A Biochemical study to evaluate the antioxidant status and antiangiogenic factor Soluble-fms-like tyrosine kinase 1 (sFlt1) in preeclamptic women.

By

Sana Ahmed

DEPARTMENT OF ZOOLOGY
FACULTY OF BIOLOGICAL SCIENCES QUAID-I-AZAM UNIVERSITY ISLAMABAD, PAKISTAN
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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF PHILOSOPHY

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"In the Name of ALLAH, the Most Beneficent, the Most Merciful"

CERTIFICATE

This dissertation **"A Biochemical study to evaluate the antioxidant status and antiangiogenic factor Soluble-fms-like tyrosine kinase 1 (sFlt1) in preeclamptic women"** submitted by **Sana Ahmed** is accepted in its present form by the Department of Zoology, Faculty of Biological Sciences, Quaid-I-Azam University, Islamabad, as satisfying the thesis requirement for the degree of Master of Philosophy in Reproductive Physiology Laboratory.

Supervisor:

_____

Prof. Dr. Sarwat Jahan

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Chairman _____

Professor Dr. Amina Zuberi

Date: _____

DECLARATION

I hereby declare that the work presented in the following thesis is my own effort and the material contained in this thesis is my original work. I have not previously presented this work elsewhere for any other degree.

Sana Ahmed

Dedicated to:

"My father and mother (late), respected supervisor, sisters, my nephews Muhammad Abu Bakar and Muhammad Abdullah, my nieces, and my friend Rehmana Aslam"

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PE	Preeclampsia
BP	Blood Pressure
CI	Confidence intervals
CVD	Cardiovascular disease
BMI	Body Mass Index
DNA	Deoxyribonucleic Acid
DBP	Diastolic Blood Pressure
SBP	Systolic Blood Pressure
FLT-1	Fms like tyrosine kinase 1
ROS	Reactive oxygen species
ELISA	Enzyme-linked Immunoassay
Eng	Endoglin
$FeCl_3$	Ferric chloride
$FeSO_4$	Ferrous sulfate
H_2O_2	Hydrogen peroxide
HDP	Hypertensive Disorders of Pregnancy.
HELLP	Hemolysis, Elevated Liver enzymes, and Low
HRP	Horse-radish Peroxidase
Kg	Kilogram
M	Meter
mM	Millimolar
NADH	Nicotine adenine dinucleotide
NO	Nitric Oxide
OD	Optical Density
VEGF	Vascular endothelial growth factor
PlGF	Placental growth factor
JMJD6	Jumanji domain-containing protein 6
HDP's	Hypertensive disorders

OS	Oxidative Stress
DNA	Deoxyribonucleic acid
SOD	Superoxide dismutase
CAT	Catalase
LPO	Lipid peroxidase
PBS	Phosphate Buffer Saline
PE	Preeclampsia
GSH	Reduced glutathione
GSR	Glutathione reductase
GPx	Glutathione peroxidase
rpm	Revolution per minute
mRNA	Messenger RNA
GST	Glutathione transferase
sEng	Soluble Endoglin
RUQ	Right upper quadrant
sFlt1	Soluble fms like tyrosine kinase 1
U/L	Unit per liter
WHO	World Health Organization
µm	Micromolar
µl	Microliter
%	Percentage
µmol	Micro molar
X^2	Chi-squared test
kDa	Kilo Dalton

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ABSTRACT

Preeclampsia (PE) is a severe hypertensive multisystem disorder characterized by sudden onset of proteinuria and hypertension by the 20th week of gestation. It is a serious condition causing maternal and fetal deaths worldwide and complicates 2-8% of pregnancies. The present study illustrated the demographic, clinical, biochemical, and hormonal data analysis of preeclamptic patients. A total of pregnant women (n=200), divided into two groups, PE (n=100) and control (n=100). The demographic data and medical history were collected from each subject with their informed consent. Blood, urine, and placenta samples were also taken from both groups. Urine samples were tested for protein concentration The blood was centrifuged, and plasma was collected. The blood and placental tissues were processed for biochemical analysis of soluble fms like tyrosine kinase 1 (sFlt1) and antioxidants levels. The odds ratio, the independent sample t-test, and the Chi-square test were used to statistically examine the data. The role of antioxidant enzymes and antiangiogenic factor sFlt1 in PE and normotensive pregnant women was investigated in the present study.

Proteinuria was found to be significantly higher in the PE group ($p<0.001$) than in the control group. Significant decrease in glutathione-s-transferase (GST) ($p<0.001$), glutathione peroxidase (GPx) ($p<0.001$), reduced glutathione (GSH) ($p<0.001$) and glutathione reductase (GSR) ($p=0.01$) levels were observed in the PE placental homogenates compared to control. Similarly, GPx ($p<0.001$), GSH ($p<0.001$), GST ($p<0.01$), and GSR ($p<0.05$) levels were decreased significantly in the plasma of PE patients as compared to control. In the current investigation, improper trophoblast invasion, poor spiral artery remodeling, placental hypoxia, and angiogenic factor imbalance were found to be key contributors to PE. The study concluded that the increased levels of sFlt1 are directly related to the severity of PE as it is the major cause of endothelial dysfunction. Decreased levels of plasma and placental antioxidants depict oxidative stress during the disease. Preeclampsia is a major public health problem in Pakistan and around the world. Obstetricians find it difficult to diagnose and treat this condition because of its severity, unclear etiology, and complex pathophysiology. There are currently no viable treatments for this pregnancy complication. Hence, PE prognostic and diagnostic biomarkers are critical for developing a proper diagnosis, treatment, and management of feto-maternal health.

INTRODUCTION

Hypertension is defined as a blood pressure greater than or equal to 140mmH (systolic) 90 mmHg (diastolic). Hypertensive diseases may increase the risk of maternal and prenatal morbidity and mortality. During pregnancy 5-10% of women are affected by hypertensive disorders of pregnancy (HDP's), which include chronic hypertension, gestational hypertension, preeclampsia, chronic hypertension with superimposed preeclampsia, and eclampsia. The most severe of these disorders is preeclampsia (Ying *et al*., 2018). Women suffering from HDP's are at the greater risk for placental abruptions, intrauterine growth restriction, cesarean birth, and preterm birth. The factors related to hypertensive disorders are obesity advancing maternal age, and pregnant females with linked comorbidities (Folk, 2018). HDP's are linked with an increased risk of lasting cardiovascular disease and vascular dysfunction in pregnancy (Ying *et al*., 2018). Severe hypertension is defined when the systolic and diastolic blood pressure goes above 160 mm Hg and 110 mm Hg, respectively (Townsend *et al*., 2016; Reddy & Jim, 2019).

Chronic hypertension:

Chronic hypertension is defined as a type of hypertension, identified before the 20^{th} week of pregnancy, and lasts for more than 12^{th} weeks postpartum. It affects almost 5% of women during pregnancy. It is characterized as preexisting or before pregnancy hypertension having a blood pressure of 140 mmHg/90 mmHg. Chronic hypertension may exist in two forms: Primary hypertension is defined as raised blood pressure when there is no end-organ damage. Secondary hypertension results from an endocrinologic or renal source. Most women suffer from primary hypertension. Some women with chronic hypertension may have endocrine or renal disorders eventually leading to secondary hypertension (Sutton *et al.*, 2018; Dhariwal & Lynde, 2017).

Gestational Hypertension:

It is defined as the early onset of hypertension having blood pressure 140 mmHg/90 mmHg after 20^{th} weeks of gestation without any symptoms of preeclampsia and normalized within 12 weeks postpartum. Gestational hypertension affects approximately 2-3% of women during pregnancy. 25-50% of gestational hypertension leads to the development of preeclampsia. Gestational hypertension is developed into preeclampsia mostly in women who experience hypertension before 32 weeks of pregnancy. Pregnant women with mild gestational hypertension may progress into preeclampsia almost within

7 to 21 days after identification. The elevated uric acid levels may be linked with the development of preeclampsia (Folk *et al*., 2018; Wisner, 2019).

Chronic hypertension with superimposed preeclampsia:

Superimposed preeclampsia may arise when chronic hypertension will lead to the development of preeclampsia. It affects almost 13- 40% of the cases. Women suffering from this type of hypertension are at great risk for both mother and fetus in developing the disease. It is characterized by the early onset of proteinuria, thrombocytopenia, and other organ dysfunctions in addition to preexisting hypertension (Ying *et al*., 2018).

Eclampsia:

Preeclampsia with neurologic involvement is termed eclampsia**.** It is defined as the existence of convulsions or coma in a pregnant woman having preeclampsia. Eclampsia is one of the serious and severe complications of pregnancy, causing high morbidity and mortality for both the mother and fetus (Fishel Bartal & Sibai, 2020). Eclampsia is the severe phase of preeclampsia, having symptoms such as severe headaches, visual changes, and over-responsive reflexes (Reddy & Jim, 2019). Eclamptic seizures complicate about 2% of preeclamptic cases (Nirupama *et al*., 2021). Eclamptic seizures can occur before childbirth (53%), during pregnancy (19%), and postpartum (28%). Eclamptic seizures are usually generalized for 60 to 90 seconds. During an eclamptic seizure, the fetus often establishes hypoxia-related bradycardia, but mostly recovers (Leeman *et al*., 2016).

Preeclampsia:

Preeclampsia (PE) is a multisystem disorder characterized by sudden onset of proteinuria and hypertension at the 20^{th} week of gestation. It complicates 2-8% of pregnancies (Ives *et al*., 2020). Previously, preeclampsia is defined as the development of hypertension along with the presence of protein in the urine after the 20^{th} week of conception. As stated by the International Society for the Study of Hypertension in Pregnancy, preeclampsia is stated as a blood pressure greater than or equal to 140mmH (systolic) 90 mmHg (diastolic) on two measurements at least in women who were previously normotensive, accompanied with the proteinuria (>300 mg of protein in 24 h), reduced liver functions, hematological problems, neurological symptoms, renal failure and uteroplacental dysfunction, such as fetal growth restriction. Almost 10% of women suffer from high blood pressure during gestation and it complicates 2–8% of pregnancies. The perinatal mortality rates are high in preeclampsia, and even higher following eclampsia (Poon *et*

al., 2019). PE develops with vascular dysfunction and if not treated, it leads to circumstances like pulmonary edema, kidney failure, stroke, liver rupture, and eclampsia. Different environmental, genetic, or immunogenic factors may involve in the pathogenesis of PE. Every year PE can cause almost 70,000 maternal and 500,000 fetal deaths worldwide and 15% of maternal deaths in developing countries (Nirupama *et al*., 2021).

Incidence rate:

Preeclampsia is a serious condition causing maternal and fetal deaths. It is responsible for 15% of maternal mortalities in developing countries every year. In the United States PE is the 3rd leading cause of maternal death. Almost 10 % of fetal deaths were reported due to preeclamptic pregnancies (Nirupama *et al*., 2021). According to World Health Organization (WHO), the occurrence rate of preeclampsia is seven times greater in underdeveloped countries in comparison to more developed countries (Machano & Joho, 2020). Due to increased mortality, it affects almost 5 to 7 percent of all expected women (Rana *et al.*, 2019). Every year 76,000 women and 500,000 babies died due to preeclampsia worldwide. Moreover, less developed states are at a greater stake in preeclampsia than developed countries (Poon *et al*., 2019). Between 1990 and 1999 the rate of preeclampsia has increased globally significantly in developed countries due to multiple births and older mothers. Annually 60,000 maternal deaths are due to preeclampsia worldwide (Shamsi *et al*., 2010; Naseeb *et al*., 2015). Preeclampsia affects almost 2%-8% of pregnant women and causes significant deaths worldwide. More than fifty thousand maternal mortalities are due to preeclampsia every year (Kara *et al*., 2021).

Risk Factors

The major key risk factors of PE involve obesity, antiphospholipid syndrome, preeclampsia history, chronic hypertension, prior preeclampsia, multiple gestations, and pregestational diabetes mellitus. However, other risk factors are nulliparity, maternal age, prior placental, history of kidney infection, advanced maternal age >35, genetic susceptibility, history of stillbirth, preexisting hypertension, and use of assisted reproductive technologies (Jim & Karumanchi, 2017; Rana et al., 2019). Women were at high risk when they have insulin-dependent diabetics, preexisting hypertension, previous early-onset preeclampsia, and chronic kidney disease. In primigravida women, preeclampsia is more common. The greater the interval among pregnancies, the greater the risk of disorder. Age greater than 40 increases the risk, previous history of

preeclampsia pregnancy, and pregnancy with a donor egg, donor insemination, or embryo donation double the risk. Also, different paternal factors can complicate pregnancy and increase the risk of preeclampsia. Preeclampsia itself is a risk for both mother and baby. Preeclampsia if not treated may develop seizures (eclampsia), placental abruption, oligohydramnios, pulmonary edema, and fetal growth restriction. Effects on the fetuses include an increased risk of hypertension and stroke. Besides health complications for baby, women also have a 3.7 times greater risk of developing hypertension in the future if they already have preeclampsia, the risk of coronary heart disease increase by 2.2 times, and the risk of stroke is 1.8 times higher (English *et al*., 2015). National Institute for health and care excellence guidelines stated that the risk of preeclampsia increases in women if there is a hypertensive disease during a past pregnancy or any maternal disorder including autoimmune diseases, kidney disease, diabetes, or any type of hypertension (Fox *et al*., 2019). The risk of stroke increases 5-fold in early-onset preeclampsia as compared to later-onset preeclampsia. For placental abruption, hypertension is a major risk factor increasing the risk 5 times compared with pregnancies that are without hypertension (Witcher, 2018).

Pathogenesis of Preeclampsia:

The pathogenesis of preeclampsia involves two stages**.** During the first stage, abnormal placentation occurs, whereas during the second stage the release of different antiangiogenic factors or cytokines in circulation by the diseased placenta.

Stage 1: Blastocyst embeds in the uterine wall during the initial stages of fetal development. Any abnormalities in this process might be the cause of the origin of preeclampsia. Immune responses related to the mother with increased macrophages as well as monocytes result in improper remodeling of spiral arteries, as a result, there is an abnormal development of the placenta and decreased perfusion of the placenta.

