation with birth outcomes, in preeclampsia - a case-control study

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ABSTRACT

Introduction and Aim: Preeclampsia (PE) results from impaired placentation, leading to placental hypoxia and dysfunction and abnormalities in the endothelial nitric oxide synthase (eNOS)-nitric oxide pathway. The present study was undertaken to estimate and compare maternal serum eNOS concentrations in women with PE and normal healthy pregnant women, to know the correlation of eNOS level with severity of PE, to find the best cut-off value for the diagnosis of PE and to study its association of eNOS levels with the birth outcome.

Material and Methods: This was a case-control study, conducted at a tertiary care hospital. The study was approved by the Institutional ethics committee. Informed consent was obtained from all the participants. Primigravida (singleton) pregnant women aged 18-35 years, 20 or more weeks of gestation, diagnosed as PE and classified as mild/severe PE based on America college of obstetricians and gynecologists guidelines were selected for the study. eNOS was estimated by the ELISA method. All the participants were visited again to note the outcomes.

Results: The maternal serum eNOS in PE was significantly lower than the control group (p=0.019). The best cut-off value was 187.25pg/ml to diagnose PE, area under the ROC curve (AUC) 0.61, sensitivity 95.1%, specificity 37.5% and diagnostic accuracy was 66,67%. Birth outcomes did not have a significant correlation with eNOS.

Conclusion: eNOS was significantly lowerer in PE, it can be used as a diagnostic marker with the best cut of value of 187.25 pg/ml.

Keywords: Preeclampsia; nitric oxide synthase; diagnosis.

INTRODUCTION

Preeclampsia (PE), constitute a unique, complex clinical condition of human pregnancy, is characterized by the development of de-novo hypertension and proteinuria after 20 weeks or above gestation in patients free from any clinical disease, but it can occur at any time during labor, or even up to 6 weeks after delivery. PE is a multi-organ involvement targets the endothelium of the brain, liver, kidneys and the coagulation system and impairs a short-term perinatal outcome and remote prognosis of cardiovascular disease for the mother. Termination of pregnancy remains the only curative treatment in severe PE. Management of patients with PE must be individualized and must balance the maternal and fetal risks (1-3).

It has been suggested that the root cause of PE is the placenta. Its functions are vascular development and blood flow, which depend on proper trophoblast growth and differentiation. According to the studies, preeclampsia results from impaired placentation early in the beginning of the pregnancy, leading to placental hypoxia and dysfunction and abnormalities in the endothelial nitric oxide synthase (eNOS)-nitric oxide pathway (4-6). eNOS is localized mainly in the plasma membrane, and a small amount is present in the cytosol also. Golgi apparatus acts as the main source of eNOS. The eNOS is a key enzyme of the cardiovascular system that contributes to vascular homeostasis through tightly regulated NO production (7,8). Endothelial cells release NO, a potent vasodilator. Nitric oxide is required for regulation of the vascular tone and hence maintains blood pressure. The placental blood flow is regulated by NO and it also participates in trophoblast invasion and development of the placenta. As NO is a highly reactive and short-lived molecule. It is clearly not known whether eNOS deficiency plays an important role in pathogenesis of preeclampsia. Many studies suggest that insufficient nitric oxide synthesis or NO bioavailability, may contribute to increased blood pressure, systemic vascular resistance, and sensitivity to the pressors. However, studies conducted among various ethnic groups, which resulted in mixed or inconclusive results (9-12).

The current study was undertaken to estimate and compare maternal serum concentrations of endothelial nitric oxide synthase (eNOS) in women with PE in comparison to healthy normotensive pregnant women. and also to find the correlation between eNOS level with severity of PE, the best

cutoff value for the diagnosis of PE and to study its association of eNOS levels with the birth outcome.

MATERIALS AND METHODS

This is a case-control study, conducted in the department of obstetrics and gynecology and department of biochemistry at tertiary care hospital, Karnataka India, from Jan 2019 to Jun 2019. The study was approved by the Institutional ethics committee. Informed consent was obtained from all the study participants.

