STUDY ON EVALUATION OF OXIDATIVE STRESS INDEX, PLASMA PLACENTAL PROTEINS AND THEIR IMPLICATION ON VASCULAR ENDOTHELIAL DYSFUNCTION IN PRE-ECLAMPSIA

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For the requirements of the degree

DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY

Under

Faculty of Medicine

by

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February 2020

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I, **Ranjeeta Gadde** here by declare that this thesis entitled **"Study on evaluation of oxidative stress index, plasma placental proteins and their implication on vascular endothelial dysfunction in pre-eclampsia"** is an original research work carried out by me for the award of **Doctor of Philosophy** in the subject of Biochemistry (Faculty of Medicine) under the supervision of **Dr.C.D.Dayanand** Professor, Department of Biochemistry, Sri Devaraj Urs Medical College, A Constituent Institute of Sri Devaraj Urs Academy of Higher Education and Research, under the Co-supervision of **Dr. S.R. Sheela** Professor and Head, Department of Obstetrics and Gynecology, Sri Devaraj Urs Medical College and **Dr. Sharath Balakrishna** Associate Professor, Department of Cell Biology and Molecular Genetics, Sri Devaraj Urs Academy of Higher Education and Research. No part of this thesis has formed the basis for the award of any degree or fellowship previously elsewhere.

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ABBREVIATIONS

ACOG	American College of Obstetrics and Gynecology
ADMA	Asymmetric Di Methyl Arginine
AOPP	Advanced Oxidation Protein Products
ATP	Adenosine Triphosphate
AT1-AA	Angiotensin 1 receptor Agonistic Antibodies
APAF-1	Apoptotic protease Activating Factor-1
$AGXT_2$	Alanine Glyoxylate Amino Transferase 2
AUC	Area Under Curve
Bcl-2	B-cell Lymphoma-2
BAX	B-cell Lymphoma-2 Associated X
BAK	B-cell Lymphoma-2 Antagonist Killer
BH_4	Tetrahydrobiopterin
BH_3	Trihydrobiopterin
CASPASE	Cysteine dependent Aspartic Specific Protease
CRD	Carbohydrate Recognition Domain
CAT	Catalase
CRP	C-Reactive Protein
CD	Cluster of Differentiation
CHOP	C/EBP homologous protein
COX	Cyclooxygenase
DNA	Deoxyribo Nucleic Acid
δ-ALA	Delta Amino Levulinic acid
DBP	Diastolic Blood Pressure
DDAH	DimethylarginineDimethyl Amino Hydrolase

ELB	Erythrocyte Lysis Buffer
FASL	First Apoptosis Signal Ligand
GPx	Glutathione Peroxidase
GADD	Growth Arrest DNA Damage
HELLP	Hemolysis, Elevated Liver enzymes, Low Platelet count
HIF	Hypoxia Inducible Factor
HRP	Horse Radish Peroxidase
IUGR	Intrauterine Growth Retardation
IMA	Ischemia Modified Albumin
IRE-1	Inositol Requiring Enzyme-1
LGALS 13	Lectin Galactoside binding Soluble 13
MAP	Mean Arterial Pressure
MDA	Malondialdehyde
MAPK	Mitogen Activated Protein Kinase
NHBPEP	National High Blood Pressure Education Program
NOX	NADPH Oxidase
NOS	Nitric Oxide Synthase
NCBI	National Center for Biotechnology Information
OSI	Oxidative Stress Index
OD	Optical Density
PP13	Placental Protein 13
PCR	Polymerase Chain Reaction
PAPP-A	Pregnancy Associated Placental Protein- A
PECAM	Platelet and Endothelial Cell Adhesion Molecule
PlGF	Placental Growth Factor

PGI_2	Prostaglandin I_2
ROC	Receivers Operating Characteristic
ROS	Reactive oxygen Species
SBP	Systolic Blood Pressure
sFlt-1	Soluble fms like tyrosine kinase-1
SGA	Small for Gestational Age
SOD	Superoxide Dismutase
SNP	Single Nucleotide Polymorphism
TOS	Total Oxidant Status
TAS	Total Antioxidant Status
TXA_2	Thromboxane A_2
TAE	Tris Acetate Ethylene Diamine Tetra Acetic acid
TNF	Tumor Necrosis Factor
tPA	Tissue Plasminogen Activator
TABRS	Thio Barbituric acid Reactive Species
UPR	Unfolded Protein Response
VCAM	Vascular Cell adhesion Molecule-1
WHO	World Health Organization
XO	Xanthine Oxidase
XOR	Xanthine Oxido Reductase
XDH	Xanthine Dehydrogenase

CHAPTER-1

INTRODUCTION

1.0. INTRODUCTION

Preeclampsia is a pregnancy-specific rapidly progressive disorder having devastating effect on the mother and developing fetus and known as one of the leading cause of maternal morbidity and mortality globally. The syndrome is mainly characterized by new onset of hypertension and proteinuria after twenty weeks of gestation. Besides, the other symptoms are nausea, epigastric/right upper quadrant pain, renal insufficiency, blurred vision, persistent headache and sudden weight gain[1].

The maternal complications are hypertension, cardiovascular diseases, kidney injury, liver failure, central nervous system damage, stroke, seizures, diabetes mellitus, pulmonary edema, cardiomyopathy, coronary artery disease and death. It also contributes to fetal complications such as Intra Uterine Growth Restriction (IUGR), placental abruption, preterm delivery, neonatal respiratory distress syndrome, cerebral palsy, retinopathy of prematurity, sepsis and still birth. The children born to preeclamptic mothers are at the risk of developing stroke, impaired cognitive function, metabolic syndrome and coronary heart disease in their adult life[2].

Even though, this disease is known from a long time, the exact etiology and development of the disease is still not known. However, there exist two variants of pregnancy hypertensive complications, pre-eclampsia and eclampsia. Preeclampsia is characterized with new onset of hypertension and eclampsia with development of seizures in women with preeclampsia[3].

Francois Mauriceau was a pioneer mentioned Obstetrics as a speciality and he illustrated preeclampsia in 1637 as high risk of seizures in primigravidas. Later on preeclampsia was described by John Lever in 1843 as the presence of albumin in

urine of women. Dr. Robert John noted symptoms of headache, proteinuria, vision changes and edema in preeclamptic patients and hence these symptoms are indicating the possibility of convulsion conditions[4].

In the 20th century, considerable progress was made in the understanding of pathophysiological changes of preeclampsia development. Several research groups explained the placental physiology between preeclampsia and non-preeclampsia. Histo-pathogical examination of placental biopsies describes inadequate invasion of placental trophoblast cells into maternal spiral arteries and convert arteries from small muscular vessels into large and low resistance vessels in preeclampsia. The lack of spiral artery conversion limits arterial lumen diameter and distensibility. As a consequence, in 1960 research evidences suggested impaired placentation and perfusion in preeclampsia connected with wide spread endothelial dysfunction. Since then, researchers demonstrated major advances in preeclampsia research in an attempt to find out the basics of pathogenesis[5].

The underlying mechanisms responsible for the etio-pathogenesis of preeclampsia are complex and poorly elucidated, however several pathological and clinical studies evidenced placental abnormality that plays as a fundamental role. Such that impaired placental implantation and elevated oxidative stress at the maternal-fetal interface leads to maternal vascular inflammation and endothelial cell activation**.** Besides impaired placentation, the involvement of maternal immune, genetic, angiogenic and metabolic factors have also been implicated in the pathogenesis of the disorder[6]. Although, removal of the placenta on delivery resolves the clinical symptoms and stress but also preeclampsia can continue after delivery or postpartum.

1.1. Epidemiology

Worldwide, the incidence of Preeclampsia complicates up to 2-10% of pregnancies[7]. Every day, approximately 800 women die from pregnancy and child birth related complications around the world8. Every year, ten million women develop preeclampsia around the world and it also responsible for about 76,000 pregnant women and 5 lakhs fetal and neonatal deaths[9]. The distribution of mortality rate of preeclampsia in the developed and developing countries varies. World Health Organization (WHO) estimates the occurrence of preeclampsia to be seven times higher in developing countries than in developed countries. Whereas in India, the frequency of preeclampsia is reported to be 8-10% among the pregnant women[10]. As per the recent study conducted in Karnataka state reported 7.9% incidence of hypertensive disorders[11].

1.2.Guidelines for the Diagnosis of Preeclampsia

1.2.1. World Health Organization (WHO)

As per the 2011 report of WHO, the Criteria for the diagnosis of preeclampsia is onset of new episode of hypertension after 20 weeks of pregnancy characterized by persistent hypertension with diastolic blood pressure ≥ 90 mmHg and substantial Proteinuria ≥ 0.3 g/24hrs[12].

1.2.2. American College of Obstetricians and Gynecologists' (ACOG) Task Force

In November 2013, ACOG guidelines described that the diagnosis of preeclampsia does not require the detection of Proteinuria. With a blood pressure cut off of ≥ 140/90 mmHg at or beyond 20 weeks of pregnancy and/or thrombocytopenia, renal insufficiency, severe headache, heart-lung compromise and impaired liver function were considered. Organ problems with the kidney and liver can also occur without signs of proteinuria and so the amount of protein in urine does not predict the

progression and severity of the disease. This information was based on the report by National High Blood Pressure Education Program (NHBPEP) Working Group in Pregnancy[13].

In January 2019, the ACOG practice bulletin number 202 stated the diagnostic criteria for preeclampsia as hypertension i.e., systolic blood pressure (SBP) of 140 mm Hg or more or diastolic blood pressure (DBP) of 90 mmHg or more on two occasions at least 4 hours apart after 20 weeks of gestation in a women with a previously normal blood pressure. Along with new onset hypertension with fresh commencement of any of the conditions like thrombocytopenia, renal insufficiency, impaired liver function, pulmonary oedema, headache and visual impairment were noticed[14].

1.3. Etiopathogenesis

Placental bed is a specialized flattened organ has two components, one at fetal side as fetal placenta (Chorion frondosum) and other at maternal side as maternal placenta (Desidua basalis) that connects the developing fetus to the uterine wall through umbilical cord. It is an interface between the mother and the fetus and provides immunological tolerance, nutrient and gaseous exchange and produces hormones essential during pregnancy. Therefore, placental abnormality plays a central role in the etiology of preeclampsia process.

The cause for pathogenesis of preeclampsia is multifactorial and several mechanisms have been proposed to explain the basis of pathogenesis. Currently considered few causes for preeclampsia are placental implantation with abnormal trophoblastic invasion of uterine vessels, immunological maladaptive tolerance between maternal and fetal tissues, maternal mal-adaptation to cardiovascular or inflammatory changes

of normal pregnancy, genetic factors including inherited predisposing genes and epigenetic influences[15].

Essentially, the etiology of preeclampsia is multifactorial and complex in nature and is represented as an interlinked two staged disorder associated with abnormal placentation and maternal syndrome as shown in Figure 1. Relative placental ischemia linked to maternal inflammatory response and endothelial cell activation via release of placental factors appears to underlie the clinical features of preeclampsia.

Spiral arteries in non-pregnant women are the terminal branches of the uterine arteries that supply blood to the endometrium. They are small, muscular arteries, richly innervated and sensitive to humoral and neural signals. During normal pregnancy, between 10-20 weeks of gestation, these vessels undergo physiological changes i.e., they are invaded by mononuclear extravillous fetal cytotrophoblasts and the muscular and elastic components of the invaded segments are replaced by fibrinoid and connective tissue transforming them into flaccid and widely dilated tubes to increase the utero-placental blood flow with low resistance.

The spiral arteries remodeling take place more in the center of the placental bed and progressively reduces towards the periphery. These spiral arteries are extensively remodeled upto the inner third of the myometrium and this depth of invasion is important for successful pregnancy. The uterine blood flow is increased considerably to ensure adequate blood supply for fetal development. Whereas in pathological conditions like preeclampsia, endovascular trophoblast invasion is restricted to the peripheral decidual segments of the spiral arteries which are incompletely remodeled and retain their smooth muscle and elastic lamina. These inadequately remodeled

arteries remain tortuous, thick-walled and less dilated than the normally transformed arteries.

These arteries further remain sensitive to humoral and neuronal vasoconstrictor influences. Retention of vasoactive smooth muscle in the vessels results in intermittent perfusion leading to oxidative stress. Many predisposing risk factors like genetic, demographic and environmental factors can cause abnormalities in integrin which are transmembrane receptor involve in cellular adhesion, matrix metalloproteinases, major histocompatibility complex and cytokines leading to superficial vascular remodeling, vasoconstriction of the uteroplacental vasculature, apoptosis of the trophoblast cell and abnormal placental development which leads to reduced utero-placental perfusion pressure resulting placental ischemia[16].

This stimulates the generation of anti-angiogenic factors (sEndoglin and soluble fms like tyrosine kinase-1 (sFlt1), inflammatory cytokines, Reactive Oxygen Species (ROS), Hypoxia Inducible Factor-1 alpha (HIF-1α) and Angiotensin 1 receptor Agonistic AutoAntibodies (AT1-AA). sFlt-1 acts as a receptor for Vascular Endothelial Growth Factor (VEGF), thus decreasing VEGF bioavailability where it is implicated in placental angiogenesis, neovascularisation, maintains the homeostasis of endothelial cell and regulates the proliferation and induces the synthesis of Nitric oxide molecule.

Episodes of uteroplacental ischemia-reperfusion injury in the intervillous space triggers placental oxidative and endoplasmic reticulum stress and finally activating the apoptotic cascade leading to trophoblast cell apoptosis[17]. This causes the shedding of toxic placental debris into the maternal circulation that incites systemic inflammatory response and endothelial cell dysfunction. An imbalance of vasodilators

like Nitric oxide and Prostaglandin I_2 (PGI_2) and vasoconstrictors like Thromboxane A_2 (TXA_2) and Endothelin leads to vasospasm.

Endothelial cell dysfunction and intense vasospasm affects the vessels of the uterus, kidney, placental bed and brain leading to the clinical complications of preeclampsia. Thus, placenta plays a crucial role in the development and progression of preeclampsia[18]. Pre-existing maternal endothelial cell dysfunction aggravates during pregnancy which could also prone to preeclampsia. The pathological findings of the preeclamptic placenta are associated with fibrin deposition, infarcts, atherosis, sclerotic narrowing of arteries, leading to placental hypoperfusion and ischemia[19].

1.4. Risk factors for Preeclampsia

Pregnancy complications are associated with the following several risk factors. They are essentially primigravida, nulliparity, extremes of maternal age, multi-fetal pregnancy, multiple gestation and prior preeclampsia. The other maternal risk factors include chronic inflammatory conditions such as systemic lupus erythematosus, chronic infections and chronic hypertension. Besides, insulin resistance, pre-existing thrombophilias, obesity (pre-pregnancy BMI>30 kg/m^2), antiphospholipid antibody syndrome, renal disease, genetic susceptibility and assisted reproductive technology are also listed[20].

1.5. Oxidative stress in preeclampsia

Oxidative stress and energy metabolism takes place in the placental tissue during pregnancy. In normal pregnancy, the ROS act as signaling molecules and induces the gene expression for many cellular activities like trophoblast invasion, proliferation, angiogenesis and apoptosis in order to maintain placental homeostasis[21]. Altered homeostasis of oxidants and antioxidants at placental and maternal level play a key

role in the pathophysiology of preeclampsia. Oxidative stress at the maternal-fetal interface is induced by repetitive ischemia-reperfusion injury due to superficial trophoblast invasion and defective spiral artery remodeling in preeclampsia.

The main sources of ROS are mitochondria and endoplasmic reticulum, besides they are also produced during metabolic process with an involvement of Cyclooxygenase 2, Lipooxygenases, Xanthine oxidase (XO), NADPH oxidases (NOX), uncoupled nitric oxide synthase (NOS) and heme oxygenase system[22] .

Nitric oxide is synthesized by NOS that catalyse the oxidation of L-arginine into citrulline. When intracellular production of superoxide radical is increased, it reacts with Nitric oxide forming peroxynitrite and thus the bioavailability of Nitric oxide is low.

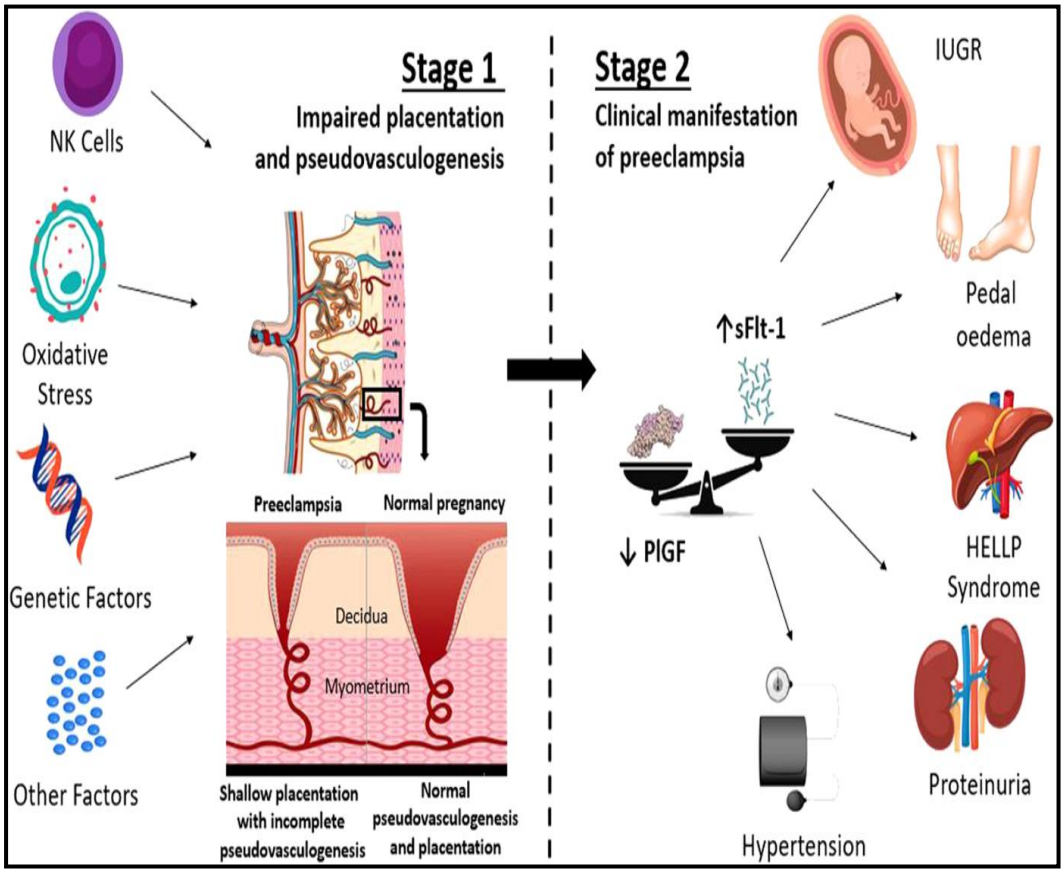


Figure 1: Stage 1 - Abnormal placentation and stage 2- Maternal syndrome in Preeclampsia

Source: Shanmugalingam R, Hennessy A, Makris A. Aspirin in the prevention of preeclampsia: the conundrum of how, who and when. J Hum Hypertens. 2019;33:1-9.

Peroxynitrite brings about nitration of lipids and proteins, damages DNA and oxidizes tetrahydrobiopterin present in its oxy–iron species essentially required for hydroxylation reaction of L-arginine during production of Nitric oxide as depicted in Figure 2. Generally, tetrahydrobiopterin (BH_4) helps in electron transfer and gets converted to trihydrobiopterin (BH_3) radical and stabilizes the dimerisation of NOS. But in the deficiency of BH_4, uncoupling of NOS generates superoxide that accentuates oxidative stress. Peroxynitrite generally acts through the p38 Mitogen Activated Protein Kinase (MAPK) pathway activating Cyclooxygenase-2 enzymes, Vascular Cell Adhesion Molecule-1 (VCAM) and release of pro-inflammatory cytokines[23].

Ischemia reperfusion injury induce XO activity and generates superoxide radical and hydrogen peroxide during catalysis of the conversion of xanthine to uric acid. It also triggers the release of cytokines and inflammatory factors like Interleukins (IL-6 & 10), Tumor Necrosis Factor-alpha (TNF-α) and C-Reactive Protein (CRP). Therefore, oxidative stress favors the redox signaling pathway to induce placental apoptosis[24].

In preeclampsia, peroxynitrite and superoxide radical stimulates vascular dysfunction by contraction of vascular smooth muscle occurring through influx of calcium intracellular stores. Accumulation of intracellular calcium induce mitochondrial permeability of the transition pore in the inner mitochondrial membrane and alters requisite inter membrane proton gradient and hence decrease ATP synthesis during oxidative phosphorylation, consequently the ionic homeostasis is lost and the cell undergoes apoptosis.

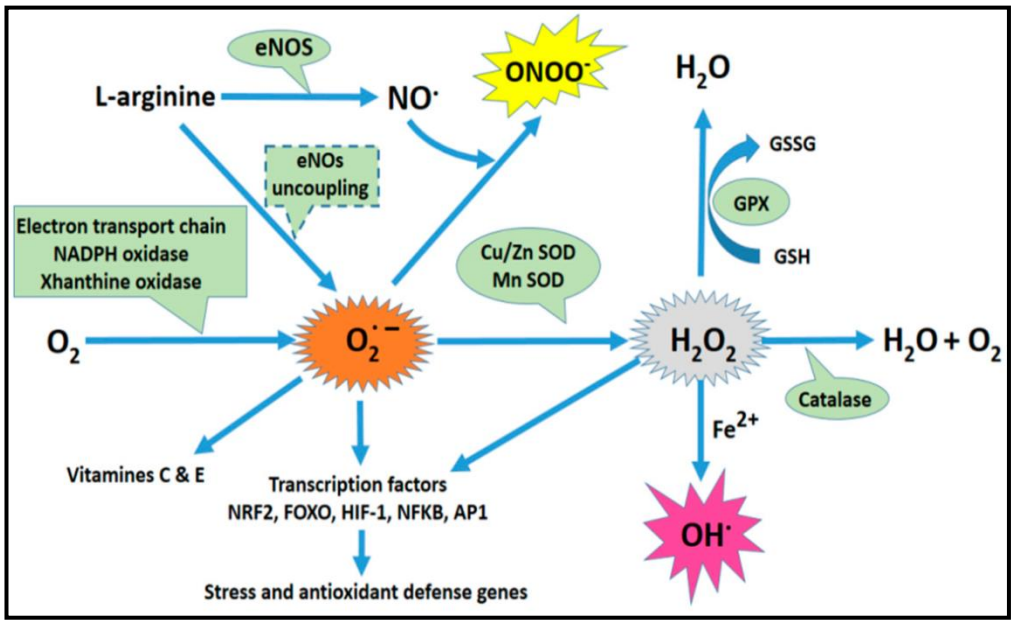


Figure 2: Schematic representation of the formation of free radicals

Source: Aouache R, Biquard L, Vaiman D, Miralles F. Oxidative Stress in Preeclampsia and Placental Diseases. Int. J. Mol. Sci. 2018;19:1496.

Similarly, HIF-1α is accumulated in the placenta due to ischemia stimulates the anti-angiogenic factor sFLT-1 and sEndoglin which are contributing to insufficient trophoblast invasion and induces angiotensin converting enzyme expression in the kidney and lungs[25].

At initial stage, the effect of hypoxia on endoplasmic reticulum results in endoplasmic reticulum stress and produce misfolded proteins, accumulation of such misfolded proteins activates unfolded protein response (UPR) to re-establish cellular homeostasis. Thus, the UPR is cytoprotective which triggers chaperones to bind to the unfolded proteins and brings about degradation of misfolded proteins. Prolonged endoplasmic reticulum stress induces apoptosis through conformational changes in the apoptotic regulator proteins like B-cell lymphoma 2-associated X (BAX) & B-cell lymphoma 2 antagonist killer (BAK) proteins. Which has direct role on outer mitochondrial membrane permeabilization and thus leading to activation of caspases through activated calcium dependent cysteine protease (Calpin) in turn cleaves inactive pro-caspases into active caspases (Caspase 12 & 9).

Further, these active casapses binds to the released mitochondrial cytochrome-c and Apoptotic Protease Activating Factor-1 (APAF-1), the combined effect activates executioner Caspase-3 that brings finally cell death[26]. In addition to the above caspase activation pathway, endoplasmic reticulum stress also triggers Inositol Requiring Enzyme 1 (IRE-1) mediated and C/EBP Homologous Protein (CHOP)/Growth Arrest DNA Damage 153 (GADD153) i.e.,(CHOP/GADD153) for cellular apoptosis in response to endoplasmic reticulum stress.

ROS also mediates mitochondrial stress and causes damage to inner mitochondrial membranes and impairs proteins and DNA function associated with the ATP synthesis. Inadequate ATP concentration affects the metabolic functions and promotes cellular apoptosis**.** The cumulative effect of endoplasmic reticulum and mitochondrial stress in response to oxidative stress in pregnancy conditions increase syncytiotrophoblast apoptosis and release of toxic placental debris (pro-inflammatory molecules and oxidative stress mediators) into the maternal circulation which provoke maternal systemic inflammation and vascular endothelial cell dysfunction[27].

1.6. Placental protein 13 (PP13)

PP13 is a glycoprotein synthesized in the syncytiotrophoblast belongs to beta-galactoside binding soluble-type galectin super-family. PP13 is one among the 56 proteins isolated from the placenta by Hans Bohn[28]. The synthesis of PP13 occurs on free ribosomes in the cytoplasm of syncytiotrophoblast and immunohistochemical studies evidenced that it is present in soluble form in the brush border apical plasma membrane of the syncytiotrophoblast. The gene LGALS13 located on the long arm of chromosome 19 at loci q13.2 encodes PP13**.**

PP13 lacks N-terminal signal peptide sequence hence it bypasses endoplasmic reticulum and Golgi bodies. So it reaches the maternal circulation by the "non-classical" secretory pathway or unconventional route either through direct translocational process or vesicular shedding in the form of microvesicles or exosomes. PP13 is highly co-localized with cytoskeletal protein actin, annexin II, placental alkaline phosphatase, a glycophosphotidyl inositol-anchored lipid raft protein and a non- raft plasma membrane protein CD71. The motor proteins, actin

cytoskeleton polymerization and with the help of lipid rafts of the syncytiotrophoblast are the driving force for the PP13 secretion[29].

PP13 is a prototype galectin with molecular weight 32 kDa consisting of 139 amino acid residues. It is a homo-dimer stabilized by disulfide bonds, having the carbohydrate content 0.6%, isoelectric point is 4.7, sedimentation co-efficient 3.1 Svedberg unit and its electrophoretic mobility is similar to albumin. It is secreted into maternal circulation from 5-6 weeks of gestation, its level gradually increase as gestational age advances and decline 4-5 weeks after delivery.

The maternal plasma PP13 concentrations depends on the trophoblastic synthesis and trophoblast mass[30]. It is reported that, the normal term placenta yields nearly 2.5mg of PP13 and makes upto approximately 7% of the total placental proteins. The primary structure of PP13 has two linear polypeptide chains with Aspargine at the amino terminal and methionine at the C-terminal. The primary structure of PP13 has 69% similarity to Galectin 10/ Human Eosinophil Charcot-Leyden crystal structure and secondary structure is similar to Galectin-7 and Galectin-10. The C-terminal carbohydrate recognition domain (CRD) is same as Galectin 3 (chimera type).

The structural alignment of PP13 consists of five stranded (F1-F5) and six stranded (S1-S6 a/S6b) β-sheets stabilized by one or two α-helices at their ends as illustrated in Fig 3A. The two subunits of PP13 are linked by disulphide bonds between four cysteine residues (Cys19, Cys92, Cys136 and Cys138) on its surface. The conserved CRD consists of 13 residues, of which eight residues (Arg53, Asn65, Trp72, Glu75, Arg55, His57, Val63 and Thr77) play important role in sugar binding. From these eight residues, four residues are similar to other galectins (Val63, Asn65, Trp72 and Glu75) and four are substituted conservatively (Arg53, Arg55, His57, Thr 77) in

PP13.The CRD is present on the concave face of S4-S5-S6 β–sheets as shown in Figure 3B.

Substrate automatic docking experimental analysis indicated binding of ligand to protein conformations. It also showed that PP13 has high affinity for N-acetyllactoseamine, lactose, mannose, N-acetyl galactosamine and Galactose. N-acetyllactoseamine has higher binding energy (-26.9kcal /mole) because of strong Van der Waals forces and stacking interactions with the aromatic ring of Tryptophan72 (Trp72) and three strong hydrogen bond interactions ($<2.5A^0$) with Arg55, Asn65 and Gln75 residues in the CRD of PP13 as illustrated in Figure 3C.

The conserved Arg53 and Arg55 in the CRD plays a key role in the interactions between PP13 and lactose moiety. Mannose has the lowest Van der Waals interaction with the PP13 but no hydrogen bond interactions are present. Mass spectrometry revealed Annexin II and beta/gamma actin are ligands for PP13 and hence these proteins play a key role in embryo implantation and placentation[31].

Computational analysis indicated sites for serine/tyrosine kinase phosphorylation at Ser48 (44-57) and Tyr41 (37-45) and Tyr80 (76-84) adjacent to CRD domain. However the carbohydrate binding affinity was same in both placental expressed phosphorylated form (PP13-B) and bacterial expressed non-phosphorylated form (PP13-R). Inadequate information is available on phosphorylation and functions of PP13 with phosphorylation[32].

PP13 display many diverse functions during pregnancy. Like other galectins, it participates in many signaling pathways and involve in regulation of biologic response like cell differentiation, cell migration and apoptosis. It binds to β and γ actin within the syncytiotrophoblast and facilitates its migration towards the placental

bed and also promotes vascular remodeling of maternal spiral arteries in early placentation by the involvement of nitric oxide or prostacyclin pathways[33]. PP13 is one of the placenta specific galectins that regulate immune responses at the maternal-fetal interface. PP13 with the help of Annexin II is secreted into the intervillous space, drains through the decidua basalis veins to form perivenous crystal like aggregates in proximity to decidual veins in the extracellular matrix. The lysophospholipase activity is thought to be implicated in this process however this mechanism needs to be elucidated.

Thus formed PP13 aggregates serve to activate, attract and bring about apoptosis of the maternal T cells to provide immune tolerance to the semi-allogeneic fetus and also to enable spiral artery remodeling. Proper transformation of the maternal vasculature is important to meet the needs of the growing fetus for oxygen and nutrients. Inadequate spiral artery remodeling is linked to immune maladaptation syndrome like preeclampsia. PP13 forms decidual crystal like aggregates within "decidual zones of necrosis"consisting of necrotic and apoptotic immune cells like CD68+ macrophages, CD45RO memory T cells and CD57+ large granular lymphocytes. The "zones of necrosis" peak at 7-8 weeks of gestation when placental circulation is still not established and lessen after the completion of spiral artery remodeling at about 10-14 weeks of gestation.

Immuno stainings reported IL-1α and IL-6 were expressed within the macrophages in the zones suggesting PP13 pro-inflammatory role[34]. PP13 purified from placenta triggers the secretion of cytokines and chemokines into the culture medium from mononuclear cells from peripheral blood of pregnant women. This in-vitro study supports PP13 with pro-inflammatory property[29]. On the other hand, truncated PP13 lacking CRD region cannot bring about T cell apoptotis and hence structural integrity

of PP13 is vital for cell-matrix interactions for successful placentation. As a result, structural derangement with altered function of PP13 results in adverse conditions during pregnancy and poor obstetric outcome[35].

PP13 is also important for differentiation and syncytialization of the villous trophoblast that is important for the release of immune proteins and placental hormones for immune tolerance and embryo development[36]. Besides, its mild Lysophospholipase A activity causes the release of Linoleic acid from the plasma-membrane contributing to implantation. PP13 with its haem-agglutination property binds to β-galactosides (Galactose, Fucose and N-acetyl galactoseamine) present on the terminal positions of ABO blood group antigens with highest affinity to AB blood group. Sugar binding assays revealed that PP13 exhibits sugar binding properties and haemagglutination activity only in its homodimer form, whereas reducing agents tend to weaken the di-merization of structure and thus alters the functional properties[32].

Animal model studies demonstrated that PP13 contributes to vasodilation and remodeling of uteroplacental vasculature and improves pregnancy outcome. However the exact molecular mechanism of vasodilation and the unknown responsive factor that regulates endothelial nitric oxide synthase and cyclooxygenase needs to be elucidated[37-39].

Further exploration to assess the therapeutic use of PP13 in humans for improving blood flow and better pregnancy outcome in hypertensive disorders of pregnancy or preeclampsia is fundamental requisite. Another in-vitro experiment, demonstrated the effect of PP13 on cultured trophoblasts and presented trophoblast membrane depolarization by calcium ions causing the release of Linoleic acid from membrane lipids that favors synthesis of prostacyclin that possess anticoagulant and vasodilatory properties[40].

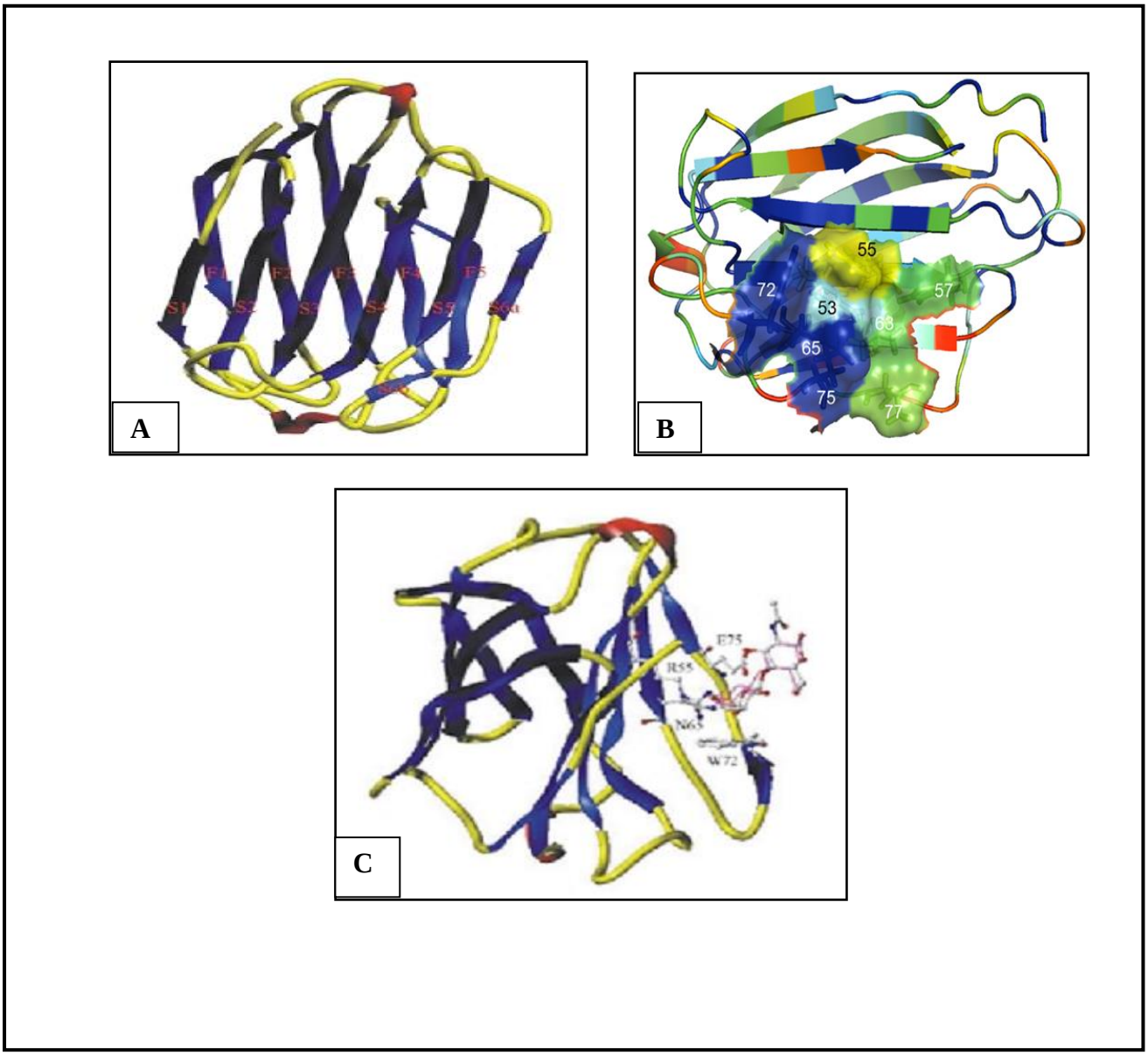


Fig 3: A) Jellyroll structure of PP13 with five stranded (F1-F5) and six stranded β- sheet (S1-S6a/S6b) linked by two α-helices at the two ends

B) The CRD of PP13 with eight amino acid residues (Arg 53, Arg 55, His 57, Thr 77, Val 63, Asn 65, Trp 72, Glu 75)

C) Crystal structure showing stacking interactions between N-acetyl Lactosamine and CRD of PP13

Source: Visegrady B, Than NG, Kilar F, Sumegi B, Bohn H. Homology modeling and molecular dynamic studies of human placental tissue protein 13 (galectin-13). Protein Eng. 2001;14(11):875-80

1.7. LECTIN GALACTOSIDE SOLUBLE 13 (LGALS13) Gene

The LGALS13 gene on chromosome 19q13.1 encoding PP13 is expressed in the syncytiotrophoblast, it comprises promoter region followed by four exons E1, E2, E3 and E4 spaced by Intronic regions with a total base pairs of 5064 as depicted in Figure 4. A part of Exon 3 and Exon 4 exclusively codes for the entire CRD[29]. The expression of the gene is down-regulated due to single nucleotide polymorphism (SNP) in promoter region, single nucleotide deletions (delT221) in exon 3 and mutations at the exon-intron boundaries (Dex-2 mutation). Impaired Lysophospholipase activity of PP13 could also be associated with intronic polymorphisms. The DNA variants leads to the formation of a truncated, misfolded, functionally altered protein that may be associated with preeclampsia by virtue of disruption of normal placentation and implantation[41,42]. The decreased measurement of mRNA level of LGALS13 provided information on its expression in first trimester of gestation period that could be associated with the pathophysiological changes in preeclampsia[43-45].