2nd Stage: In the 2nd stage, recurrent placental ischemia occurs which decreases the perfusing process of the placenta and results in the secretion of different antiangiogenic factors, cytokines, reactive oxygen species that result in endothelial dysfunction, inflammation, prothrombotic conditions, and maternal systemic inflammations. This stage is indicated by hypertension. In severe cases, organ damage, increased liver enzymes, protein in the urine, increased levels of creatinine, and neurological symptoms may have been seen (Rana *et al*., 2019; Leslie & Briggs, 2016; Sircar *et al*., 2015). During normal pregnancy, cytotrophoblast deeply invades the spiral artery into the myometrium which

can cause remodeling of the spiral arteries, increasing the capacity of blood flow and decreasing the resistance, while in preeclamptic women invasion cytotrophoblast is abnormal as a result of remodeling of spiral arteries is incomplete. Incomplete remodeling of spiral arteries leads to the narrowing of maternal vessels which can cause placental hypoxia and maternal syndrome of preeclampsia which bring the release of various antiangiogenic factors including sFlt1, sEng so creating an imbalance in circulating angiogenic factors like VEGF, PIGF, TGF-B (Poon *et al*., 2019; Rana *et al*., 2019). Preeclampsia was linked with cardiovascular disease. Women were at higher risk and experienced preeclampsia by the 34th week of gestation. Early-onset preeclampsia can cause a significantly higher risk of respiratory, cardiovascular, CNS, hepatic, renal, and other related diseases (Armaly *et al.*, 2018).

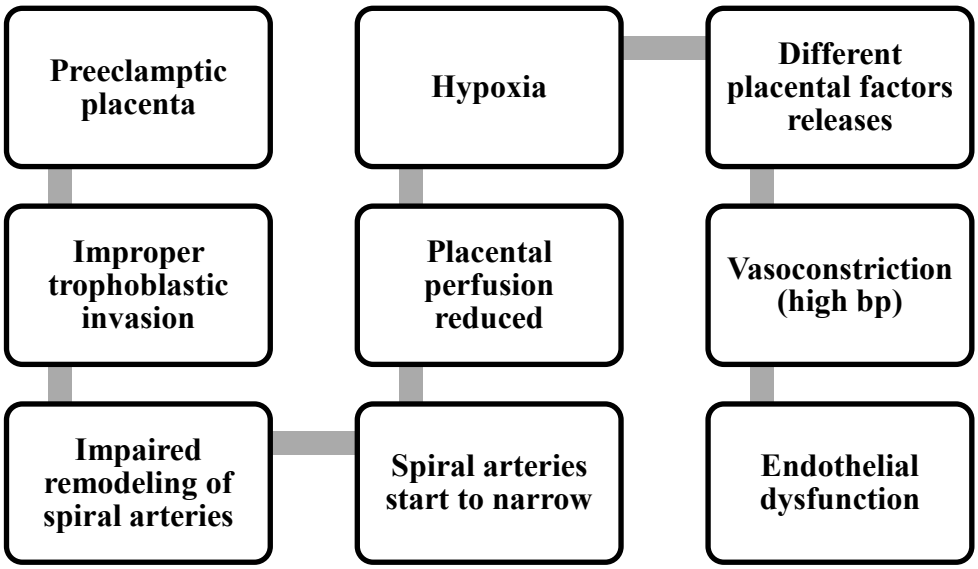


Figure 1: Pathogenesis of preeclampsia (Poon *et al*., 2019).

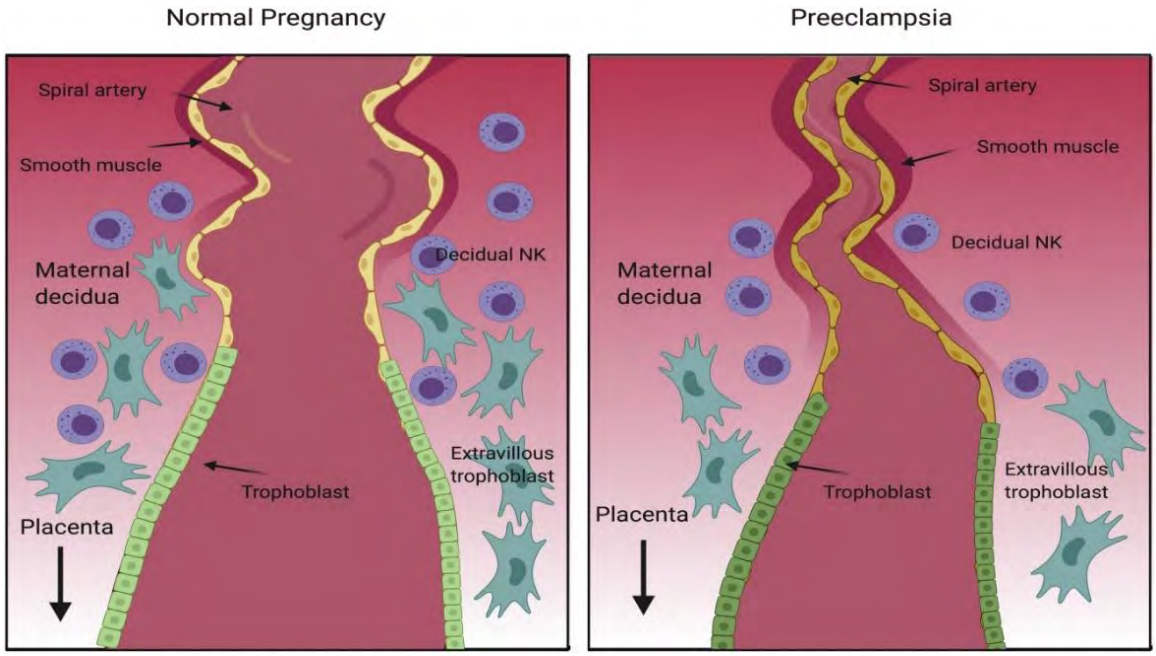


Figure 2: Spiral artery remodeling during normal pregnancy and preeclampsia (Rana *et al*., 2021).

Clinical presentation and symptoms:

PE is a multi-factorial disorder affecting multiple organ systems (Eiland *et al*., 2012). Proteinuria is the major symptom of preeclampsia. The quantity of protein excreted in the urine fluctuates. In PE the significant protein excretion is 300mg/24-hm (Poon *et al*., 2019). Proteinuria is the predominant sign in which urinary proteins are tested for early diagnosis (Myatt & Roberts, 2015). The characteristic symptoms of PE include visual disturbance, epigastric pain, headache, swelling of hands and feet, belly pain, severe headaches, dizziness, trouble breathing, dizziness, weight gain, stomach pain, changes in reflexes severe nausea and vomiting, blurred eyesight, protein in the urine, high blood pressure and seizures in severe cases. Symptoms mostly start after 34 weeks of gestation (Nirupama *et al*., 2021; Young & Karumanchi, 2016). Women with severe cases of preeclampsia can encounter the type of PE known as HELLP syndrome. It is a severe complication in PE (Guerby *et al*., 2021). Swelling of the feet is prevalent during normal pregnancy, but swelling of the face and hands may be the clinical sign of PE (Portelli & Baron, 2018). The severity of clinical presentation of PE is highly variable but when it develops after 36 weeks of gestation outcomes are usually favorable in mild PE cases (English *et al*., 2015). If the PE develops before 33 weeks of gestation then significant increase in the risk of adverse maternal and neonatal outcomes (Lai *et al*., 2014).

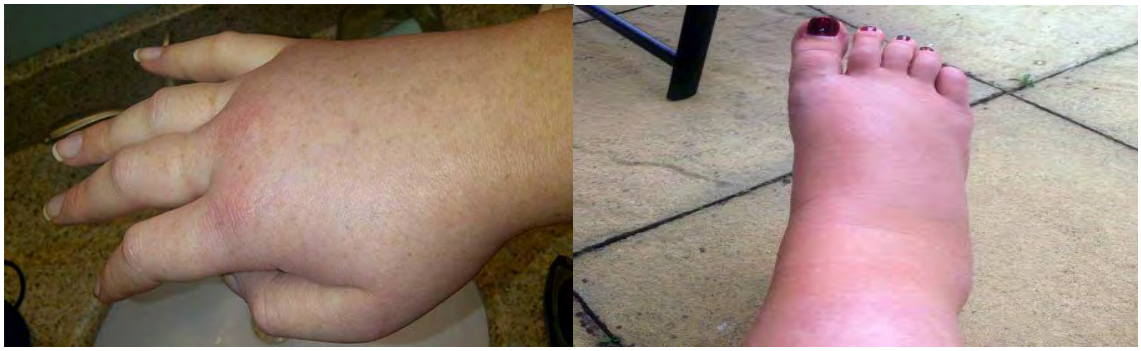


Figure 3: Showing edema of hands and feet in preeclamptic females

Types:

There are two types of PE based on the gestational period onset. The timing of preeclampsia plays a significant role in the degree of the disorder whereby maternal symptoms arise. Two types are Type I is Placental PE arises before the 34th gestational week and is also called as early onset of PE whereas Type II is Maternal PE also called as the late onset of PE, arises after the 34th gestational week. The early-onset is linked with relatively severe complications with adverse risks during gestation and postpartum to the mother and the child than the late onset of PE (Bakrania *et al*., 2021).

1. **Type I:** It can occur because of the impaired development of the placenta during early gestation. It comprises 5 to 20% of all preeclampsia cases. It is also termed as "placental preeclampsia" because it primarily involves the placenta. It is characterized by improper trophoblast invasion in early pregnancy. It decreases spiral arteries vascularization, which leads to blood flow alterations. These altered spiral arteries lead to hypoxic conditions in the placenta. The blood flow alterations in umbilical arteries and uterine arteries during early PE onset can be due to the higher peripheral resistance of vessels in the placenta. During early PE onset decreased weight of neonate at birth, altered uteroplacental perfusion, fetal growth restriction, hemolysis, neurological, and cardiorespiratory complications can be seen (Jena *et al*., 2020; Marín *et al*., 2020).
2. **Type II:** It may occur due to impaired maternal stimuli towards the end of pregnancy. Late-onset PE comprises more than 80% of all preeclampsia cases It is termed "maternal preeclampsia" as it is a maternal syndrome. Defects in the placenta in this subtype affect the maternal endothelium without affecting the development of the fetus. The fetus develops normally without any growth restrictions. Uterine spiral arteries are normal or with slight modifications. No

alterations in the flow of blood across umbilical arteries (Jena *et al.,* 2020; Marín *et al.,* 2020).

PE is further subdivided into three subtypes:

Subtypes of preeclampsia:

i. **Mild preeclampsia** can be characterized having systolic/diastolic blood pressure of 140 mmHg/90 mmHg respectively and with the presence of proteinuria exceeding 300 mg 24-h, and the ratio of protein in urine is: creatinine >0.3 or 30 mg/dl of protein in the urine sample.

ii. **Severe preeclampsia** can be characterized as blood pressure 160/110 mmHg, proteinuria >3+ and having clinical symptoms such as headache, presence of thrombocytopenia, upper abdominal pain, visual disturbances, elevation in serum of creatinine, and transaminase.

iii. **Eclampsia (E)** is defined as the presence of seizures or coma in a pregnant woman with preeclampsia. Eclampsia is one of the serious severe complications of pregnancy, causing high morbidity and mortality for both the mother and fetus (Fishel Bartal & Sibai, 2020; González-garrido *et al*., 2017; Suvakov *et al*., 2020).

Placenta and PE:

The placenta is a discoid structure that acts as a crossing point between the mother and fetus. Besides its immune barrier function, the placenta, has specialized epithelium functions to transport nutrients, gases, and waste products. It also acts as the most important synthetic organ for various peptide and steroid hormones that control placental, maternal, and fetal systems (Marín *et al*., 2020; Myatt, 2002). The placenta is made up of the chorionic disc on the side of the fetus and the basal disc on the side of the mother's placental part. Both sides are parted by the space between the villous of the placenta (Jansen *et al*., 2020). The placenta is acquiring blood vessels from both fetus and mother. The development and progression of the placenta depend on the construction of fresh blood vessels, the development of blood vessels from previously prevailing blood arteries, and the remodeling of arteries (Helmo *et al*., 2018). The placenta is responsible for PE. Syncytiotrophoblast stress, caused by impaired placental perfusion is usually the primary cause (Flint *et al*., 2019). The placenta is a complex structure that performs heterogeneous functions. In normal and abnormal placentae there is a change in intervillous blood flow has been reported. However in the normal placenta flow of blood begins in the marginal region and gradually pours in the direction of the center whereas in preeclampsia the flow

of blood is started in the central portion of the placenta (Sahay *et al*., 2018). The oxidative stress of the placenta is a primary cause for the pathogenesis of PE, as the complications are resolved as the placenta is delivered out of the body (Shaheen & Almajwal, 2020). Most of the placenta is made up of several finger-like projections known as chorionic villi where the maternal-fetal exchange takes place. These finger-like structures aid in nutrients, waste material, and gaseous exchange between mother and fetus through blood (Fisher, 2015). During PE, there is an inadequate remodeling of maternal arteries. Consequently, smooth muscle cells persist resulting in impaired trophoblast penetration detected in almost 30 to 50 percent of placental arterioles. Reduced oxygen levels are observed, generating a hypoxic environment (Guerby et al., 2021). The placental injury leads to complications such as DNA fragmentation, destruction of membranes, apoptosis, and syncytial debris released into the maternal blood, which affects multiple organ systems (Haram *et al*., 2019; Ahmed *et al*., 2016). During pregnancy flow of blood in the placenta is facilitated by the formation of new blood vessels and the remodeling of existing arteries. Trophoblast separates into inner and outer layers after implantation. Cytotrophoblast is the inner layer while the outer layer is the syncytiotrophoblast. The extravillous cytotrophoblasts spread into decidual and myometrial uterine spiral arteries and substitute the inner layer of the spiral blood vessel. Penetration of trophoblast starts at the end of the first trimester, and transforms the narrow, low capacitance, high resistance spiral blood vessels into high capacitance, low resistance arteries. This remodeling, conversion, and transformation of the maternal blood vessels are crucial to make sure a sufficient supply of oxygen and nutrients during pregnancy. Any alterations in the remodeling of spiral arteries nearby the placenta will affect the blood flow which can cause complications resulting in restriction of fetal development. Various angiogenic elements like placental growth factor and vascular endothelial growth factor perform their angiogenic activity with their co-receptors like Flt1. The processes of endothelial functioning, placental angiogenesis, and vascular growth are influenced by markers of oxidative stress (Sahay *et al*., 2018)

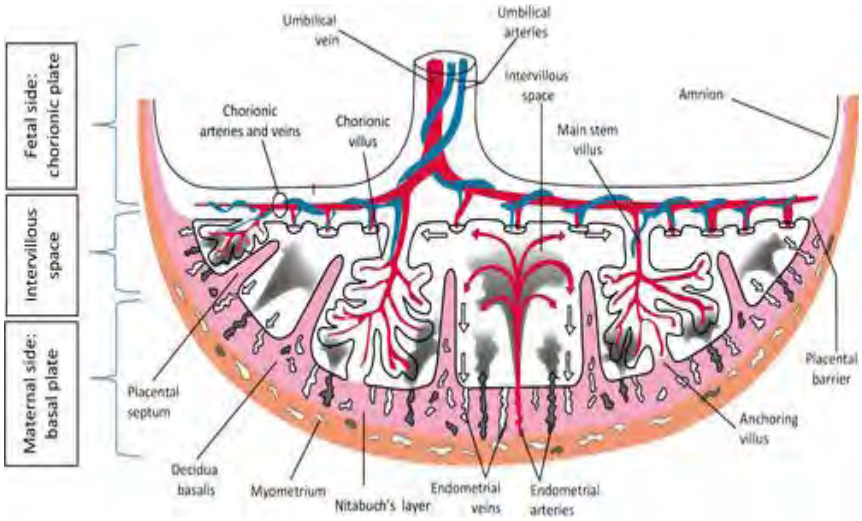


Figure 4: Anatomy of human placenta (Jansen *et al*., 2020*)*.