Primigravida (singleton) pregnant women aged 18-35 years, 20 or more weeks of gestation, diagnosed as PE and classified as mild/severe PE based on America college of obstetricians and gynecologists' guidelines (13) were selected for the study. Patients with premature rupture of membranes, chorioamnionitis, multiple gestations, Rh isoimmunization, fetal anomalies, intrauterine fetal death, chronic inflammatory diseases, history of diabetes mellitus, history of systemic hypertension, cardiovascular or renal diseases and chronic inflammatory diseases were excluded from the study. Healthy pregnant women, matched for age, gravida and gestational weeks with the cases were selected as controls.

All participants were subjected to history taking and followed by general physical examination. Abdominal examination was performed for fundal level, lie and presentation of the fetus. Blood samples were collected by venipuncture in two tubes, 2ml with anticoagulant and 2 ml without anticoagulant. Complete blood count, blood grouping and rhesus factor were done. After clotting, the samples were

centrifuged and serum was pipetted and the following parameters were measured blood glucose, liver function tests, urea. serum creatinine, uric acid and eNOS. Sample for eNOs measurement was stored at -20º C till estimation. eNOS was estimated by the ELISA method the kits were supplied by Bioassay Technologies Laboratory. Urine analysis for proteinuria was done. All the participants were visited again at the outcome of the pregnancy and the outcome findings were noted.

Sample size calculation was done using Open Epi software version 2.3:1, retrospectively with 83.47% power of the study, using case severity into consideration, the sample size calculated was 39~ 45. Hence, 45 preeclampsia cases and 45 normal gestation controls were included in the study.

Analysis was done using SPSS software version 19. Unpaired 't' test for quantitative data and Pearson's correlation tests were applied. P<0.05 will be considered statistically significant. ROC curve analysis for eNOS was done to find the optimum cutoff value for the diagnosis of PE. Tests of validity namely sensitivity, specificity, positive predictive value, negative predictive value and diagnostic accuracy of eNOS were calculated with 95% confidence intervals.

RESULTS

In the present study, there was no significant difference in maternal age, gestational weeks. The systolic, diastolic blood pressure, SGOT were significantly higher in PE patients than normal pregnant women (Table 1).

Table 1: Demographic characteristics of controls and cases

	Controls	Cases	p
Age in years	22.55±3.22	22.83±4.23	0.94
Gestational Weeks	37.88±2.87	35.83±4.77	0.085
SBP mmHg	118.51±10.65	158.45±18.85	**0.000**
DBP mmHg	75.40±8.75	102.30±9.20	**0.000**
Platelet	228792.84±49502.22	200888.45±82528.64	0.078
Creatinine mg/dl	0.72±0.14	0.76±0.10	0.600
AST IU/L	24.27±10.38	44.53±62.24	**0.044**
ALT IU/L	15.85±10.18	36.37±68.25	0.067
Birth weight in Kg	2.88±0.67	2.16±0.85	**0.000**
APGAR 1min	7.82±0.770	6.49±2.62	**0.016**
APGAR 5min	8.94±0.40	7.69±2.85	**0.020**

SBP: Systolic blood pressure, DBP: Diastolic blood pressure, AST: Aspartate transaminase, ALT: Alanine transaminase

Table 2: eNOS in controls and cases

	Controls	Cases	p
eNOS pg/ml	173.36±187.68	99.87±56.58	0.019

eNOS: Endothelial derived nitric oxide synthase

The maternal serum eNOS in PE was significantly lower than the control group(p=0.019).
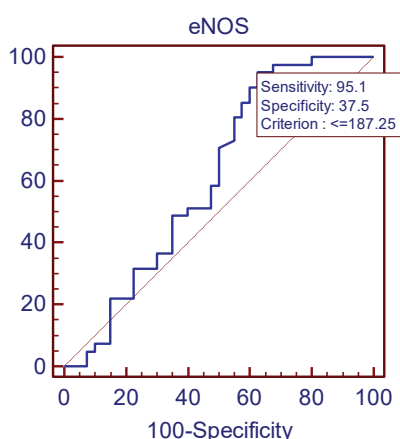
Fig. 1: The best cutoff value of eNOS for the diagnosis of PE by ROC curve