In addition to the above obvious important information few exciting information have been assembled based on the research reports with respect to importance of PP13 in consideration as early risk indicator in pregnancy complications like preeclampsia. The collective information from 2008-2018 reported measurement of PP13 as independently or in combination with Pregnancy Associated Placental Protein-A (PAPP-A), Placental Growth Factor (PlGF) and uterine artery Doppler pulsatality index[46-49]. The reported genetic incidence of preeclampsia pertaining to the screening of plasma LGALS13 gene analysis in first trimester is seldom and available within UK population in risk prediction (-98A/C) and sequence analysis of LGALS13 gene

evidenced exonic variant (221DelT) at exon 3 in South African population. Similar genetic and biochemical data availability in Indian population is limited and becomes essential hence data needs to be documented. Therefore, the present study focus is to know plasma PP13 level and its gene LGALS13 sequence in cord blood. Furthermore, the association of PP13 and other placental proteins with endothelial functions are the other parameters of interest in the study.

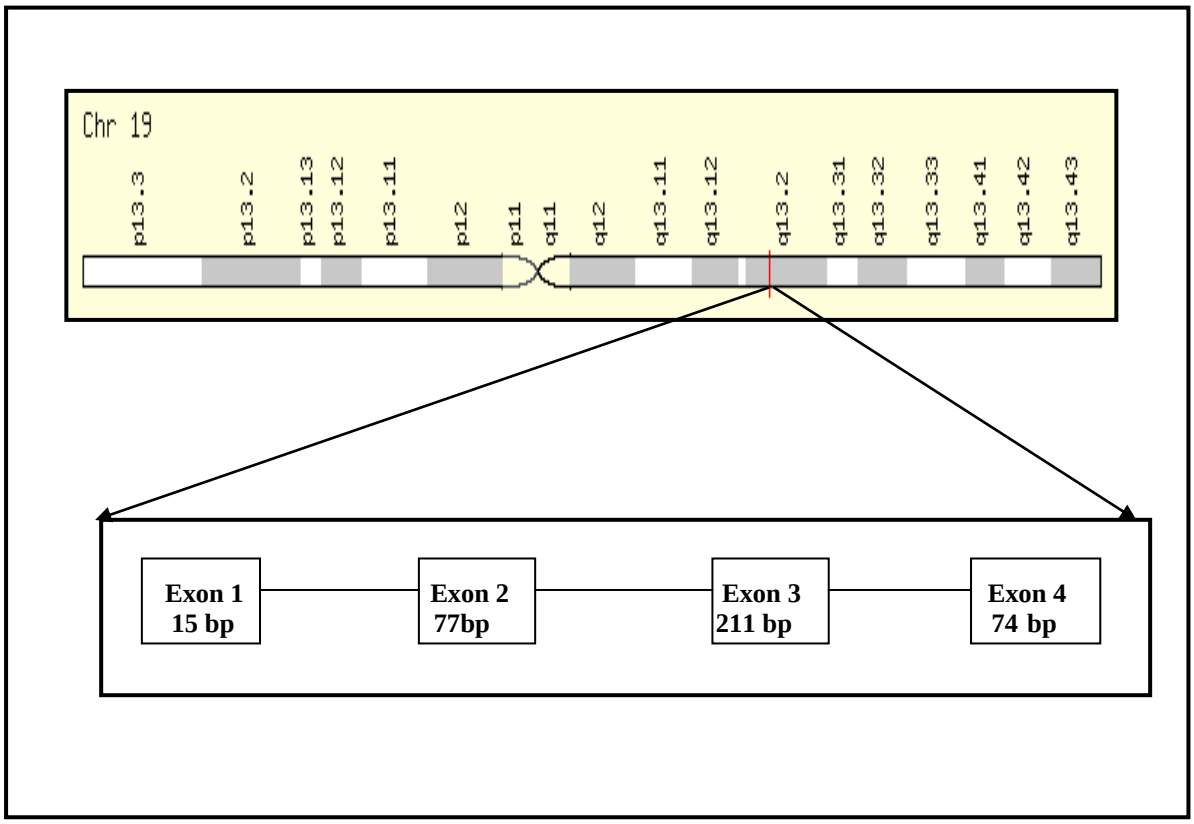


Fig 4: Location of LGALS13 gene on Chromosome 19 and structure of LGALS13 gene

Source : National Center for Biotechnology Information (NCBI)

1.8. Xanthine Oxidase (XO)

Xanthine oxido-reductase (XOR) exists in two iso-forms such as dehydrogenase and oxidase which are inter-convertible. Xanthine dehydrogenase (XDH) is a homodimer with Molecular weight 300 kDa. Each subunit consists of a single polypeptide chain with 1330 amino acids. Each subunit binds to a Flavin Adenine Dinucleotide (FAD) site, two Iron-Sulphur (Fe_2S_2) sites and molybdenum cofactor for its catalytic activity as represented in Figure 5A. Many factors such as growth factors, hormones, inflammatory cytokines and hypoxia upregulate the expression of XOR. XO is versatile in healthy tissues and is found in heart, kidney, lung, placenta and vascular endothelium[50].

Xanthine dehydrogenase (XDH) converts hypoxanthine/xanthine to uric acid by transferring electrons to NAD^+ to form NADH. During hypoxia/reperfusion injury, XDH released into circulation from the liver gets converted to XO (EC: 1.17.3.2) by either calcium mediated proteolysis or oxidation of thiol (-SH) groups of cysteine residues as shown in Figure 5B. Proteolysis of the surface exposed loops of the protein brings about conformational changes in the Flavin Adenine Dinucleotide (FAD) binding sites which pass the electrons to oxygen to form superoxide radical. XO is found in the cytoplasm of the endothelial cell and is also attached to the glycosaminoglycans on the apical surface of the endothelium. Increased breakdown of ATP in hypoxic conditions leads to accumulation of purine catabolites, adenosine and hypoxanthine. During reperfusion, XO catalyses conversion of hypoxanthine/xanthine to uric acid along with generation of ROS (superoxide radical, hydrogen peroxide and hydroxyl radical) in the endothelium, leading to endothelial dysfunction as seen in preeclampsia[51].

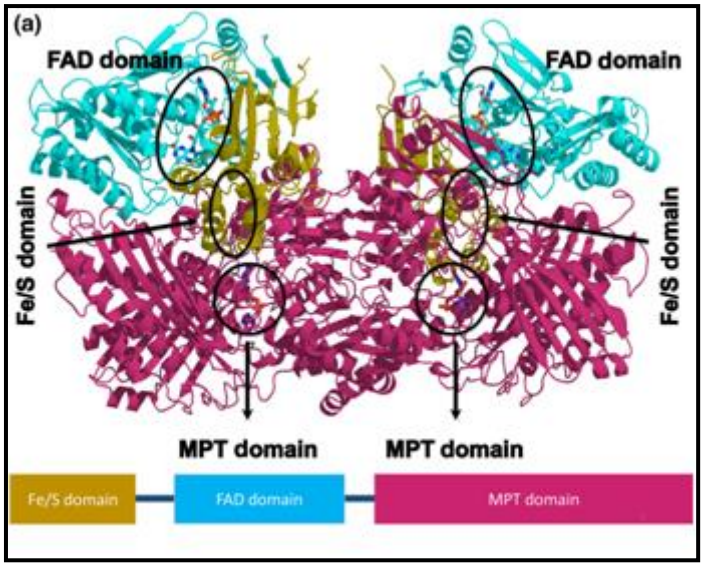
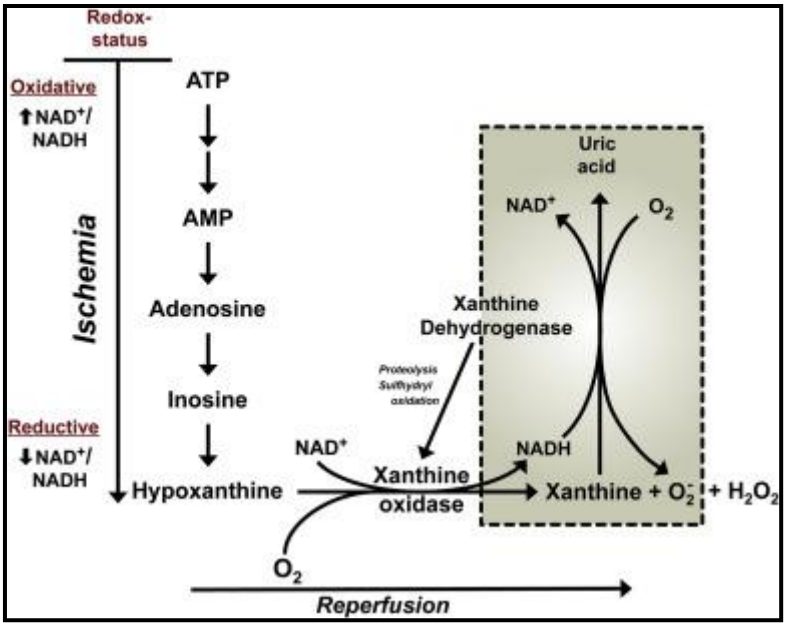


Figure 5A: Structure of Xanthine Oxidase

Source: Mehmood A, Ishaq M, Zhao L, Safdar B, Ashfaq-ur-Rehman, Munir M et al. Natural compounds with xanthine oxidase inhibitory activity: A review. Chemical Biology and Drug Design. 2018;93(4):387-418.



5B: Ischemia reperfusion pathway

Source: Granger DN, Kvietys RR. Reperfusion injury and reactive oxygen species: The evolution of a concept. Redox Biology. 2015;6:524-551

Unlike XDH, Xanthine oxidase exhibits higher affinity to oxygen than NAD^+, so the electrons generated from the conversion of hypoxanthine to xanthine are quickly accepted by oxygen and forms hydrogen peroxide and superoxide radical[52]. Very few studies reported increase of XO activity in pregnancy complications such as preeclampsia[53,54].

1.9. Cysteine dependent aspartic specific protease-3 (CASPASE-3)

Apoptosis is brought about by Caspases a family of integral protease enzymes playing essential roles in programmed cell death and inflammation. The role of Caspases in apoptosis was well characterized and established in 1993. Apoptosis is important to maintain cellular homeostasis with the degradation of cellular components in a controlled manner. There are 14 Caspases and 11 of them are present in humans. There are three type of Caspases, they are Initiator (2,8,9,10), Effector (3,6 & 7) and Inflammatory Caspases (1,4,5,12). The apoptotic cascade is stimulated by the two signaling pathways i.e., Intrinsic (mitochondrial) and Extrinsic (death receptor) pathway. Caspases are cysteine proteases attack at nucleophilic site and cleaves after the aspartate residues on the target proteins.

Caspase-3 (EC:3.4.22.56**)** is a protease formed from 32 kDa zymogen pro-Caspase-3 which is cleaved at a specific site to form p17 and p12 subunits, active heterodimer stabilized by hydrophobic bonds. The six β sheets of each heterodimer (four anti-parallel β sheets from p17 and two from p12 subunit) fuse with six β sheets of another heterodimer to form 12 stranded β sheet structure surrounded by α helices. The active enzyme is in the form of hetero tetramer. The catalytic site of the enzyme consists of Cysteine-285 and Histidine-237 responsible to cleave the aspartate residues present on the polypeptide chain[55] as illustrated in Figure 6.

In response to cellular stress, the pro-apoptotic factors like Bcl_2 induce the permeability of the outer mitochondrial membrane releasing cytochrome-c into the cytosol. The mitochondrial transmembrane potential collapses and ATP production declines. Cytochrome c forms multi protein complex apoptosome on binding to AFAP-1 and Caspase-9. This leads to activation of effector caspases (3,6 &7) for final apoptosis. In the extrinsic pathway, the ligands TNF-α, TNF-Related Apoptosis-Inducing Ligand (TRAIL) and First Apoptosis Signal Ligand (FASL) bind to death receptors TNF-α receptor, TRAIL receptor and FASL receptor on the membrane and forms death inducing signaling complex, which further cleaves pro-Caspase 8 & 10 to form Caspase 8 & 10 which inturn activates effector Caspases stimulating the destruction of the cell[56]. The two apoptotic signaling pathways are described in Figure 7.

The biochemical changes occurred in apoptotic cell is cell shrinkage, cell membrane blebbing, degeneration of nucleus, cross-linking and cleavage of proteins. Apoptosis plays a major role in all stages of pregnancy includes placental development and attachment, spiral artery transformation, trophoblast differentiation and also in immune tolerance at the feto-maternal interface[57]. In placental pathology such as apoptosis of the utero-placental tissue as seen in preeclampsia occured due to elevated hypoxia induced reperfusion injury and oxidative stress.

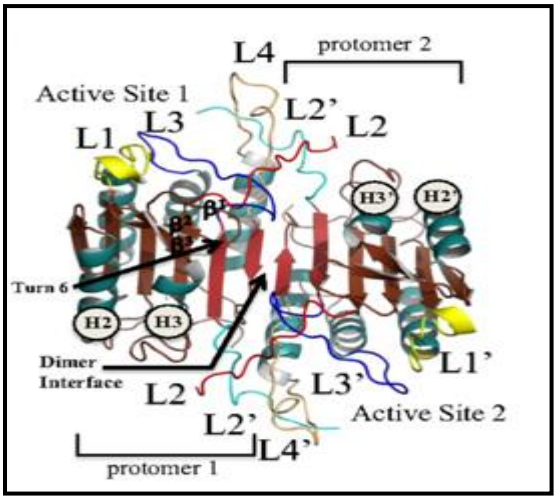


Figure 6: Structure of Casapse-3

Source: Maciag JJ, Mackenzia SH, Tucker MB, Schipper JL, Swartz P, Clark AC. Tunable allosteric library of caspase-3 identifies coupling between conserved water molecules and conformational selection. Proc Natl Acad Sci U S A. 2016;113(4):E6080-E6088.

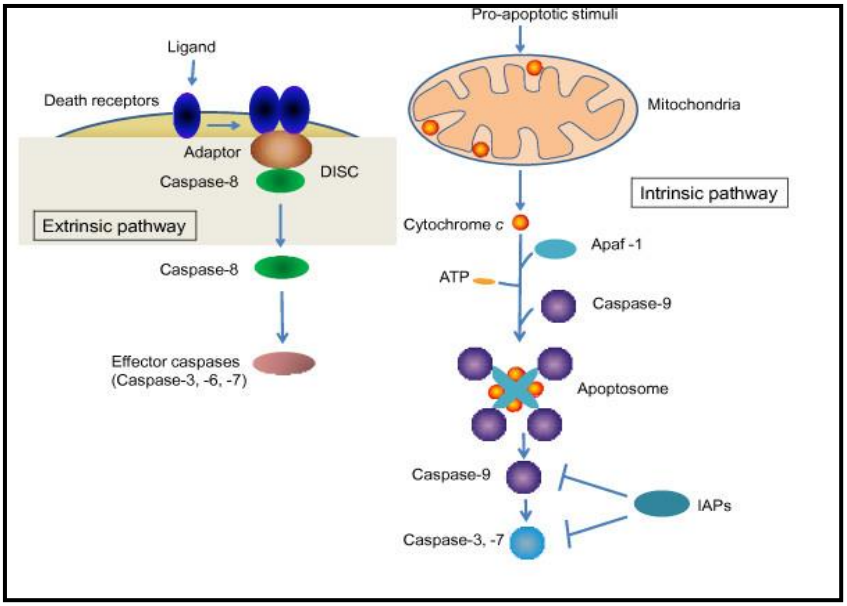


Figure 7: Cellular Apoptotic pathway

Source: Li Z, Sheng M. Caspases in synaptic plasticity. Mol Brain. 2012;5:15.

1.10. The endothelium

The endothelium is the largest endocrine organ consisting of a thin layer of a single layered squamous cells which are mesodermal in origin. The human body is made up of 1-2 trillion endothelial cells with a surface area of 400 square meters weighing 1.5 kg. It lines the interior surface of the blood and lymphatic vessels and forms a structural barrier between circulating blood or lymph in the lumen and the vessel wall.

1.10.1. Endothelial cell structure

The endothelial cells are flat, have a central nucleus with a thickness of 1-2 µm and with a diameter of 10-20 µm. These mono-nucleated cells rest on thin basal lamina separating them from surrounding tissues. The cells are flat and elongated with their long axis parallel to the direction of the blood flow in the artery. The nucleus of the endothelial cell is also elongated in the direction of blood flow. The cytoplasm consists of few organelles concentrated in the perinuclear zone. Many membrane bound vesicles and caveolae help in trans-endothelial transport of molecules Weibel-Palade bodies are rod like cytoplasmic inclusions that consists of pro-coagulant von Willebrand factor[58].

The cytoskeleton consists of microtubules and a network of actin and intermediate filaments. These organelles provide structural support and mechanism for changes in cell shape during endothelial contraction. The luminal surface of each endothelial cell is coated by negatively charged glycocalyx rich in proteoglycans and glycoproteins. Endothelial cells are linked by intercellular junctions like tight, gap, adherens and desmosomal junctions to serve as a semi permeability barrier.

Few endothelial cells are organised like the cortical web, junction associated actin filament system and striated myofibril like filament bundles or stress fibres. The cortical web provides shape and elasticity to the cells. The junction associated actin filament system is present in the intercellular space and its contraction and relaxation controls the dimension of the intercellular space. The functions of junction associated actin filament system could be altered by pro-inflammatory cytokines, thrombin, ROS, platelet activating factor, increased calcium concentration, ATP exhaustion and other toxic substances. Stress fibers play an important role in cellular contractility and also in functions such as cell adhesion, migration and morphogenesis[59].

The plasma membrane of the endothelial cell show flask shaped invaginations rich in proteins and lipids such as cholesterol and sphingolipids. They play a role in signal transduction, endocytosis, mechanoprotection, mechnosensation, oncogenesis and uptake of pathogenic bacteria and certain viruses. There are several ways of transporting plasmatic molecules through the endothelial barrier by intercellular junctions, vesicles and true transcellular channels formed from calveolae. Abnormalities in the structure and function of the endothelium play a role in the development of many diseases.

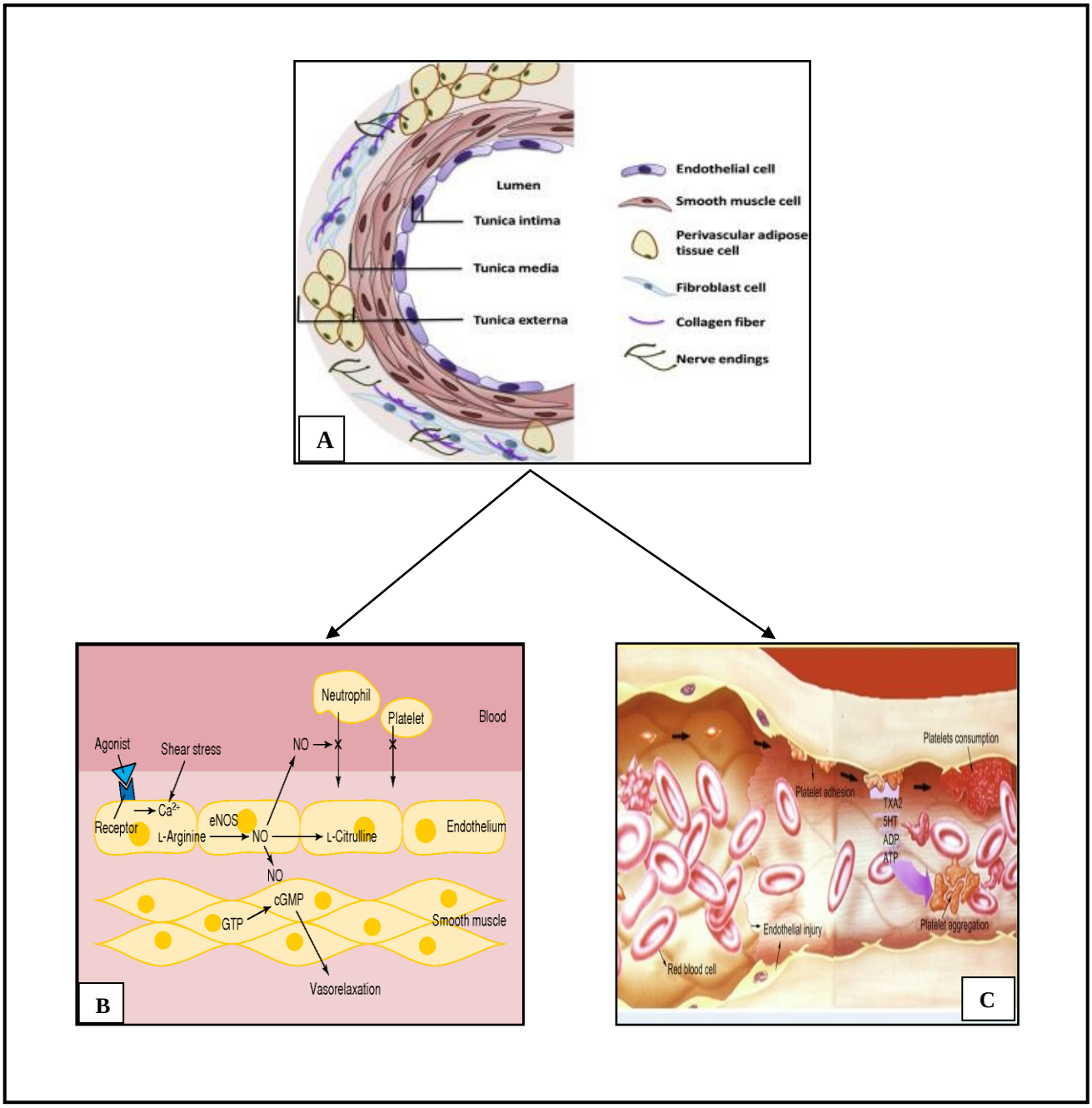


Figure 8: A) Structure of blood vessel
B) Endothelium in normal pregnancy
C) Endothelium in preeclampsia

Source: Lyall F, Greer IA. The vascular endothelium in normal pregnancy and pre-eclampsia. Reviews of Reproduction. 1996;1:107-116.

1.10.2. Functions of endothelium

The biological functions of endothelium are maintenance of extracellular matrix, transport functions, pro and antithrombotic, defense mechanism, vascular tone, regulation of cell growth and angiogenesis. Endothelium is also critical for remodeling of blood vessels and angiogenesis for tissue growth & repair. The potential ability of the endothelium is to release various chemical mediators that regulate vascular homeostasis. Factors like prostacyclin and nitric oxide acts as vasodilators and inhibits platelet and neutrophil adhesion. Besides, the enzyme Ectonucleotidases regulates vascular tone and platelet function.

Platelet activating factor activates platelets and leukocyte function, Endothelin facilitates vasoconstriction, cell adhesion molecules (VCAM-1, P-Selectin, Platelet and Endothelial Cell Adhesion Molecule-1 (PECAM) and E-Selectin) promote leukocyte adhesion and cytokines like IL 1,6 and 8 have effects on immune system. In addition, von Willebrand factor and tissue factor promotes coagulation and and plasminogen activator inhibitor inhibits fibrinolysis. Thrombomodulin, Glycosaminoglycans, Protein C and Protein S inhibits coagulation, Tissue plasminogen activator (tPA) brings Fibrinolysis.

Nitric oxide plays a crucial role in regulation of blood pressure. It is synthesized by the endothelial NOS, diffuses into the smooth muscle and mediates vasodilation through cGMP mediated process and it also inhibits neutrophil and platelet activation. Prostacyclin is synthesized from arachidonic acid by the enzymes cyclooxygenase-1 & 2 (COX). It promotes vascular smooth muscle cell relaxation through stimulation of Inositol Phosphate receptor in vascular smooth muscle cell which increases cAMP and decreases intracellular calcium resulting in vasorelaxation. Furthermore, it also

inhibits platelet aggregation, promotes angiogenesis, neovascularisation in hypoxic tissues and stimulates renin secretion. Endothelium derived hyperpolarising factor also brings about vasodilation by potassium efflux and reduction in calcium influx into the endothelial cells[60].

1.10.3. Endothelial function in normal pregnancy

During normal pregnancy, physiological and anatomical changes of the vascular endothelium in the placental, uterine and systemic vasculature are necessary to ensure proper fetal development. Maternal vasculature is adapted to an increase in blood volume and cardiac output with decrease in vascular resistance to enhance the blood flow to the utero-placental unit. Besides embryo implantation, placental vascular endothelium is essential to produce angiogenic growth factors, placental hormones like estrogen and progesterone, exchange of nutrients and gases, produces vasodilators like nitric oxide, elimination of waste materials and helps to keeps the fetus safe from harmful substances[61]. The uterine smooth muscle microvasculature, vascular smooth muscle cell hypertrophy, endothelial hyperplasia and vessel lengthening promotes increased blood flow with decreased vascular resistance to the placenta. These changes are required for adequate transport of nutrients and gases, removal of waste materials[62]. In normal pregnancy the extracellular vesicles derived from the normal turnover of the syncytiotrophoblast contains molecules that are biologically active that interact with leukocytes, platelets and endothelial cells of maternal circulation which communicate between maternal and fetal compartment. They also confer maternal immunoregulatory functions by activating innate immunity, inflammatory response, suppressing immune reactions towards the fetus and hypercoaguable state during normal pregnancy[63].

1.10.4. Endothelial dysfunction in preeclampsia

Placental vascular defects leads to placental insufficiency and IUGR in preeclampsia. All the major symptoms of preeclampsia such as hypertension, proteinuria and coagulation abnormalities are due to endothelial dysfunction. The intensity of the oxidative stress is the major contributing factor to maternal and placental vascular dysfunction in preeclampsia. Some toxic factors released from the vesicles due to enhanced apoptosis in preeclampsia induce inflammation and endothelial dysfunction[64].

Anionic phosphatidyl serine and tissue factor present on the surface of the vesicles causes hypercoaguable state in preeclampsia. Increased expression of adhesion molecules on the surface of the vesicles promotes aggregation and activation of platelets on the endothelial surface. The anti-angiogenic factor (sFlt-1) present in the vesicles stimulate endothelial dysfunction. These extracellular vesicles stimulate monocytes, neutrophils, T and B lymphocytes to generate TNF-α, and IL 1β, 6,8,12 and 18 which induce injury to endothelium causing increased vascular permeability.

The syncytiotrophoblast derived vesicles stimulate the neutrophils to produce reactive oxygen species that injures not only the placental endothelial cells but also the blood vessels of other organs such as liver, kidney and brain. Besides these extracellular vesicles also blocks endothelial NOS thus reduce the Nitric oxide synthesis. In preeclampsia, the hypercoagulable state results in fibrin deposition on the endothelium further increasing the rigidity of the blood vessel wall which induces hypertension, increased number of extracellular vesicles exaggerate inflammation[65] as depicted in Figure 9. Endothelial cell activation is defined as the pro-coagulant and pro-inflammatory state of the endothelium. Increased vascular permeability and

sensitivity to vasoconstrictors, decreased calcium signaling and vasodilator production are observed in the damaged endothelium in preeclampsia. This leads to an increase in vascular resistance contributing to hypertension in preeclampsia. The integrity of the endothelial cells is lost especially in the glomerulus (glomerular endotheliosis) and blood-brain barrier resulting in proteinuria and seizures respectively[66].

In addition, in preeclampsia hypoxia, cytokines and AT1-AA stimulate endothelial cells to produce vasoconstrictor Endothelin-1. It acts through the endothelin receptors on the vascular smooth muscle cells stimulates the release of intracellular calcium and also calcium entry through calcium channels and through Protein Kinase C pathway induces vascular smooth muscle contraction.

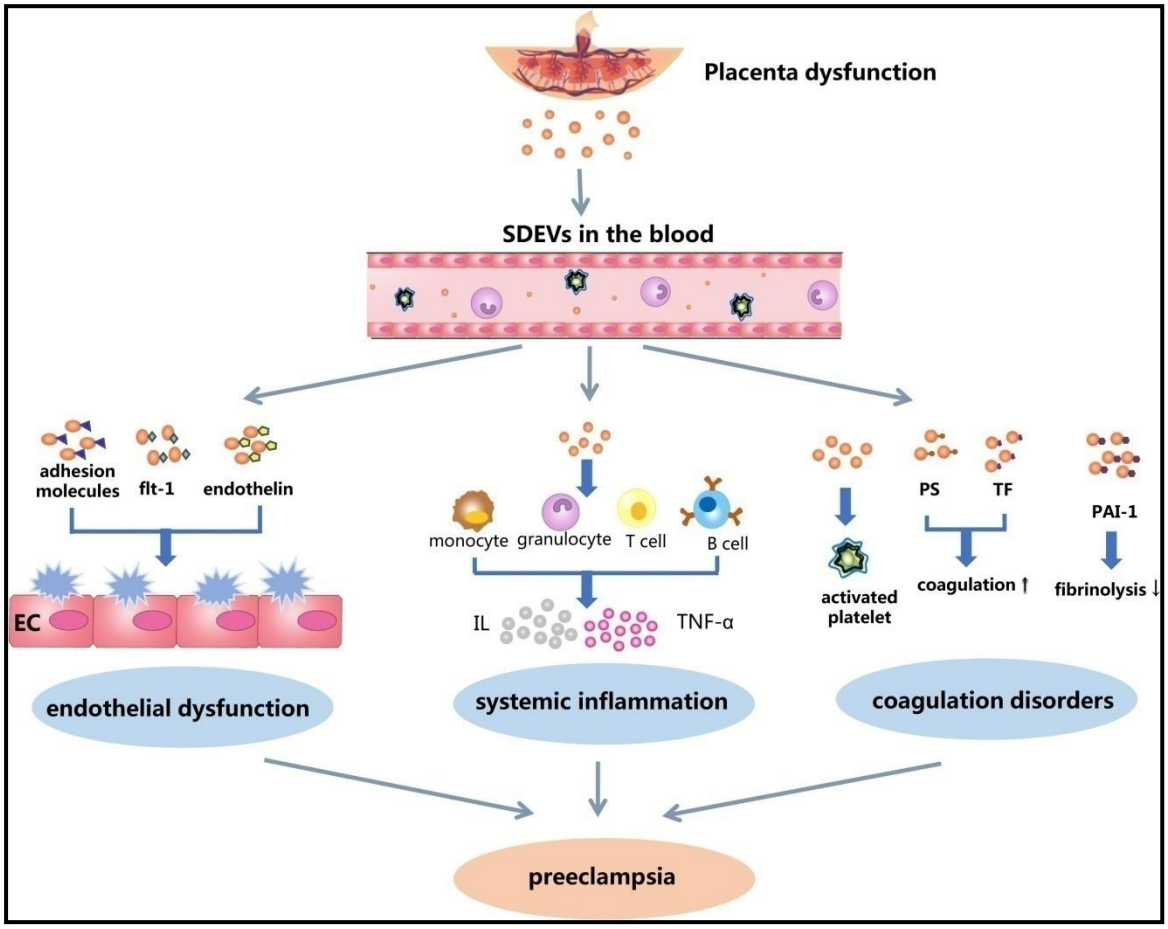


Figure 9: Schematic representation of Syncytiotrophoblast derived extracellular vesicles and their effect in preeclampsia

Source: Han C, Han L, Huang P, Chen Y, Wang Y, Xue F. Syncytiotrophoblast Derived Extracellular Vesicles in Pathophysiology of Preeclampsia. Front. Physiol. 2019;10: 1236.

Endothelin induces generation of anti-angiogenic factors, oxidants trophoblast and trophoblast apoptosis[67]. TXA_2 a prostanoid produced by COX-1 in the platelets that stimulates vascular smooth muscle cell proliferation, mitogenesis and platelet aggregation. It also causes vasoconstriction by increasing intracellular calcium and activates prostanoid receptors and protein kinases such as MAPK, Rho-kinase and Protein kinase C in vascular smooth muscle cell. Increased lipid peroxides may also increase TXA_2 production by activating COX-1 in the platelets[68]. Elevated thromboxane leads to aggregation and adherence of platelets to the damaged endothelium leading to thrombocytopenia. Inflammatory cytokines TNF-α and IL-1 produced from the damaged endothelium increase the expression of cell adhesion molecules like integrins, selectins and cadherins that mediate the adherence of neutrophils and leukocytes to the endothelium. Thus, activated neutrophils contribute the vascular damage by releasing proteases, elastase and ROS[69].

In preeclampsia neutrophils are activated in the hypoxic placenta in response to oxidative stress and inflammation. The uncontrolled neutrophil activation results in Neutrophil Extracellular Traps (NETs) which are long filaments of DNA and histones formed in the intervillous space which promotes platelet aggregation and blood coagulation. This leads to thrombus formation in the placental vasculature aggravating placental ischemia. Micro-infarctions and fibrin deposition which are common in preeclamptic placentas[70].

Hyperlipidemia is also associated with endothelial dysfunction in preeclampsia. Circulating lipid peroxide products cause increased synthesis of thromboxanes. Increased levels of oxidized low density lipoproteins accelerates the formation of atherosclerotic lesions in the placenta**.** In preeclampsia, the maternal immune

dysfunction and inflammatory reactions affect the coagulation and fibrinolytic system leading to multi-organ dysfunction and systemic metabolic disorders[71]. The inflammatory cytokines in ischemic placenta are one of the cause of endothelial dysfunction in preeclampsia. TNF-α stimulates the secretion of Endothelin-1 and cell adhesion molecules by the endothelium, destabilizes mitochondrial electron flow forming free radicals and stimulates Angiotensin II synthesis[72].

Decreased expression of endothelial NOS, increased ADMA and arginase expression are responsible for decreased Nitric oxide synthesis.The interactions between uterine natural killer cells and regulatory CD4+ T cells are crucial in accepting and recognizing fetal antigens to facilitate maternal immune tolerance in placental growth. In preeclampsia, in response to placental ischemia, CD4+ and CD8+T cells mediates oxidative stress by increasing the levels of TNF-α, IL-17, sFlt-1 and autoantibodies to Angiotensin II that induces the release of Endothelin 1 from the activated endothelium which results in hypertension and contributes to the pathophysiology of preeclampsia[73].

1.11. Nitric oxide

Nitric oxide is an endothelium derived relaxant factor and is the smallest biologically active molecule produced from its precursor, L-arginine by the enzyme Nitric oxide synthase in the presence of cofactor such as tetrahydrobiopterin (BH_4), nicotinamide adenine dinucleotide phosphate, FAD, FMN, iron protoporphyrin IX and calmodulin[74] as shown in Figure 10. The three different isoforms of NOS are endothelial, inducible and neuronal forms. Endothelial NOS is present in the caveolae on the endothelial plasma membrane and numerous mechanisms regulate its activity. BH_4 is essential for

the homodimerisation of NOS, while ROS can oxidise BH_4 to BH_3 radical leading to uncoupling of Nitric oxide synthase.

The Asymmetric dimethyl arginine (ADMA) and arginase both decrease Nitric oxide synthesis by reducing the binding of l-arginine to NOS. Nitric oxide stimulates the formation of guanosine 3',5'-cyclic monophosphate from soluble Guanyl cyclase in vascular smooth muscle cell which further activates Protein kinase A and Protein kinase G, which promotes the expulsion of intracellular calcium and uptake of cytosolic calcium by the sarcoplasmic reticulum and activation of calcium dependent potassium channels. Hence the reduced concentration of intracellular calcium inactivates calmodulin so that the myosin light chain kinase cannot phosphorylate myosin protein leading to vascular smooth muscle cell relaxation. In the same way Nitric oxide can also stimulate calcium ATPase of endoplasmic reticulum to reduce the concentration of intracellular calcium causing vascular smooth muscle cell relaxation. In hypoxic conditions, cyclic inosine monophosphate formed from soluble guanyl cyclase causes the activation of Rho-associated protein kinase that inhibits the activity of myosin light chain phosphatase which results in the process of contraction[75].

Nitric oxide inhibits the generation of superoxide radical and oxidation of low density lipoprotein -cholesterol and hence considered as an “anti-atherosclerotic molecule”. Inducible NOS is expressed at the maternal fetal interface and endothelial NOS is expressed mainly by the syncytiotriophoblast and endothelial cells plays a major role in vascular tone regulation. In the placenta, nitric oxide plays a crucial role in embryo development and implantation, trophoblast invasion, inhibits platelet aggregation in the intervillous space and promotes angiogenesis. The expression of angiogenic factors like VEGF, Angiopoietin-1 and Basic Fibroblast Growth factor are mediated

by Nitric oxide[76]. Reduced levels of Nitric oxide in the placenta leads to vasoconstriction involved in resistance and hypertension. Decreased levels of L-arginine and increased ADMA also contribute to endothelial dysfunction by decreasing Nitric oxide synthesis[77]. Till date there are conflicting results regarding the levels of Nitric oxide in preeclampsia. Some reported increased[78], decreased[79] and no change[80] in Nitric oxide levels in preeclampsia.

1.12. Asymmetric dimethylarginine (ADMA)

Protein methylation is a post translational modification catalyzed by an enzyme protein arginine methyltransferase (PRMT) which transfer the methyl group from S-adenosyl methionine. This results in the formation of methylated proteins and S-adenosyl homocysteine[81]. The cellular processes regulated by methylated proteins are RNA processing, transcriptional regulation, signal transduction and DNA repair. During protein turnover of methylated proteins, ADMA, toxic N- monomethylargine (MMA) and non-toxic Symmetric dimethyl arginine (SDMA) are formed as shown in Figure 12. Methylated proteins are present endogenously. There are two types of PRMT Type I and II. PRMT Type I are responsible for the production of N-MMA and ADMA and Type II produces SDMA as illustrated in Figure 11. The methylation of arginine residues can be blocked by peptidyl arginine deaminases.

