Potential Biochemical and Molecular Aspects in Pathophysiology of Preeclampsia: Assessment of Endothelial Nitric Oxide Synthase Gene in Preeclamptic Pakistani Women

By

Ghazala Shaheen

Department of animal sciences

Faculty of biological sciences

Quaid-i-azam university

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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Ghazala Shaheen

DEPARTMENT OF ANIMAL SCIENCES

FACULTY OF BIOLOGICAL SCIENCES

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2020

“In the Name of ALLAH, the most Beneficent, the most Merciful”

Dedicated to my Husband, In Laws, Parents, Grand Father (late)

and Family

For their endless love, patience, kindness and support

TABLE OF CONTENTS

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Ghazala Shaheen

LIST OF TABLES

LIST OF FIGURES

List of Abbreviations

ALP	Alkaline phosphatase
ALT	Alanine-aminotransferase
AP	Atheromatous plaque
aPTT	Activated partial thromboplastin time
AST	Aminotransferase
BMI	Body mass index
Ca	Calcium
CAT	Catalase
cDNA	Complementary DNA
CI	Confidence intervals
C-section	Caesarean section
DEPPD	N, N-Diethyl-p-phenylenediamine sulphate salt
Dl	Deciliter
EIA	Enzyme immunoassay
ELISA	Enzyme linked immune sorbent assay
eNOS	Endothelial nitric oxide synthase
EtBr	Ethidium bromide
$FeCl_3$	Ferric chloride

$FeSO_4$	Ferrous sulphate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
H_2O_2	Hydrogen peroxide
Hb	Haemoglobin
HCT	Haematocrit
HRP	Horse radish peroxide
INR	International normalizing ratio
IU/L	International unit per liter
IUGR	Intra uterine growth retardation
K	Potassium
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
MEIA	Microparticle enzyme immunoassay
mg/dl	Milligram per deciliter
Ml	Milliliter
mM	Milimolar
Na	Sodium
NAB	Nucleic acid builder

NADH	Nicotine adenine dinucleotide
nM	Nanometer
NO	Nitric oxide
NO_2^-)	Nitrite
NO_3^-	Nitrate
OR	Odds ratio
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PCV/HCT	Packed cell volume/hematocrit
PE	Preeclampsia
PIMS	Pakistan Institute of Medical Sciences
PLT	Platelets
POD	Peroxidase
PT	Prothrombin time
PVF	Perivillous fibrin
qRT-PCR	Quantitative real time polymerase chain reaction
RBC	Red blood cell
RDW-CV	Red blood cell distribution width
RMSD	Root mean square deviation

RMSF	Root mean square fluctuation
ROS	Reactive oxygen species
ROS	Reactive oxygen specie
RV	Reaction vessel
SK	Syncytial knots
SOD	Superoxide dimutase
STAT 3	Signal transducer and activator of transcription 3
STAT6	Signal transducer and activator of transcription 6
SVD	Spontaneous vaginal delivery
T_3	Triiodothyronine
T_4	Thyroxine
TBARs	Thiobarbituric acid reactive substances
TLC	Total leucocyte count
TMB	Tetra methyl benzidin
TSH	Thyroid stimulating hormone
U/L	Unit per liter
VP	Villous particles
WBC	White blood cell
χ^2	Chi-squared test

%	Percentage
µl	Microliter
µmol	Micro molar

GENERAL ABSTRACT

Background: Hypertensive disorders are a common complication of pregnancy that put women and their fetuses at disproportionate risk for further complications, as well as life-long sequelae. Preeclampsia (PE), in particular, is one of the most feared complications of pregnancy characterized by maternal endothelial cell dysfunction which causes symptoms including high blood pressure and proteinuria with maternal organ dysfunction and/or fetal growth restriction. Preeclampsia (PE) is one of the major causes of maternal and perinatal morbidity and mortality, particularly in resource-limited settings. Although the cause of PE remains largely unknown, the leading hypothesis strongly relies on disturbing placental function in early pregnancy. Assessment of hematological parameters, hormonal analysis, endothelial dysfunction, oxidative stress, and genetic screening is important for early prediction to allow close surveillance and preventive strategies. Unidentified genetic factors and vasodilation induced by impaired nitric oxide (NO) are thought to contribute to the syndrome's development. The endothelial nitric oxide synthase (eNOS) gene polymorphisms have an impact on NO production and were associated with hypertension and preeclampsia.

Objectives: Current study was designed for the comprehensive assessment of biochemical and molecular risk factor in the susceptibility of PE in the Pakistani population. The objectives of the study include:

- Assessment of demographic, clinical and biological markers in the pathophysiology of preeclampsia in Pakistani women.
- To describe the potent role of oxidative stress markers in susceptibility to preeclampsia in Pakistan.
- Evaluation of histomorphological and histomorphometric changes of the placenta in preeclamptic Pakistani women.
- Determination of NO and epinephrine levels in both normal and PE patients.
- To analyze the placental localization and intensity of eNOS staining in normal and PE patients.
- Estimation of mRNA expression of eNOS in the placentas of both normal and PE patients.
- Screening of the mutations and polymorphisms in the DNA sequence of the *eNOS* gene in both normal and PE patients.

- Computational analysis of identified variants in the coding and non-coding region of the *eNOS* gene.

Materials and methods: Total 400 blood (PE/controls=200), 400 urine (PE/controls=200), and 100 placental tissue samples (PE/controls=50) were recruited. History and samples were collected from each subject with informed consent after diagnosis for hematological or biochemical and molecular analysis. Biochemical analysis of blood and urine routine examination was done. Histomorphological and histomorphometric analysis were performed for placental tissues. Reactive oxygen species (ROS), thiobarbituric acid-reactive substances (TBARS), superoxide dismutase (SOD), catalase (CAT) and guaiacol peroxidase (POD) levels were analyzed through a spectrophotometer. Nitric oxide (NO), epinephrine, triiodothyronine (T_3), thyroxine (T_4) and thyroid stimulating hormone (TSH) levels were determined through spectrophotometer and enzyme linked immunosorbent assay (ELISA).

Immunohistochemistry and quantitative real-time polymerase chain reaction (qRT-PCR) was done to estimate the localization and expression of eNOS in placentas of PE patients and healthy pregnant women. *eNOS* gene variants were screened in PE patients and control group by genotyping and sequencing. Further *in silico* studies were performed to get insights into the structural and functional impact of identifies mutation on eNOS protein as well as on protein regulation. Independent sample t-test, Chi-squared test (χ^2) and Odds Ratio (OR), Hardy-Weinberg equilibrium model and Pearson's correlation was determined using IBM SPSS Statistics 21 software and package of R 3.5.1 (R Development Core Team, 2018).

Results: Experimental finding showed that several parameters including gestational age ($p < 0.001$) and weight of the child ($p < 0.001$) were significantly reduced while age (p 0.002), BMI ($p=0.047$) systolic blood pressure (SBP) and diastolic blood pressure (DBP) ($p < 0.001$) were significantly elevated in PE patients as compared to control group. Pregnancy history including headache (OR: 2.42), swelling in hands and face (OR: 4.8), excessive weight gain (OR: 3.74) urination problem (OR: 2.90), abdominal pain (OR: 1.45), shortness of breath (OR: 4.49), muscular pain (OR: 1.64) and blurring of vision (OR: 2.03), history of preeclampsia in previous pregnancy and in family (OR: 7.38; OR: 8.43) were more obvious in preeclamptic women. Significantly ($p<0.001$) elevated levels of alkaline phosphatase, serum urea, uric acid, urine proteins, total leucocyte count (TLC) ($p=0.028$) and haematocrit ($p=0.016$) were reported in PE group.

Prolonged Activated partial thromboplastin time (aPTT), prothrombin time (PT) and international normalizing ratio (INR) were recorded in both PE groups with a decrease in platelets and fibrinogen levels as compared to controls. While, total bilirubin ($p=0.019$), aspartate aminotransferase (AST) ($p=0.012$), serum calcium ($p=0.002$) and sodium ($p= 0.010$) concentrations were reduced in case patients as compared to control group.

Significantly increased concentrations of ROS ($p<0.001$) and TBARS ($p=0.04$) were determined in preeclamptic patients while non-significant difference was observed in POD ($p=0.11$), SOD ($p=0.97$) and CAT ($p=0.81$) levels among both groups. Histological changes determined abnormal villi, more syncytial knots (SK) and a significant decrease in elongated and large villi in PE placentas as compared to normal placentas.

Reduced NO ($p=0.016$), eNOS immunoreactivity ($p\leq0.001$) and mRNA abundance ($p\leq0.001$) was observed in preeclamptic group as compared to control group. Thyroid hormones demonstrated non-significant alterations in both groups. Significant positive correlation was observed in creatinine and urea levels of PE patients while SBP and DBP showed significant positive correlation with each other and with age.

The frequency of c.894T (p.298Asp) was high in the PE group ($p\leq0.001$). Likewise, a significant association of g.-786C alleles was found with the PE group ($p=0.007$) when compared to normotensive women. In addition, a novel homozygous variant g.2051G>A was also significantly associated with PE ($P=0.02$) when compared to normotensive women. Dynamic simulation studies revealed that Glu298Asp mutation destabilizes the protein molecule and decrease the overall stability of eNOS protein. Molecular docking analysis of the mutant promoter with transcription factors signal transducer and activator of transcription 3 (STAT 3) and signal transducer and activator of transcription 6 (STAT6) proposed changes in protein regulation upon these reported mutations in the upstream region of the gene.

Conclusion: Considering the results of the current study, it is concluded that present study reveals the importance of demographic and clinical risk factors in early diagnosis of PE, and should be considered in the counselling, diagnostic and therapeutic interventions to improve the reproductive health of women in Pakistan. Furthermore, oxidative stress elevated epinephrine, reduced NO, reduced placental expression of

eNOS and the functional alterations induced by the variants of *eNOS* gene results in endothelial dysfunction and histological placental alterations leading to PE in Pakistani pregnant women. These variations lead to blood pressure elevation and renal insufficiency in preeclamptic patients. However, further studies and meta-analysis are necessary to validate these findings and clarify this issue to prevent maternal and neonatal morbidity and mortality in Pakistan.

GENERAL INTRODUCTION

Introduction

Reproductive Health is the focal point for issues related to maternal and neonatal health (Gunderson *et al.,* 2018). Improving maternal health is one of the 17 Sustainable-Development-Goals (SDGs) adopted by the international community. Under SDG3 which addresses "ensure healthy lives and promote well-being for all at all ages" maternal health is the foremost priority (Norheim *et al.,* 2015). Since 1990, maternal mortality has dropped by nearly 50 percent. However, the percentage of maternal mortality, the proportion of women who do not survive childbirth compared to those in developing areas, remains 14 times greater than in developed regions (Patton *et al.,* 2009).

In developing nations, supreme maternal fatalities (99%) happen. One third of these deaths occur in South Asia and sub-Saharan Africa is facing more than half of these deaths (Sharma *et al.,* 2018). In developing nations, the maternal mortality ratio is 240 per 100 000 births compared to 16 per 100 000 in developed countries (Norheim *et al.,* 2015). There are significant disparities between regions, with some countries having exceptionally elevated maternal mortality rates of 1000 per 100 000 live births (Pasha *et al.,* 2018). Complicated pregnancy and problems in childbirth are the foremost reason of maternal and neonatal deaths in developing nations. Pregnancy related complications are common but various complications arise following pregnancy and childbirth. Some starts before pregnancy but are worsened during pregnancy (Noh *et al.,* 2019).

Hypertensive pregnancy disorders

Hypertensive disorders of pregnancy (HDPs) complicate approximately 12 - 22% of all pregnancies and remain the main source for maternal and pre-natal mortality and morbidity (Al-Ansary *et al.,* 2013; Gillon *et al.,* 2014). These disorders mainly include; Gestational hypertension which occurs during pregnancy after 20 weeks of gestation without the involvement of multisystem and returns to normal within 12 weeks postpartum; Chronic hypertension which can be characterized as pre-existing hypertension having blood pressure 140/90 mmHg (Gillon *et al.,* 2014). It is mostly diagnosed before 20 weeks of gestation or before pregnancy contrary to gestational hypertension and it persists for 12 weeks postpartum; Chronic hypertension with superimposed preeclampsia and it can be characterized as hypertension which develops

most of the symptoms or systematic features of preeclamptic syndrome like thrombocytopenia, new-onset proteinuria etc; preeclampsia syndrome occurring in first pregnancy and; preeclampsia syndrome occurring in an ensuing pregnancy as well as repeating with underlying susceptibility state (Shah, 2005; Hutcheon *et al.*, 2011).

Hypertensive issues of pregnancy comprise of a confounding as well as a clinically complex group of pregnancy intricacies which cause poor health and disease outcome in developing as well as in developed countries (Hutcheon *et al.*, 2011). However, the composition of hypertensive issues during pregnancy may have changed throughout the years (Al-Ansary *et al.*, 2013). Deferring childbearing and increasing weight as a social trend may have increased the rate of hypertension; while assertive obstetric intercession and treatment may have lessened the number of extreme cases and maternal morbidity (Gillon *et al.*, 2014). Though, the HDPs are the main source of maternal and prenatal mortality and morbidity around the world, it is foreseen that this situation will just decline, given the intensifying predominance of higher body mass and metabolic disorder among women of childbearing age (Zhang *et al.*, 2003; Gillon *et al.*, 2014). Gestational hypertension, comprises of preeclampsia and eclampsia, is accountable for 70% of cases (Rathore *et al.*, 2010).

Preeclampsia (PE)

PE is a pregnancy-related syndrome and is characterized by high blood pressure and onset of proteinuria usually after the 20^{th} week of gestation, while according to revised definition of PE new onset of hypertension in the absence of proteinuria but combined with renal insufficiency, impaired liver function, haematological complications and neurological symptoms also fulfil diagnostic criteria for PE (Kallela *et al.*, 2016). It complicates 3%–8% of pregnancies worldwide resulting in morbidity and mortality at rate of 10%-15% (Balogun and Sibai, 2017).

In the late 19th century it was documented that eclampsia, which was considered a convulsive disorder of pregnancy for decades, was headed by maternal hypertension and proteinuria, and named as PE (Chesley *et al.*, 1978). Between 1990 and 1999 prevalence of preeclampsia increased by 40% around the world particularly in developed countries (Ventura SJ *et al.*, 2001). Incidence of PE is summarized in figure 1 (Dahlstrøm *et al.*, 2006; Serrano, 2006; Hernández-Díaz *et al.*, 2009; Thornton *et al.*, 2013; Magee *et al.*, 2019). Prevalence of PE is higher in developing countries like

Pakistan where PE and eclampsia was around 19% and 1 in 89 women dies of maternal causes (Shamsi *et al.,* 2010; Naseeb *et al.,* 2015). Worldwide, estimated 76,000 maternal and 500,000 fetal and newborn fatalities happen annually as a result of PE (Duley, 2009; Carty *et al.,* 2010; Khowaja *et al.,* 2016).

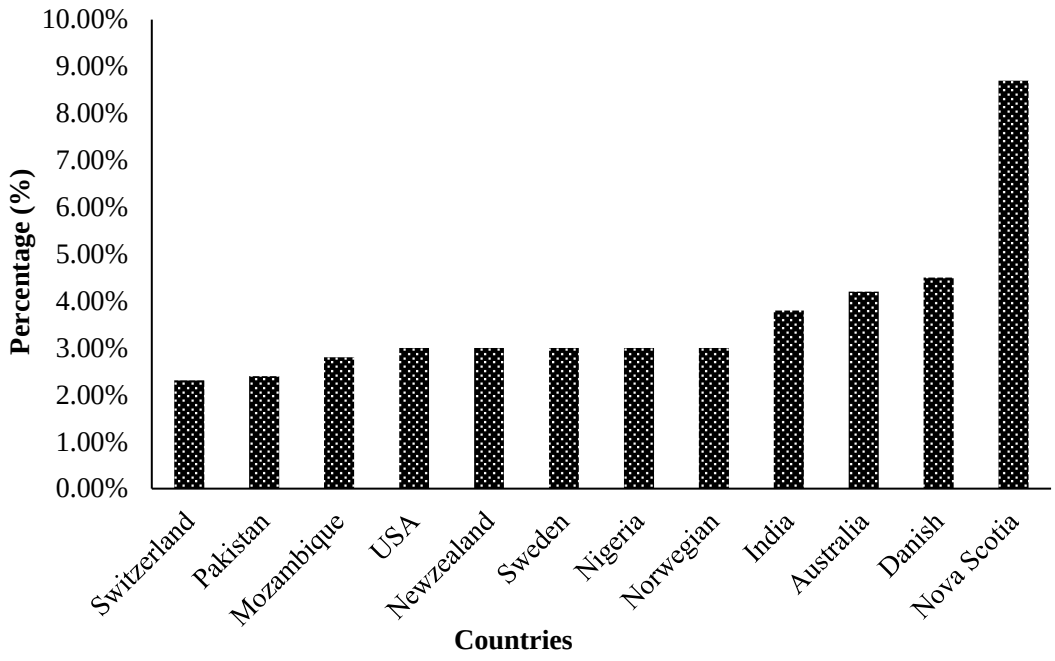


Figure 1: Incidence of preeclampsia (PE) around the world (Magee *et al*., 2019).

Epidemiology

PE is more common in women with first pregnancy. It has been evident that normal feto-maternal transfusion that takes place during gestation or predominantly during delivery, the mother exposes to product of the fetal genome, protecting her from subsequent pregnancies (de Souza Rugolo *et al.,* 2011). This protective effect of pregnancy lost with the child of women with new partner. There is also maternal predisposition to PE because risk of PE increases three to four-fold in the first degree relatives of affected women (Carty *et al.,* 2010). There are numerous pregnancy-specific factors and general medical conditions that predispose to the onset of PE (Roberts and Bell, 2013). Risk factor for PE are summarized in figure 2.

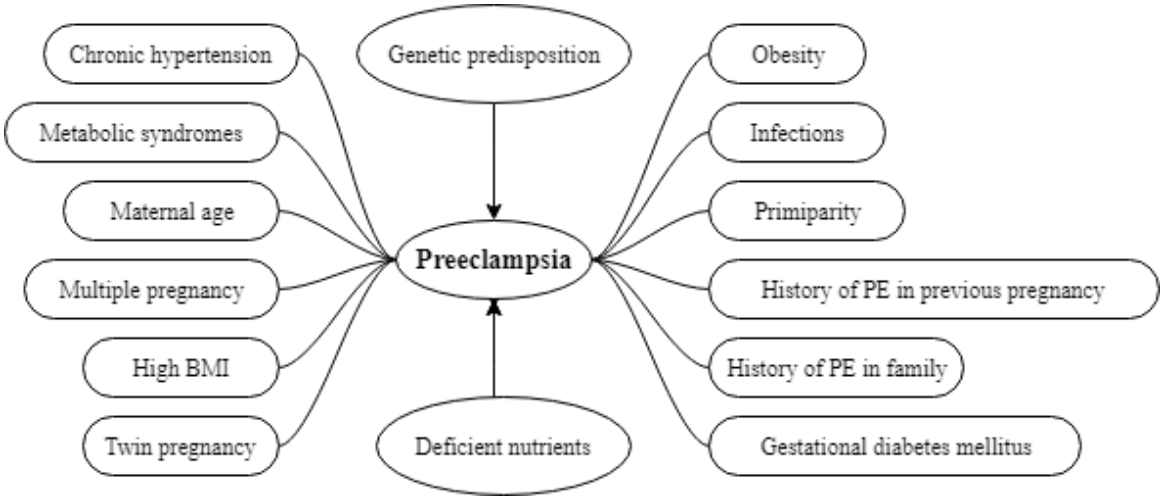


Figure 2: Risk factors for preeclampsia (PE).

Clinical presentation

The classic symptoms of PE include headache, epigastric pain, visual disturbance and seizures in severe cases (Young and Karumanchi, 2016). Clinical examination includes complete neurological and obstetric examination and hypertension which is often the first sign but sometimes absent or temporary until the late phase of the disorder (Mol *et al.,* 2016; August and Sibai, 2017). Proteinuria is the predominant symptom in which urinary proteins are tested in early diagnosis (Myatt and Roberts, 2015). Dependent feet oedema is prevalent during ordinary pregnancy, but rapidly progressive face and hand oedema may suggest PE (Figure 3) (Portelli and Baron, 2018). Epigastric tenderness is a worrying indication and suggest involvement of liver (Steegers *et al.,* 2010). Neurological examination may disclose clonus and hyperflexia in severe cases. PE is heterogeneous disorder effecting multiple organ system (Eiland *et al.,* 2012). Highly variable severity of clinical presentation of PE is present, outcomes are usually favorable in mild PE cases when it develops after 36 weeks of gestation (English *et al.,* 2015). Significant increase in the risk of adverse maternal and neonatal outcome occurs if the PE develops before 33 weeks of gestation (Duckitt and Harrington, 2005).

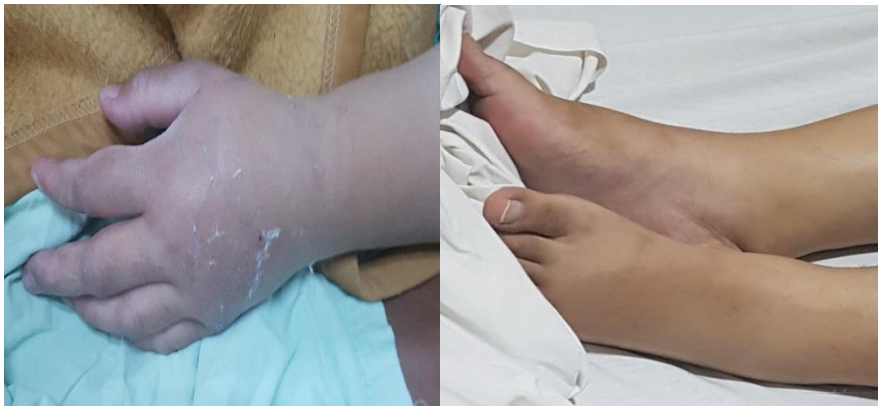


Figure 3: Oedema of hands and feet in preeclamptic patient.

Sub-Classification

PE arise from multiple pathways, it varies from evanescent disease of PE at term to the severe disease commonly evolve remote from term (Chaiworapongsa et al., 2014). PE is further subdivided into early and late-onset preeclampsia (Obstetricians and Gynecologists, 2013). It has been proposed that early onset of PE that occurs before 34 weeks and late onset of PE which occurs after 34 weeks have varied etiologies and in this way an alternate clinical expression, but still it is a subject of significant research (English *et al.*, 2015). The late onset of preeclampsia contains over 80% of all preeclampsia cases around the world. A large portion of these late beginning cases are related with normally developed child without any indications of any growth limitation, an ordinary or slight adjustment of the uterine spiral arteries behavior, No alterations in umbilical arteries' blood surge and elevated hazard for women pregnant with increased mass of placenta or surface (iron deficiency, diabetes, high elevation, multiple pregnancies etc (Myatt and Roberts, 2015).

The early onset of PE contains a small portion of all preeclampsia cases (5% to 20%, relying upon the statistics), however, includes the most extreme cases of particular clinical importance (Parra-Cordero et al., 2013). The normal features of this kind of preeclampsia included, an insufficient and inadequate trophoblast intrusion of maternal spiral arteries, alterations of blood flow in the spiral arteries as well as the uterine arteries of the placenta, one of the main reason for abnormal blood flow in the umbilical arteries can be due to high resistance in the peripheral region of placental vessels and clear indications of a fetal growth restriction (Huppertz, 2008)

PE can be classified as mild preeclampsia, severe preeclampsia and eclampsia. Mild PE has systolic (SBP) and diastolic blood pressure (DBP) of 140 mmHg/90 mmHg respectively, the existence of proteinuria exceeding a 300mg/24 hour, and protein ratio of urine: creatinine>0.3 or 30mg/dl in a random urine sample (1+ reaction on Dipstick) (Kallela *et al.,* 2016). Severe PE can be characterized as having blood pressure of 160/110 mmHg, proteinuria > 3+, irregular liver proteins in association with cerebral pain or visual disorders or upper stomach pain or presence of thrombocytopenia (platelet tally under 100,000/mm3), the rise in the serum of creatinine and transaminase, pneumonic oedema, seizures, oliguria (under 500 mL for every 24-hour time frame) (Sibai, 2003; González-Garrido *et al.,* 2017). Eclampsia can be characterized by the existence of seizures (González-Garrido *et al.,* 2017).

Etiology and pathophysiology

PE is specific to pregnancy but has also been reported in molar pregnancies (pregnancies lacking a fetus) and in ectopic pregnancies suggesting that presence of trophoblast tissue provide the stimulus for the onset of this disorder (Sasaki, 2003; Goldstein *et al.,* 2020). Placental bed biopsies have revealed that patchy trophoblast invasion occurs in PE and muscular walls of the spiral arteries retains (Fisher, 2015). This can prevent elevated blood flow and low uteroplacental circulation impedance. Less effective invasion of trophoblast is not known in these pregnancies but maternal immune systems may adapt abnormally under such conditions (Johnsen *et al.,* 2019).

It has been demonstrated that defective trophoblastic invasion leads to relative under-perfusion of the placenta which releases many factors in the maternal circulations that target the vascular endothelium (Carter *et al.,* 2015). The nature of these variables has not been accurately described, although various factors have been suggested, including a variety of cytokines, growth factors and oxidative stress products generated by placenta hypoxic reperfusion injury (Mol *et al.,* 2016).

As the target cells of the disorder process, the vascular endothelial cells are so prevalent, so PE is truly multisystem disease that affects various organs, often simultaneously including the vascular system, liver, kidney and brain (Davison *et al.,* 2004). Offspring's of preeclamptic mothers have high blood pressure, coronary heart disease, epilepsy, metabolic disorders, and other complications in childhood and adolescence (Ukah *et al.,* 2018b).

Placenta and PE

Human placenta is a discoid structure and interface between mother and fetus (Burton and Jauniaux, 2015) (Figure 4). No organ can match the placenta for its astonishing functions because it performs all major organ systems functions which differentiate and mature in fetus (Wooding and Burton, 2008). Interestingly, problems linked with PE cease after delivery, demonstrating that placenta is the reason of the onset of PE (Redman and Sargent, 2003).

After 21st day of conception vascularization of placental villi begins and syncytiotrophoblast do infiltrate between the uterine epithelial cell (Widdows, 2009). During first and second trimester of pregnancy, placental villi began to proliferate and differentiate from the cells of trophoblastic lineage into anchoring villi and floating villi considered as uteroplacental villi and fetoplacental villi. Each villous comprises of outer trophoblastic epithelial layer, fetal vascular endothelium and stromal cells (Serov *et al.*, 2015). Villous growth begins to change from branching to nonbranching angiogenesis after 26 weeks of gestation. In the third trimester these villi change into terminal villi, important structures for feto-maternal nutrients and gaseous exchange(Widdows, 2009). Maternal and fetal circulation takes place through these important structures, in case of poor vascularization the fetus is at high risk of hypoxia and low birth weight (Avagliano *et al.*, 2016).

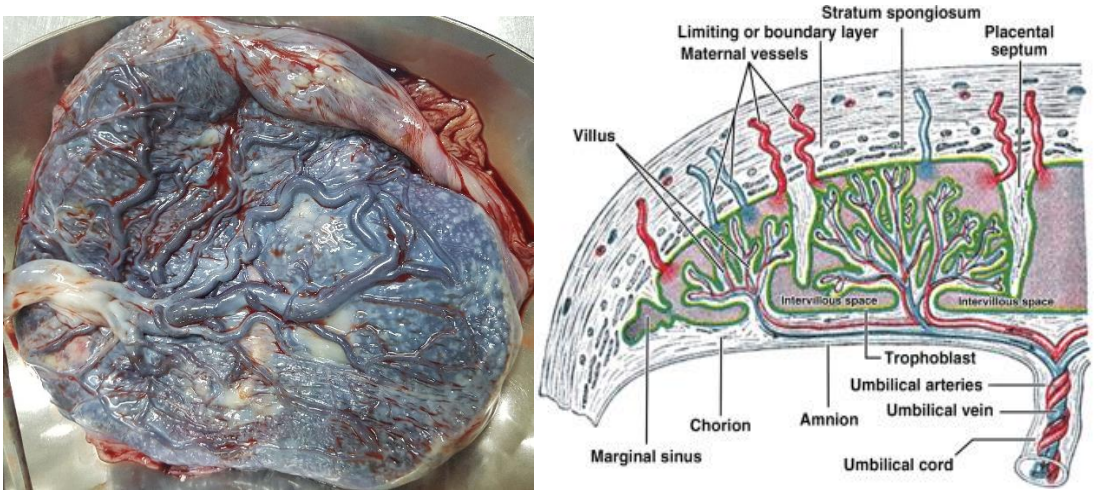


Figure 4: Anatomy of human placenta (Serov *et al.*, 2015)**.**

PE is the disorder of theories, many theories propose that the development of PE is associated with amendments in trophoblastic invasion, intolerance immunological factors between maternal and feto placental tissue, inflammatory alterations in

pregnancy and genetic variations (Clifton *et al.,* 2012). The most accepted theory about PE describes its two stages; stage1 is characterized by reduced placental perfusion while stage 2 consists of multisystem maternal syndrome (Christina *et al.,* 2006) (Figure 5).

There is evidence that reduce flow of blood from maternal side towards placenta exists in PE. This concept is supported and proved by experimentation on animals in literature (McCarthy *et al.,* 2011; Roberts and Bell, 2013; August and Sibai, 2017). Hypertension and diabetes are the main medical conditions which can also increase the risk of PE (Aardema *et al.,* 2001). Reduced blood circulation leads to reduced perfusion of placenta which is associated with onset of PE. Maternal spiral arteries failed to remodel and perfuse in the intervillous spaces in case of PE (Avagliano *et al.,* 2016). In normal pregnancy striking changes occurs during the placental formation in which diameter of the terminal arteries increase with loss of smooth muscles while, in PE this remolding is not complete, vessels are not dilated as in normal pregnancy with the presence of smooth muscles resulting in neural modification of vascular tone (Figure 6) (Bellomo, 2018).

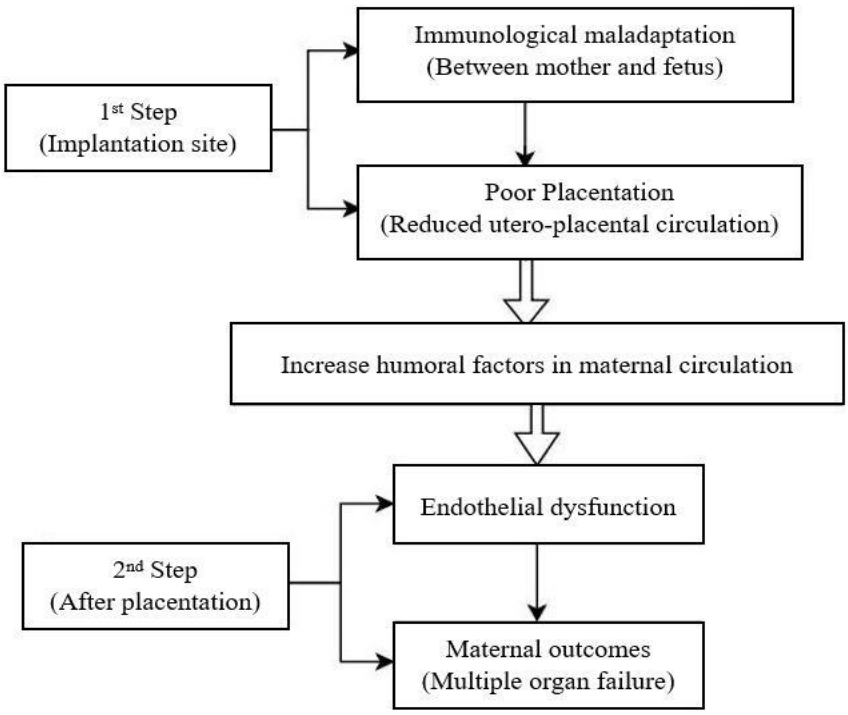


Figure 5: Two step theory in the pathogenesis of preeclampsia (PE) (Matsubara *et al.,* 2015).

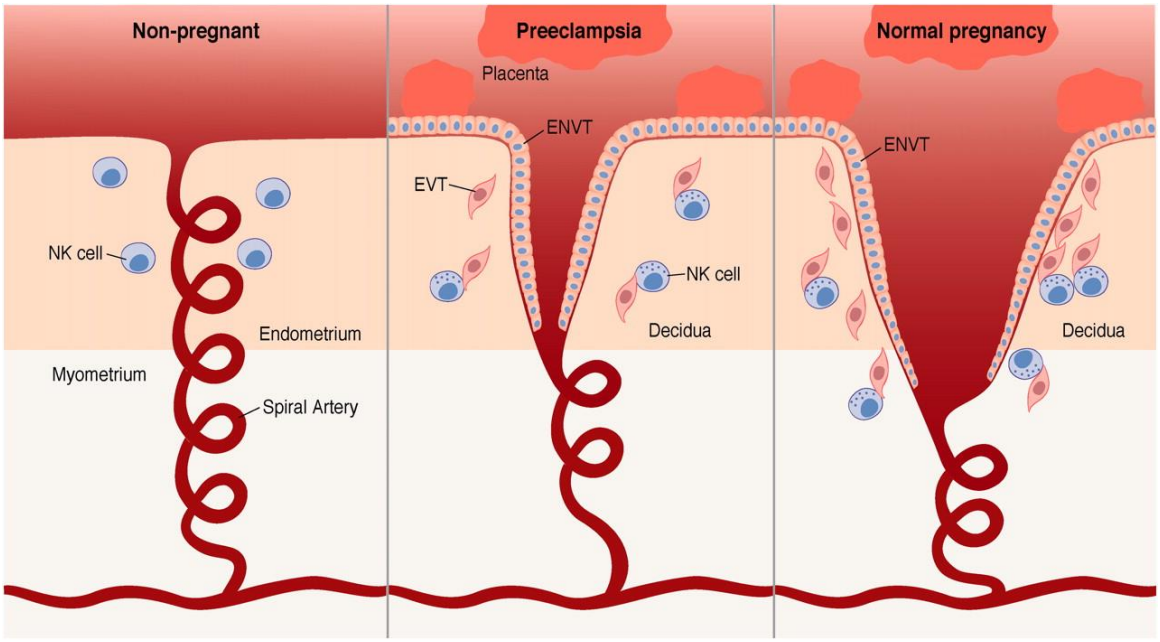


Figure 6: Spiral artery remodeling during preeclampsia, normal pregnancy and in the absence of pregnancy (Bellomo, 2018)**.**

PE is characterized by abnormal maternal spiral artery remodeling due an impaired invasion of fetal trophoblasts leading to reduced blood flow towards placenta, which disturbs the fetal and placental oxygen and nutritional need (Barker *et al.,* 2010). The mother develops hypertension at the end of the second or third trimester of gestation to compensate for the blood flow deficiency towards placenta. Interestingly, problems linked with PE cease after delivery, demonstrating that placenta is the reason of the onset of PE (Redman and Sargent, 2005).

Oxidative stress and PE

Oxidative stress plays a significant role in the pathogenesis of PE in which key humoral factors are generated by poor perfused placenta (Matsubara *et al.,* 2015). Oxidative stress is the imbalance between antioxidants defence mechanism and formation of oxidative substances such as reactive oxygen species (ROS) and peroxides (Touyz, 2004). Antioxidants are the agents that inhibit oxidation of biomolecules such as DNA, proteins and lipids preventing oxidative damage (Matsubara *et al.,* 2015). In case of impairment of this antioxidant system impaired placentation could be initiated (Williams and Pipkin, 2011). In normal conditions nuclear membrane, endoplasmic reticulum and mitochondria generate anions as byproduct of auto-oxidation (Bhat *et al.*,

2019). ROS are also produced through various sources such as NADPH oxidase (NOX) and metabolism of arachidonic acid. Endothelial nitric oxide synthase (eNOS) can produce $O_2^{\bullet-}$ and H_2O_2 specially when its substrate L-arginine concentration reduces, results in loss of vasodilation due to reduced nitric oxide (NO) production and increase oxidative stress due to generation of $O_2^{\bullet-}$ ion (Aouache *et al.,* 2018). Oxidative stress can also cause elevation in thiobarbituric acid reactive substances (TBARS), a product of lipid peroxidation (Bhat *et al.,* 2019). Oxidative stress can also be caused due to reduced antioxidant enzyme activity. Antioxidant enzymes comprises of super oxide dismutase (SOD), catalase (CAT), guacol peroxidase (POD) and glutathione peroxidase (GPX), they metabolize ROS into innocuous byproducts (Mannaerts *et al.,* 2018). In the placental-umbilical unit eNOS is expressed in the microvasculature and microvasculature of umbilical cord (UC) and placenta (Bailey *et al.,* 2017). NO is responsible for maintenance of vascular homeostasis for better uterine blood flow. In case of elevated ROS levels or oxidative stress placental endothelial function disrupt and it oxidize the cofactor of eNOS results in the production of $O_2^{\bullet-}$ ions instead of NO. $O_2^{\bullet-}$ ions induce the production of per oxynitrate ($ONOO^-$) by reacting with H_2O as represented in figure 7 (Chiarello *et al.,* 2018).

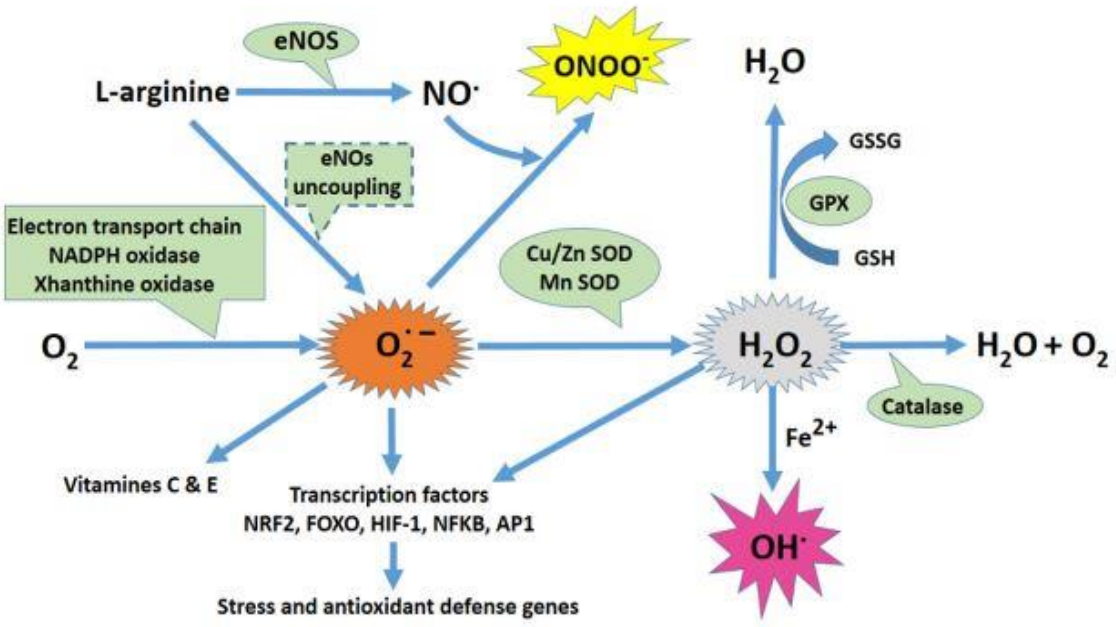


Figure 7: Schematic representation of oxidative stress induction in PE (Aouache *et al.,* 2018).

Hormones and PE

Pregnancy is associated with many hormonal changes. There is unknown mechanism behind preeclamptic hypertension. Pregnancy related stress derived from pregnancy such as fear of giving birth, lack of support from friends and family and unplanned pregnancy and sympathetic nervous system has been suggested to be involved in PE onset , also effect maternal and neonatal health through various mechanisms (Øian *et al.*, 1984; Zhang *et al.*, 2013). Catecholamines such as epinephrine released by hypothalamic adrenal axis during stress may interfere with the vasodilator action of NO leading to endothelium impairment (Toda and Nakanishi-Toda, 2011). As a result, physiological changes in maternal vessels do not complete normally. Therefore, responsiveness of these vessels increased to vasoconstrictors such as epinephrine or angiotensin (Van der Graaf *et al.*, 2013).

Excess or deficiency of maternal thyroid hormones can also influence on reproductive health of women and fetal outcomes at all stages of gravidity. These hormones can also interfere with ovulation and fertility (Sardana *et al.*, 2009). Function of thyroid that are unique to pregnancy has been reported to be altered in numerous conditions (Khadem *et al.*, 2012). The mechanism of hypothyroidism and its clinical significance is controversial in PE, while, increased levels of endothelin and decreased protein concentrations in plasma may be related to this condition (Harshvardhan *et al.*, 2017). Hypothyroidism can leads to contraction of vascular smooth muscle both in renal and systemic vessels, which results in increased diastolic hypertension, decreased tissue perfusion and peripheral vascular resistance (Satyanarayan *et al.*, 2015). Proteinuria can also be associated with thyroid dysfunction, which results in increased excretion of thyroid-binding globulins and thyroxine (Negro and Mestman, 2011). One of the pathophysiologic causes of PE has been suggested to be the physiological changes in thyroid gland in all stages of pregnancy (Muraleedharan and Janardhanan, 2017).

Role of *eNOS* gene in PE

Various mediators are involved in controlling endothelial dysfunction in PE but the role of *eNOS* gene appears most significant in the development of PE (Devendran *et al.*, 2015). NO is synthesized by the NOS family of enzymes which consist in three isoforms: nNOS or neuronal isoform, eNOS which is endothelial NOS and iNOS, the inducible (Bernatchez *et al.*, 2012). NO is formed from the reduction of L-arginine to

L-citruline by the action of eNOS (Palmer *et al.,* 1988; Moncada *et al.,* 1991; Moncada and Higgs, 1993). *eNOS* gene is located at the 7q36.1 region as shown in figure 8.

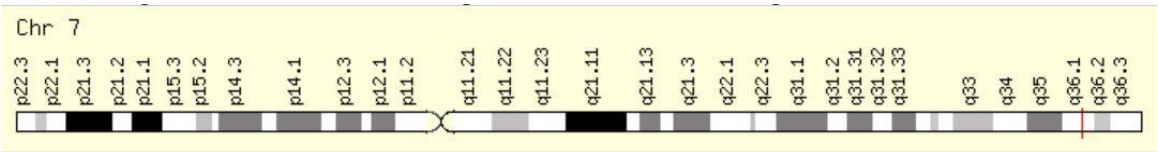


Figure 8: Assembly of chromosome 7 having location of *eNOS* gene at q36.1 region (Ensemble, 2019).

NO is a low molecular weight mediator and highly reactive free radical, it mediates function of endothelium by regulating platelet aggregation, vascular tone, smooth muscle cells development and leukocyte adhesion (Figure 9) (Qian and Fulton, 2013). Endothelial dysfunction is defined as the decreased bioavailability of NO in many ways such as through reduced production or increased consumption by oxidative stress (Dias *et al.,* 2018). It has been reported that women with PE have decreased concentrations of NO both in plasma and in the placenta, it is postulated that reduced concentrations of NO might effect on uteroplacental blood flow through lack of vasodilatory effect, otherwise the the role of NO in the pathophysiology of PE has not been well defined (Brosens *et al.,* 2002; Dikensoy *et al.,* 2009).

Methylated amino acids can also be produced by endothelial cells, such as asymmetric dimethylarginine, an endogenous competitive inhibitor of NO synthase, which has been found to be elevated in women with PE (Nishizawa *et al.,* 2009). In placenta, cytotrophoblast is connected with eNOS expression to differentiate into syncytotrophoblast (Eis *et al.,* 1995). In addition *eNOS* is an important vascular tone regulator and play an crucial role in reduction of the uteroplacental resistance during normal pregnancy. That's why *eNOS* gene has emerged as a logical candidate gene in the development of preeclampsia (Magness *et al.,* 1997; Norris *et al.,* 1999; Xu *et al.,* 2019).

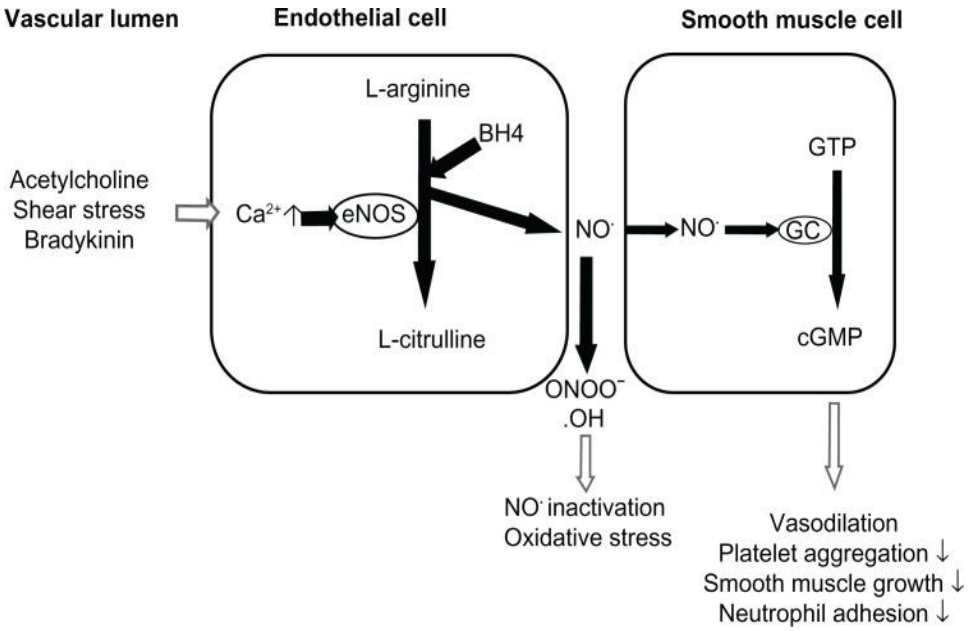


Figure 9: Role of nitric oxide (NO) in regulation of endothelial function (Williams and Morgan, 2012).