Oxidative Stress:

It is an imbalance between the levels of oxidant and antioxidant, which leads to an interruption in the signaling pathways of redox resulting in molecular damage. OS plays a key role in the pathology of preeclampsia. It occurs when free radical production exceeds the buffering capacity by cellular defense mechanisms. The antioxidants regulate the endogenous defense system and reduce the damaging effects of ROS. During pregnancy, proper placental oxygenation is required. Oxidative stress is caused by low oxygen pressure ensued by proper oxygenation of maternal blood which results in the normal development of the placenta. Recurrent hypoxia and oxygenation are triggered by reduced development of spiral arteries. Oxidative stress may stimulate the production of antiangiogenic factors for example sFlt1 and sEng. Oxidative stress is considered as one of the central key factors in PE, created by the poorly perfused placenta (Matsubara *et al*., 2015). There is an increase in oxidative metabolism throughout pregnancy due to the increased oxygen demand of the mother and fetus, which leads to the production of free oxygen radicals. These free radicals then increase the utilization of antioxidants which results in placental ischemia (Kaur *et al*., 2008). Oxidative stress is the consequence of a deficiency of antioxidants. Two types of antioxidants are non-enzymatic and enzymatic antioxidants. Furthermore, placental enzymatic antioxidants include SOD, CAT, GSR, GST, and Gpx which protect the endothelium from reactive oxygen species damage and maintain normal endothelial functions. However, hypoxic conditions in the placenta during PE reduce the antioxidant activity (Dsouza *et al*., 2016). Glutathione peroxidase (GPx) is an enzyme that plays an important role in reducing hydrogen and LPO by using reduced glutathione as a cofactor to reduce hydrogen peroxide, consequentially producing

oxidized glutathione (Aouache *et al*., 2018). Reduced uteroplacental perfusion leads to the production of radicals in the vascular space. Placental hypoxia is increased due to decreased activity of antioxidants which in turn increase oxidative stress. In endothelial cells of the placenta, oxidative stress is an underlying cause of preeclampsia (Haram *et al*., 2019; Witcher, 2018).

Endothelial dysfunction:

Endothelial dysfunction and abnormal placentation may play a key role in the development of PE. The placenta helps in nutrient exchange and removal of waste among mother and fetus. During the first trimester of gestation, this crossing point is developed in the mother and fetus. After its formation trophoblasts of the placenta overcome the maternal decidual part. Throughout this phase remodeling of spiral arterioles occur which is essential to approach the maternal supply of blood. Remodeling of spiral arteries results in vascularization. It is then characterized by the replacement of smooth muscle and elastic tissue of arteries with fibrinoid tissues which ensures vasodilation and low resistance. In PE there is an impaired fetal trophoblasts invasion. It results in reduced maternal spiral arteries remodeling, leading to vasoconstriction and a decreased inflow of blood towards the placenta. It interrupts the oxygen supply towards the fetus leading to hypoxia. Moreover, it also alters the nutritional status of the placenta leading to the development of preeclampsia (Boeldt & Bird, 2017; Sánchez-Aranguren *et al.*, 2014). The hypoxic placenta leads to the secretion of antiangiogenic factors into the circulation, eventually resulting in the persisting extensive endothelial dysfunction (Witcher, 2018).

Fms-like tyrosine kinase (FLT-1):

Fms like tyrosine kinase 1 (Flt-1) also identified as vascular endothelial growth factor receptor 1 (VEGFR1) is of 185kDa transmembrane glycoprotein receptor. FLT-1 contains 3 domains: an extracellular domain that binds a ligand, a distinct segment that is membrane-spanning, and an intracellular part carrying two domains of tyrosine kinase. Flt1 is a membrane-bound tyrosine kinase co-receptor for both vascular endothelial growth factor (VEGF) and (PlGF). These receptors are localized majorly on the surface of activated monocytes, the membrane of endothelium, vascular endothelial cell, syncytial and expressed all over the placenta, mostly in the inner layer of the trophoblast. Flt1 is 7.4kb mRNA coding for an Flt1 which is 150kDa protein. As a result of glycosylation of Flt1, 185kDa membrane-bound protein is formed on the surface of the cell (Sahay *et al*., 2018) (Helmo *et al*., 2018; Jung *et al*., 2012; Plymire & Jeyabalan, 2009). FLT-1 is made

up of 1,338 amino acids. The extracellular domain contains seven Immunoglobulin-like domains and can bind ligands (Shibuya, 2006). Flt1 gene encodes tyrosine kinase receptors Flt1 and sFlt1. It is reported that VEGF bind to Flt1 receptors, which may serve to dimerize tyrosine kinase receptors and activates signaling pathways linked with proliferation, endothelial mitogenesis, vascular permeability, initiation of angiogenesis, vasculogenesis (Keyt *et al*., 1996).

Soluble fms like tyrosine kinase 1:

A soluble form of Flt-1 which lacks a membrane-spanning and intracellular regions is an antiangiogenic protein produced in the placenta. It is produced through the splicing of mRNA of the Flt-1 gene in syncytiotrophoblast. sFlt1 is a 100kDa membrane-bound protein. It can interact with the free growth factors VEGF or PlGF in maternal blood circulation. But it has antiangiogenic characteristics, when it interacts with PLGF and VEGF it inhibits their interaction with the angiogenic Flt-1 receptor and acts as an antagonist of circulating VEGF and PLGF's receptors (Helmo *et al*., 2018; Jung *et al*., 2012). It has a unique 31-aa C-terminus segment derived from splicing. Endothelial dysfunction and vasoconstriction were among the effects of sFlt1 (Maynard *et al*., 2005)**.** The placenta has been shown to produce several different isoforms of sFlt1. humans and primates express one of these isoforms, which is known as sFlt1 (Steinberg *et al.*, 2009). sFlt1 is released in significant amounts in pregnancies affected by preeclampsia.

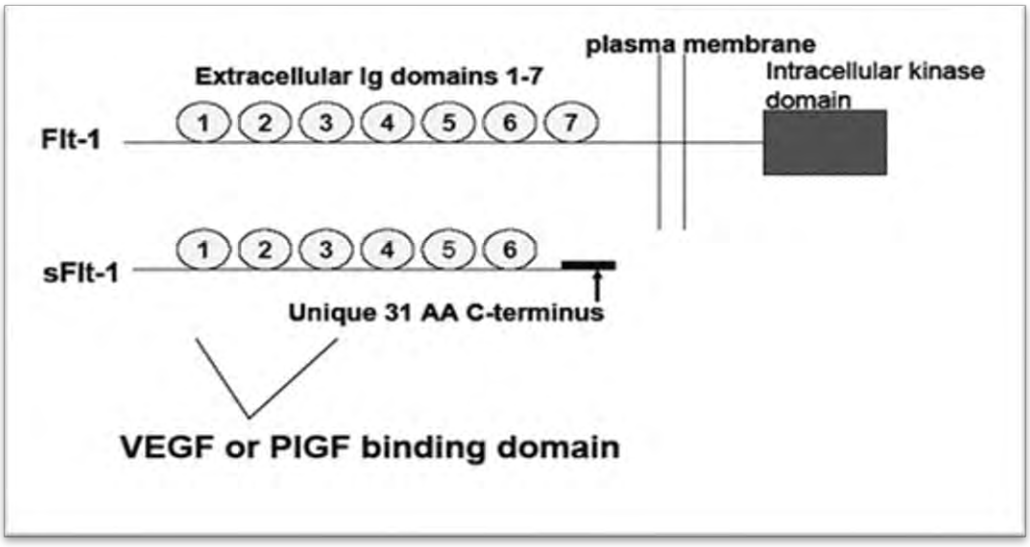


Figure 5: Protein structure of Flt1 and its splice variant sFlt1 (Maynard *et al.*, 2005).

Five weeks before the development of preeclampsia, maternal serum levels of sFlt1 are raised, indicating that sFlt1 is a significant factor in the clinical manifestation of preeclampsia. Endothelial dysfunction is linked to an elevated sFlt1 level (Jung *et al*.,

2012). Preeclampsia causes glomerular endothelial damage, hypertension, and proteinuria by overexpressing soluble sFlt1. Higher sFlt1 concentrations are related to lower levels of free VEGF and PlGF in the blood, leading to endothelial dysfunction (Sircar *et al*., 2015).

Splicing mechanism:

In endothelial cells, the oxygen-sensitive Jumanji domain-containing protein 6 (JMJD6) altered the splicing pathway of FLT-1. It is an oxygen-sensing protein that relates to the superfamily of 2 oxoglutarate-dependent oxygenases. The enzymatic activity of this protein is dependent on the presence of oxygen. JMJD6 was able to complete its usual catalytic activity under normoxic situations and hydroxylates U2 small nuclear ribonucleoprotein which is a 65-kDa subunit of the splicing machinery. As a result, the splicing machinery is unable to splice FLT1 and produce the membrane bound Flt1 receptor. However, because JMJD6 is an oxygen-sensing protein, its catalytic activity was reduced during hypoxic conditions as a result, JMJD6 was unable to hydroxylate the splicing machinery U2AF65. Consequently, the splicing machinery secreted relatively shorter spliced variant sFlt1 from the Flt1 protein (Palmer *et al*., 2016; Jena *et al*., 2020).

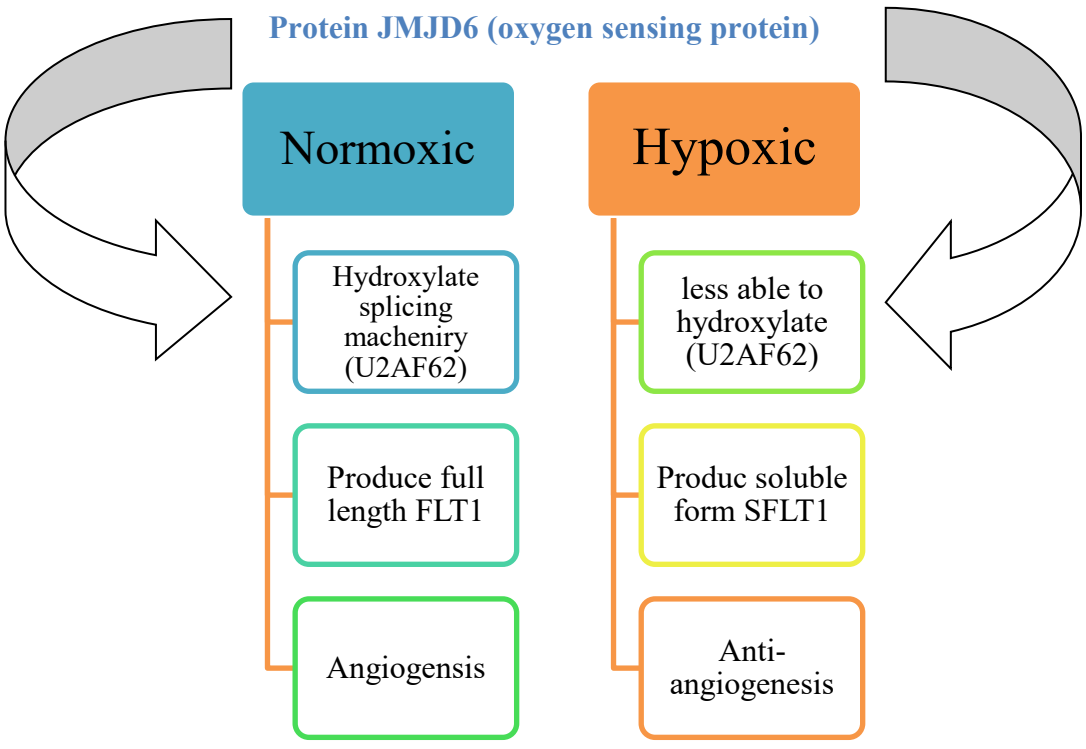


Figure 6: Splicing mechanism of Sflt-1 (Palmer *et al*., 2016).

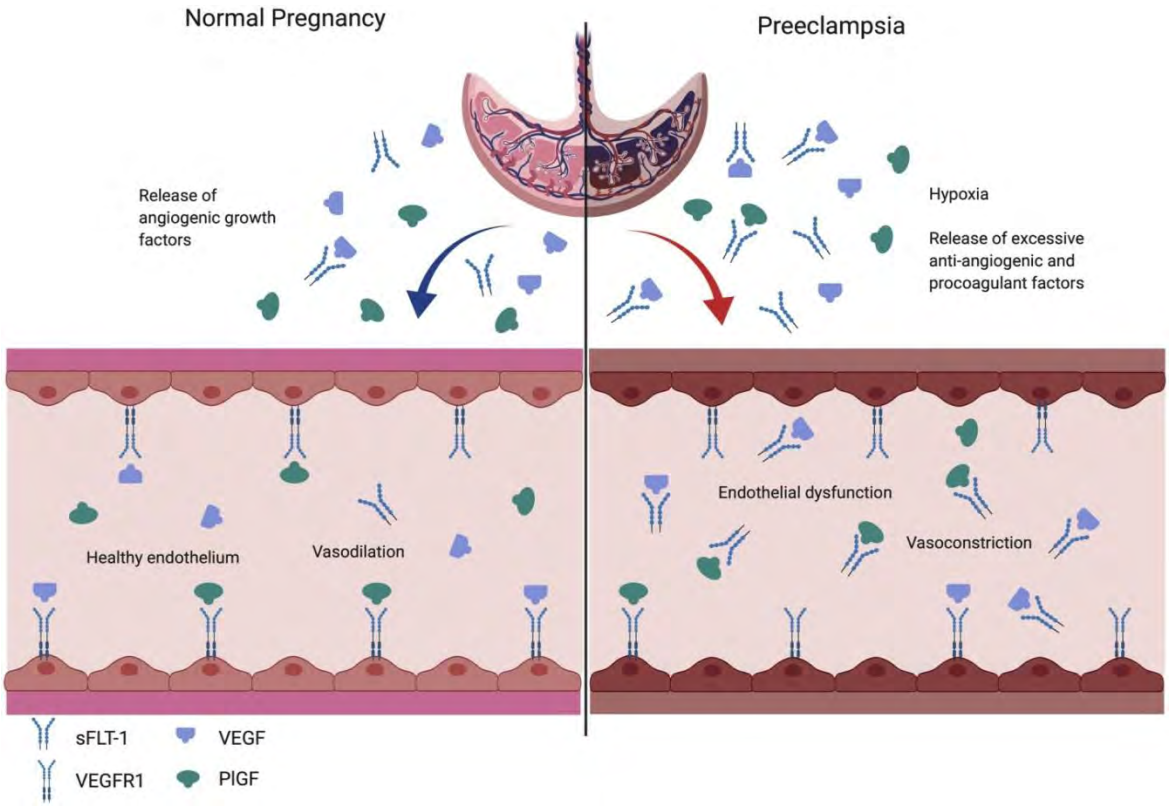


Figure 7: Role of sFlt1 in PE pathology (Rana *et al*., 2021).