ADMA is released out of the cell by cationic amino acid transporters that determine the distribution of ADMA between extracellular fluid and cytosol in kidney, liver and brain for degradation. ADMA is metabolized by three enzymes, Dimethylarginine Dimethyl Amino Hydrolase (DDAH) 1 & 2 and Alanine-Glyoxylate Amino Transferase 2 (AGXT2)[82]. DDAH-1 is present in tissues that express neuronal NOS and DDAH-2 is prevalent in tissues expressing endothelial NOS. DDAH catalyses

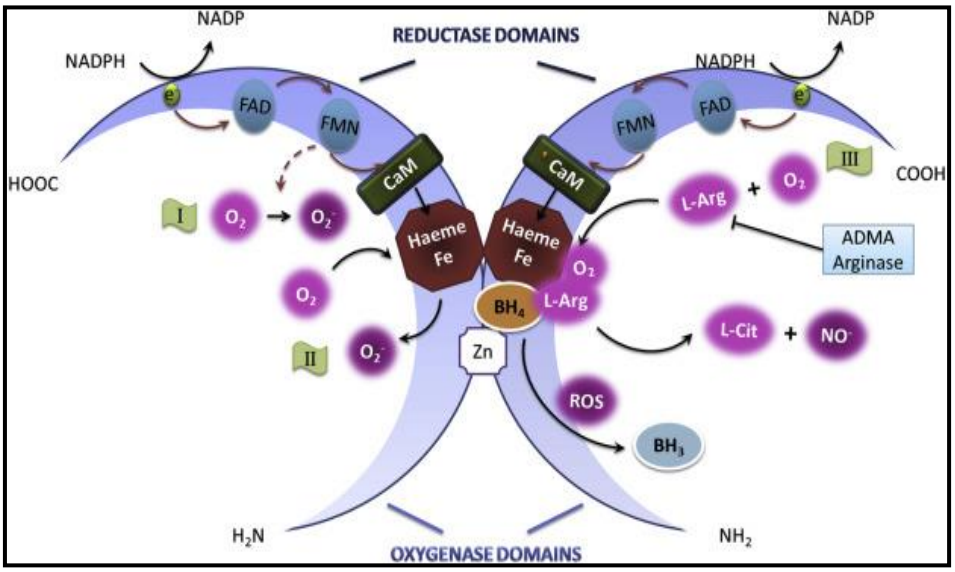


Figure 10: Showing reaction catalyzed by endothelial Nitric oxide synthase

Source: Zhao Y, Vanhoutte PM, Leung SWS. Vascular nitric oxide: Beyond eNOS. Journal of Pharmacological Sciences. 2015;129(2):83-94

ADMA to form L citrulline and dimethylamine, AGXT2 breaks down ADMA to form α-keto-δ-(NG,NG-dimethylguanidino) valeric acid. AGXT2 is an amino transferase expressed in the mitochondria present abundantly in the kidney. The levels of ADMA in the tissues is regulated by the activities of AGXT2 and DDAH. β- amino isobutyric acid is known to inhibit AGXT2. SDMA is eliminated from the circulation mainly by urinary excretion, and N-MMA in much lower concentration in circulation inhibits NOS.

Oxidative stress, TNF-α, hypertension, hypercholesterolemia, hyperhomocysteinemia, Angiotensin II, hyperglycemia and Oxidized LDL inhibit DDAH activity[83]. Under oxidative stress conditions, the Cysteine (Cys)-249 in the active site of DDAH undergoes reversible nitrosylation thereby inactivating the enzyme. Histidine (His) 162, Glutamate (Glu)-114 and Cys-249 forms a catalytic triad in the active site of DDAH. Cys-249 is responsible for the catalytic activity of the enzyme and His-162 and Glu-114 keep the cysteine in active state by forming thiolate-imidazolium ion pair.

By feedback inhibitory mechanism, increased nitric oxide causes the accumulation of ADMA by bringing about the S-nitrosylation of DDAH[84]. DDAH brings about catabolism of N-MMA and ADMA to citrulline and mono/di methylamine. Both N-MMA and ADMA inhibit the three isoforms of NOS competitively. Less information is available on the degradative pathway of ADMA by AGXT2. Approximately 20% of ADMA is excreted by kidney and 80% is metabolized by enzyme DDAH to L citrulline and dimethylamine as shown in Figure 13.

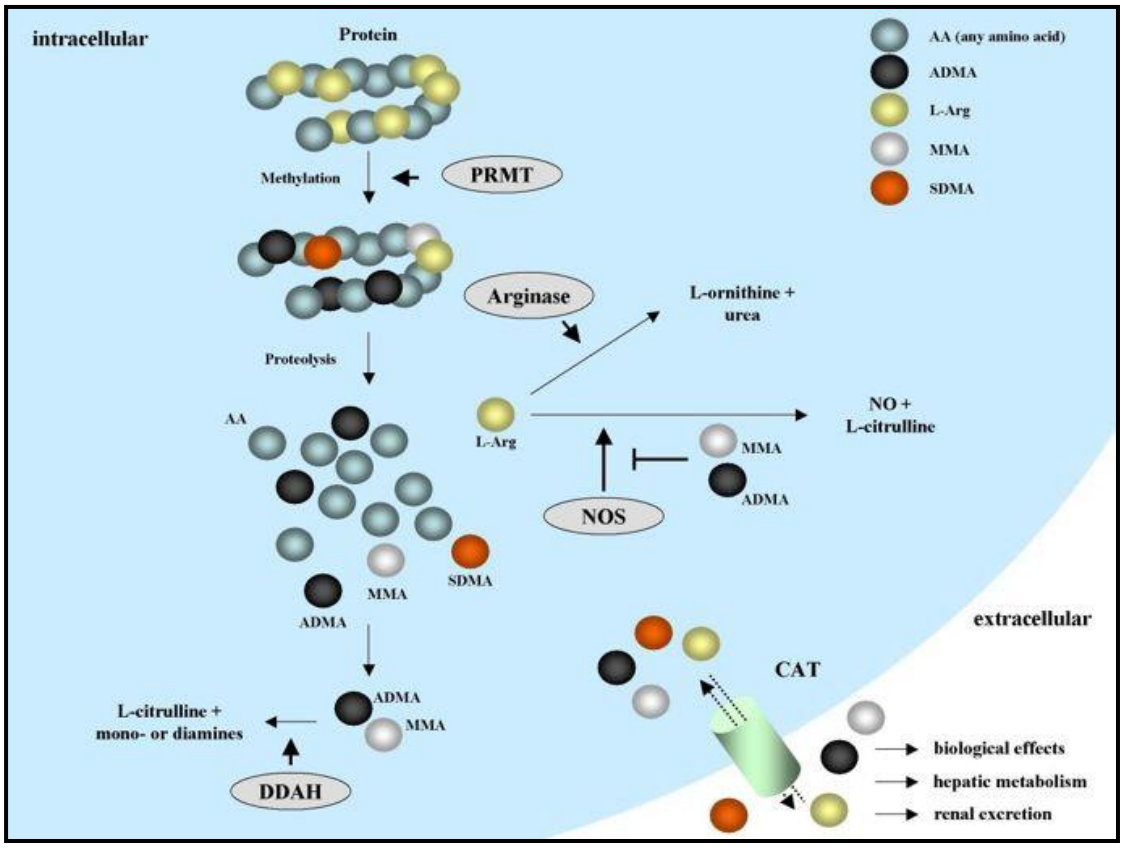


Figure 11: Showing protein methylation and formation of methyl arginines
PRMT: Protein arginine methyl transferases
ADMA: Asymmetric dimethylarginine
NMMA: N- Monomethyl arginine
SDMA: Symmetric dimethylarginine
DDAH: Dimethyl arginine dimethyl amino hydrolase

Source: Zhao Y, Vanhoutte PM, Leung SWS. Vascular nitric oxide: Beyond eNOS. Journal of Pharmacological Sciences. 2015;129(2):83-94.

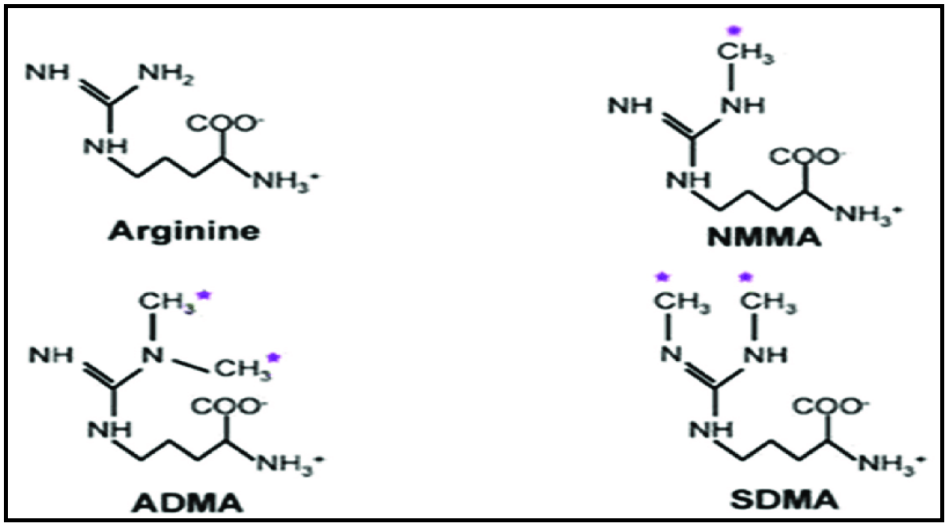


Figure 12: Structure of MMA, ADMA and SDMA.

Source : Relfold R, Robertson J, Clements C. Symmetric Dimethylarginine. Vet Clin Small Anim. 2016;46:941-960.

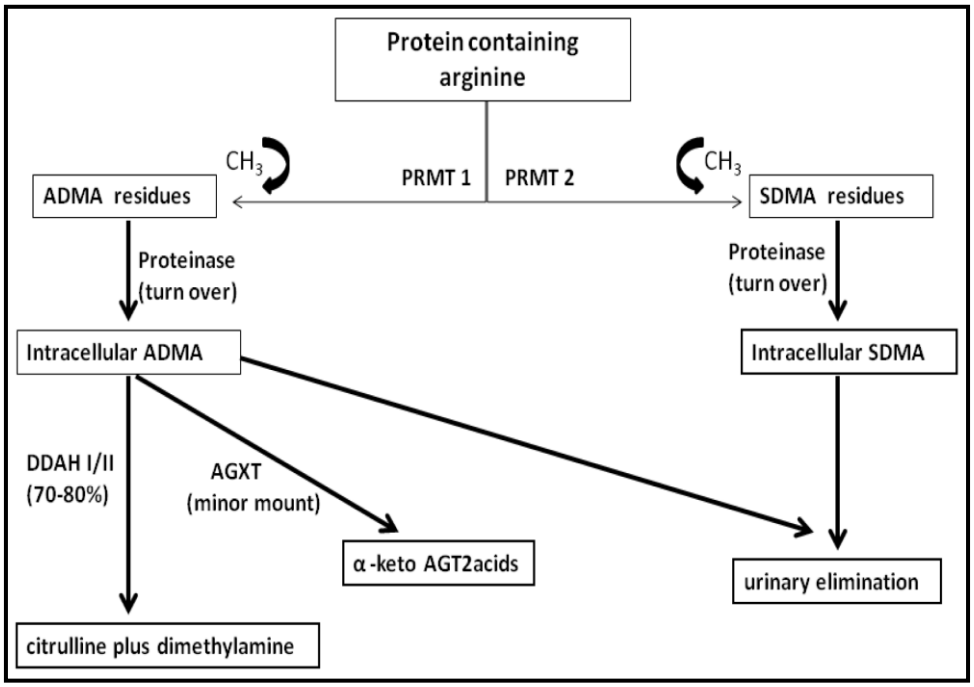


Figure 13: Pathway showing the degradation of ADMA

Source: Dimitroulas T, sandoo A, Kitas GD. Asymmetric Dimethylarginine a a Surrogate Marker of Endothelial Dysfunction and Cardiovascular Risk in Patients with Systemic Rheumatoid Diseases. International Journal of Molecular Sciences. 2012; 13(10): 12315-335

In normal pregnancy, the levels of ADMA increases as pregnancy advances. Increased ADMA levels in the third trimester increases the contractile activity of uterine muscle fibres before labor to antagonize the effects of Nitric oxide to promote successful delivery. The placenta expresses DDAH-2 subtype which is responsible for the breakdown of ADMA. In preeclampsia, DDAH-2 is sensitive to oxidative stress[85].

ADMA is a competitive inhibitor of L- arginine for NOS thereby decreases the synthesis of Nitric oxide. Besides, it induces endothelial NOS uncoupling, transport of electrons are impaired within the catalytic domains of endothelial NOS, oxygen accepts the electrons instead of arginine to produce superoxide, which further reacts with Nitric oxide to produce peroxynitrites. The latter further promotes NOS uncoupling either by inactivating the cofactor BH_4 or by releasing Zinc from the enzyme[86-88]. ADMA is reported to have the function of decreasing the concentrations of cGMP, reduction in renal plasma flow and glomerular filtration rate (GFR) at pathophysiological conditions.

Studies have also reported the role of ADMA in the pathophysiology of vascular dysfunction related to cardiovascular diseases. Elevated levels of ADMA were associated with the thickness of carotid artery intima, predicts the progression of intimal thickness during follow-up and is also associated with 4 fold risk for acute coronary syndrome and thus can be considered as an independent risk factor for cardiovascular disorders[89]. ADMA inhibits the cellular uptake of L-arginine by cationic amino acid transporter system thus decreasing Nitric oxide synthesis. In hypercholesterolaemia ADMA is also known to exhibit anti-angiogenic effects induces vascular lesions and increases the activity of Angiotensin converting enzyme[90]. Besides ADMA was also found to be elevated in severe Liver failure,

Renal disorders, Hyperthyroidism, Insulin resistance, Metabolic syndrome, Preeclampsia and Neuro degenerative disorders.

1.13. Lacunae of the knowledge

Preeclampsia is a disease of placental origin has become challenging to health care system. This leading disorder has more impact on maternal and perinatal morbidity and mortality especially in the developing countries. The early understanding of preeclampsia is much needed and important goal in obstetrics for its management. Preeclampsia usually breaks out in the second trimester but the underlying pathological processes in the placenta develop very early. Thus early detection of preeclampsia risk during pregnancy can provide a means to prevent the disease or reduce the severity of the symptoms. Therefore, identification of early indicators for detection, understanding the mechanism of the disorder and management of preeclampsia is crucial. Hence large prospective studies are required to identify new markers with good predictive value to understand the aetiology of preeclampsia.

Oxidative stress index (OSI) represents the ratio of total oxidant status (TOS) and total antioxidant status (TAS). Information on oxidants and antioxidants are available in terms of oxidative stress separately or in combination in several research reports. This study focus on TOS and TAS represented as OSI during normal pregnancy and pregnancy complications through all trimesters and its influence on placenta.

PP13 was studied internationally as single or in association with other markers like Pregnancy associated placental protein-A (PAPP-A), Placental growth factor (PlGF) and uterine artery Doppler pulsitality index for risk assessment at early stage.

As per the international research reports, decreased PP13 level in first trimester pregnancy and then after consistently increased PP13 level was reported and it is

unclear from these reports about the reason for low PP13 level in first trimester. Addressing the exact reason for low PP13 levels is challenging. Besides, obtaining baseline data of PP13 level in normal pregnancy and in pregnancy complication in our population and also to find any association with endothelial dysfunction is an essential requirement.

The data on plasma PP13 level in Indian women during pregnancy and in preeclampsia is inadequate and the frequency of any genetic polymorphism in PP13 (LGALS13) gene in South Indian population is not known. Hence, this research gap has become the need for the study.

XO activity during pregnancy trimesters in longitudinal study was not documented. Hence, assay of XO activity in first and second trimesters of pregnancy in preeclampsia and normotensive pregnant women is essential.

Caspase-3 is a terminal enzyme in cellular apoptotic pathway. Measurement of Caspase-3 in circulation indicate apoptosis of the placental trophoblast destruction in response to oxidative stress environment. Since no data is able regarding plasma Caspase-3 levels in pregnancy condition. Therefore it is desirable to explore the possibility of Caspase-3 in normal pregnancy and pregnancy complications to establish as a marker for early assessment of preeclampsia.

ADMA regulates NOS by inhibitory action and controls synthesis of the Nitric oxide in the endothelium. Studies have reported elevated ADMA and decreased Nitric oxide levels under oxidative stress conditions in pregnancy and its complications. However the data available on ADMA:Nitric oxide ratio during pregnancy is requisite and also to find an association of PP13 with respect to Nitric oxide and ADMA synthesis becomes essential.

Therefore, current research is prioritized to find out the effect of oxidative stress on placental cellular integrity measured in terms of PP13 level, XO activity and Caspase-3 levels and their possible association with endothelial function in first and second trimesters with genetic screening of LGALS-13 gene sequence from cord blood to know the frequency of polymorphism has become the newer aspect of the study.

CHAPTER-2

REVIEW OF LITERATURE

2.0. REVIEW OF LITERATURE

The following are the recent literature reviewed in respect of the study on oxidative stress, placental proteins, endothelial function and LGALS13 gene.

2.1. OXIDATIVE STRESS

In 2016, Ostaz E et al conducted a case control study in Turkey on determination of maternal serum TAS, TOS and OSI along with paraoxonase (PON) and arylesterasein severe preeclampsia and preeclampsia related perinatal morbidity.The results showed significant increase in oxidative stress markers along with TAS in preeclampsia compared to the healthy pregnant group. However, OSI did not show and significant difference between the two groups. In preeclampsia, Oxidative stress markers were positively correlated with adverse perinatal outcomes and a significant negative correlation was observed between serum TAS levels and gestational weeks and also between PON and birth weight. This study concluded that elevated oxidative stress is associated with severe preeclampsia and positively correlated with perinatal outcomes. Limitation of the study is small sample size of the groups[91].

In 2016, De Lucca and co-workers conducted a case control study in Santa Maria to investigate the levels of Delta-aminolevulinate (δ-ALA) dehydratase activity and oxidative stress markers in mild and severe preeclampsia. Thiobarbituric acid – reactive species (TABRS) was significantly higher and thiol groups, levels of vitamin C, catalase and δ-ALA dehydratase activity were significantly lower in preeclampsia compared with healthy women. In oxidative stress conditions, the sulfhydryl groups in δ-ALA dehydratase is inhibited leading to accumulation of the substrate 5-ALA which generates excessive ROS with depletion of antioxidant levels.

The study concluded iron supplementation may promote a protective effect against oxidative damage produced during pregnancy. However, the iron levels were not measured and studies in a larger cohort should be carried to confirm these findings[27].

Draganovic D and co-workers in 2016 conducted a case-control study to determine the levels of oxidative stress markers like thiobarbituric acid reactive substance to analyze correlation with ultrasound and cardiotocography parameters in women with pregnancy induced hypertension especially preeclampsia. The study results showed statistically significant high values of TABRS and also strong association between TABRS levels and ultrasound (amount of amniotic fluid and fetus biophysical profile) and cardiotocography recording. This study concluded TABRS levels could be used as risk assessment of pregnant women with pregnancy induced hypertension (PIH), however further research is required on larger population[92].

Ferguson KK and co-workers in 2017 conducted a longitudinal study in USA on oxidative stress and inflammation biomarkers in preeclamptic and normotensive pregnancies. Oxidative stress and inflammatory markers were analyzed in plasma samples at 10, 18, 26 and 35th weeks of pregnancy. The hazard ratios in preeclampsia were significantly elevated in association with oxidative and inflammation biomarkers in second trimester. The study concluded that this data can be useful in understanding the maternal inflammatory profile in pregnancy in early second trimester so that preventive measures can be undertaken. The limitation of the study was small sample size and confounders such as diet were not included [93].

D'Souza JMP and co-workers in 2017 conducted a case-control study in India to evaluate ischemia modified albumin (IMA) and advanced oxidation protein products (AOPP) in different hypertensive disorders in pregnancy and investigated their

association with antioxidant parameters such as albumin, total antioxidant activity and total thiols. The study results showed a significant increase in advanced oxidation protein products and IMA and also a significant decrease antioxidant levels in every hypertensive disorder of pregnancy compared with controls. A significant positive correlation was observed between IMA and AOPP and a significant negative correlation was observed between protein oxidation and antioxidant status in all hypertensive disorders of pregnancy. These results evidenced imbalance between pro-oxidants and antioxidants as an underlying factor in the pathogenesis of the pregnancy hypertension. The limitation of the study is the parameters could have been measured in the placenta and cord blood which could probably give an indication on the origin of oxidative stress[94].

Al- Kuraishy HM and co-workers in 2018 conducted a cross sectional study in Iraq to investigate serum levels of free radicals and also endothelial dysfunction in preeclampsia. Preeclamptic women showed significant increase in the levels of peroxynitrite and Malondialdehyde (MDA) and significant decrease in paraoxonase and Nitric oxide levels. These observations evidenced that oxidative stress plays a significant role in the pathogenesis of preeclampsia and could also induce endothelial dysfunction. The main limitation of the study is small sample size, cross-sectional design and no placental hormones were measured to study the placental function. Longitudinal studies should be carried out to assess the effect of anti-hypertensive agents on oxidative stress during preeclampsia[95].

Mannaerts D and co-workers in 2018 conducted a longitudinal study in Belgium to elucidate the correlation between endothelial dysfunction and vascular stiffness in preeclampsia with oxidative stress and inflammatory markers. Levels of superoxide,

systemic inflammation and iron status are much higher in preeclampsia compared to healthy pregnancy. In preeclampsia superoxide was correlated to micro-vascular endothelial dysfunction. These results support the hypothesis that vascular dysfunction is linked to oxidative stress and could be used in the prediction of preeclampsia. The limitations of the study were a significant difference was observed in the parity between the groups which could influence the results, small sample size and iron supplementation was not recorded[96].

Ekun OA and co-workers in 2018 conducted a cross-sectional study in Nigeria to evaluate the interrelationship between oxidative stress, hematological and inflammatory markers in preeclamptic women. Levels of Superoxide dismutase (SOD), Catalase (CAT) and Glutathione were reduced and increased levels of MDA, Cardiac-Specific Troponin I, Prothrombin Time and Activated Partial ThromboplastinTime were observed in preeclampsia. Preeclampsia is associated with elevated oxidative stress along with imbalance in hematological and coagulation homeostasis which may have deleterious effects on the cardiovascular system[97].

Chiarello DI and co-workers in 2018 in their comprehensive analysis reviewed the current literature on the role of oxidative stress in pathophysiology of normal pregnancy and in preeclampsia. In preeclampsia, oxidative stress results from abnormal placentation, intermittent blood flow to intervillous space and placental ischemia-reperfusion leading to enhanced inflammatory response and endothelial dysfunction. The review also addressed that more clinical trials should be carried out aiming to prevent preeclampsia with antioxidant supplementation[98].

Barneo-Caragol and co-workers in 2019 conducted an observational study to evaluate serum strontium levels and oxidative stress markers and compare it with other

complications like IUGR and gestational hypertension. The mean strontium, sFlt-1/PlGF, uric acid, lipid peroxidation/ total antioxidant activity were significantly higher and estimated glomerular filtration rate and total antioxidant activity were significantly lower in early preeclampsia compared with other complications. A significant positive correlation was observed between strontium with estimated glomerular filtration rate and sFlt-1/PlGF, a significant negative correlation was observed between total antioxidant activity with gestational week of sampling and uric acid. These observations hypothesized that elevated oxidative stress markers like uric acid and lipid peroxides and lowered total antioxidant activity suggested the significance of oxidative stress in preeclampsia. The study is limited in being an observational study and future research is required to elucidate the pathophysiological mechanisms by which strontium and oxidative stress are involved in the disease[99].

Bhat PV and co-workers in 2019 conducted a case-control study in India to evaluate the effects of metabolic syndrome components and oxidative stress factors among preeclamptic women. The results showed abnormal lipid metabolism, raised lipid peroxide concentrations and increased antioxidant status which may result in oxidative stress and vascular dysfunction in preeclampsia. The study also hypothesized that metabolic syndrome components play an important role in pregnancy hypertension. The study is limited to small geographical area, the role of raised lipoproteins in preeclampsia needs to be explored and future studies with uniform methodologies must be carried out in larger number of patients at varying gestational ages[100].

In 2019, Ahmad IM et al conducted a prospective longitudinal study in USA to measure oxidative stress in early week of gestation (12-20 weeks) and to identify

women who are at risk for developing preeclampsia. Oxidative stress was measured in terms of measuring the levels of superoxide, SOD, CAT, oxidized and reduced glutathione. The study results showed decreased levels of antioxidant enzymes and increased superoxide. CAT was associated significantly with the reduced preeclampsia risk and could predict preeclampsia. Besides it can also be used as a therapeutic agent in prevention trials for preeclampsia. Based on these results, it is known that oxidative stress plays a key role preeclampsia pathogenesis[101].

Haram K et al in 2019 conducted a case control study in Norway to determine the levels of adhesion molecules, oxidative stress ,markers and antioxidant levels in the etiopathogenesis of preeclampsia. This study reported low levels of serum antioxidants, such as Glutathione peroxidase (GPx), SOD, CAT, Lycopene, Coenzyme A and other non-enzymatic antioxidants. Besides increase in serum oxidative stress markers like thromboxanes, MDA and F2 isoprostanes the expression of adhesion molecules is increased in the placentas of preeclampsia. The study concludes that oxidative stress in the placenta is an important feature in preeclampsia[102].

2.2. PLACENTAL PROTEIN 13

Moslemi Zadeh et al in 2012 conducted a prospective nested case-control study in Iran to study the predictive values of biochemical markers like PP13 and PAPP-A at 11-13 and 24-28 weeks of pregnancy in early diagnosis of preeclampsia. Serum PP13 and PAPP-A levels were significantly lowered in women who developed preeclampsia. Since both these placental proteins play a key role in placentation it is advantageous to measure their concentrations in first and second trimesters of pregnancy. The study concluded that the declined levels of these markers in first

trimester could be good predictive markers for preeclampsia. The limitation of the study is the reason for the reduced levels of PP13 from the placenta was not reasoned[47].

In 2014 Ceylan N et al conducted a prospective case-control study in Turkey to assess if first trimester PP13 and PAPP-Acould predict preeclampsia. The serum levels of PAPP-Awas lower in early and late preeclampsia when compared to the normal pregnancies, however no significant differences in the levels of PP13 were observed between the three groups. The predictive capacity of PAPP-A and PP13 was assessed by plotting Receivers operating characteristic (ROC) curve. The area under curve (AUC) for PAPP-A was 0.751 and for PP13 was 0.543. Odds ratio (OR) showed the risk for the development of Preeclampsia was decreased as the concentrations of PAPP-A increased however no significant differences were observed in the comparison of risk between the three groups. This study concluded that neither PAPP-A nor PP13 can be used as reliable screening markers for preeclampsia in first trimester. The limitation of the study is small sample size[103].

In 2014, Meiri H et al conducted a prospective case-control study to determine whether preeclampsia can be predicted by PP13, mean arterial pressure (MAP) and risk factors such as history of preeclampsia, contraception by assisted fertility , BMI >35 or maternal age > 40. The detection rate of preeclampsia was 80% in PP13 alone with or 14.6, 55% with only risk factors with OR 14.6, 85% with PP13 and risk factors with OR 32.1 and 93% with PP13+MAP+risk factors with OR 119.6. The study concluded the predictive capacity of preeclampsia is increased by combining PP13 with MAP and risk factors with an Area under curve (AUC) of 0.93. Benefit of

using aspirin in the prevention of preeclampsia when the risk was due to low PP13 alone[104].

In 2014, Sammar M et al conducted an animal model experiment to demonstrate the role of full length PP13 (wild type) and truncated variant ($DelT_{221}$ PP13 variant) in pregnant rats. The study reported that both wild type and truncated variant reduced blood pressure but only the wild type PP13 cause expansion of utero-placental vasculature. The truncated variant lacked a part of exon 3, entire exon 4 and 2 amino acids forming the CRD indicating that CRD is necessary for lowering blood pressure and also for vasculature expansion. The study was carried out to demonstrate the importance of PP13 in regulation of blood pressure and also in vasculature expansion during pregnancy. The limitation of the study was the experiment was not carried out in preeclamptic rat models and the future perspective was that full length PP13 could be replenished to patients at risk for preeclampsia[105].

De Muro et al in 2016 conducted a prospective study in Italy to evaluate the levels of plasma PP13 in combination with urinary glycosaminoglycans/proteoglycans as early markers for preeclampsia as early as 11-13 weeks of pregnancy to predict pregnancy complications such as preeclampsia, Proteinuria and hypertension. The results showed statistically decreased PP13 levels, no differences in urinary glycosaminoglycans /proteoglycans, increase in total urinary trypsin inhibitor, reduced excretion of heparin and chondroitin sulfate in women with pregnancy complications compared with controls. PP13 plays a crucial role in placental implantation and maternal spiral artery remodeling, so low levels of PP13 in early weeks of pregnancy could be a risk factor in the for the development of preeclampsia. The study concluded that early prediction of preeclampsia in first trimester is important to facilitate therapeutic interventions.

An increase in total urinary glycosaminoglycans and proteoglycanscould is related to the inflammatory conditions in preeclampsia. The study limits in small sample size and no other renal or endothelial dysfunction markers were measured[106].

In 2017, Luo Q et al conducted a prospective study in China to predict the risk for preeclampsia. They measured PP13, PAPP-A Pentraxin-3, sFlt-1, Myostatin and Follistatin-like-3 in second trimester. The results showed significant increase in PP13 and sFlt-1 and decrease in Follistatin-like-3 but no significant changes in the levels of Myostatin, PAPP-A and Pentraxin-3. The detection rate for PP13 was 61.3%, 48.1% for sFlt-1, and 39.1% for Follistatin-like-3 and when three markers were combined the detection rate was increased to 69.8% with an AUC of 0.813. Thus these serum markers play an important role in predicting preeclampsia. The study limited in measuring more serum markers in second trimester of pregnancy whaich could predict preeclampsia[107].

N Yu et al in 2017 conducted a nested case-control study in China to determine the concentrations of first trimester PP13, PAPP-A, β-human Chorionic Gonadotropin (β-hCG), a distintegrin and metalloproteas and uterine Doppler in second trimesterto predict preeclampsia and IUGR. The study results showed PAPP-A and ADAM12 were lower in first trimester with increased uterine artery Doppler in second trimester which could predict preeclampsia and IUGR. However no significant changes were observed in the levels of PP13 and β-hCG in both the groups[108].

Beljan et al in 2017 conducted a prospective study in Croatia to detect if first trimester serum PP13 and Copeptin can predict preeclampsia in advanced age nulliparous women. The results showed insignificant decreased levels of PP13 and significant increased levels of Copeptin between preeclampsia and unaffected pregnancies. The

calculated odds ratio for PP13 was 0.93 and for Copeptin are 2.62. Since PP13plays a key role in placentation and Copeptin in increasing blood pressure and water retention, so decreased levels of PP13 and increased levels of Copeptin provides effective first trimester screening for preeclampsia before clinical diagnosis. The limitation this study was small sample size (n=3) of the preeclampsia cases[109].

De Villiers et al in 2017 conducted a nested case control study in Denmark in the first trimester samples obtained from a prenatal screening study conducted to detect preeclampsia and HELLP syndrome with preterm births. The PP13 concentration was decreased in preeclamptic women and in women with HELLP syndrome compared to controls in first trimester. These results suggest that PP13 could be a marker in first trimester to detect the risk for developing the pregnancy complications and pre –term delivery. The study also concludes the diagnostic utility of PP13 was increased when combined with free Leptin index The results of the study reported that the detection rate of PP13 combined with free Leptin index was improved than with PP13 alone. The study also reported poor fetal outcome in preeclamptic mothers[48].

Asiltas B et al in 2018 conducted a case-control study in Turkey to investigate the predictive value of PAPP-A, PP-13, β-hCG, and oxidative stress marker MDA individually or in combination in risk assessment of preeclampsia in the first trimester. The study reported significantly lower PP13 and higher MDA levels inwomen who developed preeclampsia. The combined model "MDA + PP-13 + PAPP-A + β-hCG" exhibited the best predictive outcome with AUC of 0.91 with 97% sensitivity and 75% specificity. The study concluded oxidative stress markers along with placental markers could be used as predictive markers in early pregnancy for the prediction of later development of preeclampsia. The limitation of the study is

small sample size of the preeclampsia group and limited parameters were measured in this study [49].

2.3. XANTHINE OXIDASE

Bambrana V and her co-workers in 2015 conducted a case control study in India to evaluate the plasma levels of XO activity, uric acid and Nitric oxide levels in preeclampsia and normal pregnant women during antenatal and post-partum period. Results showed significant elevated levels of XO activity and uric acid and decreased levels of Nitric oxide in preeclampsia compared to healthy pregnant women. Postpartum, there was decrease in the levels of XO activity and uric acid levels and increased Nitric oxide levels in preeclamptic women compared to controls. Correlation analysis in preeclampsia, revealed a non-significant negative correlation between XO activity and Nitric oxide level before and after delivery. Based on these results it can be hypothesized that increase in XO activity and uric acid is due to hypoxia reperfusion injury in the preeclamptic placenta leading to elevated oxidative stress which suppresses the expression of endothelial nitric oxide synthase. Limitation of the study is measurement of XO activity in all trimesters of pregnancy would help us to understand the pathological changes at an early stage and also to know if XO activity could be a marker for early identification of women at risk for preeclampsia[53].

In 2016, Elmas et al conducted a case-control study in Turkey to study the relationship between the levels of plasma Allantoin, Uric acid and XO activity and hypertension and their predictive role in preeclampsia. The results showed uric acid, XO activity and Allantoin levels were related to arterial blood pressure in preeclampsia. Higher levels of uric acid, XO activity and Allantoin levels were seen

in preeclampsia compared to controls. These increased levels reflect high cell death due to abnormal trophoblastic activity observed in preeclampsia. ROC showed highest AUC for XO activity 0.92 compared with uric acid (0.88) and Allantoin (0.73). Uric acid, XO activity and Allantoin showed positive correlations with blood pressure, however nitrate levels didnot show any changes in cases of preeclampsia. Limitation of the study is there was no data available on the levels of these markers before the appearance of preeclampsia symptoms, a prognosis follow-up was not done and gestational age at delivery and birth weight of all pregnant women were not recorded[110].

2.4. CASPASE-3

In 2012, Hsu CD and his co-workers conducted a case control study in China on the placentas of preeclamptic and normotensive women to determine if gene polymorphism of Caspase 3 proteins differs between patient with and without preeclampsia. Their findings indicate that preeclamptic placentas expressed significantly more genotype of TT of Caspase-3 SNP (+567) than normotensive patients when compared to CC. These findings indicate that placental caspase-3 gene polymorphisms are associated with preeclampsia and suggested that preeclampsia is a trophoblastic disorder[111].

In 2013, Cali U and his co-workers conducted aprospective case control in Turkey to examine the placental expression of Caspase-3 and Bcl2 (inner mitochondrial membrane protein which inhibits apoptosis) in preeclampsia, IUGR and Hemolysis, Elevated Liver enzymes and Low Platelet Count (HELLP) syndrome. The results showed significantly higher Caspase-3 immunostaining score indicating increased placental apoptosis in each group compared with the control group. Placental

apoptosis may be initiated by a variety of stimulus like hypoxia and oxidative stress. Increased apoptosis in the trophoblast may also reduce the nutrient transport across the trophoblast resulting in IUGR. Limitation of the study is small sample size and maternal serum Caspase-3 levels were not compared between preeclampsia and healthy pregnant women[112].

In 2014, Mendilcioglu I and his co-workers conducted a case-control study on placental tissue to determine whether preeclampsia is associated with an increase in placental apoptosis by measuring the placental expression of apoptotic mediators such as Fas, Fas ligand, Caspase-3, p53, B-cell lymphoma 2 (Bcl-2) and BAX protein. The study concluded increased apoptosis in preeclampsia may not be associated with significant alterations in the expression of apoptotic mediators[113].

In 2016, Dagdelen M et al conducted a case control study in Turkey to demonstrate if placental apoptosis is increased in preeclampsia as compared to normal pregnant women. Apoptosis was evaluated by Caspase-3, BAX protein and terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick end-labelling (TUNEL) immunohistochemical methods in syncytiotrophoblasts, syncytial cluster, extravillous cytotrophoblast, decidual and stromal cells. The three methods showed significant apoptosis in preeclamptic group compared to normal group. Apoptosis in the placental bed is increased in preeclamptic woman.Limitation of the study is evaluation of apoptosis in the tissue samples was done by semi-quantitative method and not by quantitative method like flow cytometry and maternal serum Caspase-3 levels could have been measured to know the early occurrence of increased apoptosis[114].

In 2018, Suparman E et al conducted a cross-sectional studyin Indonesia to see the difference of expression of apoptotic markers LC3 and Caspase-3 in normal pregnancy, early onset and late onset preeclampsia. The study results showed no difference in the expression of LC3 between the three groups however expression of Caspase-3 differed significantly. Expression of Caspase-3 in early onset preeclampsia was higher than normal pregnancy.Weakness of the study was confounding factors such as nutritional factors and gestational age were not considered during sampling which could affect the validity of the study and also examination methods used in this study was another limitation[115].

2.5. LGALS13 GENE

In 2009, Gebhardt S et al in South Africa conducted a prospective cohort study to investigate the role of novel and known sequence variations in the LGALS13 gene in association with adverse pregnancy outcomes (preterm labor). The gene variants identified in the group were -98 A/C (rs: 3764843), IVS2-36 G/A, IVS2-22 A/G (rs: 2233706), IVS2-15 G/A, 130A-G, 221delT, Hotspot, 260A-G, IVS3+ 72T/A (rs:2233708) and 351G-A. The novel exonic variant, 221delT was found to be associated with adverse pregnancy outcome (preterm labor) with a relative risk of 2.27. The deletion of a single thymine base at the nucleotide position 221 in the exon 3 of LGALS13 gene, resulting in substitution of a leucine with the tryptophan residue at amino acid position 74. This result in production of altered and truncated protein in which 37 amino acids are removed which may be important for its function. Limitation of the study is PP13 were not measured inorder to understand whether if the variant could be associated with the altered protein level and also would be helpful to know if PP13 could be used as an early biomarker for pregnancy related complications[41].

In 2018, Madar Shapiro L etalconducted a case-control study in London to predict the risk of developing preeclampsia in first trimester combining the promoter region variants and maternal risk factors. The study reported lower LGALS13 expression with the "A" nucleotide replaced by "C" in the -98 promoter region and adjusted OR calculated for A/A genotype combined with history of preeclampsia, BMI >35, advanced maternal age>40, black ethnicity could serve to predict preeclampsia in first trimester. This was the first study to demonstrate genetic analysis in maternal plasma samples and suggested polymorphism as a risk factor for the assessment of preeclampsia. However more studies are required to study the role of polymorphisms in the development of the disease so as to find novel therapeutic measures[42].