Three clinically important polymorphisms in the eNOS gene have been widely investigated: a variant in the gene promoter area: T to C conversion at position 786 (Fatini et al., 2006; Aggarwal et al., 2010; Sandrim et al., 2010), in intron 4(4b/a) a variant variable number of 27 bp tandem repeats (Tempfer et al., 2001; Zeng et al., 2016), exon 7 variant G to T replacement at nucleotide position 894 resulting in codon 298 replacement of glutamic acid (Glu) with aspartic acid (Asp) (Yoshimura et al., 2000; Hillermann et al., 2005; Chen et al., 2007; Maria Procopciuc et al., 2018). Conflicting and contentious findings among various population-based research have been documented, however, among the several agents that control vascular dysfunction in PE, the most important appears to be the altered synthesis of nitric oxide (NO) arising from particular mutations in the eNOS gene. However, the precise mechanism by which altered NO synthesis affects vascular growth and placental function remains uncertain (Krause et al., 2011; Pisaneschi et al., 2012).

Management of PE

The only effective treatment available for PE is early delivery to avoid serious maternal complications before 34 weeks of gestation, but this approach may involves serious neonatal damage (Pennington *et al.,* 2012; Lisonkova and Joseph, 2013). Adequate prenatal care is required in this condition. The management of PE depends on its severity review, assessment and admission to the hospital. Risk reduction for women at risk of PE needs a series of strategies such as standardized assessment and surveillance, avoidance and management of blood pressure through various hypertensive drugs such as Labetalol which is alpha and beta blocking agent and Nifedipine which is calcium channel blocker (Duro-Gómez *et al.,* 2017), prevention and treatment of seizures if predicted by the use of drug of choice which id magnesium sulphate which reduce the convulsion (Ueda *et al.,* 2016; Adrianes Bachnas *et al.,* 2019), and aggressive rehydration should be avoided in women with severe PE (Steegers *et al.,* 2010).

Precisely predicting high-risk women to develop PE will facilitate targeting increased antenatal observation while allowing low-risk women to participate in community-based antenatal care (Ukah *et al.,* 2018a). In addition, novel therapeutic preventive interventions can be developed through predictive tests. Unfortunately, at present there is no screening test for PE (Magee *et al.,* 2014). In spite of intensive research on PE, No single blood biomarker has been found to have adequate sensitivity and specificity to be clinically helpful either alone or in conjunction with other biomarkers or clinical information (Wu *et al.,* 2015).

In pregnancy with incomplete trophoblastic remodeling of spiral arteries doppler ultrasound uterine artery waveform analysis can help to identify the risk of PE (Khong *et al.,* 2015). This form of screening test could play a part in pregnant females who have already been recognized as at risk of PE due to medical or post-obstetric history (Khong *et al.,* 2015). However, it is not of valuable for screening low risk women.

Established preventive intervention includes low-dose aspirin, which modestly decreases the risk of PE in females at high risk, and additional calcium may also decrease the risk, but only in females with nutritional consumption (Mohammad-Alizadeh-Charandabi *et al.,* 2015; Szymański *et al.,* 2015). Despite promoting preliminary research, it now seems certain that vitamin C and E will not reduce the danger of PE (Mohammad-Alizadeh-Charandabi *et al.,* 2015).

Severe PE often required premature delivery of the fetus often by Caesarean section (C-section). Post-natally, proteinuria and blood pressure will resolve, but in minority of cases due to the existence of renal disease or underlying chronic hypertension one or both persist beyond 6 weeks (Magee *et al.*, 2014). In addition, careful post-natal search for underlying medical disorders in females who develop severe PE before 34 weeks of pregnancy should be undertaken (Nathan *et al.,* 2018). Further research is crucial for the identification of causes and mechanisms involved in the pathogenesis of PE to prevent maternal and neonatal morbidity and mortality.

Based on the above literature review, the current study was designed for the assessment of demographic, biochemical and molecular risk factors in the onset of PE. Considering the potent role of polymorphism and mutations in eNOS gene in causing clinical symptoms of PE, the present study was directed to study the localization, expression and role of eNOS gene variants in PE patients and normal healthy women in Pakistani population to improve the reproductive health of women. Moreover, information gathered from current data could be used by doctors/clinical practitioners and researchers which could be of great benefaction for society to improve the management of women destined to develop PE.

Aims and objectives

As PE is poorly understood pregnancy complication and there is no gold standard for the diagnosis of PE. There is the constant search of prognostic factors to predict the progression and severity of the disease. Various clinical, epidemiological and genetic risk factors should be identified before it threatens both maternal and fetal survival. Pakistan is an economically developing country in Asia, studies have been conducted on PE but there are many gaps to be filled. It is important to identify the women who are more likely to be at high risk of developing PE so that they can benefit from intervention. Therefore, the present study was designed to prospectively evaluate the predictive role of several demographic, clinical, biological and genetic markers in the pathophysiology of preeclampsia in Pakistani women.

The objectives of the study include:

- Assessment of demographic, clinical and biological markers in the pathophysiology of preeclampsia in Pakistani women.
- To describe the potent role of oxidative stress markers in susceptibility to preeclampsia in Pakistan.
- Evaluation of histomorphological and histomorphometric changes of the placenta in preeclamptic Pakistani women.
- Determination of NO and epinephrine levels in both normal and PE patients.
- To analyze the placental localization and intensity of eNOS staining in normal and PE patients.
- Estimation of mRNA expression of eNOS in the placentas of both normal and PE patients.
- Screening of the mutations and polymorphisms in the DNA sequence of the *eNOS* gene in both normal and PE patients.
- Computational analysis of identified variants in the coding and non-coding region of the *eNOS* gene.

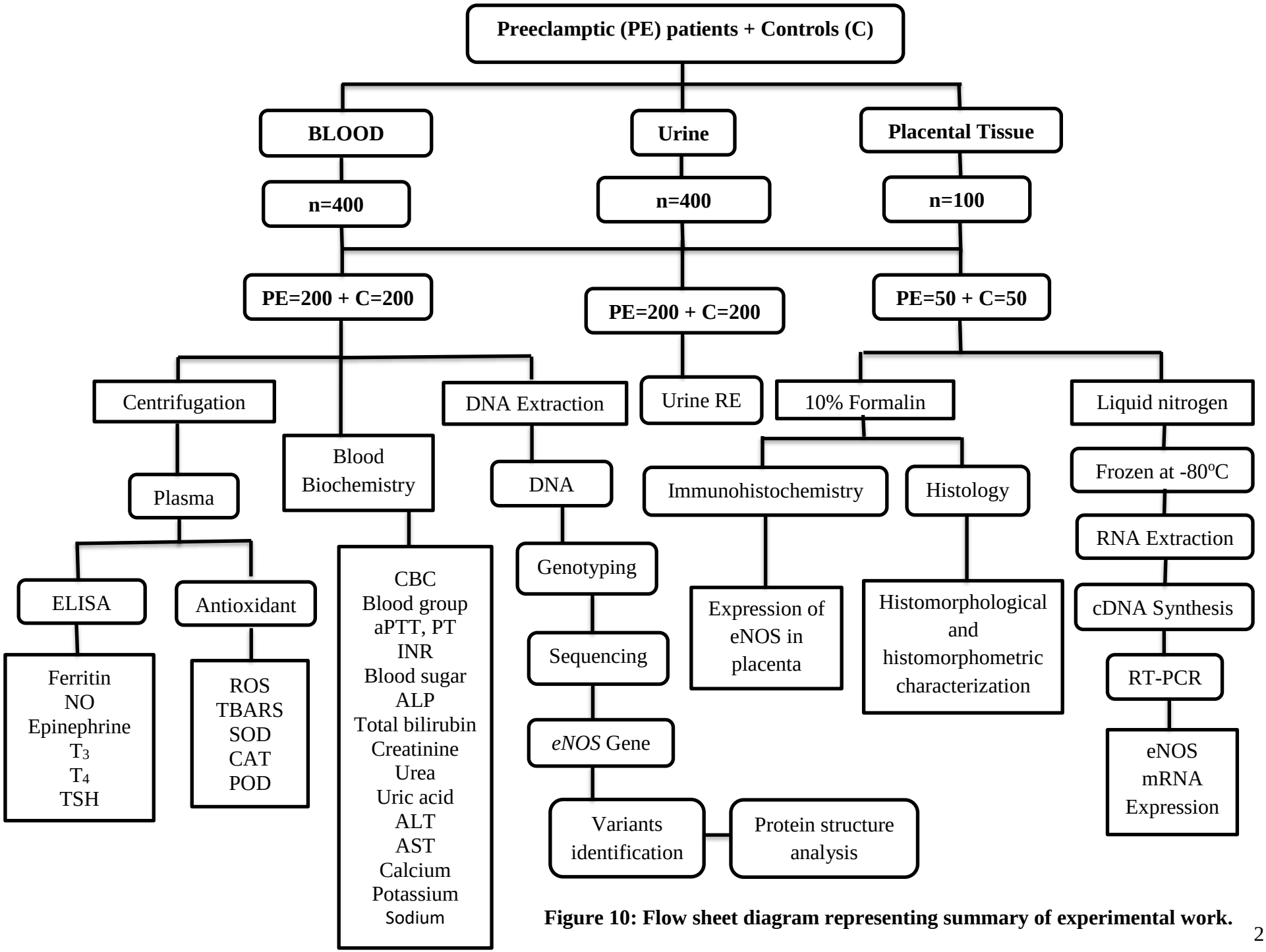


Figure 10: Flow sheet diagram representing summary of experimental work.

CHAPTER 1

Analysis of demographic and clinical risk factors in preeclamptic Pakistani women.

ABSTRACT

Preeclampsia (PE) is a pregnancy-related multi-factorial disorder, characterized by increased hypertension and proteinuria after 20 weeks of gestation. It is important to identify women at high risk of PE in order to reduce this complication and to develop possible treatment modalities. The use of biomarkers in early pregnancy would allow adequate monitoring during pregnancy and administer interventions. The present study elaborated demographic, clinical and biochemical risk components for PE in Pakistani pregnant women. Total 400 pregnant women were included in this study, 200 with PE and 200 with normal pregnancy. History and blood samples were collected from each subject with informed consent after diagnosis for hematological or biochemical analysis. Data was analyzed and laboratory tests were performed. Data were statistically analyzed by Independent sample t-test, Chi-square test and the odds ratio. Several parameters including gestational age ($p < 0.001$) and weight of child ($p < 0.001$) were reduced while age (p 0.002), BMI ($p=0.047$) and blood pressure ($p < 0.001$) were elevated in PE patients as compared to control group. Pregnancy history including headache (odds ratio (OR): 2.42), swelling in hands and face (OR: 4.8), excessive weight gain (OR: 3.74) urination problem (OR: 2.90), abdominal pain (OR: 1.45), shortness of breath (OR: 4.49), muscular pain (OR: 1.64) and blurring of vision (OR: 2.03), history of preeclampsia in previous pregnancy and in family (OR: 7.38; OR: 8.43) were more obvious in preeclamptic women. Elevated levels ($p<0.001$) of alkaline phosphatase, serum urea, uric acid, urine proteins, total leukocyte count (TLC) ($p=0.028$) and haematocrit ($p=0.016$) were reported in PE. Prolonged activated prothrombin time (aPTT), prothrombin time (PT)and international normalization ratio (INR) were recorded in both PE groups with fibrinogen levels as compared to controls. While, total bilirubin ($p=0.019$), aspartate aminotransferase (AST) ($p=0.012$), serum calcium ($p=0.002$) and sodium ($p= 0.010$) concentrations were reduced in case patients as compared to control group. In conclusion, the present study reveals the importance of demographic and clinical risk factors in early diagnosis of PE, and should be considered in the counselling diagnostic and therapeutic interventions to improve the reproductive health of women in Pakistan.

INTRODUCTION

PE, a hypertensive disorder of pregnancy, is a major contributor to intrauterine growth retardation, perinatal and maternal morbidity and mortality, yet its exact pathogenesis remains elusive (Kelly *et al.,* 2017). Improving the outcomes of PE necessitates early prediction of the disorder to identify women at high risk (Kirbas *et al.,* 2015). It poses several problems to both child and mother and the risk of adverse perinatal and maternal outcomes increase with the progression of PE (English *et al.,* 2015; Meher *et al.,* 2017).

The only effective treatment available for PE is setting up premature delivery before 34 weeks of gestation to avoid severe maternal complications, considering that this attitude involves serious neonatal damage (Pennington *et al.,* 2012; Lisonkova and Joseph, 2013). The disease can be predisposed to many maternal causes, which can be hereditary, psychological or environmental (Roberts and Cooper, 2001). Factors related to the increased prevalence of PE are maternal age, pre-pregnancy obesity, diabetes, multifetal gestations, and chronic hypertension but the main underlying cause of PE remains unknown. PE seems to have multifactorial cause and has no specific marker for diagnosis (Duckitt and Harrington, 2005; Poon *et al.*, 2010; Paré *et al.*, 2014).

The direct connection between these risk factors and preeclampsia is poorly understood, but most hypotheses explaining PE etiology indicate that the disease is a cascade caused by the combination of irregular maternal inflammatory response, endothelial cell activation / damage with a disrupted hemodynamic environment, and immunity deranged. (Steinberg *et al.*, 2009; Clifton *et al.*, 2012). Changes in coagulation factors increase the risk of pre-eclampsia bleeding complications (Norwitz *et al.,* 2002). Haemorrhages are a major problem and main cause of maternal mortality, usually during operational delivery or regional anesthesia (Lakshmi, 2016). In addition, Serum ferritin is a reliable indicator of the iron status of the whole body in non-diseased individuals. Iron deficiency is diagnosed by low levels of ferritin, while its high levels not always signify iron excess (Lakshmi, 2016).

Women with previous history of PE are more vulnerable to cardiovascular diseases leading to death. Many studies have been conducted to gain insight to the pathophysiology of the disease state and to identify various biological markers in many disciplines including obstetrics (Chaiworapongsa *et al.,* 2013). Many hemostatic abnormalities have been associated with pregnancy hypertensive disorder (FitzGerald

et al., 1996). PE can also contribute to a number of hematological aberrations and thrombocytopenia is the most common hematological abnormality in pre-eclampsia. (Chen and Lin, 2017). Measuring blood cell sub types could provide prognostic and diagnostic clues to disease (Zenclussen, 2018). Predictive tests should be performed timely for the identification of women for whom the risk of PE is high enough to justify the application of preventive measures.

Contemporary studies show that various risk factors are linked to PE, the identified risk factors are: higher BMI, maternal age, nulliparity, autoimmune diseases, pre-existing diabetes, multiple pregnancies, pregnancy pause greater than10 y, PE family history and history of PE in a prior pregnancy (Bellomo, 2018; Sheikhi *et al.,* 2018). Strong risk factor for PE is the first pregnancy (Pridjian *et al.,* 2002). Significant difference had been found in these risk factors in Asians as compared to other ethnic groups including obesity and blood pressure (Lee *et al.,* 2000).

As PE is a disease of theories and it is poorly understood pregnancy complication and there is no gold standard for its diagnosis. There is the constant search of prognostic factors to predict the progression and severity of the disease. Various clinical and epidemiological risk factors should be identified before it threatens both maternal and fetal survival. Pakistan is an economically developing country in Asia, studies have been done on PE but there are many gaps to be filled. It is important to identify the women who are more likely to be at high risk of PE so that they can benefit from intervention. Therefore, the present study was designed to prospectively evaluate the predictive role of several demographic, clinical and biological markers in the pathophysiology of preeclampsia in Pakistani women.

MATERIALS AND METHODS

Ethical Compliance

The present study was conducted with prior approval from ethical committees of Quaid-i-Azam University, Islamabad and collaborating hospitals including Pakistan Institute of Medical Sciences (PIMS), Islamabad and Quaid-e-Azam International Hospital, Islamabad. All participants were informed about the study objectives and signed an informed consent. The study protocol was done in accordance with the principles of the Declaration of Helsinki.

Subjects

Total of 400 individuals were included, out of which 200 were patients suffering from PE and 200 were healthy controls from respective hospitals, during the period of September 2015 to July 2017. All patients were <35 years of age with 24-40 weeks of gestation. Informed consent and a detail proforma including history and clinical examination were filled before sample collection.

Inclusion and exclusion criteria

Diagnosed patients with PE which is defined as new onset of elevated blood pressure >140/90mmHg along with proteinuria ≥1+ on dip stick on two occasions at least 6 h apart or >300mg per day on two occasions (American Society of Obstetrics and Gynecology Technical Bulletin # 91, 1986) were selected for this study (Kallela *et al.*, 2016). The normotensive control group have women with uncomplicated gestation and blood pressure <125/85mmHg and no proteinuria. Subjects were excluded if they had one or more of the following: diabetes, asthma, kidney disease, autoimmune disease, urinary tract infection, current or past history of smoking and eclampsia.

Measurement of Body Mass Index (BMI)

Height was measured by using Stadiometer and weight by weighing machine according to the criteria described by Kamal, 2010 (Kamal, 2010). Height of women of both groups was recorded in meters and weight in kilogram during their visit to the hospital and BMI was calculated in Kg/m^2 from height and weight by the following formula:

$$BMI = \frac{\text{weight in kilogram}}{\text{height in meters}^2}$$

Blood Sampling and Storage

Blood and urine samples were collected during the antepartum period before the onset of delivery in labelled Ethylene diamine tetra acetic acid (EDTA) tubes, and blood divided into two parts, serum was separated from half of blood and stored at -80 ^{0}C, and second part was stored for further analysis.

Urine Examination

Dipstick Test:

At the time of diagnosis urine dipstick test was performed through Combur test strips (Roche, Combas®, USA). Test strip was dipped in the urine sample. Colour of the stick was changed based on the presence of certain substances like protein concentration or specific gravity, pH levels or acidity and urobilinogen (by product of bilirubin reduction).

Procedure

1. The specimen was collected and transferred in a test tube for even mixing.
2. The test strip was dipped in the urine for one second.
3. The strip was drawn, and its edge was ran over the rim of container for removal of excess liquid.
4. After 60 seconds the reaction colour in the test area was compared against the colour scale on the label
5. Results were recorded.

Biochemical analysis of Blood

Complete Blood profile on haematology analyser

Half of the blood was used for complete blood profile. Complete blood profile was performed on Automated Haematology Analyser (pocH-100i, Japan). Each profile presented the data of TLC, white blood cell count (WBC), red blood count (RBC), platelets count (PLT), measurement of haemoglobin (Hb), mean corpuscular volume (MCV), measurement of haematocrit (HCT), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), packed cell volume/hematocrit (PCV/HCT), red blood cell distribution width (RDW-CV), neutrophils and lymphocytes percentage.

Determination of blood group by slide method

Blood groups were identified by slide method using ABO Antisera. Red cells in the specimen were reacted with the reagent antisera (anti-A and anti-B). Presence of corresponding antigen was indicated by the agglutination of red cells.

Procedure

1. A clean and dry glass slide with a glass pencil was divided into two parts. To classify the antisera, the sections are marked as anti-A and anti-B.
2. One drop of each anti erum was put on the center of the conforming section of the slide.
3. One drop of blood from each sample was added to each drop of antiserum which has to be tested.
4. Blood and antiserum were mixed by using a separate stick for each section on the slide.
5. Slide was tilted forwards and backwards and after 2 minutes agglutination was examined.
6. Interpretation of results was done.

Coagulation factors analysis

APTT, PT, INR andfibrinogen levels were checked by automated coagulation analyzer (pocH-100i, Japan).

Determination of Serum Ferritin

Quantitative measurement of ferritin concentrations (ng/mL) was estimated by ferritin Enzyme-Linked Immunosorbent Assay (ELISA) kit (cat# 1601-16, AccuDiag ™, California, USA).

Principle of the Assay:

Solid phase ELISA was the basis of Ferritin Quantitative Test Kit. The assay system employed one anti-ferritin antibody for solid phase (microtiter wells) immobilization and the other one for mouse monoclonal anti-ferritin antibody in the antibody-enzyme (horseradish peroxidase) conjugate solution. The test samples were given time for simultaneous reaction with the antibodies, leading to sandwiching of ferritin molecules between the solid phase and enzyme-linked antibodies. Solution was incubated for 60

minutes at room temperature. After incubation, wells were washed with water to detach unbounded labelled antibodies. Following washing, a TMB solution was added. The solution was again incubated for 20 minutes which results into a development of blue color. Stop solution was added to stop color development and a yellow color was appeared. Solution was measured spectrophotometrically at 450 nm. There was a direct relation of ferritin concentration to the color intensity of test sample.

Plasma Creatinine level

Determination of plasma creatinine level was done by AMP diagnostic kit (AMEDA labordiagnostik Gmbh, Austria) and analysed on chemistry analyser (picco 5).

Procedure

The test samples were analysed on chemistry analyser at the wavelength of 510 nm in 1cm light path of cuvette and incubation at 37°C. The blank contained 1000 µl of working reagent, sample solution contained 1000 µl of working reagent and 50 µl of test sample, while standard solution was composed of 1000 µl of working reagent and 50 µl of standard.

Plasma Blood Urea level

Estimation of plasma urea level was done by using Gesan urea kit (GESAN Production s.r.i, Italy) and were analysed on chemistry analyser (picco 5).

Procedure:

The test samples were analysed on chemistry analyser at the wavelength of 340 (334-365) nm in 1cm light path of cuvette and incubation at 37°C. The blank contained 1000 µl of working reagent, sample solution contained 1000 µl of working reagent and 10 µl of test sample, while standard solution was composed of 1000 µl of working reagent and 10 µl of standard. All reagents were mixed and incubation was done at 37°C. Absorbance of each sample was determined after 30 seconds and after 60 seconds.

Determination of Alanine-Aminotransferase (ALT):

Determination of plasma ALT level was done by using AMP diagnostic kit (AMEDA labordiagnostik Gmbh, Austria) and were analysed on chemistry analyser (picco 5).

Procedure:

The reagents were analysed on chemistry analyser at the wavelength of 340 (334-365) nm in 1cm light path of cuvette and incubation at 37°C. Reagents were brought at 15-25°C before testing. The blank contained 1000 µl of working reagent, sample solution contained 1000 µl of working reagent and 50 µl of test sample, while standard solution was composed of 1000 µl of working reagent and 50 µl of standard. All reagents were mixed and incubation was done at 37°C for 1 minute. Absorbance of each sample was determined at time 0 and after 1, 2 and3 minutes. Then, the absorbance variation ΔE/min was obtained by performed readings and calculated as:

$$ALT\ (U/L) = \Delta E/min \times 1746$$

AST:

Estimation of plasma AST was done by using AMP diagnostic kit (AMEDA labordiagnostik Gmbh, Austria) and were analysed on chemistry analyser (picco 5).

Procedure:

The reagents were analysed on chemistry analyser at the wavelength of 340 (334-365) nm in 1cm light path of cuvette and incubation at 37 °C.

- All the reagents were pre-incubated to reaction temperature.
- The photometer was brought to zero with distilled water.
- 1ml of working reagent and 50 µl of sample was pipetted at reaction temperature of 37°C.
- Mixture was gently mixed by inversion and then incubated for 1-minute.
- Absorbance reading was repeated three times exactly 1 minute after the previous reading and the difference between the absorbance values was determined.
- The average of the results was estimated to obtain the average change of absorbance per minute as:

$$AST\ (U/L) = \Delta A/min \times 1746$$

Estimation of Alkaline Phosphatase (ALP):

Plasma AST was determined by using Gesan ALP kit (GESAN Production s.r.i, Itly) and were analysed on picco 5 chemistry analyser.

Procedure:

The test samples were analysed on chemistry analyser at the wavelength of 404 (400-410) nm in 1cm light path of cuvette and incubation at 37°C. The blank contained 1000 µl of working reagent, sample solution contained 1000 µl of working reagent and 10 µl of test sample, while standard solution was composed of 1000 µl of working reagent and 10 µl of standard. All reagents were mixed and incubation was done at 37°C. Absorbance of each sample was determined after 30 seconds and after 60 seconds.

- Absorbance was measured after every 60 second and determine the ΔA/min.
- ALP concentration was calculated as:

$$\text{ALP concentration (U/L)} = \Delta A/\text{min} \times 5454$$

Estimation of plasma Glucose:

Plasma glucose was determined by using Gesan glucose kit (GESAN Production s.r.i, Itly) and were analysed on picco 5 chemistry analyser.

Procedure:

The test samples were analysed on chemistry analyser at the wavelength of 510 (500-550) nm in 1cm optical path and incubation at 37°C. The blank contained 1000 µl of working reagent, sample solution contained 1000 µl of working reagent and 10 µl of test sample, while standard solution was composed of 1000 µl of working reagent and 10 µl of standard. All reagents were mixed and incubation was done at 37°C. Absorbance of each sample was determined after 30 seconds and after 60 seconds.

- Serum glucose was then calculated by following formula

$$\text{Glucose (mg/dl)}=(\text{A sample/A STD}\times\text{STD value}$$

Estimation of Total Bilirubin

Determination of plasma total bilirubin was done by using Gesan Total biluribun kit (GESAN Production s.r.i, Itly) nd were analysed on chemistry analyser (picco 5).

Procedure

The test samples were analysed on chemistry analyser at the wavelength of 570 (550 – 580) nm in 1cm optical path and incubation at 37°C. The blank contained 1000 µl of

working reagent, sample solution contained 1000 µl of working reagent and 50 µl of test sample, while standard solution was composed of 1000 µl of working reagent and 50 µl of standard.

- After mixing, it was incubated for 5 minutes at 37°C.
- Absorbance of sample (EC) and blank against water (ECB) was measured.
- Total Bilirubin concentration was calculated as:

Total bilirubin (mg/dl) = (EC-ECB) x 14.5

Estimation of Uric Acid

Etimation of plasma blood urea level was done by using Gesan uric acid kit (GESAN Production s.r.i, Itly) and were analysed on chemistry analyser (picco 5).

Procedure

The test samples were analysed on chemistry analyser at the wavelength of 510 (500-550) nm in 1cm optical path and incubation at 37°C. The blank contained 1000 µl of working reagent, sample solution contained 1000 µl of working reagent and 50 µl of test sample, while standard solution was composed of 1000 µl of working reagent and 50 µl of standard. All reagents were mixed and incubation was done at 37°C. Absorbance of each sample was determined after 30 seconds and after 60 seconds.

- After mixing, mixture was incubated at 37°C for 5 minutes and absorbance of each sample was measured.

Determination of electrolyte concentration

Electrolytes concentrations were determined by Flame Atomic Absorption Spectrophotometer (FAAS) (Varian AA240 FS, USA). Specific lamps were used, and calibration curves preparation was done by various standard solutions to analyse the unknown concentration of electrolytes in the serum.

Standard solutions

Standard solution for each electrolyte was prepared at the concentration of 1000 ppm by addition of or 0.1 g/100 ml of salt in distilled water. Therefore, Instrument was calibrated by preparing standard solutions of known concentrations.

Procedure

1. Serum was exposed to fast sequential FAAS. Concentrations of Calcium (Ca), Sodium (Na) and Potassium (K) were determined by adjusting specific wavelength of 422.7, 766.5 and 589 for each electrolyte.
2. FAAS was done by using hollow cathode lamps and air acetylene flame.
3. Aspiration of the serum sample was done then aerosolized and then mixed with combustible gases. temperature was maintained at maximum ranges from 2100 to 2800°C at which during combustion sample was ignited.
4. Atom of electrolyte absorb specific wavelength on reduction and when they came to ground state atom.
5. Wavelength was precise for all electrolytes absorbed from hollow cathode lamps and was accurate up to 0.01 to 0.1 nm.
6. Monochromator was used to determine the light wavelength absorbed by each sample.
7. Light chosen by monochromator was absorbed on the photomultiplier tube detector, which detects the quantity of reduction in light intensity through absorption by analyte.

Statistical Analysis

All the data were expressed as a median along with 95% confidence intervals (CI). Quantitative data was expressed as mean ± SEM. Odds Ratios (OR) were calculated as a measure of the degree of relative risk for PE. Significant difference between groups for clinical, demographic data and biochemical results was determined by applying Independent sample t-test and Chi-squared test (χ^2) using IBM SPSS Statistics 21 software.

RESULTS

Demographic characteristics

Percentage of the study individuals belonging to different areas of Pakistan has been summarized in Figure 11. Characteristics of study population are summarized in table 1 with figures 12-17. Demographic results of current study revealed significant difference in age (p<0.01), BMI (p=0.04), gestational age (p=<0.001) systolic and diastolic blood pressure (p=<0.001) (Fig. 12-14). Although, case patients have 3 weeks short pregnancy period, but weight gain was higher in case patients as compared to control group (Fig. 16). Similarly, significant difference was observed in infant's weight (p <0.001) of mothers with PE and normotensive mothers as shown in Figure 17. No significant difference was observed in rate of abortion (p=0.09), average age at marriage (p=0.09) and first child birth (p=0.13) in both control and preeclamptic group as shown in figure 12 and 15.

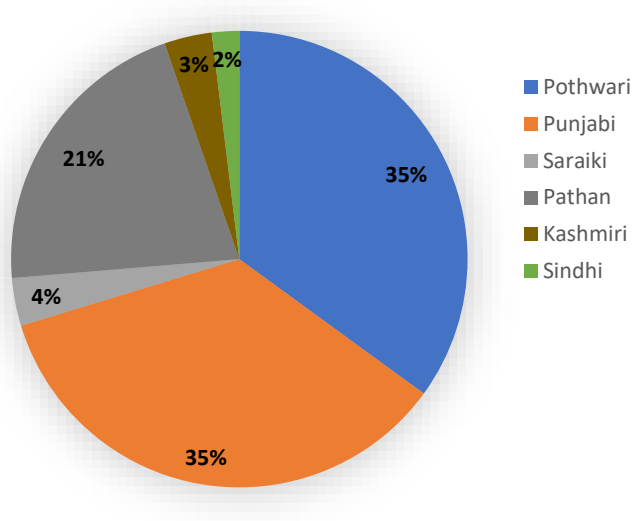


Figure 11: Pie chart showing percentage of subjects included in the study from different areas of Pakistan.

Table 1: Characteristics of the study population.

Parameters	Controls	PE Patients	p value
Age (Years)	27.017±0.32	28.52±0.34	<0.01
BMI (kg/m^2)	27.67±0.28	28.60±0.36	0.04
Gestational Age (Weeks)	36.32±0.36	33.12±0.15	<0.001
Systolic blood pressure (mmHg)	114.71±0.51	154.36±1.54	<0.001
Diastolic blood pressure (mmHg)	73.88±0.45	100.95±1.12	<0.001
Abortions	0.51±0.064	0.71±0.033	0.09
Age at marriage (Years)	22.13±0.286	22.90±0.305	0.09
Age at first child birth (Years)	23.45±0.315	24.52±0.333	0.13
Weight of child (kg)	3.03±0.03	2.41±0.021	<0.001

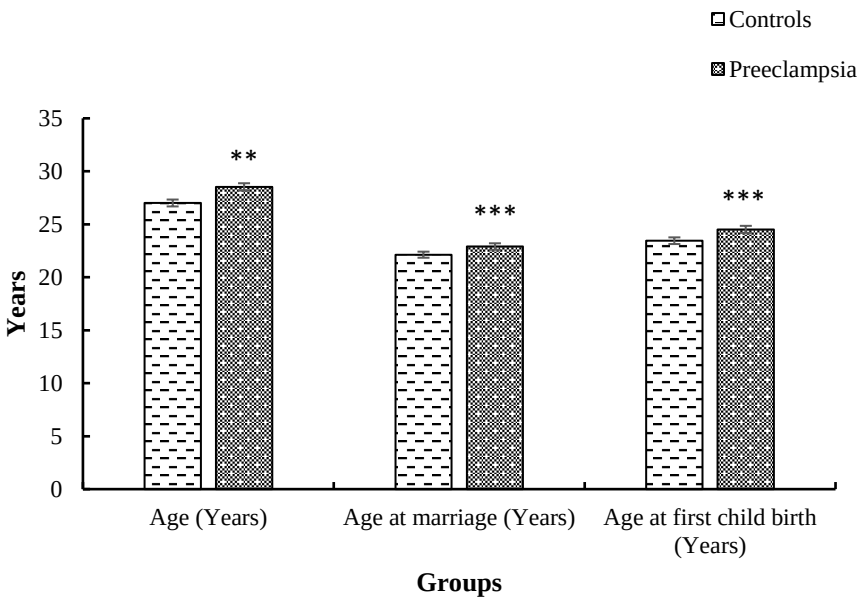


Figure 12. Mean ± SEM difference of age, age at marriage and first child birth in control and preeclamptic group.

, * indicate significant difference in PE group at probability of $p<0.01$ and $p<0.001$ respectively when compared to control.

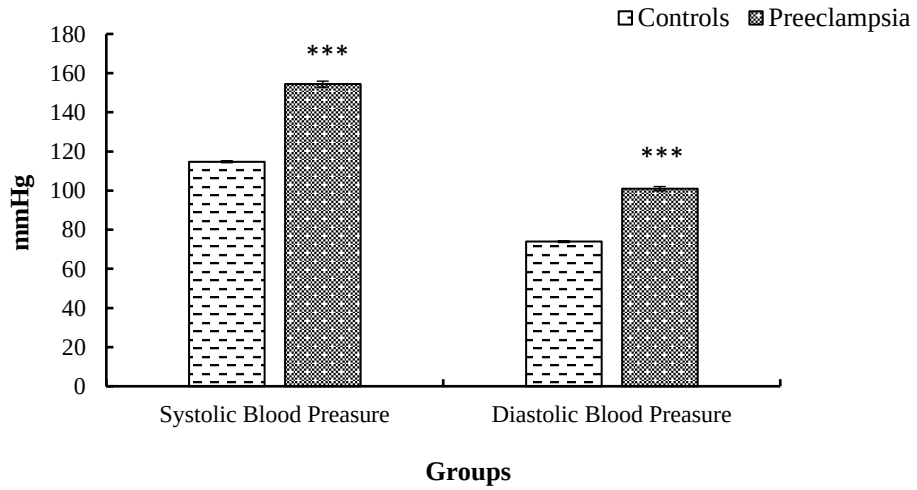


Figure 13. Mean ± SEM difference of systolic and diastolic blood pressure among control and preeclamptic subjects.

*** indicate significant difference in PE group at probability of $p<0.001$ when compared to controls.

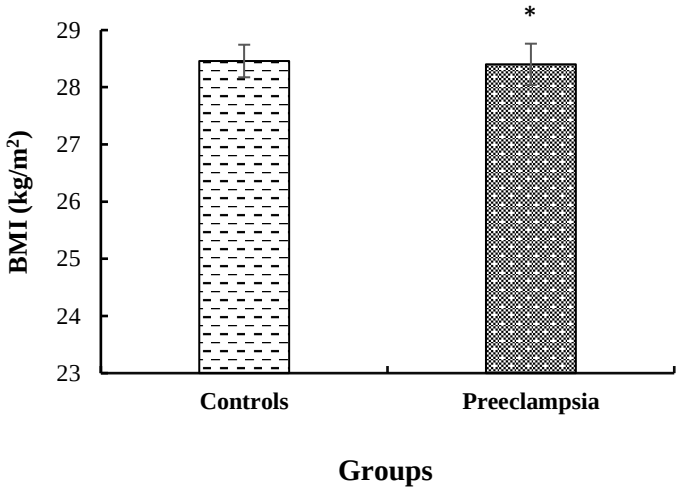


Figure 14. Mean ± SEM difference of body mass index (BMI) among control and preeclamptic subjects.

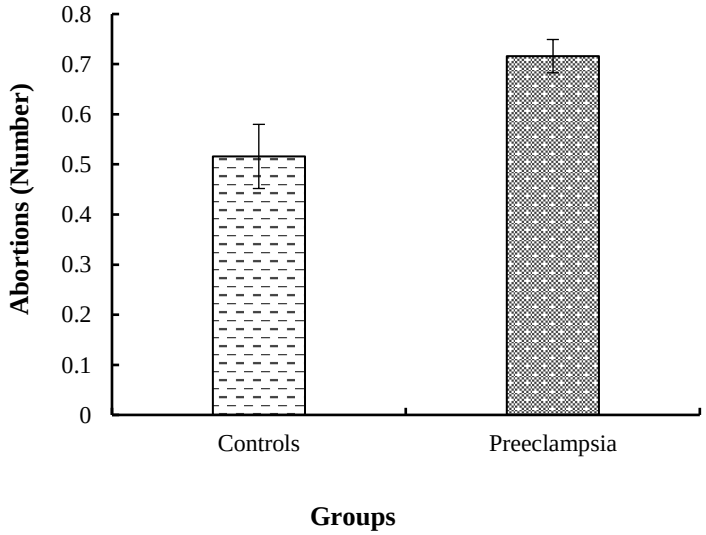


Figure 15. Number of abortions in healthy women and preeclamptic pregnant women.

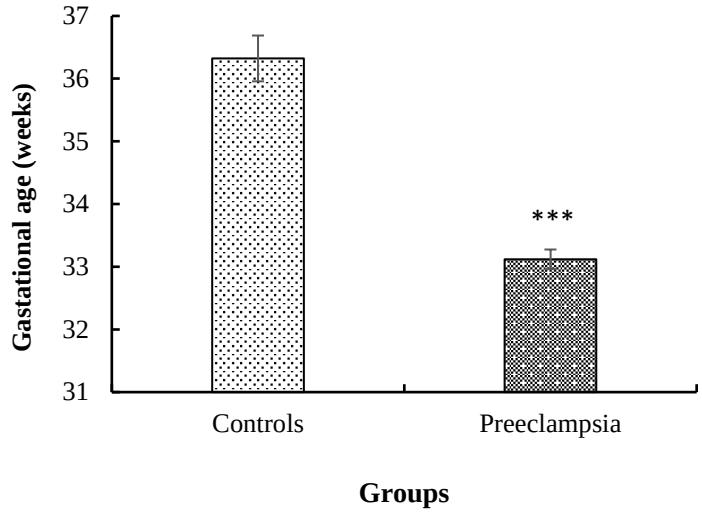


Figure 16. Mean ± SEM difference of gestational age in control and preeclamptic group.

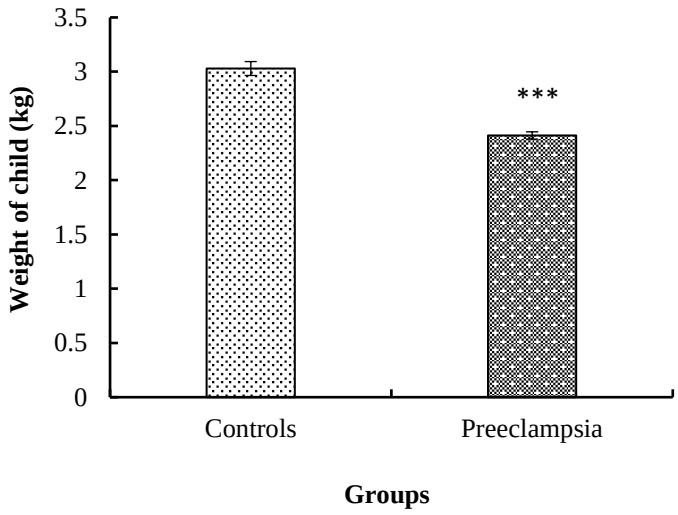


Figure 17. Mean ± SEM difference of child weight after birth among control and preeclamptic subjects.

Pregnancy and medical history

Pregnancy and medical history revealed the expected results, as case patients were more likely to be suffering from various symptoms as compared to control group. Gender difference of child and malnourishment between both groups was non-significant ($p=0.10$ and $p=0.56$). More obvious symptoms including headache, swelling in hands and face, excessive weight gain, urination problem and shortness of breath was significantly ($p<0.001$) prominent in women with PE as compared to control group. Nausea and vomiting were non-significantly ($p=0.64$) different while, abdominal pain with significance of ($p=0.02$) was present in both groups. No women in control group experienced seizers while 1.5% of them experienced loss of consciousness however, 0.5% of preeclamptic women experienced seizers and 2% of patients experienced loss of consciousness, while significance was p=32 and $p=0.70$. Muscular pain ($p=0.02$) and blurring of vision($p<0.01$) was also significantly prominent in preeclamptic women as compared to normotensive women. Non-significant difference was observed in consanguineous marriage ($p=0.06$) and pregnancy with twins (0.10) between control and preeclamptic group. Large proportion of case patients have history of preeclampsia in previous pregnancy and in family (28%, OR: 7.38; 26%, OR: 8.43; $p<0.001$) as compared to control group. Proportion of twin pregnancies was also high in case patients as thirteen pregnancies ended in twin birth, whereas six control women gave birth to twins. (Table 2, Fig. 18).

Table 2: Medical and pregnancy history of control group and preeclamptic group.

Parameters	**Controls**	**PE patients**	**Total**	**X^2**	**OR (CI)**	**p value**
Gender of child (%)						
Male	64%	56%				
	128	112	240	2.66	0.71	0.10
Female	36%	44%			(0.47-1.07)	
	72	88	160			
Headache	26%	46%				
Yes	52	92	144	17.36	2.42	<0.0001
No	148	108	256		(1.59-3.69)	
Swelling in face or	19%	53%				
hands	38	106	144	50.17	4.8	<0.0001
Yes	162	94	256		(3.06-7.53)	
No						
Excessive weight gain	7%	22%				
Yes	14	44	58	18.14	3.74	<0.0001
No	186	156	342		(1.98-7.09)	
Nausea and vomiting	24%	26%				
Yes	48	52	100	0.213	1.11	0.64
No	152	148	300		(0.70-1.75)	
Urination problem	22%	45%				
Yes	44	90	134	23.74	2.90	<0.0001
No	156	110	266		(1.87-4.48)	
Abdominal pain	37%	46%				
Yes	74	92	166	3.33	1.45	0.02
No	120	108	228		(0.97-2.16)	
Shortness of breath	12%	38%				
Yes	24	76	100	36.05	4.49	<0.0001
No	176	124	300		(2.69-7.50)	

Seizures	0.00%	0.50%	1	1.00	2.0	0.32
Yes	0	1	399		(1.8-2.21)	
No	200	199				
Loss of consciousness	1.5%	2.00%				
Yes	3	4	7	0.14	1.34	0.70
No	197	196	393		(0.29-6.06)	
Agitation	1%	2.5%				
Yes	2	5	7	1.35	2.57	0.24
No	198	195	393		(0.49-13.4)	
Muscle pain	28%	39%			1.64	
Yes	56	78	134	5.43	(1.08-2.50)	0.02
No	144	122	266			
Blurring of vision	0.00%	3.50%				
Yes	0	7	7	7.12	2.03	<0.01
No	200	193	393		(1.84-2.25)	
Consanguineous	39%	48%				
marriage	78	96	174	3.29	1.44	0.06
Yes	122	104	226		(0.97-2.14)	
No						
Preeclampsia in	5%	28%				
previous pregnancy						
Yes	10	56	66	38.39	7.38	<0.0001
No	190	144	334		(3.64-14.98)	
Preeclampsia in	4%	26%				
Family	8	52	60	37.96	8.43	<0.0001
Yes	192	148	340		(3.88-18.29)	
No						
Pregnant with Twins	3%	6.5%				
Yes	6	13	19	2.70	2.24	0.10
No	194	187	381		(0.83-6.03)	

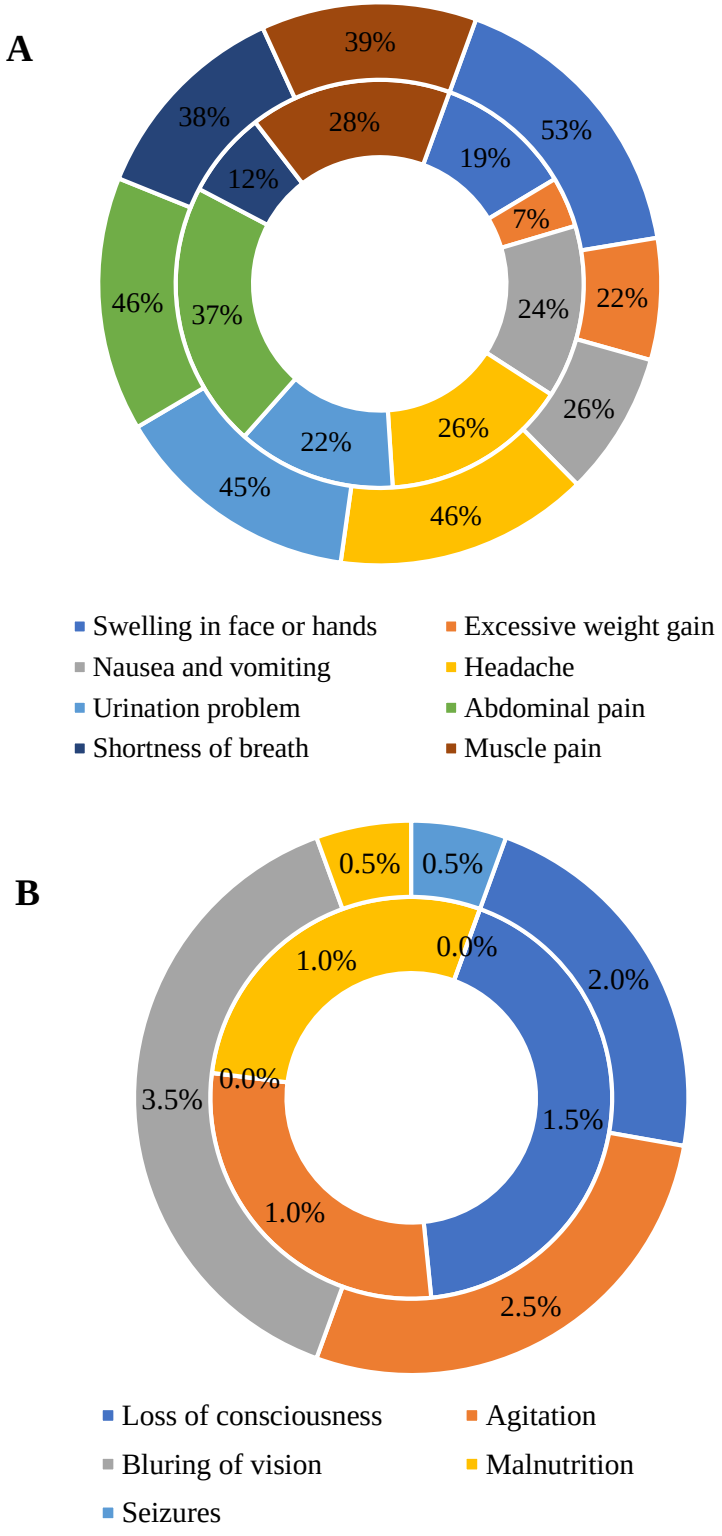


Figure 18. A and B: Pregnancy history of control group (inner circle) and preeclamptic group (outer circle).