Preeclampsia Complications:

During the first and second trimesters of gestation, preeclampsia is asymptomatic as the symptoms are extremely diverse. It can only be diagnosed during regular prenatal analysis. The primary symptoms are proteinuria (300 mg/24 h) and high blood pressure of 140mm Hg/90 mm. In severe cases, right upper quadrant pain due to severe hepatotoxicity, eclamptic seizures, and hemolysis occur if not treated instantly. Fetal growth restriction, premature delivery, iatrogenic prematurity, and perinatal death are complications related to fetus occur due to insufficiency of the placenta, abnormal uteroplacental blood flow, and abnormal placentation (Nirupama *et al*., 2021). The most common complications of preeclampsia are eclampsia, pulmonary edema, and intracranial hemorrhage. Edema of the liver is one of the frequent cardiopulmonary complications of preeclampsia. It is a severe character of the disease and occurs almost in 3% of women with preeclampsia. Severe kidney damage is an uncommon complication of preeclampsia. Intrauterine growth restriction, placental abruption, and fetal demise are obstetric complications (Witcher, 2018). If PE is not treated it can result in severe problems like eclampsia and HELPP syndrome, a condition in which red blood cells break down, the

number of platelets decreases, and liver enzymes increases (Guerby *et al*., 2021; Leeman *et al*., 2016) During gestation, 10% of women suffer from high blood pressure (Sidani & Siddik-Sayyid, 2011).

Preeclampsia Diagnosis:

The presence of specific markers may be used for diagnosis to avoid maternal and neonatal morbidity and mortality (Steegers *et al*., 2010). Nowadays, different tests are recommended for the diagnosis of preeclampsia. These tests include kidney function tests, blood tests, blood platelet count, urinalysis to determine the quantity of protein in the urine (protein: creatinine), biophysical profile to determine the baby's heart rate, and ultrasound to determine the fetus's breathing rate, size, and weight. Both ultrasound and laboratory testing are used to monitor the severity of the infection in the mother. (Nirupama *et al*., 2021).

Management of PE:

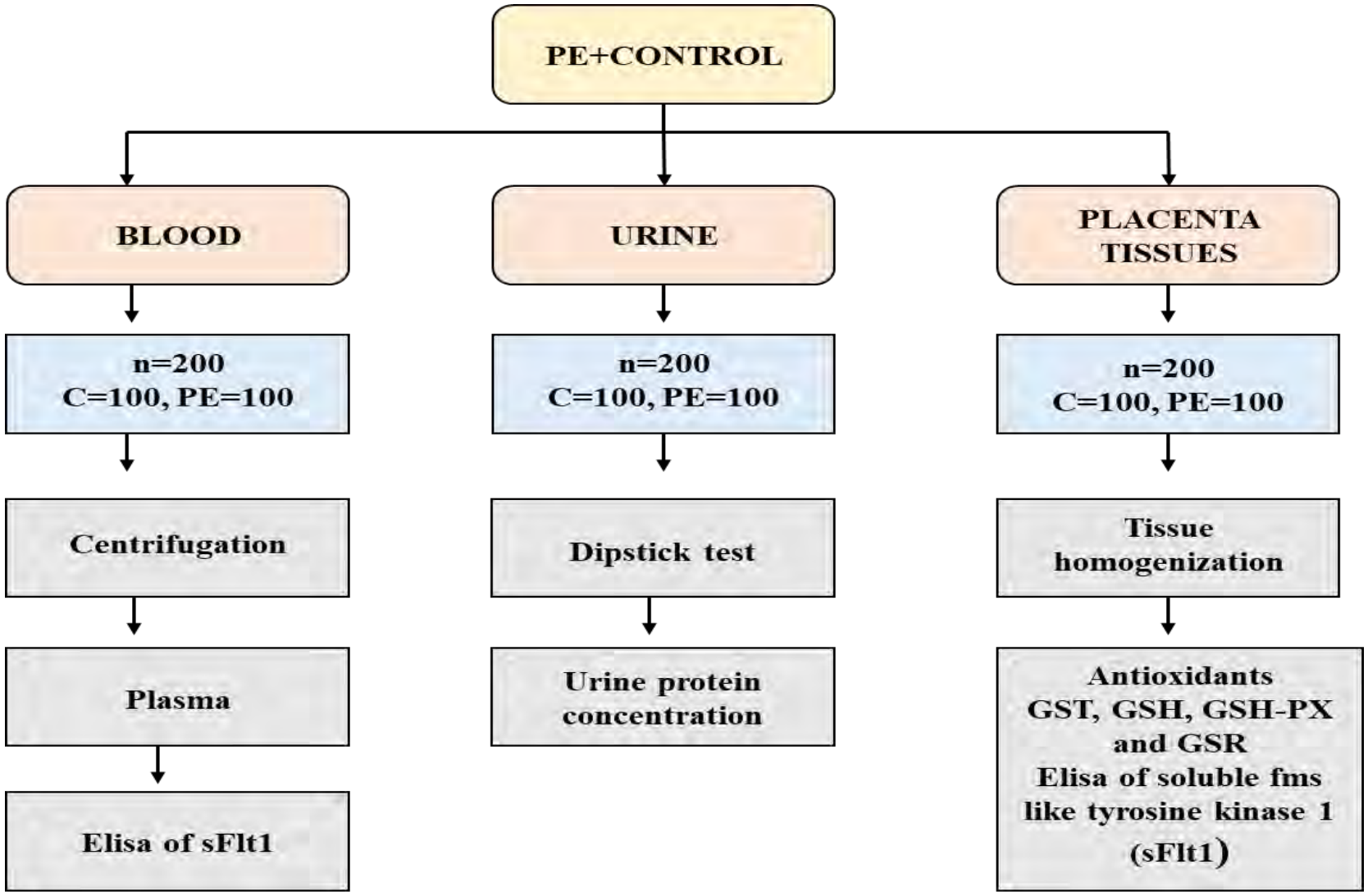
Administration of antihypertensive drugs are highly recommended by international guidelines for severe hypertension and preeclampsia. It prevents the mother from serious complications such as cerebral eclampsia and hemorrhage. Antihypertensive drugs included hydralazine, β- blockers, acetylsalicylic acid, methyldopa, and nifedipine (Nirupama *et al*., 2021). Administration of heparin and aspirin is recommended to prevent preeclampsia (Winer & Tsasaris, 2008). Doppler ultrasound uterine artery waveform analysis can help to identify the risk of PE in pregnancy with the incomplete remodeling of maternal spiral arteries (Khong *et al*., 2015). Current management strategies of preeclampsia are based on the identification of the disease, severity of the disease, antihypertensive therapy, and the timing of delivery. Recent management strategies may stop the pathological processes of preeclampsia or prevent its incidence in high-risk patients; hence controlling the disease (El-sayed, 2017). Premature delivery before 34 weeks of gestation is the only promising treatment for PE to avoid severe maternal complications, but it may result in severe neonatal damage (Snead *et al*., 2019). Low dose usage of aspirin has a temperate effect preventing preeclampsia, but the consequence is higher in women who are at the stake of developing the disease. To prevent hypertension and preeclampsia calcium supplementation may be recommended. In high-risk women, the death rate is prevented with low consumption of calcium. Intake of magnesium sulfate is recommended to prevent convulsions and maternal injury (Leeman *et al*., 2016).

Objectives

The aim of the current study is the collection and analysis of demographic, clinical, biochemical, and hormonal data of PE patients and to evaluate the important risk factors involved in pathogenesis and susceptibility to preeclampsia (PE). The study objectives are:

- To determine the urine protein concentration in pathogenesis of PE.
- To investigate the role of circulating and placental enzymatic and nonenzymatic antioxidants in susceptibility to PE.
- To evaluate the role of soluble fms like tyrosine kinase 1 in the pathophysiology of PE.

EXPERIMENTAL DESIGN



MATERIALS AND METHODS

Ethical approval

This study was carried out at the Reproductive Physiology Laboratory, Department of Zoology, Faculty of Biological Sciences, with the consent of the ethical committee of Quaid-i-Azam University, Islamabad. All the subjects involved in this study were informed about the objectives of the present study and a consent form was signed. Patients were from the Pakistan Institute of Medical Sciences (PIMS), Islamabad, and Quaid-e-Azam International Hospital, Islamabad.

Patient identification and selection

The Patients were selected and identified according to the criteria given below. A total of 200 subjects were involved in the current study, where the number of control subjects was 100 while, PE women were 100.

Inclusion and exclusions criteria

The current study includes preeclamptic females having the systolic and diastolic BP ≥140/90 mmHg and early onset of proteinuria (≥300mg/24 hours Urine collection) and normotensive females with the normal blood pressure ≤120/85 mmHg without proteinuria and uncomplicated gestation but excluded the subjects if they are suffering from any of the following chronic diseases including asthma, renal, diabetes, hepatic or hematological disorders, autoimmune disease, infection of the urinary tract, eclampsia and any infection related to smoking.

Data and Sample Collection

Collection of demographic and clinical data

Demographic data were collected using structured questionnaires. Information regarding personal health, medical history, and clinical features was obtained. The history of the patient was taken based on their height, weight, age, menstrual cyclicity, gestational age, gravidity (number of times to conceive), history of abortions or stillbirths, parity (number of times given birth to a fetus), history of PE in family, the ratio of consanguineous marriages and duration of the marriage.

Sample collection and Storage

Blood and urine samples were collected. Approx. 3-5ml of blood samples were taken from the antecubital vein of the patient in heparinized syringes. The blood was taken into a 3ml tube, subjected to centrifugation at 13000 revolutions/minute (rpm) for 20 minutes to separate plasma, and stored at -20°C for further analysis. The urine samples were collected in glass vials just before the delivery period. The fetal placenta tissue samples

were obtained and stored immediately in dry ice. Placental tissue samples were collected in cryo-Eppendorf and stored immediately in dry ice. The remaining samples were stored at -80°C for further analysis.

Blood pressure measurement

The Blood pressure from all the study subjects was noted with a Sphygmomanometer before the delivery.

Height measurements

The height of each subject was evaluated by using a device 'Stadiometer' and the readings were recorded in meters(m) to calculate the BMI.

Weight measurements

The weight of each subject was evaluated by using a 'Weighing scale' and the evaluations were recorded in kilograms(kg).

Measurement of Body Mass Index

Body mass index for both groups under study was estimated as:

Body mass index= weight in kilogram/height in meters^2

Urine Examination

Dipstick Test

Urine examination was performed by urine dipstick test strips. Combur test strips (Roche, Combas. ®, USA) were used for the test. The test was done by dipping the Test strips in the urine sample. The stick color becomes changed due to the presence of specific constituents in the urine, such as protein concentration, urobilinogen conc (a by-product of bilirubin reduction), specific gravity, pH levels, or acidity

Procedure

- The urine samples were collected and transferred to a test tube.
- After dipping the test strip in the urine for one second, the strip was drawn.
- While withdrawing the strip, wipe the edge on the vessel rim to remove any excess urine.
- The color of the test strip changes after about 60 seconds.
- The color of the reaction in the testing area was then compared to the color scale on the label, and the results were recorded.

Biochemical Analysis:

In the control and preeclamptic groups, various antioxidant enzymes were measured in plasma and placental tissues. The frozen placental tissues were thawed and homogenized in 3 mL of phosphate buffer (pH 7.4). After that, the homogenate was centrifuged at 4°C for 30 minutes at 12000 rpm. The supernatant was taken to determine the antioxidant status of the placental tissues. Heparinized tubes were used to collect blood during the antepartum period before birth. Blood samples were centrifuged for 15 minutes at 3000 rpm to separate the plasma and then stored at -80°C for further biochemical analysis.

Glutathione reductase (GSR) activity

Glutathione reductase levels were calculated by using Carlsberg and Mannervik's method (Mannervik, 1967)

Reagents:

NADPH	0.1 ml (0.1 mM)
PBS	1.65 ml (0.1M; pH=7.6)
Glutathione Oxidized	0.05 ml (1 mM)
EDTA	0.1 ml (0.5 mM)
Sample	0.1ml

Procedure:

To evaluate GSR levels 0.1ml NADPH (0.1 mmol), 0.1 ml EDTA (0.5 Mm) 1.65 ml phosphate buffer (0.1mol; pH=7.6), 0.1 ml samples, and 0.05ml of oxidized glutathione (1 Mm) were mixed in a cuvette. NADPH activity was noted and expressed as nmol of NADPH oxidized/min/mg protein at 25°C with the help of coefficient of molar extinction of $6.22\times10\times_3$ M-1 cm-1 at a wavelength of 340nm.

Glutathione –S- Transferase Activity (GST):

Reagents

PBS	1.475ml (0.1mol; pH=6.5)
2, 4- Dinitrochlorobenzene	0.025 ml (1mmol)

Reduced Glutathione	0.2 ml (1 mmol)
Sample	0.3 ml

Procedure:

To measure Glutathione-S-Transferase activity the reaction mixture consisted of 1.475ml phosphate buffer (0.1 mol, pH 6.5), 0.025ml (CDNB; 1 mmol), 0.2ml reduced glutathione (1 mmol), and 0.3 ml of sample in a total volume of 2.0ml. Absorbance was recorded at the wavelength of 340nm and activity of the enzyme was calculated as nM CDNB conjugate formed/min/mg protein by using a molar extinction coefficient of $9.6\times10_3$ M-1 cm-1.

Reduced Glutathione Activity (GSH)

Reagent

PBS	2.7 ml (pH 7.4)
Ellman	0.2 ml (100 NM)
sulfosalicylic acid	1 ml (4%)
Sample	1.0ml

Procedure:

A 1.0 ml of sample was precipitated with 1.0 ml of (4%) sulfosalicylic acid. For 1 hour the Sample was kept at 4 degrees and then subjected to centrifugation at 1200×g for 20 minutes at 4°C0.1 ml filtered aliquot, 2.7 ml phosphate buffer (0.1 mol; pH=7.4), and 0.2 ml Ellman's reagents (100mM) made up the entire volume of the 3.0 ml assay mixture. The yellow color developed, and absorbance was measured immediately at a wavelength of 412 NM on a smart Spec TM plus Spectrophotometer.