2.6. NITRIC OXIDE AND ADMA

In 2012, Rizos D et al in Greece conducted a prospective study to measure the levels of ADMA in all trimesters in uncomplicated pregnancies, women who developed preeclampsia and also in women who had small for gestational age babies (SGA). The study results showed lower levels of ADMA throughout gestation in normal pregnancies and in women who had SGA babies compared to women who had complicated pregnancies. However the levels of ADMA were significantly elevated in women who developed preeclampsia especially in the second trimester which could be due to reduced activity of DDAH, an enzyme which is sensitive to oxidative stress. ADMA also showed positive correlation with fetal birth weight in pregnancies with SGA and in uncomplicated pregnancies. Limitation of the study is small sample size, missing data on the pregnant women who had IUGR and also on their BMI. This limited the authors to find out the difference between the levels of ADMA in women

who had SGA babies and who developed IUGR, and also any role of BMI and ADMA on the development of preeclampsia[116].

In 2013, Alpoim PN and her co-workers in Brazil conducted a case-control study to evaluate the levels of ADMA in early-onset and late-onset (severe) preeclampsia. The results showed that ADMA levels were increased in the early severe preeclampsia compared to late severe preeclampsia and normotensive pregnant women. No difference was observed comparing late severe preeclampsia and normotensive pregnant. This study concluded that oxidative stress occurs during in early placental development in preeclampsia causing inactivation of the enzyme DDAH II leading to accumulation of ADMA. ADMA is known to affect angiogenesis and decreases the synthesis of nitric oxide thus impairing placentation and causing endothelial dysfunction as seen in preeclampsia. The limitation of the study was ADMA was not measured longitudinally to know if ADMA could be used as a marker for preeclampsia in early pregnancy[117].

In 2013, Laskowska M et al conducted a case control study in Poland. The levels of serum endothelial nitric oxide synthase, ADMA and homocysteine levels were measured in normal and preeclamptic pregnancies. The study reported higher levels of ADMA and homocysteine levels in preeclampsia compared with controls, however there was no significant differences in the levels of these molecules in early and late preeclampsia. Endothelial NOS level was lower in both groups of preeclampsia compared to controls but was non-significant. The study concluded that the elevated levels of ADMA and not the decreased levels of endothelial NOS is responsible for the decreased levels of Nitric oxide in preeclampsia. The study limits in measuring Nitric oxide levels and small sample size. They study also suggested reducing the

levels of ADMA and homocysteine may help in prevention and treatment of preeclampsia[118].

In 2014, Tayal D et al conducted a case control study in India to find out the association of inflammatory cytokines, lipid peroxidation end products and NO with clinical severity and fetal outcome in preeclampsia and also to assess their role as prognostic determinants. The results showed the parameters MDA, Nitric oxide, IL-6 and TNF were significantly higher and fetal birth weight were significantly lower in subjects with preeclampsia. A significant positive correlation was observed between Nitric oxide and uric acid, MDA and urinary proteins and a significant negative correlation was observed between levels of Nitric oxide, MDA and severe Proteinuria with fetal birth weight. ROC curve analysis showed AUC for Nitric oxide and MDA as 0.995 and 0.981 respectively. According to multivariate analysis, Tumor necrosis factor had superior predictive role in assessing the severity of preeclampsia. The study concluded that preeclampsia is a disease of multifactorial etiology associated with oxidative stress, inflammation and endothelial dysfunction. Limitation of the study is the study design, because longitudinal studies would help in early detection and aid in better management of this disorder[119].

In 2015, Zheng Bian and his co-workers in China conducted a prospective nested case-control study to investigate whether first trimester serum levels of sFLT1, PlGF and ADMA and second trimester pulsatility index could be markers to predict preeclampsia. The results showed significantly higher sFLT1 and ADMA levels and lower PlGF levels in first trimester of women who developed preeclampsia compared with normal pregnancies. ROC showed an AUC for PlGF + ADMA was 0.902, BMI+ PlGF+ ADMA was 0.909 which were better than second trimester uterine

artery pulse index 0.836, but was not significant. Limitation of the study was no sub group analysis was done; women with only no pregnancy and obstetrics complications were included in the control group which could introduce bias and small sample size. Further studies in larger populations are needed[120].

In 2015, Zeng et al conducted a case-control study in China to investigate the change of levels of serum Homocysteine, Endothelin-1 and Nitric oxide in preeclampsia and normotensive women. They reported significantly high concentrations of Homocysteine and Endothelin-1 and low levels of Nitric oxide in patients with hypertensive disorders complicating pregnancy compared to the control group. Correlation analysis showed level of serum Homocysteine and Endothelin-1 was positively correlated and Nitric oxide was negatively correlated with severity of the disease. Increased Homocysteine levels could be due to insufficient intake or malabsorption of B group vitamins leading to elevated levels of Homocysteine resulting in endothelial cell injury. This results in increased synthesis of Endothelin-1 and decreased synthesis of Nitric oxide. The limitation of the present study is the patients were not followed up and could not get data of serum Homocysteine, Endothelin-1 and Nitric oxide levels after recovery and pregnancy outcomes[121].

In 2015, Lopez –Alarcon et al in Mexico conducted a prospective cohort study to evaluate whether serial determinations of ADMA and Homocysteine levels could be useful during pregnancy to predict preeclampsia. Vitamins B_6, B_{12} and Folic acid were also measured. The study reported that elevated levels of ADMA and Homocysteine precede the clinical manifestations of preeclampsia and monitoring their levels help to identify women at risk for preeclampsia[122].

In 2015, Zheng JJ et al conducted a case control study in China to study the relationship between the steroid hormones such as estradiol and progesterone and ADMA and their role in causing endothelial dysfunction in severe preeclampsia. This study results showed significantly increased serum and placental levels of ADMA in severe preeclampsia compared to normal pregnant women. Significantly increased levels of progesterone, decreased estradiol and decreased progesterone/estradiol ratio was observed in severe preeclampsia. Significant positive correlation was observed between systolic blood pressure (SBP), MAP and progesterone/estradiol ratio with serum ADMA levels in both severe preeclampsia and control group. Estradiol is known to increase the expression of endothelial NOS to maintain the placental vascular tone and also to regulate blood pressure. Imbalance in the levels of steroid hormones and increase in ADMA levels is thought to result from ischemic placenta which is responsible for the clinical manifestations of preeclampsia. The study also suggested that estradiol therapy could reduce ADMA levels as it can reverse the effect of oxidized low density lipoprotein on the activity of DDAH. The study limits in not measuring the expression of DDAH in the placenta and correlating it with progesterone/estradiol ratio[123].

Hodzic J et al in 2017 conducted a prospective cross sectional study in Zenica to investigate the biosynthesis of Nitric oxide in preeclampsia and in normal pregnancy. The results showed that levels of Nitric oxide increased as gestation progressed compared to non-pregnant women. However the late third trimester of normal pregnant showed non-significant decrease in Nitric oxide levels compared to the non-pregnant women. A statistically non-significant decrease in serum levels of Nitric oxide was seen in preeclampsia compared to non-pregnant and control group. A significant positive correlation was also seen between Nitric oxide concentrations and

systolic blood pressure, diastolic blood pressure, creatinine clearance and uric acid. A significant negative correlation was observed between Nitric oxide levels and platelet count. Limitation of the study is small sample size[124].

Darkwa E.O et al in 2018 conducted a case-control study in to compare the levels of Nitric oxide in healthy pregnant women and preeclampsia in Ghana. This study reported that there was a significant difference in the blood pressure between the preeclampsia and normotensive group, however there was no significant reduction in plasma Nitric oxide levels between the two groups. A non-significant negative correlation was observed between plasma Nitric oxide levels and MAP. The study concluded that plasma Nitric oxide may not play a role in the pathophysiology of preeclampsia and changes in Nitric oxide levels are a poor predictor of mean arterial pressures in preeclampsia[80].

Deniz R et al in 2019 conducted a case-control study in Turkey to evaluate the levels of Elabela, Apelin and Nitric oxide levels in maternal blood of normal pregnant women, pregnant women with preeclampsia, severe preeclampsia and umbilical arteries and venules of new born. The study results demonstrated low levels ofthese molecules in preeclampsia and severe preeclampsia and their newborns' venous-arterial blood and also their relation with low birth weight. The possible reason for the reduced levels of endothelial dysfunction markers could be due to pathological changes in preeclampsia. Limitation of the study is low sample size and since Apelin and Nitric oxide results were controversial in preeclampsia more studies are needed to evaluate this relationship[125].

Abraham AJM et al in 2019 conducted a cross sectional study in India to evaluate the predictive capacity of ADMA, TAS and time of onset of hypertension for adverse

neonatal outcomes like low birth weight, fetal and infant mortality, pre-term births and with low apgar score in hypertensive disorders of pregnancy. The study reported ADMA with an AUC of 0.752 could predict fetal mortality and with an AUC of 0.731 can predict babies with low apgar score. TAS was also reported to be a predictor of preterm births with an AUC of 0.718. The study also reported the role of blood pressure and time of onset of hypertension to predict adverse fetal outcomes. Although oxidative stress parameter MDA was measured however, the study limits in measuring the levels of folic acid, homocysteine and DDAH to know the other reasons for the elevated levels of ADMA[126].

CHAPTER 3

AIM & OBJECTIVES

3.0. AIM AND OBJECTIVES

3.1 Aim

Measurement of Oxidative Stress Index, Caspase-3 and Placental protein 13 during placentation and their possible relation with endothelial dysfunction in first and second trimesters of preeclampsia and normotensive pregnancy with LGALS-13 gene analysis in the cord blood.

3.2. Objectives

1. To determine the Oxidative Stress Index in terms of total oxidant status and total antioxidant status in the first and second trimesters of normotensive pregnancy and in preeclampsia cases.
2. To determine the concentration of plasma Placental protein 13, Caspase-3 and Xanthine oxidase activity in first and second trimesters of normotensive pregnancy and in preeclampsia cases.
3. To estimate the ratio of Asymmetric dimethyl arginine to Nitric oxide in relation to endothelial dysfunction in first and second trimesters of normotensive pregnancy and in preeclampsia cases.
4. To find out the frequency of occurrence of genetic polymorphism of LGALS13 gene from the cord blood of preeclamptic and normotensive women postpartum.

CHAPTER-4

MATERIALS&METHODS

4.0. RESEARCH METHODOLOGY

4.1. MATERIALS

Study designed was prospective nested case-control carried out in R L Jalappa Hospital and Research Centre, Tamaka, Kolar. The duration of the study was three years (August 2016 to August 2019), and study was conducted by the Department of Biochemistry in collaboration with Department of Obstetrics and Gynaecology of Sri Devaraj Urs Medical College, Tamaka, Kolar. The study design and the patients recruitment criteria was approved by the University Central Ethics Committee of Sri Devaraj Urs Academy of Higher Education and Research Centre in a letter with vide number SDUAHER/KLR/R&D/47/2017-18 dated on 7-7-2017. The participants of the study were recruited on obtaining patient written informed consent.

4.1.1. Calculation of sample size

Sample size was calculated based on the difference found in PP13 levels between cases and controls. To detect the concentration difference of 16.2pg/mL in PP13 between the cases and controls with 90% power, 95% confidence interval with an average variance estimate of PP13 as 243.36 pg/mL[47].

Based on the following equation, the estimated total sample size was 268 pregnant womenwith 10% dropout rate during the follow-up.

$$n = \frac{2\sigma^2[z_\alpha + z_{1-\beta}]}{(d)^2}$$

Where Z_α = standard normal deviate for α 95% = 1.96, Z_β = standard normal deviate for β with power 90% = 1.28 and d= difference in means.

4.1.2. Sample collection

Four ml of whole blood was collected in EDTA tubes from pregnant women in first and second trimesters of pregnancy during their antenatal check-up visit to the Department of Obstetrics and Gynecology. Blood samples were centrifuged at 3000 rpm for 10 minutes at 4^0C to obtain the clear plasma. Thus obtained clear plasma was stored at-80^0C until analysis. The umbilical cord was clamped, wiped with antiseptic, needle was inserted into the vein in the umbilical cord at fetal side and 2ml of cord blood was drawn in an EDTA tube which was stored at 4^0C for extraction of the DNA. Soon after the sample was collected both the mother and the neonate were treated in R.L.Jalappa Hospital as per the standard care adapted by the hospital.

4.1.3. Inclusion criteria

Pregnant women who visited for prenatal check-up in the first trimester to the Department of Obstetrics and Gynaecology were enrolled in the study. Based on the criteria women who were primigravida with healthy single fetus without any anomalies confirmed by ultrasonography were included in the study. They were also

followed at second trimester and until delivery for record of fetal outcome and collection of cord blood.

4.1.4. Exclusion criteria

Pregnant women with any history of chronic hypertension and on treatment, renal disease, thyroid disorder, gestational diabetics, epilepsy, hypertensive encephalopathy, cardio vascular disease, twin pregnancies and those having the habit of tobacco chewing/consumption were excluded from the study.

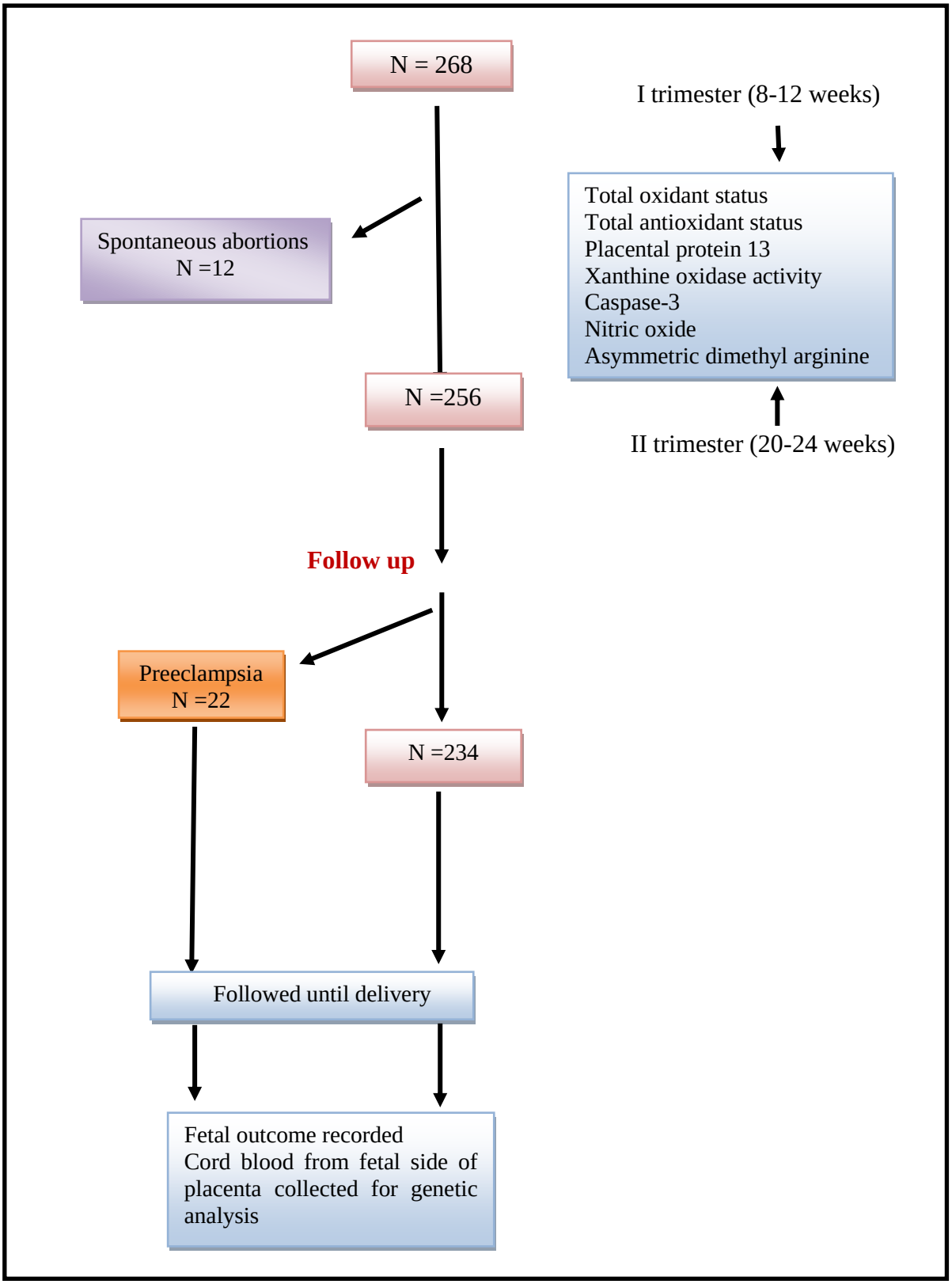


Figure 14: Schematic representation of study design

4.2. METHODS

The following Table 1 describes various methodologies and instruments used in the study.

Table 1: Methods and Instruments used for biochemical analysis

S.No.	Parameter	Method	Make/Catalog no.	Instrument
1.	Total Oxidant Status (U/mL)	Double antibody sandwich ELISA	Sunred, Shanghai, China 201-12-5538	Merilyzer EIAQuant
2.	Total Antioxidant Status (U/mL)	Double antibody sandwich ELISA,	Sunred, Shangai China 201-12-7412	Merilyzer EIAQuant
3.	Placental protein 13 (pg/mL)	Competitive inhibition Enzyme immunoassay technique	Cusabio, China CSB-E12733h	Merilyzer EIAQuant
4.	Xanthine Oxidase activity (mU/mL)	Colorimetric Assay	Biovision, USA K710-100	Merilyzer EIAQuant
5.	Caspase-3 (pg/mL)	Quantitative sandwich enzyme immunoassay technique	Cusabio, China CSB-E08856h	Merilyzer EIAQuant
6.	Nitric oxide (nmol/µL)	Colorimetric Assay	Biovision, USA K262-200	Merilyzer EIAQuant
7.	Asymmetricdimethylarginine (ng/mL)	Quantitative sandwich enzyme immunoassay technique	Cusabio, China CSB-E09298h	Merilyzer EIAQuant

4.2.1. DETERMINATION OF PLASMA HUMAN TOTAL OXIDANT STATUS

Method: Human Total oxidant status (hTOS) measured by enzyme-linked immunosorbent assay (ELISA) technique as per the procedure supplied by Shanghai Sunred Biological Technology Company Ltd, China.

Principle: This quantitative technique based on the principle of double-antibody sandwich ELISA method.The antigen which is to be analyzed in plasma sample is incubated with specific antibody coated to the well. Then a second antibody which is labeled with horse radish peroxidase (HRP) is added which binds to the coupled antigen-antibody to form antibody-antigen/antibody complex and thus the antigen is now sandwiched between two antibodies. After washing procedure, substrate was added and allowed to react by an enzyme conjugated with antibody that can catalyze the conversion of colourless substrate into coloured product. The intensity of the colour produced is directly proportional to the antigen/total oxidant capacity in the sample measured at 450 nm.

Reagents:

1. Standard solution (480 U/mL)
2. Standard diluents
3. Streptavidin-HRP conjugate
4. 30X Wash solution
5. Biotin-(TOS) antibody
6. Chromogen solution A
7. Chromogen solution B
8. Stop solution

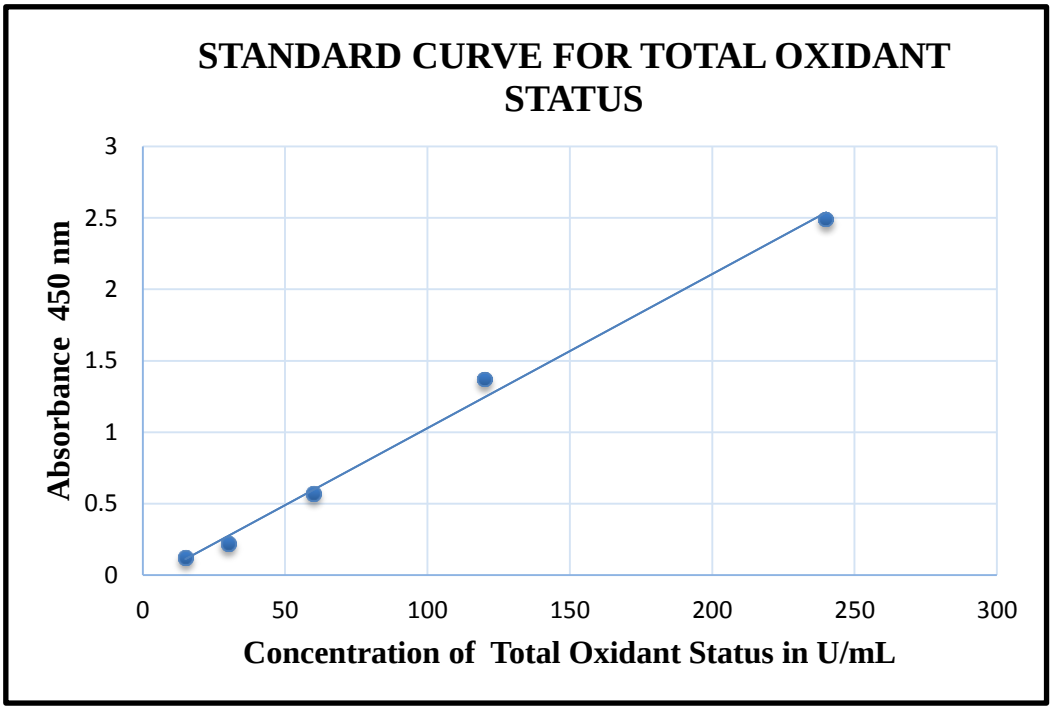
Procedure: To the test wells 50 µL of patients serum sample and 50 µL of standard (S1-S5) was added. 10 µL of Biotin-(TOS) antibody was added to test wells and not to the standard wells because standards already had combined biotin antibody. 50 µL of Streptavidin –HRP- conjugate reagent was added to test wells and standard

wells. The plate was sealed with the sealing membrane and incubated for 60 minutes at 37^0C. After washing procedure, 50 µL chromogen A and 50 µL chromogen B solution was added. The sealed plate was incubated for 10 minutes at 37^0C and 50 µL of stop solution was added to all the wells. The blank and standards were run in duplicates. The blank was adjusted to zero and optical density (OD) was measured at 450 nm.

Calculation: After plotting the standard concentration on the X-axis and the OD values on the Y-axis, the standard curve was drawn on excel sheet. According to standards' concentration and corresponding OD values, the standard curve linear regression equation was calculated. The OD values of the sample was applied in the regression equation ($y= 0.018x- 0.0487$, $R^2= 0.994$) and corresponding TOS in the sample was calculated. The sensitivity of the test was1.362 U/mL. The assay range was 1.5– 400 U/mL. The result was represented as U/mL.

Preparation of standard curve for Total Oxidant Status

Standard	S1	S2	S3	S4	S5
Concentration U/mL	15	30	60	120	240
Absorbance 450 nm	0.12	0.22	0.57	1.37	2.49



4.2.2. DETERMINATION OF PLASMA HUMAN TOTAL ANTIOXIDANT STATUS

Method: Human Total antioxidant status (hTAS) measured by enzyme-linked immunosorbent assay (ELISA) technique as per the procedure supplied by Shanghai Sunred Biological Technology Company Ltd, China.

Principle: This quantitative technique based on the principle of double-antibody sandwich ELISA method. The total antioxidant status measured by considering antioxidant enzymes in a sample.The antigen which is to be analyzed in plasma sample is incubated with specific antibody coated to the well. Then a second antibody which is labelled with HRP is added which binds to the coupled antigen-antibody to form antibody-antigen/antibody complex and thus the antigen is now sandwiched between two antibodies. After washing procedure, substrate is added and allowed to react by an enzyme conjugated with antibody that can catalyze the conversion of colorless substrate into coloured product. The intensity of the colour produced is directly proportional to the antigen/total oxidant capacity in the sample measured at 450 nm.

Reagents:

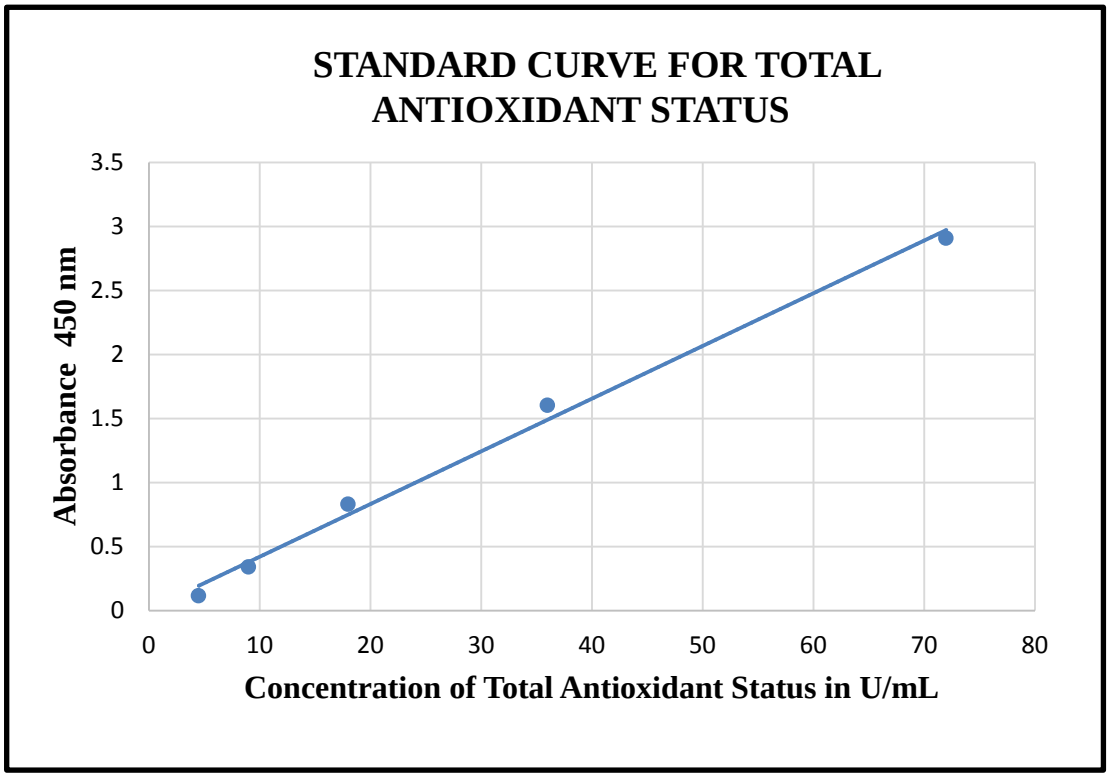
1. Standard -144U/mL
2. Standard Diluent
3. Streptavidin-HRP conjugate
4. 30X Wash solution
5. Biotin-(TAS) antibody
6. Chromogen solution A and B
7. Stop solution

Procedure: 50 µL of serum sampleand 50 µL of standard (S1-S5) were added to the wells. The blank and standards were run in duplicates. The 10 µL of Biotin-(TAS) antibody was added to the test wells and not to the standard wells because standards already had combined biotin antibody. 50 µL of Streptavidin–HRP- conjugate reagent was added to test and standard wells. The plate was sealed with the sealing membrane and incubated for 60 minutes at 37 ^{0}C. After washing process, 50µL chromogen A and 50µL chromogen B was added to all the wells. Theplate was sealed and incubated again for 10 minutes at 37 ^{0}C and 50 µL of stop solution was added. Taking blank as zero, OD was measured under 450 nm wave length within 15 min after adding the stop solution.

Calculation: Taking the standard concentration on the X-axis and the OD values on the Y-axis, the standard curve was drawn on excel sheet. According to standards' concentration and corresponding OD values, the standard curve linear regression equation was calculated. The OD values of the sample was applied in the regression equation ($y= 0.041x-0.010$, $R^2= 0.993$) to calculate the corresponding TAS in the sample. The sensitivity of the test was 0.411 U/mL. The assay range was 0.5–120 U/mL. The result was represented as U/mL.

Preparation of standard curve for Total Antioxidant Status

Standard	S1	S2	S3	S4	S5
Concentration U/mL	4.5	9	18	36	72
Absorbance 450 nm	0.114	0.337	0.829	1.601	2.906



4.2.3. DETERMINATION OF PLASMA PLACENTAL PROTEIN 13

Method: Placental protein 13 (PP13) is measured by enzyme-linked immunosorbent assay (ELISA) technique as per the procedure supplied by Cusabio Biotech Co. Ltd, China.

Principle: This assay employs the competitive inhibition enzyme immunoassay technique. Antibody specific for PP13 has been pre-coated onto a micro plate. A competitive inhibition reaction launched between PP13 (standards or samples) and HRP-conjugated PP13 with the pre-coated antibody specific for PP13. The more amount of PP13 in the samples, the less antibody bound by HRP-conjugated PP13. Following washing procedure, substrate was added to the wells, colour develops is inversely proportional to the concentration of PP13 in the sample and measured at 450 nm.

Reagents

1. Standard
2. HRP-Conjugate
3. 20 X Wash buffer
4. Substrate A
5. Substrate B
6. Stop Solution

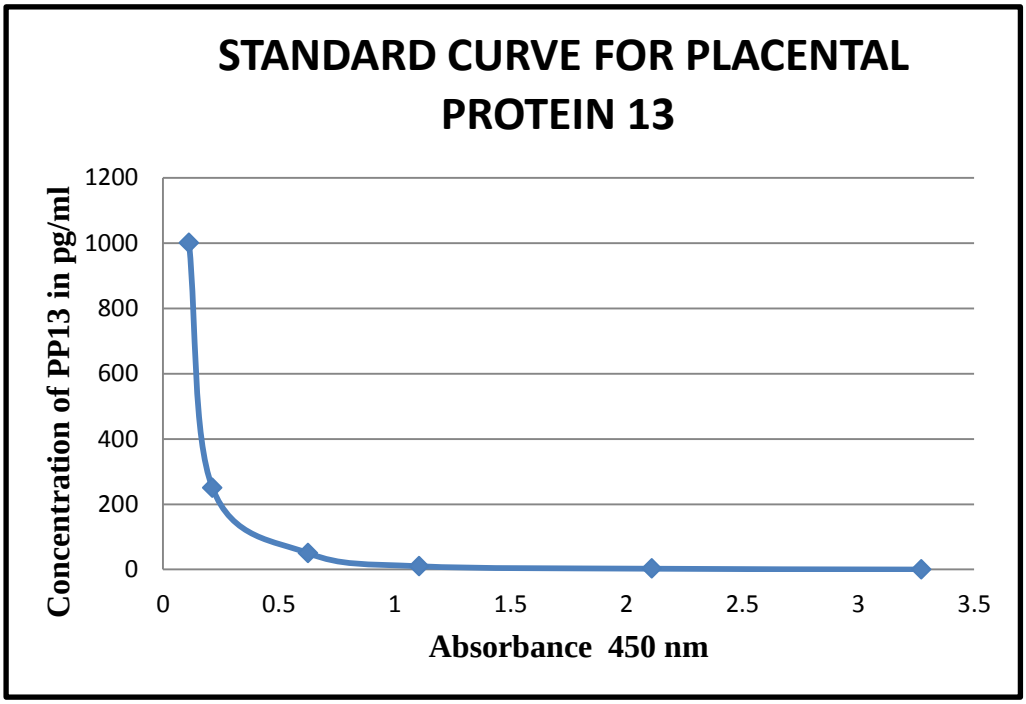
Procedure: 50 µL of patients serum sample and 50 µL of standards (S1-S5) were added to the wells. The standards and blank were run in duplicates. 50 µL of HRP-conjugate was added to both standards and sample wells but not to blank well. The plate was gently shook and incubated for 1hour at 37 ^{0}C. Each well was aspirated and washed thrice with the wash buffer. 50 µL of Substrate A and 50 µL of Substrate B was added to each well and mixed well. The plate was incubated for 15 min at 37 ^{0}C. 50 µL of stop solution was added to each well and the plate was tapped gently to

ensure thorough mixing. The optical density was determined within 10 minutes using a micro plate reader set to 450 nm.

Calculation: The standard curve was plotted by taking the mean absorbance for each standard on the x-axis against the concentration on the y-axis. With the sample OD value in the equation ($y= 0.041x + 0.010$, $R^2= 0.996$), the PP13 concentration in the sample was calculated. The detection range was 2.5 -1000 pg/mL. The sensitivity was less than 1 pg/mL.The result was represented as pg/mL.

Preparation of standard curve for PP13

Standard	S1	S2	S3	S4	S5	S6
Concentration pg/mL	0	2.5	10	50	250	1000
Absorbance 450 nm	3.27	2.10	1.10	0.62	0.21	0.11



4.2.4. DETERMINATION OF PLASMA XANTHINE OXIDASE ACTIVITY

Method: Xanthine oxidase activity is measured by colorimetric assay as per the procedure supplied by BioVision Incorporation, USA.

Principle: Xanthine oxidase oxidizes xanthine to hydrogen peroxide (H_2O_2) which reacts stoichiometrically with OxiRed (chromogen) probe to generate pink colour measured at 570 nm.

Reagents:

1. Xanthine oxidase assay buffer
2. OxiRed^{TM} probe
3. Xanthine oxidase enzyme mix
4. Xanthine oxidase substrate mix
5. Xanthine oxidase positive control
6. H_2O_2 standard

Procedure: To the standard wells 10, 20, 30, 40, 50µL of the 0.2 mM H_2O_2 standard was added. The total volume was made up to 50 µL in each well with distilled water to generate 0,2,4,6,8,10 nmol/H_2O_2 standards. 50 Ml test samples wasadded to sample wells. For the positive control, 5µL of positive control solution was added and the volume was made upto 50 µL/well with distilled water. 50 µL of reaction mix (Assay buffer+ substrate mix +enzyme mix+OxiRed^{TM}) was added to each well containing standard, positive control and test samples. The plate was measured immediately at 570 nm at T_1 and $A_1$was recorded. The plate was incubated away from light at 25^0C for 10-20 minutes and measured again at T_2 to read A_2 . The signal generated by XO is $\Delta A = A_2 - A_1$. The kit detects 1-100 mU XO in 100µl reaction volume.

Calculation: The background absorbance was subtracted from all the values. The H_2O_2 standard curve was plotted. The sample ΔA was applied to the H_2O_2 standard curve to get B nmol of H_2O_2. The result was represented as mU/mL.

$$\text{XO activity (mU/mL)} = \frac{\text{B x sample dilution factor}}{\text{(T2-T1) x V}}$$

Where B = amount of H_2O_2generated by xanthine oxidase from standard curve (nmol)

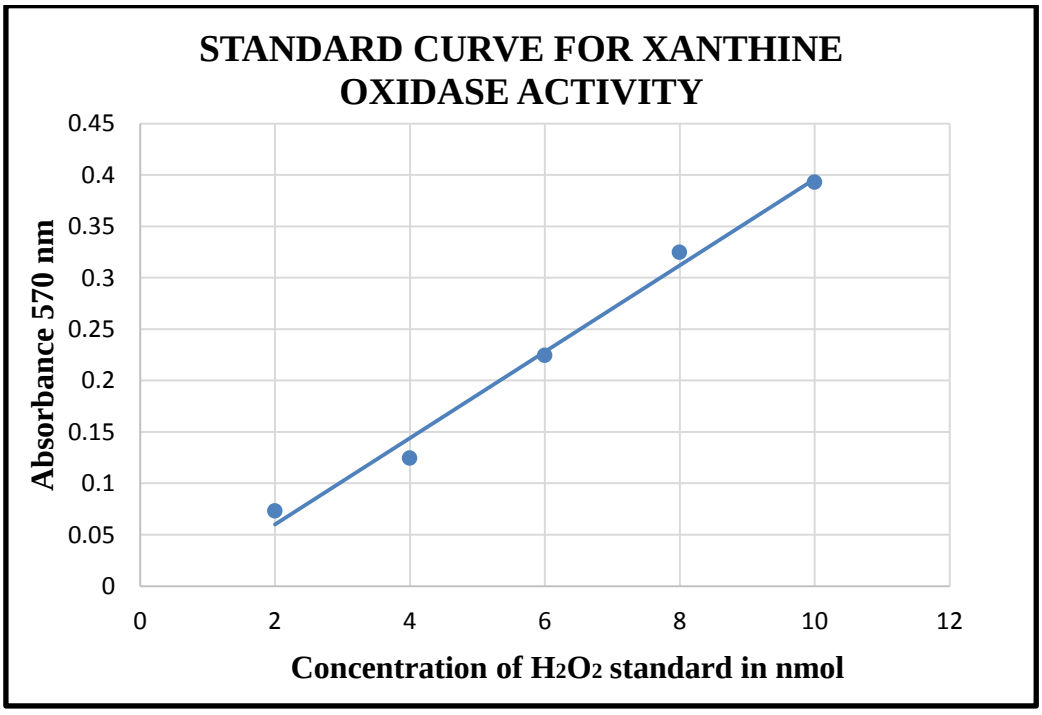
T_1 = time of the first reading (A_1) (in min)

T_2= time of the second reading (A_2) (in min)

V = pre-treated sample volume added into the reaction well (in ml)

Preparation of standard curve for XO activity

Standard	S1	S2	S3	S4	S5
Concentration mU/mL	2	4	6	8	10
Absorbance 450 nm	0.073	0.124	0.224	0.325	0.392



4.2.5. DETERMINATION OF CONCENTRATION OF PLASMA CASPASE 3

Method: Caspase-3 concentration measured by enzyme-linked immunosorbent assay (ELISA) technique as per the procedure supplied by Cusabio Biotech Co. Ltd, China.

Principle: The assay employs the quantitative sandwich enzyme immunoassay technique. Antibody specific for Caspase-3 pre-coated onto wells of micro plate. Standards and samples are added into the wells. Caspase-3 present in plasma bound to immobilized antibody. After removing any unbound substances during washing procedure, HRP-conjugated antibody specific for Caspase-3 was added into the wells. On addition of substrate solution, HRP catalyses conversion of substrate into yellow coloured product measured at 450nm. The intensity of the colour is proportionate to the amount of Caspase-3 content.