Table 3: Sensitivity, Specificity, Positive Predictive Value, Negative Predictive Value and Diagnostic Accuracy of eNOS in PE patients

Sensitivity	95.1%	(48.69, 71.94[1])
Specificity	37.5%	(65.66, 96.71[1])
Positive Predictive Value	95.12%	(83.86, 98.65[1])
Negative Predictive Value	37.50%	(24.22, 52.97[1])
Diagnostic Accuracy	66.67%	(55.85, 75.97[1])

The best cut-off value was 187.25 pg/ml to diagnose PE, area under the ROC curve (AUC) 0.61, sensitivity 95.1%, specificity 37.5% and diagnostic accuracy was 66.67% (Fig. 1, Table 3).

Table 4: eNOS in mild and severe PE patients

	Mild PE	**Severe PE**	**p**
eNOS pg/ml	101.18±60.63	95.76±40.94	0.824

eNOS: Endothelial derived nitric oxide synthase, PE: Preeclampsia.
There was decrease in eNOS in severe PE cases than mild PE cases but it was not statistically significant (p=0.824) (Table 4).

Table 5: Correlation of eNOS with the severity of PE and birth outcomes

	Severity of PE	**Birth weight**	**APGAR 1min**	**APGAR 5min**
r	0.036	-0.029	-0.112	-0.142
p	0.824	0.868	0.530	0.422

eNOS: Endothelial derived nitric oxide synthase, PE: Preeclampsia
Birth outcomes did not have a significant correlation with eNOS (Table 5).

DISCUSSION

NO is synthesized from the reduction of L-arginine to L-citrulline by the NOS enzyme, which has three isoforms: nNOS or neuronal, iNOS, the inducible and eNOS endothelial NOS, here BH4 acts as a coenzyme, promotes dimerization and activity of NOS. Decreased concentration of the substrate L-arginine and increased concentration of the endogenous NOS inhibitor asymmetric dimethylarginine (ADMA) may interfere with eNOS activity in preeclampsia (14,15).

In the present study, there was a significant decrease in eNOS in PE patients compared to the normal pregnant women (p=0.019). Kim *et al.,* and Khalil *et al.,* found lower expression of eNOS in the syncytiotrophoblast, reduced concentrations of L-arginine, and unchanged ADMA in the serum of women with pregnancies complicated by preeclampsia (16,17). A cohort study conducted in Greek showed that the plasma ADMA levels in PE women were found to be higher (18) than the control group. Maranzana *et al.,* and Laskowska *et al.,* showed that the levels of serum eNOS were lower in women with PE than in the healthy women from the control group, but it was not statistically significant (p¼0.238899). The mean values of serum eNOS were 181.30±178.44 U/ml in the PE group and 217.74±265,11 U/ml in the control group (19,20). The levels of serum endothelial nitric oxide synthase were lower in women with pregnancies complicated by severe PE than in the healthy women from the control group, but these differences were not statistically significant (p = 0.118770). The mean values of serum eNOS were 134.06 ± 76.73 U/ml in the Pre group and 187.70 ± 165.41 U/ml in the Control group (21). Myatt *et al.,* (5) observed intense expression of eNOS in placentas from pregnancies

complicated by preeclampsia. Schiessl *et al.,* found significantly increased placental expression of endothelial nitric oxide synthase in pregnancies complicated by preeclampsia (22). In a Brazilian cohort study, plasma ADMA concentration was more in PE women and plasma NO concentration was found lower in comparison with normotensive women (23).

The uncoupling of eNOS has also been shown as a source of superoxide formation and this leads to reduce NO production. When eNOS cofactor, tetrahydrobiopterin(BH4) is low or when post-translational changes regulate eNOS function. It has been demonstrated that various inflammatory makers like TNF-α and CRP are increased in plasma and placenta from PE women. TNF-α an inflammatory marker increased in PE, which downregulates eNOS and mitochondrial biogenesis leading to mitochondrial dysfunction and elevated ROS. Conversely, CRP is another inflammatory marker also increased in PE, indirectly downregulates BH4 production, leading to uncoupling of eNOS catalysed reaction and leads the formation of peroxynitrite. Animal studies have shown that BH4 supplementation in pE increased the concentration of NO (14,15,24).