Glutathione peroxidase assay (GSH-px) activity

Glutathione peroxidase activity was observed by the method of (Mohandas *et al*., 1984).

PBS	1.49 ml (0.1 M; pH=7.4)
Sodium azide	0.1 ml (1mM)
EDTA	0.1 ml (1 mM)

Glutathione Reductase	0.05 ml (1 mM)
H2O2	0.01 ml (0.25Mm)
Reduced Glutathione	0.05 ml (1 mM)
NADPH	0.1 ml (0.2 mM)
Sample	0.2ml

Procedure:

In a total volume of 2 ml, the reaction mixture contained 1.49 ml phosphate buffer (0.1 M; pH 7.4), 0.1 ml Ethylenediaminetetraacetic acid (1mM), 0.1 mm Sodium Azide (1Mm), 0.05 ml glutathione reductase (1 IU/m), 0.05 ml GSH (1Mm), 0.1 ml NADPH (0.2 mM), 0.01 A Smart Spec TM plus Spectrophotometer was used to measure the disappearance of Nicotinamide adenine dinucleotide phosphate at the wavelength of 340 nm at 25°C. NADPH oxidized was used to calculate enzyme activity.

ELISA of Soluble fms like tyrosine kinase 1 (sflt1)

The ELISA of Soluble fms like tyrosine kinase 1 was carried out by using the Human Elisa kit of MyBioSource, Catalog No: MBS2601616. The concentration of sflt1 in plasma and placental homogenate samples was determined.

Preparation of sample

The EDTA-Na2 tubes were used to separate the plasma from collecting blood. Then centrifuge the blood samples for 15 minutes at 1000xg. After centrifugation layers were formed, the upper layer (Supernatant) was collected and used for analysis. Homogenized the placental tissues by using a glass homogenizer, the process of homogenization was performed at the ice. The buffer solution (PBS) with 1:9 was used for this motive. Then centrifuge the homogenates at 5000xg for 5 minutes and place in refrigerator at 4^{o}C.

Principle of Test

The current ELISA technique is the double antibody sandwich ELISA. The detection antibody is a biotinylated polyclonal antibody while the pre-coated antibody is an anti-Human salt-1 monoclonal antibody. After adding samples and biotinylated antibody to ELISA plate wells, they are rinsed off with PBS or TBS. After that, the wells are filled with Avidin-peroxidase conjugates. After the enzymes conjugate has been properly rinsed out of the wells with PBS or TBS, the TMB substrate is utilized for coloring. TMB

combines with peroxidase activity to produce a blue product, which then becomes yellow after the stop solution is added (Color reagent C). The amount of target analyte in the sample and its color intensity are positively associated.

Procedure of the Assay:

Steps:

1. Remove as many strips as you want and set them aside to acclimatize to room temperature. The desiccant and unused strips should be returned to the sealed aluminum foil bag and stored at 2-8°C.
2. Make a list of blank wells and sent them away (if measuring at dual-wavelength, the blank wells can be ignored)
3. Pour 100 mL of water into each well to hold standards or samples. Keep in mind that the 0ng/mL well should be filled with 100L of Standard Diluent. Incubate at 37°C for 90 minutes after sealing the wells/plate with the adhesive tape strip.
4. Prepare 30 minutes ahead of time the needed quantity of Biotinylated Antibody.
5. Wash the ELISA plate twice.
6. Pour 100 mL of produced Biotinylated Antibody into each well. Incubate at 37°C for 30 minutes after sealing reaction wells with adhesive tape strips. Add prepared Enzyme Conjugate to each well other than the blank wells (100µl for each). Seal the wells with the adhesive tape strip and incubate at 37℃ for 30min.
7. Rinse the ELISA plate five times before using it.
8. Pour 100L of the prepared Color Reagent into separate wells (as well as the blank well) and incubate at 37°C in a light-protected environment. The incubation can be halted when the coloration of the highest standards becomes darker, and the color gradient occurs. Within 30 minutes, the chromogenic process should be under control.
9. Fill each well with 100L stop solution (Color Reagent C) (also into the blank well).
10. Spectrophotometry with a microplate reader at 450 nm was used to determine the optical density (OD value) for each well within 10 minutes. The values of the blank should be subtracted from the OD values of each sample and standard. (Weel *et al*., 2016; Khalil *et al*., 2008)**.**

Statistical Analysis

All of the data was presented as a mean standard error of the mean. To analyze the degree of risk variables and clinical presentations implicated in PE, data with 95 percent confidence intervals (CI) and odds ratios (OR) were generated. The difference in demographic and clinical data results between the two study groups was examined using Graph Pad Prism and an unpaired T-test or chi-square (2). Graph Pad Prism version 5 was used to evaluate the biochemical data differences between the groups using Welch's two-sample t-test.

RESULTS

Demographic characteristics

The present study involved subjects belonging to various areas of Pakistan, their details have been summarized in figures 1 and 2. Most of the subjects involved in our study belonged to Islamabad (40%) and Rawalpindi (8%). Analysis of demographic data showed a significant increase in gestational age ($p<0.01$) of PE women as compared to control subjects. A significant decrease in maternal age ($p<0.05$) was evident, whereas a significant increase in body mass index ($p=0.02$) among the two groups was seen. A highly significant increase was observed in systolic ($p<0.001$), and diastolic blood pressure ($p<0.001$) among preeclamptic women as compared to control subjects. A nonsignificant difference was observed in age at marriage ($p=0.23$) as well as age at first childbirth ($p=0.46$) in both control and preeclamptic groups. The demographic characteristics of the present study have been summarized in Table 1.

Table 1: Mean ± SEM of demographic parameters of control and preeclamptic study groups.

Parameters	Control	PE	p-value statistics
Gestational age (weeks)	35.97 ± 0.71	32.78 ± 0.71	< 0.01
Maternal age (years)	25.11 ± 0.85	27.92 ± 0.76	<0.05
BMI (kg/m^2)	26.23 ± 0.64	28.72 ± 0.85	<0.05
Age at time of marriage	21.06 ± 0.52	22.09 ± 0.68	0.23
Age at first childbirth	22.90 ± 1.11	23.87 ± 0.77	0.46
Systolic blood pleasure (mmHg)	112.6 ± 1.02	153.9 ± 3.97	< 0.001

Diastolic blood pleasure (mmHg)	72.05 ± 0.83	100.1 ± 2.91	< 0.001

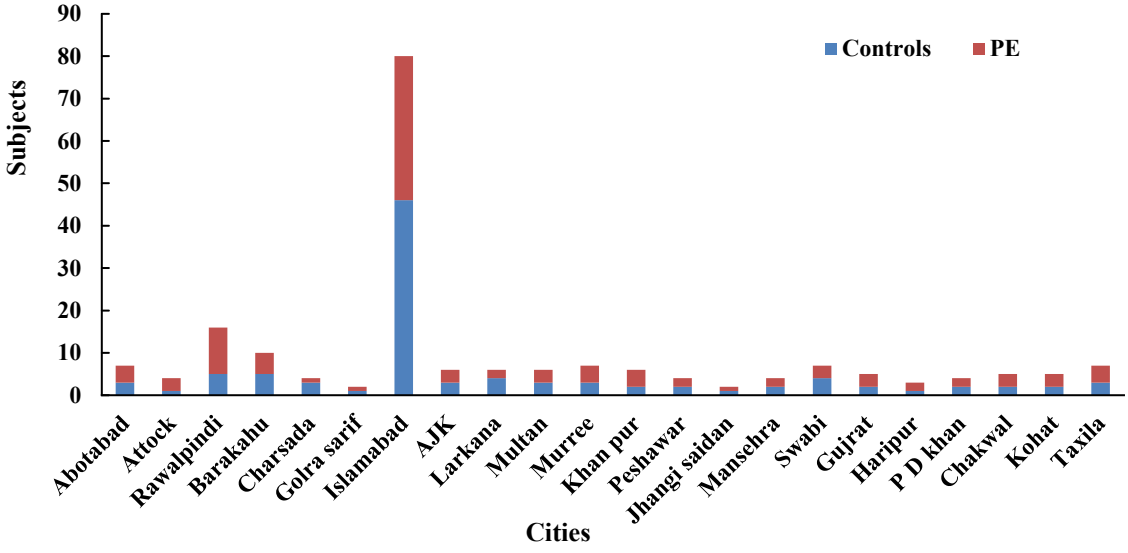


Figure 8: Graph showing the number of the control and preeclamptic subjects involved in the current study.

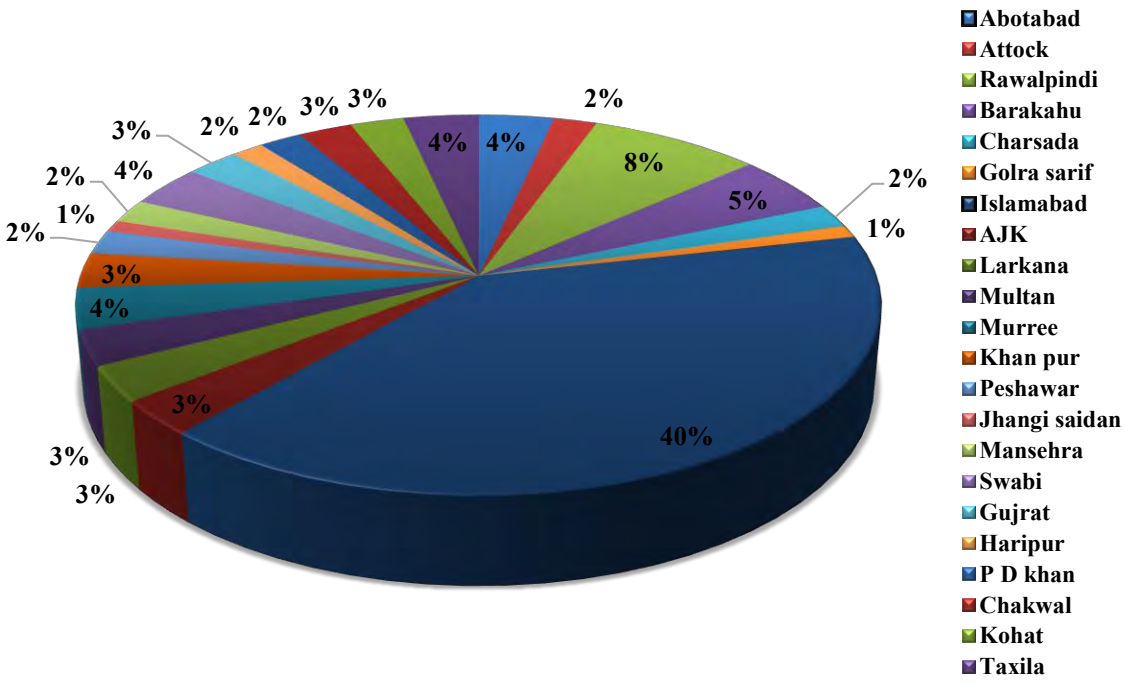


Figure 9: Study subjects from the population of Pakistan

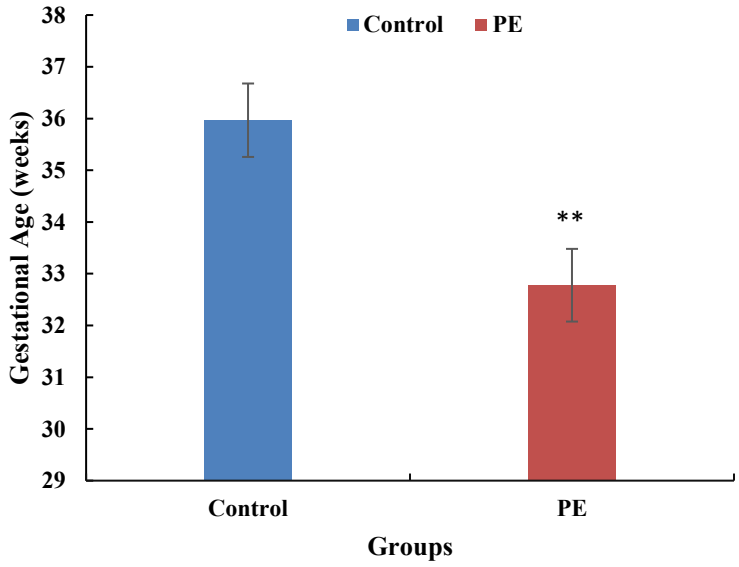


Figure 10: Mean ± SEM difference of Gestational age (weeks) in the control and preeclamptic group.

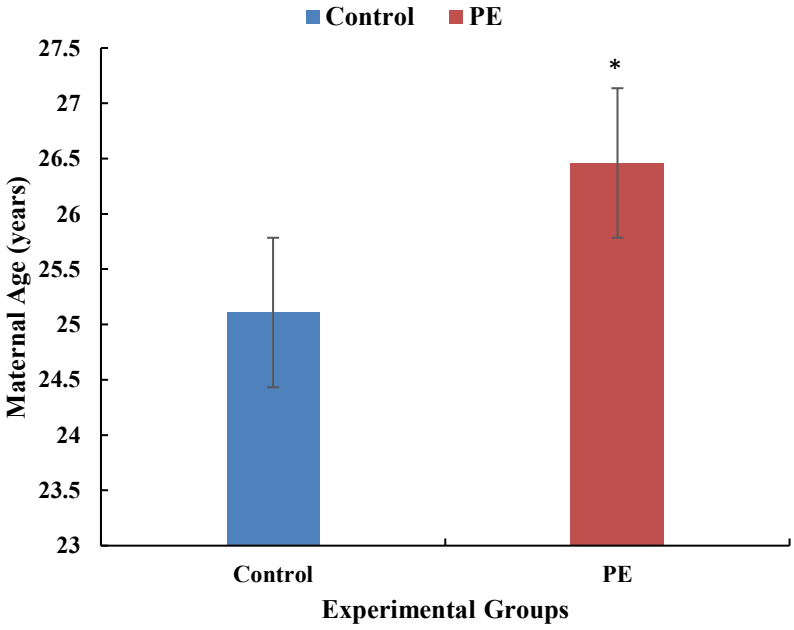


Figure 11: Mean ± SEM difference of maternal age (years) in the control and preeclamptic group.