Reagents

1. Standard
2. HRP-Conjugate
3. HRP-Conjugate Diluent
4. Sample Diluent
5. Wash buffer
6. TMB Substrate
7. Stop Solution

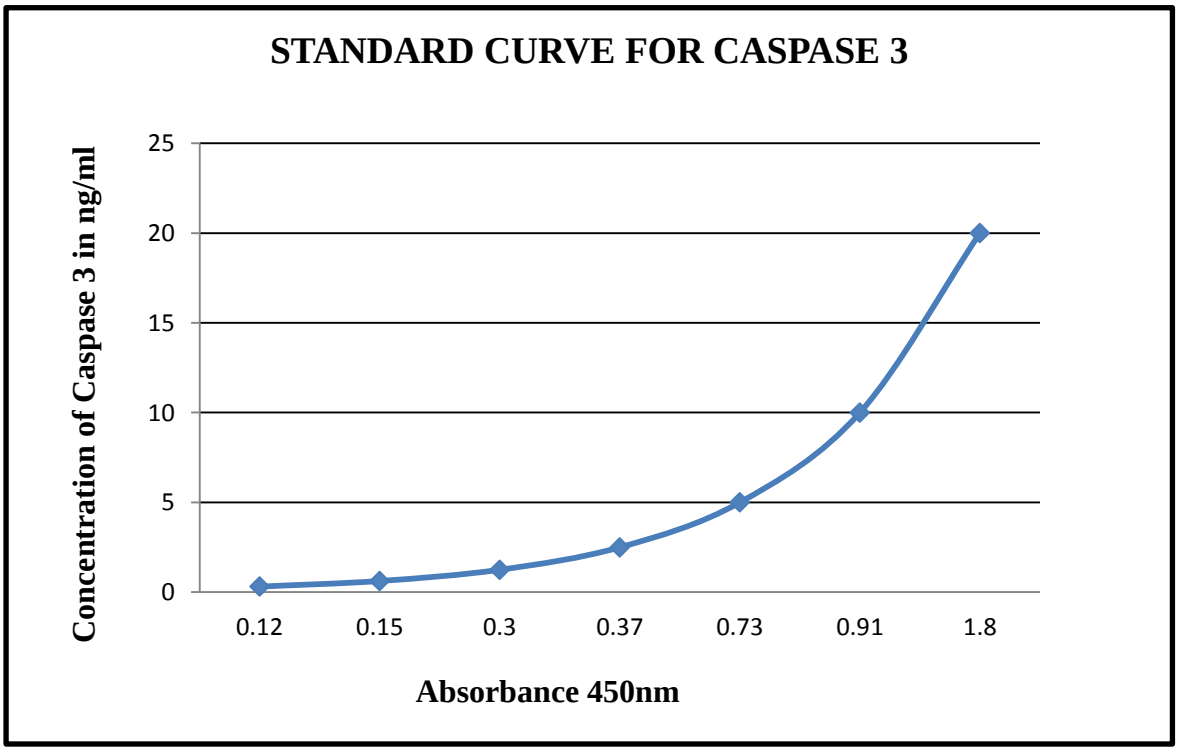
Procedure: 100 µL of standards (S0-S1) and samples were added per well. The plate was covered with adhesive strip and incubated for 30 minutes at 37 ^{0}C followed by washing procedure.100µL of HRP-conjugate was added to each well and the plate was covered with a new adhesive strip and incubated for 30 minutes at 37 ^{0}C and again subjected for wash process. 90 µL of TMB Substrate was added to each well

and incubated for 30 minutes at 37 ^{0}C. 50 µL of stop solution was added to each well and OD was measured at 450nm.

Calculation: The standard curve was constructed by plotting mean absorbance for each standard on x-axis against the concentration on the y-axis. With the sample OD value in the equation ($y= 0.682x^2 – 3.729x + 4.335$, $R^2 = 0.960$) the caspase-3 concentrationin the sample was calculated. The detection range was 0.312 -20 ng/mL. The sensitivity of the assay was less than 0.078 ng/mL.The result was represented as ng/mL.

Preparation of standard curve for Caspase-3

Standard	S0	S1	S2	S3	S4	S5	S6	S7
Concentration ng/mL	0	0.312	0.625	1.25	2.5	5	10	20
Absorbance at 450 nm	0.1001	0.1233	0.1547	0.3049	0.3761	0.7361	0.9197	1.8015



4.2.6. DETERMINATION OF PLASMA NITRIC OXIDE

Method: Nitric oxide in plasmawas measured by colorimetric assay as per the procedure supplied by BioVision Incorporated, USA.

Principle: The method based on the principle of reduction of nitrate to nitrite by the action of nitrate reductase. The nitrite produced is estimated by diazotization of sulphanilamide in acidic medium and then coupling with naphthyethylene diamine dihydrochloride to produce pink coloured azo compound. The absorbance was measured spectrophotometrically at 540nm.

Reagents:

1. Assay Buffer
2. Enzyme cofactor
3. Enhancer
4. Nitrate Reductase
5. Nitrate standard
6. Griess Reagent R1
7. Griess Reagent R2

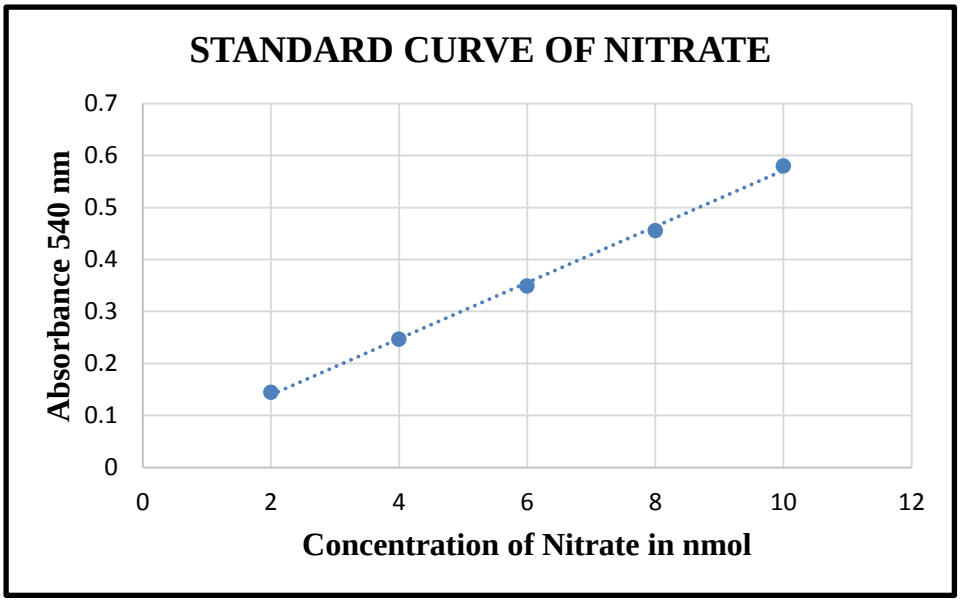
Procedure: 5µL of the 100 mM Nitrate standard was reconstituted with 495µL of Assay Buffer to generate 1 mM standard working solution. Into a series of wells 0,2,4,6,8,10 µL of standards were added. The volume was adjusted to 85 µL with Assay Buffer to generate 0,2,4,6,8,10 nmol/well of Nitrate Standard. The standards were run in duplicates. 85 µL of sample was added to each well. To the Blank well, 115 µL of Assay Buffer was added. 5 µL of the Nitrate Reductase mixture and 5 µL of the enzyme cofactor was added to standards and test wells. The plate was covered and incubated at room temperature for 1 hour to convert nitrate to nitrite. 5µL enhancer was added to each well and incubated for 10 minutes. 50 µL Griess Reagent

R1 and 50 µL Griess Reagent R2 was added to each well. The colour was developed for 10 minutes at room temperature and the absorbance was read at 540 nm.

Calculation: Taking the concentration of Nitrate in nmol on x axis and absorbance at 540 nm on y axis, the standard curve was plotted. The absorbance of each sample was applied in the equation ($y=0.157x + 0.009$, $R^2 = 0.996$) and the concentration of nitrate inthe sample was calculated. The detection range was 1 nmol nitrate/well or 10µM. The result was represented as nmol/µL.

Preparation of standard curve for Nitrate

Standard	S1	S2	S3	S4	S5
Concentration ng/mL	2	4	6	8	10
Absorbance at 450 nm	0.145	0.247	0.349	0.456	0.580



4.2.7. DETERMINATION OF PLASMA ASYMMETRIC DIMETHYL ARGININE

Method: Asymmetric dimethylarginine (ADMA) concentration measured by enzyme-linked immunosorbent assay (ELISA) technique as per the procedure supplied by Cusabio Biotech Co. Ltd, China.

Principle: The assay was based on the principle of quantitative sandwich technique. The antibody specific for ADMA was pre-coated onto micro plate wells.Standards and samples wereadded into the wells. ADMA present in plasma was bound to the immobilized antibody. After removing any unbound substances by washing procedure, biotin-conjugated antibody specific for ADMA was added to the wells. On washing, avidin conjugated HRP was added to the wells. After wash process, substrate solution was added into the wells and yellow colour developed is proportion to the amount of ADMA measured at 450nm.

Reagents

1. Standard
2. Biotin-antibody
3. HRP-avidin
4. Biotin-antibody Diluent
5. HRP-avidinDiluent
6. Sample Diluent
7. Wash Buffer
8. TMB substrate
9. Stop solution

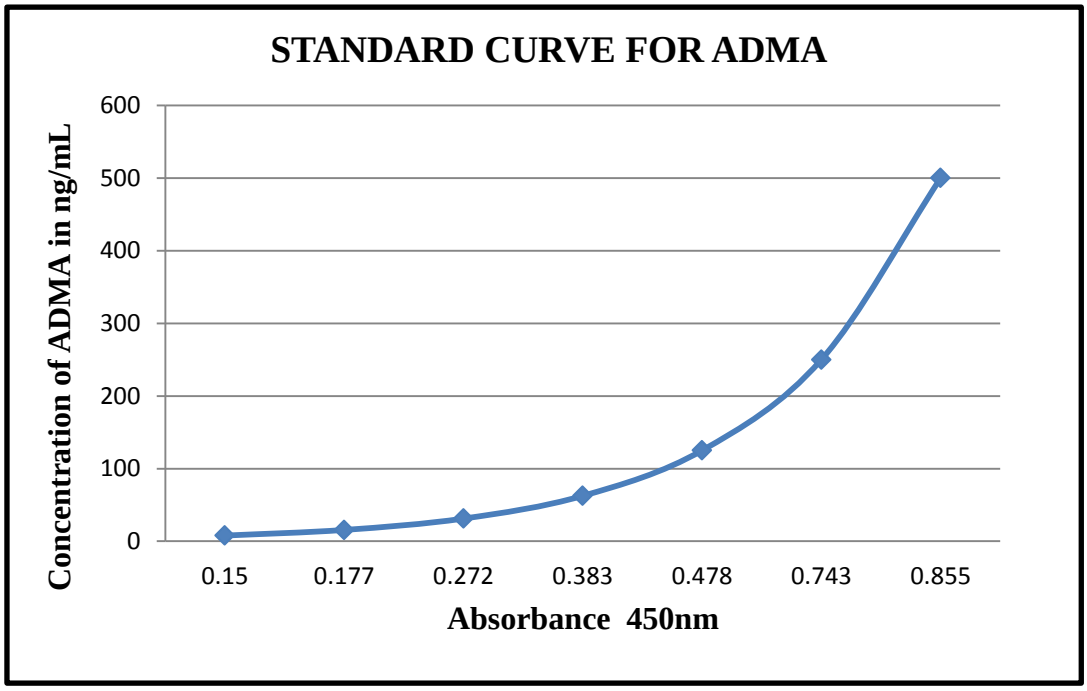
Procedure: 100 µL of standards (S1-S7) and samples were added to the micro titre plate. The plate was covered with adhesive strip and incubated for 2 hours at 37 ^{0}C. Liquid in each well was removed but not washed. 100 µL of Biotin-antibody was

added to each well (standards and test wells). The microtiter plate was sealed with adhesive strip and incubated for 1 hour at 37 ^{0}C. After washing procedure, 100 µL of HRP-avidin was added to each well and the plate was incubated for 1 hour at 37 ^{0}C. After washing procedure, 90 µL of TMB Substrate was added to each well and the plate was again incubated for 15-30 minutes at 37 ^{0}C. 50 µL of Stop Solution was added and OD was determined at 450 nm.

Calculation: The standard curve was constructed by plotting mean absorbance for each standard on x-axis against the concentration on the y-axis. With the sample OD value in the equation ($y=905.5x^2 – 280.7x +30.93$, $R^2= 0.960$), the concentration of ADMA in the sample was calculated. The detection range was 7.8 -500 ng/mL. The sensitivity was less than 1.95 ng/mL.The result was represented as ng/µL.

Preparation of standard curve for ADMA

Standard	S1	S2	S3	S4	S5	S6	S7
Concentration ng/mL	7.8	15.6	31.2	62.5	125	250	500
Absorbance at 540 nm	0.150	0.177	0.272	0.383	0.478	0.743	0.855



4.2.8. ANALYSIS OF LGALS13 GENE

The LGALS13 gene encoding for PP13 is located on chromosome 19q13.1. It consists of promoter region and four exons E1-E4. The cord blood at fetal side was collected for isolation of DNA, amplification and sequencing of the gene.

Analysis of LGALS13 gene consists of the following procedures.

1. Isolation of genomic DNA
2. Determination of purity of the DNA
3. Standardization of the exons of LGALS13 gene
4. Amplification of DNA by polymerase chain reaction (PCR)
5. Purification of amplified product
6. Sequencing

1. Isolation of Genomic DNA

Genomic DNA extraction protocol consisted of treating 2 ml of cord blood sample by salting out procedure[127].

Reagents:

i. Erythrocyte Lysis Buffer (ELB): (1L, pH 7.4 set with concentrated HCl, stored at 4^0C) - 0.155M 8.3g NH_4Cl, 0.01M 0.03g EDTA and 0.0001M 1.1g $KHCO_3$.

ii. 20% sodium dodecyl sulphate (SDS): (100mL, stored at room temperature to prevent precipitation): 20 g of SDS dissolved in 80mL of milli-Q water which was further made upto 100mL.

iii. Proteinase K (stored at -20^0C): 20mg/mL dissolved in milli-Q water, aliquot in 1.5mL Eppendorf tubes

iv. NaCl : (500mL, saturated solution): 5M 146.1g NaCl

v. 80% Ethanol (freshly prepared): 80mL of 100% ethanol in 20mL of milli-Q water

vi. Tris EDTA buffer (100mL, pH 8)–1M 121.14g Tris-Cl& 0.5M 292.25g EDTA Measure 1mL of 1M Tris-Cl and 0.2mL of 0.5M EDTA and add to Duran bottle. Make up the volume to 100mL by adding 98.8mL distilled water. Close the lid and invert the bottle for few minutes to mix thoroughly.

vii. Isopropyl alcohol

Procedure: This method was based upon salting out of cellular proteins by dehydration and precipitation by saturated NaCl solution.The stored samples were brought to room temperature and vortexed for 1-2 minutes and later transferred to 15 mL falcon tube. The volume was made upto 12 mL with ELB and vortexed vigorously for 2-3 minutes. Any debris or clumps formed in the tube was discarded. The tubes were kept in the refrigerator for 30 minutes. The tubes were centrifuged for 10 min at 3000 rpmand supernatant was disposed, without disturbing the pellet. The pellet was dissolved in 10mL ELB, centrifuged at 3000 rpm for 10 min, supernatant was discarded keeping the pellet. The pellet was dissolved again in 8mL ELB, centrifuged at 3000 rpm for 8 min. 5mL of ELB was added and mix thoroughly. 270 µL of 20% SDS and 30 µL of proteinase K (20mg/mL) were added. The tubes were swirled slowly so that no foam was formed and incubated overnight in 37^{o}C water bath.

500µL of 5M NaCl was added in the falcon tubes drop by drop. Equal volume of Isopropyl alcohol was added, mixed, and swirled gently until the DNA strands are visible. DNA was carefully transferred to an eppendorf containing 500µL of 80% ethanol, air-dried at room temperature for half an hour and centrifuged at 12,000 rpm for 7 minutes. The supernatant was discarded without disturbing the pellet. 500µL of 80% ethanol was added and centrifuged for 5 minutes at 12,000 rpm.

The supernatant was again discarded and centrifuged for 2 min. The supernatant was discarded and the pellet was air dried for 45 minutes. 500µL of Tris EDTA buffer was added and kept in 65^{o}C water bath for half an hour. Eppendorfs were parafilmed and placed in the rotator so that the DNA was solubilised in the TE buffer. DNA sample was stored at -80^{0} C till further analysed.

2. Determination of DNA purity

The DNA obtained from the above procedure was subjected for checking purity by using spectrophotometer against TE buffer as blank. The absorbance of DNA sample mixed with TE buffer was measured at 260nm and 280nm. The purity was representedby the ratio between 260/280 nm, the DNA samples having the ratio between 1.7-1.9 were considered for PCR reactions. The DNA samples with expected purity were used for PCR reactions.

3. Standardization of the Exons of LGALS13 gene

Reference sequence for LGALS13 gene (Accession No: NC_000019.10) was retrieved from the NCBI website. The gene consists of 4 exons spaced by introns. The primer pairs (forward and reverse) for each coding exon were designed using the software IDT (Integrated DNA technologies, Inc, Coralville, IO, USA) and was also checked for hair pin loops, presence of homo and heterodimers, Primer melting temperature Tm (^{o}C), Primer GC content and Primer size. The primers were purchased from Sigma Merck company.

The software In-silico PCR was used to check if the designed primer was amplified. LGALS13 primers details were given in the Table 2.

Reagents for PCR

i. PCR purification kit (Genei Pvt. Ltd)
ii. Taq DNA polymerase buffer
iii. Taq DNA polymerase
iv. dNTP (10mM)
v. Primers (Sigma Aldrich chemicals USA)

Reagents for agarose gel electrophoresis

i. Agarose
ii. Loading dye: 0.042% (W/V) Bromophenol blue powder, 2.5% Ficoll and 0.042% (W/V) Xylene Cyanol FF.
iii. Ethidium bromide
iv. Tris Acetate EDTA buffer (TAE): 50X stock solution: Dissolve 242g Tris base in water adding 57.1mL glacial acetic acid and 100mL of 500mM EDTA (pH 8.0) solution and the volume was made upto 1 liter. 1X TAE buffer was prepared by diluting 20mL of stock into 980mL of deionised water.

Procedure: The gene was standardized using gradient PCR reactions for each exon using forward and reverse primer. The annealing temperatures were calculated based upon the GC and AT content by the formula 4GC+2AT. To know the exact annealing temperature, gradient PCR was run. The amplified products were run on Agarose gel electrophoresis. 2% agarose gels were prepared by dissolving 2g of agarose in 100mL of 1X TAE buffer in a conical flask.

Table 2: Details of LGALS13 primers

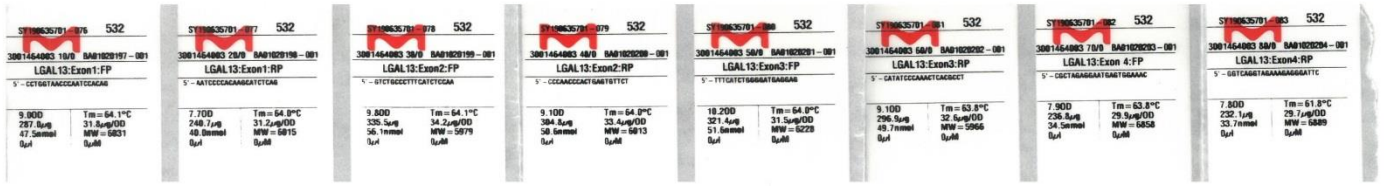
SALES ORDER NO: 3001464003
CUSTOMER NO: 2035582539
SHIPMENT DATE: 25/06/2019

Technical Datasheet

This is computer generated report and needs no signature

INSTITUTE: Orbit Science
RESEARCHER: Orbit Science
PURCHASE ORDER NO: ORBIT/PO/181/SDUAHER

Batch #	Oligo Name	Oligo #	Len	Pur	Scale	MW	Tm°	µg/OD	OD	µg	nmol	Epsilon (/mMcm)	Dimer	2ndry	GC %	µl for 100µM	Sequence(5'-3')
BA01020197	LGAL13:Exon1:FP	3001464003-10/0	20	DST	0.025	6031	64.1	31.8	9.0	287.0	47.5	89.1	No	Weak	55	475	CCTGGTAACCCAATCCACAG
																	Desalt
BA01020198	LGAL13:Exon1:RP	3001464003-20/0	20	DST	0.025	6015	64.0	31.2	7.7	240.7	40.0	92.4	No	None	50	400	AATCCCCACAAGCATCTCAG
																	Desalt
BA01020199	LGAL13:Exon2:FP	3001464003-30/0	20	DST	0.025	5979	64.1	34.2	9.8	335.5	56.1	74.6	No	None	50	561	GTCTGCCCTTTCATCTCCAA
																	Desalt
BA01020200	LGAL13:Exon2:RP	3001464003-40/0	20	DST	0.025	6013	64.0	33.4	9.1	304.8	50.6	79.5	No	Weak	55	506	CCCAACCCACTGAGTGTTCT
																	Desalt
BA01020201	LGAL13:Exon3:FP	3001464003-50/0	20	DST	0.025	6228	64.0	31.5	10.2	321.4	51.6	97.6	No	Moderate	50	516	TTTCATCTGGGGATGAGGAG
																	Desalt
BA01020202	LGAL13:Exon3:RP	3001464003-60/0	20	DST	0.025	5966	63.8	32.6	9.1	296.9	49.7	82.8	No	None	50	497	CATATCCCAAACTCACGCCT
																	Desalt
BA01020203	LGAL13:Exon 4:FP	3001464003-70/0	22	DST	0.025	6858	63.8	29.9	7.9	236.8	34.5	128.7	No	None	50	345	CGCTAGAGGAATGAGTGGAAAC
																	Desalt
BA01020204	LGAL13:Exon4:RP	3001464003-80/0	22	DST	0.025	6889	61.8	29.7	7.8	232.1	33.7	131.4	No	Very Weak	50	337	GGTCAGGTAGAAAGAGGGATTC



SLS/14/12

* Calculation is provided for oligos shipped dry.

Centrifuge tube prior to opening to prevent loss of pelleted oligonucleotide.
For R&D use only. Not for drug, household, or other uses.
Key to Symbols: R = A + G, Y = C + T, M = A + C, K = G + T, S = G + C, W = A + T, H = A + T + C, B = G + T + C, D = G + A + T, N = A + C + G + T, V = G + A + C.
Phosphorothioate linkages = *, 2'O-Methyl RNA = [mA], [mG], [mC], [mU], LNA Bases = [+A], [+G], [+C], [+T], DNA Bases within RNA oligos = [dA], [dC], [dG], [dT]

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Agarose was melted for 45 seconds in oven and allowed to cool at room temperature. 4µL of ethidium bromide was added to cooledagarose and was poured into the gel caster and comb was placed. The bubbles were removed from the surface of the agarose and ensured no bubbles were trapped beneath the comb. The gel was allowed to solidify at room temperature for 30-45 minutes. Gel was transferred to the electrophoresis tank, sufficient 1XTAE was added to cover the gel to the depth of approximately 1mm and the comb was removedcarefully.

4 µL PCR product was mixed with 1µL of loading buffer and loaded into the wells on the submerged gel with a micropipette. 3µL of an appropriate DNA molecular weight marker was also loaded into a well on the gel. The lid of the electrophoretic tank was closed and the run was allowed to take place at 100 volts and 400mA for 45 minutes. The DNA bands were visualized on the gel under UV illumination in Biorad Gel Documentation system and captured in Gel Doc system. The observed annealing temperature for Exon 1 (61.3^{o}), Exon 2 (61.3^{o}), Exon 3 (61.3^{o}) and Exon 4 (64.3^{o}).

4. Amplification of DNA by Polymerase Chain Reaction (PCR)

The isolated DNA from each cord blood sample was subjected to PCR reactions by using both forward and reverse primer of the respective exons at their annealing temperatures. The amplification was assessed by running the amplicons along with the DNA markers on Agarose gel electrophoresis and the bands were visualised in Bio Rad Gel Doc System. The amplified DNA products that exhibited prominent and clear bands on gel were subjected for further purification. The composition of PCR mix is given in Table 3.

5. Purification of PCR amplified products

Purification of the PCR product was carried out according to the protocol provided in the GeneiPure^{TM}Quick PCR purification kit manual (Cat No. 2115300021730) for the removal of primer, primer-dimers and low molecular weight DNA fragments generated by non-specific amplification. 100µl PCR product was added to 500µl of binding buffer and mixed thoroughly. GeneiPure^{TM} Column was placed into 2 ml Collection tube and the sample was loaded into the column and subjected to centrifugation for 1 minute at 11,000 rpm. The flow through was discarded and the GeneiPure^{TM} Column was placed back in the Collection tube.

The GeneiPure^{TM} Column was washed with 500µl with Wash Buffer I, centrifuged at 11,000 rpm for 1 minute and flow through is discarded and the column was placed back in the Collection tube. 150µl of Wash Buffer II is diluted with 600µl of absolute ethanol (1:4) and mixed thoroughly. GeneiPure^{TM} Column was subjected for washing with 700 µl of diluted Wash Buffer II, centrifuged at 11,000 rpm for 1 minute, flow through was discarded and the GeneiPure^{TM} Column was placed back to the Collection Tube. It is again subjected for centrifugation for 2 minutes at 11,000 rpm to remove the traces of Wash Buffer. The Collection Tube was discarded.

The GeneiPure^{TM} Column was opened and placed in a 1.5 sterile vial and incubated for 2 minutes at 70^{o}C to ensure the complete removal of ethanol. In another sterile 1.5ml vial, required amount of Elution Buffer was taken and pre-warmed in a dry bath set at 70^{o}C for 5 minutes. For elution of DNA, 50µl of Elution Buffer was added to the centre of the column and incubated at room

temperature for 1-2 minutes to increase the yield of DNA. The eluted DNA centrifuged for 1 minute at 11,000rpm and stored at -20^{o}C.

6. Sequencing

The purified DNA used for sequencing all the four exons of LGALS13 gene. The procedure consists of a) Amplification of the target DNA b) post-sequencing clean up c) analysis.

a) **Amplification of the target DNA:** The PCR purified products were subjected to sequencing reactions with only the forward primer of the respective exon. The components of the PCR mix for the sequencing reactions are shown in the Table 4.

b) **post-sequencing clean up :**

Reagents:

1. EDTA 125mM (pH 8.0) :
2. Sodium Acetate 3M (pH 5.2):

Master mix 1 consists of 2 µl of 125 mM EDTA and 10 µl sterile Milli Q water. The total reaction volume was 12 µl. Master mix 2 was prepared by adding 2 µl of sodium acetate and 50 µl of ethanol. The total reaction volume was 52 µl. 10 µl of PCR sample was taken in a sterile eppendorf and 12 µl of master mix 1 and 52 µl of master mix 2 was added. The eppendorf was vortexed and incubated at room temperature for 15 minutes followed by a spin at 14 thousand rpm for 5 minutes. The supernatant was discarded by blotting on the tissue paper. 250 µl of 70% ethanol was added and the eppendorf was incubated at room temperature for 5 minutes and spun down for 10 minutes at 12 thousand rpm followed by the removal of supernatant by blotting on the tissue paper.

Table 3: Composition of PCR mix

S.No.	Components	Final Volume(µl)
1.	Forward primer	1.0
2.	Reverse primer	1.0
3.	DNA sample	3.0
4.	1X Taq Buffer with $MgCl_2$	4.0
5.	dNTPs	2.5
6.	Taq polymerase	0.3
7.	Milli Q water	13.2
	Total reaction volume	**25.0**

Table 4: Composition of PCR mix for sequencing PCR

S.No.	Components	Final Volume (µl)
1.	Forward primer	2.0
2.	DNA sample	2.0
3.	Dilution Buffer	1.5
4.	Dideoxy nucleotides	1.0
5.	Milli Q water	4.0
	Total reaction volume	**10.0**

The pellet was dried at room temperature for 15 minutes and 12 µl of Hi-Di™ Formamide (Applied Biosystems). The solution was mixed with the micropipette properly along the walls of the eppendorf and subjected to denature in PCR at 96^{o}C for 2 minutes and snap chilled at 4^{o}C.

c) Sequencing of exons: On completion of post-sequencing clean up of sequencing DNA of all the exons were subjected for sequencing. DNA sequencing was performed for all the four exons of the LGALS13 gene with BigDye® Terminator v3.1 Cycle Sequencing Kit using ABI-3500 Genetic Analyzer (Applied Biosystems, USA) according to the manufacturer's instructions. The results obtained from sequencing of all the exons were recorded and subjected for data analysis to identify any polymorphism.

Data analysis

Chromas software was used to get the FASTA sequence. Mutational analysis was performed using clustal omega multi-sequencing software by comparing with the reference LGALS13 gene sequence.

According to WHO, in pregnancy ≥ 11g/dL is non-anemia, < 11g/dL as anemia, <10 g/dL as moderate anemia and < 7g/dL as severe anemia [128]. The criteria for the diagnosis of preeclampsia was systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90mmHg recorded with 4 hours apart on two occasions, plus dipstick Proteinuria ≥ 2+ after 20th week of pregnancy. Gestational age (weeks) was calculated based on the first day of the last menstrual period. According to WHO, the infants are classified as Low Birth Weight (LBW) with <2500 grams and normal birth weight as 2500-4000 grams[129].

4.3. STATISTICAL ANALYSIS

The research data of the study was not normally distributed and hence the results were represented as median (inter-quartile range) and the level of significance between the measured parameters in first and second trimesters was derived from Wilcoxon signed rank test. To assess the correlation between the measured parameters in preeclampsia group and control group, Spearman's rank correlation was used. ROC curve was plotted to know the diagnostic performance of the parameters. Statistical analysis was done using SPSS licensed version IBM 22.0 and p-value<0.05 was considered statistically significant.

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CHAPTER-5

RESULTS &DISCUSSION

5.0. RESULTS AND DISCUSSION

5.1. Results

The study population comprised of the normotensive pregnant women (n=268) at their first trimester in the age group of 20-30 years. The demographic details were recorded and blood samples were collected during their first trimester (8-11 weeks) visit for antenatal check up. The same group were followed in a longitudinal manner to second trimester and up to delivery.

During follow-up, 12 women underwent spontaneous abortions after their first trimester. The results observed from the 12 abortive cases were also recorded. So the number of subjects entered into second trimester were n=256. Blood samples were collected in the second trimester (20-24 weeks). The number of subjects that developed preeclampsia during follow up were recorded (n=22). The basal first trimester data and the second trimester data of the preeclamptic cases (n=22) and normotensive group (n=248) were compared to find out any research evidence of the study parameters that can serve as a predictive marker.

The haematological and biochemical characteristics of normotensive group and preeclampsia were illustrated in Table 5. The investigations were screened as general characteristics during patient visit to OBG department for antenatal check-up were presented. The women who developed preeclampsia had median maternal age 21 years with hemoglobin levels < 11g/dL.

Table 5: Comparison of I trimester maternal characteristics, hematological and biochemical parameters in preeclampsia and normotensive women

Variables	Trimester-I (n = 22)		Trimester-I (n = 246)		**Spontaneous abortions (n=12)**	
	Median	**IQR**	**Median**	**IQR**	**Median**	**IQR**
Maternal age (years)	21	20-22	24	22-25	21	20-22
Body weight (kg)	51	47-54	53	51-54	52	51-54
Height (m)	1.56	1.48-1.6	1.56	1.49-1.6	1.53	1.5-1.57
BMI (kg/m^2)	20.31	18.59-23.03	21.33	18.1-22.65	21.22	18.32-22.1
SBP (mmHg)	110	100-200	110	100-110	110	100-200
DBP (mmHg)	70	65-80	70	70-80	70	65-80
MAP(mmHg)	83.33	78.33-93.33	83.33	76.66-90	83.33	78.33-93.3
Haemoglobin (gm%)	10.4	9.2-11.35	12.2	11.5-12.7	12.15	11.5-12.7
Platelets ($10^3/\mu L$)	265	238-280	266	254-315	266	254-315
Total Count (mm^3)	8.21	7.40-8.8	8.2	7.6-8.6	8.0	7.6-8.6
MCV (Fl/red cell)	73.11	72.8-77.6	87.1	85.4-89.9	87.1	85.4-89.9
MCH (pg/cell)	24.31	20.21-26.13	28.42	26.44-30.22	27.22	26.44-30.2
RBS (mg/dL)	99	95.75-108.2	100.5	95.75-110	97.5	95.75-110
Creatinine (mg/dL)	0.7	0.6-0.8	0.7	0.6-0.8	0.7	0.6-0.8
Blood Urea (mg/dL)	22	20.5-25.5	24	21-27	25	21-27
Calcium (mg/dL)	9	8-10	9.9	8.9-10.8	9.9	8.9-10.8
Uric acid (mg/dL)	3.2	2.9-3.45	3.3	3.1-3.8	3.3	3.1-3.8

*$p<0.05$ statistically significant.BMI – body mass index; SBP- systolic blood pressure; DBP- diastolic blood pressure; MAP - mean arterial pressure.

Table 6 depicts the median and inter-quartile range of TOS, TAS and OSI in first and second trimesters of normotensive women and the cases developed preeclampsia.

In normotensive pregnant women, even though TOS was elevated, simultaneously to counteract the oxidative stress, TAS was also elevated. Therefore OSI was marginal between trimesters. However, the basal values of the preeclampsia group indicated 2.4 fold increase of oxidative stress evinced minimal increase of antioxidant status. The OSU in normotensive pregnant group is marginally declined between first and second trimesters whereas the same indicator in second trimester of preeclampsia developed cases elevated to 2.6 fold when compared basal values in the first trimester. The research resultsdenotes the characteristic cause in onset of preeclampsia is linked to elevated oxidative stress with inadequate TAS.

Thereby concentration of free radical species in a hypoxic environment play predominant role as one of the causative factor in onset of preeclampsia.

Table 6: Oxidative Stress Index in I & II trimesters of normotensive and cases developed preeclampsia

Variables	Trimester- I (n = 246) Normotensive		Trimester-II (n = 234) Normotensive		
	Median	**IQR**	**Median**	**IQR**	**P**
Total Oxidant Status (U/mL)	33.08	30.53-42.8	55.5	50.15-60.4	<0.001*
Total Antioxidant Status (U/mL)	15.47	14.22-22.3	34.67	8.80-50.0	<0.001*
Oxidative Stress Index (TOS/TAS)	2.1	2-2.20	1.61	1.12-5.67	0.191
	Trimester- I (n = 22) Preeclampsia		**Trimester-II (n = 22) Preeclampsia**		
	Median	**IQR**	**Median**	**IQR**	**p**
Total Oxidant Status (U/mL)	37	34.5-38	89.67	76.1-107.26	<0.001*
Total Antioxidant Status (U/mL)	14.65	13.93-18.1	20	6.22-24	0.300
Oxidative Stress Index (TOS/TAS)	2.02	1.85-2.23	5.29	3.69-16.28	<0.001*

*p<0.05 statistically significant, IQR inter-quartile range

Table 7 illustrates the values of Placental protein 13, Caspase 3 and XO activity in I& II trimesters of normotensive and preeclampsia group. PP13 level observed considerably lowered by 1.54 fold in first trimester compared to the second trimester in the normotensive pregnant group. Whereas fourfold decrease of PP13 value observed in the baseline data of the cases developed preeclampsia in the study group.

The overall research results emphasize that the predominant role of PP13 during placentation process and also signifies the key importance of considering as a biochemical indicator during pregnancy period to understand early the possible implication of disease process and the probability of such subjects develops preeclampsia.

In addition to this, elevated XO enzyme activity also contributes in predicting preeclampsia. XO activity was not increased to an appreciable level in first and second trimester of normotensive pregnant. However in cases of preeclampsia, its activity doubled. Therefore screening of XO activity along with oxidative stress index provides useful information in diagnosis of preeclampsia. XO activity at first trimester of pregnancy irrespective of the vulnerability of the subjects towards preeclampsia implies almost same value. While the normotensive subjects in their pregnancy period exhibited almost similar value in first and second trimester. The notable research outcome is that the cases prone towards preeclampsia and developed preeclampsia at second trimester indicated significant elevated XO activity. Hence, study propose that screening of XO activity has diagnostic importance in predicting preeclampsia.

Cellular Caspase-3 is a terminal enzyme in a cascade process of apoptosis. In pregnancy, the involvement of placenta and itsexposure to oxidative stress alters the cellular integrity and releases to blood. Caspase-3 concentration at first and second

trimesters gradually increases by 2 fold in normotensive and 3 fold in preeclampsia. Therefore oxidative stress index, PP13, XO activity and Caspase-3 levels are elevated in the same manner in preeclampsia condition. Therefore, Caspase-3 level observed increase in second trimester of normotensive (11 ng/ml) and further higher value in preeclampsia (18 ng/ml). The data of Caspase-3 measurement facilitates to diagnose the involvement of placental pathology in preeclampsia and serves as a cost effective marker.

Table 8 narrates the parameters linked to vascular function in normotensive pregnancy and in preeclampsia. The Nitric oxide plays a predominant role in the regulation of blood pressure. Therefore adequate concentration of Nitric oxide is of paramount importance. However generation of Nitric oxide by NOS in the endotheliAL cell is regulated by various factors. ADMA is of one such factor alters the activity of NOS and thus alters Nitric oxide level. Most commonly Nitric oxide is detected in the plasma as a stable form of nitrate. The Nitric oxide level in second trimester increases by two fold in normotensive group and also cases developed preeclampsia. Since the Nitric oxide level is unaltered in the second trimester of normotensive group and in preeclampsia cases, determination of Nitric oxide as a single parameter is not reliable and conclusive in early prediction of preeclampsia.

Table 7: Placental protein 13, Caspase-3 and Xanthine oxidase activity in I & II trimesters of normotensive and cases that developed preeclampsia

Variables	Trimester- I (n = 246) Normotensive		Trimester-II (n = 234) Normotensive		
	Median	**IQR**	**Median**	**IQR**	**P**
Placental protein 13 (pg/mL)	205.91	102-296	323.38	189-463	<0.001*
Xanthine oxidase activity (mU/mL)	12.5	10.8-14.7	13.5	12.3-16.4	<0.001*
Caspase-3 (ng/mL)	5.56	5-6.20	10.9	9.88-12.07	<0.001*
	Trimester- I (n = 22) Preeclampsia		**Trimester-II (n = 22) Preeclampsia**		
	Median	**IQR**	**Median**	**IQR**	**P**
Placental protein 13 (pg/mL)	110.98	99-221	429.78	344-540	<0.001*
Xanthine oxidase activity (mU/mL)	12.2	10.8-13.7	33.9	22.15-42.5	<0.001*
Caspase-3 (ng/mL)	5.8	4.9-6.4	17.99	10.11-19.35	<0.001*

*p<0.05 statistically significant, IQR inter-quartile range

Whereas ADMA serves as a significant marker by virtue of its diagnostic importance. As per the research results ADMA level in second trimester of normotensive is increased by 2 fold (45 ng/ml). The same parameter increased by 4 fold in preeclampsia (84.69 ng/mL) determines ideal conditions to serve as a marker in assessment of preeclampsia at early stage and found to be statistically significant. In the second trimester, the normotensive group showed marginal increase in ADMA: Nitric oxide where as preeclampsia group showed 2 fold increases from its base line first trimester values. Two fold increase in ADMA:Nitric oxide was also observed between normotensive group and preeclampsia in second trimester.