In the present also there was low levels of maternal serum concentration of eNOS in severe PE as compared to mild PE but it was not statistically significant (p=0,824). The unchanged levels of eNOS in severe PE. A small increase in eNOS in mild hypertension PE patients, whereas in a subgroup with severe hypertension, concentrations of these markers were greatly reduced. Studies have shown elevated ADMA levels in early-onset PE may suggest a relationship between the severity of the disease and determining the time of PE clinical manifestations (2,12,25).

In the current study the best cut-off value was 187.25 pg/ml to diagnose PE, area under the ROC curve (AUC) 0.61, sensitivity 95.1%, specificity 37.5% and diagnostic accuracy was 66.67% (Figure 1, Table 3). Wender-Ozegowska *et al.,* in their study, found that eNOS AUC was 0.57 best cut-off was 0.34 with a sensitivity of 78.6% specificity 40% (1). Our study did not show any correlation of eNOS with the severity of PE and birth outcome viz birth weight and APGAR score.

Further studies are required with gene expression along with serum estimation of NO, eNOS, L-arginine, ASDA, BH4, oxidative stress markers and inflammatory markers may give the role of NO and eNOS in the pathogenesis of PE.

CONCLUSION

The eNOS was significantly lower in PE patients than normal healthy pregnant women, it can be used as tool diagnose PE with the best cut-off value of

187.25 pg/ml, with sensitivity 95.1%, specificity 37.5% and diagnostic accuracy of 66.67%.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1. Wender-Ozegowska, E., Zawiejska, A., Lciek, R., Brązert, J. Concentrations of eNOS, VEGF, ACE PlGF in maternal blood as predictors of impaired fetal growth in pregnancy complicated by gestational hypertension/preeclampsia. Hypertension in Pregnancy 2015;34(1):17-23.
2. Laskowska, M., Laskowska, K., Terbosh, M., Oleszczuk, J. A comparison of maternal serum levels of endothelial nitric oxide synthase, asymmetric dimethylarginine, and homocysteine in normal and preeclamptic pregnancies. Med Sci Monit 2013;19: 430-437.
3. Uzan, J., Carbonnel, M., Piconne, O., Asmar, R., Ayoubi, J. M. Pre-eclampsia: pathophysiology, diagnosis, and management. Vasc Health Risk Manag 2011;7: 467-474.
4. Roberts, J.M., Escudero, C. The placenta in Preeclampsia. Pregnancy Hypertens 2012;2(2):72-83.
5. Myatt, L. Review: Reactive oxygen and nitrogen species and functional adaptation of the placenta. Placenta 2010;31(Suppl.): S66-S69.
6. Mislanova, C., Martsenyuk, O., Huppertz, B., Obolenskaya, M. Placental markers of folate-related metabolism in preeclampsia. Reproduction, 2011;142(3):467-476.
7. Sessa WC. eNOS at a glance. J Cell Sci 2004;117: 2427-2429.
8. Shaul, P.W. Regulation of endothelial nitric oxide synthase: location, location, location. Annu Rev Physiol 2002; 64: 749-774.
9. Demir, B., Demir, S., Pasa, S., Guven, S., Atamer, Y., Atamer, A., *et al.,* The role of homocysteine, asymmetric dimethylarginine and nitric oxide in pre-eclampsia. J Obstet Gynaecol 2012;32(6):525-528.
10. Huang, L.T., Hsieh, C.S., Chang, K.A., Tain, Y.L. Roles of nitric oxide and asymmetric dimethylarginine in pregnancy and fetal programming. Int J Mol Sci 2012;13(11):14606-14622.
11. Kulandavelu, S., Whiteley, K.J., Qu, D., Mu, J., Bainbridge, S.A., Adamson S.L. Endothelial nitric oxide synthase deficiency reduces uterine blood flow, spiral artery elongation, and placental oxygenation in pregnant mice. Hypertension 2012;60(1):231-238.
12. Zawiejska, A., Wender-Ozegowska, E., Iciek, R., Brazert, J. Concentrations of endothelial nitric oxide synthase, angiotensin-converting enzyme, vascular endothelial growth factor and placental growth factor in maternal blood and maternal metabolic status in pregnancy complicated by hypertensive disorders. Journal of Human Hypertension 2014;28:670-676.
13. American College of Obstetricians and Gynecologists; Task Force on Hypertension in Pregnancy. Hypertension in pregnancy. Report of the American College of Obstetricians and Gynecologists' Task Force on Hypertension in Pregnancy. Obstet Gynecol 2013;122: 1122-1131.
14. Sánchez-Aranguren, L.C., Prada, C.E., Riaño-Medina, C.E., Lopez, M., Endothelial dysfunction and preeclampsia: role of oxidative stress. Frontiers in Physiology 2014 Oct;5(Article372):1-11.
15. Qian, J., Fulton, D. Post-translational regulation of endothelial nitric oxide synthase in vascular endothelium. Front Physiol 2013; 4:347. doi:10. 3389/fphys.2013.00347.
16. Kima, Y.J., Parkc, H.S., Leed, H.Y., Hac, E.H., Suhe, S.H., Ohf, S.K., *et al.,* Reduced L-arginine Level and Decreased Placental eNOS Activity in Preeclampsia. Placenta 2006);27:438-444.
17. Khalil, A.A., Tsikas, D., Akolekar, R., Jordan, J., Nicolaides, K.H. Asymmetric dimethylarginine, arginine