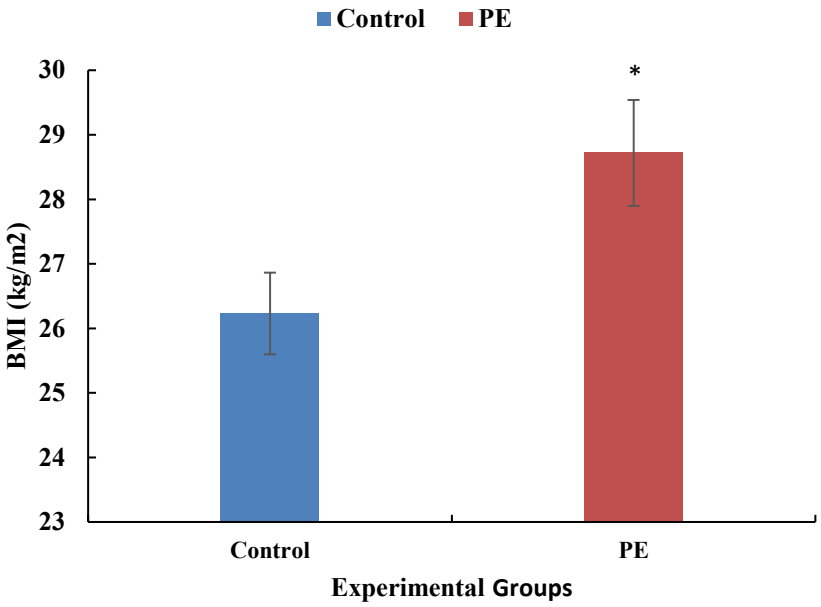


Figure 12: Mean ± SEM difference of BMI in the control and preeclamptic group.

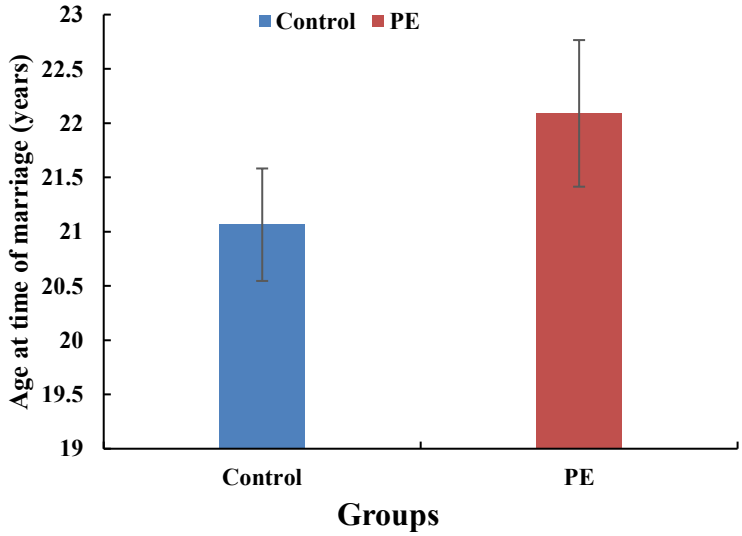


Figure 14: Mean ± SEM difference of Age at marriage (years) in the control and preeclamptic group.

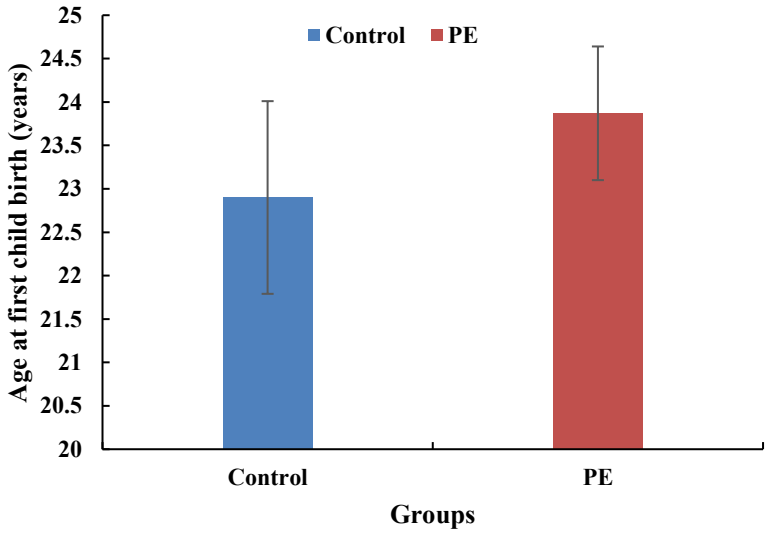


Figure 13: Mean ± SEM difference of age at first childbirth in control and preeclamptic group

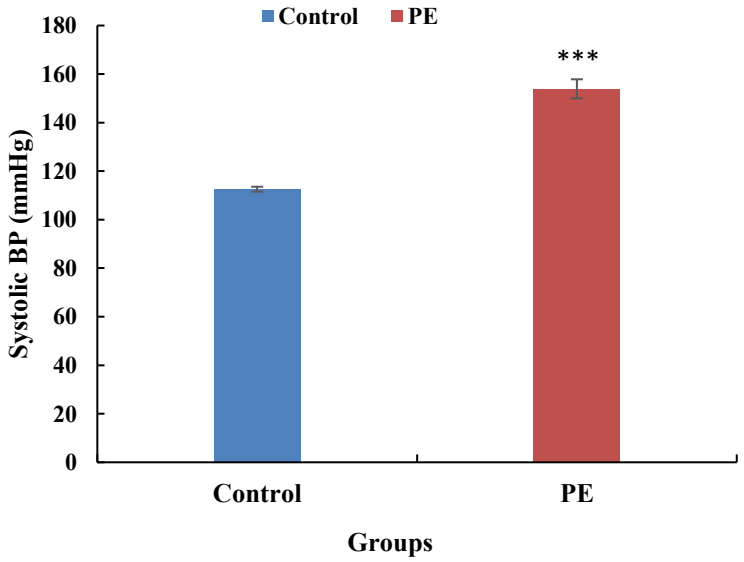


Figure 15: Mean ± SEM difference of Systolic BP (mmHg) in the control and preeclamptic group.

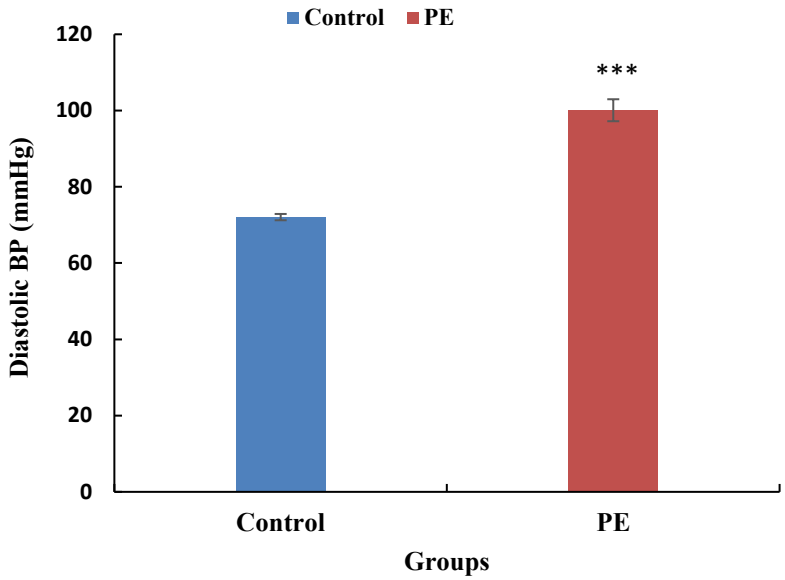


Figure 16: Mean ± SEM difference of Diastolic BP (mmHg) in the control and preeclamptic group.

Pregnancy and medical history:

The medical history taken from pregnant females showed that PE patients were at greater risk of suffering from severe signs and symptoms than the control group. The apparent symptoms include swelling in hands and face ($p<0.001$), headache ($p<0.01$), urination problem ($p<0.01$), shortness of breath ($p<0.01$), excessive weight gain ($p<0.05$), these all were significantly present in women suffering from PE as compared to the control group. Agitation was non-significant ($p=0.5101$). Nausea and vomiting were also nonsignificant in both groups ($p=0.7263$). Abdominal pain was significant in both groups ($p<0.05$). No single subject experienced seizures in the control group whereas only a single subject from the PE group suffered from loss of consciousness ($p=0.3062$). However, 2 PE subjects and 1 subject from the control group experienced the loss of consciousness, though significance was ($p=0.5579$). A significant difference was seen in symptoms like muscle pain ($p<0.05$), blurring of vision ($p<0.05$) in subjects. A nonsignificant difference was observed in consanguineous marriage($p=0.197$), and twin pregnancies($p=0.414$) between the control and preeclamptic subjects. The case-patients were having a previous history of PE ($p<0.01$) during their previous pregnancies. Furthermore, the PE group data revealed that case patients were having PE family histories ($p<0.01$) as depicted in table 2.

Table 2: Medical history from control and preeclamptic study groups

Parameters	Control	PE	X^2	OR (CI)	p-value
Swelling in hands or face					
Yes	9	27	14.91	0.17 (0.06-0.43)	<0.001
No	40	21			
Headache					
Yes	13	25	7.51	0.30 (0.13-0.72)	<0.01
No	37	22			
Excessive weight gain					
Yes	4	12	4.96	0.26 (0.07-0.90)	<0.05
No	46	37			
Urination problem					
Yes	11	23	7.26	0.30 (0.12-0.73)	<0.01
No	39	25			
Abdominal pain					
Yes	17	29	3.84	0.44 (0.19-1.00)	<0.05
No	29	22			
PE in a previous pregnancy					
Yes	3	13	7.66	0.17 (0.04-0.67)	<0.01
No	48	37			
Shortness of breath					

Yes	6	19	9.80	0.21 (0.07-0.58)	<0.01
No	44	29			
Seizures					
Yes	0	1	1.05	0.31 (0.01-7.89)	0.30
No	50	47			
Family history of PE					
Yes	2	13	9.49	0.11 (0.02-0.55)	<0.01
No	48	37			
Blurring vision					
Yes	0	4	3.92	0.10 (0.005-2.07)	<0.05
No	49	48			
Loss of consciousness					
Yes	1	2	0.34	0.49 (0.04-5.58)	0.55
No	50	49			
Twin pregnancy					
Yes	2	4	0.66	0.48 (0.08-2.80)	0.41
No	48	47			
Consanguineous marriages					
Yes	20	26	1.66	0.59 (0.27-1.31)	0.19
No	31	24			

Nausea and vomiting					
Yes	12	13	0.12	0.85 (0.34-2.11)	0.72
No	38	35			
Agitation					
Yes	1	2	0.43	0.45 (0.03-5.13)	0.51
No	50	45			
Muscle pain					
Yes	12	23	4.66	0.39 (0.16-0.92)	<0.05
No	37	28			

Urine Examination:

For the urine examination protein content and urine pH were measured summarized in table 3. There was a significant elevation observed ($p<0.001$) in the concentration of urine protein among the preeclamptic group. No significant change was observed for pH ($p= 0.34$) in both preeclamptic and control subjects.

Table 3: Mean ± SEM of urine parameters of control and preeclamptic group.

Parameters	Control	PE	p-value
Protein conc (mg/dl)	0.07 ± 0.04	2.19 ± 0.10	<0.001
pH	6.059 ± 0.08	5.942 ± 0.09	0.34

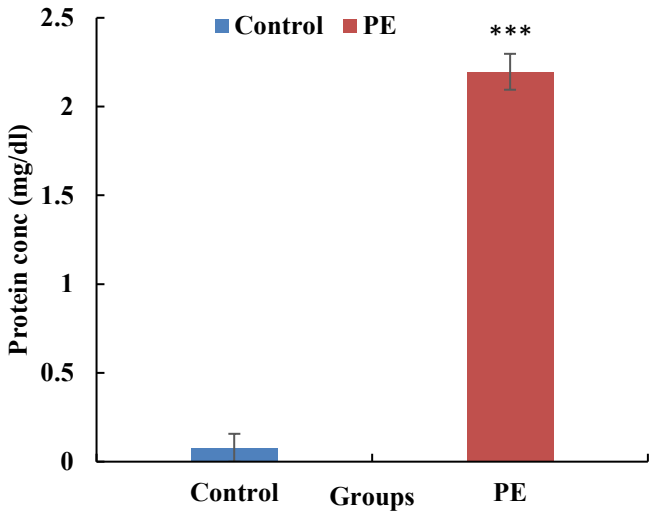


Figure 17: Mean ± SEM difference of protein concentration (mg/dl) of control and preeclamptic group.

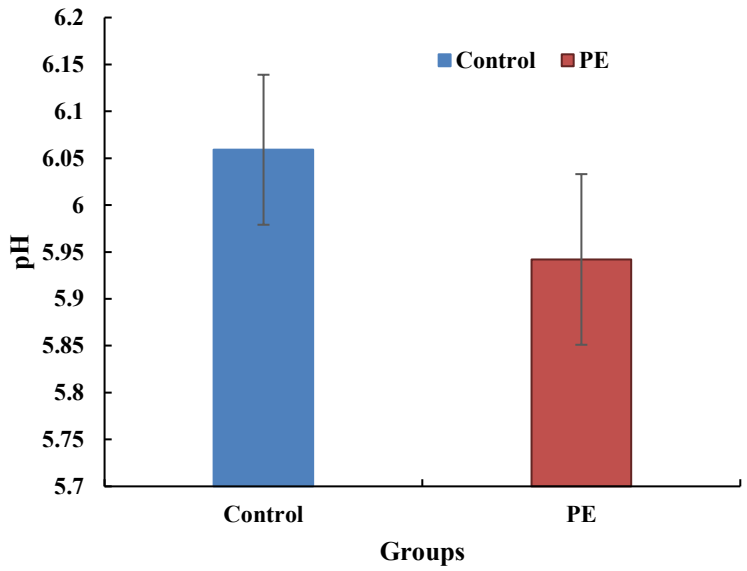


Figure 18: Mean ± SEM difference of pH in control and preeclamptic group.

Biochemical analysis

Placental Antioxidants:

Different antioxidant enzymes including GST, GSR, GPx, and GSH were evaluated in both control and preeclamptic subjects summarized in Table 4. Decrease in GSR ($p<0.05$), and GSH ($p<0.01$) levels were observed in the placenta of the PE group than the control group. Furthermore, GST ($p<0.001$) and GPx ($p<0.001$) were decreased significantly in preeclamptic placentae as compared to controls.

Table 4: Mean ± SEM of placental antioxidants of control and preeclamptic group.