Table 9 reflects fetal outcome and gestational age at delivery. Normotensive group and cases of preeclampsia reveals lower gestational age and slightly lowered birth weight in preeclampsia. Significant difference was noted with respect to gestational age by 2 weeks difference without contributing much for fetal weight.

However, the preeclampsia developed cases have less haemoglobin level <11gm/dl compared to the normotensive group <14gm/dl. Thereby there is direct evidence linked between maternal anemia and low birth weight. Based on the available information, feto-maternal circulation of oxygen and increased oxidative stress is associated with impaired placentation and vascularisation.

Table 8: Showing the concentrations of Nitric oxide, ADMA and ADMA:Nitric oxide in relation to endothelial functionin I& II trimesters of normotensive and cases developed preeclampsia

Variables	Trimester- I (n = 246) Normotensive		Trimester-II (n = 234) Normotensive		
	Median	**IQR**	**Median**	**IQR**	**P**
Nitric oxide (nmol/µL)	2.43	2.02-3.12	4.45	4.14-4.78	<0.001*
ADMA (ng/mL)	20.99	16.43-24.77	45.65	37.99-51.02	<0.001*
ADMA:Nitric oxide	9:1		10:1		
	Trimester- I (n = 22) Preeclampsia		**Trimester-II (n = 22) Preeclampsia**		
	Median	**IQR**	**Median**	**IQR**	**P**
Nitric oxide (nmol/µL)	2.22	1.83-3.01	4.33	4.09-4.8	<0.001*
ADMA (ng/mL)	21.6	19.61-23.38	84.69	74.57-96.37	<0.001*
ADMA:Nitric oxide	10:1		20:1		

*p<0.05 statistically significant, IQR interquartile range

Table 9: Showing fetal outcome in study population

Parameter	Normotensive (n = 234)	Preeclampsia (n = 22)
Gestational age at delivery (weeks)	39 [38-40]	37 [38-39]
Infant birth weight (kgs)	3.0[2.60-3.0]	2.40[2.2-2.4]

*p<0.05 statistically significant, IQR inter-quartile range

Table 10 shows the correlation between OSI, Caspase-3 and PP13 with endothelial function markers at first trimester in normotensive group and preeclampsia. Among normotensive group, OSI is very weak negative correlated with Nitric oxide and it was found to be statistically significant ($p=0.030$). Whereas ADMA has a moderate correlation which was found to be statistically significant ($p<0.001$). Caspase-3 has a positive correlation with Nitric oxide which was not statistically significant but has a fair degree of negative correlation with ADMA and was also found to be statistically significant ($p<0.001$). But PP13 has a very weak non-significant positive correlation with Nitric oxide and ADMA.

Among preeclampsia, OSI shows a positive correlation with Nitric oxide and ADMA which is a weak correlation and was not statistically significant. Whereas Caspase-3 has a negative weak correlation with Nitric oxide but has weak positive correlation with ADMA which was not found to be statistically significant.Whereas PP13 has a weak positive correlation with Nitric oxide but positive correlation with respect to ADMA.

Table 11 shows the correlation between OSI, Caspase-3 and PP13 with endothelial function markers at second trimester in normotensive group and preeclampsia. In normotensive group, in the second trimester OSI and Nitric oxide shows weak correlation which was statistically significant. Similarly with ADMA, OSI showed a positive non-significant positive correlation. Whereas Nitric oxide and ADMA has no significant correlation with Caspase 3. Nitric oxide and ADMA did not show any significant correlation.

Table 10: Correlation between Oxidative Stress Index, Caspase-3 and PP13 with endothelial function parameters at first trimester in normotensive pregnant women and in preeclampsia

Normotensive group	Spearman's rho (ρ)	P	Preeclampsia	Spearman's rho (ρ)	P
OSI& Nitric oxide	-0.139	0.030*	**OSI& Nitric oxide**	+0.026	0.901
OSI& ADMA	+ 0.230	<0.001*	**OSI& ADMA**	+0.176	0.399
Caspase-3 &Nitric oxide	+0.108	0.093	**Caspase-3 &Nitric oxide**	-0.201	0.335
Caspase-3& ADMA	-0.250	<0.001*	**Caspase-3& ADMA**	+0.015	0.943
PP13 & Nitric oxide	+0.103	0.108	**PP13 & Nitric oxide**	+0.119	0.570
PP13 & ADMA	+0.106	0.099	**PP13 & ADMA**	+0.346	0.090

*p<0.05 = statistically significant

Table 11: Correlation between Oxidative Stress Index, Caspase-3 and PP13 with endothelial function parameters at second trimester in normotensive pregnant women and in preeclampsia

Normotensive group	Spearman's rho (ρ)	p	Preeclampsia	Spearman's rho (ρ)	p
OSI& Nitric oxide	+0.242	<0.001*	**OSI& Nitric oxide**	-0.034	0.879
OSI& ADMA	+0.329	0.109	**OSI& ADMA**	+0.410	<0.001*
Caspase-3 & Nitric oxide	+0.010	0.875	**Caspase-3 & Nitric oxide**	- 0.339	0.097
Caspase-3& ADMA	-0.023	0.729	**Caspase-3& ADMA**	+0.308	0.134
PP13 & Nitric oxide	-0.074	0.265	**PP13 & Nitric oxide**	-0.487	0.016*
PP13 & ADMA	-0.121	0.068	**PP13 & ADMA**	+0.405	0.044*

*p<0.05 = statistically significant

In second trimester among the preeclampsia group, OSI and Nitric oxidehas a very weak negative correlation, OSI and ADMA has a moderately high correlation which was found to be statistically significant ($p < 0.001$). Caspase-3 shows a negative correlation with Nitric oxide which was not statistically significant. ADMA has a positive correlation with Caspase-3 and not found to be statistically significant. PP13 was negatively correlated with Nitric oxide and showed significance ($p=0.016$). ADMA has a significant positive correlation with PP13 ($p=0.044$).

Table 12 illustrates the sensitivity, specificity and area under curve of the parameters analyzed in first trimester. The data showed good area under curve for PP13, ADMA, TAS and OSI in preeclampsia.

Table 13 illustrates the sensitivity, specificity and area under curve of the parameters analyzed in second trimester. The data showed good area under curve for PP13, Caspase-3, XO activity, ADMA and TOS in second trimester in women who developed preeclampsia.

Table 12: Receivers operating characteristic curves for the parameters in first trimester of pregnancy

Trimester-I						
Parameter	**Cut-off**	**Sensitivity (%)**	**Specificity (%)**	**AUC**	**95% CI Range**	**p**
PP13 (pg/mL)	117.77	95	78	0.932	0.855- 1.00	<0.001
ADMA(ng/mL)	19.7	72	78	0.733	0.921-1.00	0.008
TAS (U/mL)	20.06	91	78	0.807	0.657-0.95	<0.001
OSI	1.86	73	82	0.747	0.586-0.90	0.005

Table 13: Receivers operating characteristic curves for the parameters in second trimester of pregnancy

Trimester-II						
Parameter	**Cut-off**	**Sensitivity (%)**	**Specificity (%)**	**AUC**	**95% CI Range**	**p**
PP13 (pg/mL)	371.25	95	87	0.955	0.887-1.00	<0.001
Caspase-3 (ng/mL)	12.9	64	82	0.704	0.53-0.877	0.021
XO activity (mU/mL)	17.25	95	78	0.969	0.921-1.00	<0.001
ADMA(ng/mL)	50.36	100	100	1.00	1.00-1.00	<0.001
TOS (U/mL)	53.76	95	82	0.971	0.928-1.00	<0.001

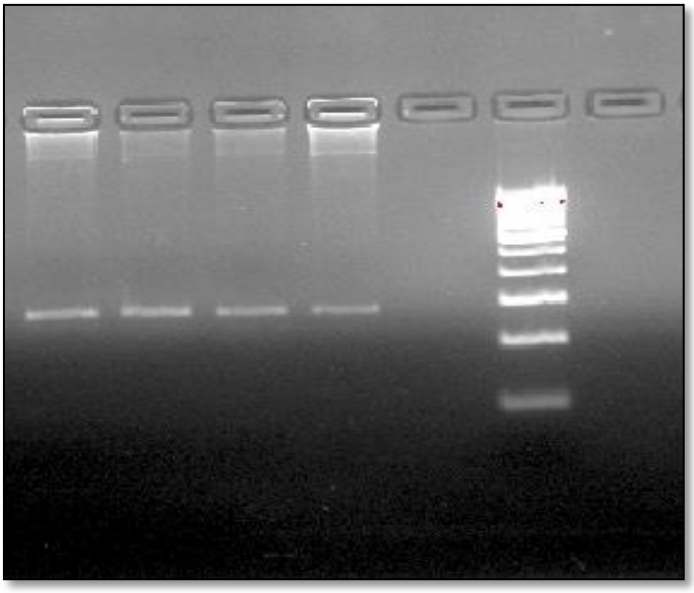

Figure 15: Gel picture of standardization of the four Exons of LGALS13 gene

```
Reference_Promoter      AAGCCCGAGGGCAAGGCTGAAGTGGGTCATTTAAATGCTGCAACTCAGAGATTCACTCAG    60
S2                      AAGCCCGAGGGCAAGGCTGAAGTGGGTCATTTAAATGCTGCAACTCAGAGATTCACTCAG    60
S4                      AAGCCCGAGGGCAAGGCTGAAGTGGGTCATTTAAATGCTGCAACTCAGAGATTCACTCAG    60
S5                      AAGCCCGAGGGCAAGGCTGAAGTGGGTCATTTAAATGCTGCAACTCAGAGATTCACTCAG    60
S6                      AAGCCCGAGGGCAAGGCTGAAGTGGGTCATTTAAATGCTGCAACTCAGAGATTCACTCAG    60
S8                      AAGCCCGAGGGCAAGGCTGAAGTGGGTCATTTAAATGCTGCAACTCAGAGATTCACTCAG    60
S13                     AAGCCCGAGGGCAAGGCTGAAGTGGGTCATTTAAATGCTGCAACTCAGAGATTCACTCAG    60
S17                     AAGCCCGAGGGCAAGGCTGAAGTGGGTCATTTAAATGCTGCAACTCAGAGATTCACTCAG    60
S19                     AAGCCCGAGGGCAAGGCTGAAGTGGGTCATTTAAATGCTGCAACTCAGAGATTCACTCAG    60
S20                     AAGCCCGAGGGCAAGGCTGAAGTGGGTCATTTAAATGCTGCAACTCAGAGATTCACTCAG    60
S22                     AAGCCCGAGGGCAAGGCTGAAGTGGGTCATTTAAATGCTGCAACTCAGAGATTCACTCAG    60
S1                      AAGCCAGAGGGCAAGGCTGAAGTGGGTCATTTAAATGCTGCAACTCAGAGATTCACTCAG    60
S3                      AAGCCAGAGGGCAAGGCTGAAGTGGGTCATTTAAATGCTGCAACTCAGAGATTCACTCAG    60
S7                      AAGCCAGAGGGCAAGGCTGAAGTGGGTCATTTAAATGCTGCAACTCAGAGATTCACTCAG    60
S9                      AAGCCAGAGGGCAAGGCTGAAGTGGGTCATTTAAATGCTGCAACTCAGAGATTCACTCAG    60
S10                     AAGCCAGAGGGCAAGGCTGAAGTGGGTCATTTAAATGCTGCAACTCAGAGATTCACTCAG    60
S11                     AAGCCAGAGGGCAAGGCTGAAGTGGGTCATTTAAATGCTGCAACTCAGAGATTCACTCAG    60
S12                     AAGCCAGAGGGCAAGGCTGAAGTGGGTCATTTAAATGCTGCAACTCAGAGATTCACTCAG    60
S14                     AAGCCAGAGGGCAAGGCTGAAGTGGGTCATTTAAATGCTGCAACTCAGAGATTCACTCAG    60
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S16                     AAGCCAGAGGGCAAGGCTGAAGTGGGTCATTTAAATGCTGCAACTCAGAGATTCACTCAG    60
S18                     AAGCCAGAGGGCAAGGCTGAAGTGGGTCATTTAAATGCTGCAACTCAGAGATTCACTCAG    60
S21                     AAGCCAGAGGGCAAGGCTGAAGTGGGTCATTTAAATGCTGCAACTCAGAGATTCACTCAG    60
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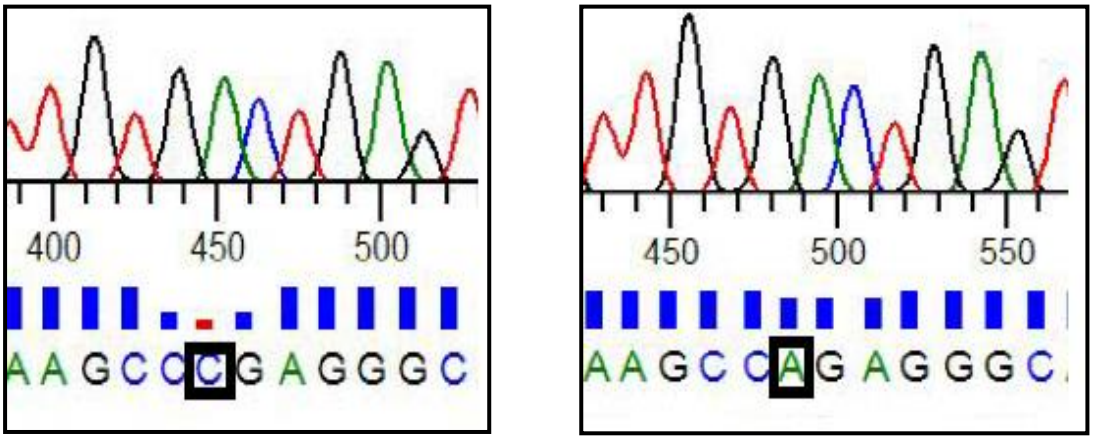
Figure 16: Alignment of promoter sequence of LGALS13 gene of samples with NCBI reference sequence of the promoter region. Samples S1 to S21 showing the -98 SNP (Cytosine replaced by Adenine)



-98 C

-98 A promoter variant

Figure 17: Sequencing Chromatograms showing SNP at -98 position in promoter region of LGALS 13 gene in preeclampsia in comparison with promoter region of normal pregnant group

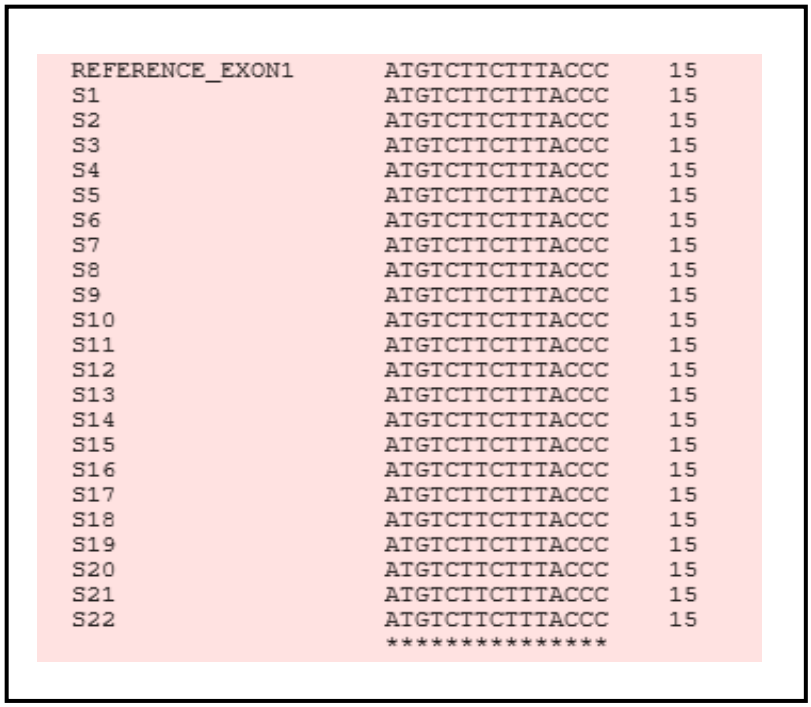


Figure 18: Alignment of Exon 1 sequence of LGALS13 gene of samples with NCBI reference sequence of Exon 1

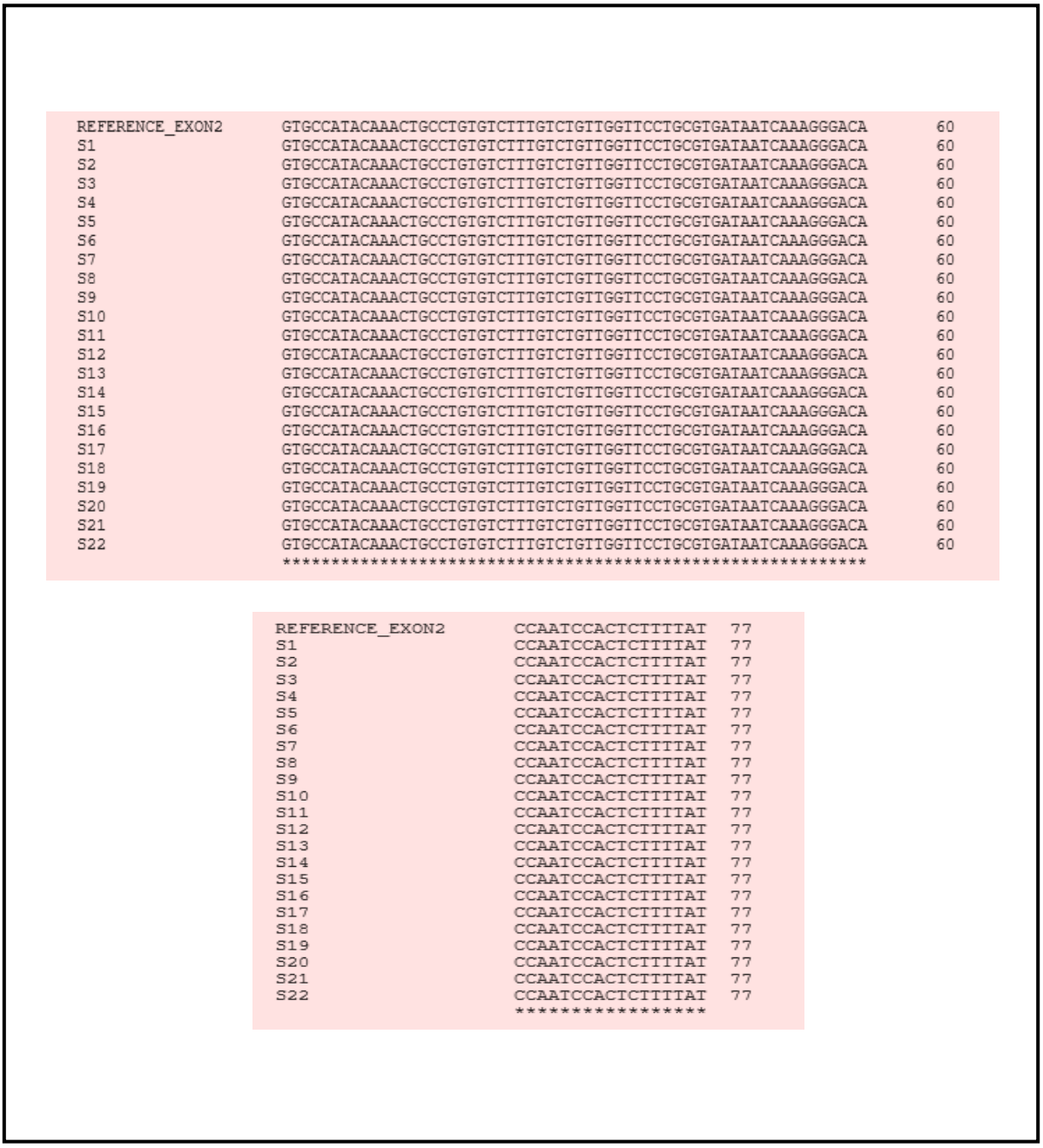


Figure 19: Alignment of Exon 2 sequence of LGALS13 gene of samples with NCBI reference sequence of Exon 2

```
REFERENCE_EXON3      CAATGACCCACAGCTGCAGGTGGATTTCTACACTGACATGGATGAGGATTCAGATATTGC      60
S1                   CAATGACCCACAGCTGCAGGTGGATTTCTACACTGACATGGATGAGGATTCAGATATTGC      60
S2                   CAATGACCCACAGCTGCAGGTGGATTTCTACACTGACATGGATGAGGATTCAGATATTGC      60
S3                   CAATGACCCACAGCTGCAGGTGGATTTCTACACTGACATGGATGAGGATTCAGATATTGC      60
S4                   CAATGACCCACAGCTGCAGGTGGATTTCTACACTGACATGGATGAGGATTCAGATATTGC      60
S5                   CAATGACCCACAGCTGCAGGTGGATTTCTACACTGACATGGATGAGGATTCAGATATTGC      60
S6                   CAATGACCCACAGCTGCAGGTGGATTTCTACACTGACATGGATGAGGATTCAGATATTGC      60
S7                   CAATGACCCACAGCTGCAGGTGGATTTCTACACTGACATGGATGAGGATTCAGATATTGC      60
S8                   CAATGACCCACAGCTGCAGGTGGATTTCTACACTGACATGGATGAGGATTCAGATATTGC      60
S9                   CAATGACCCACAGCTGCAGGTGGATTTCTACACTGACATGGATGAGGATTCAGATATTGC      60
S10                  CAATGACCCACAGCTGCAGGTGGATTTCTACACTGACATGGATGAGGATTCAGATATTGC      60
S11                  CAATGACCCACAGCTGCAGGTGGATTTCTACACTGACATGGATGAGGATTCAGATATTGC      60
S12                  CAATGACCCACAGCTGCAGGTGGATTTCTACACTGACATGGATGAGGATTCAGATATTGC      60
S13                  CAATGACCCACAGCTGCAGGTGGATTTCTACACTGACATGGATGAGGATTCAGATATTGC      60
S14                  CAATGACCCACAGCTGCAGGTGGATTTCTACACTGACATGGATGAGGATTCAGATATTGC      60
S15                  CAATGACCCACAGCTGCAGGTGGATTTCTACACTGACATGGATGAGGATTCAGATATTGC      60
S16                  CAATGACCCACAGCTGCAGGTGGATTTCTACACTGACATGGATGAGGATTCAGATATTGC      60
S17                  CAATGACCCACAGCTGCAGGTGGATTTCTACACTGACATGGATGAGGATTCAGATATTGC      60
S18                  CAATGACCCACAGCTGCAGGTGGATTTCTACACTGACATGGATGAGGATTCAGATATTGC      60
S19                  CAATGACCCACAGCTGCAGGTGGATTTCTACACTGACATGGATGAGGATTCAGATATTGC      60
S20                  CAATGACCCACAGCTGCAGGTGGATTTCTACACTGACATGGATGAGGATTCAGATATTGC      60
S21                  CAATGACCCACAGCTGCAGGTGGATTTCTACACTGACATGGATGAGGATTCAGATATTGC      60
S22                  CAATGACCCACAGCTGCAGGTGGATTTCTACACTGACATGGATGAGGATTCAGATATTGC      60
                     ************************************************************
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REFERENCE_EXON3      CTTCCGTTTCCGAGTGCACTTTGGCAATCATGTGGTCATGAACAGGCGTGAGTTTGGGAT      120
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S2                   CTTCCGTTTCCGAGTGCACTTTGGCAATCATGTGGTCATGAACAGGCGTGAGTTTGGGAT      120
S3                   CTTCCGTTTCCGAGTGCACTTTGGCAATCATGTGGTCATGAACAGGCGTGAGTTTGGGAT      120
S4                   CTTCCGTTTCCGAGTGCACTTTGGCAATCATGTGGTCATGAACAGGCGTGAGTTTGGGAT      120
S5                   CTTCCGTTTCCGAGTGCACTTTGGCAATCATGTGGTCATGAACAGGCGTGAGTTTGGGAT      120
S6                   CTTCCGTTTCCGAGTGCACTTTGGCAATCATGTGGTCATGAACAGGCGTGAGTTTGGGAT      120
S7                   CTTCCGTTTCCGAGTGCACTTTGGCAATCATGTGGTCATGAACAGGCGTGAGTTTGGGAT      120
S8                   CTTCCGTTTCCGAGTGCACTTTGGCAATCATGTGGTCATGAACAGGCGTGAGTTTGGGAT      120
S9                   CTTCCGTTTCCGAGTGCACTTTGGCAATCATGTGGTCATGAACAGGCGTGAGTTTGGGAT      120
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S11                  CTTCCGTTTCCGAGTGCACTTTGGCAATCATGTGGTCATGAACAGGCGTGAGTTTGGGAT      120
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S19                  CTTCCGTTTCCGAGTGCACTTTGGCAATCATGTGGTCATGAACAGGCGTGAGTTTGGGAT      120
S20                  CTTCCGTTTCCGAGTGCACTTTGGCAATCATGTGGTCATGAACAGGCGTGAGTTTGGGAT      120
S21                  CTTCCGTTTCCGAGTGCACTTTGGCAATCATGTGGTCATGAACAGGCGTGAGTTTGGGAT      120
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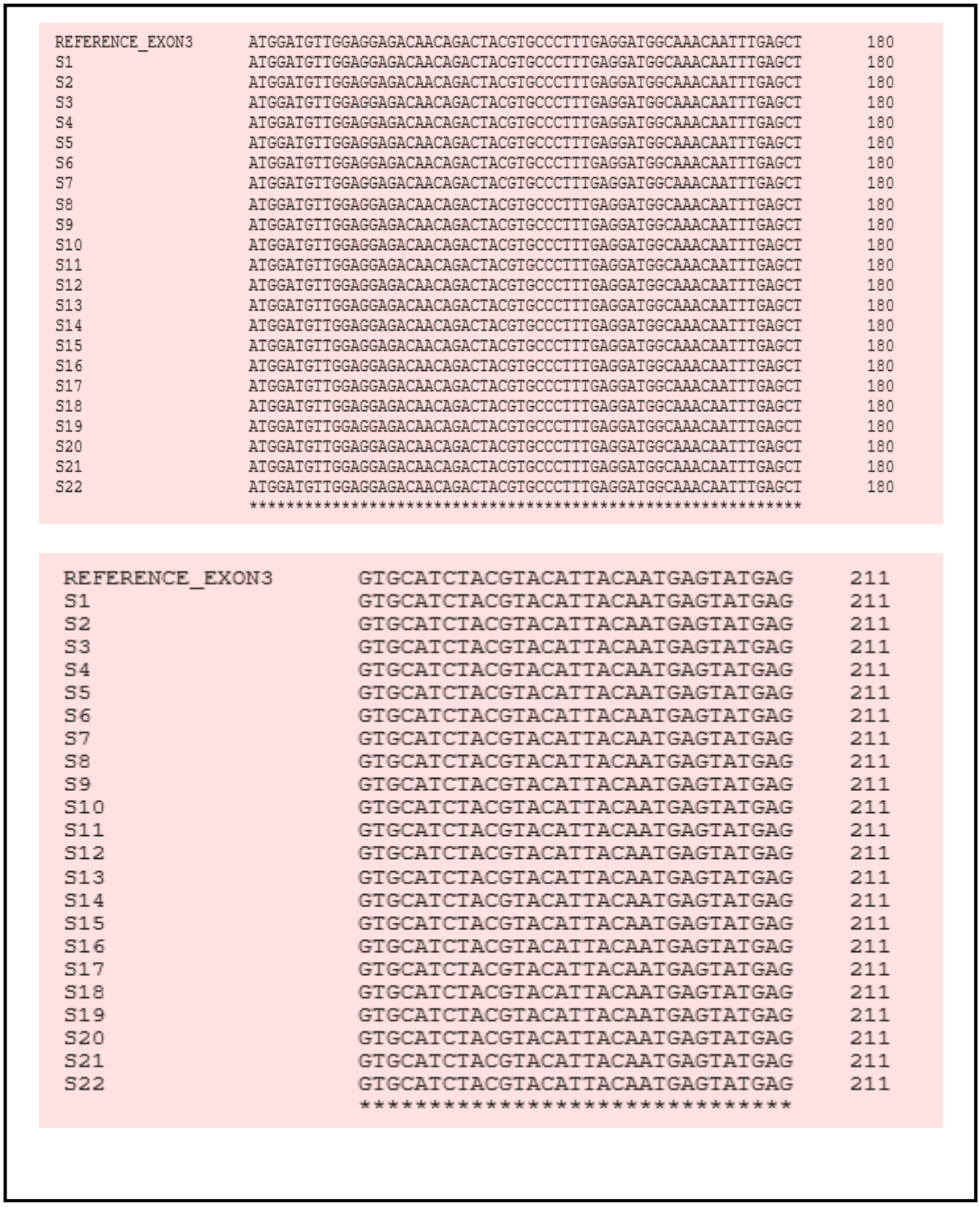

Figure 20: Alignment of Exon 3 sequence of LGALS13 gene of samples with NCBI reference sequence of Exon 3

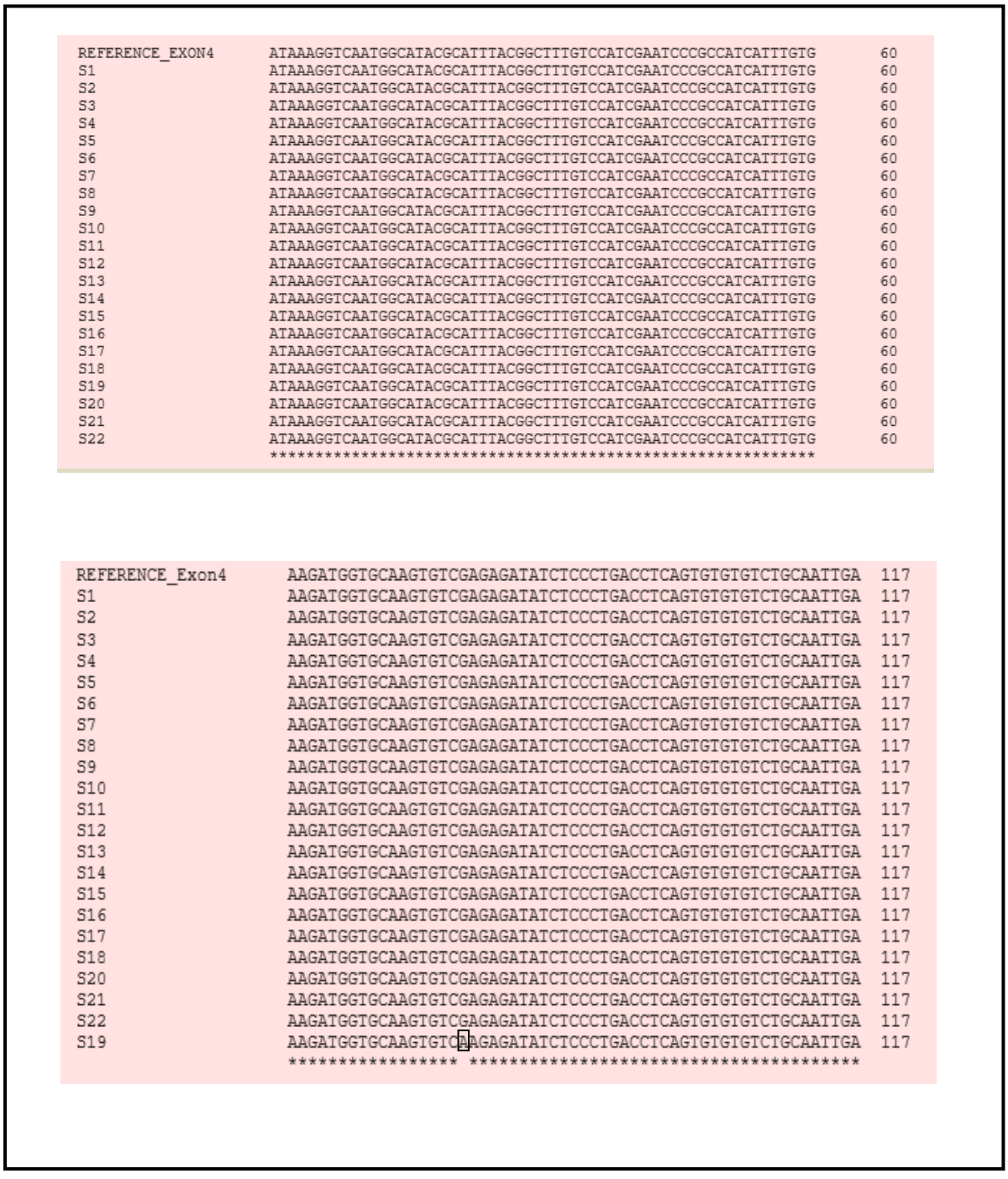

Figure 21: Alignment of Exon 4 sequence of LGALS13 gene of samples with NCBI reference sequence of Exon 4. Silent mutation inC.78 G>A; P.26 serine–serine (S19)

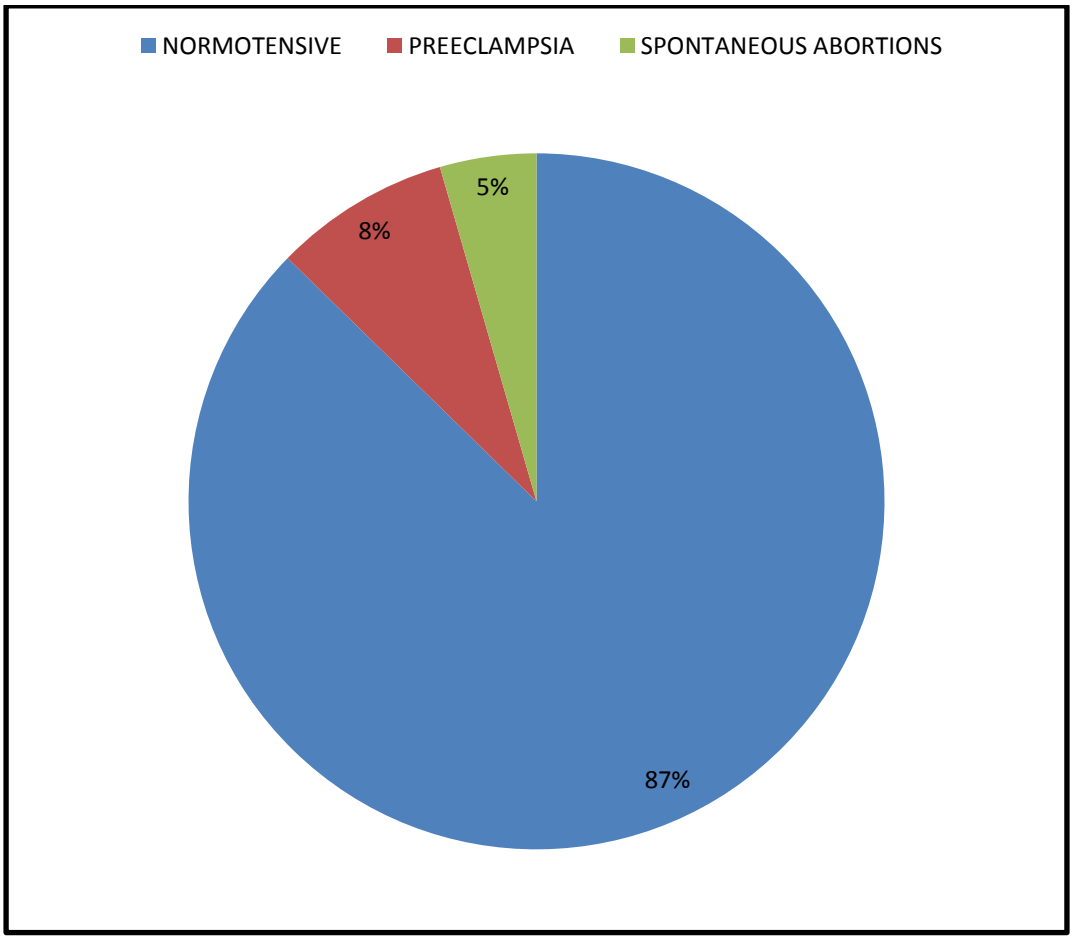

Figure 22: Pie-chart Showing distribution of sub groups as Normotensive/ Preeclampsia/Spontaneous abortions in percentage

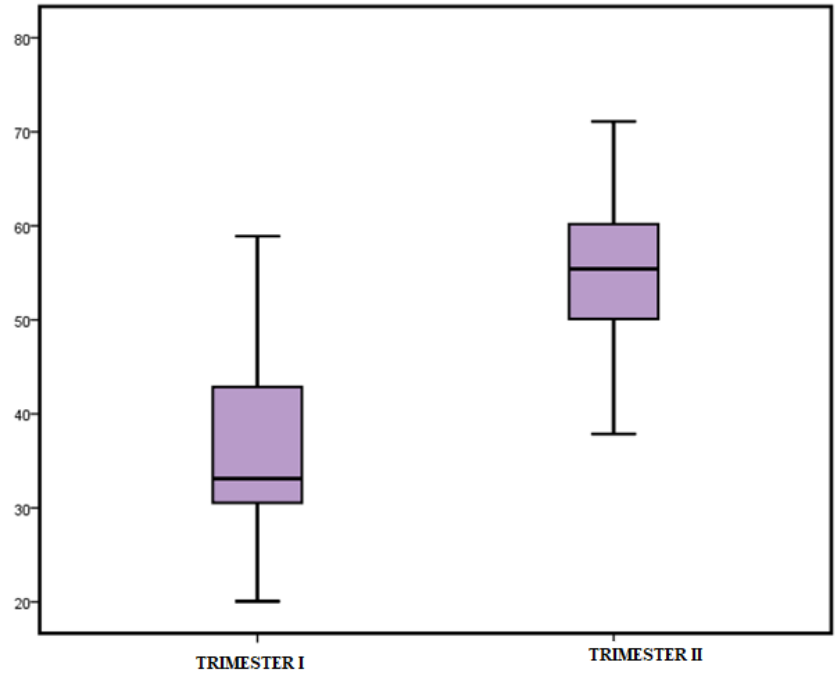

Figure 23: Depicting the median TOS concentration in normotensive pregnant women at first and second trimesters of pregnancy