Blood biochemistry

To determine whether blood cell count is a reliable predictor of PE, complete blood profile was analysed. Significant increase in TLC (p=0.02) and Haematocrit (p=0.01) among preeclamptic subjects as compared to control group was noticed while, non-significant change was observed in Haemoglobin (p=0.44), WBC (p=0.23), RBC (p=0.72), Platelets (p= 0.08), PCV/HCT (p=0.20), MCV (P=0.09), MCH (P=0.32), MCHC and RDW-CV (p=0.41) among these two groups (Fig. 19-24). Non-significant change in Neutrophils (p=0.88) and lymphocyte (p=0.57) percent was evident when comparison was done between control and preeclamptic groups (Table 3, Fig. 21). Blood group types were also analyzed but there was no significant (p=0.16) difference in blood groups between control and Preeclamptic subjects (Table 4, Fig. 25).

Table 3: Levels of various blood parameters in normotensive group and preeclamptic group.

Parameters	Controls	PE Patients	p value
TLC (10^9/L) (4-10)	10.03±0.17	11.43±0.36	0.02
Hemoglobin (g/dl) (12-15)	11.88±0.76	11.29±0.10	0.44
WBC (10^9/l) (4-10)	10.41±0.31	9.00±0.28	0.23
RBC (10^12/l) (3.8-4.8)	4.18±0.05	4.21±0.04	0.72
PLT (10^9/l) (150-400)	259.41±5.53	242.86±6.58	0.08
Hematocrit (%) (36-46)	32.85±0.38	35.45± 0.25	0.01
PCV/HCT (%) (35-55)	36.06±0.90	34.66±0.33	0.20
MCV (fL) (80-99)	80.39±1.05	82.90±0.90	0.09
MCH (pg) (29-32)	26.64±0.29	27.08±0.30	0.32
MCHC (g/dl) (30-35)	32.39±0.20	32.03±0.14	0.17
RDW-CV (%) (11.6-14)	16.39±0.46	15.97±0.23	0.41
Neutrophils (%) (40-70)	69.20±1.03	68.45±1.35	0.88
Lymphocytes (%) (20-40)	21.76±0.58	21.21±0.66	0.57

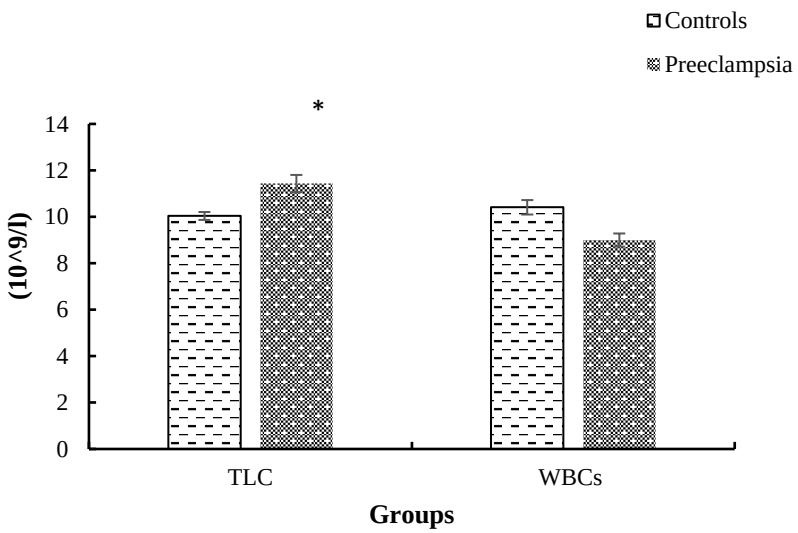


Figure 19. Number of TLC and WBCs in normotensive pregnant and preeclamptic women.

* indicate significant difference in PE group at probability of $p<0.05$ when compared to controls.

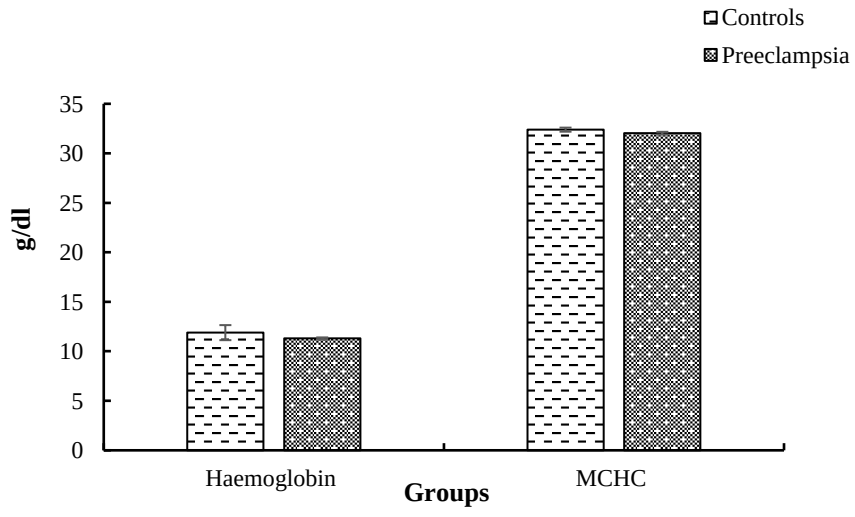


Figure 20. Concentration of haemoglobin and MCHC in normotensive pregnant and preeclamptic women.

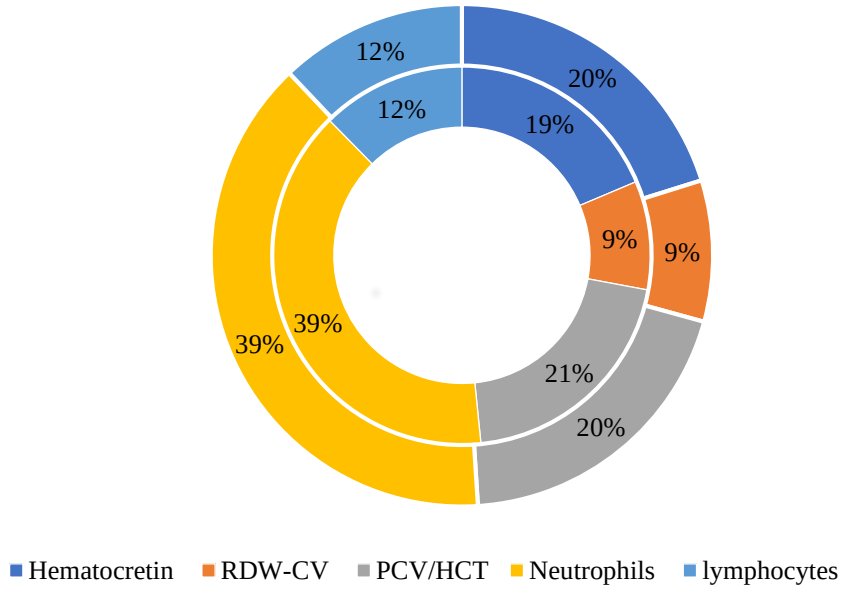


Figure 21. Percentage of hematocretin, RDW-CV, PCV/HCT, neutrophils and lymphocytes in healthy pregnant women (inner circle) and women with PE (outer circle).

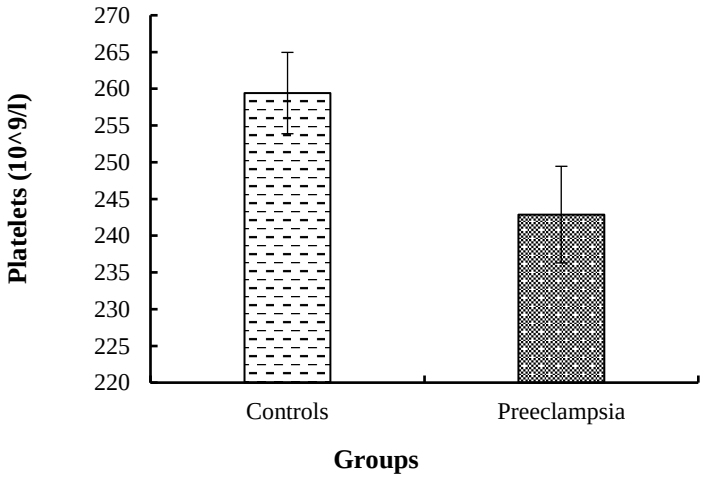


Figure 22. Platelets count difference between controls and preeclamptic patients.

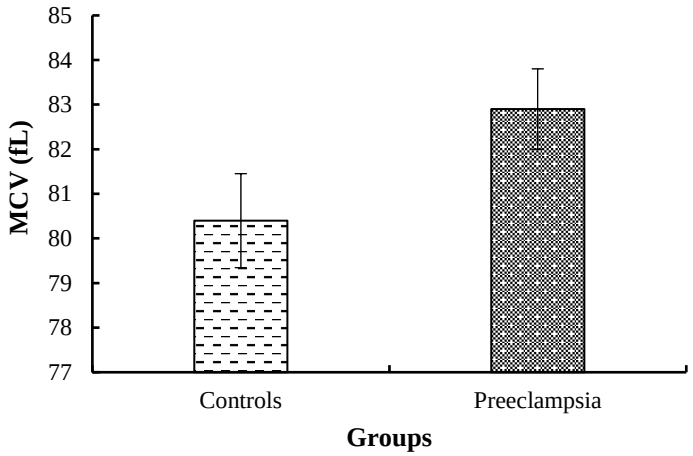


Figure 23. Difference of MCV in controls and preeclamptic patients.

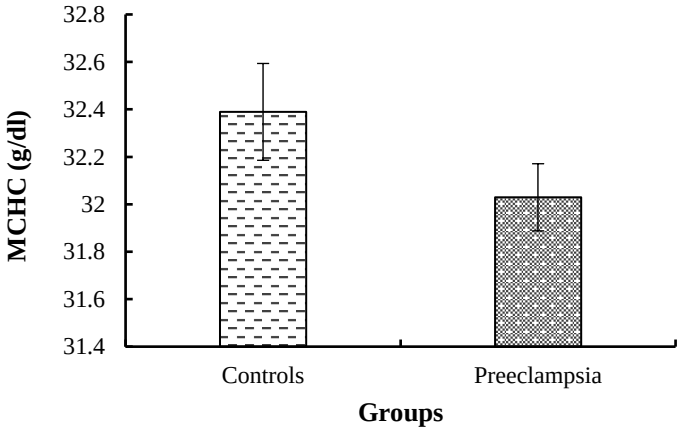


Figure 24. Levels of MCHC in normotensive women and preeclamptic patients.

Table 4: Percentage of different blood groups in control and preeclamptic group.

Blood Group (%)	Controls	PE Patients	Total
A+	13.23%	19.79%	
	9	18	27
AB+	14.70%	16.66%	
	11	17	28
B-	1.47%	4.16%	
	1	4	5
B+	23.52%	21.25%	
	17	30	47
O-	8.82%	6.25%	
	0	6	6
O+	32.35%	21.87%	
	23	22	45

X^2=7.912, p=0.16

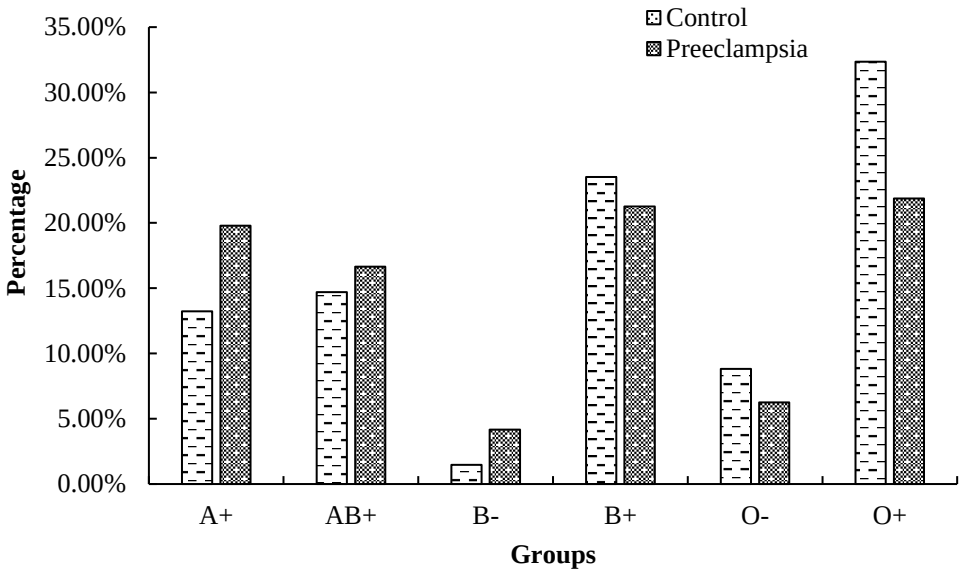


Figure 25. Percentage of ABO blood groups between controls and preeclamptic patients.

Mean and statistical significance of coagulation parameters was observed and tabulated in table 5. A significantly ($p<0.001$) prolonged aPTT, PT and INR were observed in PE group with the decrease in PLT and fibrenogen ($p<0.01$) (Fig. 26-28). Non-significant ($p=0.23$) decrease was observed ferritin levels in PE groups as compared to control groups (Fig. 29).

Table 5. Coagulation profile of control and preeclamptic groups.

Parameters	**Controls**	**PE group**	p value
aPTT (s)	31.54±0.52	34.66±0.62	<0.001
PT (s)	12.10±0.23	13.25±0.28	0.001
INR (s)	0.81±0.01	0.88±0.01	<0.001
Fibrinogen (mg/dl)	279.40±5.04	260.4±3.71	<0.01
Ferritin (ng/ml)	56.24±3.23	54.58±2.77	0.23

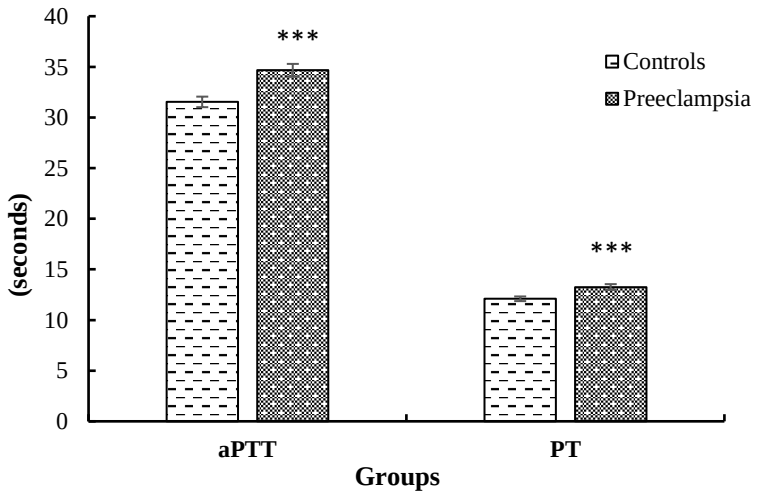


Figure 26. Difference of activated prothrombin time (aPTT) and prothrombin time (PT) timings in controls and preeclamptic patients.

*** indicate significant difference in PE group at probability of $p<0.001$ when compared to controls.

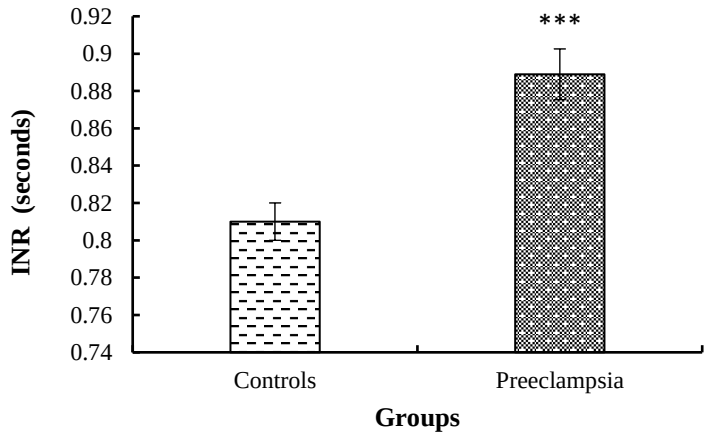


Figure 27. Difference of international normalization ratio (INR) in controls and preeclamptic patients.

*** indicate significant difference in PE group at probability of $p<0.001$ when compared to controls.

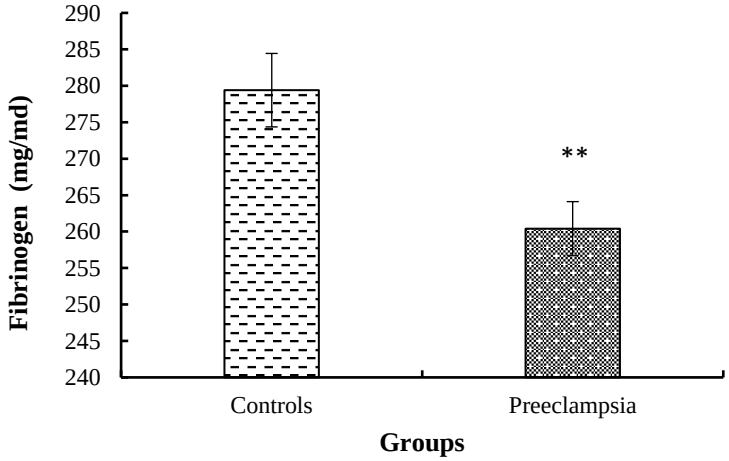


Figure 28. Concentration of fibrinogen in normotensive pregnant and preeclamptic women.

** indicate significant difference in PE group at probability of $p<0.01$ when compared to controls.

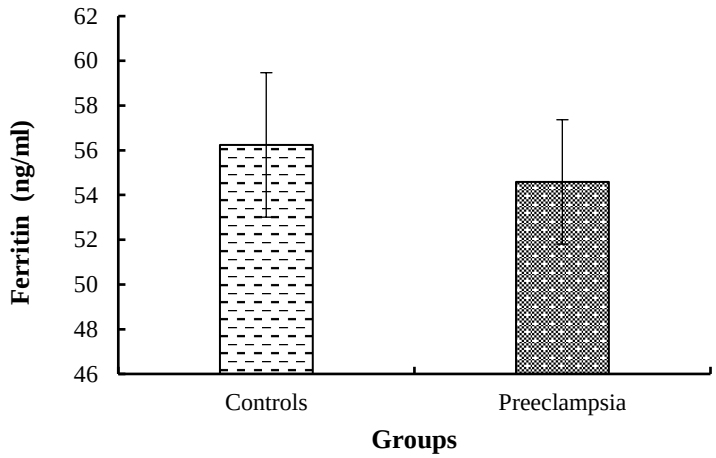


Figure 29. Concentration of ferritin in normotensive pregnant and preeclamptic women.

Blood biochemical parameters including blood sugar, liver function tests and electrolytes of both groups have been summarized in table 6 and figure 30-34. Non-significant increase in blood sugar random (p=0.07), serum creatinine (p=0.12) and ALT (p=0.06) was observed in PE subjects as compared to control. While significant increase was determined in levels of alkaline phosphatase (p<0.001), total bilirubin (p=0.02), serum urea (<0.001) AST (p=0.012) and urea (p<0.001) in preeclamptic patients as compared to normal pregnant women. Serum calcium (p<0.01) and sodium (p=0.01) were significantly decreased while potassium levels were non-significantly (p=0.15) decreased in women with PE as compared to normal pregnant women.

Table 6: Blood biochemistry and urine examination of healthy and preeclamptic women.

Parameters	Controls	PE Patients	p value
Random blood sugar (mg/dL)	86.56±1.20	90.22±1.41	0.07
ALP (U/L)	95.77±4.47	147.33±4.92	<0.001
Total bilirubin (mg/dl)	0.39±0.03	0.29±0.01	0.02
Serum creatinine (mg/dL)	0.77±0.01	0.81±0.01	0.128
Serum urea (mg/Dl)	17.08±0.56	21.25±0.74	<0.001
ALT (U/L)	20.50±2.29	21.94±1.45	0.06
AST (U/L)	21.63±0.64	30.44±1.78	0.01
Uric Acid (mg/dL)	3.53±0.08	5.67±0.18	<0.001
Calcium (nmol/l)	1.87±0.05	1.26±0.01	p<0.01
Potassium (nmol/l)	3.97±0.02	3.67±0.05	0.15
Sodium (nmol/l)	138.8±0.17	134.5±0.44	0.01

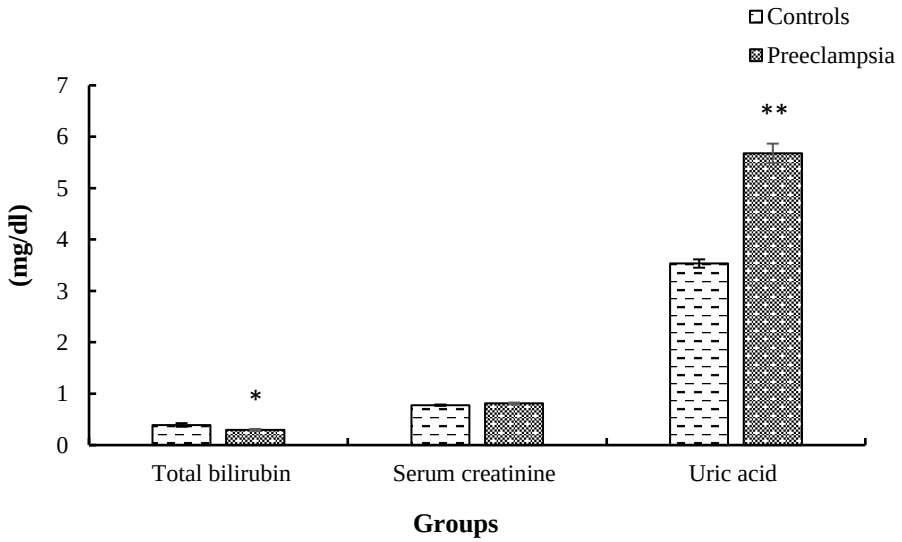


Figure 30. Levels of total bilirubin, serum creatinine and uric acid in control and preeclamptic group.

*, *** indicate significant difference in PE group at probability of $p<0.05$ and $p<0.001$ respectively when compared to controls.

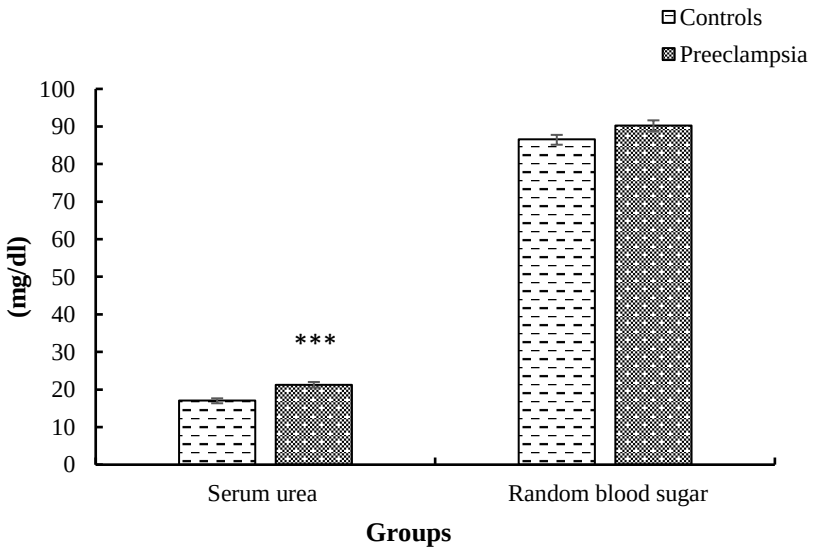


Figure 31. Levels of serum urea and random blood sugar in healthy pregnant and preeclamptic women.

*** indicate significant difference in PE group at probability of $p<0.001$ when compared to controls.

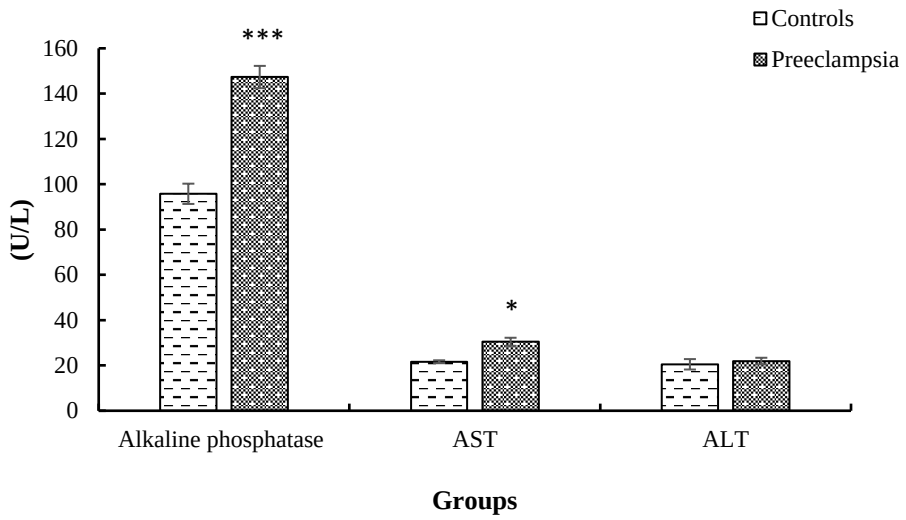


Figure 32. Mean levels of ALP, AST and ALT in healthy pregnant and preeclamptic women.

*, ** indicate significant difference in PE group at probability of $p<0.05$ and $p<0.01$ respectively when compared to controls.

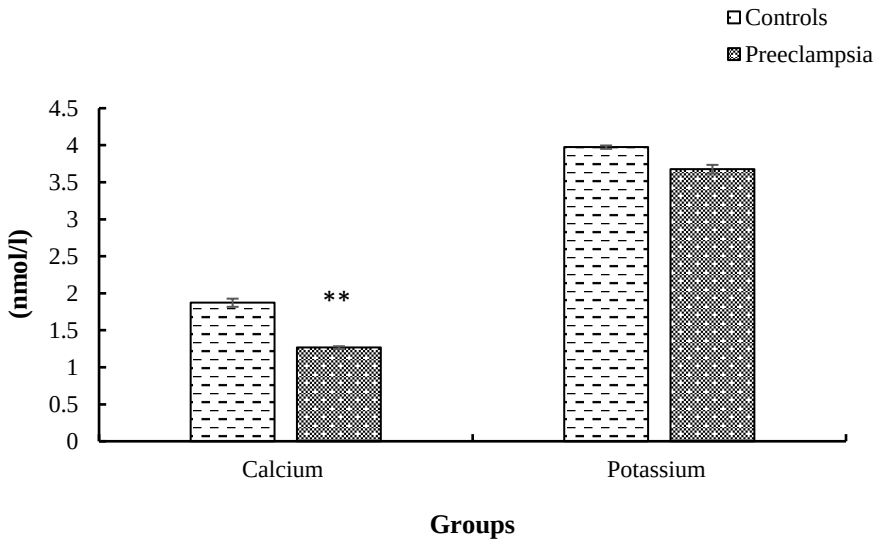


Figure 33. Mean levels of calcium and potassium in normotensive pregnant and preeclamptic women.

** indicate significant difference in PE group at probability of $p<0.01$ when compared to controls.

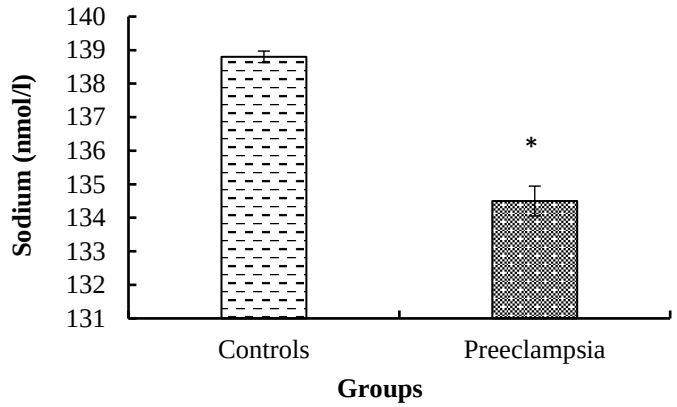


Figure 34. Concentration of sodium in controls and preeclamptic women.

* indicate significant difference in PE group at probability of $p<0.05$ when compared to controls.

Urine Examination

At the time of diagnosis urine examination was done. A highly significant elevation (p=<0.001) in urine protein levels were observed among preeclamptic subjects (Fig. 36) while no significant change was observed in specific gravity (p=0.37), PH (p=0.14) and urobilinogen (p=0.74) between control group and preeclamptic group (Table 7, Fig. 35, 37 and 38).

Table 7: Urine examination of control group and preeclamptic group.

Parameters	Controls	PE Patients	t value	F value	p value
Sp. Gravity	1.01±0.00	1.01±0.00	0.893	0.683	0.37
Proteins (mg/dl)	0.08±0.03	2.29±0.05	25.427	105.899	<0.001
pH	6.10±0.06	5.96±0.04	1.473	2.188	0.14
Urobilinogen (mg/dl)	0.22±0.03	0.22±0.01	0.323	0.441	0.74

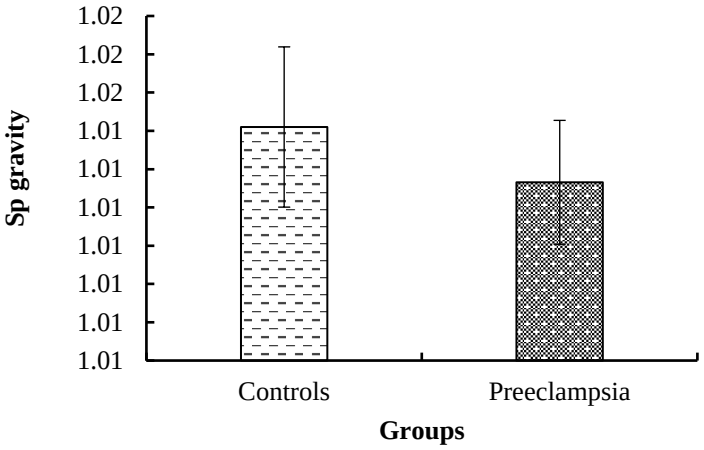


Figure 35. Mean levels of Specific gravity in urine of controls and preeclamptic women.

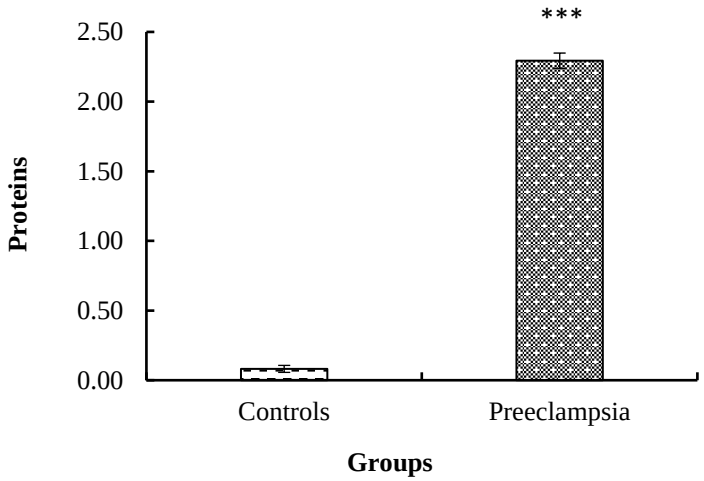


Figure 36. Mean levels of proteins excreted in urine of healthy and preeclamptic women.

*** indicate significant difference in PE group at probability of $p<0.001$ in comparison to controls.

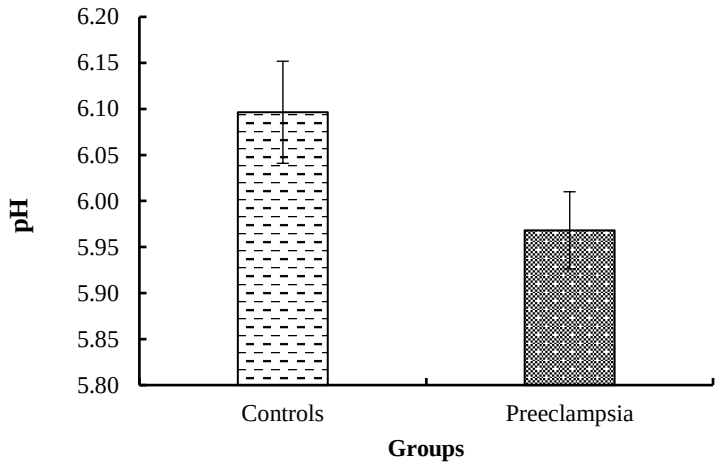


Figure 37. Mean pH of urine in control and preeclamptic group.

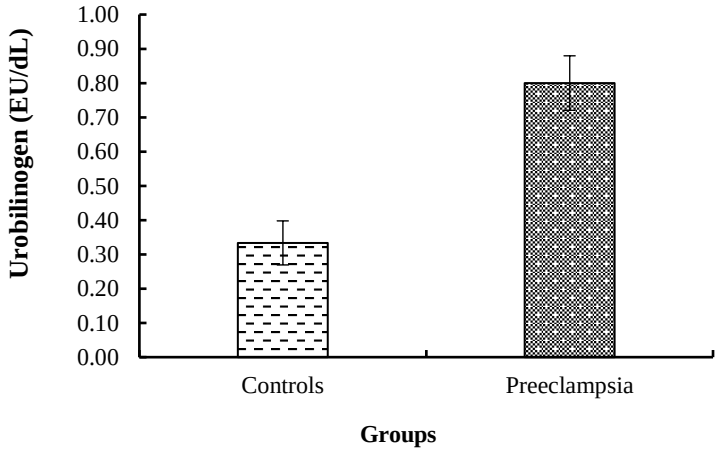


Figure 38. Mean levels of urobilinogen in urine of normotensive and women with PE.

DISCUSSION

Pregnancy hypertensive disorders remain a major health concern worldwide for women and infants (Balogun and Sibai, 2017). Maternal Mortality is extremely high in Pakistan essentially due to complications related to pregnancy, it is approximately predicted to be 500 per 100,000 live births (Shamsi *et al.,* 2010). PE is a great challenge to obstetricians because it has complex pathophysiology with unknown cause (de Souza Rugolo *et al.,* 2011; Madoglio *et al.,* 2016). It involves several organs systems involving kidney, liver, placenta, hemopoietic, vasculature, coagulation system and brain. Determination of multiple risk factors and evaluation of specific markers can predict this condition before the manifestation of maternal syndrome and can show that PE have several different reasons and presentations with the involvement of different organs and systems (Madoglio *et al.,* 2016). The presence of specific markers eventually be used for diagnosis and management of this disorder to avoid maternal and neonatal morbidity and mortality (Steegers *et al.,* 2010).

In present study various risk factors and clinical markers were investigated to find the possible cause of PE in Pakistan. According to current study possible predictors of PE are maternal age ($p=0.002$), gestational age ($p<0.001$) and blood pressure ($p<0.001$), however there was no significant association of BMI, previous abortions and maternal age at time of marriage with the development of PE. These findings correspond to previous finding showing elevated SBP (odds ratio, 2.66) and DPB (odds ratio, 1.72) but no change in BMI and maternal age between groups (Duckitt and Harrington, 2005). Underlying mechanism involves the changes in blood pressure during pregnancy due to various factors initiated by the placenta, in case of uteroplacental insufficiency blood flow reduces toward placenta, to fulfil such need maternal blood pressure rises to accommodate the blood supply towards placenta (Pridjian and Puschett, 2002; Alpoim *et al.,* 2011). Transportation of nutrients from maternal to fetal circulation through placenta depends on the blood delivery to the uterus, which is directly related to maternal cardiac output and local factors controlling uterine perfusion (Pridjian and Puschett, 2002).

Women with increased age had twice the risk of developing PE and its risk increases up to 30% in every additional year of age past (Saftlas *et al.,* 1990; Bianco *et al.,* 1996; Stamilio *et al.,* 2000); (Myatt and Miodovnik, 1999; Abalos *et al.,* 2013). In present study weight of child after birth was lower in preeclamptic patients as compared to

normotensive women. Uteroplacental insufficiency, low gestational age and placental abruption are considered the leading factors associated with low child birth weight and poor perinatal outcome (Backes *et al.*, 2011; Madoglio *et al.*, 2016; Shah *et al.*, 2016).

There was no association of blood groups noticed in present study with respect to PE. Previous literature also revealed no relationship of blood group with PE (Clark and Wu, 2008; Alpoim *et al.*, 2013; Mishra and Pradhan, 2013; Aghasadeghi and Saadat, 2017). According to present study headache, swelling in hands and face, excessive weight gain and urination problem have increased frequency in PE patients as compared to control subjects. Previous literature demonstrated the same findings which evaluated the accuracy of these symptoms in predicting adverse maternal outcomes (Witlin *et al.*, 1999; Black, 2007; Menzies *et al.*, 2007).

In current results abdominal or epigastric pain, muscular pain and blurring of vision was determined to be more frequent in PE subjects as compared to control group as observed in various previous studies that predict maternal complications (Harms *et al.*, 1991; Thangaratinam *et al.*, 2011).

Family history was also observed in this study. According to these findings' history of PE in preceding pregnancy and history of PE in family predicts the association with the onset of PE. These findings were supported by previous literature showing that family history of PE is associated with a fourfold increased risk of PE in women, underlying mechanism may involve the predisposition of various genetic factor involved in the pathophysiology of PE (Duckitt and Harrington, 2005; Thangaratinam *et al.*, 2011).

Clinical manifestation in the current study involved the presence of protein in the urine of PE women with high significance as compared to Controls. There was no significant change in urobilinogen, Sp. gravity and PH of urine in PE group as compared to healthy controls. These findings were supported by previous studies resulting in either ≥1+ dipstick proteinuria on two separate occasions or ≥2+ dipstick proteinuria on one occasion or ≥300 mg proteinuria over 24 hours (Wagner, 2004; Thangaratinam *et al.*, 2011; English *et al.*, 2015; Kurt *et al.*, 2015).

Considering that large differences that have been reported earlier (Hays *et al.*, 1985), surprising that no significant changes are seen in a number of blood parameters, RBCs number, haemoglobin concentration, MCV, MCH, MCHC, RDW-CV and neutrophil percentage as well as platelet numbers, but significant change in TLC and hematocrit

in preeclamptic women as compared to healthy control was observed. Canzoneri et al. also reported a significant increase in TLC in preeclamptic group which may results in certain infections or other inflammatory response (Canzoneri *et al.*, 2009). Wagner also reported high hematocrit level as a risk associated with Preeclampsia (Wagner, 2004).

Measuring aPTT appears to be important for early detection of coagulation abnormalities in PE-risk patients with normal platelet count. Our results are in line with the results of (Jahromi and Rafiee, 2009) and against the concept of platelet count < 100,000/mm for all preeclamptic patients with coagulation abnormality (Metz *et al.*, 1994). Ferritin concentrations were not statistically distinct in both groups, but regular inquiry of the serum ferritin status of pregnant females with high probability of PE as part of antenatal screening may assist to identify PE diagnosis before clinical manifestations appear and unnecessary iron use can be prevented in a non-anemic pregnant woman (Lakshmi, 2016).

Damage to the hepatic and renal systems was confirmed by a significant increase of several well-established blood markers such as ALP, total bilirubin, urea, AST and uric acid. Our results for liver function tests were consistent with those obtained by Hay, Wagner, Kenny et al. and FitzGerald et al. (FitzGerald *et al.*, 1996; Wagner, 2004; Hay, 2008; Kenny *et al.*, 2015).

It was demonstrated in present study that serum electrolytes including calcium and sodium levels decreased significantly in preeclamptic group as compared to control group with no significant change in potassium level. These results correlate with several studies demonstrated that calcium and sodium are essential in pregnancy which plays an significant role in metabolic activities at cellular level (Adewolu, 2013; Guo *et al.*, 2017). Decreased level of calcium results in increased parathyroid hormone levels, which further increase the intracellular calcium levels, that leads to an increased vascular smooth muscle contraction and finally raise the blood pressure (Indumati *et al.*, 2011).

Contemporary studies showed that serum sodium levels significantly decreased in the preeclamptic patients as compared to controls, because of altered sodium transport across the cell membrane leads to the deposition of sodium in the extravascular spaces and a decrease in the plasma sodium levels (Adewolu, 2013). Sodium retention

increased sensitivity to angiotensin which leads to hypertension, oedema and proteinuria, the diagnostic triad of preeclampsia (Anuradha and Shamshad, 2016).

Conclusion

All the findings of this study showed the importance of gaining a comprehensive medical history from the women early in their pregnancy. The manifestation of clinical risk factors and assessment of laboratory parameters are necessary for early diagnosis and improved management of the condition before it threatens the survival of mother and fetus. Baseline laboratory investigation should be conducted early in pregnancy in women who are at high risk of PE. The timing of tests may affect the predictive power of tests and may invalidate its use as an early predictor of the disease if the results become abnormal just before the expression of clinical manifestations. Future research on preeclampsia might provide valued information in understanding its pathophysiology and help in the development of preventive and therapeutic strategies. Most of the risk factors for PE are not modifiable, that's why better prenatal care is required to assure the timely diagnosis and management of this complication so that maternal and perinatal deaths due to PE might be avoided more easily as the ultimate long-term goal in Pakistan.

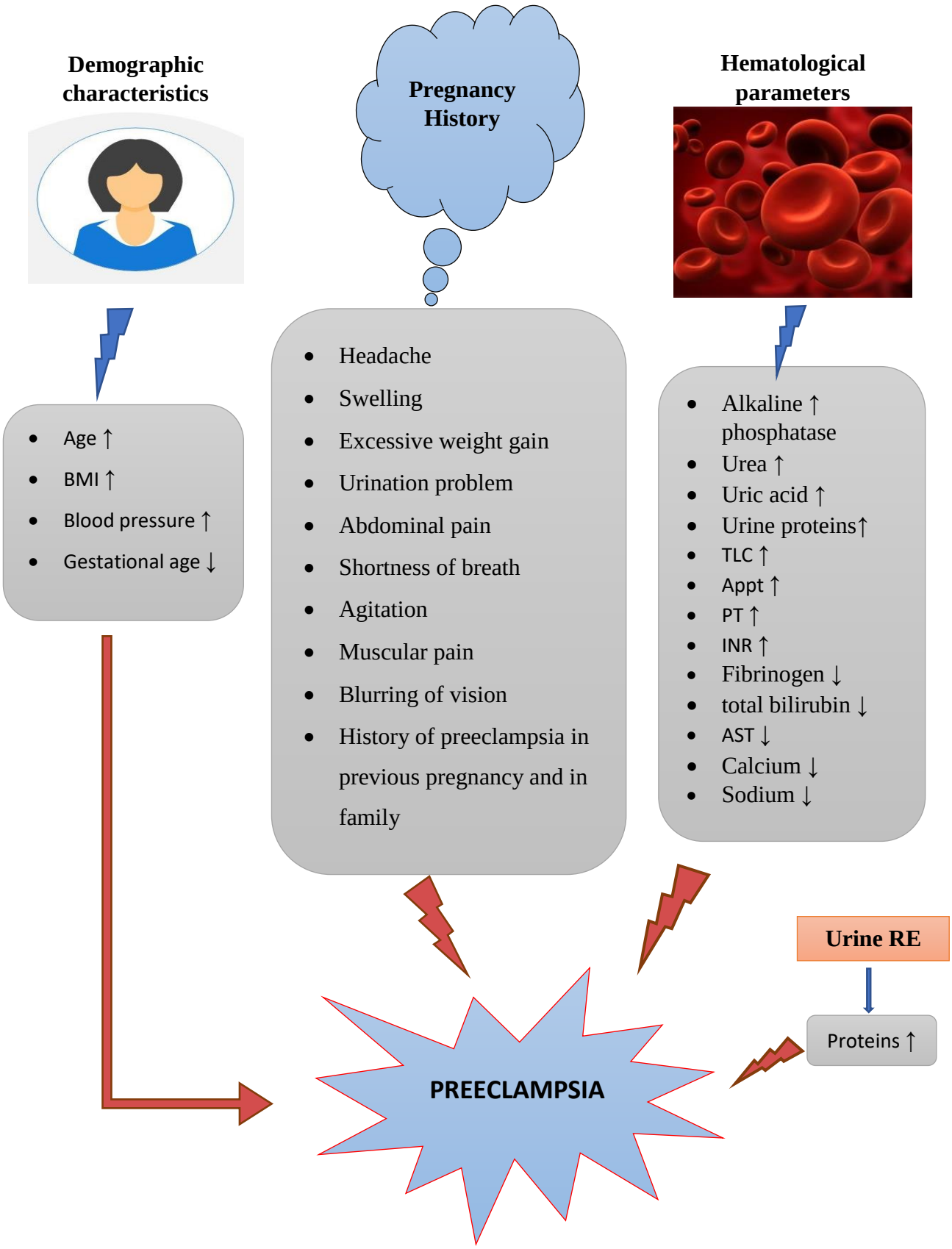


Figure 39: Graphical representation of role of demographic and clinical risk factors in susceptibility to preeclampsia in Pakistani women.

CHAPTER 2

Histological aspects of placenta: role of oxidative stress in susceptibility to Preeclampsia in Pakistan.