Homogenates Antioxidants	Control	PE	p-value
Glutathione Transferase	2.06±0.141	1.25±0.087	< 0.001
Glutathione Reductase	2.45±0.26	1.47±0.296	<0.05
Reduced Glutathione	1.14±0.09	0.77±0.089	<0.01
Glutathione Peroxidase	1.05 ± 0.07	0.59 ± 0.03	<0.001

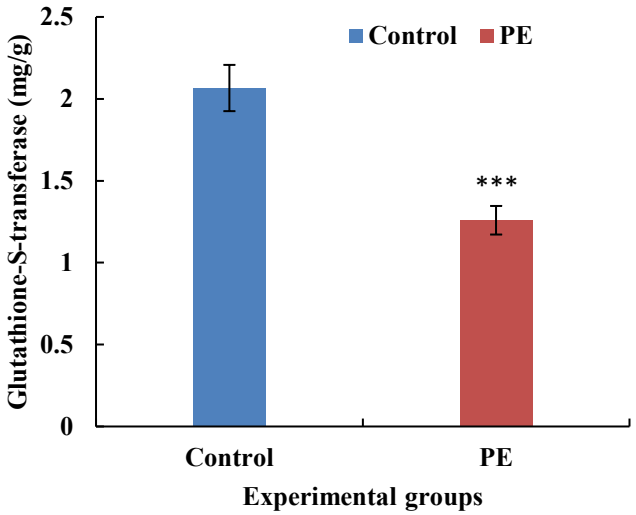


Figure 19: Mean ± SEM difference of Glutathione-S-transferase in the placenta of control and preeclamptic group.

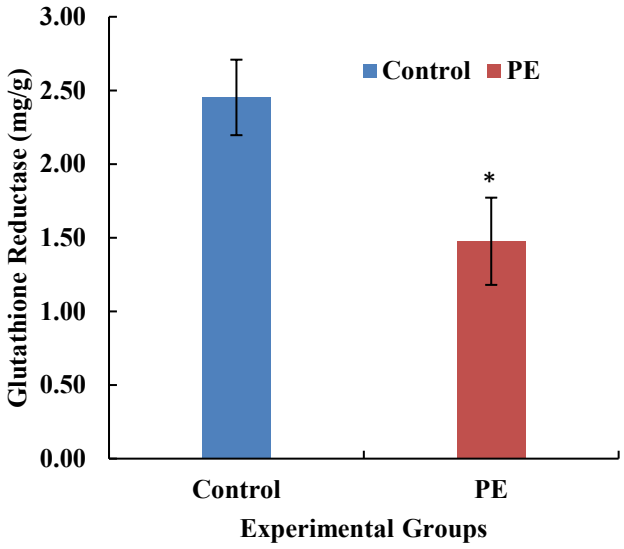


Figure 20: Mean ± SEM difference of Glutathione Reductase in the placenta of control and preeclamptic group.

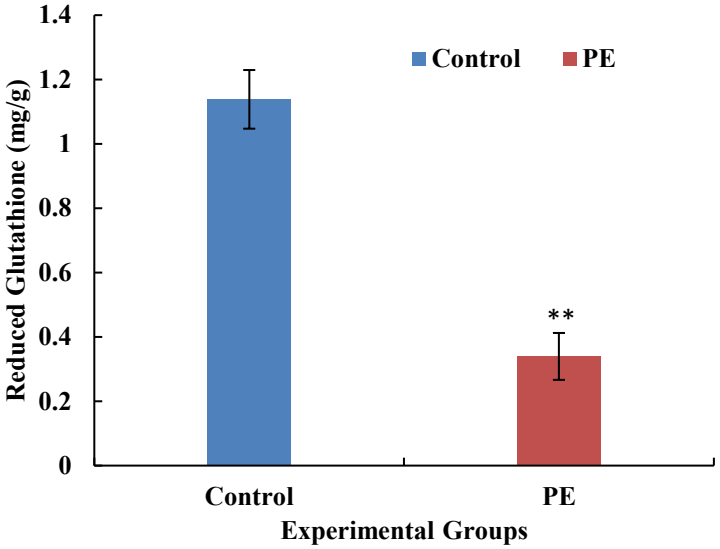


Figure 21: Mean ± SEM difference of Reduced Glutathione in the placenta of control and preeclamptic group.

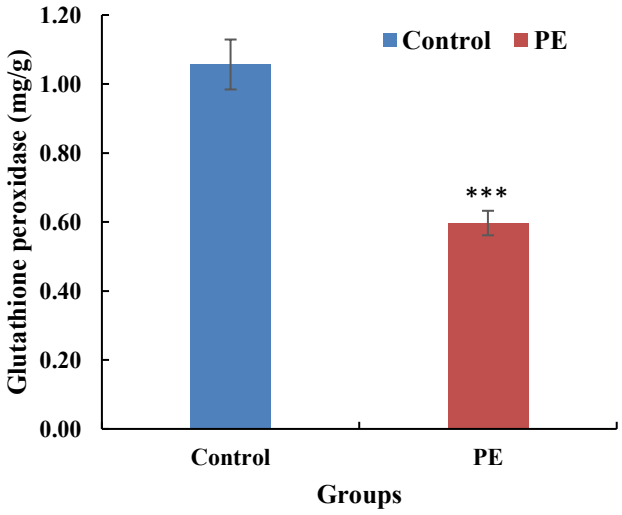


Figure 22: Mean ± SEM difference of Glutathione peroxidase in the placenta of control and preeclamptic group.

Plasma Antioxidants:

Different antioxidant enzymes including GST, GSR, GPx, and GSH were also evaluated in the plasma of both PE and normotensive groups summarized in Table 5. A decrease in GST ($p<0.01$) and GSR ($p< 0.05$) levels in the PE group than the control group was observed. Moreover, GSH ($p<0.001$) and GPx ($p<0.001$) were decreased significantly in preeclamptic subjects.

Table 5: Mean ± SEM of plasma antioxidants of control and preeclamptic group.

Plasma Antioxidants	Control	PE	P-value
Glutathione transferase	6.642±0.671	3.81±0.62	<0.01
Glutathione reductase	10.48 ± 1.42	6.142 ± 0.84	<0.05
Reduced glutathione	9.84±0.90	4.80±0.778	<0.001
Glutathione peroxidase	5.20 ± 0.27	2.63 ± 0.21	<0.001

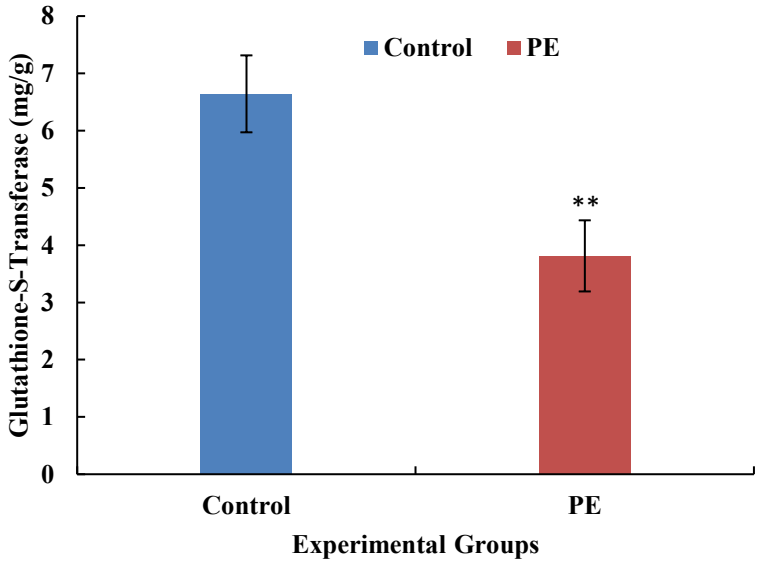


Figure 23: Mean ± SEM difference of Glutathione-S-transferase in the plasma of control and preeclamptic group.

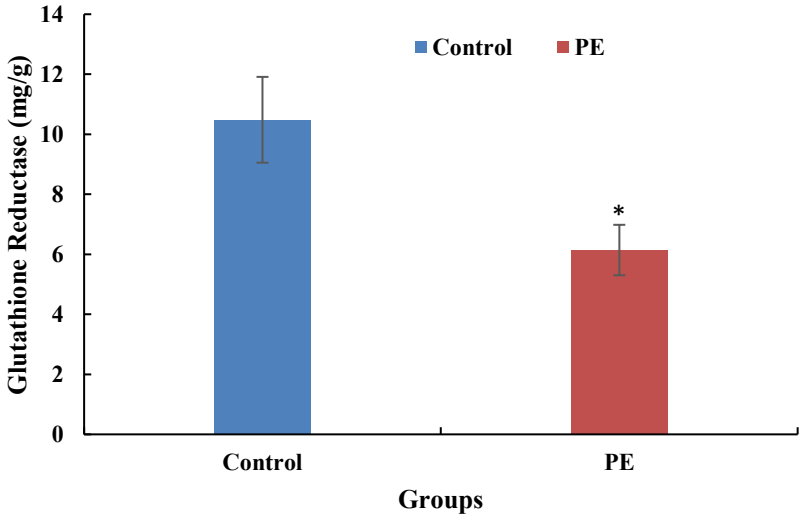


Figure 24: Mean ± SEM difference of Glutathione Reductase in the plasma of control and preeclamptic group.

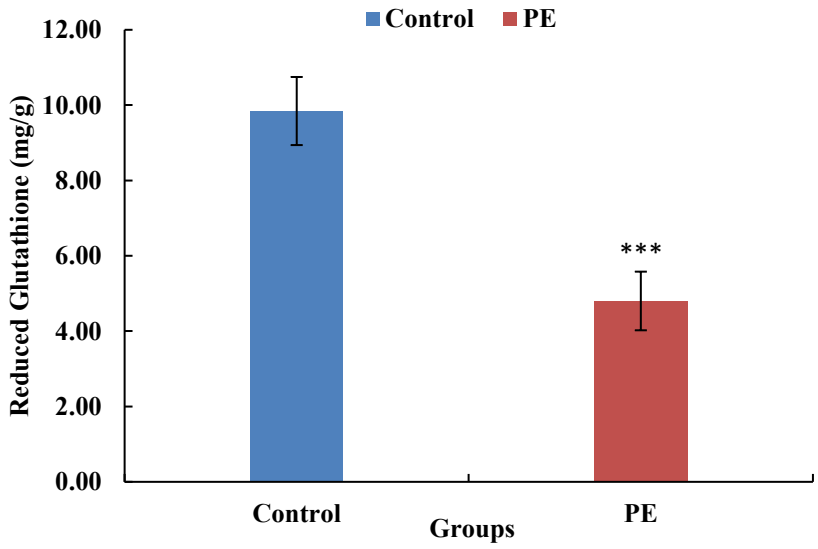


Figure 25: Mean ± SEM difference of Reduced Glutathione in the plasma of control and preeclamptic group

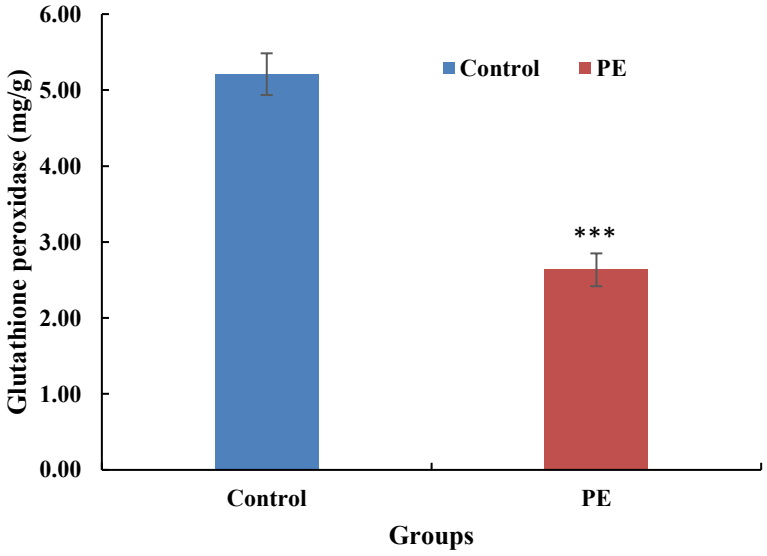


Figure 26: Mean ± SEM difference of Glutathione Reductase in the plasma of control and preeclamptic group.

Hormonal Analysis

Soluble fms like tyrosine kinase 1 levels were determined in the plasma and placental tissues of both study groups. A significant difference was observed in the circulating sFlt1 levels between the two groups. sFlt1 shows a significant increase (p=0.01) in the plasma of preeclamptic pregnant women than the normotensive controls (Fig 27). sFlt1 levels were also observed in the placental homogenates of both study groups. Placental sFlt1 levels were significantly higher (p<0.001) in PE subjects (Fig 28).

Table 6: Mean ± SEM of soluble FMS like tyrosine kinase 1 in control and preeclamptic group.

sFlt1	Control	PE	p-value
Plasma sFlt1	0.17 ± 0.04	1.42 ± 0.47	0.01
Placental sFlt1	0.22 ± 0.03	2.41 ± 0.53	<0.001

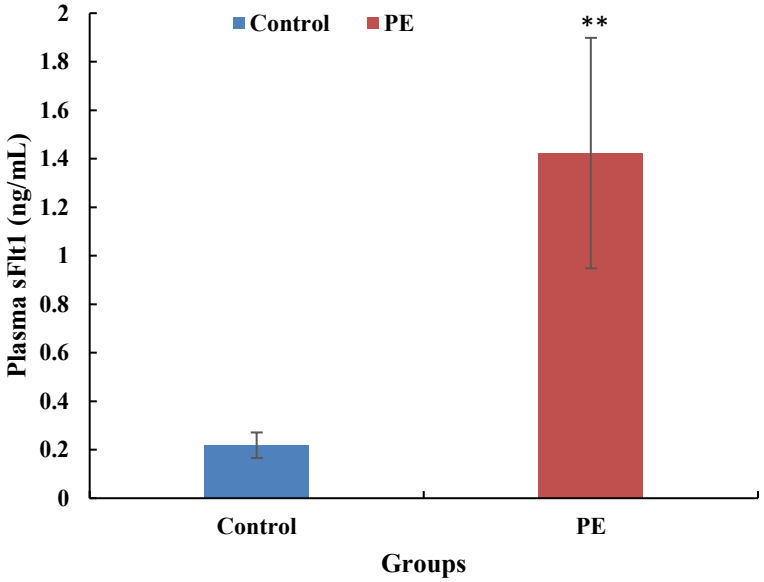


Figure 27: Mean ± SEM difference of plasma sFlt1 (ng/ml) in the control and preeclamptic group.