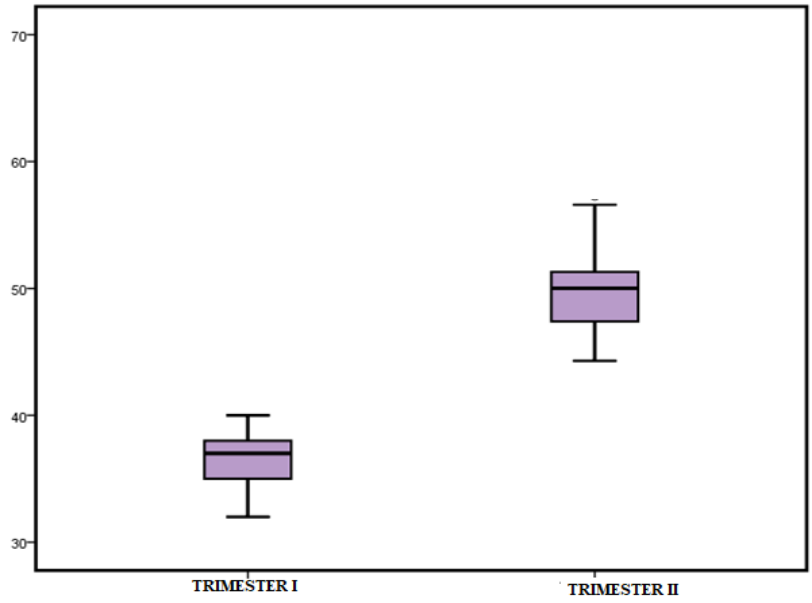

Figure 24: Depicting the median TAS concentration in cases developed preeclampsia at first and second trimesters of pregnancy

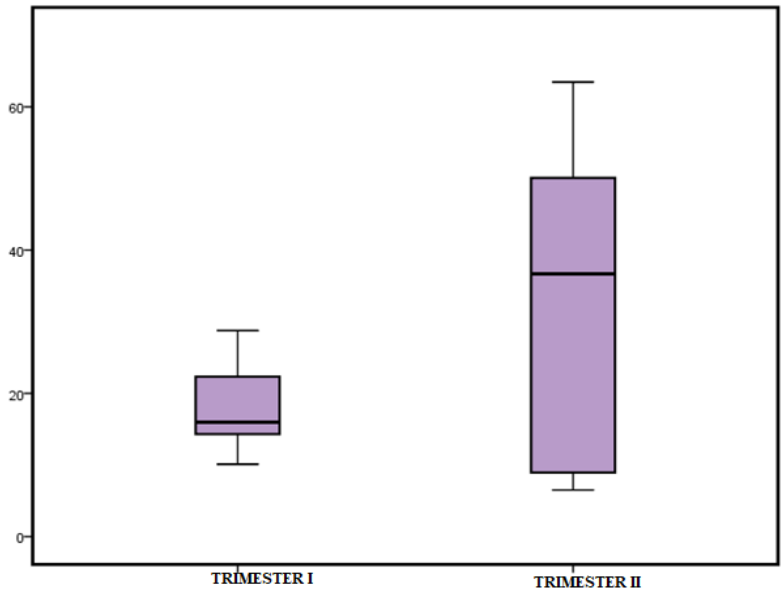

Figure 25: Depicting the median TAS concentration in normotensive pregnant women at first and second trimesters of pregnancy

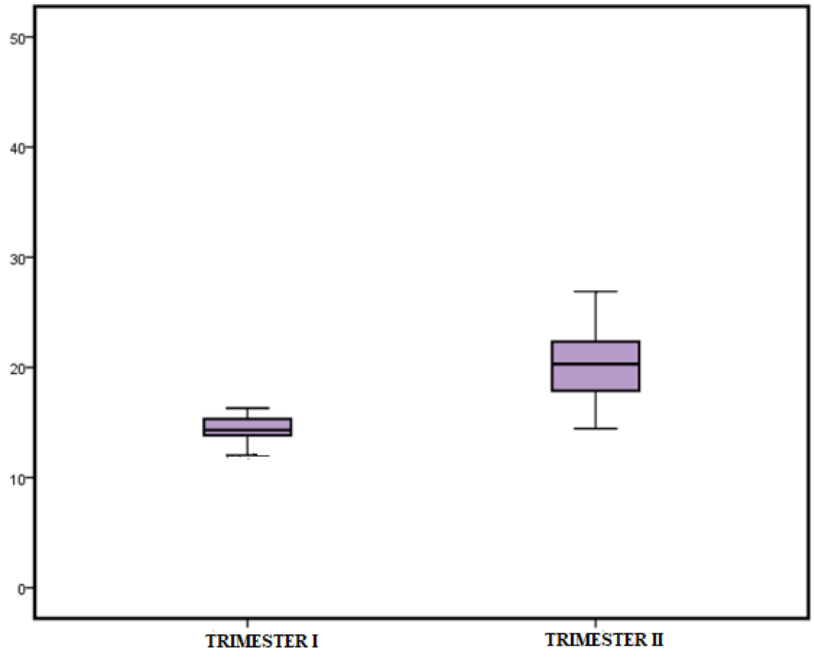

Figure 26: Depicting the median TAS concentration in cases that developed preeclampsia at first and second trimesters of pregnancy

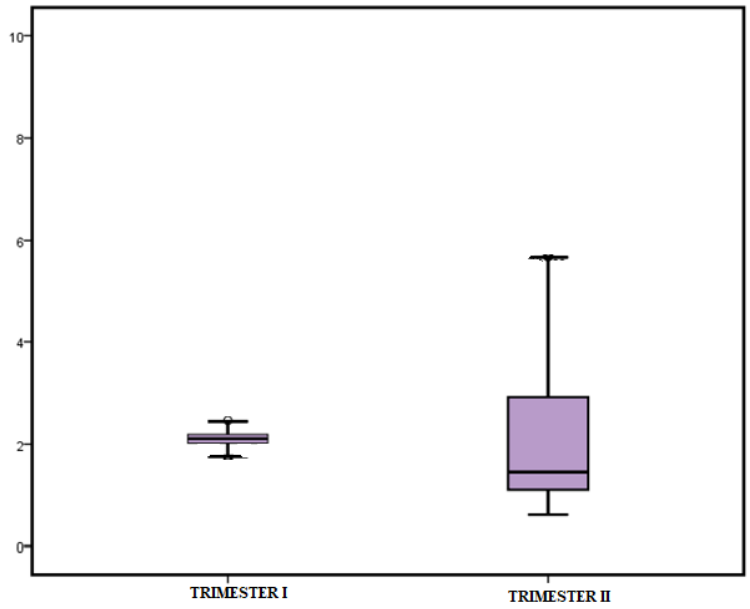

Figure 27: Depicting the median OSI in normotensive pregnant women at first and second trimesters of pregnancy

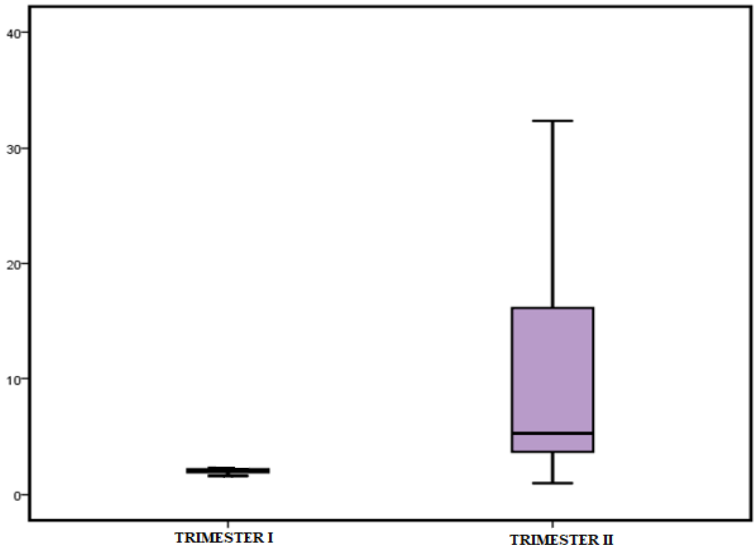

Figure 28: Depicting the median OSI in cases that developed preeclampsia at first and second trimesters of pregnancy

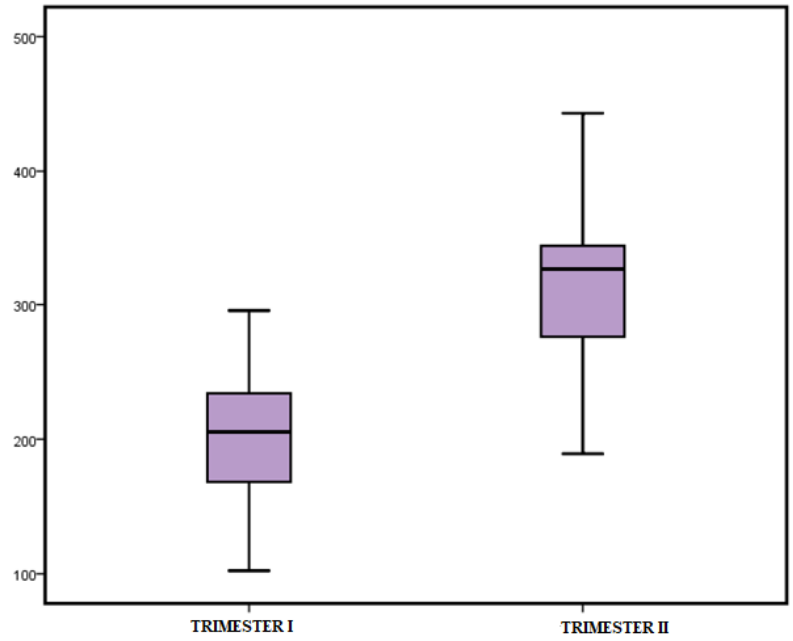

Figure 29: Depicting the median PP13 in normotensive pregnant women at first and second trimesters of pregnancy

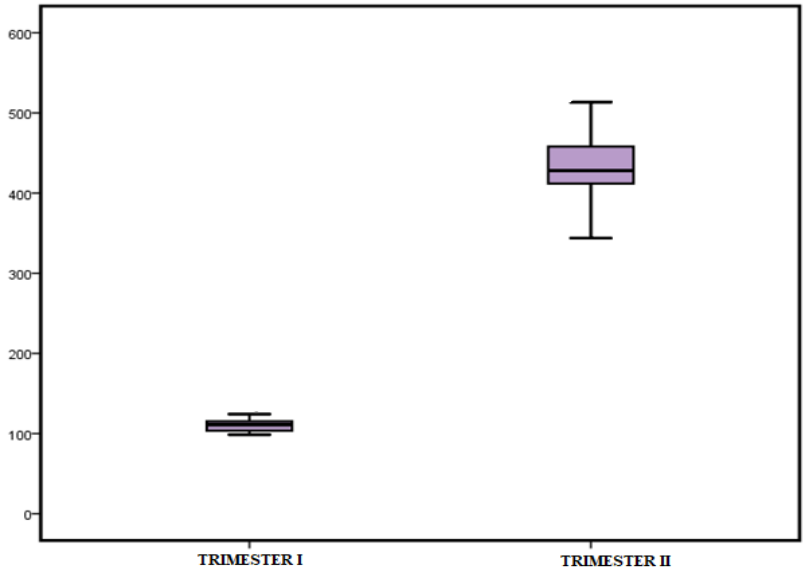

Figure 30: Depicting the median PP13 in cases developedpreeclampsia at first and second trimesters of pregnancy

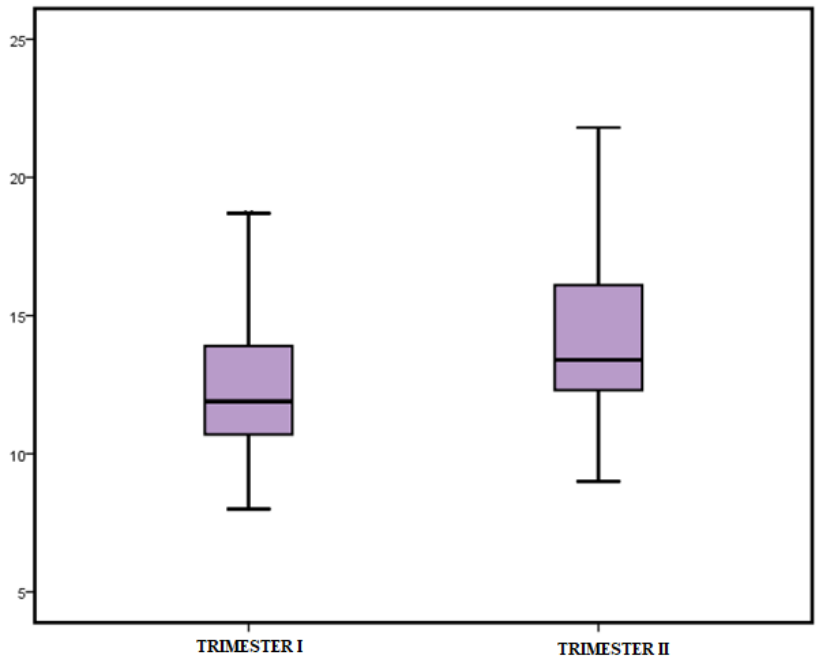

Figure 31: Depicting the median XO activity in normotensive pregnant women at first and second trimesters of pregnancy

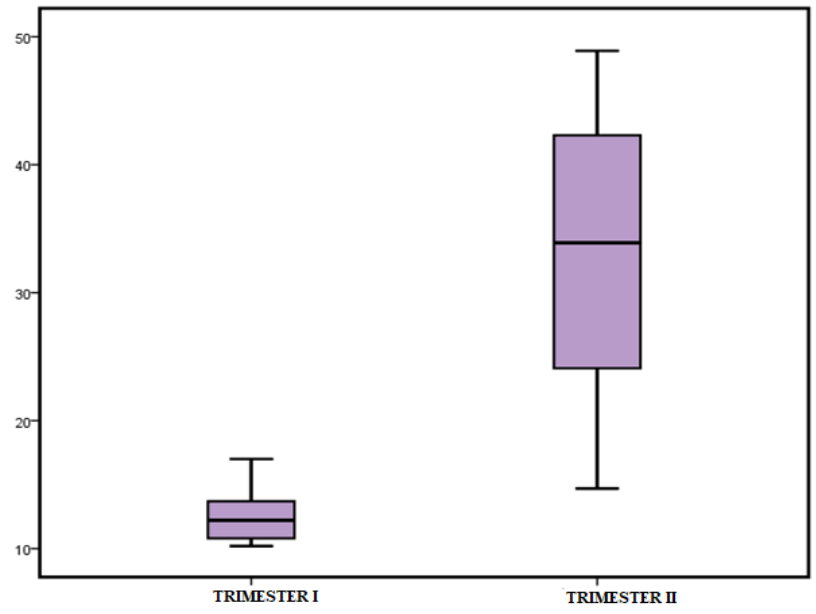

Figure 32: Depicting the median XO activity in cases developed preeclampsia at first and second trimesters of pregnancy

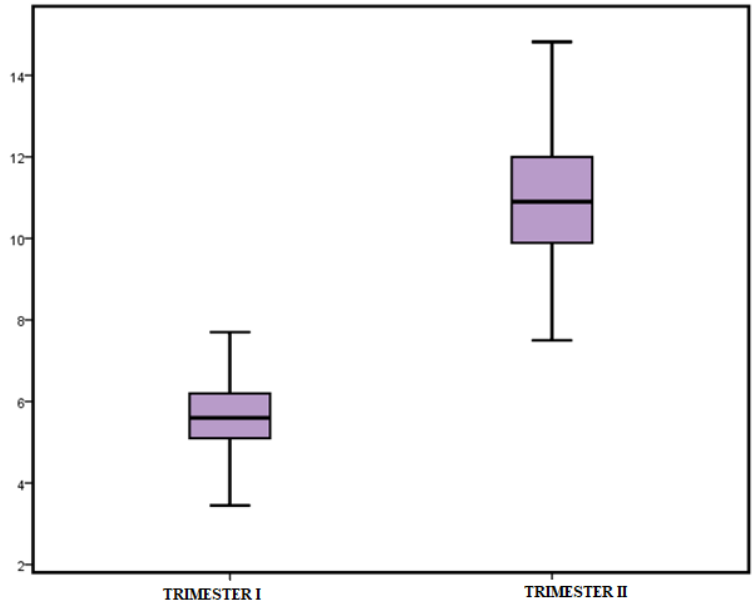

Figure 33: Depicting the median Caspase-3 in normotensive pregnant women at first and second trimesters of pregnancy

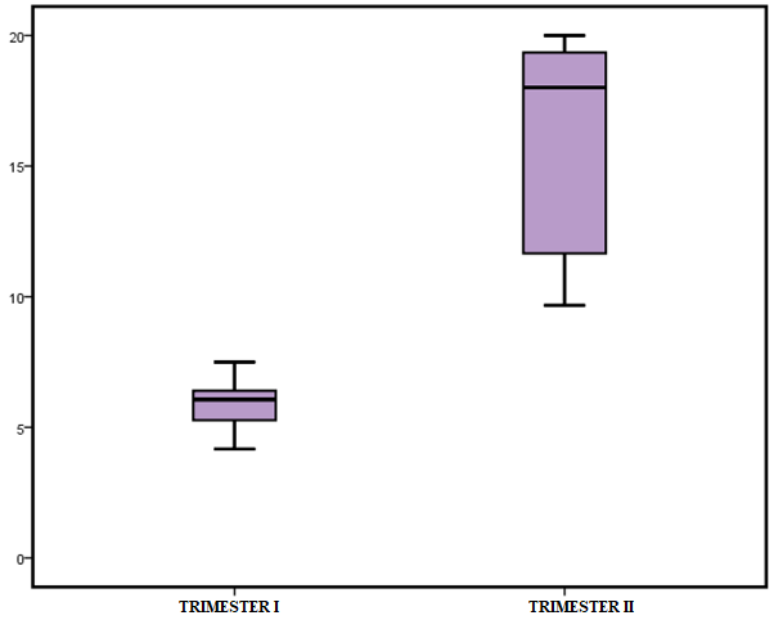

Figure 34: Depicting the median Caspase-3 in cases developed preeclampsia at first and second trimesters of pregnancy

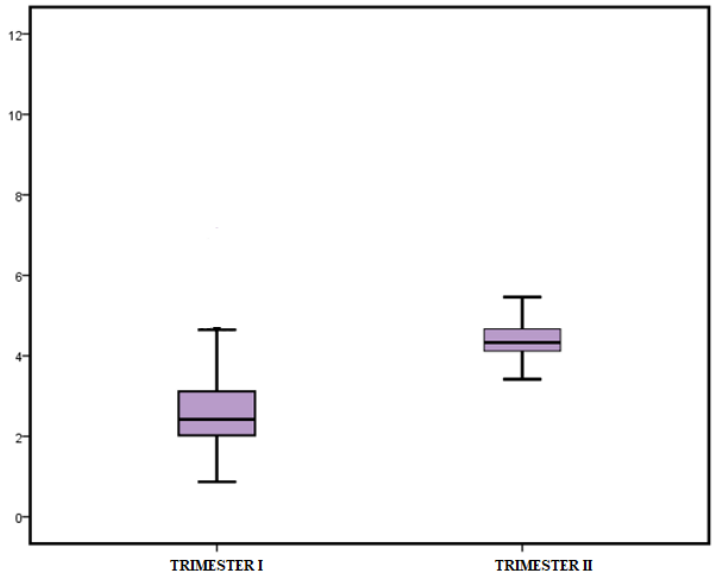

Figure 35: Depicting the median Nitric oxide in normotensive pregnant women at first and second trimesters of pregnancy

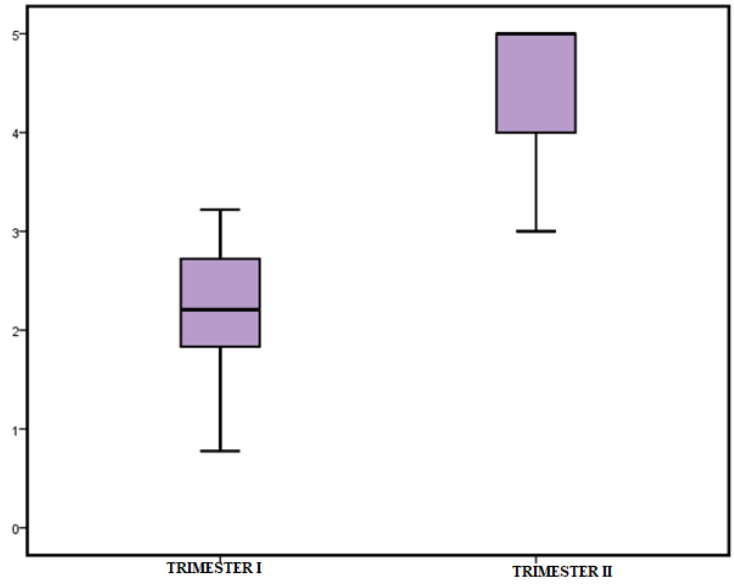

Figure 36: Depicting the median Nitric oxide in cases developed preeclampsia at first and second trimesters of pregnancy

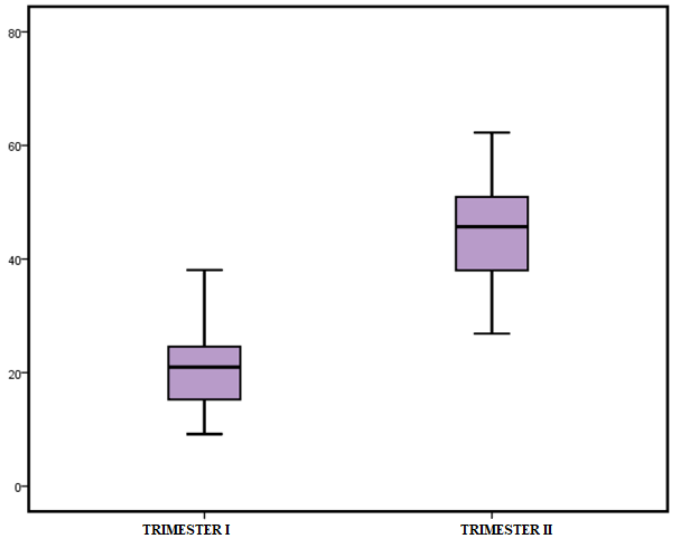

Figure 37: Depicting the median ADMA in normotensive pregnant women in first and second trimesters of pregnancy

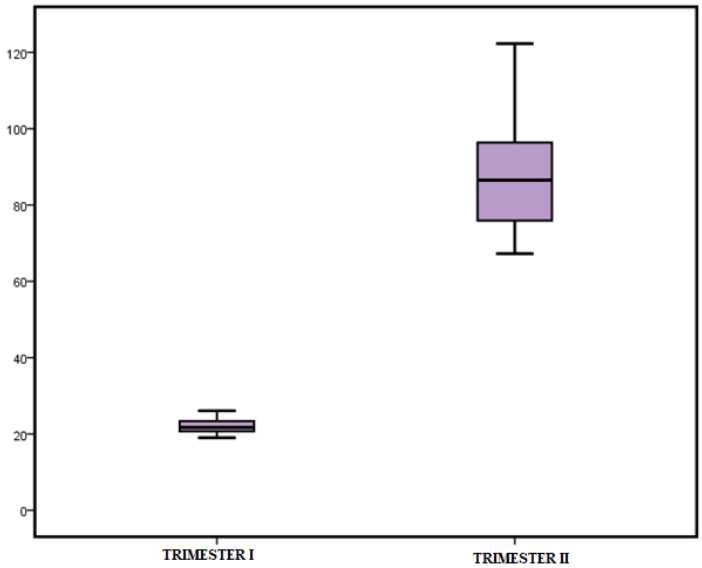

Figure 38: Depicting the median ADMA in cases developed preeclampsia in first and second trimesters of pregnancy

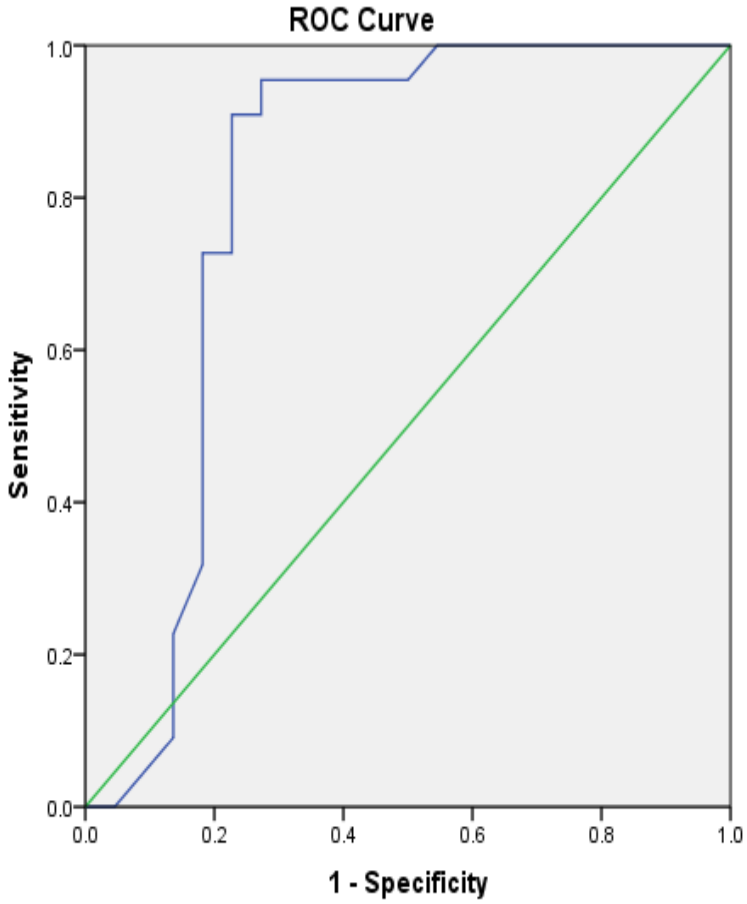

Figure 39: Receiver operating characteristic curve showing the area under curve for TAS in first trimester of pregnancy

The diagnostic performance of first trimester TAS was represented by receiver operating characteristic curvewith False Positive Rate (1- specificity) on X -axis and True Positive Rate (sensitivity) on Y-axis. Cut-off of 20.06 U/mL showed sensitivity (91%), specificity (78%), and area under curve (AUC) (0.807), 95% confidence interval range (0.657-0.956) and p <0.001. Hence, area under curve showed good accuracy for the test parameter in first trimester.

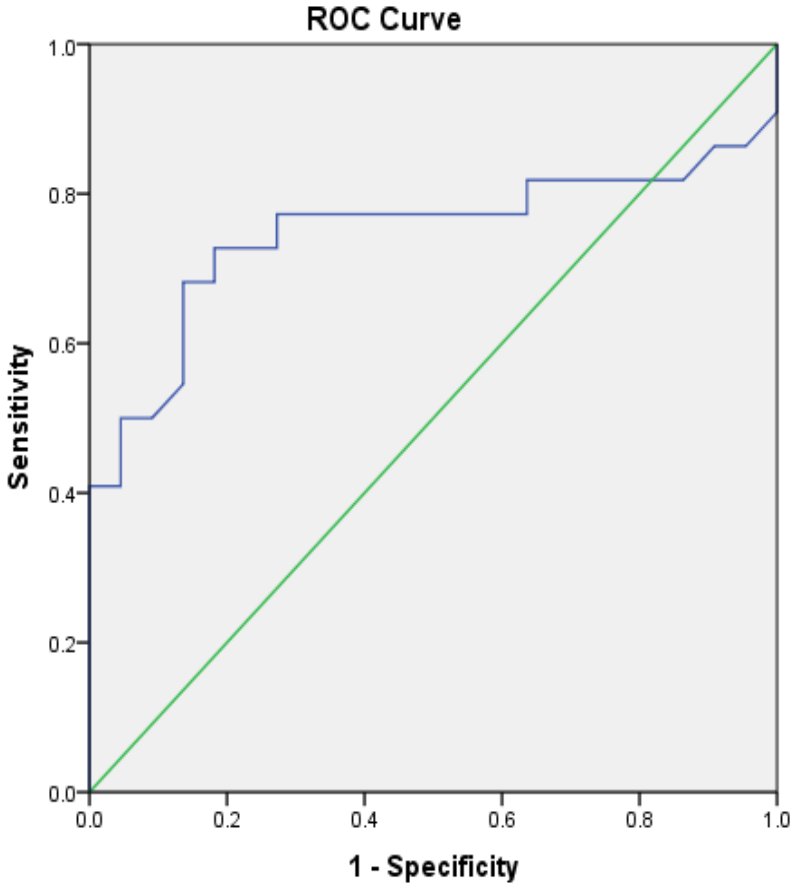

Figure 40: Receiver operating characteristic curve showing the area under curve for OSI at first trimester of pregnancy

The diagnostic performance of first trimester OSI was represented by receiver operating characteristic curve with False Positive Rate (1- specificity) on X -axis and True Positive Rate (sensitivity) on Y-axis. Cut-off of 1.86 showed sensitivity (73%), specificity (82%), AUC (0.747), 95% confidence interval range (0.586-0.908) and p=0.005. The AUC showed moderate accuracy for the test parameter in first trimester.

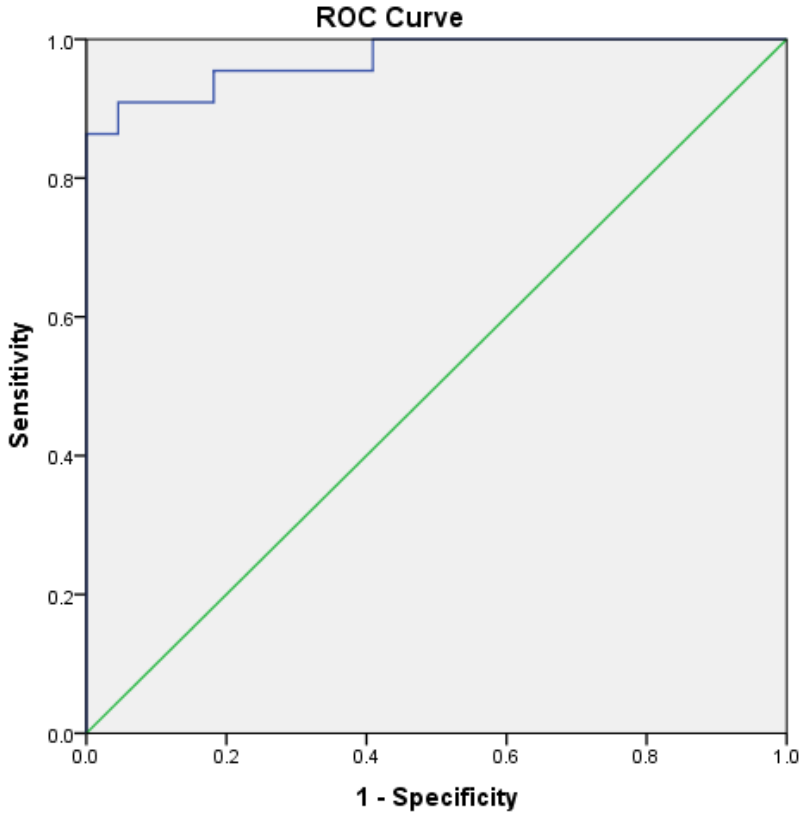

Figure 41: Receiver operating characteristic curve showing the area under curve for TOS at second trimester of pregnancy

The diagnostic performance of second trimester TOS was represented by receiver operating characteristic curve with False Positive Rate (1- specificity) on X -axis and True Positive Rate (sensitivity) on Y-axis. Cut-off of 53.76 U/mL showed sensitivity (95%), specificity (82%), AUC (0.971), 95% confidence interval range (0.928-1.000) and $p<0.001$. AUC showed good accuracy for the test parameter in second trimester.

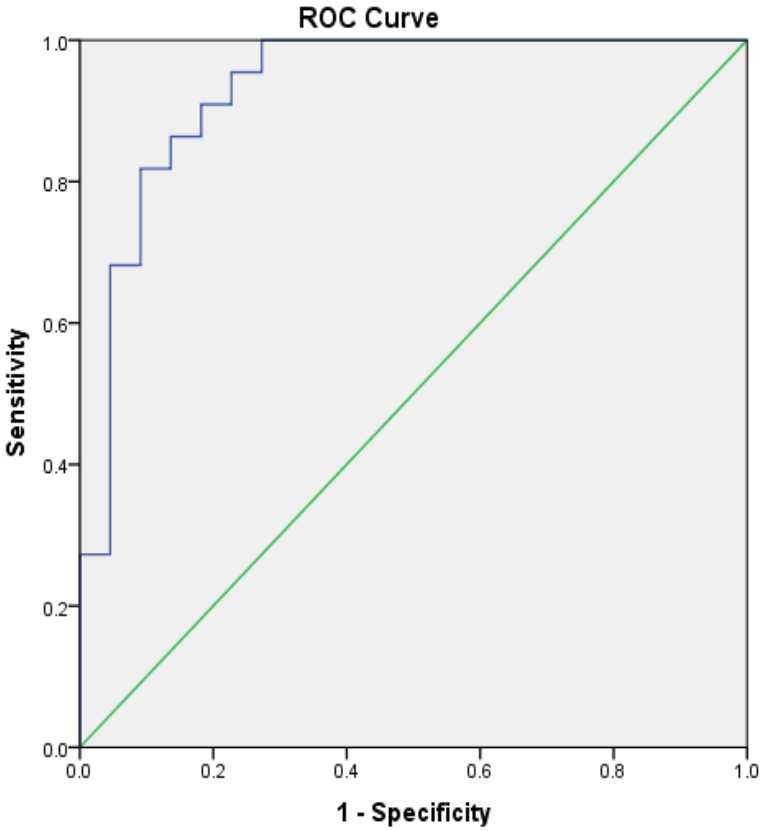

Figure 42: Receiver operating characteristic curve showing the area under curve for PP13 at first trimester of pregnancy

The diagnostic performance of first trimester PP13 was represented by receiver operating characteristic curve with False Positive Rate (1- specificity) on X -axis and True Positive Rate (sensitivity) on Y-axis. Cut-off of 117.77 pg/mL showed sensitivity (95%), specificity (78%), and AUC (0.932), 95% confidence interval range (0.855-1.000) and $p<0.001$. AUC showed good accuracy for the test parameter in first trimester.

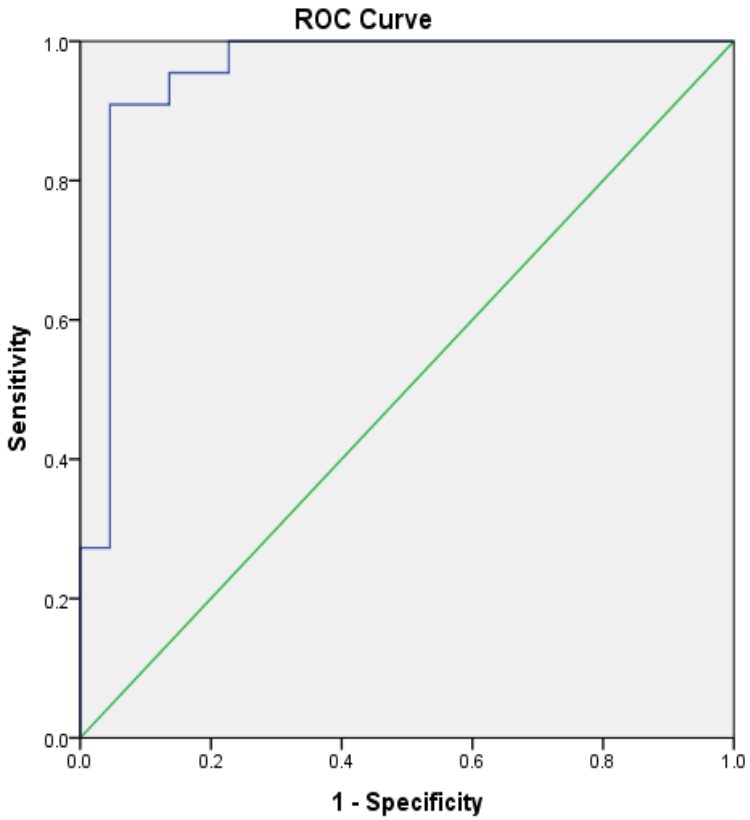

Figure 43: Receiver operating characteristic curve showing the area under curve for PP13 at second trimester of pregnancy

The diagnostic performance of second trimester PP13 was represented by receiver operating characteristic curve with False Positive Rate (1-specificity) on X -axis and True Positive Rate (sensitivity) on Y-axis. Cut-off of 371.25 pg/mL showed sensitivity (95%), specificity (87%), AUC (0.955), 95% confidence interval range (0.887-1.000) and $p<0.001$. AUC showed good accuracy for the test parameter in second trimester.

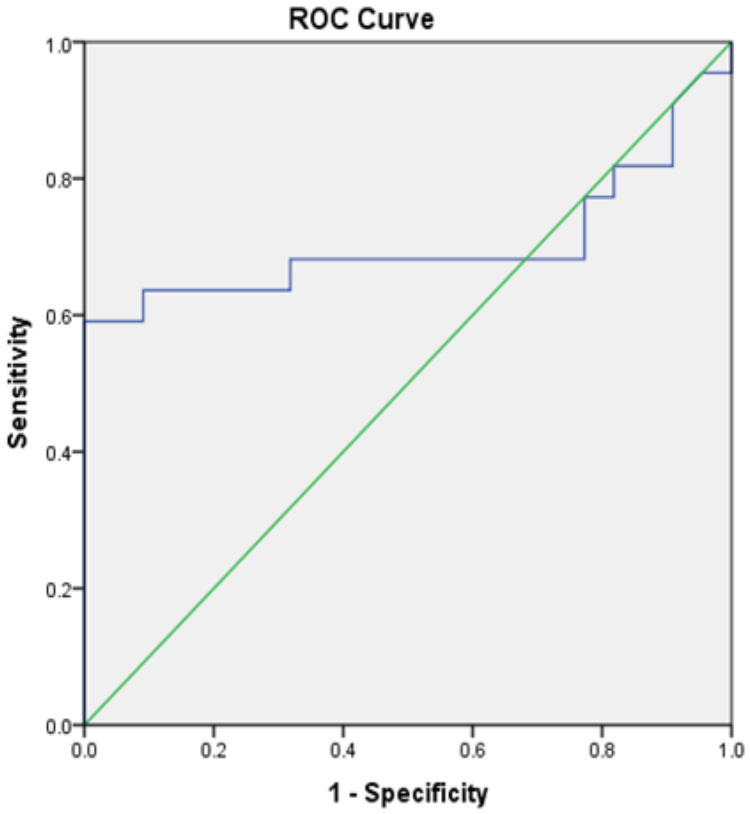

Figure 44: Receiver operating characteristic curve showing the area under curve for Caspase-3 at second trimester of pregnancy

The diagnostic performance of second trimester Caspase-3 was represented by receiver operating characteristic curve with False Positive Rate (1-specificity) on X -axis and True Positive Rate (sensitivity) on Y-axis. Cut-off 12.90 ng/mL showed sensitivity (64%), specificity (82%), AUC (0.704), 95% confidence interval range (0.530-0.877) and p = 0.021. AUC showed moderate accuracy for the test parameter in second trimester.