and homoarginine at 11-13weeks' gestation and preeclampsia: a case-control study. J Hum Hypertens 2013;27:38-43.

18. Savvidis, A., Daniilidis, A., Giannoulis, C., Tantanasis, T., Koiou, K., Zournatzi, V., *et al.*, Correlation between asymmetric dimethylarginine maternal plasma levels and preeclampsia. Clin Exp Obstet Gynecol 2011; 38:134-136.

19. Maranzana, E., Barbero, G., Falasca, A.I., Lenaz, G., Genova, M.L. Mitochondrial respiratory super complex association limits production of reactive oxygen species from complex I. Antioxid Redox Signal 2013;19:1469-1480.

20. Laskowska, M., Laskowska, K., Oleszczuk, J. The relation of maternal serum eNOS, NOSTRIN and ADMA levels with aetiopathogenesis of preeclampsia and/or intrauterine fetal growth restriction. J Matern Fetal Neonatal Med 2014; DOI:10.3109/14767058.2014.900036.

21. Laskowska, M., K. Laskowska, K., Oleszczuk, J. Pregnancy Hypertension: An International Journal of Women's Cardiovascular Health 2012;2:240-339.

22. Schiessl, B., Mylonas, I., Hantschmann, P., Kuhn, C., Schulze, S., Kunze, S., *et al.,* Expression of endothelial NO synthase, inducible NO synthase, and estrogen receptors alpha and beta in placental tissue of normal, preeclamptic, and intrauterine growth-restricted pregnancies. J Histochem Cytochem, 2005; 53(12): 1441-1449.

23. Sandrim, V.C., Palei, A.C., Metzger, I.F., Cavalli, R.C., Duarte, G., Tanus Santos, J.E. Interethnic differences in ADMA concentrations and negative association with nitric oxide formation in preeclampsia. Clin Chim Acta 2010; 411:1457-1460.

24. Yzydorczyk, C., Comte, B., Huyard, F., Cloutier, A., Germain, N., Bertagnolli, M., *et al.,* Developmental programming of eNOS uncoupling and enhanced vascular oxidative stress in adult rats after transient neonatal oxygen exposure. J Cardiovasc. Pharmacol 2013; 61: 8-16.

25. Dymara-Konopka,W., Laskowska, M. The Role of Nitric oxide, ADMA, and homocysteine in the etiopathogenesis of preeclampsia-Review. Int J Mol Sci 2019; 20:2757.