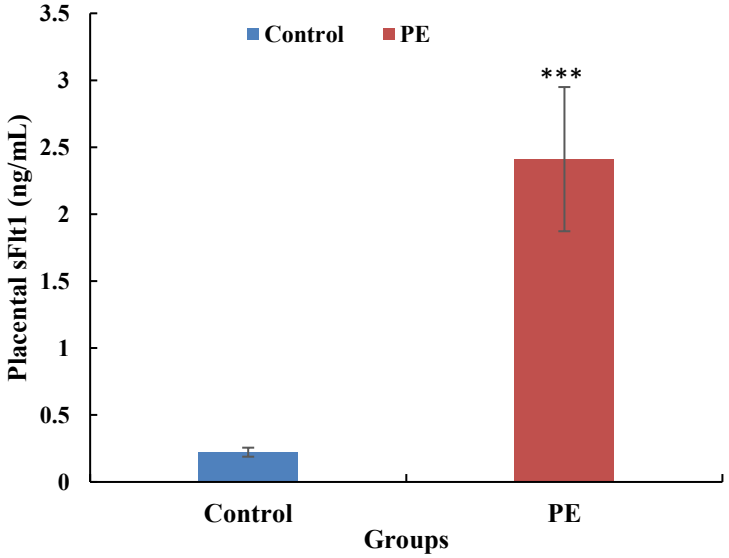


Figure 28: Mean ± SEM difference of Placental sFlt1 (ng/ml) in the control and preeclamptic group.

DISCUSSION

Hypertensive disorders are a major cause of maternal and prenatal morbidity and mortality. During pregnancy, 5-10% of women were affected by hypertensive disorders, including gestational hypertension, chronic hypertension, chronic hypertension with superimposed preeclampsia, preeclampsia, and eclampsia. The women suffering from HDP's are at the greater risk for, placental abruptions, intrauterine growth restriction, cesarean birth, and preterm birth. HDP's are associated with increased maternal and prenatal morbidity and mortality (Sutton *et al*., 2018; Madoglio *et al*., 2016). The most severe of these disorders is preeclampsia (Ying *et al*., 2018). Preeclampsia is a multisystem disorder characterized by the sudden onset of proteinuria and hypertension at the 20th week. It complicates 2-8% of pregnancies (Ives *et al*., 2020; Wilkerson & Ogunbodede*,* 2019). PE develops with vascular dysfunction and if not treated, it leads to circumstances like pulmonary edema, kidney failure, stroke, liver rupture, and eclampsia (San Juan-Reyes *et al*., 2020). PE can cause almost 70,000 maternal and 500,000 fetal deaths worldwide every year. Preeclampsia can cause 15% of maternal deaths every year (Nirupama *et al*., 2021). In the development of PE endothelial dysfunction and abnormal placentation are key events (Boeldt & Bird, 2017). PE is characterized as an imbalance in angiogenic factors and oxidative stress (Flint *et al*., 2019).

In the present study blood pressure, maternal age, BMI, and gestational age were all found to be significant predictors of preeclampsia, while there was no significant link between age at first childbirth and age at marriage and the development of PE. In the current study, SBP and DPB were significantly higher in PE than in the normotensive group. Our findings are consistent with prior findings that showed higher SBP and DPB in the PE as compared to normotensive groups (Shaheen & Almajwal, 2019; Korkes *et al*., 2017; Benian *et al*., 2004)

In the current study, clinical signs such as swelling in the hands and face, as well as urination problems, were found to be more prevalent in preeclamptic women than in control women. Previous research has found the same results, which can help in predicting severe maternal outcomes. (Alhozali *et al*., 2012; Black*,* 2007; Shaheen & Almajwal*,* 2019)

According to our findings, abdominal or epigastric pain, muscular pain, headache, excessive weight gain, and blurred vision were more common in PE individuals compared to controls, as evidenced by previous studies which can predict maternal abnormalities and disease severity (Shao *et al*., 2017; Thangaratinam *et al.,* 2011).

The PE history in families and past pregnancies predicts the relationship with the disease onset. Our current findings are consistent with the previous findings (Poon *et al*., 2010; Iii *et al*., 2007; Carr *et al.*, 2005; English *et al.,* 2015). Some epidemiological studies have indicated that PE is linked to an underlying mechanism that plays a role in the predisposition of certain genetic immunological factors in the pathophysiology of the disease. Females with a maternal history of PE are at a 2 to 5 fold increased risk of developing the disease. (Uzan *et al*., 2011; Duckitt & Harrington, 2005).

In the current study, the presence, and concentration of protein in the urine of PE individuals were considerably higher than those of control subjects. The pH of urine in PE and control subjects did not show any significant change. The current findings were consistent with the previous studies that showed 300 mg proteinuria every 24 hours (or 0.30 mg/mmol protein/creatinine ratio) in either 1+ dipstick proteinuria or 2+ dipstick proteinuria (Shaheen & Almajwal, 2019; Shaheen & Almajwal, 2020; Kurt *et al*., 2015) (Nischintha *et al*., 2014). According to recent studies, the number of urine podocytes in PE women has increased as compared to the women suffering from gestational hypertension or having a normal pregnancy. Antiangiogenic factors were found to be higher in the plasma of preeclamptic subjects while angiogenic molecules were decreased. The development of both podocyte and endothelial damage in the glomerular filtration barrier may be linked to this angiogenic imbalance in preeclampsia (Fishel Bartal *et al*., 2020).

Preeclampsia is a serious pregnancy complication. Oxidative stress (OS) is a condition associated with ischemia and preeclampsia is assumed to be linked with placental ischemia and increased markers of oxidative stress (Holland *et al.*, 2018) In the development of PE, the placenta plays an important role. Placental dysfunction occurs as a result of abnormal remodeling of the uterine spiral arteries and hypoxia in the placenta, which results in the disturbance of antioxidants levels and ROS which causes systemic maternal endothelial dysfunction and severe inflammation (Ferreira *et al.,* 2020; Kolialexi *et al*., 2015)

In the current study glutathione levels in maternal plasma and placental tissue have also been found to be significantly lowered. Glutathione peroxidase (GPx), and glutathione transferase (GST) activities were decreased significantly in the preeclamptic placentas and plasma as compared to normotensive pregnant women. Similarly, a significant reduction in the levels of reduced glutathione (GSH) was also observed in the preeclamptic placenta as compared to the normal placenta. Previous literature demonstrated the same findings

the decrease in GSH, (Rani *et al.*, 2010; Haram *et al.*, 2019) GPx (Walsh & Wang, 1993), and a decrease in GST levels (Mutlu-Türkoğlu *et al*., 1998) in plasma and placenta of PE subjects.

GPx is the principal enzymatic antioxidant in tissues, a decrease in its activity in the placenta would increase oxidative stress. In preeclamptic placentas, GPx activity, as well as GSH, were shown to be lower than in normal placentas. These findings suggest that the antioxidant system of the preeclamptic placentas is deficient. GST, on the other hand, is a multipurpose enzyme that conjugates electrophilic molecules and acts as peroxidase to break down lipid peroxides. The preeclamptic placenta may suffer from an electrophilic compound detoxification deficit, resulting in a substantial drop-in GST activity. Finally, reduced antioxidant levels in the placenta and plasma reveal the presence of oxidative stress in preeclampsia (Mutlu-Türkoğlu *et al*., 1998; Perkins, 2006).

Normal pregnancy leads to an increase in oxidative stress and lipid peroxidation, but it also increases antioxidant defense. In contrast to normal pregnancy, the increase in antioxidants in PE is insufficient to offset the increased oxidative stress. The lack of antioxidants in the placenta contributes to OS. Glutathione levels dropped dramatically. Glutathione levels in maternal plasma and placental tissue have also been found to be significantly lower. Glutathione is a major antioxidant system found within cells. Reduced glutathione is abundant in red blood cells, which account for over 98 percent of total blood content. Glutathione and other thiols regulate the redox balance of cells, limiting oxidative damage, in addition to their detoxifying role of conjugating with toxic chemicals (Benian *et al.*, 2004).

The antioxidant enzymes (GSR and GPx) showed a decrease in their activities in preeclamptic women as compared to the normal women, indicating the loss in their antioxidant capacity (Suhail *et al*., 2008). GPx deficiency is thought to be involved in the pathophysiology of PE because its decreased activity is associated with the synthesis of lipid peroxides which are increased in the PE placenta (Matsubara *et al*., 2015).

In the current study, the concentration of sFlt1 in plasma and placental tissues were significantly higher in PE subjects as compared to control subjects. Our results are in accordance with previous findings showing higher sFlt1 levels in preeclamptic than controls (Yonekura Collier *et al.*, 2019; Nikuei *et al*., 2020; Shibata *et al*., 2005).

sFlt1 secreted by the placenta may induce endothelial dysfunction and may have a major role in the pathophysiology of PE (Müller *et al*., 2019). VEGF binds to Flt1 receptors, which may serve to dimerize tyrosine kinase receptors activates signaling

pathways linked with proliferation, endothelial mitogenesis, vascular permeability, initiation of angiogenesis, vasculogenesis during normal pregnancy. sFlt1, an alternative mRNA splice variant of the FMS-like tyrosine kinase gene (Flt1) and antagonist of vascular endothelial growth factor, may play a key role in the clinical presentation of PE because of a reduction of circulating free VEGF. The production of soluble fms like tyrosine kinase 1 protein is stimulated by placental hypoxia. The presence of higher amounts of sFlt1 in placental tissues than in serum demonstrated that the placenta is the primary source of sFlt1 production. Serum sFlt1 levels can be used to monitor adverse outcomes and complications in women having PE and can also be used as an early diagnostic marker for PE in pregnancy. Previous studies demonstrated that all symptoms of PE including hypertension, proteinuria, and renal impairment, may be easily generated in pregnant mice or rats by overexpressing sFlt1 in their placentas (Bergmann *et al*., 2010; Maynard *et al.*, 2003). When sFlt1 interacts with VEGF and PLGF it inhibits their interaction with the angiogenic Flt-1 receptor and acts as an antagonist of circulating VEGF and PLGF's receptors (Helmo *et al*., 2018) Endothelial dysfunction and vasoconstriction are considered as consequences of sFlt1 (Maynard *et al*., 2005)**.** According to previous studies, sFlt-1 expression is markedly enhanced in preeclamptic placentas at the 11th week of pregnancy, and it begins to increase 5 weeks before the onset of clinical PE symptoms indicating that sFlt1 is a significant factor in the clinical manifestation of preeclampsia. Its concentration increased during the ischemic and hypoxic environment. Endothelial dysfunction is linked to elevated sFlt1 levels (Xiao *et al.,* 2018; Kita & Mitsushita, 2008). Preeclampsia causes glomerular endothelial damage, hypertension, and proteinuria by overexpressing soluble sFlt1. Higher sFlt1 concentrations are related to lower levels of free VEGF and PlGF in the blood, leading to endothelial dysfunction (Zhou *et al.*, 2010).

Anti-angiogenic factors with increased relative concentrations are thought to cause vascular endothelial cell damage in the placenta. Hypertension, proteinuria, and other systemic symptoms of the syndrome are most likely caused by endothelial dysfunction. Preeclamptic pregnancies are characterized by hypoxic and hypoperfused placentas. Hypoperfusion of the placenta leads to the placental secretion of soluble factors such as sFlt1 and sEng, both of which have been linked to maternal endothelial dysfunction (Ryu *et al*., 2008). The severity of preeclampsia was linked to circulating sFlt1 concentrations. Elevations in sFlt1 can be seen by 5 to 8 weeks before the onset of preeclampsia symptoms (Steinberg *et al*., 2009). Endothelial dysfunction was amplified by sFlt1 in

combination with sEng, resulting in more severe preeclampsia symptoms such as HELLP syndrome and cerebral edema that resembled eclampsia (Masoura *et al*., 2014; Szpera-Gozdziewicz & Breborowicz, 2014).

The underlying reason for the development of PE could be inadequate trophoblastic invasion leading to impaired spiral artery remodeling resulting in poor uteroplacental perfusion (Weel *et al*., 2016). Changes in placental perfusion can lead to the release of several placental factors, cytokines, reactive oxygen species, increased vascular resistance, increased platelet aggregation, initiation of the coagulation system, and an imbalance of angiogenic and antiangiogenic factors into the maternal blood. All these factors enter the maternal circulation and contribute to the vasoconstriction of maternal vessels, raising maternal blood pressure to improve placental perfusion. As a result, maternal systemic inflammation and endothelial dysfunction may occur (Rana *et al.*, 2019). The fetus is at risk of hypoxia and low birth weight due to inadequate vascularization (Mecacci *et al.,* 2016).

PE is a leading cause of fetal growth retardation, neonatal and maternal morbidity, and mortality, although the specific pathophysiology of the disease is unknown (Kelly, Croteau-Chonka, *et al.*, 2017). As diagnostic and prognostic biomarkers for preeclampsia are still lacking, it is a major public health concern around the world (Kelly, Giorgio, *et al*., 2017).

Conclusion:

The current study concluded that increased levels of an antiangiogenic factor, sFlt1 during PE linked with the severity of the disease as it is one of the major causes of endothelial dysfunction. Hypoxia stimulates the production of sFlt1 which in turn generates a more hypoxic environment resulting in increased OS.

In the present study decreased levels of plasma and placental antioxidants depict oxidative stress during the disease. After examining demographic, clinical, biochemical, and hormonal markers in the preeclamptic population of Pakistan, the current study indicated that placental dysfunction and altered angiogenesis are the most significant factors in preeclampsia.

Future perspectives:

The early diagnosis of the disease can help in understanding disease management. These early detection parameters can help to lower the mortality rate for both the mother and the fetus. The tests can be used as an early predictor of disease as abnormal results

right before the onset of clinical manifestations can provide information about the disease's prognosis

Regular laboratory tests and the assessment of risk factors are critical in the early detection of disease. As a result, mother and fetus health are maintained, and PE-related death rates can be reduced. Urine proteinuria was discovered to be an early diagnostic indication in PE after a urine examination. To determine the disease outcome, the subjects' medical histories and symptoms were obtained and examined.

In the current study complete and detailed medical history from the women early in their pregnancy were collected. The demonstration of clinical risk factors and assessment of clinical signs and symptoms are necessary for early diagnosis. Early diagnosis requires the identification of clinical risk factors as well as the evaluation of clinical signs and symptoms.

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<u>This is where I describe the study and let people know that their participation is voluntary and their data would be arranged anonymous and confidential.</u>

SampleNo. **Date:** _____

Name:

Contact: **Age** _____

PermanentAddress:

- ✓ **Body Mass Index (BMI):**
  - Weight _____
  - Height _____
- ✓ **Blood Pressure:** _____
- ✓ **Gestational Age:** _____
- ✓ **Last menstrual periods:** _____
- ✓ **Blood Group:** _____

MEDICATION:

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

- Inter pregnancy interval _____
- History of abortion _____
- History of stillbirth _____

REPRODUCTIVE HEALTH

- Number of visits for antenatal care? _____
- Family status and home environment _____
- The place you have given birth to a 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 a newborn 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 _____

✓ **CLINICAL 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**

_____

_____

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_____