ABSTRACT

Preeclampsia (PE) is hypertensive pregnancy disorder which is one of the leading causes of maternal and neonatal morbidity and mortality. Various risk factors including histopathological studies are crucial for disease management. Therefore, it is important to identify PE early in pregnancy, so that women can benefit from intervention. The present study elaborated the role of antioxidant enzymes and histological examination of placental villi in PE and normotensive pregnant women. Total 400 blood (PE/controls=200) and 100 tissue samples of placenta (PE=50, controls=50) were recruited for antioxidants and histological analysis. Reactive oxygen species (ROS), thiobarbituric acid-reactive substances (TBARS), superoxide dismutase (SOD), catalase (CAT) and guaiacol peroxidase (POD) were analyzed through blood samples. Morphology of stem villi was studied through histology and histomorphometric characteristics of villous particles (VP) were determined by ImageJ software. Data was statisticaly analyzed by Independent sample t-test, Chi-square test and the odds ratio. Significantly increased concentrations of ROS ($p<0.001$) and TBARS ($p=0.04$) were determined in preeclamptic patients while non-significant difference was observed in POD ($p=0.11$), SOD ($p=0.97$) and CAT ($p=0.81$) levels among both groups. Histological changes determined abnormal villi, more syncytial knots (SK) and a significant decrease in elongated and large villi in PE placentas as compared to normal placentas. The present study reveals that oxidative stress and abnormal placentation are important risk factors in susceptibility to PE. Moreover, oxidative stress markers and histological placental evaluation provide valuable features that are useful in understanding the cause of an adverse outcome and provide probable clinical evidence of PE occurrence and new methods for PE screening and prediction for clinicians and doctors. Such risk factors should be considered in the counseling, diagnostic and therapeutic interventions to improve the reproductive health of women and infants in Pakistan.

INTRODUCTION

PE is pregnancy specific multisystem disorder, affecting several organs and maternal systems including the vascular system, kidney, liver and brain (Davison *et al.,* 2004). The most accepted theory about PE describes its two stages; stage1 is characterized by reduced placental perfusion while stage 2 consists of multisystem maternal syndrome (Christina *et al.,* 2006). The principal contributor to the pathogenesis of PE is placenta, as the syndrome is resolved when placenta is delivered (Raghupathy, 2013). The placenta is an ephemeral endocrine organ developed between the mother and fetus, producing many hormones that affect the status of the pregnancy and maternal physiology (Hannan *et al.,* 2018). Proper development and function of placenta is crucial for fetal growth because it serves for the exchange of blood gases, nutrients and waste through maintained maternofetal interference (Hansson *et al.,* 2015). In hypertensive pregnancy disorder altered architecture of the placenta has been observed (Ronco *et al.,* 2009; Barker *et al.,* 2010; Sankar *et al.,* 2013). Interestingly, problems linked with PE cease after delivery, demonstrating that placenta is the reason of the onset of PE (Redman and Sargent, 2005; Ukah *et al.,* 2018).

Placental and systemic oxidative stress is thought to be key aspects in pathogenesis of PE. Oxidative stress is the imbalance between antioxidants defense mechanism and formation of oxidative substances such as ROS and peroxides (Touyz, 2004). Antioxidants are the agents that inhibit oxidation of biomolecules such as proteins, lipids and DNA preventing oxidative damage (Matsubara *et al.,* 2015). CAT, POD and SOD are major enzymatic antioxidants, various studies reported reduced concentrations of these antioxidants in PE and high levels of free radicles such as ROS and lipid peroxidation (Hung, 2007). Oxidative stress holds a central position in onset of PE and the clinical manifestations of this disorder (Hansson *et al.,* 2015). Due to imbalanced antioxidant activity, abnormal spiral arteriole remodeling and uteroplacental perfusion, placental ischemia occurs causing fetal hypoxia resulting in morphological and histological changes leading to PE in addition to premature delivery and fetal death (Ilie *et al.,* 2011; Pennington *et al.,* 2012).

Interrelationship between histomorphometrical changes and placental functions has been experimentally determined by various authors with conceivable results (Almasry and Elfayomy, 2012; Sankar *et al.,* 2013; Haeussner *et al.,* 2015; Kidron *et al.,* 2017).

Trophoblastic cells invasion and proliferation is the key step of placentation, PE is characterized by abnormal maternal spiral artery remodeling due an impaired invasion of fetal trophoblasts leading to reduced blood flow towards placenta, which disturbs the fetal and placental oxygen and nutritional need (Widdows, 2009). Therefore, the regeneration of the intermediate villi stops, and the remaining intermediate villi continue to differentiate into stem villi until the term (Roberts, 2008). Although placenta is a vital organ, but its histological examination has been neglected (Sankar *et al.*, 2013). However, in recent times more ideas have provoked the systemic studies of placenta for determination of disease cause such as PE and IUGR. (Kidron *et al.*, 2017). PE is linked with high maternal and neonatal morbidity and mortality accompanied with gross pathological changes in placenta (Pennington *et al.*, 2012). The pattern of placental villi varies considerably in histological sections and are divide into different types according to their structure, diameter, trophoblastic layer, stroma and capillaries (Corrêa *et al.*, 2008; Kidron *et al.*, 2017).

Until now the pathogenecity of PE is poorly understood and further research is required to understand the pathologies of PE. Therefore, the present study attempted to describe the potent role of oxidative stress markers in susceptibility of preeclampsia and to evaluate the histomorphological and histomorphometric changes of the placenta in preeclamptic Pakistani women.

MATERIALS AND METHODS

Patient identification and sample collection

This study was carried out with the prior approval ethical committees of both Quaid-i-Azam University, Islamabad and collaborating hospitals. Total 400 blood samples and 90 placental tissues were obtained. Among 400, 200 blood samples were collected from females suffering from Preeclampsia and 200 from healthy controls. While 50 placental tissues were collected from PE women and 50 from normal individuals at PIMS Islamabad and Quaid-e-Azam International Hospital, Islamabad. Informed consent and a detail performa were filled before sample collection. Sociodemographic characteristics, pregnancy and medical history were recruited from all studied subjects.

Inclusion and exclusion criteria

PE was considered with blood pressure ≥140/90 mmHg and new onset of proteinuria. The normotensive control group have women with uncomplicated gestation and blood pressure <125/85mmHg and no proteinuria (Kallela *et al.,* 2016). Exclusion criteria involved diabetes, asthma, kidney disease, hematological disorder, autoimmune disease, urinary tract infection, current or past history of smoking and eclampsia.

Sampling and Storage

Blood samples were collected in labeled tubes, serum was separated and stored at -80 ^{0}C for oxidative stress markers and antioxidant enzymes analysis. Placental tissues were obtained after termination of pregnancy according to the protocol described by Burton et al. (Burton *et al.,* 2014). Tissues were washed in phosphate buffer saline (PBS) and fixed in 10% formaldehyde for histological processing.

Antioxidant enzymes

Antioxidant enzymes were estimated in serum samples of patients having PE and healthy pregnant women.

ROS assay

Hayashi et al. protocol was followed for ROS detection (Hayashi *et al.,* 2007).

Procedure:

0.1 M sodium acetate buffer was prepared by dissolving 4.1 g of sodium acetate in 500 ml of distilled water. The pH was maintained at 4.8. Then 10 mg of N, N-Diethyl-p-phenylenediamine sulphate salt (DEPPD) in 100 ml of sodium acetate buffer was dissolved and a second solution was prepared by adding 50 mg of ferrous sulphate ($FeSO_4$) in 10 ml of sodium acetate buffer. Both the solutions were mixed in a ratio of 1:25 and incubated in dark for 20 min at room temperature. Then 20 µl was taken from the solutions mixture, 1.2 ml of buffer and 20µl of sample were taken in a cuvette and absorbance was checked at 505 nm by using Smart Spec TM plus Spectrophotometer. Three readings were taken for each sample after every 15 seconds.

TBARS assay

Method given by Wright et al. was used to estimate lipid peroxidation (Wright *et al.*, 1981).

Procedure:

Reaction solution consisted of 0.02 mM ferric chloride ($FeCl_{3)}$ of 100mM, 0.2 ml ascorbic acid of 100mM, 0.2 ml of sample and 0.58 ml phosphate buffer (0.1 M) with pH value of 7.4 made the total volume 1 ml. In water bath at temperature of 37 °C, final mixture was incubated for 1 hour and 1 ml of 10% trichloroacetic acid was used to end the reaction. Then addition of 1ml of 0.67 % thiobarbituric acid was done in water bath of boiling water, all the tubes were kept for 20 minutes and finally moved to crushed ice-bath. After that, centrifuged for 15 minutes at 25000 rpm and at 535 nm readings were noted from spectrophotometer.

CAT activity

With small modification, method of Chance and Maehly was used to find out the activities of CAT (Chance and Maehly, 1955).

Procedure:

To measure CAT levels in samples, 0.1 ml sample, 2.5ml of 50 mM phosphate buffer (pH 5.0) and 0.4ml of 5.9 mM H_2O_2 were added in a cuvette. Absorbance of solution was noted after one minute at the wavelength of 240nm. One unit of CAT activity was considered to be the change of absorbance at 0.01 units in one minute.

SOD activity

By using the protocol of Kekkar et al. activity of SOD was determined (Kakkar *et al.*, 1984).

Procedure:

For this purpose, 0.3 ml of sample, 1.2 ml of sodium pyrophosphate buffer (0.052 mM; pH 7.0) and 0.1 ml of phenazine methosulphate (186 μM) were mixed together and reaction was initiated by the addition of 0.2 ml of NADH (780 μM). Finally, after 1 minute by the addition of of glacial acetic acid (1 ml) reaction was ended and at 560 nm readings were noted.

POD activity

Determination of POD activity was done by using Chance and Maehly method (Chance and Maehly, 1955).

Procedure:

Reaction was carried out by adding 0.3 ml of 40 Mm H_2O_2, phosphate buffer (2.5 ml of 50 mM, pH= 5.0) and 0.1 ml of 20 mM guaiacol into 0.1 ml of sample. Change in absorbance was noted after one-minute at 470 nm. One unit of POD activity was considered to be the change of absorbance at 0.01 units in one minute.

Tissue Histology

Histology of placental tissues was done in order to determine the histomorphological and histomorphometric changes in placenta. Following collection of placental tissue, following steps were performed:

Fixation

10% formalin was used for fixation of placental tissues for 2 days.

Dehydration

Tissues were dehydrated after fixation at 25°C in the subsequent grades of ethyl alcohol. Tissues were placed in 70% ethanol for 120 minutes followed by 80% ethanol, then 90% ethanol. Tissues were then transferred to 100% ethanol. Three changes in 100% ethanol were given for 120 minutes each.

When dehydration was completed tissues were placed in xylene for the next 2 hours. Xylene cleared the tissues.

Embedding

Embedding of tissues was done by transferring the tissues from xylene to paraplast. Following sequence of steps is followed to get the tissues properly embedded.

1. Benzol + Paraplast (1:1) for 2 hours at 60 ºC
2. Paraplast for 14 hours at 62 ºC
3. Paraplast 2 for 4 hours at 62 ºC
4. Paraplast 3 for 4 hours at, 62 ºC

Following the above steps, tissues were shifted to a paper boat with molten paraffin. Bubbles were removed from it and let it harden. To cut the hardened wax blocks, a knife or blade was used, and these blocks were fixed on wooden wedges used for further process.

Microtomy

Tissue sectioning was done using microtome (Thermo, Shandon finesse 325, UK). Thin slices of ovaries and uteri (5-7 µm) thick were cut and placed in warm water to stretch, then fixed to clean glass slides coated with albumen placed on a slide warmer whose temperature is maintained up to 60 ºC. After drying, these slides were moved to the incubator (45 ºC) for 14-15 hours for complete stretching of sections and to remove any bubbles remained.

Staining

For staining of the slides, following procedure was performed:

Hydration of sections

Soon after removing the slides from the incubator, the slides were deparaffinized by placing in xylene twice for 5 minutes each time. After removing the slides from xylene, the slides were rehydrated by placing them in descending grades of ethanol.

At first, 100% of ethanol was given to each slide for up to 3 minutes. Then slides were moved to 90% ethanol for 2-5 minutes. After removing them from 90% ethanol, the same step was repeated in 70% ethanol which was followed by moving the slides to 30% ethanol for 2-5 minutes. The slides were then washed with water and placed in hematoxylin for 7-8 minutes. After 7-8 minutes tap water was run over each slide to remove the extra stain. After proper washing, the tissue turned blue. The slides were then placed in 95% acidified alcohol for 2 minutes. Then, slides were moved to the

bluing solution already prepared by mixing water with few drops of ammonium hydroxide and kept intact for 1 min. The slides were then washed with tap water for 1-2 min. After washing, the g slides were transferred to 70% ethanol for 2 minutes. After 2 minutes, Slides were placed in the eosin stain for 2 minutes and then washed with water for 1 minute. Then, each slide was placed in 90% ethanol for 2 minutes and then moved to 100% ethanol and placed in it for 2-5 minutes. Finally, each slide was given two changes in xylene for 5 minutes each wash.

For permanent mounting, two to three drops of mount DPX were added on the slides and the slides were covered with cover slips.

Light Microscopy

Stained slides were observed under Leica Microscope. (New York, USA) connected with a digital camera (Canon, Japan). Every 10th section was examined under microscope at (20 or 40X). The number of follicles were counted. Morphometric analysis of villous particles was done using Image J software.

Statistical Analysis

All data were expressed as a median along with 95% confidence intervals (CI). Quantitative data will express as mean ± S.E. Significant differences between groups for antioxidant levels were determined by performing Welch two sample t test by using package of R 3.5.1 (R Development Core Team, 2018). Histomorphometric characteristics between both groups were compared by performing Independent sample t test using IBM SPSS Statistics 21 software.

RESULTS

Anti-Oxidants

Oxidative stress markers such as antioxidant enzymes including SOD, CAT, POD, ROS and TBARS were evaluated in both groups (Table 8). There was no difference in SOD (*P*=0.20), CAT (0.81) and POD (0.11) levels in PE group as compared to control group (Fig. 40 and 41). While, significantly elevated levels of TBARS (0.04) and ROS (<0.001) were observed in preeclamptic women as compared to normotensive pregnant women. (Fig. 42-43).

Table 8: Oxidative stress markers in Control and Preeclamptic group.

Parameters	Controls	PE Patients	P value
SOD (U/ml)	14.74±0.07	14.75±0.01	0.97
CAT (U/ml)	10.49±1.10	10.14±1.04	0.81
POD (nmole)	12.71±0.56	13.72±0.47	0.11
TBARS (nM/ml)	27.80±1.78	33.41±2.09	0.04
ROS (U/ml)	1.77±0.02	2.1±0.07	<0.001

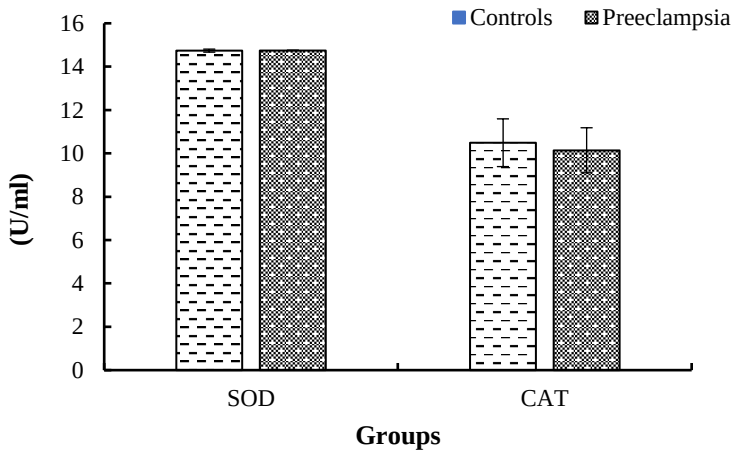


Figure 40. Mean ± SEM levels of SOD and CAT in control and preeclamptic group.

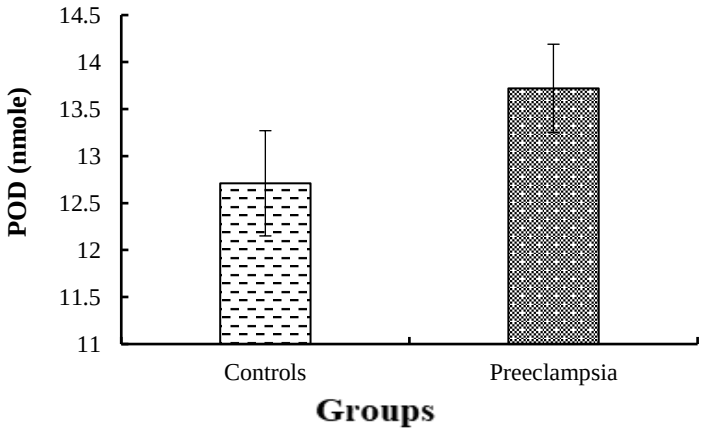


Figure 41. Mean ± SEM levels of POD in normotensive and preeclamptic women.

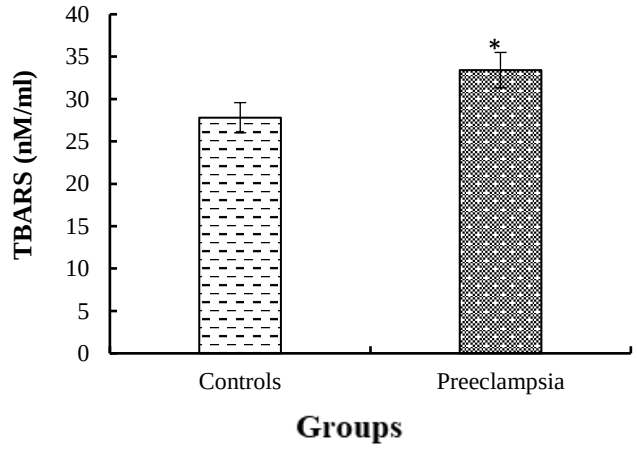


Figure 42. Mean ± SEM levels of TBARS in control and preeclamptic women.

* indicate significant difference in PE group at probability of $p<0.05$ in comparison to control group.

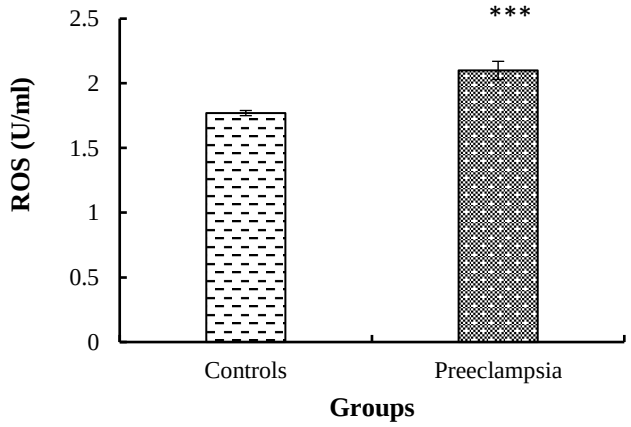


Figure 43. Mean ± SEM levels of ROS in control and preeclamptic group.

***indicate significant difference in PE group at probability of $p<0.001$ when compared with control group.

Histological analysis

Results of histomorphological findings indicated various abnormal villi in PE placentas as compared to control group. Preeclamptic placentas showed Stem villous with contracted vessels (Fig. 44A), perivillous fibrin (PVF) deposition (Fig. 44B), obliteration of vascular lumen (Figure 44C) and atheromatous plaque (AP) (Fig. 44D). Similarly, syncytial knots (SK) (aggregated syncytial nuclei in the outer surface placental villous) along with thin and elongated villi were more in PE placentas than control group (Fig. 44E & F).

Results of histomorphometric analysis of placenta are given in Table 9. Significant decrease in total area (<0.05) and circularity (<0.01) of VP was observed in PE placentas as compared to control group (Fig. 47 and 48). However non-significant difference was observed in no. of villi, perimeter, ferret diameter, minimum ferret diameter and percentage of villi per image between both groups (Fig. 46, 48-50). VP were further divided into three types small, elongated and large according to the criteria given by Kidron et al.(Kidron *et al.*, 2017) (Table 10). Small VP were rounded with greater than 0.3 circularity (i.e. VP 16, 19, 22, 23, Fig. 45C), elongated VP have circularity of 0.2-0.3 (VP 14, Fig. 45C) and large VP were determined by irregular outlines with less than 0.2 circularity (VP 5, Fig. 45C).

A significant decrease in percentage area (<0.001) of small VP was reported in PE placentas and approximately 47% area was occupied by small VP (Fig. 57). While no. of villi, area, perimeter, ferret diameter and minimum ferret diameter determined no significant change in both groups. Elongated villi determined significant decline in area (<0.05), perimeter (<0.05), circularity (<0.001), ferret diameter (<0.05) and minimum ferret diameter (<0.01) in PE placentas as compared to control group (Fig. 52-56). However no of villi and percentage area per image of elongated VP remain unchanged in both groups. Large VP determined significant decrease in area (<0.01), perimeter (<0.05) and ferret diameter (<0.001), circularity (<0.001) and percentage area (<0.01) of preeclamptic placenta as compared to normotensive pregnant women (Table 10, Fig. 52-57).

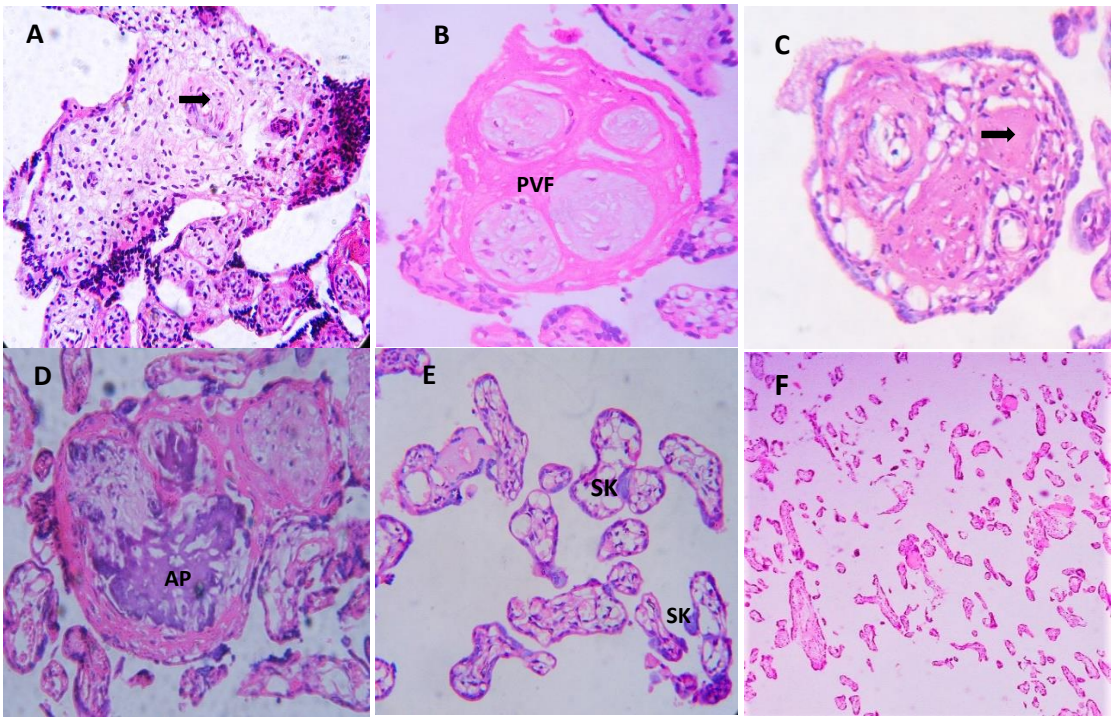


Figure 44: Hematoxylin and eosin stained sections of PE placentas. (A) Stem villous having contracted vessels. (B) Peri villous fibrin (PVF) deposition in stem villous. (C) Obliteration of vascular lumen in stem villous. (D) Atheromatous plaque (AP) formation in stem villous (E) syncytial knots (SK) (F) Thin and elongated villi in PE. (A-E, 40X, F, 20X)

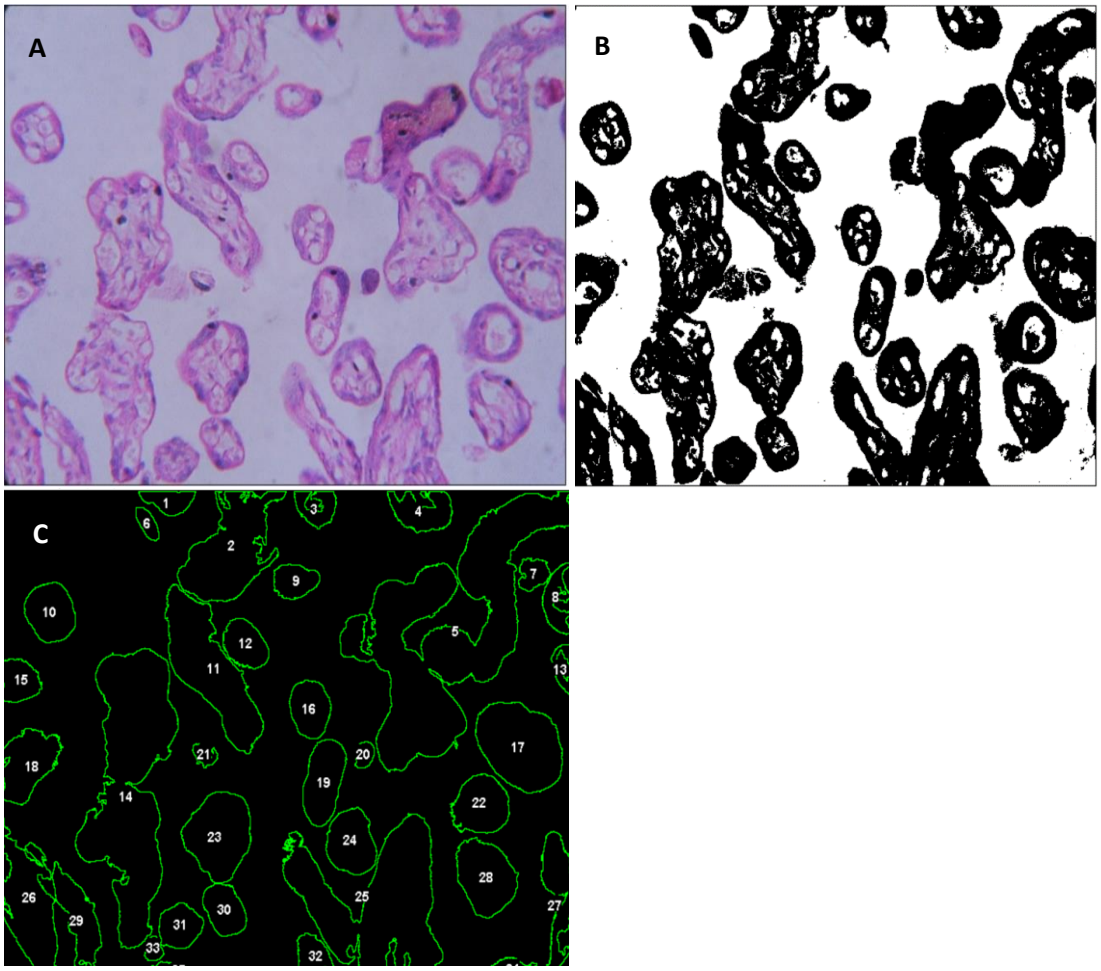


Figure 45: Analysis of placental villi by ImageJ software. A: Normal appearance of villi (H&E, 40 X). B: Villi with adjusted threshold. C: Outlines of villi after particle analysis.

Table 9: Measurements of villous particles in control and preeclamptic group.

Parameters	Controls	PE	p value
No. of villi	2080	2968	
Area ($x10^4$µm)	12.66±0.79	10.62±0.92	0.02
Perimeter ($x10^2$µm)	23.24±1.17	24.98±1.49	0.36
Circularity	0.3±0.012	0.21±0.013	<0.01
Feret diameter (µm)	441.92±12.78	421.62±16.72	0.34
Minimum feret diameter (µm)	248.19±7.66	233.65±11.25	0.29
percentage area per image	36.69±1.46	35.30±1.64	0.52
No. of villi per image	20.75±1.12	18.7473±0.81103	0.15

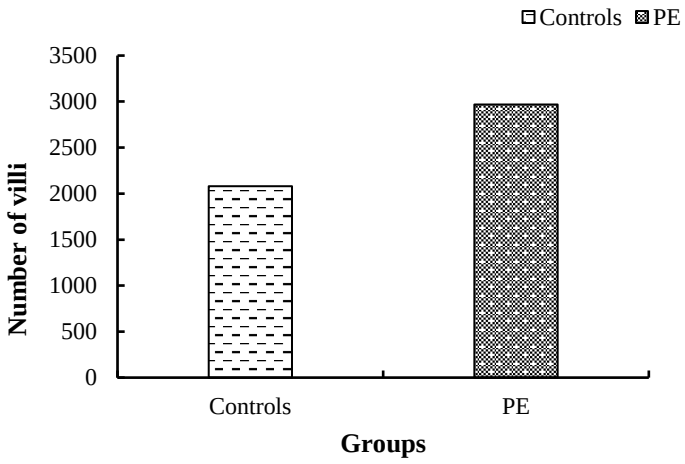


Figure 46. Mean number of villi in control group and preeclamptic women.

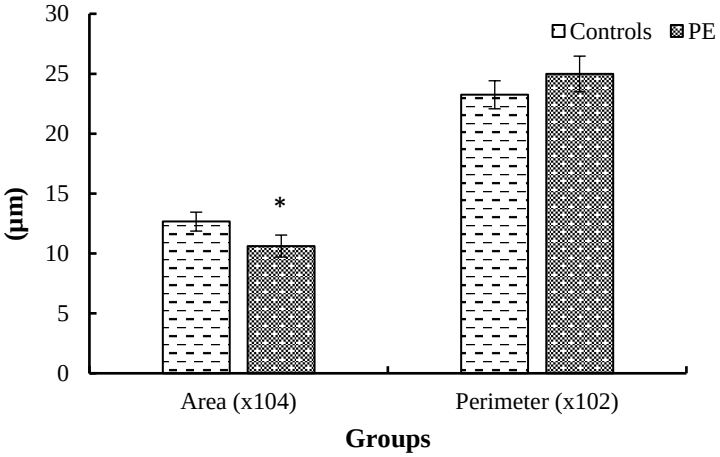


Figure 47. Mean ± SEM area and perimeter of villous particles (VP) in normotensive pregnant and preeclamptic women.

* indicate significant difference in PE group at probability of $p<0.05$ in comparison to control group.

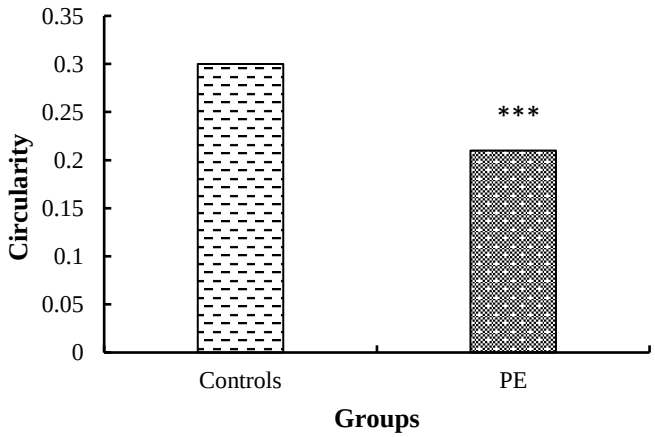


Figure 48. Mean ± SEM circularity of villous particles (VP) in control and preeclamptic group.

*** indicate significant difference in PE group at probability of $p<0.001$ when compared to controls.

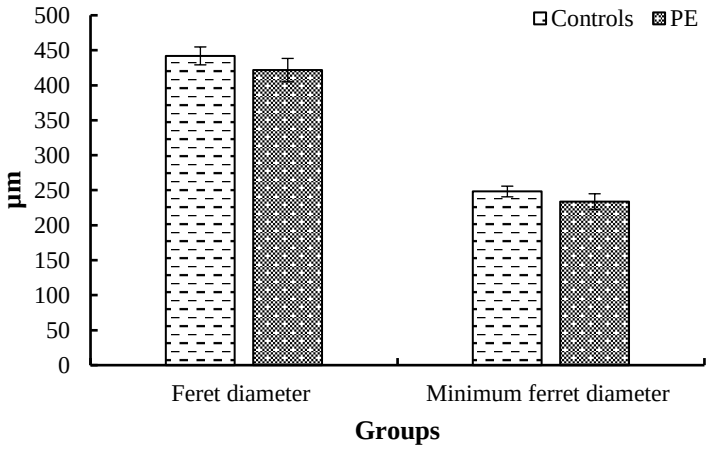


Figure 49. Mean ± SEM feret diameter and minimum ferret diameter of VP in normotensive pregnant and preeclamptic women.

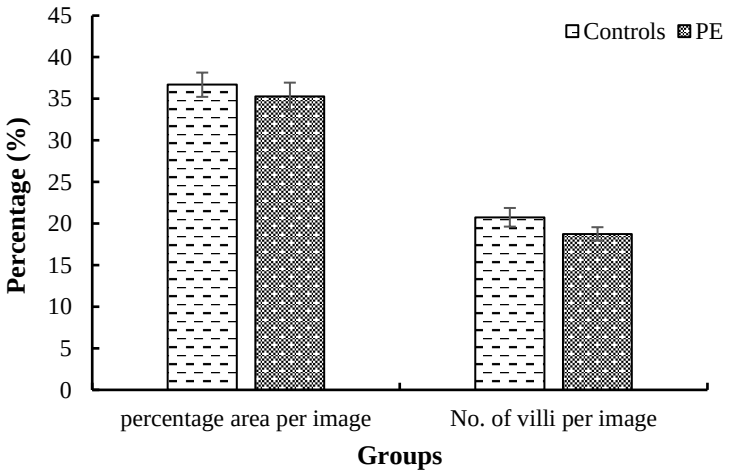


Figure 50. Mean ± SEM percentage area and number of villi per image in control and preeclamptic group.

Table 10: Measurements of villous particles conferring to size in normotensives and preeclamptic group.

Size	Parameters	Controls	PE	p value
Small	**No. of villi (%)**	922 (44.32)	1390 (46.83)	
	Area (x10^{4}µm)	1.41±0.20	1.11±0.23	0.50
	Perimeter (x10^{2}µm)	10.00±0.21	9.43±0.23	0.59
	Circularity	0.50±0.01	0.49±0.01	0.23
	Feret diameter (µm)	147.71±4.58	142.92±5.22	0.48
	Minimum feret diameter (µm)	148.66±2.001	145.83±2.71	0.39
	% area per image	88.01±0.38	84.43±0.62	<0.001
Elongated	**No. of villi (%)**	713 (34.27)	845 (28.47)	
	Area (x10^{4}µm)	7.88±0.79	5.74±0.48	0.01
	Perimeter (x10^{2}µm)	16.60±0.78	14.32±0.63	0.02
	Circularity	0.24±0.00	0.23±0.00	<0.001
	Feret diameter (µm)	400.73±21.26	362.65±16.00	0.02
	Minimum feret diameter (µm)	223.66±10.54	185.24±8.62	<0.01
	% area per image	89.45±0.71	89.24±0.85	0.86
Large	**No. of villi (%)**	445 (21.39)	733 (24.69)	
	Area (x10^{4}µm)	13.80±1.04	11.65±1.09	<0.01
	Perimeter (x10^{2}µm)	38.92±2.14	33.27±1.28	0.01
	Circularity	0.11±0.00	0.08±0.00	<0.001
	Feret diameter (µm)	593.70±20.40	459.85±12.58	<0.001
	Minimum feret diameter (µm)	308.19±12.21	237.83±7.66	<0.001

% area per image	89.13±0.40	90.66±.0.29	<0.01

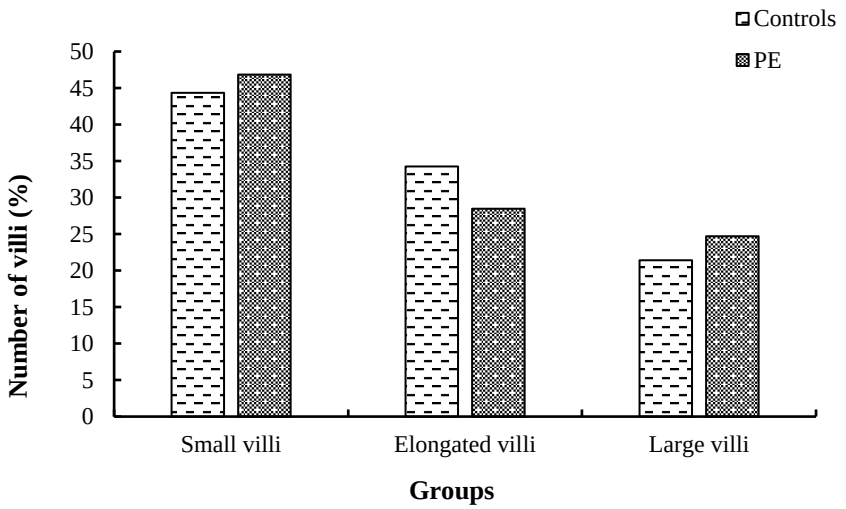


Figure 51. Mean number of small, elongated and large villi in placenta of normotensive pregnant and preeclamptic women.

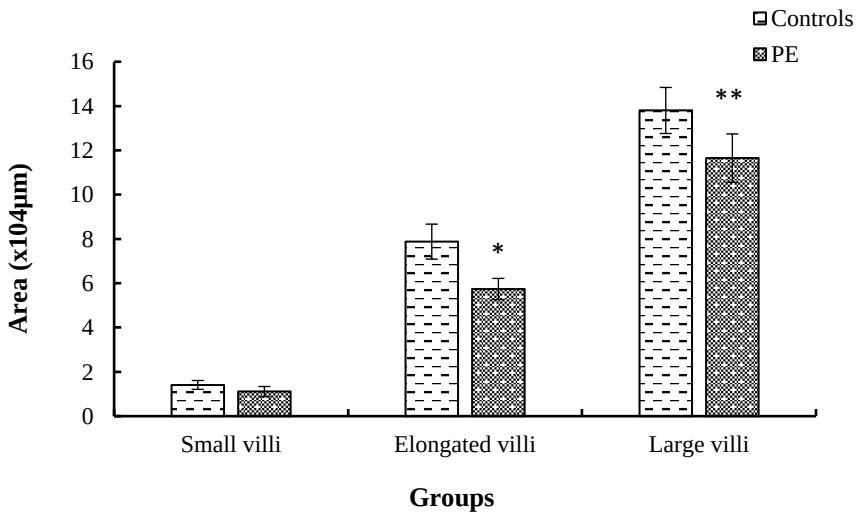


Figure 52. Mean ± SEM area of small, elongated and large villi in placenta of healthy pregnant females and women with PE.

* and** indicate significant difference in PE group at probability of p<0.05 and p<0.01 when compared to control group.

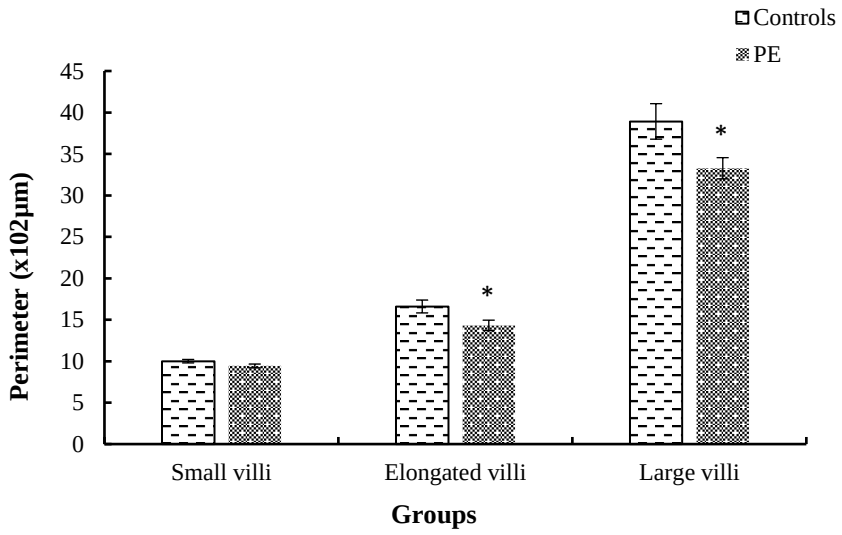


Figure 53. Mean ± SEM perimeter of small, elongated and large villi in placenta of control and preeclamptic group.

* indicate significant difference in PE group at probability of $p<0.05$ in comparison to control group.

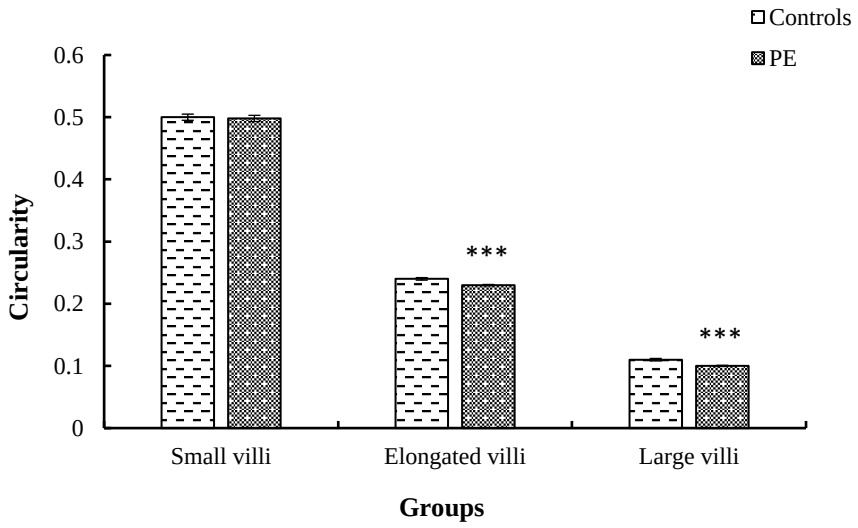


Figure 54. Mean ± SEM circularity of small, elongated and large villi in placenta of healthy pregnant and preeclamptic women.

*** indicate significant difference in PE patients at probability of $p<0.001$ when compared to controls.

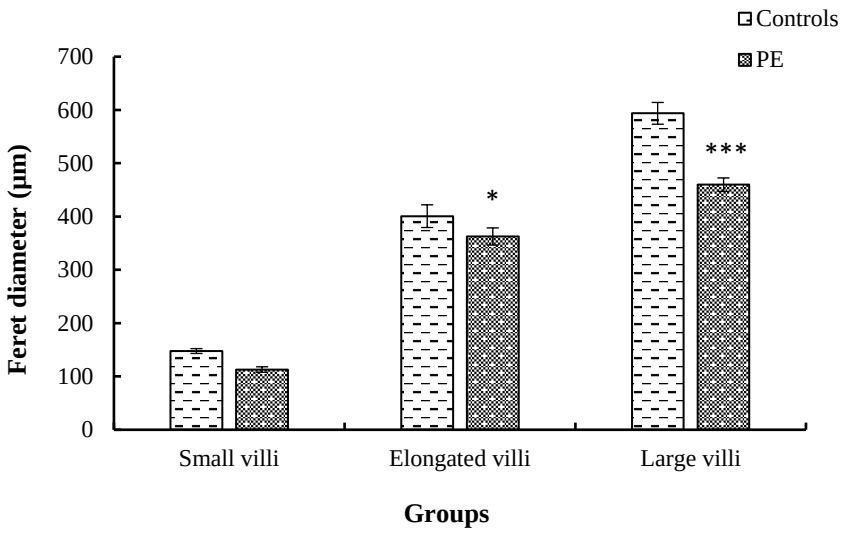


Figure 55. Mean ± SEM feret diameter of small, elongated and large villi in placenta of normotensive pregnant and preeclamptic women.

* and*** indicate significant difference in PE group at probability of $p<0.05$ and $p<0.001$ respectively when compared to controls.

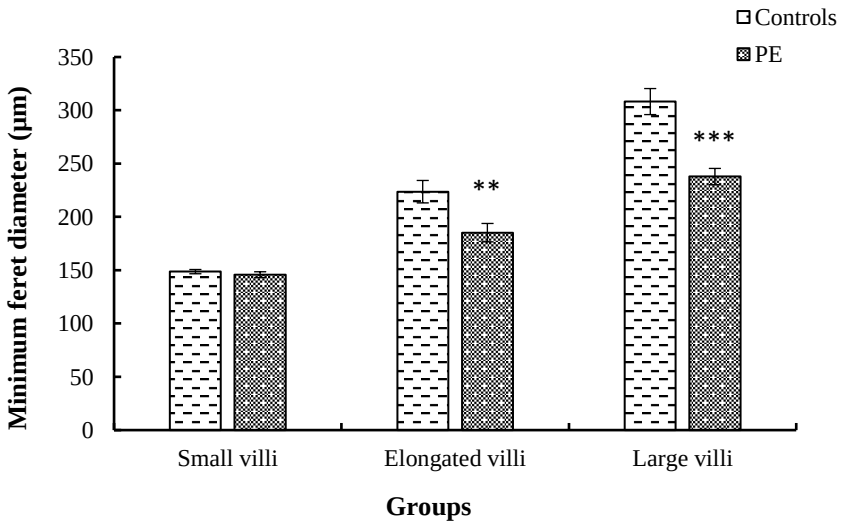


Figure 56. Mean ± SEM minimum feret diameter of small, elongated and large villi in placenta of control and preeclamptic group.

** and *** indicate significant difference in PE women at probability of $p<0.01$ and $p<0.001$ respectively in comparison to healthy women.

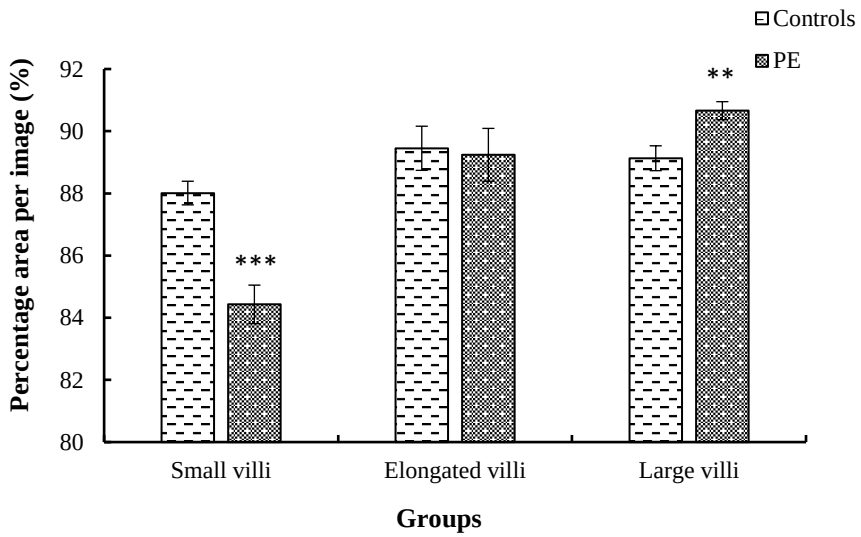


Figure 57. Mean ± SEM percentage area per image of small, elongated and large villi in placenta of control and preeclamptic group.

** and *** indicate significant difference in PE group at probability of $p<0.01$ and $p<0.001$ respectively in comparison to control group.

DISCUSSION

Pregnancy hypertensive disorders remain a major health concern worldwide for women and infants (Balogun and Sibai, 2017). PE is a great challenge to obstetricians because it has complex pathophysiology with unknown cause and involvement of several organs systems (Madoglio *et al.,* 2016). The presence of specific markers may be used for diagnosis of disease as an alternative to the syndrome of hypertension, proteinuria, and edema (Steegers *et al.,* 2010). Pathophysiological roots of the foremost complications of pregnancy, PE have roots in poor placentation because placenta signifies the platform for a healthy life, as it is the interface between the mother and fetus (Burton, 2012).

Possible underlying mechanism involves the changes in maternal blood pressure during pregnancy through various factors initiated by placenta. In case of uteroplacental insufficiency, reduced blood flow towards placenta results rise in maternal blood pressure to accommodate the blood supply towards placenta (Pridjian and Puschett, 2002; Alpoim *et al.,* 2011). Transportation of nutrients from maternal to fetal circulation through placenta depends on the blood delivery to the uterus, which is directly related to maternal cardiac output and local factors controlling uterine perfusion (Pridjian and Puschett, 2002).

In PE poor placental perfusion is a result of abnormal development of the placenta. The abnormal and poor trophoblastic invasion is the defined characteristic of placenta of women with PE (Eiland *et al.,* 2012). It was demonstrated that this results in oxidative stress, hypoxia, and the release of factors that promote endothelial dysfunction, inflammation, and other possible reactions (Ilie *et al.,* 2011; Ukah *et al.,* 2018).

In present study elevated ROS and TBARS levels were observed in PE women as compared to normotensive controls. Similar studies showed that increased ROS and TBARS bioactivity exceeding antioxidant activity leads to oxidative stress (Matsubara *et al.,* 2015). It can possibly be one of the risk factors for the development of PE through endothelial dysfunction and increased contractility resulting in placental hypoxia and ischemia (Touyz, 2004; Matsubara *et al.,* 2010). It was demonstrated that free radicals attack free fatty acids in cell membrane leads to the production of lipid peroxides which may cause endothelial dysfunction in PE patients (Gupta *et al.,* 2005; Gupta *et al.,* 2009).

According to histological perspectives of this study, abnormalities in stem villi were detected. Preceding studies also exhibited such results in which contracted vessels, PVF deposition and obliteration of vascular lumen AP formation in stem villous were observed in PE placentas (Allaire *et al.,* 2000; Sankar *et al.,* 2013; Khong *et al.,* 2016). In present observations SK were more in PE patients as shown in study conducted by Sankar et al. proposing that the functional impairment of the placenta is due to structural alterations of the villous syncytiotrophoblast (Sankar *et al.,* 2013). Thin and elongated villi were also more in PE as detected by Khong et al. (Roberts, 2008). These changes affect the utero-placental blood flow and significantly decrease the birth weight of the neonate. Placental villi represented significant decrease in area and circularity of PE as compared to controls. The difference was more prominent in elongated VP (150-250 µm in diameter) which is probably similar to intermediate villi, and large villi (>250 µm in smaller diameter) representing aggregates of adherent or closely approximated villi or immature intermediate villi. Terminal villi were characterized as small VP (50-150 µm in diameter) showing non-significant difference in both groups.

After 21st day of conception vascularization of placental villi begins. Villous growth begins to change from branching to nonbranching angiogenesis after 26 weeks of gestation. Maternal and fetal circulation takes place through these important structures, in case of poor vascularization the fetus is at high risk of hypoxia and low birth weight (Teasdale, 1987; Krielessi *et al.,* 2012). Here terminal villi are not lesser in PE which determine the natural compensation of placental dysfunction and feto maternal nutrients exchange. Quality of life can be improved, and disease burden can be potentially lightened by understanding the underlying mechanisms of placental maturation and perfusion (Avagliano *et al.,* 2016).

Conclusion

Findings of the present study showed that oxidative stress markers can play a causative role in pathogenesis of PE so, it is important to improved management of the condition before it threatens the survival of mother and fetus. This study also contributed to the histomorphological and histomorphometric characteristics of preeclamptic women placental tissues as compared to normotensive women, allowing for a greater agreement that pathological examination of the placenta is an important parameter for detection of placental alterations during PE. This approach may facilitate grading placentas for diagnostic and research purposes. Further work must be done on histological

examination of placenta which can provide useful information in the determination of various reasons and mechanisms involved in poor pregnancy outcomes and useful to health care providers both for parent counselling and as a legal defence in cases of medical malpractice allegations.

Future recommendations

Longitudinal studies are required to detect biomarkers of oxidative stress or a combination of these biomarkers with high sensitivity and early PE prediction specificity. This will assist in defining high-risk females who need close pregnancy monitoring. Large, randomized and controlled antioxidant supplementation studies are needed to further strengthen the efficacy of these approaches in overcoming oxidative stress and prevent PE.

SUMMARY

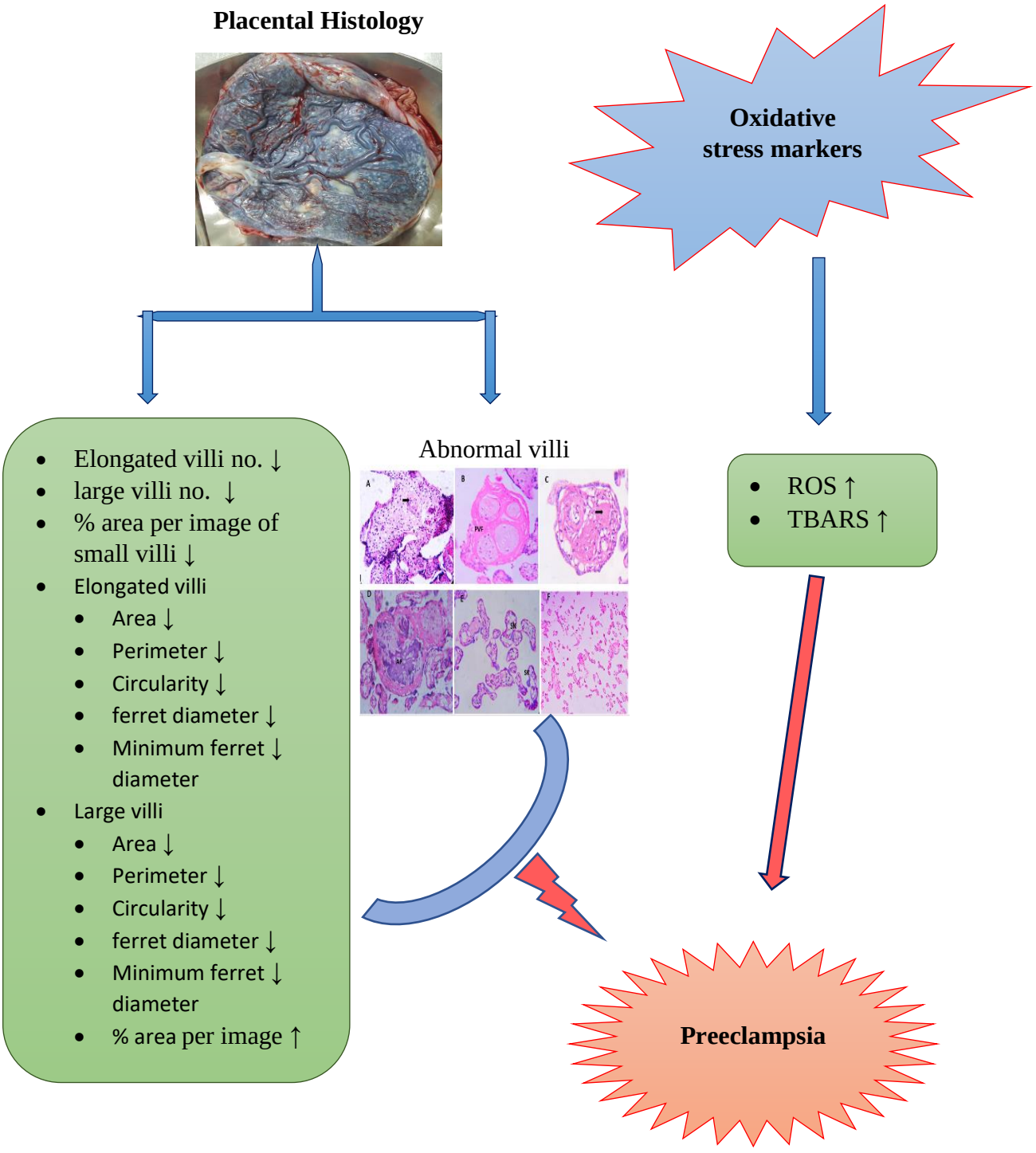


Figure 58: Graphical representation of histological aspects of placenta and role of oxidative stress in susceptibility to PE in Pakistan

CHAPTE 3

Placental expression of eNOS and hormonal analysis of preeclamptic women in Pakistan.

ABSTRACT

Preeclampsia (PE) is a hypertensive disorder of pregnancy characterized by de-novo development of concurrent hypertension and proteinuria. The prevailing theory determined that PE starts from the placenta and ends in the maternal endothelium. Role of endothelial dysfunction in the onset of PE has been reported in different populations. Alterations in hormones released from hypothalamic adrenal axis such as epinephrine which released during stress condition and thyroid hormones may interferes in vasodilation leading to endothelial impairment. Therefore, present study was designed to investigate the localization and expression of endothelial nitric oxide synthase (eNOS) and role of epinephrine and thyroid hormones in preeclamptic Pakistani women. A total of 400 blood samples (PE=200, controls=200) and 100 placental tissues (PE=50, controls=50) were recruited from pregnant women. Nitric oxide (NO), epinephrine, triiodothyronine (T_3), thyroxine (T_4) and thyroid stimulating hormone (TSH) levels were analyzed through spectrophotometer and enzyme linked immunosorbent assay (ELISA). Immunohistochemistry and quantitative real time polymerase chain reaction (qRT-PCR) was done to estimate the localization and expression of eNOS in placentas of PE patients and healthy pregnant women. Reduced NO ($p=0.016$), eNOS immunoreactivity ($p\leq0.001$) and mRNA abundance ($p\leq0.001$) was observed in preeclamptic group as compared to control group. Thyroid hormones demonstrated non-significant alterations in both groups. Significant positive correlation was observed in creatinine and urea levels of PE patients while systolic blood pressure (SBP) and diastolic blood pressure (DBP) showed significant positive correlation with each other and with age. In conclusion, our data suggested that elevated epinephrine, decreased NO levels and placental eNOS expression might play a role in the pathology of PE seen both in placenta and ultimately in maternal endothelium. These alterations lead to high blood pressure and renal insufficiency in preeclamptic patients. However, further studies are necessary to validate these findings to prevent maternal and neonatal morbidity and mortality in Pakistani population.

INTRODUCTION

PE is a hypertensive disorder of pregnancy characterized by de-novo development of concurrent hypertension and proteinuria, is a major contributor to maternal mortality, intrauterine growth retardation, premature birth, and perinatal mortality (Steegers *et al.,* 2010). Setting up premature delivery before 34 weeks of gestation is the only effective treatment available for PE to avoid severe maternal complications, but this procedure have chances of serious neonatal damage (Pennington *et al.,* 2012; Lisonkova and Joseph, 2013).

Placenta is the principal contributor to the pathophysiology of PE, as this syndrome resolved when placenta is delivered (Raghupathy, 2013). Mechanistically, it was suggested that main contributors towards onset of PE are reduced placental perfusion and endothelial dysfunction which occurred due to poor trophoblastic invasion and abnormal spiral arteries remodeling (George and Granger, 2010). Central to the symptoms of PE, uteroplacental hypoxia, angiogenic and anti-angiogenic protein deficiency (Wang *et al.,* 2009), oxidative stress (Gupta *et al.,* 2005), maternal endothelial dysfunction (Gilbert *et al.,* 2008) and elevated systemic inflammation are the main outcomes (Manuel-Apolinar *et al.,* 2013).

Pregnancy related stress derived from pregnancy such as fear of giving birth, lack of support from friends and family and unplanned pregnancy, also effect maternal and neonatal health through various mechanisms (Zhang *et al.,* 2013). Catecholamines such as epinephrine released by hypothalamic adrenal axis during stress may interfere with the vasodilator action of NO leading to endothelium impairment (Toda and Nakanishi-Toda, 2011).

Pregnancy is associated with many hormonal changes. Excess or deficiency of maternal thyroid hormones can also influence on reproductive health of women and fetal outcomes at all stages of gravidity. These hormones can also interfere with ovulation and fertility (Sardana *et al.,* 2009). Function of thyroid that are unique to pregnancy has been reported to be altered in numerous conditions (Khadem *et al.,* 2012). The mechanism of hypothyroidism and its clinical significance is controversial in PE, while, increased levels of endothelin and decreased protein concentrations in plasma may be related to this condition (Harshvardhan *et al.,* 2017).

Hypothyroidism can leads to contraction of vascular smooth muscle both in renal and systemic vessels, which results in increased diastolic hypertension, decreased tissue perfusion and peripheral vascular resistance (Satyanarayan *et al.,* 2015). Proteinuria can also be associated with thyroid dysfunction, which results in increased excretion of thyroid-binding globulins and thyroxine (Negro and Mestman, 2011). One of the pathophysiologic causes of PE has been suggested to be the physiological changes in thyroid gland in all stages of pregnancy (Muraleedharan and Janardhanan, 2017).

Not all free radicals cause disturbances in the organism, NO is an example of such radicals (Palmer *et al.,* 1988; Moncada *et al.,* 199; 1Kalyanaraman, 2013). NO is a low molecular weight mediator and highly reactive free radical, it mediates function of endothelium by regulating platelet aggregation, leukocyte adhesion, vascular tone and smooth muscle cells development (Qian and Fulton, 2013). Endothelial dysfunction is defined as the decreased bioavailability of NO in many ways such as through decreased production or enhansed consumption by oxidative stress (Chen *et al.,* 2014).

It has been reported that women with PE have decreased concentrations of NO both in plasma and in the placenta, it is postulated that reduced concentrations of NO might effect on uteroplacental blood flow through lack of vasodilatory effect, otherwise the the role of NO in the pathophysiology of PE has not been well defined (Brosens *et al.,* 2002; Dikensoy *et al.,* 2009). NO is synthesized by the eNOS enzyme (Qian and Fulton, 2013).

NO is formed from the reduction of L-arginine to L-citruline (Palmer *et al.,* 1988; Moncada *et al.,* 1991; Moncada and Higgs, 1993). In the placenta, cytotrophoblast is connected with eNOS expression to differentiate in syncytiotrophoblast (Eis *et al.,* 1995). The activity of eNOS in PE is still debatable; eNOS has been shown to be unchanged, decreased or increased in activity in women with PE as compared with normotensive pregnant women (Sánchez-Aranguren *et al.,* 2014).

Biologically and clinically, PE-complicated pregnancy has been extensively studied, but it is unfortunate that the mechanism of this complication is not clear yet. Considering clinical symptoms of PE, different mega projects and meta-analysis were conducted to better understand the underlying mechanisms but there are many gaps to be filled.

The present study was directed to determine the role of epinephrine, NO and thyroid hormones in susceptibility to preeclampsia, in addition, to elaborate the localization and expression of eNOS in the placenta of preeclamptic Pakistani women.

MATERIALS AND METHODS

Identification of patients and collection of samples

This research was carried out with the initial permission of both ethical boards including Quaid-i-Azam University, Islamabad and collaborating hospitals. Approximately 400 blood samples of pregnant women (200=controls, 200=PE) were recruited according to the diagnostic criteria for PE before delivery. Total 100 placental tissues (PE=50, controls=50) were collected after spontaneous vaginal delivery (SVD) and Caesarean section (C-section) at PIMS Islamabad and Quaid-e-Azam International Hospital, Islamabad. Informed consent and a detail performa were filled before sample collection.

Inclusion and exclusion criteria

Diagnostic criteria for preeclampsia was blood pressure ≥140/90mmHg, and proteinuria ≥+1 on Dipstick test. The normotensive control group have women with uncomplicated gestation and blood pressure <125/85mmHg and no proteinuria (Kallela *et al.*, 2016). Women with less than 35 years of age were included in the study. Exclusion criteria involved diabetes, asthma, kidney disease, hematological disorder, autoimmune disease, urinary tract infection, current or past history of smoking and eclampsia.

Sampling and Storage

Blood samples were collected in labelled EDTA tubes, serum was separated and stored at -80 ^{0}C for further analysis. After vaginal delivery or caesarean section in less than ten minutes, fresh placental villous biopsies were drawn from different locations of each placenta according to the protocol described by Burton *et al.*,2014. Tissues were washed in phosphate buffer saline (PBS) and half tissue was fixed in 10% formaldehyde for immunohistochemical processing and half tissue was snap-frozen immediately in liquid nitrogen and maintained at -80 °C for subsequent mRNA aanalysis using qRT-PCR.

Epinephrine

Epinephrine concentrations was determined quantitatively by Adrenaline/Epinephrine ELISA Kit (Catalog # E4359-100, Biovision, USA).

Principle:

The principle of epinephrine hormone ELISA is based on the competitive incubation binding with epinephrine specific antibody, between biotin labeled epinephrine in the test samples.

Procedure:

For the measurement of epinephrine in the tested samples, serum was coagulated for 2 hours at room temperature and then centrifuged at approximately 1000×g for 20 min. Supernatant was gathered and immediate assay was performed. According to the manufacturer instruction, all reagents, specimens and norms were prepared. With 1X Wash Solution, the plate was washed twice before adding standards, sample and control to the wells. 50 µl of each standard or sample was added to the suitable wells and 50 µl of the antibody work solution for biotin detection was added to the wells instantly. Plate was sealed and incubated at 37ºC for 45 min. After the incubation wells were carefully washed three times by auto washer and dried by gently tapping on filter paper. Followed by washing, 100 µl of of SABC working solution was dispensed in each well, again microplate was covered and incubated at 37ºC for 30 min. With 1X Wash Solution, the plate was washed five times. In each well, 90 µl of TMB substratum was subsequently dispensed, coated the plate, and incubated in dark for 15-30 minutes at 37ºC. The shades of blue have been seen, the intensity of the color in the sample is inversely proportional to the quantity of epinephrine. After incubation, Stop Solution (50 µl) was dispensed after incubation. The plate was gently shaken to mix thoroughly and read result at 450 nm within 20 minutes by microplate reader (Platos R 496, USA).

Estimation of NO

For the quantitative determination of NO, total NO kit (Cat# EMSNO), Thermo Scientific, USA) was used to convert nitrate to nitrite according to the manufacturer's guidelines.

Principle

NO decomposes from nitrite (NO_2^-) and nitrate (NO_3^-). Nitrite is the stable end product and, using Greiss reaction, it is estimated as an index of NO. The Griess reaction, in which visible light has been absorbed at 540 nm, detects nitrite as a colored azo dye product.

Nitrite assay procedure

Standard solution for nitrite has been allowed to warm up to room temperature. The standard's serial dilutions were then made by adding 1X Reagent Diluent. All samples and standards were executed in duplicate. In blank wells, about 200 µl of 1X Reagent Diluent was added. 50 µl of nitrite standards and specimens were added to suitable wells, while 50 µl of 1X Reagent Diluent was added to zero standard, standard and sample wells. Afterword, Griess Reagent 1 (50 µl) was added to each well, except for the blank wells followed by 50 µl of Griess Reagent 2 being added to each well, except for the blank wells. To mix the contents, the plate was gently tapped. Plate was incubated for 10 minutes at room temperature. Plate reader was blanked against the blank wells after incubation and read the optical density at 540 nm ±20 nm.

Nitrate assay procedure

Standard nitrate solution was allowed warming up to room temperature. Serial dilutions of the standard were then made by the addition of 1X Reagent Diluent. Approximately 200 µl of 1X Reagent Diluent was added in blank wells. 50 µl of nitrite standards and test samples were added to suitable wells, while 50 ul of 1X Reagent Diluent was added to zero standard, standard and sample wells. 25 µl of diluted NADH was added into all zero standard, standard and sample wells followed by the addition of 25 µl of diluted nitrate reductase enzyme into all wells. Plate was tapped gently to mix the contents. Plate was sealed and incubated at 37°C for 30 minutes. After incubation 50 µl of Griess Reagent 1 and 50 µl of Griess Reagent 2 was added into each well, except the blank wells. Plate was then tapped gently to mix the contents and incubated at room temperature for 10 minutes. Plate reader was blanked against the blank wells after incubation and read the optical density at 540 nm ±20 nm.

Calculation of results

The nitrite concentration was calculated corresponding to the nitrite standard curve mean absorbance.

To determine the sample nitrate concentration:

a. The concentration of endogenous nitrite (X µmol/L) was assessed using nitrite Assay.

b. Total nitrite concentration (Y µmol/L) was estimated using the nitrate reduction assay procedure after converting nitrate to nitrite.

c. The total sample NO concentration was determined by subtracting the endogenous concentration of nitrite from the total concentration of nitrite.

Total NO = (Y-X) μmol/L

Since samples were diluted, the dilution factor was multiplied by the concentration read from the normal curve.

Quantitative determination of T_3

Estimation of T_3 (ng/ml) was done by using ELISA kit (Cat# 108685, abcam, USA) according to the manufacturer's guidelines.

Principle of the Assay:

1. All materials and prepared reagents were equilibrated to room temperature ahead to use.
2. All standards, controls and samples were assayed in duplicates.
3. Respected wells were added 50 μL of standards and samples. Diluted T_3 HRP (100 Ml) was added and conjugated to each well. Blank well was left for substrate blank.
4. Wells were covered with the foil supplied in the kit and were incubated at room temperature for 1 hour.
5. Foil was removed and content of the wells were aspirated, and each well was washed five times with 300 μL of 1X washing solution.
6. TMB substrate solution in the quantity of 100 μL was added to all wells. This solution was incubated at room temperature (18–25°C) in the dark for straight 15 minutes.
7. Afterwards, 100 μL of stop solution was added into all well in exactly the same pattern and at the same rate as it were for substrate solution. Microplate was gently shaken. Any blue color developed was gradually turned to yellow after being incubated. Read the microplate at 450nm within 5 minutes.

Quantitative determination of T_4

Quantitative estimation of T_4 (ng/dl) was done by using ELISA kit (Cat# RE55261, IBL International, Germany) according to the manufacturer's guidelines.

Principle of the Assay:

1. A required number of microtiter wells were assured in the frame holder.
2. Then, 10 µL of each standard, control and samples were dispense out with the new disposable tips into suitable wells.
3. These were incubated at room temperature for 5 minutes.
4. In each well 100 µL Enzyme Conjugate was dispensed. It was thoroughly mixed for 10 seconds.
5. Incubation was undergone for 80 minutes at room temperature (18–25 °C).
6. Swift shaking of content of wells were carried out.
7. With diluted wash solution (400 µL per well) wells were rinsed 5 times. After that wells were striked on absorbent paper to drain out any leftover droplet.
8. Substrate solution at the quantity of 100 µL was added to each well. Incubation was then carried out at room temperature (18–25 °C) for 10 minutes.
9. Enzymatic reaction was ceased by adding 100 µL of stop solution to each well and read at 450 ± 10nm with a microtiter plate reader within 10 minutes after stop solution was added.

Quantitative determination of TSH

Quantitative determination of TSH (µIU/ml) was done by using ELISA kit (Cat# SE120135, Sigma-Aldrich, USA) according to the manufacturer's guidelines.

Principle of the Assay:

1. Prior to assay, all the reagents were allowed to stand at room temperature and were mixed gently before use.
2. Coated strips were placed into the holder
3. Designated wells were pipetted with 50 µL of TSH standards, control, and specimens.
4. After words, 100 µL of ready to use conjugate reagent was added to all
5. Wells and shaken for 10–30 seconds.
6. Plate was covered and incubated minutes at room temperature for 60
7. Liquid was removed from all wells and then wells were washed three times with 300 µL of 1x wash buffer.
8. TMB substrate in the quantity of 100 µL was added to all wells and incubation was done at room temperature for 15 minutes.

9. Followed by incubation 50 µL of Stop Solution was dispensed to all wells and shaken well to gently mixed the solution and read at 450 nm within 15 minutes.

Immunohistochemistry

For immunohistochemistry, according to conventional procedure, one full-thickness section was placed in 10% buffered formalin for 12–24 h before embedding in paraffin wax and cut into 5 µm sections in series and put on super frosted glass slides. (Micro slides, Santa Cruz Biotechnologies, Dallas, Texas USA). Tissue sections were incubated overnight for dewaxing. Antigen retrieval was done by heating slides for 2 minutes in tris-buffer saline TBS. After drying slides were washed with phosphate-buffer saline (PBS) and incubated for one hour with incubation solution (0.05% bovine serum albumin, 0.01% Triton X and 10% goat serum). Again, slides were washed with PBS and then incubated with specific rabbit polyclonal primary antibody against eNOS (Catalogue no. ab-5589; Abcam Biotechnology, Inc., Cambridge, United Kingdom) for 48 hours at 4°C. Slides were washed with PBS after incubation and then incubation was done with Goat Anti-Rabbit IgG (Alexa Fluor® 488) antibody (Catalogue no. ab-150077; Abcam Biotechnology, Inc., Cambridge, United Kingdom) for 2 hours. Slides were then washed with PBS and kept for drying, mount with mounting medium and observed under fluorescent microscope (Bx51, Olympus, Tokyo, Japan). Cells with positive immunofluorescence signals were then counted manually to minimize specificity errors occurs through automated image analyzers.

eNOS mRNA Expression

qRT-PCR was used to determine the expression of eNOS mRNA in placental tissues. Results were then analyse by Qiagen tool for mean expression by comparing with Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as housekeeping gene.

mRNA Extraction

Placental tissues were homogenized in lysis buffer containing β-mercaptoethanol by using VWR® Deluxe Universal 200 Homogenizer, USA. RNA was extracted with and Pure Link^{TM} RNA isolation kit (Invitrogen, Thermo Fisher Scientific, USA).

Procedure

For the extraction of mRNA, 1 ml 70% ethanol has been introduced to each cell volume homogenate and vortexed to carefully mix and disperse any noticeable precipitate that

may appear after ethanol has been added. Afterword, 700 µl of the sample was transferred to the spin cartridge (with the same collection tube, including any remaining precipitate). Centrifugation was performed at room temperature at 12,000 ×g for 15sec. Flow-through was removed and reinserted the spin cartridge in the same collection tube. Repeated above steps until the whole sample was processed. After that, wash buffer 1 (700ul) was added to the spin cartridge. Centrifuged at room temperature for 15 sec at 12,000 xg. The collection tube has been removed and the spin cartridge has been put in fresh collection tube. Wash buffer 2 (500 µl) with ethanol was added to the spin cartridge. Centrifuged at room temperature for 15 seconds at 12000 rpm. The flow has been discarded. The spin cartridge was centrifuged to dry the membrane with bound RNA for 1-2 minutes at 12000 xg and the collection tube was removed, and the spin cartridge was inserted into the retrieval tube. The spin cartridge center was pipetted with 30-100 µl RNase-free water. Incubation was then performed for 1min at room temperature. At room temperature, the spin cartridge was centrifuged at ≥12000 ×g for 2 min to elute the RNA from the membrane into the recovery tube. Purified RNA concentration was determined by nanodrop 1000^{TM} UV/VIS spectrophotometer (Thermo Scientific, nanodrop technologies, USA) and stored at -80°C.

Complementary DNA (cDNA) synthesis

cDNA was synthesized from RNA by Protoscript® cDNA synthesis kit (New England Biolabs, USA).

Procedure

Two sterile RNA-free microfuge tubes were used to mix the primary d(T) VN and RNA sample:

Component	**Volume**
Total RNA	1 µl
Primer d(T) VN (50Um)	2 µl
Nuclease-free H_2O	5 µl
Total Volume	**8 µl**

DNA has been denatured at 70 °C for 5 minutes. Centrifuged shortly, putting it on ice quickly. This is an optional step. However, for longer messenger RNAS and GC-rich RNA areas, it increases the output of cDNA. Each tube contained following components:

- M-MuLV Reaction Mix 10 µl
- MpMuLV Enzyme Mix 2 µl

20ul cDNA synthesis reaction was subsequently incubated for one hour at 42 °C. Before the 42 °C incubation, an incubation step for 5 min at 25 °C was suggested when using Random Primer Mix. Enzyme was inactivated for 5 minutes at 80 ° C, then the PCR reaction was diluted to 50ul with 30ul of H2O. The product cDNA was stored at -20 °C.

Conventional PCR

To confirm the cDNA synthesis conventional PCR was done. Different primer sets of candidate genes were used to amplify the specific region od cDNA. The reagents are listed in table 11 and conditions in table 12. All reagents were added to sterile 0.2 ml PCR tubes, mixed well and transferred to thermocycler (Biometra Tpersonal, Germany). The 40 cycles of PCR were completed by using thermal parameters given in table 3. After PCR amplification, sizes of the amplified products were confirmed on 2% agarose gel with 100 bp ladder (Cat. No. SM0323, Thermoscientific, USA) to determine amplified product sizes.

Table 11. List of all reagents used in conventional PCR.

Sr. No	**Reagents**	**Quantities**
1	DreamTaq Green PCR Master mix (2X)	5 µl
2	Forward primer	1 µl
3	Reverse primer	1 µl
4	cDNA	1 µl
5	Nuclease free water	2 µl
	Total	**10 µl**

Table 12. Conditions for conventional PCR thermo cycling

Steps	No. of Step	Temperature	Time	No. of Cycles
Initial denaturation	Step 1	94 °C	4 Minutes	01 Cycle
Denaturation	Step 1	94 °C	45 Seconds	40 Cycles
Annealing	Step 2	Tm	45 Seconds	
Extension	Step 3	72 °C	45 Seconds	
Final extension	Step 1	72 °C	10 Minutes	01 Cycle

qRT-PCR

MyGo Pro real time PCR Thermocycle r(IT-IS International Ltd, UK) was used to amplify the resulting cDNA in with the reagents summarized in Table 13. Primers efficiency was determined by cDNA amplification for eNOS and GAPDH with their respective primers and product was run on 2.5% agarose gel product size confirmation. The primer sequence used are summarized in table 14:

Table 13. List of reagents used in qRT-PCR.

Sr. No	Reagents	Quantities
1	Maxima SYBR Green/ROX qPCR Master Mix (2X)	5 µl
2	Forward primer	1 µl
3	Reverse primer	1 µl
4	cDNA	1 µl
5	Nuclease free water	2 µl
	Total	**10 µl**

Table 14. Conditions of qRT-PCR analysis of *eNOS* and *GAPDH* gene.

Variants	Primers	Temperature (°C)	Bands patterns (bp)
eNOS	F: 5′-CCTCGTCCCTGTGGAAAGAC-3′, R: 5′-TGCTTCATGAAAGAGGCCGT-3	60	121
GAPDH	F: 5′-GCTCTCTGCTCCTCCTGTTC-3, R: 5′-CCATGGTGTCTGAGCGATGT-3	58	80

Table 15 represented the conditions used for qRT-PCR. PCR analysis was performed at annealing temperature of 60°C for eNOS and 58°C for GAPDH for 40 cycles.

Table 15. Conditions used for qRT-PCR.

Sr. No.	Steps		Incubation temperature (°C)	Time (seconds)	Ramp (°C/s)	Cycle
1	Hold		95	600	4	No
2	3 step amplification		95	15	5	40
			60	30	4	
			72	30	5	
3	Pre-melting hold		95	10	5	No
4	High resolution melting	Initial stage	60	60	4	No
		Final stage	97	1	0.05	

Data analysis

Data was analyzed by Pfaffl method (Pfaffl, 2001). Fold change of gene expression was determined by following formula

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta CP_{\text{target}}\,(\text{control-sample})}}{(E_{\text{ref}})^{\Delta CP_{\text{ref}}\,(\text{control-sample})}}$$

In this formula: symbol Δ is called delta which represent the difference between values of two articles and CP termed as PCR cycles determined the cycle number where the florescence signal intensity over background noise was considerably detectable. The REST-384 Tool version 2 (Qiagen, USA) was used for this analysis in the study.

Statistical Analysis

All data were presented as mean ± S.E.M (standard error of mean) with 95% confidence intervals (CI). Quantitative data will express as mean ± S.E.M. Significant differences between groups for clinical, demographic data, antioxidant levels, NO concentration, epinephrine, thyroid hormones levels and immunohistochemistry results were determined by performing Welch two sample t test, while, Paired t-test was used for comparison of fold expression of gene by using package of R 3.5.1 (R Development Core Team, 2018), While IBM SPSS Statistics 21 software was used to determine the Pearson correlation. Statistically significant values of $p<0.05$ were considered.

RESULTS

Demographic and clinical history

Mode of delivery and gravidity has been summarized in table 16. Rate of delivery through C-section was pronounced in PE patients as compared to controls and PE incidence was more in primary gravida patients as compared to healthy pregnant women (Fig. 59).

Table 16: Percentage of mode of delivery and gravidity in control and preeclamptic group.

Parameters	Controls n (%)	PE Patientsn (%)	Total n (%)	χ^2	OR	95% CI	p value
Mode of delivery							
C-Section	8 (16)	36(72)	44 (22)	31.81	13.5	5.087-35.83	<0.001
SVD	42(84)	14(28)	56 (28)				
Gravidity							
Primary Gravida	118 (59)	150 (75)	268 (67)	11.57	2.08	1.361-3.193	<0.001
Multiple Gravida	82 (41)	50 (25)	132 (33)				

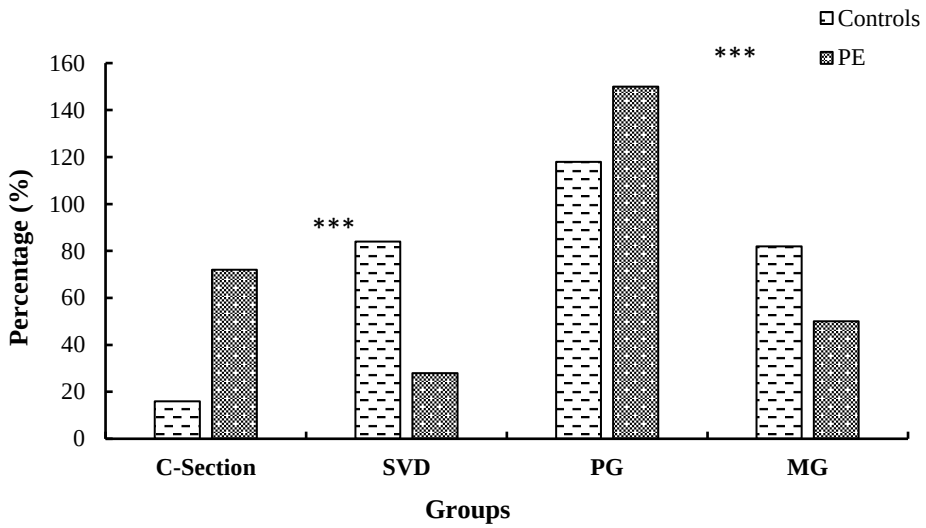


Figure 59: Percentage of mode of delivery and gavidity in normotensive pregnant and preeclamptic women.

*** indicate significant difference in PE group at probability of $p<0.001$ when compared to controls.

Hormonal analysis

NO, epinephrine and thyroid hormones including T_3, T_4 and TSH levels were determined in both groups (Table 17). Concentration of NO was reduced significantly (p=0.016) in preeclamptic pregnant women as compared to normotensive controls (Fig. 60). Epinephrine levels were significantly higher (p=0.011) in women with PE as compared to healthy pregnant women (Fig.61). There was non-significant difference in T_3, T_4 and TSH levels among both groups (Fig. 62-64).

Table 17: Levels of epinephrine and nitric oxide (NO) in Control and Preeclamptic group.

Parameters	Controls	PE Patients	P value
NO (µmol/L)	60.92±4.64	44.59±4.47	0.02
Epinephrine (pg/ml)	88.44±3.15	101.42±3.77	0.01
T_3 (ng/ml)	1.22±0.05	1.24±0.05	0.67
T4 (ng/dl)	1.20±0.03	1.24±0.04	0.45
TSH (µIU/ml)	2.35±0.13	2.38±0.11	0.88

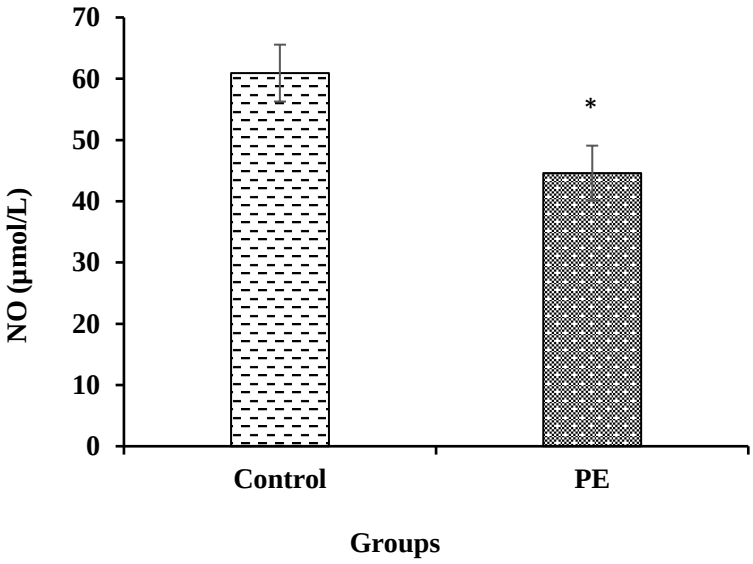


Figure 60. Mean ± SEM levels of nitric oxide (NO) in normotensive and preeclamptic group.

* indicate significant difference in PE group at probability of $p<0.05$ in comparison to control group.

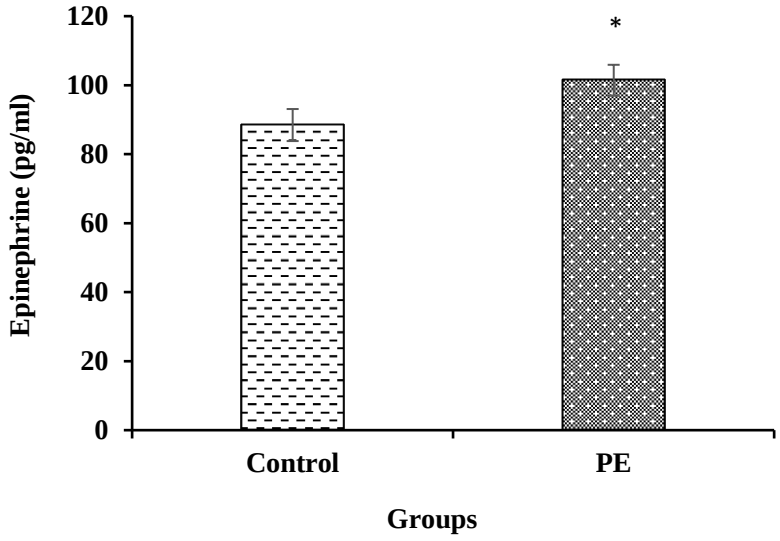


Figure 61. Mean ± SEM levels of epinephrine in control and preeclamptic women.

* indicate significant difference in PE group at probability of $p<0.05$ in comparison to control group.

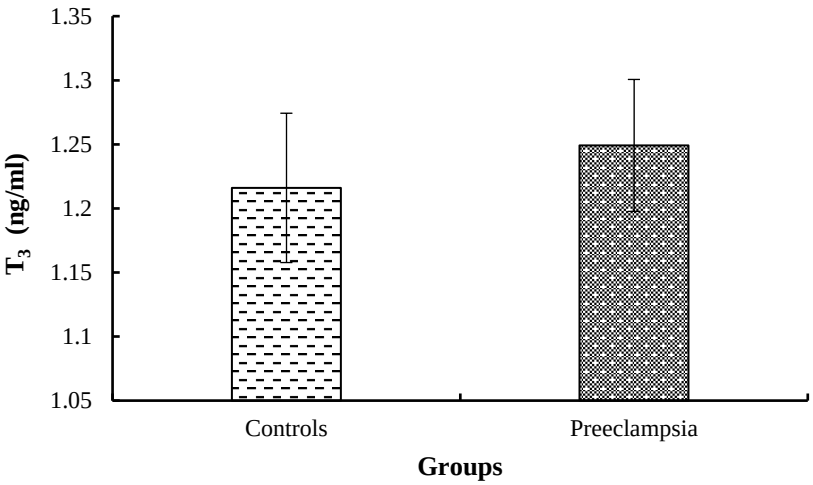


Figure 62. Mean ± SEM levels of triiodothyronine (T_3) in controls and preeclamptic women.

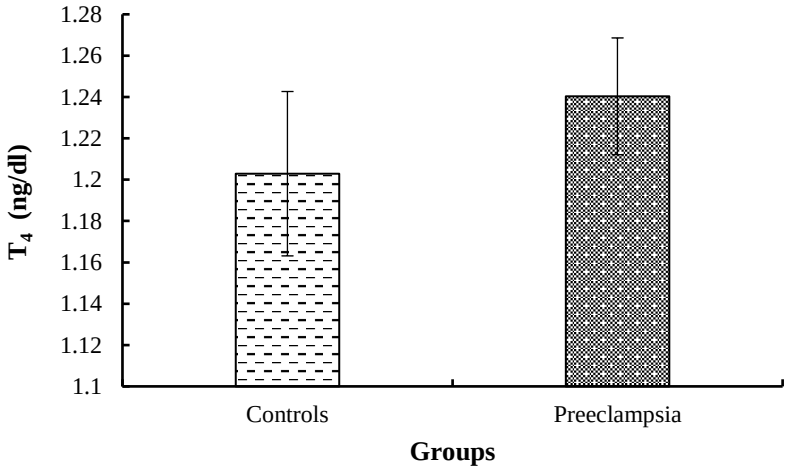


Figure 63. Mean ± SEM levels of thyroxine (T_4) in controls and preeclamptic group.

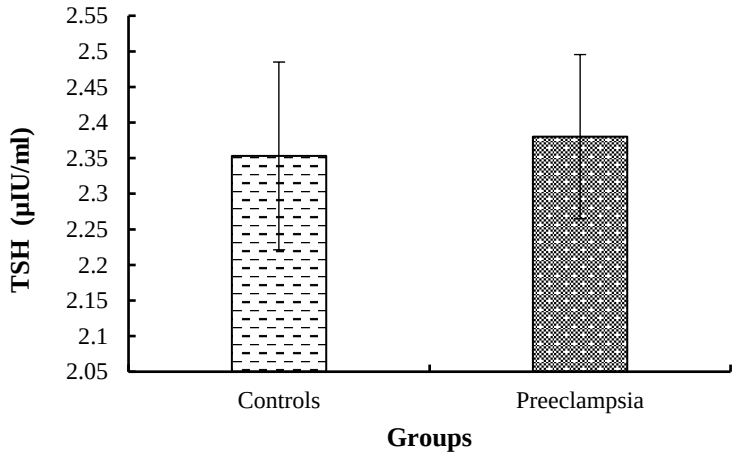


Figure 64. Mean ± SEM concentration of thyroid stimulating hormone (TSH) in normotensive and preeclamptic women.

Immunoreactivity

The immunohistochemical localization of eNOS was identified by green immunofluorescence seen only in the presence of primary antibody (Fig. 65). Omitted or negative control with no primary antibody was used to assess the sensitivity and the specificity of assay showing no green immunofluorescence (Fig. 65E). In the terminal villi of placenta difference in eNOS immunostaining in both villous vascular endothelium and the syncytiotrophoblast were seen quantitatively between the two groups of placentas when compared to histological sections (Fig. 65). Control group exhibit significantly ($p<0.001$) greater number of immunoreactive cells as compared to preeclamptic group (Table 18, Fig. 66).

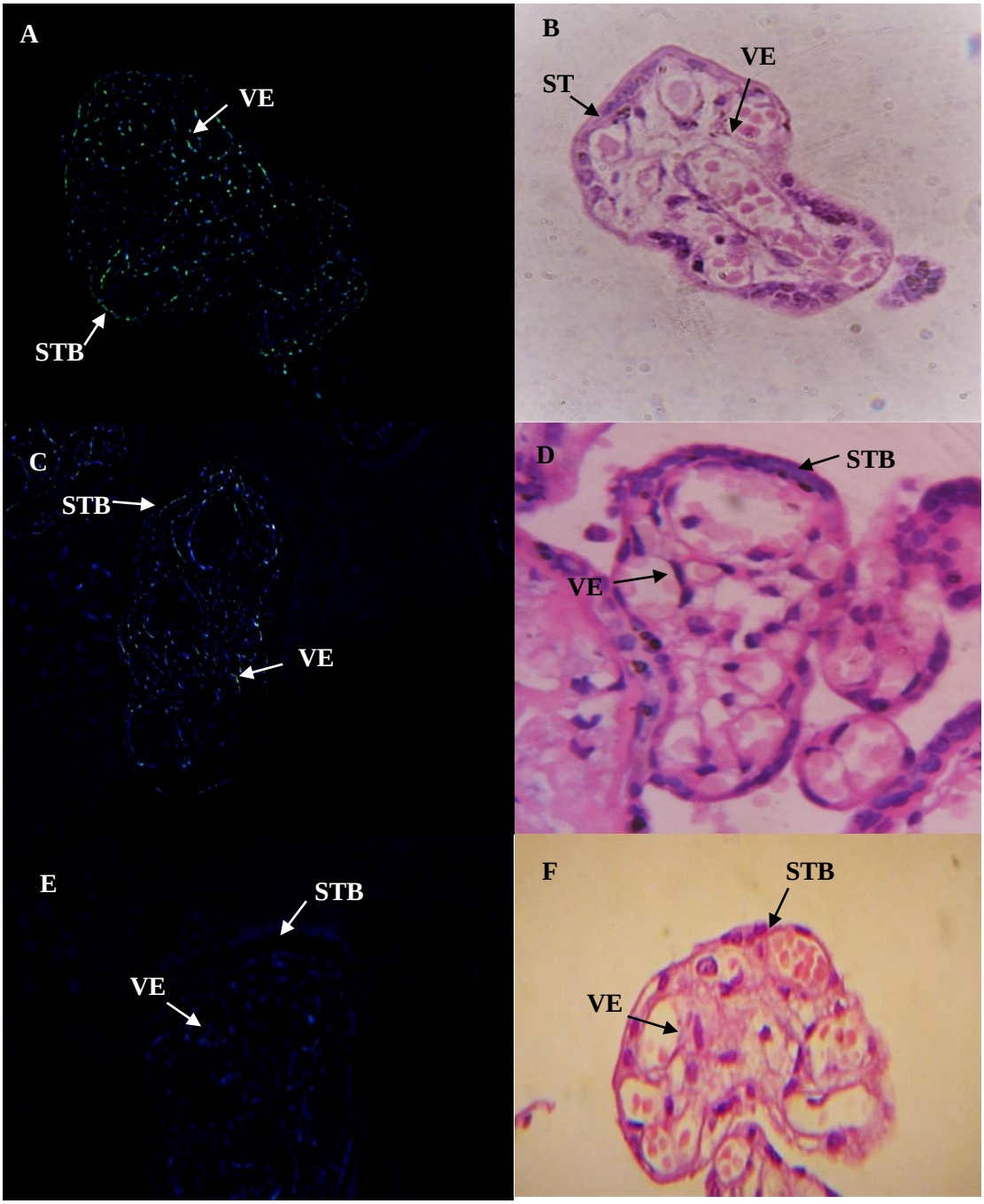


Figure 65. Fluorescent microscopic images of eNOS immunoreactivity in green (left) in placental villi when compared to H&E stained microscopic images of placental villi (right). A and B. Control group showed significant (<0.001) increase of eNOS immunoreactive cells in villi of placenta as compared to Preeclamptic group (C and D). E and F showed negative control images. Photographs were taken at 100X magnification. Syncytiotrophoblat (STB), Vascular Epithelium (VE).

Table 18. Number of eNOS immunoreactive cells based on random sections observed in placental trophoblastic villi in control and preeclamptic group.

Groups	eNOS immunoreactive cells	p value
Controls	120±3.84	<0.001
PE Patients	91±2.30	

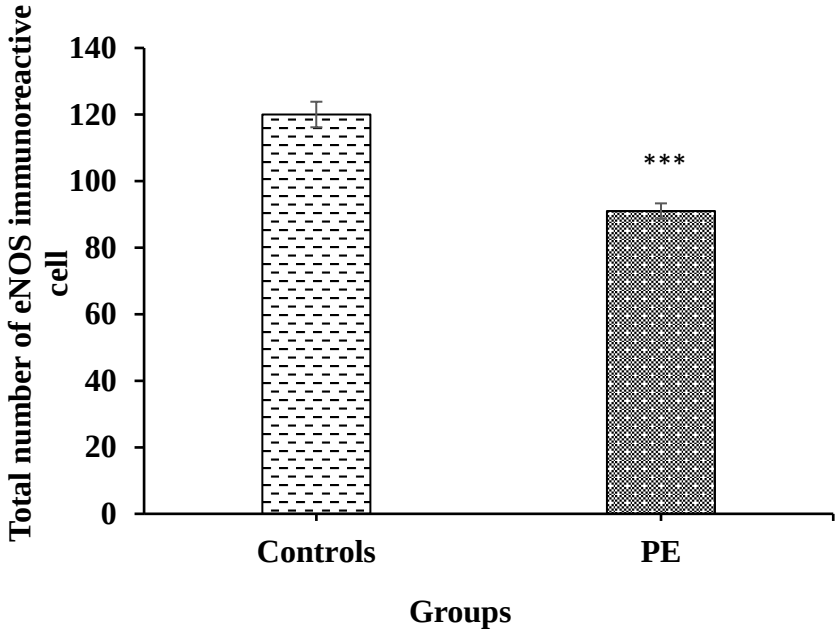


Figure 66. Number of eNOS immunoreactive cells based on random sections observed in placental trophoblastic villi. eNOS immunoreactive cells were significantly (<0.001) reduced in PE group in comparison to control group.

***indicate significant difference in PE patients at probability of p<0.001 when compared to controls.

RT-PCR

Relative RNA abundance of eNOS in placentas of preeclamptic (n=50) and normotensive (n=50) Pakistani women was determined by qRT-PCR. The qRT-PCR reaction produced a product of 121 bp for eNOS and 80 bp for GAPDH (Fig. 67). The expression of eNOS was compared in both groups and results were described as fold change. There was significant (P<0.001) downregulation of eNOS in preeclamptic group as compared to control group. Expression of eNOS was lowered in preeclamptic group by approximately 0.514 folds, as shown in figure 68 (Table 19).

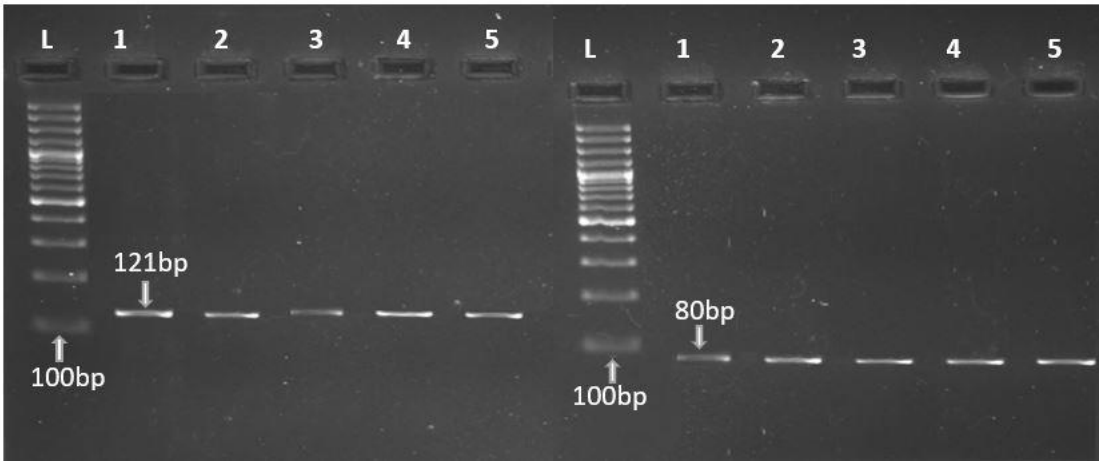


Figure 67. Gel images predicting the PCR product of eNOS primers (A) with product size of 121bp and GAPDH primers (B) with product size of 80bp compared with 100bp ladder in cDNA samples of preeclamptic women (lane 1-3) and normotensive pregnant women (lane 4, 5).

Table 19. Mean ± SEM number of eNOS relative mRNA abundance in placenta of control and PE group.

Groups	eNOS relative mRNA abundance	p value
Controls	1	<0.001
PE Patients	0.51±0.09	

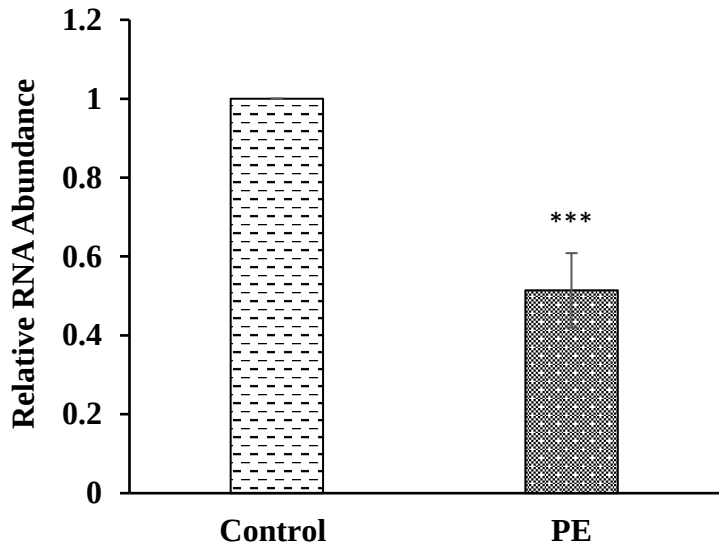


Figure 68. Data obtained after qRT-PCR on placental samples of control (n=50) and preeclamptic group (n=50), was analyzed for relative RNA abundance of eNOS as mean ± SEM. There was significant (p<0.001) decrease in abundance of eNOS mRNA.

*** indicate significant difference in PE patients at probability of p<0.001 in comparison to controls.

Correlation analysis

Correlation of different hormones and anthropometric parameters was determined in preeclamptic patients as shown in Table 20. In PE group urea determined significant ($p<0.01$) positive correlation ($r=402$) creatinine (Fig. 69). Age demonstrated significant ($P<0.05$) positive correlation with SBP ($r=0.173$) and DBP ($r=0.155$) while SBP has also positive correlation with DBP ($r=0.835$, $p<0.01$) (Fig. 70 and 71). There was no correlation found in all other parameters shown in table 20.

Table 20: Correlation analysis of hormonal and anthropometric parameters of preeclamptic group.

		epinephrine	NO	T3	T4	TSH	Ferritin	Sodium	Potassium	Calcium	Urea	Uric acid	creatinine	Age	BMI	SBP	DBP
epinephrine	r	1															
	p value																
NO	r	-.046	1														
	p value	.628															
T3	r	-.158	.045	1													
	p value	.233	.734														
T4	r	.043	-.108	-.216	1												
	p value	.745	.415	.100													
TSH	r	-.111	.036	.047	.058	1											
	p value	.402	.785	.722	.664												
Ferritin	r	-.180	.052	-.023	-.241	.124	1										
	p value	.121	.656	.869	.074	.362											
Sodium	r	.190	.053	-.145	.051	.091	-.192	1									
	p value	.160	.697	.285	.712	.504	.168										
Potassium	r	.060	-.141	-.049	-.156	-.018	.119	-.017	1								
	p value	.660	.299	.722	.252	.894	.397	.903									
Calcium	r	.263	-.056	-.119	-.083	-.037	-.044	.259	.195	1							
	p value	.050	.679	.380	.542	.788	.753	.053	.149								
Urea	r	-.001	.067	.151	.036	-.132	-.083	-.157	.022	.038	1						
	p value	.995	.468	.254	.789	.321	.480	.247	.871	.781							
Uric acid	r	-.026	.140	.033	-.211	-.051	-.058	-.015	-.067	.014	-.012	1					
	p value	.797	.161	.803	.109	.703	.621	.914	.622	.918	.905						
creatinine	r	.054	.050	.028	.050	-.075	-.089	-.137	-.007	.175	.402**	-.024	1				
	p value	.569	.590	.835	.707	.574	.449	.314	.958	.197	.000	.809					
Age	r	-.062	.031	-.085	-.089	.020	.076	-.148	.085	-.034	.040	.158	-.014	1			
	p value	.516	.739	.521	.502	.879	.517	.277	.533	.803	.661	.113	.875				
BMI	r	.053	.004	.151	-.063	.226	.050	-.111	.072	-.179	-.091	-.059	.001	.118	1		
	p value	.579	.963	.255	.634	.086	.668	.414	.600	.186	.315	.557	.994	.111			
SBP	r	-.018	-.049	-.048	.032	.228	.087	.023	.111	-.040	-.015	-.006	.004	.173*	.086	1	
	p value	.847	.595	.718	.809	.083	.459	.869	.416	.769	.869	.949	.964	.019	.250		
DBP	r	-.033	-.083	-.078	.174	.143	.068	.028	.048	-.090	.095	.013	.059	.155*	.104	.835**	1
	p value	.728	.367	.558	.188	.281	.560	.836	.723	.507	.294	.900	.513	.036	.161	.000	

* and **, Correlation is significant at the 0.05 and o.01 level (2-tailed).

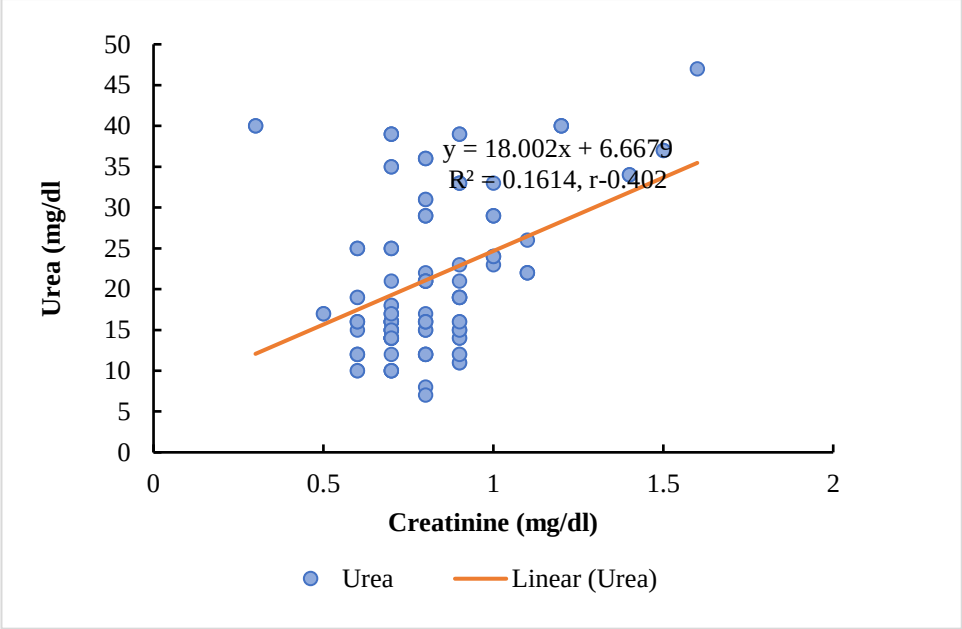


Figure 69: Scatter gram showing positive correlation of urea with creatinine in preeclamptic group.

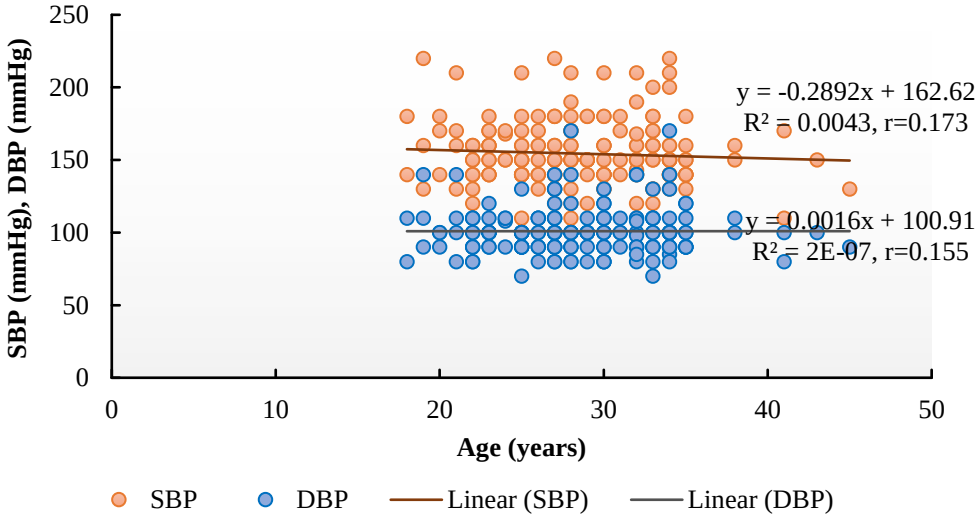


Figure 70: Scatter gram showing positive correlation of systolic blood pressure (SBP) and diastolic blood pressure (DBP) with age in preeclamptic group.

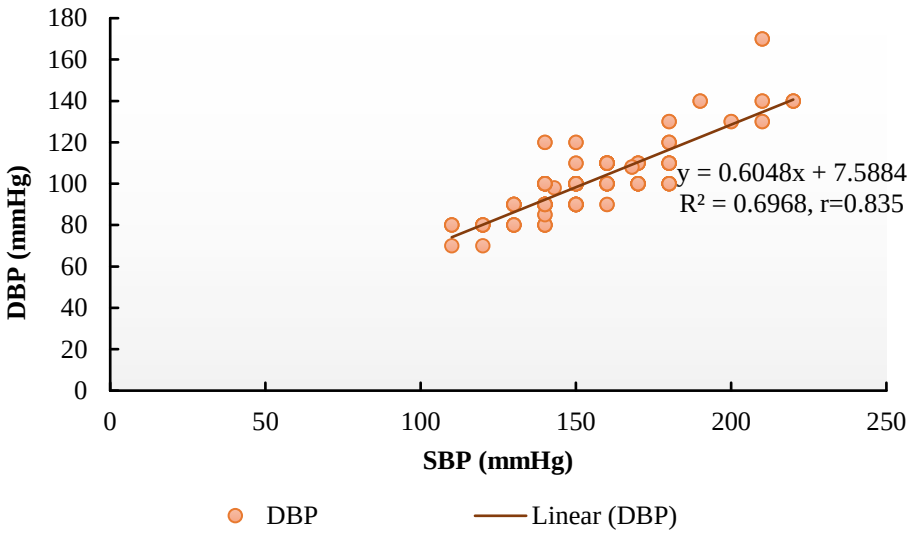


Figure 71: Scatter gram representing positive correlation of DBP with SBP in preeclamptic group.

DISCUSSION

Biologically and clinically, the pregnancy complicated by PE has been extensively studied, unfortunately the cause of this complication is not yet defined. PE is an important maternal health problem, especially in developing countries like Pakistan. Identification of various factors could help in understanding this complication and provide clues for its management and treatment. Endothelial dysfunction is linked with susceptibility to PE (Aggarwal *et al.,* 2010; Rahimi *et al.,* 2013; Gannoun *et al.,* 2015). This study was designed to assess the eNOS localization, expression and hormonal analysis in preeclamptic females compared to healthy pregnant women in Pakistan.

Results of current study revealed that levels of NO were significantly lower in preeclamptic groups as compared to controls in accordance with previous findings (Var *et al.,* 2003; Bernardi *et al.,* 2008; Singh *et al.,* 2010; Bernardi *et al.,* 2015; Aouache *et al.,* 2018). It was evident previously that impair NO levels attenuated the acetylcholine-induced relaxation in arteries of preeclamptic women placentas causing vasoconstriction leading to increased mother's blood pressure (Moncada and Higgs, 1993; Buhimschi *et al.,* 1995). Therefore, circulating NO is likely to be important for fetoplacental hemodynamics ensuring adequate placental blood flow and fetal oxygenation (Turan *et al.,* 2010).

In current study levels of epinephrine were higher in PE patients as compared to control group but there was no correlation of NO and epinephrine found. Epinephrine is the main catecholamine produced from the adrenal medulla by low blood glucose, exercise and stress (From Jastak *et al.,* 2016). It has a profound impact on the vascular system, depending on the vascular structures and the chemical concentration, epinephrine can either cause vascular dilation through eNOS activation or contraction causing hypertension (Shen *et al.,* 2008). Epinephrine in blood narrows blood vessels and increases pressure and inflammation results in endothelial dysfunction leading to PE (Holzman *et al.,* 2009). Pregnancy related stress might be the cause of epinephrine elevation (Toda and Nakanishi-Toda, 2011).

Thyroid hormones T_3, T_4 and TSH were non-significantly altered in preeclamptic group as compared to control group in this study. These results are in accordance with previous studies (Qublan *et al.,* 2003; Khadem *et al.,* 2012). Controversial findings were found in relation of thyroid hormones with preeclampsia (Sardana *et al.,* 2009; Harshvardhan *et al.,*

2017). Basbug et al. reported a significant decrease in both total and free thyroid hormones (Başbuğ *et al.,* 1999). Sardana et al. found a significant decrease in T3 (Sardana *et al.,* 2009). Tolino et al. and Lao et al. found a significant decrease in T4 in preeclamptic women (Tolino *et al.,* 1985; Lao *et al.,* 1990). Kumar et al. tested FT3 and FT4 only, but they were within the expected range (Ashok *et al.,* 2005). Qublan et al. Khadem et al. and Gulaboglu et al. did not get a significant change (Qublan *et al.,* 2003; Gulaboglu *et al.,* 2007; Khadem *et al.,* 2012)

In current study correlation was determined between biochemical and anthropometric parameters, significant positive correlation was found in urea and creatinine, as they are the important markers for assessing renal insufficiency and predicting the adverse renal outcome in PE (Manjareeka and Nanda, 2013). Glomerular filtration can be measured indirectly by serum creatinine concentration. Reduced glomerular filtration rate results in elevation of concentrations of creatinine and urea. This elevation indicates progression of kidney disease (Pandya *et al.,* 2016). In addition, significant correlation was found in SBP and DBP with age and among them (Rockwood and Howlett, 2011). Human heart and vasculature structure and function alter with age. Structural changes in the vasculature boost arterial rigidity, reducing arterial buffering ability and resulting in age-related modifications in systolic and diastolic blood pressure (Banegas *et al.,* 2018).

In present study reduced eNOS activity has been observed in preeclamptic patients, these results are in consistent with the results of kim et al. and Schofelder et al. (Schönfelder *et al.,* 2004; Kim *et al.,* 2006). These results support the notion that, there will be adverse effect on placental hemodynamic functioning if the activity of eNOS become reduced *in vivo* (Kim *et al.,* 2006). Reduced eNOS will decrease the bioavailability of NO leading to endothelial dysfunction which is the feature of this disorder (Motta-Mejia *et al.,* 2017). Fetal placental hemodynamics is likely to influenced by NO production from endothelium of placental villi. However, platelets and leukocytes aggregation in intervillous spaces might be prevented by NO produced by syncytiotrophoblast (Brennecke *et al.,* 1997). Some conflicting findings have been reported with regard to eNOS activity in preeclamptic patient's placenta showing no significant difference in eNOS activity in preeclamptic women (Matsubara *et al.,* 2001; Orange *et al.,* 2003; Matsubara *et al.,* 2015). In addition to eNOS reduced expression and activity, lower substrate levels for eNOS and elevated

inhibitors of eNOS can also contribute towards reduce NO bioavailability (Myatt and Roberts, 2015; Motta-Mejia *et al.*, 2017).

There are presently restricted therapeutic choices, but knowing the factors associated with endothelial dysfunction may assist develop fresh approaches to predicting and managing preeclampsia. Umbilical-placental vascular resistance has been improved and reduced *in vivo* by NO donating drugs (Schiessl *et al.*, 2005). Therefore, for NO donating drugs and antioxidants therapy can be given for better management of PE which will reduce the maternal and neonatal morbidity and mortality in Pakistan.

Conclusion

In conclusion, our data suggested that thyroid hormones have no significant alterations among both groups while elevated epinephrine and decreased NO levels and reduced placental eNOS expression might constitute a characteristic finding in the preeclamptic placenta. This supports the hypothesis that complicated mechanisms involving eNOS pathways and oxidative stress may encourage microvascular oxidative damage and favor abnormal placental perfusion, likely by contributing to decreased placental blood flow and enhanced flow resistance in the feto-maternal bloodstream.

Future recommendations

NO and epinephrine concentration in maternal serum could be used as a biomarker to predict PE for practitioners, which could contribute to interfere PE development in the early period in preeclamptic Pakistani women. Thyroid profile must be examined in the third trimester in PE patients. There are presently restricted therapeutic choices, but knowing the factors associated with endothelial dysfunction may assist develop fresh approaches to predicting and managing preeclampsia. Umbilical-placental vascular resistance has been improved and reduced *in vivo* by NO donating drugs (Schiessl *et al.*, 2005). Therefore, NO donating drugs can be given for better management of PE which will reduce the maternal and neonatal morbidity and mortality in Pakistan.

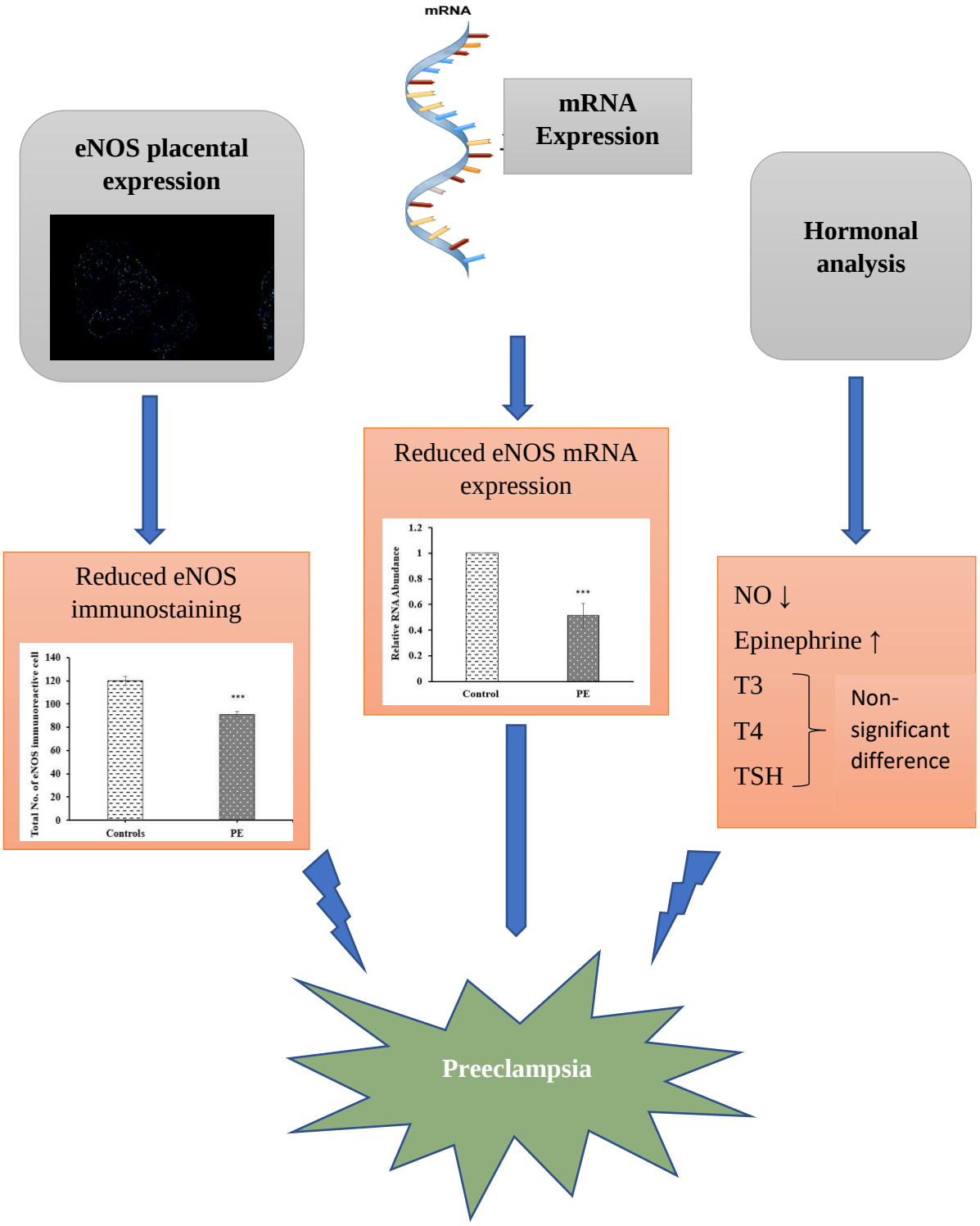


Figure 72: Graphical representation of placental localization and expression of eNOS and hormonal analysis of preeclamptic women.

CHAPTER 4

Association of *eNOS* gene variants with preeclampsia in Pakistan.

ABSTRACT

Preeclampsia (PE) is a complex pregnancy hypertensive disorder with multifaceted etiology. The endothelial nitric oxide synthase (*eNOS*) gene has been reported to be associated with PE predisposition in various populations. Therefore, the present study was designed for the first time in Pakistan to investigate the role of *eNOS* gene variants (c.894G>T (p.(Glu298Asp)), intron 4b/4a and g.-786T>C) in preeclamptic Pakistani women. A total of 400 women were evaluated, 200 with PE along with 200 normotensive pregnant women. *eNOS* gene variants were screened in PE patients and in control group by genotyping and sequencing. Further *in silico* studies were performed to get insights into the structural and functional impact of identifies mutation on eNOS protein as well as on protein regulation. The frequency of c.894T (p.298Asp) was high in the PE group ($p \leq 0.001$). Likewise, a significant association of g.-786C alleles was found with PE group ($p=0.007$) when compared to normotensive women. In addition, a novel homozygous variant g.2051G>A was also significantly associated with PE when compared to normotensive women. Dynamic simulation studies revealed that Glu298Asp mutation destabilizes the protein molecule and decrease the overall stability of eNOS protein. Molecular docking analysis of the mutant promoter with transcription factors signal transducer and activator of transcription 3 (STAT 3) and signal transducer and activator of transcription 6 (STAT6) proposed changes in protein regulation upon these reported mutations in the upstream region of the gene. Considering the results of current study, the functional alterations induced by these variants may influence the bioavailability of NO and PE onset. However, large studies or meta-analysis are necessary to validate these findings and clarify this issue to prevent maternal and neonatal morbidity and mortality.

INTRODUCTION

PE is a complex pregnancy hypertensive disorder with multifaceted etiology (Chen *et al.*, 2014; Du *et al.*, 2017). It is leading cause of maternal and prenatal morbidity and mortality in developing countries (Dai *et al.*, 2013). Annually 63000 maternal and 50000 infant's deaths are predictable to be associated with preeclampsia (Myatt *et al.*, 2014; Goulopoulou and Davidge, 2015; Lisowska *et al.*, 2018).

Studies have shown various factors involved in susceptibility of preeclampsia, but etiological details are still being debated (Singh *et al.*, 2010). Pathogenesis of preeclampsia is multifactorial involving environmental and genetic factors (Gannoun *et al.*, 2015). It was determined that defective placentation is associated with the progression of preeclampsia (George and Granger, 2010). Disturbed trophoblastic invasion and spiral arteries remodeling results in endothelial dysfunction which leads to vasoconstriction (Gannoun *et al.*, 2015).

Various mediators are involved in controlling endothelial dysfunction in preeclampsia but role of *eNOS* gene located at the 7q35-q36 region appears most significant in the development of preeclampsia (Devendran *et al.*, 2015). *eNOS* is an significant vascular tone regulator and contributes to the reduced uteroplacental resistance seen during normal pregnancy through nitric oxide (NO) production by reducing L-arginine to L-citruline. (Palmer *et al.*, 1988). Polymorphisms of *eNOS* impair NO availability which is crucial for maternal vascular vasodilation during pregnancy, leading to susceptibility of preeclampsia (Serrano *et al.*, 2004; Rahimi *et al.*, 2013).

Several variants in *eNOS* gene were identified effecting its functions or production levels (Rahimi *et al.*, 2013). These variants comprise of functional c.894G>T (p.(Glu298Asp)) variant in exon 7 of *eNOS* gene.(Hocher *et al.*, 2008; Turan *et al.*, 2010; Rahimi *et al.*, 2013; Maria Procopciuc *et al.*, 2018), an insertion deletion variant in intron 4 (4b/4a) consisting of 27bp tendem repeats, 4b allele comprises of 5 repeats and 4a allele with 4 repeats (Singh *et al.*, 2010; Dai *et al.*, 2013; Alpoim *et al.*, 2014) and g.-786T>C variant present in promotor region of *eNOS* gene (Serrano *et al.*, 2004; Zdoukopoulos *et al.*, 2011; Chen *et al.*, 2014).

Considering the potent role of polymorphism in the *eNOS* gene in causing clinical symptoms of PE different mega projects and meta-analysis were conducted to better

understand the underlying mechanisms but there are many gaps to be filled. Under a detailed study projected towards this important issue related to maternal health, for the first time, the genetic cause of PE was highlighted in Pakistan. The present study was directed to determine the association of c.894G>T (p.(Glu298Asp)), intron 4b/4a, g.-786T>C and other possible variants of *eNOS* gene with preeclampsia in Pakistani population.

MATERIALS AND METHODS

Participants

The present study was conducted with a prior approval from ethical committees of Quaid-i-Azam University, Islamabad, PIMS Islamabad and Quaid-e-Azam International Hospital, Islamabad. The studied individuals comprised of 200 pregnant women with preeclampsia and 200 normotensive pregnant women as controls. Informed consent and a detail performa were filled before sample collection.

Inclusion and exclusion criteria

The control group have women with uncomplicated gestation and blood pressure <125/85mmHg and no proteinuria. Diagnostic criteria for preeclampsia was blood pressure ≥140/90mmHg, and proteinuria ≥+1 on Dipstick test (Kallela *et al.,* 2016). Exclusion criteria involved diabetes, asthma, kidney disease, hematological disorder, autoimmune disease, current or history of smoking and eclampsia.

Sampling and Storage

Blood samples were collected in labelled EDTA tubes and stored at -4 ^{0}C for DNA extraction, genotyping and sequencing.

Genetic analysis

DNA Extraction

DNA extraction was performed in two days. Steps are as following.

Day 1

1- In 1.5 ml of eppendorf tube 0.5 ml of blood and 0.5 ml of solution A was taken.
2- Blood and solution A were assorted by upturning tubes 4-6 times and tubes were put at room temperature for 20-40 minutes.
3- Then mixture was centrifuged at 13000 rpm for 3-4 minutes.
4- Nuclear pellet was deferred again in 0.5 ml of solution A after disposal of supernatant.
5- After the clearance of pallets supernatant was disposed and nuclear pellets were suspended again in solution B (400 µl), 20 % SDS (12 µl) and proteinase kinase (2-4 µl).
6- They were incubated overnight at 37˚C for or 65˚C for 3 hours.

Day 2

7- fresh mixture of PCI (0.5 ml) was added to tubes and was assorted. Then tubes were centrifuged at 13000 rpm for 10 minutes.
8- The aqueous phase (top layer) was collected in a fresh tube and 500 µl solution C was added to the tubes and centrifuged for 10 minutes at 13000 rpm.
9- The aqueous phase was placed in a fresh tube and precipitated by the addition of sodium acetate (3M) (55 µl) and Isopropanol (500 µl) and tubes were upturned several times for precipitating the DNA.
10- Centrifugation was done for 10 minutes to settle the DNA and supernatant was discarded.
11- Addition of 0.5 ml of 70% ethanol was done to the tubes and centrifuged tubes for 5 minutes to settle the DNA and discarded supernatant
12- Ethanol was vaporized by inverting tubes over tissue paper.
13- DNA was dissolved in 60 µl of TE buffer and was kept at 37˚C overnight and then was kept in freezer at -20.

Yield gel electrophoresis

1% Agarose gel was used to quantify DNA. The heating of 1 gm of agarose in 100 ml of 1X TBE (Tris-Borate electrophoresis buffer) produced 1% gel. Heating was performed in the oven for 2 minutes by heating the mixture. After heating, the gel solution was supplemented with 5 µl of ethidium bromide (EtBr). Gel solution was poured into the double-comb casters gel tray. Then, at room temperature, the solution was permitted to solidify. The combined mixture of 2 µl diluted DNA and 2 µl 10X loading dye was loaded into the wells when the gel was solidified. Control samples were also run in some wells. The gel was run at 90V and 500A for 40 minutes (Life Technologies, USA). The UV trans-illuminator (Bio-Rad, UK) was then used to visualize the gel.

Polymerase Chain Reaction (PCR)

Primer were selected from previous study (Kidron *et al.,* 2017). Genotyping conditions for eNOS variants have been summarized in table 21. Conventional PCR was done according to the conditions described in chapter 3. c.894G>T (p.(Glu298Asp)) (rs1799983) variant in exon 7 of *eNOS* gene was detected by PCR and product was

digested using 5U MboI restriction endonuclease enzyme by overnight incubation at 37°C. Intron 4b/4a (rs1722009) allele was detected by PCR method while, g.-786T>C (rs2070744) variant in promoter region of *eNOS* gene was detected by Tetra ARMS-PCR method. Target gene was amplified, double reaction was conducted for each sample with respective inner allele primer and PCR product was subjected to 2% agarose gel electrophoresis.Genotypes were determined by separation of fragments on 2% agarose gel.

PCR gel electrophoresis

2% Gel was used for PCR amplified product. The gel was prepared in 100ml of 1XTBE buffer by mixing the 2gm of agarose and then heating the mixture. The mixture was retained at room temperature after heating for a while, then addition of 4µl of ethidium bromide was done to the mixture was poured into a gel plate with a comb to solidify the gel. After the gel was solidified at the room temperature, combs were removed carefully, and the gel was put into the gel tank also filled with 1X TBE buffer. The gel was loaded with 2µl of 100bp ladder in the first well and 4µl of amplified product in both rows. The gel was run at 90V and 500A for 50 minutes. After the gel was run, UV trans-illuminator (Bio-Rad, UK) was used to visualize the gel.

Table 21. Conditions of genotyping assays of selected variants of eNOS gene.

Variants	Primers	Genotyping method	Temperature (°C)	Bands patterns (bp) in AGE
c.894G>T	F: 5′-CATGAGGCTCAGCCCCAGAAC-3′	PCR-RFLP	60	GG=206
(p.(Glu298Asp))	R: 5′-AGTCAATCCCTTTGGTGCTCAC-3′	*MboI* with cutting site		GT=206+119+87
(rs1799983)		5′...GATC...3′ 3′...CTAG...5′		TT=119+87
Intron 4b/4a	F: 5′-AGGCCCTATGGTAGTGCCTTG-3′	AP-PCR	54	4b/4b= 420
(rs1722009)	R: 5′-TCTCTTAGTGCTGTGGTCAC -3′			4a/4b=420+93
g.-786T>C	FP: 5′-TTTCTCCAGCCCCTCAGATG -3′	AS-PCR	59	TT= 387+250
(rs2070744)	RP: 5′-AGGCCCAGCAAGGATGTAGT -3′			TC= 387+250,
	CP: 5′-CATCAAGCTCTTCCCTGGCC -3′			387+250
	TP: 5′-CATCAAGCTCTTCCCTGGCT-3′			CC= 387+250

PCR product purification

Purification of PCR product was done by using Monarch PCR cleanup kit (NEB# T1030, NEW ENGLAND BioLabs). Sample was diluted with 80 µl of DNA cleanup binding buffer. The mixture was well mixed and then transferred to the column, closing the cap, spun for 1 minute and discarding the flow-through. Column was reinserted into the collection tube and a DNA buffer of 500 µl was introduced, spun for 1 minute and this step was done again. Flow-through was removed and spun tube to dry the column and to elute all wash buffer. Afterward, column was transferred to 1.5 ml microfuge tube. 18 µl of Elution buffer (heated to 50°C prior to use) was added to the center of the matrix. After 3-5 minutes tube was spun for 1 minutes to elute DNA.

Sequencing

After the purification single band purified product was obtained which was then sequenced using automated ABI PRISM® 3130 Genetic Analyzer. The sequenced data was analyzed to reference DNA sequence from Ensemble genome browser by BioEdit version 7.0.5.3 software. Furthermore, to detect identified variants mutation Taster (*http//www.mutationtaster.org/*) was used.

Computational analysis

***In silico* site directed mutagenesis of *eNOS* protein**

The mutant ($eNOS^{Glu298Asp}$) 3D protein structure was built in Chimera using normal eNOS protein 3D structure (PDBID: 1m9q) as template. The stereochemical properties and environmental profile of the mutant model was evaluated using PROCHECK (Laskowski *et al.,* 1996) and ERRAT (servers for structure verification and analysis) respectively. Mutant model was minimized using Chimera 1.5.6 (Pettersen *et al.,* 2004) and NOMAD-Ref (http://lorentz.immstr.pasteur.fr/nomad-ref.php) further the root-mean-square deviation (RMSD) of normal and mutant structure was also calculated.

Molecular dynamic simulations

Molecular dynamic (MD) simulation experiments were conducted with $eNOS^{WT}$ and mutant $eNOS^{Glu298Asp}$ to evaluate eNOS protein folding, stability, conformation modifications and dynamic behaviors. For simulations, the Amber03 force field embedded in the GROMACS 4.5 package (Duan *et al.,* 2003) running on the high-performance linux OpenSuse system was used. Throughout simulation studies, water model TIP4P (Zlenko,

2012) solved both eNOS3^{WT} and mutant *eNOS*^{Glu298Asp} schemes in a regular box. Na^+ and Cl^- counter ions were added to neutralized the system. Energy minimization (steepest downward algorithm for 500 steps) was performed by 1000 kJ/mol $Å^2$ tolerance to eliminate original steric conflicts. Simulations were subjected for 20ns time scale under constant temperature (300 K) and pressure (1 atm) after completion of the minimization steps. Using the Particle Mesh Ewald (PME) algorithm, electrostatic interactions were calculated to this end. Use of VMD (Humphrey *et al.*, 1996), PyMol (http:/www.pymol.org) and GROMACS tools to investigate the stability behavior of *eNOS*^{WT} and mutant *eNOS*^{Glu298Asp} systems.

Transcription factor binding site prediction in the promoter region

Transcription factors are key to the regulation of genes and form the basis of gene regulation studies and understanding of gene on and off mechanism. ConTraV3 (http://bioit2.irc.ugent.be/contra/v3) and JASPER database were used for the prediction of transcription factor binding site in the *eNOS* gene promoter region.

DNA model building

DNA model was generated for promoter region with three sequences (Seq1: CCCTCAGATG**G**CACAGAACTAC, Seq2: CTTCCCTGGC**T**GGCTGACCCTG, Seq3: CCCGGGAAGC**G**TGCGTCACTG) of *eNOS* Nucleic Acid Builder (NAB) program (Pant *et al.*, 2017; Pant *et al.*, 2019) and 3D-Dart server (van Dijk and Bonvin, 2009). All the generated models were minimized for phosphate backbone geometry optimization.

Protein DNA molecular docking

Protein-DNA interactions are key to in several biological processes, DNA repair, gene regulation and chromatin structural organization. DOT 2.0 program suite (Roberts *et al.*, 2013) (http://www.sdsc.edu/CCMS/DOT) was used to calculate the potential binding site and binding energies of the promoter region of *eNOS* and its putative transcription factor protein. Grid was set large enough for smooth movement of molecule around the stationary molecule. Additionally, at the grid boundaries stationary potential was near zero to reduce the artifacts from fast Fourier calculations. In present docking experiments rotational sets of 28,800 (7.5°) and 54,000 (6°) were used.

Statistical Analysis

The frequency of alleles and genotypes were compared between study and control group by Independent sample t-test, Chi-squared test (χ^2) and Odds Ratio (OR) with values predicted by Hardy-Weinberg equilibrium model using IBM SPSS Statistics 21 software and package of R 3.5.1 (R Development Core Team, 2018). Values of $p<0.05$ were considered statistically significant.

RESULTS

eNOS variants distribution

The distribution of *eNOS* c.894G>T (p.(Glu298Asp)), intron 4b/4a and g.-786T>C variants were studied in all groups as presented in Table 22. The genotypic distribution was in Hardy-Weinberg equilibrium. For c.894G>T (p.(Glu298Asp)) variant electrophoresis of digested product yield band sizes of 206bp for GG, 206bp/119bp/87bp for GT and 119bp/87bp for TT genotype respectively (Fig. 73). Frequency of GG, GT and TT genotypes was 66%, 12% and 22% in preeclamptic women as compared to controls (75%, 14.5% and 10.5%) respectively. While frequency G and T alleles was 72% and 28% in preeclamptic individuals as compared to control group (82.25% and 17.75%) respectively. Significant difference in frequency of c.894T allele preeclamptic patients ($p \leq 0.001$) was observed likewise c.894TT (p.298AspAsp) genotype showed higher frequency in preeclamptic patients (p=0.008) as compared to control group (Fig. 76 and 77).

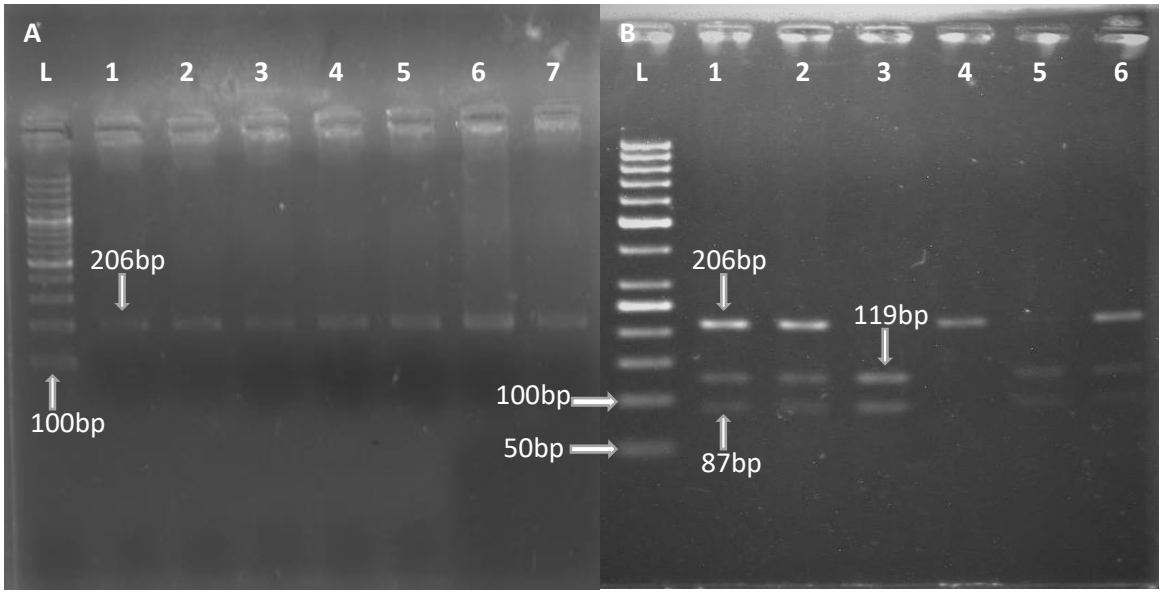


Figure 73: **Electro photomicrograph of c.894G>T (p.(Glu298Asp)) variant. (A) PCR product of 206 bp with 100 bp Ladder (L). (B) PCR-RLFP product with 50 bp Ladder, lane 1,2 and 6 represents GT genotype, lane 4 represents GG genotype and lane 3 and 5 represents TT genotype.**

As a result of electrophoresis *eNOS* 4b allele produced band of 420bp and 4ba allele produced bands of 420bp and 393bp (Fig. 74). Intron 4bb and 4ba genotypes were found in 86% and 14% individuals of preeclamptic group as compared to control subjects (90.5% and 9.55). aa genotype was found in none of the patients or control subjects. Frequency of b and a allele was 93% and 7% in total preeclamptic individuals as compared to control group (95.25% and 4.75%) (Fig. 78 and 79). Frequency of intron 4b/4b and intron 4b/4a showed no significant difference (p=0.1) in PE group as compared to normotensive pregnant females (Table 22).

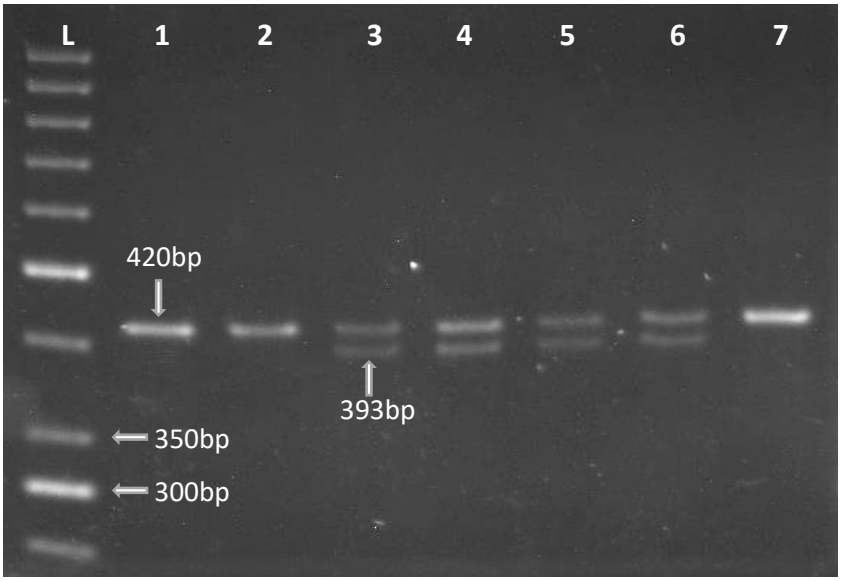


Figure 74: **Electro photomicrograph of intron 4b/4a variant with 50 bp Ladder. PCR product of 420 bp (lane 1,2 and 7) represents 4b allele while, PCR product of 420 + 393 represents 4ba allele (lane 3-6).**

Electrophoresis for g.-786T>C variant showed first band of 387bp which was control band in both PCR reaction (T and C) and second band of 250bp, corresponds to T and C allele indicating homozygous genotype (TT and CC), and heterozygous genotype (TC) in case of both bands in both reactions (Fig. 75). Genotypic distribution of g.-786TT, TC and CC was 56.5%, 34.5% and 9% in preeclamptic patients when compared to control group (68%, 26.5% and 5.5%) (Fig. 80 and 81). The frequency of g.-786C allele was significantly different in preeclamptic group ($p=0.007$) as compared to healthy controls. Likewise, g.-786TC genotype determined significant difference in preeclamptic group ($p<0.01$) when compared to normotensive control group. G.-786CC genotype showed significant difference preeclamptic group ($p<0.05$) as compared to control group (Table 22).

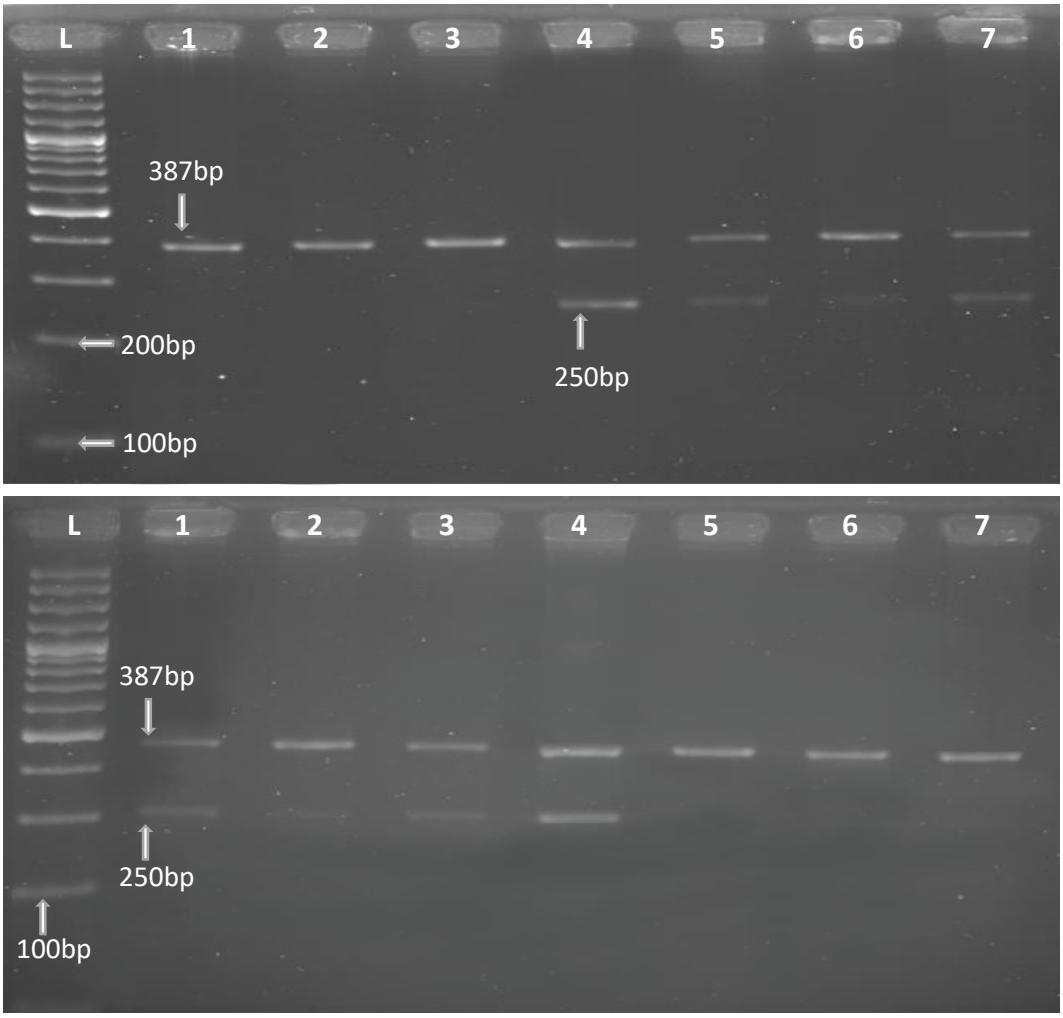


Figure 75: **Electro photomicrograph of g.-786T>C variant bp with 100 bp Ladder. (A) PCR product with inner T allele primer shows 387 bp (lane 1-3) representing TT genotype, lane 5-7 with 387 bp+250 bp product size represent CC genotype. (B) PCR product with inner C allele primer shows 387 bp (lane 1-3) representing CC genotype, lane 5-7 with 387 bp+250 bp product size represent TT genotype. While lane 4 give product size of 387 bp+250 bp in both reactions representing TC genotype.**

Table 22: Genotype distribution of *eNOS* 894 G/T, Intron 4b/4a and -786T/C haplotypes in controls and preeclamptic patients.

***eNOS* Variants**	**Genotype and Alleles**	**Controls n (%)**	**Preeclamptic patients. n (%)**
c.894G>T or p.(Glu298Asp)	GG	150 (75)	132 (66)
rs1799983	TC	29 (14.5)	24 (12)
	TT	21 (10.5)	44 (22)*
			(χ^2=9.75, P=0.008)
	Allele Frequency		
	G (Glu)	329 (82.25)	288 (72)
	T (Asp)	71 (17.75)	112 (28)
			(χ^2=11.91, p≤0.001)
			OR=1.80 (1.28-2.52)
Intron 4a/4b	bb	181 (90.5)	172 (86)
rs1722009	ba	19 (9.5)	28 (14)
	aa	0	0
			(χ^2=1.95, p=0.1)
	Allele Frequency		
	b	381 (95.25)	372 (93)
	a	19 (4.75)	28 (7)
			(χ^2=2.07, p=0.09)
			OR=1.54 (0.85-2.82)
g.-786T>C	TT	136 (68)	109 (56.5)
rs2070744	TC	53 (26.5)	73(34.5)**
	CC	11 (5.5)	18 (9)*
			(χ^2=7.84, P=0.02)
	Allele Frequency		
	T	325 (81.25)	295 (73.75)
	C	75 (18.75)	105 (26.25)
			(χ^2=6.45, p=0.007)
			OR=1.54 (1.10-2.15)

*p=<0.05 and **p=<0.01 is significant for Pearson chi-square test to identify which genotype presents significant different frequencies.

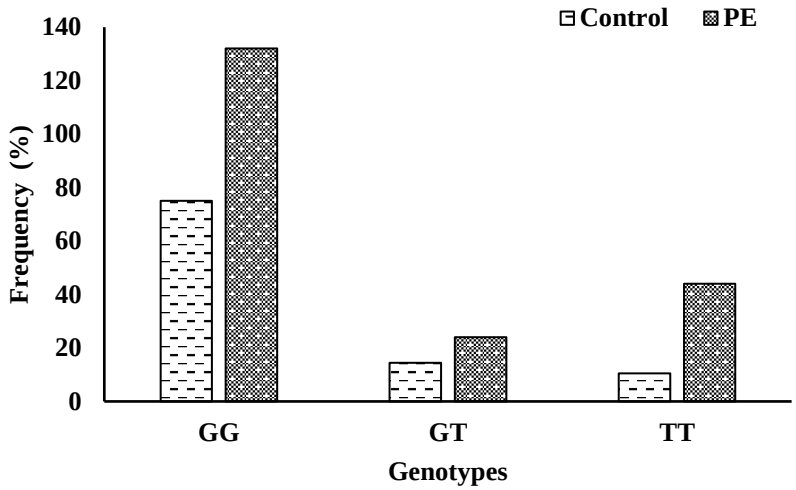


Figure 76: Percentage of genotypic frequencies of *eNOS* c.894G>T or p.(Glu298Asp) variant in control and preeclamptic patients.

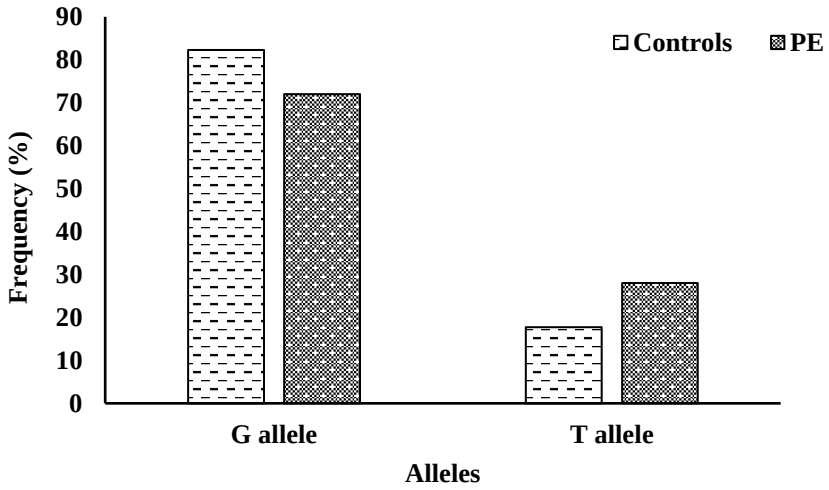


Figure 77: Percentage of allelic frequencies of *eNOS* c.894G>T or p.(Glu298Asp) variant in control and preeclamptic group.

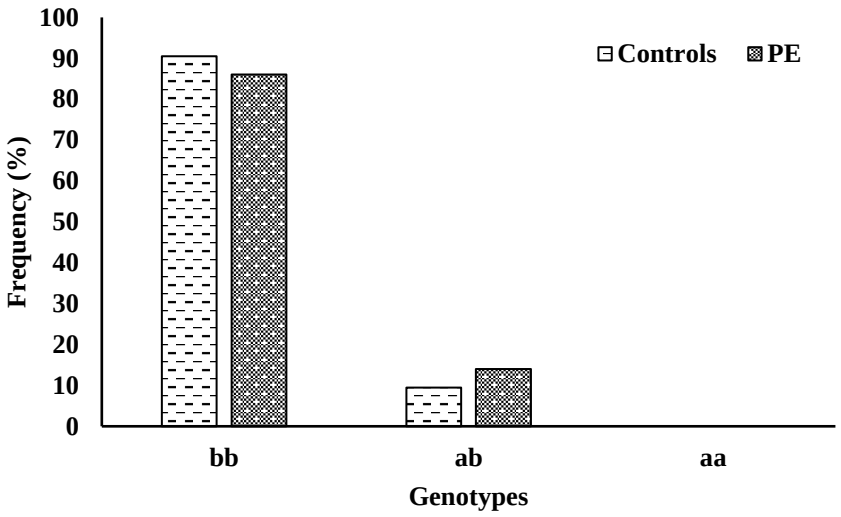


Figure 78: Percentage of genotypic frequencies of *eNOS* Intron 4b/4a variant in control and preeclamptic group.

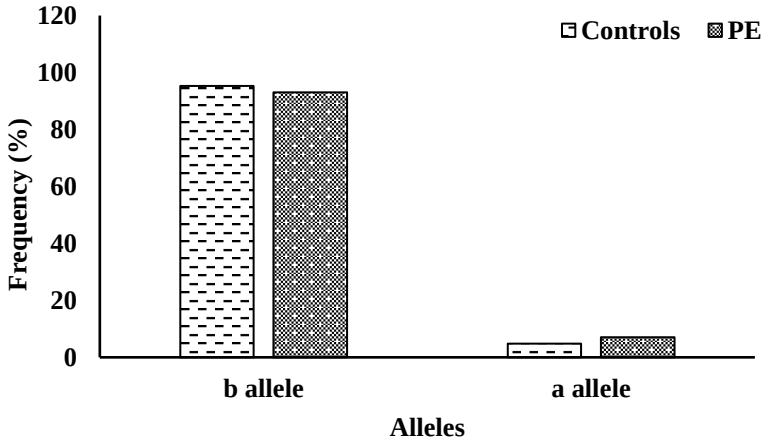


Figure 79: Percentage of allelic frequencies of *eNOS* Intron 4b/4a variant in controls and preeclamptic patients.

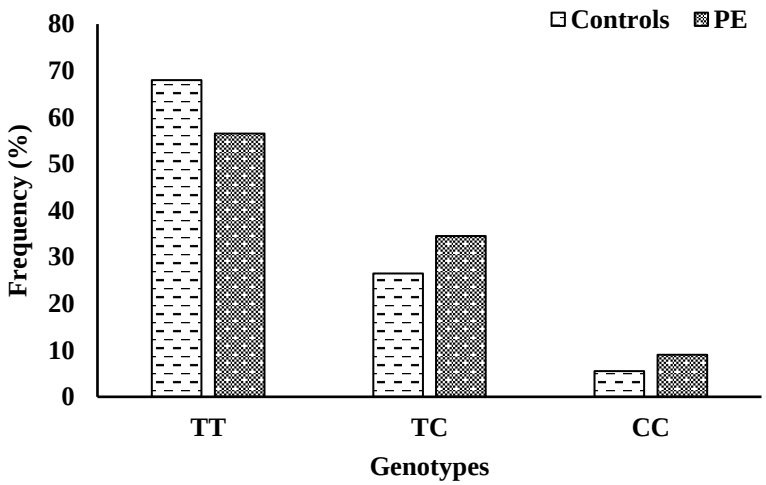


Figure 80: Percentage of genotypic frequencies of *eNOS* g.-786T>C variant in control and preeclamptic patients.

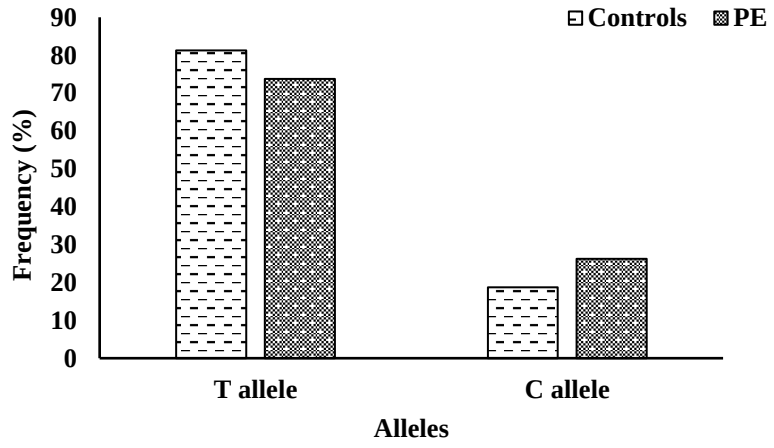


Figure 81: Percentage of allelic frequencies of *eNOS* g.-786T>C variant in control and preeclamptic group.

Sequencing

Results obtained from genotyping were confirmed by sequence analysis (Fig. 86, 87 and 88). Except all three targeted variants, other variants g.-2051G>A (rs553827594, in novel homozygous AA genotype) and g.-1861G>A (rs1800779) were also found in preeclamptic patients in homozygous GG, heterozygous GA and homozygous AA genotypes (Fig. 89 and 90). Frequencies of GG, GA and AA genotypes for g.-2051G>A variant were 65.5%, 15.5% and 19% in preeclamptic patients when compared with control group (76.5%, 17%, 6.5%) as shown in figure 79. While allelic frequencies of G and A alleles were 73.25% and 26.75% in PE group and 85% and 15% in control group. g.-2051G>A showed significant association with PE (p=0.02) group as compared to control group (Table 23, Fig. 82 and 83).

g.-1861G>A variant genotypic distribution for GG, GA and AA genotypes was 67%, 28.5% and 4.5% in preeclamptic women as compared to healthy pregnant women (71%, 27.5% and 1.5%) (Fig. 84 and 85). While allelic frequency of G and A allele was 81.25% and 18.75% in PE group when compared with control group (84.75% and 15.25%). However, the non-significant (p=0.19) association of g.-1861G>A was found between groups as shown in Table 23.

Table 23: Genotype distribution of *eNOS* -2051 G/A, Intron 4b/4a and -1861G/A variants in control and preeclamptic groups.

***eNOS* Variants**	**Genotype and Alleles**	**Controls n (%)**	**Preeclamptic patients. n (%)**
g.-2051G>A rs553827594	GG	153 (76.5)	131 (65.5)
	GA	34 (17)	31(15.5)
	AA	13 (6.5)	38 (19)*** (χ^2=7.45, P=0.02)
	Allele Frequency		
	G	340 (85)	293 (73.25)
	A	60 (15)	107 (26.75) (χ^2=6.45, p=0.007) OR=1.85 (1.17-2.94)
g.-1861G>A rs1800779	GG	142 (71)	134 (67)
	GA	55 (27.5)	57 (28.5)
	AA	3 (1.5)	9 (4.5) (χ^2=3.26, P=0.19)
	Allele Frequency		
	G	339 (84.75)	325 (81.25)
	A	61 (15.25)	75 (18.75) (χ^2=1.73, p=0.18) OR=1.28 (0.88-1.85)

*p=<0.05, **p=<0.01 and ***p=<0.001 is significant for Pearson chi-square test to identify which genotype presents significant different frequencies

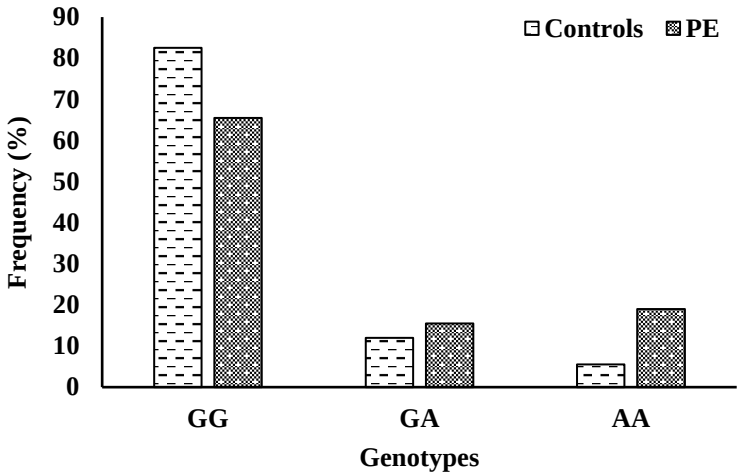


Figure 82: Percentage of genotypic frequencies of *eNOS* g.2051G>A variant in healthy pregnant and preeclamptic women.

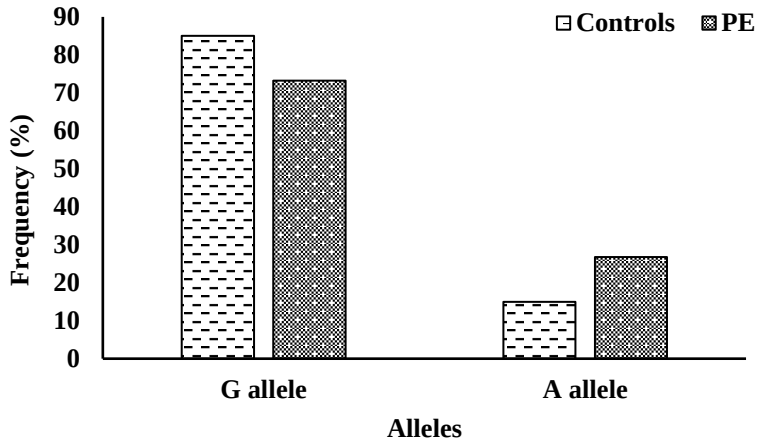


Figure 83: Percentage of allelic frequencies of *eNOS* g.2051G>A variant in control and preeclamptic group.

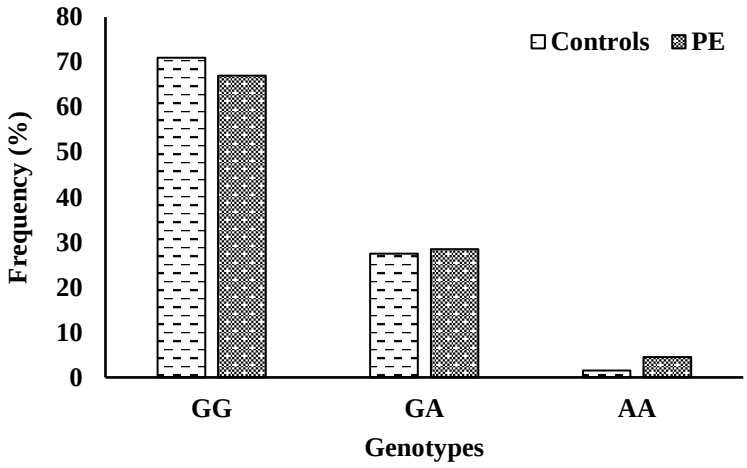


Figure 84: Percentage of genotypic frequencies of *eNOS* g.1861G>A variant in control and preeclamptic group.

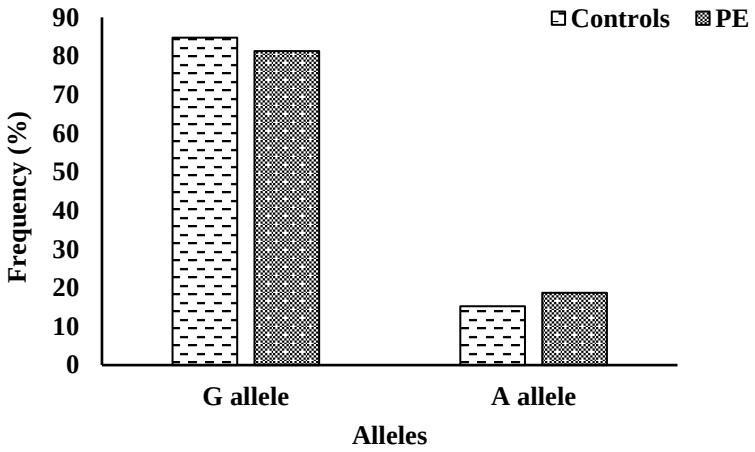


Figure 85: Percentage of allelic frequencies of *eNOS* g.1861G>A variant in normotensive pregnant and preeclamptic women.

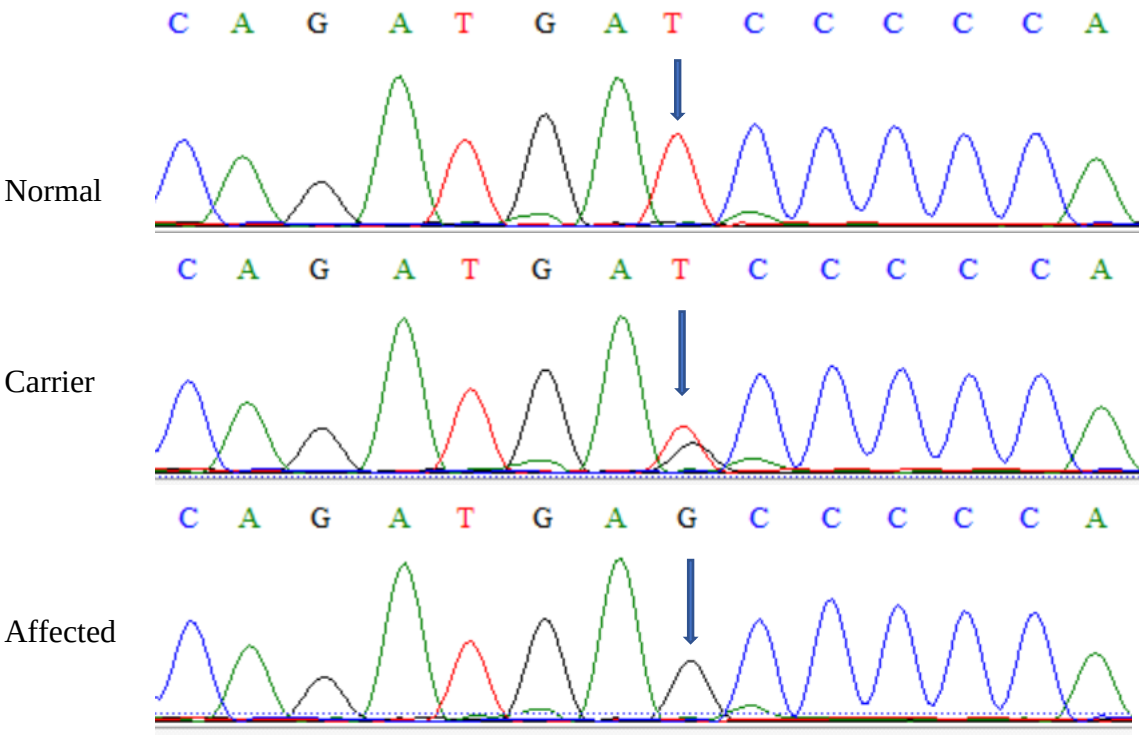
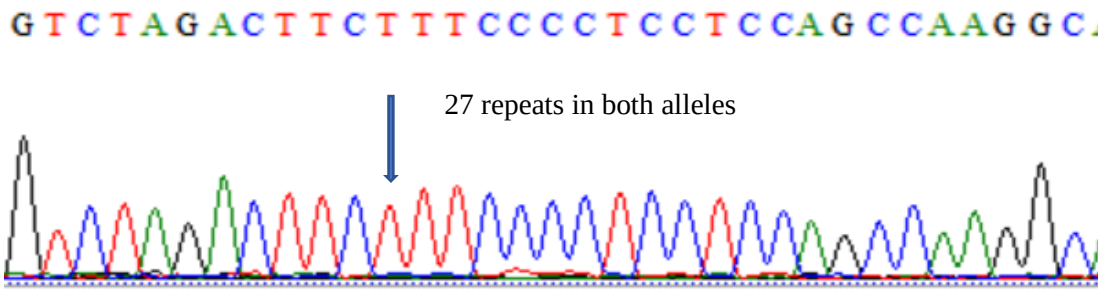


Figure 86: Sequence chromatogram of *eNOS* c.894G>T or p.(Glu298Asp) variant. Arrow indicates site of change.

4bb



4ba

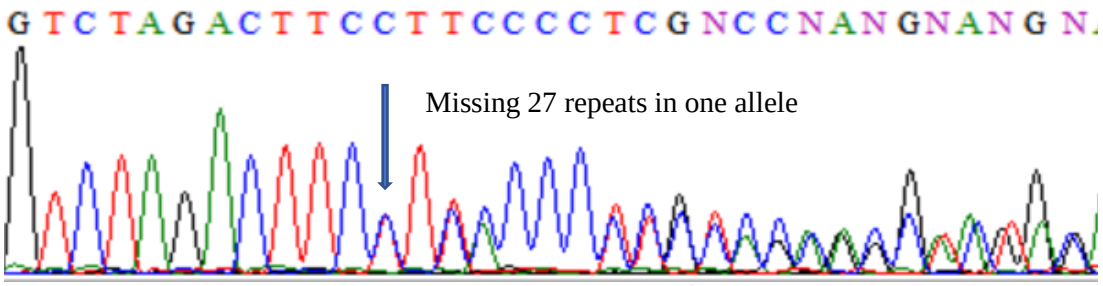


Figure 87: Sequence chromatogram of *eNOS* Intron 4b/4a variant. Arrow indicates site of change.

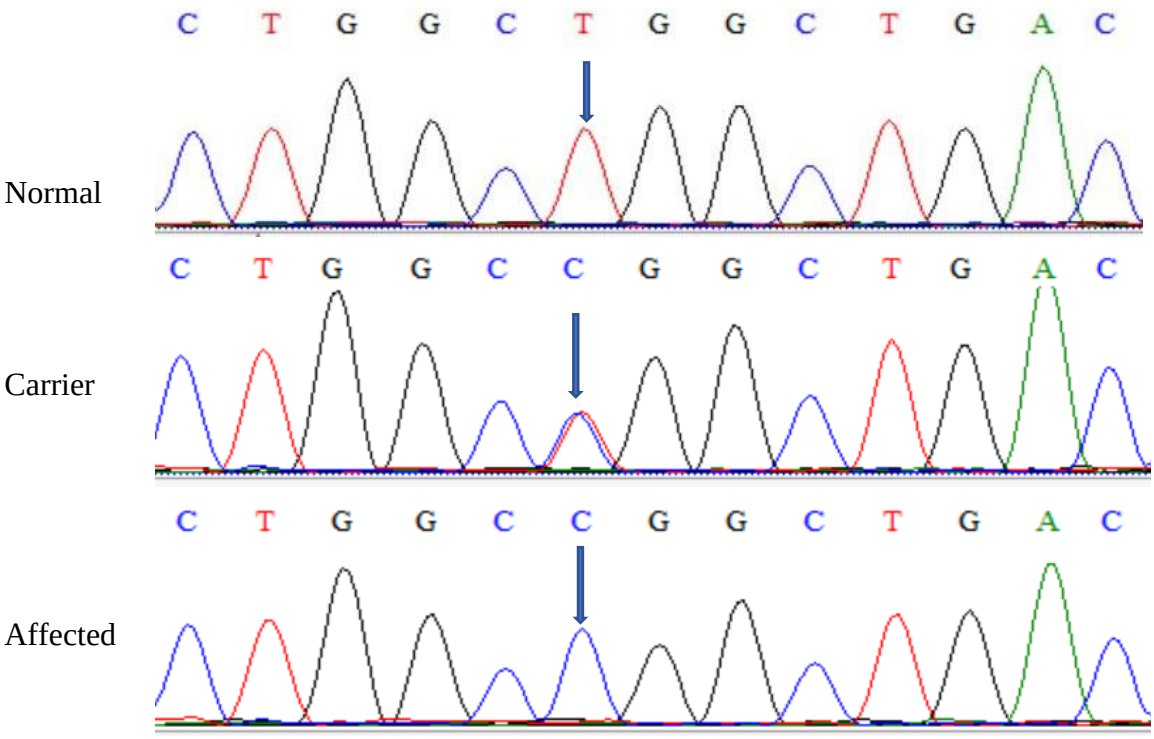


Figure 88: Sequence chromatogram of *eNOS* g.-786T>C variant. Arrow indicates site of change.

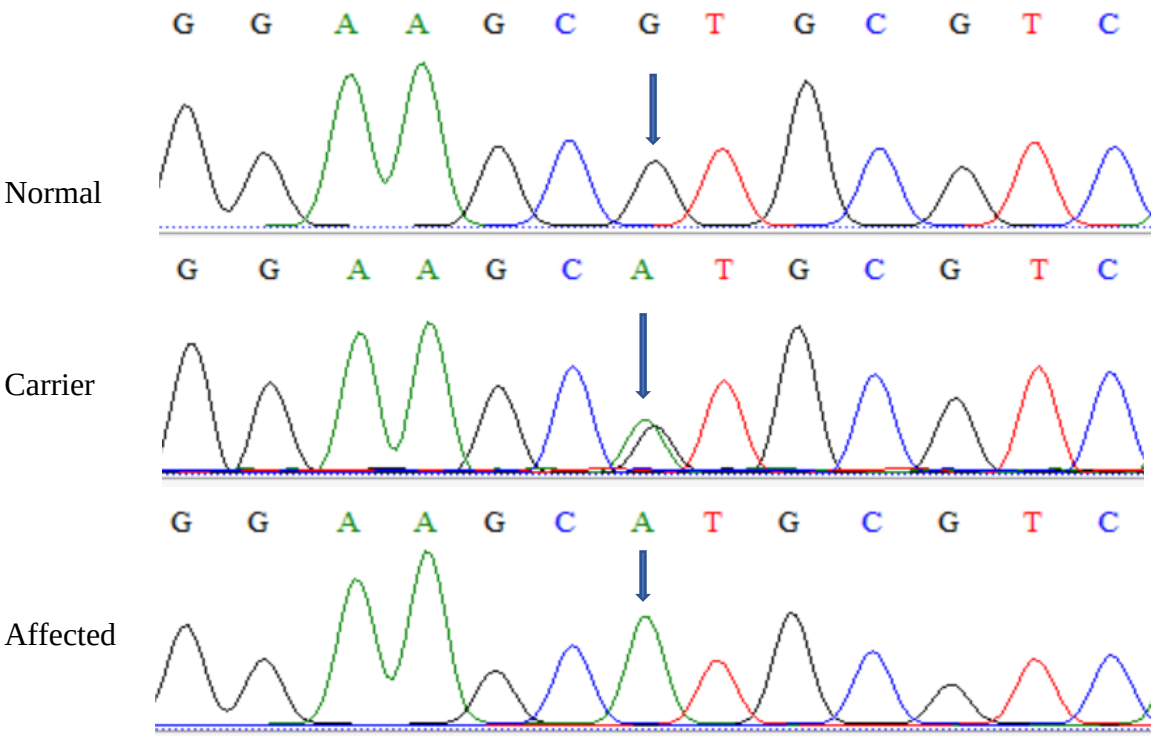


Figure 89: Sequence chromatogram of *eNOS* g.2051G>A variant. Arrow indicates site of change.

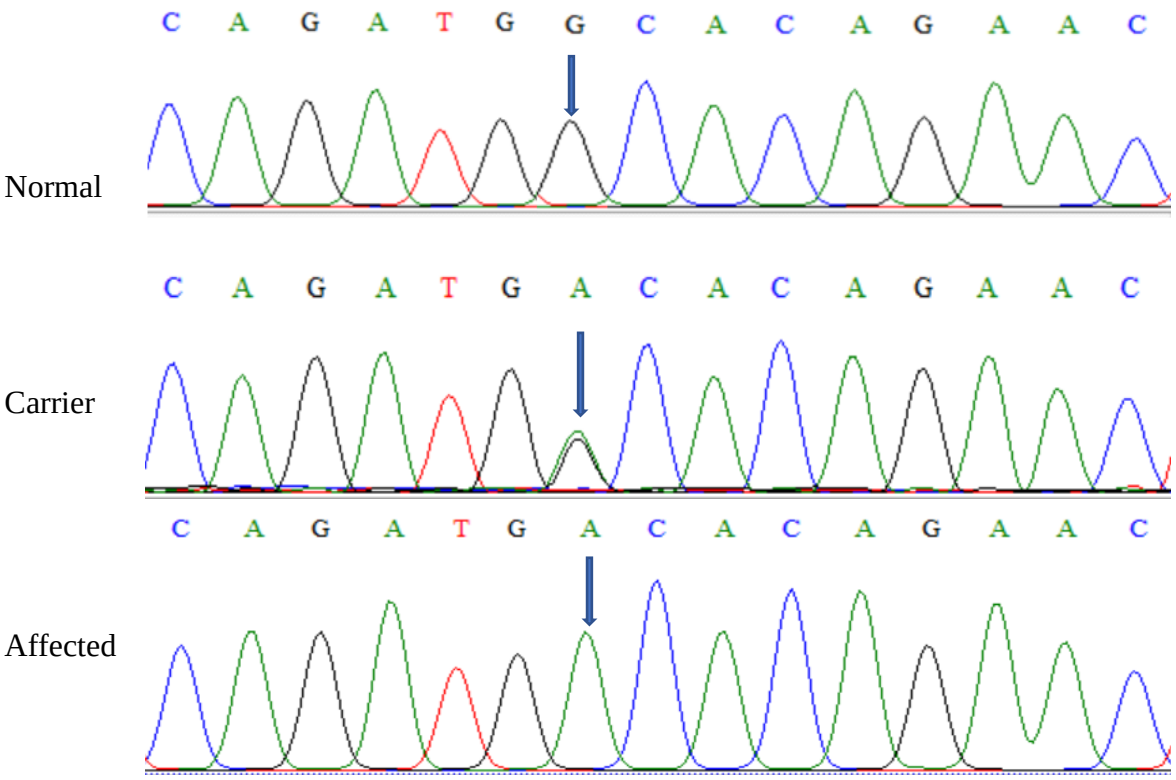


Figure 90: Sequence chromatogram of *eNOS* g.1861G>A variant. Arrow indicates site of change.

Structural Analysis

Ramachandran plot for eNOS3^{Glu298Asp} structure indicated overall >93% of residues in the allowed region (Fig. 91). This shows that structure is of good quality and can be used for the structure-activity study. Structural analysis revealed the fact that this substitution changed the overall topology of the *eNOS* protein. Upon mutation stability of the protein changes with destabilizing energy of ΔΔG: -0.071 kcal/mol. The flexibility of the protein was also changed with vibrational entropy change of $\Delta\Delta S_{Vib}$ ENCoM: -0.396 kcal.mol^{-1}.K^{-1} . Several atomic fluctuations were observed throughout the eNOS protein (Fig. 92).

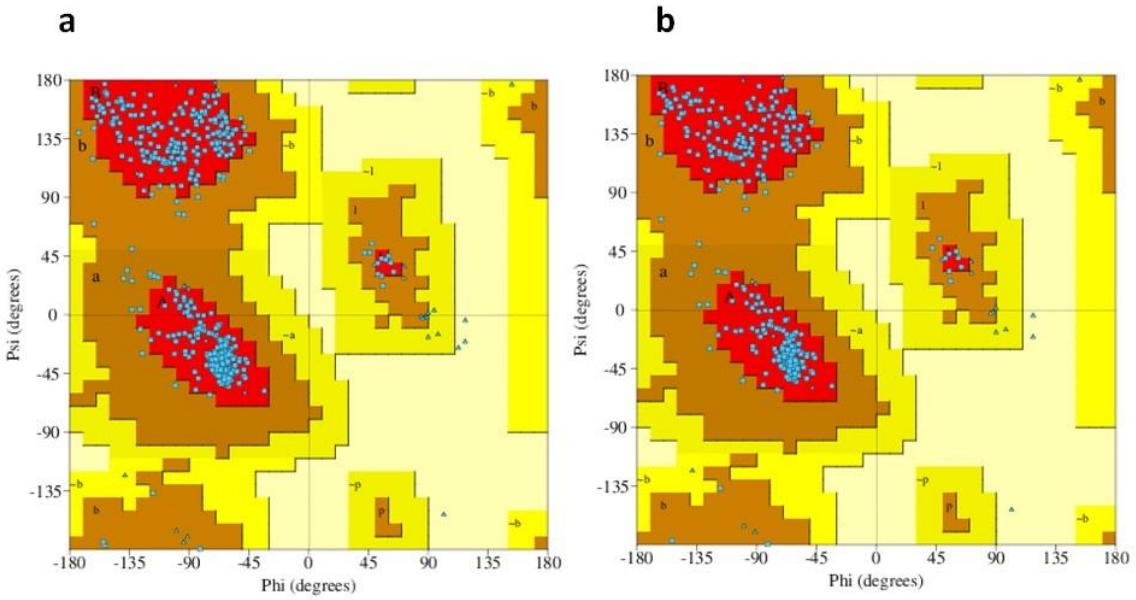


Figure 91: Ramachandran plot. (a) eNOS^{WT} (b) eNOS^{Glu298Asp}

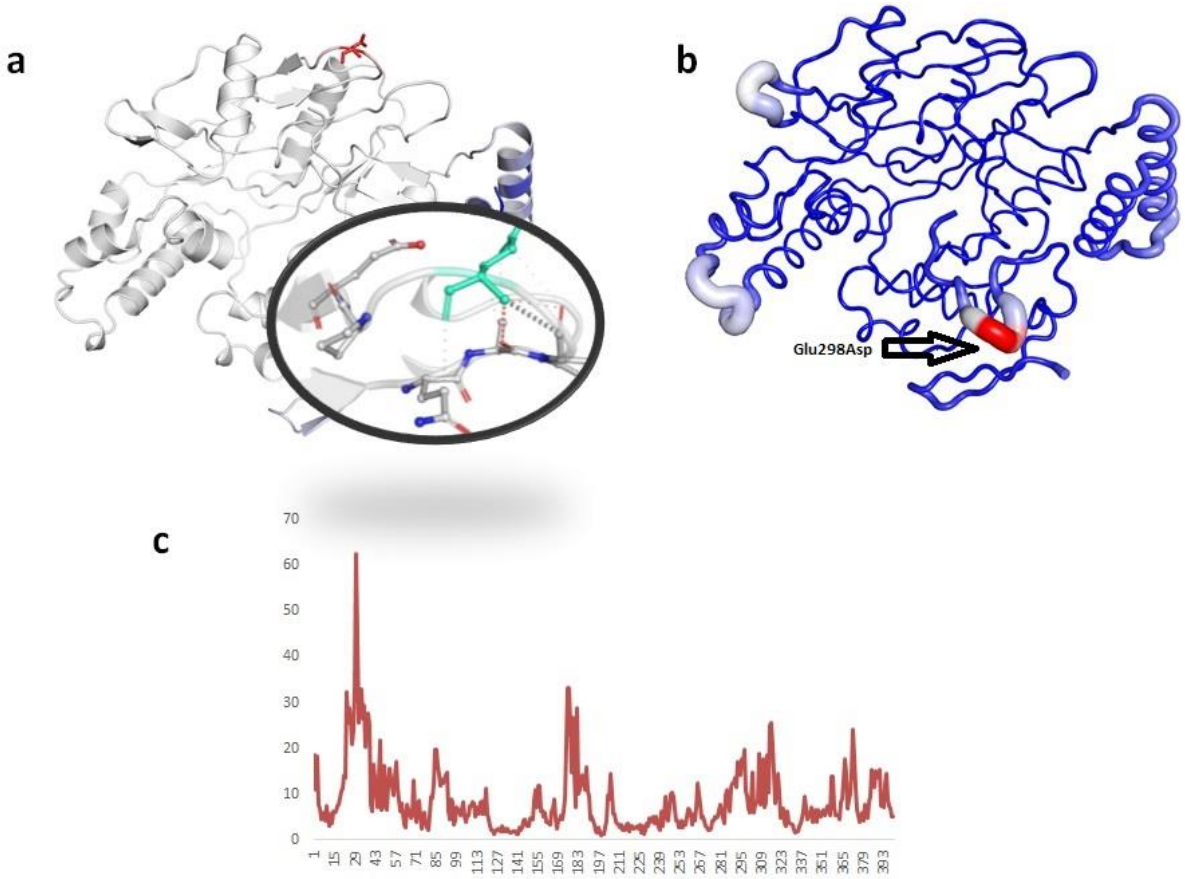


Figure 92: Structural changes upon Glu298Asp substitution in *eNOS* protein. (a) Amino acid substituent shown in cyan color (b) Amino acids coloured according to the vibrational entropy change upon mutation. Rigidification of the structure is represented by blue and a gain in flexibility is shown as red. (c) Plot showing deformities in protein upon mutation.

Molecular dynamics simulation analysis

The *eNOS*^{WT} and *eNOS*^{Glu298Asp} structures were further analyzed by molecular dynamics (MD) simulation assay to study the time-dependent behavior and overall stability of the system. The root mean square fluctuation (RMSF) and root mean square deviation (RMSD) were plotted using secondary structural elements stability and conformational deviations of all atom trajectories. The high fluctuation between 150-300 residues was observed in *eNOS*^{WT} and the systems remain stable throughout the length of the protein. The RMSF analysis of eNOS^{Glu298Asp} indicated fluctuation in the loops near mutant residue at 298 position. The high RMSF fluctuation rate of the eNOS^{Glu298Asp} indicated high fluctuation rate per residue in the C-terminus protein regions. Glue298Asp stated more fluctuation up to 5A$^{\circ}$ to our surprise region. This elevated fluctuation showed that this replacement has a adverse effect on the stability of proteins (Fig. 93).

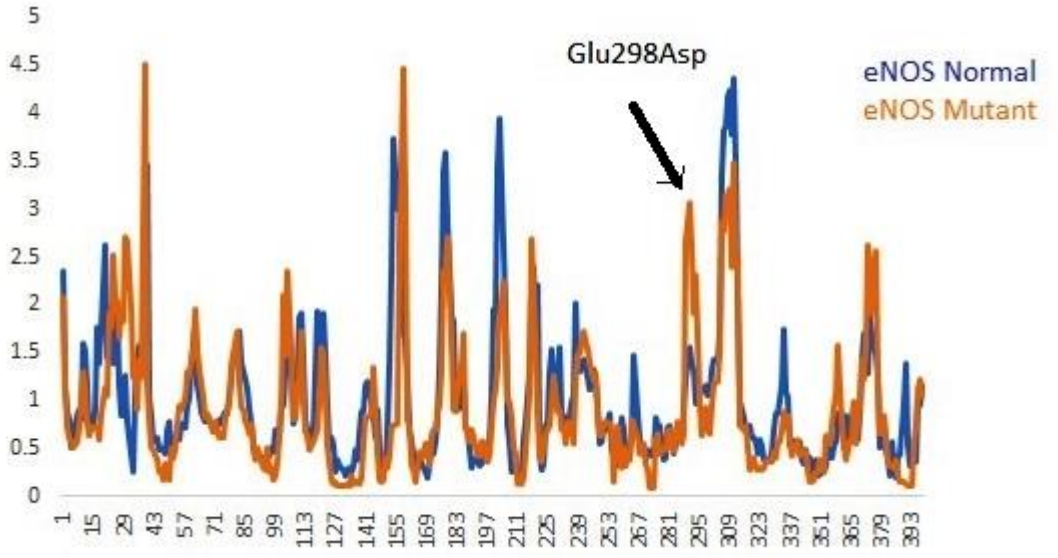


Figure 93: Root mean square fluctuation (RMSF) plot of *eNOS* normal and mutant protein.

Transcription factor (TF) binding Site prediction and DNA modeling

ConTraV3 (http://bioit2.irc.ugent.be/contra/v3) and JASPER database revealed Signal transducer and activator of transcription 3 (STAT 3) and Signal transducer and activator of transcription 6 (STAT6) transcription binding site in the *eNOS* gene promoter region (Fig. 94). STAT3 is a cell proliferation, differentiation, inflammation and survival transcription factor (Qi QR and Yang ZM., 2014). STAT6/IL-4 pathway is important for normal pregnancy (KR bound., 2017). Variants in the promoter region (-786T>C and -2015G>A) abolished the binding site for Stat3 and Stat 6 respectively. Normal and mutant DNA molecules were modeled for the promoter region of *eNOS* gene (Fig. 95).

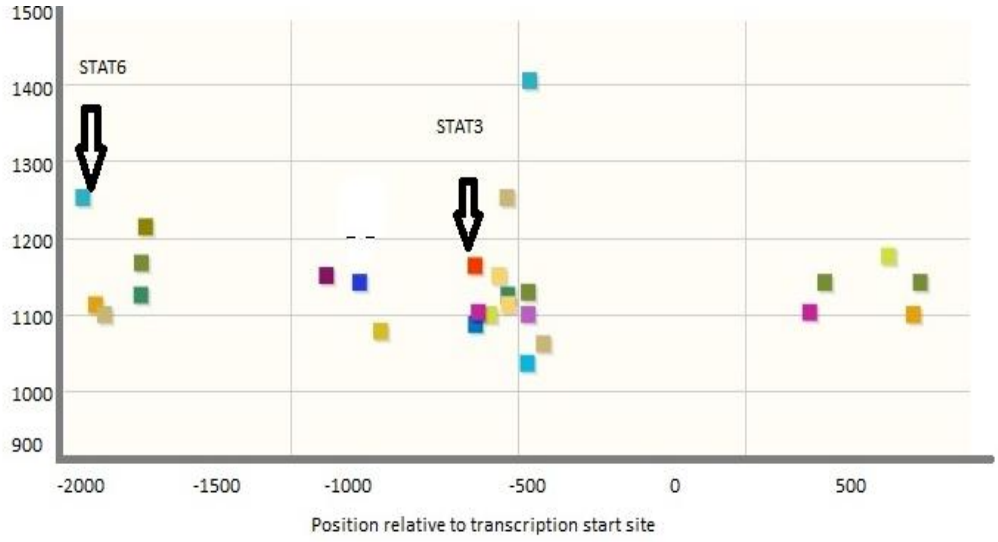


Figure 94: **Scatter plot of Transcription factor (TF) binding sites on eNOS promoter.**

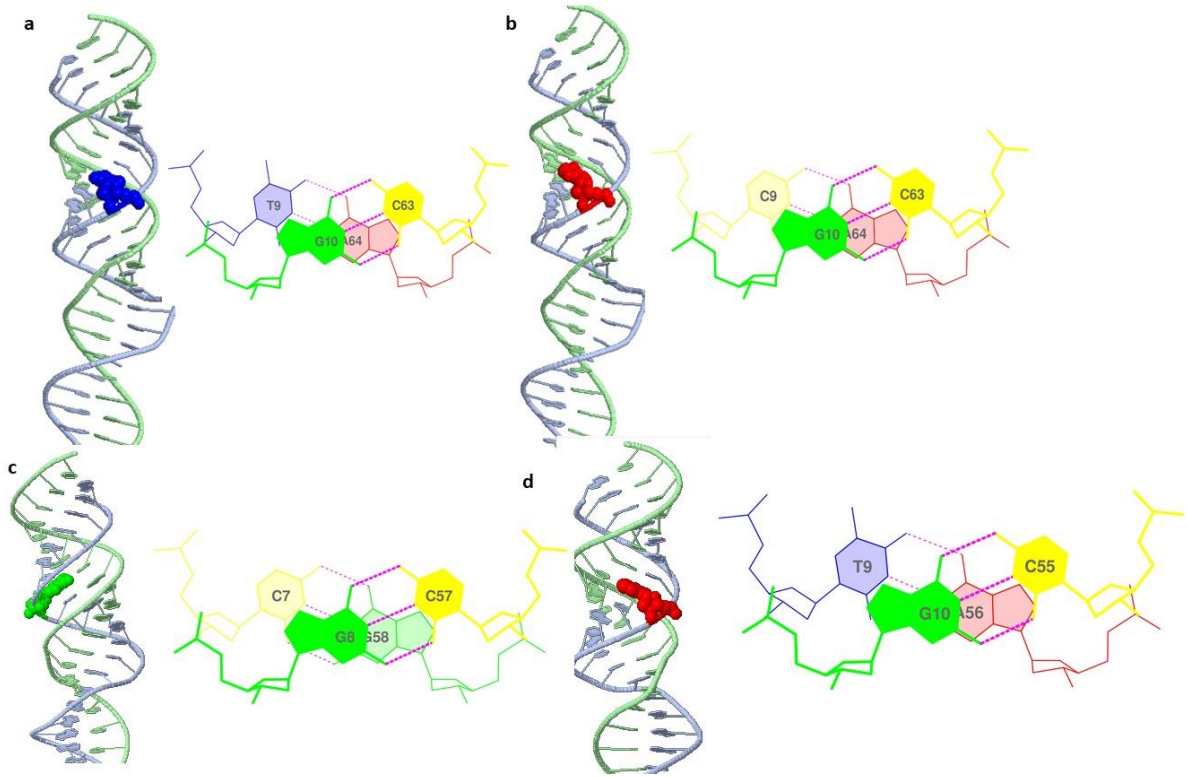


Figure 95: DNA modeling and stacking interaction of normal and mutant DNA molecules.

Docking analysis of *eNOS* promoter with STAT3 and STAT6

STAT3 and STAT6 protein's surface were assessed through molecular docking to investigate its potential for *eNOS* promoter region. Rigid body docking of eNOS DNA with STAT3 and STAT6 revealed important structural insight into the binding of DNA major and minor grooves. Mixture of major and minor grooves are involved in binding with respective transcription factor. Computational docking gave a perfect fit of binding of normal DNA with STAT3 and STAT6 protein while mutant promoter DNA moved away from the binding interface of both (STA3 and STAT6) transcription factor (Fig. 96).

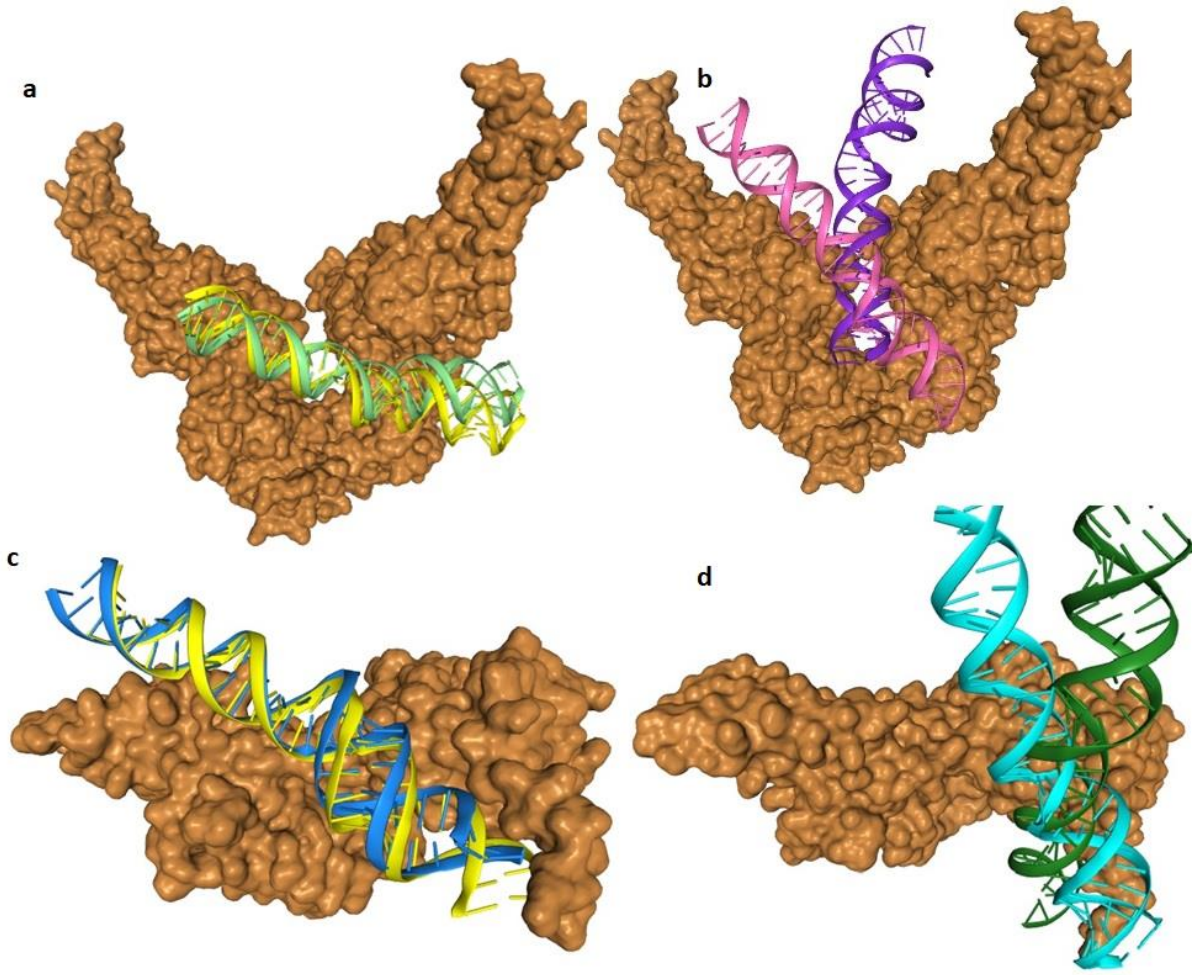


Figure 96: **Molecular docking analysis of eNOS promoter with STAT3 and STAT6 transcription factor. a (WT) and b (mutant) represents the binding of STAT3 with *eNOS* promoter encompassing -786T>C variant. Protein is shown in brown surface model while DNA in cartoon model. c (WT) and d (mutant) represents the binding of STAT6 with *eNOS* promoter encompassing -2015G>A variant. Protein is shown in brown surface model while DNA in cartoon model.**

DISCUSSION

PE is an important maternal health problem, especially in developing countries like Pakistan. Identification of various factors and candidate genes could help in understanding this complication and provide clues for its management and treatment. Endothelial dysfunction and decreased NO production has been reported to be associated with PE (Aggarwal *et al.,* 2010; Rahimi *et al.,* 2013; Gannoun *et al.,* 2015). This study was the first to evaluate the potential role of eNOS gene variants in preeclamptic females relative to healthy pregnant normotensive females in Pakistan.

In present study common variants of *eNOS* gene including c.894G>T (p.(Glu298Asp)), intron 4b/4a and g.-786T>C were analyzed by genotyping and confirmed through sequencing. Significant association was found for c.894G>T (p.(Glu298Asp)), variant. It was demonstrated that *eNOS* c.894T (p.298Asp) was significantly associated with PE. Our findings are in correspondence with previous studies (Serrano *et al.,* 2004; Alpoim *et al.,* 2014; Chen *et al.,* 2014; Maria Procopciuc *et al.,* 2018). A probable mechanism by which NO bioavailability has been reported to be reduced by c.894 T (p.298Asp). Several experiments have shown that p.298Asp is subjected to selective proteolytic cleavage in vascular tissues and endothelial cells leading to decreased NO generation in homozygous subjects for this variant (Tesauro *et al.,* 2000; Persu *et al.,* 2002; Alpoim *et al.,* 2014). In addition, p.(Glu298Asp) interacts with other proteins playing role in its product degradation to modulates *eNOS* activity, and hence its intracellular distribution (Tesauro *et al.,* 2000). Thus, women with homozygous Asp298 allele are more vulnarable for endothelial dysfunction which might increase risk of PE, observed in present study as well.

Regarding the *eNOS* intron 4b/4a variant, non-significant association has been found in current study as demonstrated by previously in different population-based case control studies (Chen *et al.,* 2007; Sandrim *et al.,* 2010a; Ozturk *et al.,* 2011; Zdoukopoulos *et al.,* 2011; Rahimi *et al.,* 2013; Gannoun *et al.,* 2015). Possible reason might be its intronic region which is unlikely to be functional in its own right (Serrano *et al.,* 2004).

The association of g.-786T>C variant with PE observed here agreed with the results presented earlier for different regions of the world (Kim *et al.,* 2008; Aggarwal *et al.,* 2010; Sandrim *et al.,* 2010b; Ozturk *et al.,* 2011; Zdoukopoulos *et al.,* 2011). Present study demonstrated that g.-786C allele was prevailing in PE patients as compared to control. The g.-798C allele in *eNOS* promoter region has been linked with reduced mRNA expression

and lower serum nitrite/nitrate levels, causing unavailability of NO (Nakayama *et al.,* 2006). It was also suggested that 50% of *eNOS* activity can be reduced if its transcription is repressed by targeting g.-786C allele through single stranded DNA binding transcription factor replication protein, A1 which results in lower NO production (Senthil et al., 2005; Dosenko1½ et al., 2006).

Sequencing revealed the association of another variant with the disease phenotype that is g.2051G>A which was present in the *eNOS* gene promoter region. Genetic analysis showed that previously this variant was associated with the disease phenotype in heterozygous form but in accordance with recent study it was also determined to be homozygous for g.2051AA. This variant might affect the transcription of the *eNOS* gene through unknown mechanism and results in reduced NO levels leading to endothelial dysfunction.

Through our detailed *in silico* structural assessment, we elucidated the structural and functional behavior of *eNOS* protein upon Glu298Asp substitution. The reported substitution resulted in the loss of flexibility and deformities were reported that alter the conformation and configuration of the protein and ultimately decrease protein stability. Molecular dynamic simulation analysis revealed that due to Glu298Asp substitution there was changes in protein structural behavior and fluctuations have been noted in the mutation-related loop area, and this change may contribute to preeclampsia. Detailed illustration of eNOS's structural and functional behavior reveals the underlying molecular mechanism and can help develop a powerful therapeutic drug. Secondly the mutations reported in the *eNOS* gene promoter region changed the binding of transcription factor STAT3 and STAT6 to the 5’ site of the *eNOS* gene. These reported variants might change the regulation of *eNOS gene* and responsible reduced NO level and leading to endothelial dysfunction.

Conclusion

The present study revealed substantial association of c.894G>T (p.(Glu298Asp)) and g.-786T>C variants while no association of intron 4b/4a variant in Pakistani population. Data from the current study suggest that there might be other risk variants of *eNOS* gene (g.2051G>A and g.1861G>A) that confers in increased risk of PE. The detailed computational investigation further confirmed the deformities and changes in protein flexibility upon Glu298Asp. These structural alterations might be involved in causing preeclampsia. Variants in the *eNOS* gene promoter region further validate the change in

gene regulation for the onset of disease. Identification of key structural and functional features in eNOS protein and gene regulatory region might be used for designing specific drugs for therapeutic purpose.

Future recommendations

Large study or meta-analysis and follow-up studies are required to determine the contribution of *eNOS* variants in PE pathogenesis and related features. By conducting genetic association studies and genome-wide association studies with appropriate credibility, further cooperative study on PE may assist to elucidate the contributory role of *eNOS* variants. In order to avoid maternal and neonatal morbidity and mortality, understanding of PE etiology and the role played by variations in exogenous factors in advanced and developing nations should be a study concern

SUMMARY

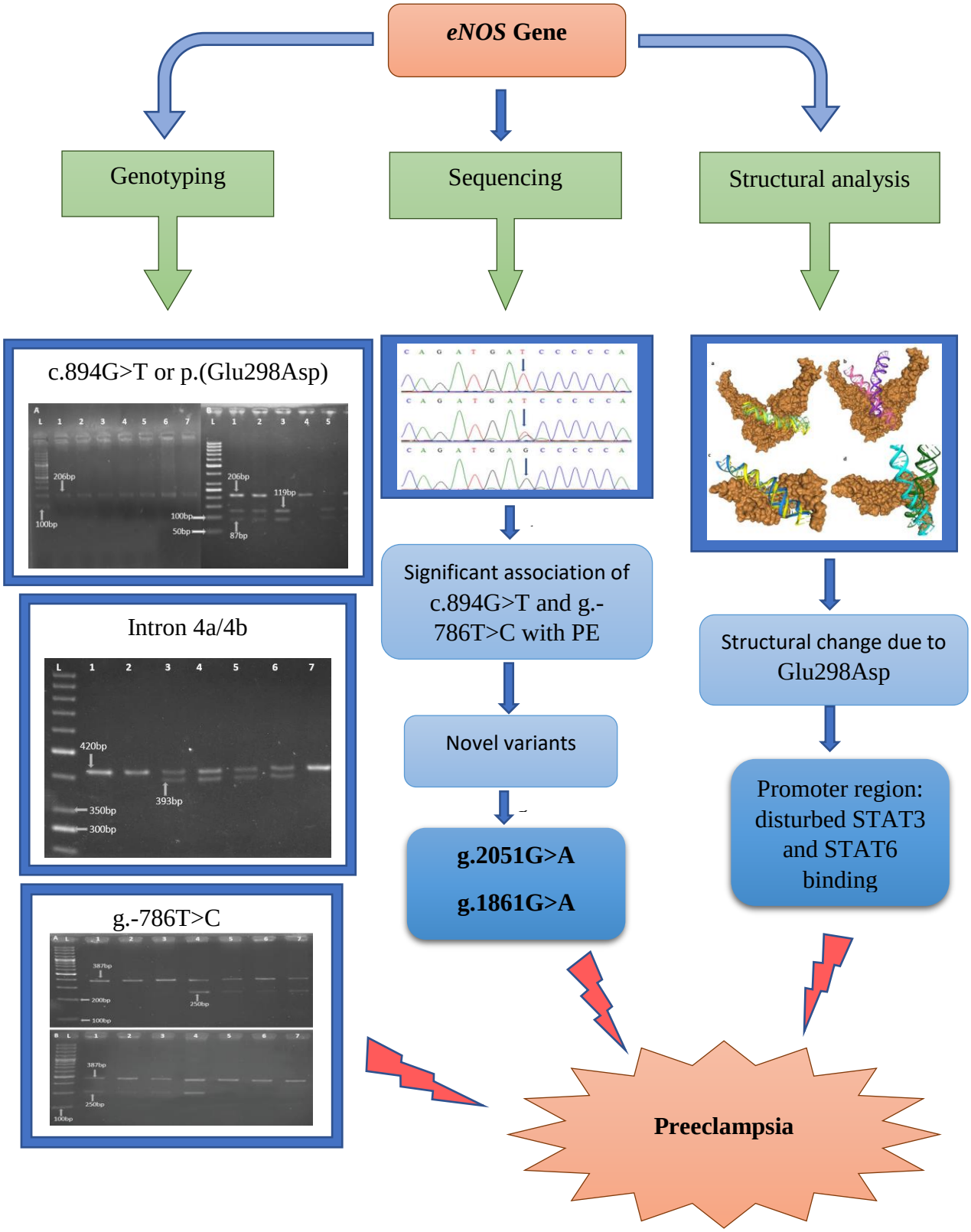


Figure 97: Graphical representation of association of *eNOS* gene variants with PE in Pakistan.

GENERAL DISCUSSION

Pregnancy hypertensive disorders remain a major health concern worldwide for women and infants (Balogun & Sibai 2017). Maternal mortality in Pakistan is exceptionally high primarily owing to pregnancy-related complications ; roughly 500 per 100,000 live births are estimated due to PE (Shansi *et al.,* 2010). PE is a great challenge to obstetricians because it has complex pathophysiology with unknown cause (Rugolo *et al.,* 2016). It involves several organs systems including kidney, placenta, liver, hemopoietic, vasculature, brain and coagulation system. Determination of multiple risk factors and measurement of particular markers for the participation of each of these systems may not only indicate the contribution of organs before the complete maternal manifestation of the syndrome but may also indicate that there are various causes and presentations leading to preeclampsia. (Rugolo *et al.,* 2016). The presence of particular markers can eventually be used for disease diagnosis. (Steegers *et al.,* 2010).

Pregnancy is associated with many hormonal changes. Catecholamines such as epinephrine released by hypothalamic adrenal axis during stress may interfere with the vasodilator action of NO leading to endothelium impairment (Toda and Nakanishi-Toda, 2011). Thyroid function was reported to be altered in several circumstances that are unique to pregnancy (Khadem *et al.*,2012). Hypothyroidism can trigger smooth vascular muscle contraction in both systemic and renal vessels, resulting in enhanced diastolic hypertension, peripheral vascular resistance and reduced tissue perfusion. (Satyanarayan *et al.*,2015). Proteinuria can also be associated with thyroid dysfunction, which results in increased excretion of thyroid-binding globulins and thyroxine (Negro and Mestman, 2011).

The principal contributor to the pathogenesis of PE is placenta, as the syndrome is resolved when placenta is delivered (Raghupathy, 2013). In PE, abnormal placenta development results in poor placental perfusion. The placenta of females with PE is abnormal and has abnormal trophoblastic invasion (Eiland *et al.,* 2012). This is believed to result in oxidative stress, hypoxia and the release of factors promoting endothelial dysfunction, inflammation and other possible responses (Eiland *et al.,* 2012). Various mediators are involved in controlling endothelial dysfunction in preeclampsia but role of *eNOS* gene appears most significant in the development of preeclampsia (Devendran *et al.,* 2015). *eNOS* is an significant vascular tone regulator and helps to reduce the

uteroplacental resistance observed during normal pregnancy by producing NO (Aggarwal *et al.,* 2010; Rahimi *et al.,* 2013; Gannoun *et al.,* 2015).

Demographic and clinical presentation

Pregnancy hypertensive disorders remain a major health concern worldwide for women and infants (Balogun and Sibai, 2017). Maternal Mortality is extremely high in Pakistan essentially due to complications related to pregnancy, it is approximately predicted to be 500 per 100,000 live births (Shamsi *et al.,* 2010). PE is a great challenge to obstetricians because it has complex pathophysiology with unknown cause (de Souza Rugolo *et al.,* 2011; Madoglio *et al.,* 2016). It involves several organs systems involving kidney, liver, placenta, hemopoietic, vasculature, coagulation system and brain. Determination of multiple risk factors and evaluation of specific markers can predict this condition before the manifestation of maternal syndrome and can show that PE have several different reasons and presentations with the involvement of different organs and systems (Madoglio *et al.,* 2016). The presence of specific markers eventually be used for diagnosis and management of this disorder to avoid maternal and neonatal morbidity and mortality (Steegers *et al.,* 2010).

In present study various risk factors and clinical markers were investigated to find the possible cause of PE in Pakistan. According to current study possible predictors of PE are maternal age ($p=0.002$), gestational age ($p<0.001$) and blood pressure ($p<0.001$), however there was no significant association of BMI, previous abortions and maternal age at time of marriage with the development of PE. These findings correspond to previous finding showing elevated SBP (odds ratio, 2.66) and DPB (odds ratio, 1.72) but no change in BMI and maternal age between groups (Duckitt and Harrington, 2005). Underlying mechanism involves the changes in blood pressure during pregnancy due to various factors initiated by the placenta, in case of uteroplacental insufficiency blood flow reduces toward placenta, to fulfil such need maternal blood pressure rises to accommodate the blood supply towards placenta (Pridjian and Puschett, 2002; Alpoim *et al.,* 2011). Transportation of nutrients from maternal to fetal circulation through placenta depends on the blood delivery to the uterus, which is directly related to maternal cardiac output and local factors controlling uterine perfusion (Pridjian and Puschett, 2002).

Women with increased age had twice the risk of developing PE and its risk increases up to 30% in every additional year of age past (Saftlas *et al.,* 1990; Bianco *et al.,* 1996; Stamilio *et al.,* 2000); (Myatt and Miodovnik, 1999; Abalos *et al.,* 2013). In present study weight of child after birth was lower in preeclamptic patients as compared to normotensive women. Uteroplacental insufficiency, low gestational age and placental abruption are considered the leading factors associated with low child birth weight and poor perinatal outcome (Backes *et al.,* 2011; Madoglio *et al.,* 2016; Shah *et al.,* 2016).

There was no association of blood groups noticed in present study with respect to PE. Previous literature also revealed no relationship of blood group with PE (Clark and Wu, 2008; Alpoim *et al.,* 2013; Mishra and Pradhan, 2013; Aghasadeghi and Saadat, 2017). According to present study headache, swelling in hands and face, excessive weight gain and urination problem have increased frequency in PE patients as compared to control subjects. Previous literature demonstrated the same findings which evaluated the accuracy of these symptoms in predicting adverse maternal outcomes (Witlin *et al.,* 1999; Black, 2007; Menzies *et al.,* 2007).

In current results abdominal or epigastric pain, muscular pain and blurring of vision was determined to be more frequent in PE subjects as compared to control group as observed in various previous studies that predict maternal complications (Harms *et al.,* 1991; Thangaratinam *et al.,* 2011).

Family history was observed in this study. According to these findings' history of PE in preceding pregnancy and history of PE in family predicts the association with the onset of PE. These findings were supported by previous literature showing that family history of PE is associated with a fourfold increased risk of PE in women, underlying mechanism may involve the predisposition of various genetic factor involved in the pathophysiology of PE (Duckitt and Harrington, 2005; Thangaratinam *et al.,* 2011).

Clinical manifestation in the current study involved the presence of protein in the urine of PE women with high significance as compared to Controls. There was no significant change in urobilinogen, Sp. gravity and PH of urine in PE group as compared to healthy controls. These findings were supported by previous studies resulting in either ≥1+ dipstick proteinuria on two separate occasions or ≥2+ dipstick proteinuria on one occasion or ≥300 mg proteinuria over 24 hours (Wagner, 2004; Thangaratinam *et al.,* 2011; English *et al.,* 2015; Kurt *et al.,* 2015).

Considering that large differences that have been reported earlier (Hays *et al.,* 1985), surprising that no significant changes are seen in a number of blood parameters, RBCs number, haemoglobin concentration, MCV, MCH, MCHC, RDW-CV and neutrophil percentage as well as platelet numbers, but significant change in TLC and hematocrit in preeclamptic women as compared to healthy control was observed. Canzoneri et al. also reported a significant increase in TLC in preeclamptic group which may results in certain infections or other inflammatory response (Canzoneri *et al.*, 2009). Wagner also reported high hematocrit level as a risk associated with Preeclampsia (Wagner, 2004).

The measurement of aPTT seems to be important for early detection of coagulation abnormalities in patients with the risk of PE whose platelet count is normal. Our results are in agreement with the results of (Jahromi and Rafiee, 2009) and against the concept that all preeclamptic patients with a coagulation abnormality have platelet count <100,000/mm (Metz *et al.*, 1994). Ferritin levels were not statistically different in both groups but routine investigation of serum ferritin status of pregnant women with high risk for PE as part of antenatal checkup may help to establish diagnosis of PE before appearance of its clinical manifestations and unnecessary use of iron in a non-anaemic pregnant woman can be avoided.

Damage to the hepatic and renal systems were confirmed by a significant increase of several well-established blood markers such as ALP, total bilirubin, urea, AST and uric acid. Our results for liver function tests were consistent with those obtained by Hay, Wagner, Kenny et al. and FitzGerald et al. (FitzGerald *et al.*, 1996; Wagner, 2004; Hay, 2008; Kenny *et al.,* 2015).

It was demonstrated in present study that serum electrolytes including calcium and sodium levels decreased significantly in preeclamptic group as compared to control group with no significant change in potassium level. These results correlate with several studies demonstrated that calcium and sodium are essential in pregnancy which plays an significant role in metabolic activities at cellular level (Adewolu, 2013; Guo *et al.,* 2017). Decreased level of calcium results in increased parathyroid hormone levels, which further increase the intracellular calcium levels, that leads to an increased vascular smooth muscle contraction and finally raise the blood pressure (Indumati *et al.*, 2011).

Contemporary studies showed that serum sodium levels significantly decreased in the preeclamptic patients as compared to controls, because of altered sodium transport across the cell membrane leads to the deposition of sodium in the extravascular spaces and a decrease in the plasma sodium levels (Adewolu, 2013). Sodium retention increased sensitivity to angiotensin which leads to hypertension, oedema and proteinuria, the diagnostic triad of preeclampsia (Anuradha and Shamshad, 2016).

Oxidative stress placental alterations

In present study elevated ROS and TBARS levels were observed in PE women as compared to normotensive controls. Similar studies showed that increased ROS and TBARS bioactivity exceeding antioxidant activity leads to oxidative stress (Matsubara *et al.,* 2015). It is one of the risk factors for the development of PE through endothelial dysfunction and increased contractility resulting in placental hypoxia and ischemia (Touyz, 2004; Matsubara *et al.*, 2010). It was demonstrated that free radicles attack free fatty acids in call membrane leads to the production of lipid peroxides which may cause endothelial dysfunction in PE patients (Gupta *et al.,* 2005; Gupta *et al.,* 2009).

According to histological perspectives of this study, abnormalities in stem villi were detected. Preceding studies also exhibited such results in which contracted vessels, PVF deposition and obliteration of vascular lumen AP formation in stem villous were observed in PE placentas (Allaire *et al.,* 2000; Sankar *et al.,* 2013; Khong *et al.,* 2016). In present observations SK were more in PE patients as shown in study conducted by Sankar et al. proposing that the functional impairment of the placenta is due to structural alterations of the villous syncytiotrophoblast (Sankar *et al.,* 2013). Thin and elongated villi were also more in PE as detected by Khong et al. (Roberts, 2008). These changes compromise utero-placental blood flow and significantly reduce the neonatal birth weight. Placental villi represented significant decrease in area and circularity of PE as compared to controls. The difference was more prominent in elongated VP (150-250 µm in diameter) which are probably similar to intermediate villi, and large villi (>250 µm in smaller diameter) representing aggregates of adherent or closely approximated villi or immature intermediate villi. Terminal villi were characterized as small VP (50-150 µm in diameter) showing no significant difference in both groups.

After 21st day of conception vascularization of placental villi begins. Villous growth begins to change from branching to nonbranching angiogenesis after 26 weeks of

gestation. Maternal and fetal circulation takes place through these important structures, in case of poor vascularization the fetus is at high risk of hypoxia and low birth weight (Teasdale, 1987; Krielessi *et al.,* 2012). Here terminal villi are not lesser in PE which determine the natural compensation of placental dysfunction and feto maternal nutrients exchange. Quality of life can be improved, and disease burden can be potentially lightening by Understanding the underlying mechanisms of placental maturation and perfusion (Avagliano *et al.,* 2016).

Hormonal analysis

Results of current study revealed that levels of NO were significantly lower in preeclamptic groups as compared to controls in accordance with previous findings (Var *et al.,* 2003; Bernardi *et al.,* 2008; Singh *et al.,* 2010; Bernardi *et al.,* 2015; Aouache *et al.,* 2018). It was evident previously that impair NO levels attenuated the acetylcholine-induced relaxation in arteries of preeclamptic women placentas causing vasoconstriction leading to increased mother's blood pressure (Moncada and Higgs, 1993; Buhimschi *et al.,* 1995). Therefore, circulating NO is likely to be important for fetoplacental hemodynamics ensuring adequate placental blood flow and fetal oxygenation (Turan *et al.,* 2010).

In current study levels of epinephrine were higher in PE patients as compared to control group but there was no correlation of NO and epinephrine found. Epinephrine is the main catecholamine produced from the adrenal medulla by low blood glucose, exercise and stress (From Jastak *et al.,* 2016). It has a profound impact on the vascular system, depending on the vascular structures and the chemical concentration, epinephrine can either cause vascular dilation through eNOS activation or contraction causing hypertension (Shen *et al.,* 2008). Epinephrine in blood narrows blood vessels and increases pressure and inflammation results in endothelial dysfunction leading to PE (Holzman *et al.,* 2009). Pregnancy related stress might be the cause of epinephrine elevation (Toda and Nakanishi-Toda, 2011).

Thyroid hormones T_3, T_4 and TSH were non-significantly altered in preeclamptic group as compared to control group in this study. These results are in accordance with previous studies (Qublan *et al.,* 2003; Khadem *et al.,* 2012). Controversial studies are found in relation of thyroid hormones with preeclampsia (Sardana *et al.,* 2009; Harshvardhan *et al.,* 2017). Basbug et al. reported a significant decrease in both total

and free thyroid hormones (Başbuğ *et al.,* 1999). Sardana et al. found a significant decrease in T3 (Sardana *et al.,* 2009). Tolino et al. and Lao et al. found a significant decrease in T4 in preeclamptic women (Tolino *et al.,* 1985; Lao *et al.,* 1990). Kumar et al. tested FT3 and FT4 only, but they were within the expected range (Ashok *et al.,* 2005). Qublan et al. Khadem et al. and Gulaboglu et al. did not get a significant change (Qublan *et al.,* 2003; Gulaboglu *et al.,* 2007; Khadem *et al.,* 2012).

In current study correlation was determined between biochemical and anthropometric parameters, significant positive correlation was found in urea and creatinine, as they are the important markers for assessing renal insufficiency and predicting the adverse renal outcome in PE (Manjareeka and Nanda, 2013). Glomerular filtration can be measured indirectly by serum creatinine concentration. Reduced glomerular filtration rate results in elevation of concentrations of creatinine and urea. This elevation indicates progression of kidney disease (Pandya *et al.,* 2016). In addition, significant correlation was found in SBP and DBP with age and among them (Rockwood and Howlett, 2011). Human heart and vasculature structure and function alter with age. Structural changes in the vasculature boost arterial rigidity, reducing arterial buffering ability and resulting in age-related modifications in systolic and diastolic blood pressure (Banegas *et al.,* 2018).

Placental eNOS expression

In present study reduced eNOS activity has been observed in preeclamptic patients, these results are in consistent with the results of kim et al. and Schofelder et al. (Schönfelder *et al.,* 2004; Kim *et al.,* 2006). These results support the notion that, there will be adverse effect on placental hemodynamic functioning if the activity of eNOS become reduced in vivo (Kim *et al.,* 2006). Reduced eNOS will decrease the bioavailability of NO leading to endothelial dysfunction which is the feature of this disorder (Motta-Mejia *et al.,* 2017). Fetal placental hemodynamics is likely to influenced by NO production from endothelium of placental villi. However, platelets and leukocytes aggregation in intervillous spaces might be prevented by NO produced by syncytiotrophoblast (Brennecke *et al.,* 1997). Some conflicting findings have been reported with regard to eNOS activity in preeclamptic patient's placenta showing no significant difference in eNOS activity in preeclamptic women (Matsubara *et al.,* 2001; Orange *et al.,* 2003; Matsubara *et al.,* 2015). In addition to eNOS reduced expression

and activity, lower substrate levels for eNOS and elevated inhibitors of eNOS can also contribute towards reduce NO bioavailability (Myatt and Roberts, 2015; Motta-Mejia *et al.,* 2017).

Functional alterations by eNOS variants

In present study common variants of *eNOS* gene including c.894G>T (p.(Glu298Asp)), intron 4b/4a and g.-786T>C were analyzed by genotyping and confirmed through sequencing. Significant association was found for c.894G>T (p.(Glu298Asp)), variant. It was demonstrated that *eNOS* c.894T (p.298Asp) was significantly associated with PE. Our findings are in correspondence with previous studies (Serrano *et al.,* 2004; Alpoim *et al.,* 2014; Chen *et al.,* 2014; Maria Procopciuc *et al.,* 2018). A probable mechanism by which NO bioavailability has been reported to be reduced by c.894 T (p.298Asp). Several experiments have shown that p.298Asp is subjected to selective proteolytic cleavage in vascular tissues and endothelial cells leading to decreased NO generation in homozygous subjects for this variant (Tesauro *et al.,* 2000; Persu *et al.,* 2002; Alpoim *et al.,* 2014). In addition, p.(Glu298Asp) interacts with other proteins playing role in its product degradation to modulates *eNOS* activity, and hence its intracellular distribution (Tesauro *et al.,* 2000). Thus, women with homozygous Asp298 allele are more vulnarable for endothelial dysfunction which might increase risk of PE, observed in present study as well.

Regarding the *eNOS* intron 4b/4a variant, non-significant association has been found in current study as demonstrated by previously in different population-based case control studies (Chen *et al.,* 2007; Sandrim *et al.,* 2010a; Ozturk *et al.,* 2011; Zdoukopoulos *et al.,* 2011; Rahimi *et al.,* 2013; Gannoun *et al.,* 2015). Possible reason might be its intronic region which is unlikely to be functional in its own right (Serrano *et al.,* 2004).

The association of g.-786T>C variant with PE observed here agreed with the results presented earlier for different regions of the world (Kim *et al.,* 2008; Aggarwal *et al.,* 2010; Sandrim *et al.,* 2010b; Ozturk *et al.,* 2011; Zdoukopoulos *et al.,* 2011). Present study demonstrated that g.-786C allele was prevailing in PE patients as compared to control. The g.-798C allele in *eNOS* promoter region has been linked with reduced mRNA expression and lower serum nitrite/nitrate levels, causing unavailability of NO (Nakayama *et al.,* 2006). It was also suggested that 50% of *eNOS* activity can be reduced if its transcription is repressed by targeting g.-786C allele through single

stranded DNA binding transcription factor replication protein, A1 which results in lower NO production (Senthil et al., 2005; Dosenko1½ et al., 2006).

Sequencing revealed the association of another variant with the disease phenotype that is g.2051G>A which was present in the *eNOS* gene promoter region. Genetic analysis showed that previously this variant was associated with the disease phenotype in heterozygous form but in accordance with resent study it was also determined to be homozygous for g.2051AA. This variant might affect the transcription of the *eNOS* gene through unknown mechanism and results in reduced NO levels leading to endothelial dysfunction.

Through our profound *in silico* structural assessment, we elucidated the structural and functional behavior of *eNOS* protein upon Glu298Asp substitution. The reported substitution resulted in the loss of flexibility and deformities were reported that alter the conformation and configuration of the protein and ultimately decrease protein stability. Molecular dynamic simulation analysis revealed that due to Glu298Asp substitution there was changes in protein structural behavior and fluctuations have been noted in the mutation-related loop area, and this change may contribute to preeclampsia. Detailed illustration of eNOS's structural and functional behavior reveals the underlying molecular mechanism and can help develop a powerful therapeutic drug. Secondly the mutations reported in the *eNOS* gene promoter region changed the binding of transcription factor STAT3 and STAT6 to the 5’ site of the *eNOS* gene. These reported variants might change the regulation of *eNOS gene* and responsible reduced NO level and leading to endothelial dysfunction.

GENERAL CONCLUSION

After the prospective evaluation of several demographic, clinical, biological and genetic markers in the pathophysiology of preeclampsia in preeclamptic Pakistani women, the following points are concluded from the present study:

- The manifestation of clinical risk factors and baseline laboratory investigation are necessary for early diagnosis and improved management of the condition before it threatens the survival of mother and fetus.
- Histopathological examination of placenta is an important parameter for detection of placental alterations and the relevance of their identification for the patient suffering PE. This approach may facilitate grading placentas for diagnostic and research purposes.
- Current data suggested that oxidative stress, elevated epinephrine, decreased NO levels and reduced placental eNOS localization and expression might favour abnormal placenta perfusion, contributing to the reduced placental blood flow results in endothelial dysfunction leading to PE.
- The present study revealed substantial association of c.894G>T (p.(Glu298Asp)) and g.-786T>C variants while no association of intron 4b/4a variant in Pakistani population.
- The detailed computational investigation further confirmed the deformities and changes in protein flexibility upon Glu298Asp.
- Novel variants in the promoter region of the *eNOS* gene (g.2051G>A) further validate the change in gene regulation for the onset of disease.

FUTURE RECCOMENDATIONS

In current study biochemical aspects and role of *eNOS* gene in susceptibility to PE has been investigated comprehensively in Pakistani population. However, further studies are required as several gaps remained unfilled and questions remained unanswered that need to be investigated.

- Future studies on preeclampsia might provide valuable information in understanding its pathophysiology and help in development of preventive and therapeutic strategies.
- Most of the risk factors for PE are not modifiable, that's why better prenatal care is required to assure the timely diagnosis and management of this complication, so that maternal and perinatal deaths due to PE might be avoided more easily as the ultimate long-term goal in Pakistan.
- Further work must be done on histological examination of the placenta which can provide useful information in the determination of various reasons and mechanisms involved in poor pregnancy outcomes and useful to health care providers.
- Genetic testing and NO concentration in maternal serum could be used as a biomarker to predict PE for practicing clinicians, which could also help in interfering PE development in the early period in preeclamptic Pakistani women.
- NO-donating drugs and antioxidants therapy can be given for better management of PE which will reduce the maternal and neonatal morbidity and mortality in Pakistan
- It is necessary to conduct large sample sized studies in the future to elucidate the association of *eNOS* gene with the severity of PE.
- Identification of key structural and functional features in eNOS protein and gene regulatory region might be used for designing specific drugs for therapeutic purpose.
- The genetic polymorphisms in both mother and fetus need to be investigated together.

- Understanding of etiology of PE and the role played by the differences in the environmental factors in developed and developing countrievs should be a research priority to prevent maternal and neonatal morbidity and mortality.

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Potential Biochemical and Molecular Aspects in Pathophysiology of Preeclampsia: Assessment of Endothelial Nitric Oxide Synthase Gene in Preeclamptic Pakistani Women

This is where I describe the study and let people know that their participation is voluntry and their data would be arranged annonymus and confidential.

Sample No._____ Date: _____

Name: _____

Contact # _____ Age _____

Permanent Address:

_____

_____

- **BMI:**
  - Height _____ Weight _____
- Blood Pressure: _____
- Gestational age (Weeks) _____
- Last menstrual periods _____
- Expected date of delivery _____
- Blood Group _____

MEDICATION:

_____

- **Pregnancy Status:**
  - Age at first marriage : _____
  - Is your marriage consanguineous? Yes / No
  - Age at birth of first child? _____
  - Primary Gravida _____
  - Multiple Gravida _____
  - History of preeclampsia in previous pregnancy_____
  - Having a mother or sister who had preeclampsia_____
  - Pregnant with twins _____

- Inter pregnancy interval _____
- History of abortion _____
- History of still birth _____

REPRDUCTIVE HEALTH

- Number of visits for antenatal care? _____
- Family status and home environment _____
- The place you have given birth to child is: Government hospital/Private hospital/Home/Other
- Assistance during delivery is provided by? (Doctor/Nurse/Lady health worker/Dai/Relative/Other)
- Postnatal checkup for new born is done? Yes/No
- The weight and size of child at birth (Small/Very small/Average)
- Mortality: Neonatal mortality/ postnatal mortality/ infant mortality/ under five mortality
- Vaccination after birth _____
- Smoking _____

❖ CLINAICAL FEATURES

- Headaches _____
- History of poor diet or malnutrition_____
- Swelling in your face or hands _____
- Excessive weight gain (obesity) _____
- Nausea and vomiting _____
- Problems urinating _____
- Abdominal pain _____
- Shortness of breath _____
- Seizures _____
- Loss of consciousness _____
- Agitation _____
- Muscle pain _____

❖ MEDICAL HISTORY

- History of diabetes _____
- Renal disease _____
- Rheumatoid arthritis _____
- Cardiovascular disease _____
- Visual disturbances _____
- Asthama _____
- Sexually transmitted infections_____

❖ **Laboratory tests**

_____

_____

_____

_____

Recipes of Solutions used in DNA extraction:

Solution A for 250 ml

Sucrose 27.36 gm

Tris 0.303 gm

MgCl2 0.254 gm

Then the mixture was autoclaved. After autoclave 2.5 ml triton X-100 was added.

Solution B

Tris 0.182 gm

NaCl 3.52 gm

EDTA 0.11 gm

All the reagents were dissolved in 200 ml of distilled autoclaved water and were mixed well before use.

Solution C

Iso-amyl alcohol 2 ml

Chloroform 48 ml

The two reagents mentioned above were taken in a 50 ml falcon tube and were mixed well.

PCI (Phenol, Chloroform, Iso-amyl alcohol)

Iso-amyl alcohol 1 ml

Phenol 25 ml

Chloroform 24 ml

PCI is always freshly prepared. PCI was prepared by mixing an equal volume of solution C and phenol.

20% SDS

12.5 gm of SDS (Sodium dodecyl sulfate) was dissolved in 50 ml of autoclaved water and then chilled for 2 days.

TE Buffer

Tris 0.08 gm

EDTA 0.014 gm

Dissolved in 50 ml of autoclaved water, then raised the volume to 70ml, then autoclaved.

10x TBE Buffer

Tris 109 gm

Boric acid 55 gm

EDTA 9.3 gm

All the ingredients mentioned above were taken in a reagent bottle and were dissolved in distilled autoclaved water. The final volume of the solution was raised to 1 litre.

Ethidium Bromide

For making Ethidium Bromide, 0.1 gm ethidium bromide was taken in 10 ml of distilled autoclaved water and mixed well. It was then stored at 4˚C.