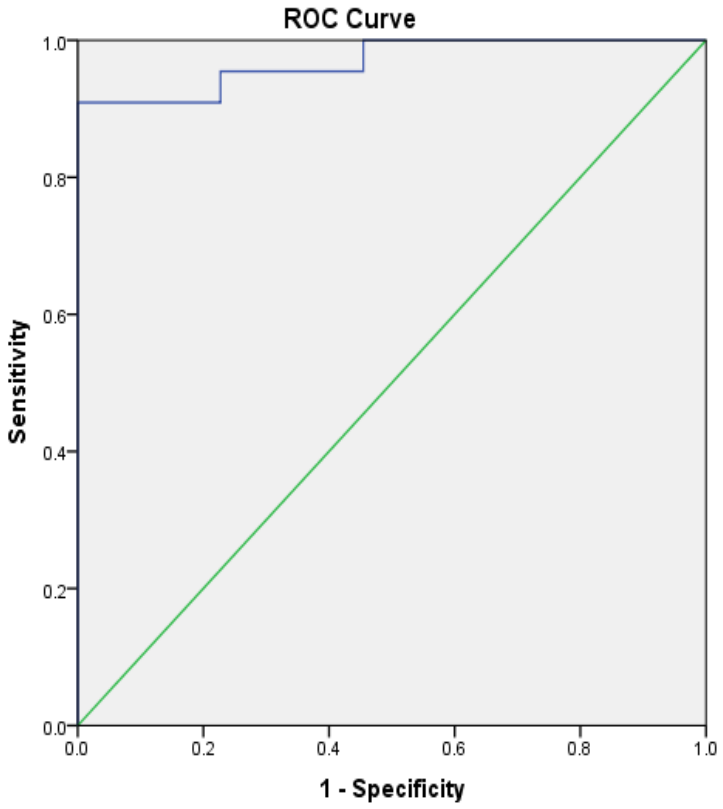

Figure 45: Receiver operating characteristic curve showing the area under curve of XO activity in second trimester of pregnancy

The diagnostic performance of second trimester XO activity was represented by receiver operating characteristic curve with False Positive Rate (1-specificity) on X - axis and True Positive Rate (sensitivity) on Y-axis. Cut-off of 17.25 mU/mL showed sensitivity (95%), specificity (78%), AUC (0.969), 95% confidence interval range (0.921-1.000) and $p<0.001$. AUC showed good accuracy for the test parameter in second trimester.

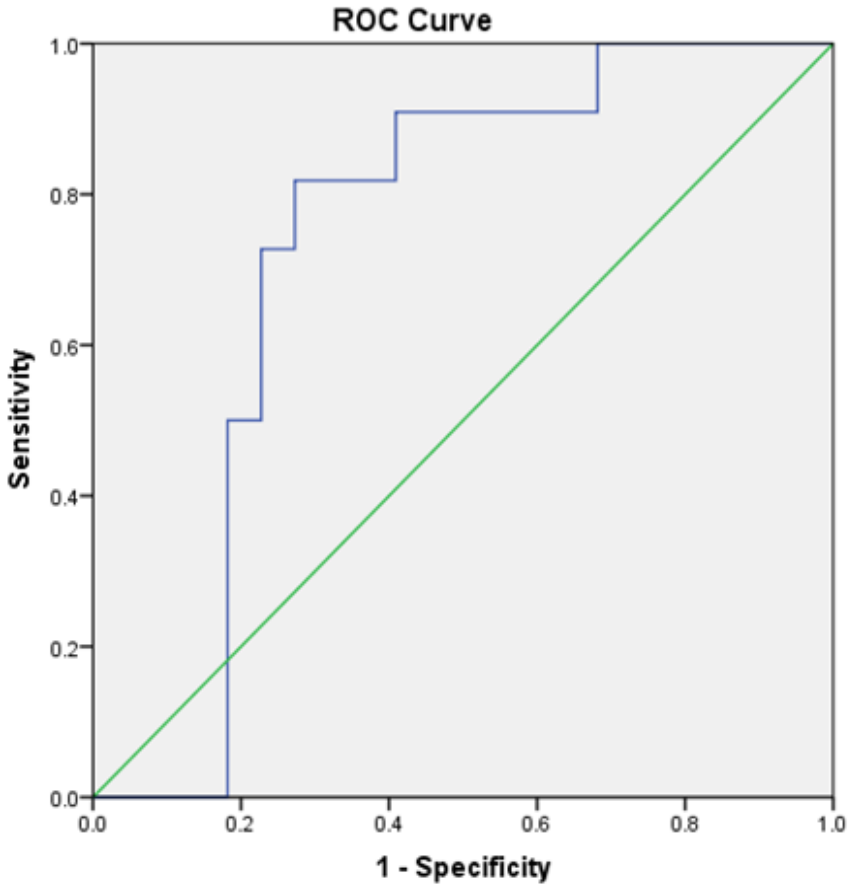

Figure 46: Receiver operating characteristic curve showing the area under curve of ADMA in first trimester of pregnancy

The diagnostic performance of first trimester ADMA was represented by receiver operating characteristic curve with False Positive Rate (1-specificity) on X -axis and True Positive Rate (sensitivity) on Y-axis. Cut-off of 19.70 ng/mL showed sensitivity (72%), specificity (78%), AUC (0.733), 95% confidence interval range (0.921-1.000) and p =0.008. AUC showed good accuracy for the test parameter in first trimester.

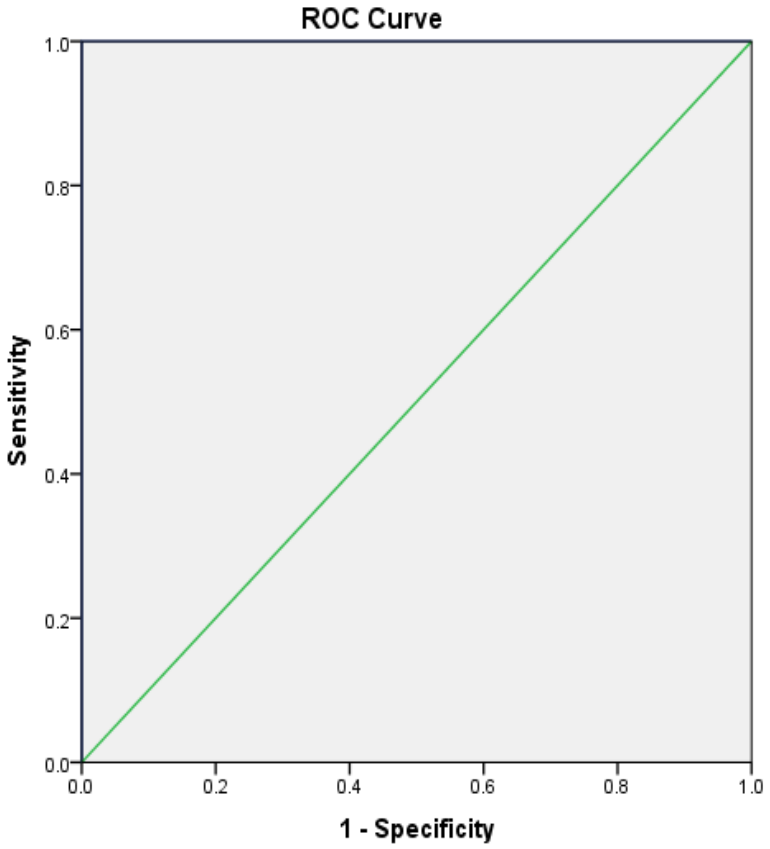

Figure 47: Receiver operating characteristic curve showing the area under curve of ADMA in second trimester of pregnancy

The diagnostic performance of second trimester ADMA was represented by receiver operating characteristic curve with False Positive Rate (1-specificity) on X -axis and True Positive Rate (sensitivity) on Y-axis. Cut-off of 50.36 ng/mL, showed sensitivity (100%), specificity (100%), AUC (1.000), 95% confidence interval range (1.000-1.000) and $p<0.001$. AUC showed best accuracy for the test parameter in second trimester.

5.2. DISCUSSION

Our findings on the demographic characteristics of the study population revealed that pregnant women with 21 years of age group with primigravida were more tend to develop preeclampsia in comparison with the normal pregnant women with age of 24-25 years. Generally, advanced maternal age (>30 years) is a risk factor for pre term delivery and early onset of preeclampsia[130]. Few studies have reported teenage pregnancy adds to the risk of preeclampsia[131,132]. On the other hand, a study also reported young maternal age (<25 years) is a risk to pre-term birth, low birth weight and preeclampsia[133]. In the current study demographic details are also in line with the above findings and serves as one of the cause for maternal complications.

Maternal anemia in first trimester provides a basis for adverse pregnancy outcomes like preeclampsia, low birth weight and still birth. The prevalence of anemia is 65-70% in pregnancy in India[134]. A prospective community based observational study conducted in Kolar, Karnataka with a sample size of 446 pregnant women reported 62.3% incidence of anemia in the age group of 21-30 years with low literacy and were associated with pregnancy and fetal complications[135]. In similar to this report, our study also indicated 45.14 % of incidence of anemia at the age group of 20-30 years.

In the same way, maternal anemia was also recorded in a retrospective study conducted on 4012 sample size[136], whereas a large prospective study with 72,750 pregnant women in Maharashtra reported maternal anemia in second trimester is associated with low birth weight, still birth and neonatal deaths[137].

Maternal anemia has the molecular basis of insufficient placental oxygen transport, results placental hypoxia and leads to free radical stress which stimulates inflammatory, anti-angiogenic and endothelial dysfunction factors in the process of

development of preeclampsia[138]. Furthermore, maternal anemia is also linked to altered endothelial NOS activity and is responsible for poor placental and fetal development[139].

In contrast to this, few studies reported elevated hemoglobin at first trimester with adverse pregnancy outcome[140-142]. Phaloprakarn C et al in his retrospective study indicated no association between low birth weight and first trimester hemoglobin levels in pregnant women[143]. However, Cakmak BD et al in his retrospective observational study reported both increase (>13g/dL) and decrease (<11g/dL) hemoglobin levels in first trimester that could be associated with poor pregnancy outcome[144].

We confirm that, in support of the above observations, our study demographic data also presented a link between maternal anemia, preeclampsia and poor fetal outcome. Thereby, the awareness between maternal anemia and preeclampsia in younger age pregnancy advocated for anemia management for improvementof maternal and fetal health status.

Many etiological factors implicated in pathogenesis of preeclampsia, and associated with more amount of ROS and low levels of antioxidants[145]. Oxidative stress in preeclampsia is influenced by ischemia reperfusiondue to inadequate trophoblast invasion and linkedto intra uterine growth restriction. Hypoxicated trophoblastic cells with endoplasmic reticulum and mitochondrial stress affected by free radical injury irreversibly damages the cellular components and cellular functions like enzyme activity, signal transduction andapoptosis. These cytotoxic factors released from the ischemic placenta into the maternal circulation could be causative for the onset of maternal inflammatory response and endothelial cell activationin preeclampsia.

Although there is no direct link between oxidative stress and antioxidant status in pathogenesis of preeclampsia and its complications, however numerous research reports indicated elevated oxidative stress, oxidation of cellular components and low antioxidant status were associated with early risk factors of preeclampsia and eclampsia[99,101,146,147]. A recent study also reported a similar observation, elevated levels of protein carbonyls and MDA in maternal plasma and cord blood were reported in preeclampsia women with adverse neonatal outcomes like IUGR, low birth weight and respiratory distress syndrome[148]. Increased lactate dehydrogenase levels reflect hypoxia and oxidative stress in preeclampsia which is associated with greater incidence of poor perinatal outcome[149].

In contrary, increase of antioxidants level wasobserved over well-known decreased levels of antioxidants in preeclampsia[150.151]. Though a larger study population reported that supplementation of antioxidants has no protective role in development of preeclampsia nevertheless, this aspect needs to be elucidated[152,153].

However, in the second trimester of the current prospective study, women who developed preeclampsia showed increased TOS, marginally increased TAS with significantly increased OSI. This kind of unique observation was not seen in normotensive group. This fact suggest that restoring total antioxidants level in normotensives is beneficial to combat the effect of oxidative stress by the scavenging action that might facilitate for minimizing the deleterious effects in preeclampsia. Very few studies have prospectively measured oxidative stress markers during pregnancy to link with the onset of preeclampsia. Literature on the longitudinal measurement of oxidative stress is scarce.

A longitudinal study conducted in Spain with a sample size of 140 in which 22 were reported as preeclamptic, 9 IUGR, 32 Gestational hypertension and 77 normotensive pregnant women. Study showed increased oxidative stress at 25 weeks of gestation i.e., elevated sFlt-1/PlGF ratio, Strontium, uric acid and lipid peroxidation/Total antioxidant activity were observed in early onset preeclampsia complications like defective remodeling of spiral arterioles and vascular endothelial damage[99].

Yet another longitudinal study conducted by Ferguson KK et al in 2017 in USA with 441 subjects in which 50 were preeclamptic and 391 were normotensives. They measuredplasma inflammatory markers and urinary oxidative stress markersat interval of 10, 18, 26 and 35 weeks. Elevated urinary oxidative stress markers (8-isoprostane and 8-hydroxydeoxyguanosine and plasma inflammatory markers (IL-1β, IL-6, IL-10 and TNF-α) were seen at the start of second trimester (18 weeks). In the study, increased interleukins suggest an elevated inflammatory response at early pregnancy and decreased urinary 8-isoprostane and 8-hydroxy deoxyguanosine suggest high placental oxidative stress. However the fetal complications like preterm birth and low fetal birth weight were reported in the study[93].

In an Indian prospective case-control study with a sample size of 211 pregnant women with 148 controls reported elevated oxidative stress in 63 preeclampsiacases. Oxidative stress markers (MDA, SOD, GPx and reduced glutathione) were measured at 16-20 weeks, 26-30 weeks and at delivery. Preeclamptic women has increased oxidative stress at 16-20 weeks of gestation and decreased which may impair placental development. Decreased geneexpression of endothelial nitric oxide synthase, GPx and SOD were also found in placentas of preeclamptic women [146]. A case-control study mentioned oxidative stress (arylesterase levels) andelevated TAS

in 30 preeclampsia compared to 30 healthy controls[91]. De Luca et al also measured decreased antioxidants vitamin c, catalase, thiol groups and increased delta-amino levulinic acid activity as oxidative stress marker in Brazilian population [27]. Similarly Al Kuraishy et al also reported increased oxidative stress marker peroxynitrite and MDA and decreased antioxidant activity of PON and Nitric oxide levels in preeclampsia compared to healthy controls (n=28)[95]. Conflicting data was also reported in a study where decreased oxidative stress markers like PON and arylesterase measured in severe preeclampsia[154].

Several markers have been identified in the literature in order to assess preeclampsia. Even then, there is a scope to screen new biomarkers which facilitates the early diagnosis procedure of preeclampsia. PP13, Caspase-3 and XO are biomarkers that secretes into maternal circulation during pregnancy. PP13 secretes before 6th week of gestation from exosomes or micro-vesicles and remains even after 2-5 weeks of delivery inmaternal circulation. The functional role of PP13 is embryoimplantation and for proper placental development. Recently, PP13 attracted importance as probable marker for early diagnosis of preeclampsia.

To the best of our knowledge, PP13 and its importance as likely a marker for early diagnosis of preeclampsia in Indian population is seldom. However, few studies conducted in Bulgaria, Austria, Florida and Israel population reported measuring PP13 level at first trimester has some predictive value in preeclampsia[49,106,155-157].

A typical observation with respect to serum PP13 levels in preeclampsia cases at second trimester was four fold higher values compared to its basal value in the first trimester of normotensive pregnant women. Whereas two fold increase of serum levels of PP13 was observed in second trimester in the normotensive group, reflects

the impact of oxidative stress and elevated placental apoptosis in preeclampsia. Therefore screening for low PP13 serum levels at first trimester and/or elevated serum PP13 level at second trimester indicate the more possibility of development of preeclampsia. Our research findings demonstrate that maternal anemia; oxidative stress on placenta links to derangement of placental integrity and serves as the basis of contribution to the pathophysiology of the disease.

In contrary to the above, Ceylan N et al reported no differences in the first trimester PP13 or PAPP-Alevel in the preeclamptic women compared to control group[103]. Seravalli V et al also reported that measuring PP13 in first trimester doesn't improve the prediction of preeclampsia nor small for gestational age[158]. Contradictory findings were reported in a longitudinal study conducted in Bulgarian population showed that no significant differences were observed in the PP13 levels between first trimester and second trimester in both preeclampsia cases and control[159].

Cellular Caspase-3 an apoptotic enzyme triggered by endoplasmic reticulum stress under oxidative environment and XO an enzyme heightens the oxidative stress by release of hydrogen peroxide and thus regarded as an enzyme oxidant. The levels of these enzymes in plasma are associated with placental trophoblastic cell integrity. Their levels are increased in second trimester than first trimester and further elevated in cases developed with preeclampsia. Amongst, Caspase-3 level was significantly increased compared to XO activity. Thereby, these plasma enzymes levels could be considered as enzyme markers in earlyrisk assessment of placental aberration in preeclampsia. As per the literature search, not much data/information is available about maternal plasma Caspase-3 and its significance relevant to preeclampsia.

Whereas, few studies have reported more expression of Caspase-3 in placental tissues in preeclampsia[112,115].

The possible explanation for the increased XO activity could be due to release of xanthine and hypoxanthine substrates. Therefore increased substrate availability in placental hypoxia due to increased break down of ATP and also conversion of cytokine activated xanthine dehydrogenase to its oxidase form increases XO activity that releases into maternal circulation from placenta. Research on XO and its relation to pregnancy complications are limited and hence scarce data is available. However, limited studies highlights increased plasma XO activity in preeclampsia[52,53,110].

Hyperuricemia in preeclampsia is well documented, even in the present study also Hyperuricemia in second trimester of women who were prone to develop preeclampsia was noticed compared to women who had healthy pregnancies. Few studies are available in support of this observation in preeclampsia cases[160-162]. Whereas, contradictory to these findings, reports also mentioned about the uric acid that has no role in the abnormal placentation and hence cannot predict preeclampsia[163-165].

ADMA is a metabolic byproduct of protein methylation process produced in-vivo into cytoplasm and known as inhibitor of endothelial NOS that catalyze the conversion of arginine into Nitric oxide. We have measured plasma ADMA levels of normotensive pregnant womenand also in preeclampsiaat first and second trimesters during pregnancy under oxidative stress conditions. ADMA concentration generally gets cleared by the hydrolytic reaction catalyzed by DDAH. Oxidative stress has an impact on the DDAH expressed in placenta because the thiol group of the active site cysteine residue is more sensitive to oxidative inactivation leading to accumulation of

ADMA[166]**.** Therefore in the present study, high ADMA levels recorded in first trimester (21 ng/ml), second trimester (46 ng/ml) and in preeclampsia cases (85 ng/ml). This suggested that increased ADMA levels in the system compromises nitric oxide synthesis and hence contributes to vascular endothelial dysfunction leading to angiogenesis.

The research findings directly links the role of ADMA between Nitric oxide synthesis and oxidative stress in pregnancy and its complications. When considering ADMA and Nitric oxide together in terms of ADMA:Nitric oxide ratio in preeclampsia cases was 19:1 whereas in normotensive pregnant women at first trimester was found to be 9:1 and in second trimester 10:1. Therefore, ADMA:Nitric oxide ratio may be regarded as one of the factor to be considered for early assessment of preeclampsia and partly accountable in understanding the biochemical basis contributing to the etiology of preeclampsia. Thus, this current study creates scope for determination of factors/ therapeutic strategy to reduce ADMA levels for better women and child health.

Number of longitudinal studies showed elevated ADMA prior to the development of clinical symptoms of preeclampsia[116,120,122,167]. And few case-control studies also reported high ADMA levels resultin endothelial dysfunction in support of our study results[85,117,118,122,123].

In the present study, Nitric oxide levels were increased by 2 fold in second trimester than first trimester basal value innormotensive pregnant women and also in preeclampsia. There was no prominent difference in the Nitric oxide levels between first and second trimester of normotensive and cases with preeclampsia. Hence Nitric oxide could be a poor predictor of preeclampsia in early pregnancy. Similar results

were also observed in few studies that supports the present Nitric oxide results which also explain that Nitric oxide has no role in thepathophysiology and cannot be the main reason of placental injury in preeclampsia[80,168,169]. But small case control studies[170-173] reported increased Nitric oxide level in preeclampsia than the control group. However few longitudinal studies[174,175] and case-control studies[62,85,176,] reported decreased level of Nitric oxide in preeclampsia. As per our study results, measurement of Nitric oxide in combination with ADMA represented as ADMA:Nitric oxide ratio is more precise and beneficial to know preeclampsia onset than normal pregnants.

Statistical analysis exhibited positive correlation of PP13 with ADMA in both first and second trimesters in cases that developed preeclampsia. This observation was scientifically answered by hypothesis of relationship between PP13 and ADMA may be indirectly involved in endothelial function. The effect of placenta purified PP13 on the isolated mononuclear cells from peripheral blood of pregnant women showed increased secretion of IL-1α & 6 into the culture medium[34]. Inflammatory cytokines that induce oxidative stress in endothelial cells can inactivate the active site of the enzyme DDAH, leading to accumulation of ADMA[177,178]. By these studies it can be proposed that concentration of PP13 and its association with increased ADMA level. Our study results are in support of the above observation regarding importance of PP13 concentration during pregnancy condition for better obstetric management.

Preeclampsia is complex genetic disorder comprising numerous genetic variants occurs as a result of mutation or genetic polymorphisms. Many candidate genes were studied in providing genetic evidences related to preeclampsia complications. Wider scope exist to study the products expressed by the genes and also to know the

functional roles in relation to preeclampsia. Therefore understanding the pattern of gene structure, expression in exerting molecular mechanism is essential to address etiopathogenesis linked to genetic causes. The targeted pharmacological agents for any genetic disorder is useful as an additional therapeutic approach in the diagnosis and management of the pregnancy associated complications. The involvement of many genes such as Factor V Leiden, Methylene tetrahydrofolate, Endothelial NOS3, VEGF receptor 1, Angiotensinogen, Apolipoprotein E, Tumor necrosis factor α and Glutathione-S-transferase with respect to preeclampsia have been studied [179]. LGALS13 is one such gene and its expressed protein PP13 studied to limited extent.

The low concentration of plasma PP13 was reported as a risk factor in placentation process and in later development of preeclampsia in pregnancy.As per the data, the low levels of PP13 was noticed in first trimester subsequently its levelincreased in second trimester onwards. The exact reason for low PP13 level in first trimester is not clearly known. Our study findings are also in align with the studies which reported low levels of PP13 and different from other studies by collecting its gene LGALS13 sequence analysis data in an attempt to find the possible explanation. LGALS13 gene was subjected for sequence analysis by employing molecular techniques. The analysis of sequence data of LGALS13 gene of preeclampsia cases was done by comparing to reference sequence as obtained from NCBI data base. The sequence analysis of 22 preeclampsia cases revealed-98C/A SNP in the promoter region of LGALS13 gene of 12 cases that amounts to 54.54%.

Whereas the same data compared to the total subjects of the study amounts to 4.48% as direct genetic evidences from 8.2% of preeclampsia cases.

Promoter variant -98C/A of LGALS13 gene responsible for low levels of PP13 in early pregnancy (South Africa and London) i.e., in first trimester of the subjects prone to develop preeclampsia is evident by few studies [41,42]. Even though the reason for the low levels of PP13 is a challenge since PP13 is involved in placentation, inflammation and immune defense during trophoblast invasion during spiral artery remodeling. PP13 decreases blood pressure by causing vascular dilation and diameter expansion. However the exact functional role of PP13 is directly linked to decrease of blood pressure and the mechanism is also not clear.

Sequence analysis results of the preeclampsia DNA sample exhibited -98C/A mutation at the promoter region in South India population. We report this observation for the first time in the South Indian population as per the available data to the best of our awareness. From 22 DNA samples, 12 DNA samples showed SNP at promoter region which have the mean value of PP13 in first trimester 108.09 pg/mL and in second trimester 404.74 pg/mL. Whereas the preeclampsia cases without the promoter variant exhibited 117.25 pg/mL in first trimester and 455.49 pg/mL in second trimester.The major finding of the study concludes that a narrow range variation of PP13 i.e., (8%) in DNA sample with promoter variant compared with DNA sample without SNP helps for preeclampsia prediction at an early stage.

There is a paucity of information on similar SNPs in study population or Indian population as a whole. So there is a scope to evaluate the structure of PP13 expressed from normotensive group and preeclampsia in order to know the role of PP13 function at early placentation process has creates a scope for further research.

CHAPTER-6

SUMMARY & CONCLUSION

6.0. SUMMARY& CONCLUSION

6.1. Summary

Every effort has been made and careful attention was given during analysis of the research data and our research findings are summarized as below.

In the first trimester of the study population, TOS, OSI & TAS measured as base line values in normotensive pregnant in the age group of 20-30 years with gestational period of 8-12 weeks. Whereas the same parameters measured during follow up in second trimester (20-24 weeks) showed increased OSI and marginally increased total antioxidant status in cases that developed preeclampsia. These subjects showed poor fetal outcome in terms of low birth weight. However, in the normotensive women at second trimester showed increased TOS and TAS but remarkably decreased OSI.

Similarly, plasma levels of PP13 and Caspase-3 & XO activity were determined in the first and second trimester during follow up of the study. The cases developed preeclampsia prominently revealed an increase of the above parameters compared to baseline first trimester values. Low plasma PP13 level in the first trimester appraises as marker to distinguish preeclampsia in the study population. PP13 concentration in second trimester was elevated both in normotensive and preeclampsia cases; even then persistent high value was noticed in preeclampsia cases than normotensive in the second trimester. Thereby recording drastic reduction of PP13 in first trimester to the half of the PP13 level in the normotensive group and persistent high value in second trimester preeclampsia cases (30%) compared to normotensive group serves as a marker to predict early onset of preeclampsia.

XO an enzyme oxidant contributes to oxidative stress and its activity is not in appreciable level in first and second trimester of normotensive pregnant. However its activity was elevated in preeclampsia cases. Therefore screening of XO activity along with OSI helps for diagnosis of preeclampsia.

Plasma Caspase-3 level during pregnancy under prevailing oxidative stress was elevated. Its concentration is associated with trophoblastic destruction and integrity. Elevated oxidative stress affects the placental trophoblastic integrity and consequently sheds placental proteins into maternal circulation. Therefore, Caspase-3 level was high in second trimester of normotensive and further elevated in preeclampsia. Hence, Caspase-3 measurement adds usefulness to diagnose the involvement of placental pathology in preeclampsia and can be considered as a cost effective marker.

ADMA and Nitric oxide were measured in the study group and presented as ADMA: Nitric oxide ratio in the first and second trimesters. The data of the second trimester compared to the baseline levels of ADMA and Nitric oxide in first trimester. ADMA concentration increased in second trimester of normotensive by two fold where as in preeclampsia attained fourfold elevation. Hence screening of ADMA and ADMA:Nitric oxide ratio has become a new tool to understand early the development of preeclampsia.

The cord blood was collected postpartum from the fetal side of placenta from the cases that developed preeclampsia (n=22) and from normotensive pregnant women. The DNA wasextracted and subjected for analysis of whole gene sequencing of LGALS13 gene consists of E1-E4 exons.Sequence analysis indicated a single nucleotide polymorphism (SNP) i.e., the cytosine at the -98 position in the promoter region is replaced by adenine (-98C/A) noticed.

This promoter variant was observed in 12 cases which amount to 55 percent. However, SNPs were not observed in the promoter region and other exons of LGALS13 gene in the normotensive pregnant women.

6.2. Conclusion

The longitudinal study presents measurement of biochemical markers like PP13, XO activity, Caspase-3, Nitric oxide and ADMA involved in placentation and endothelial function during first and second trimesters of pregnancy. Which provide information on change in the pattern of the markers under oxidative stress conditions (OSI) in normotensives and women at risk for preeclampsia.The study results provides the relation to the low levels of plasma PP13 and preeclampsia. Elevated ADMA: Nitric oxide and OSI represents vascular endothelial dysfunction. Genetic analysis of LGALS13 on South Indian population was to analyze LGALS13 gene sequence using cord blood DNA from normotensive and cases developed preeclampsia. LGALS13 gene from preeclampsia DNA samples showed -98C/A promoter variant single nucleotide polymorphism. None of the DNA samples from normotensive group exhibited any SNP at promoter and in other exons region.

Furthermore, study can be extended using larger population for finding any other genotype polymorphism in the LGALS13 gene, expression of PP13 and its functional aspect associated to the regulation of blood pressure.

CHAPTER-7

BIBLIOGRAPHY

7.0. BIBLIOGRAPHY

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APPENDIX-I

List of reprints of the publications pertaining to the Ph.D. topic

S.No.	Title	Journal name	Indexation
1.	Maternal Serum Biochemical Indicators of Trophoblastic Cell and Endothelial Function at First Trimester of Pregnancy	Open Journal of Obstetrics and Gynecology. 2018; 8, 867-881.	Web of science, Google Scholar, Pubmed, Pubmed central
2.	Relationship between Mean Arterial Pressure, Uric Acid and Calcium with Xanthine Oxidase Activity and Fetal Outcome in Normotensive and Preeclampsia in a Nested Study	Open Journal of Obstetrics and Gynecology. 2018; 8: 1532-1548.	Web of science, Google Scholar, Pubmed, Pubmed central
3.	Placental protein 13: An important biological protein in preeclampsia	Journal of Circulating Biomarkers. 2018;7:1849454418786159	Pubmed DOAJ
4.	Placental protein 13 and asymmetric dimethyl arginine for early assessment of preeclampsia	Biomedical Research. 2019;30(2).	Scopus EMBASE
5.	Relevance of Placental protein 13 in combination with Asymmetric dimethylarginine, Caspase 3 and Xanthine oxidase activity in early prediction of Preeclampsia and an evidence of LGALS13 gene promoter variant	Journal of Medical Sciences.	Scopus Under review

Scientific Research Publishing

Open Journal of Obstetrics and Gynecology, 2018, 8, 867-881
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Maternal Serum Biochemical Indicators of Trophoblastic Cell and Endothelial Function at First Trimester of Pregnancy

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Abstract

Background: Preeclampsia is a worldwide pregnancy complication, and early identification of patients with an increased risk is one of the key goals in obstetrics. First trimester screening is crucial over the second trimester for understanding the early onset of the disorder, with basal levels of the biochemical parameters associated with the underlying placentation process. **Objective:** The study aims to assess the levels of serum biochemical markers in pregnant women at first trimester, to evaluate statistical significance and correlation of the values in support of trophoblastic cell integrity, endothelial function and oxidative stress. **Materials and Methods:** A longitudinal study was conducted on 86 pregnant women of age group 20 - 35 years, Primigravida with singleton pregnancy who visited prenatal check up between 11 - 13 weeks of gestation. Maternal sera was collected for screening Placental protein 13 (PP13), Caspase 3, Asymmetric dimethylarginine (ADMA), Nitric oxide (NO) by ELISA. Xanthine oxidase (XO) activity was assayed spectrophotometrically. Calcium and Uric acid (UA) were measured by dry chemistry analyser. **Results:** The mean ± SD values for mean arterial pressure (MAP) are 108.4 ± 18.9, UA 2.01 ± 0.85, Total oxidant status (TOS) 12.83 ± 5.17, Total antioxidant capacity (TAC) 24.10 ± 14.28, XO 1.01 ± 2.67, Caspase-3 1.76 ± 2.22, PP13 489.77 ± 53.6, Calcium 10.88 ± 1.97, ADMA 19.03 ± 17.08 and NO 1.16 ± 0.75. The statistical analysis by SPSS package version 20 revealed positive correlation between ADMA & Caspase-3 (r = +0.435), PP13 & NO (r = +0.241), TOS & TAC (r = +0.176), UA & ADMA (r = +0.176), UA & TAC (r = +0.168) and negative correlation between PP13 & ADMA (r = −0.158), NO & TOS (r = −0.114), UA & XO (r = −0.173), UA & NO (r = −0.186), UA & Caspase 3 (r = −0.106) and MAP & Calcium (r = −0.303). **Conclusion:** The study concludes that first trimester biochemical markers

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Relationship between Mean Arterial Pressure, Uric Acid and Calcium with Xanthine Oxidase Activity and Fetal Outcome in Normotensive and Preeclampsia in a Nested Study

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Abstract

Preeclampsia is a pregnancy complication; early identification with increased risk is one of the key goals in obstetrics. In a nested case control study, serum uric acid and calcium measured in first and second trimesters of pregnancy were correlated with Xanthine oxidase (XO) activity, mean arterial pressure (MAP) and fetal birth weight. The mean ± SD of uric acid (2.01 ± 0.85, 4.8 ± 1.93), calcium (10.88 ± 1.97, 9.72 ± 2.04), MAP (84.32 ± 6.71, 78.40 ± 8.53) and XO activity (11.96 ± 1.91, 14.05 ± 3.09) of the study group (n=86) were observed in the first and second trimesters respectively. First trimester normotensive group (n=79) and preeclampsia cases (n = 7), showed a mean ± SD of uric acid (1.93 ± 0.80, 2.9 ± 0.88), Calcium (10.92 ± 1.9, 10.6 ± 1.72), MAP (84.19 ± 6.75, 85.71 ± 6.58) XO activity (11.82 ± 1.83, 13.57 ± 2.21). In the second trimester, normotensive group and preeclampsia cases showed a mean ± SD uric acid (4.6 ± 1.75, 7.3 ± 2.19), Calcium (9.4 ± 1.85, 12.9 ± 1.04), MAP (76.41 ± 5.41, 100.95 ± 2.52) and XO activity (13.37 ± 1.93, 21.70 ± 3.50). Statistical analysis revealed a non-significant positive correlation in first trimester between uric acid and MAP ($r = +0.116$, $p = 0.288$), negative correlations between uric acid and fetal birth weight ($r = -0.118$, 0.279) and between calcium and MAP ($r = -0.288$, $p = 0.007$). In the second trimester, significant positive correlations were observed between uric acid ($r = +0.246$, $p = 0.022$), calcium ($r = +0.326$, $p = 0.007$) with MAP along with a significant negative correlation between uric acid ($r = -0.641$, $p = 0.000$), calcium ($r = -0.316$, $p = 0.003$), Proteinuria ($r = -0.514$, $p = 0.000$) with fetal birth weight. The screening of first and second trimesters XO activity, uric acid, calcium and MAP during pregnancy is beneficial in identifying women likely to develop

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Placental protein 13: An important biological protein in preeclampsia

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Abstract

Placental protein 13 (PP13), a glycan binding protein predominantly expressed in syncytiotrophoblast, dimeric in nature, lacks N-terminal signal peptide, bypasses the endoplasmic reticulum, and secretes into maternal circulation as exosomes or microvesicles. PP13 has jelly roll fold conformation with conserved carbohydrate recognition domain which specifically binds to β-galactosides of the glycan receptors during placentation. PP13 binds to glycosylated receptors on human erythrocytes and brings about hemagglutination by the property of lectin activity; other functions are immunoregulation and vasodilation during placentation and vascularization. The gene LGALS13 located on 19q13.2 comprising four exons expresses a 32-kDa protein with 139 amino acid residues, PP13. Impaired expression due to mutation in the gene leads to a nonfunctional truncated PP13. The low serum levels predict high risk for the onset of preeclampsia or obstetric complications. Hence, PP13 turned to be an early marker for risk assessment of preeclampsia. The recombinant PP13 and monoclonal antibodies availability help for replenishing PP13 in conditions with low serum levels and for detection and prevention of preeclampsia, respectively.

Keywords

Placental protein 13, preeclampsia, eclampsia, jelly roll fold, syncytiotrophoblast

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Introduction

Preeclampsia is a pregnancy-specific disorder characterized by hypertension and proteinuria after 20 weeks of gestation.[1] Although the exact etiology of the disorder is still not known, the impairment in the early placentation is associated with onset of preeclampsia that complicates up to 2–8% of all the pregnancies.[2] Prediction, understanding of underpinning mechanism and prevention of preeclampsia, is still not clear. Hence, preeclampsia and eclampsia are the leading causes of maternal, perinatal morbidity, and mortality.[3,4] Placental protein 13 (PP13) is a carbohydrate binding protein synthesized in the syncytiotrophoblast, which is involved in early placentation process.[5,6] It is a member of galectin family with a conserved carbohydrate recognition domain (CRD).[7–9] The specificity of this site for β-galactosides-containing glycoconjugates[10–12] is established and plays a significant role in biological events such as implantation and embryogenesis.[13,14] The biological specificity of the PP13 present in the apical membrane of the syncytiotrophoblast to the glycans of the membrane and extracellular matrix proteins such as annexin II is a primary requisite for the placental implantation to the endometrium.[15]

PP13 also binds to β- and γ-actin within trophoblasts, which facilitates the migration of trophoblasts toward the placental bed and also increases the release of prostacyclins for vascular remodeling of maternal spiral arteries in early placentation.[16] From the immunological point of view, for an effective placentation, PP13 induces the apoptosis of

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Placental protein 13 and asymmetric dimethyl arginine for early assessment of preeclampsia.

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Abstract

Objective: To evaluate serum levels of biomarkers as early indicators of onset of preeclampsia during pregnancy.

Method: A nested case-control study was conducted among the pregnant women who visited for antenatal check-up at R.L. Jalappa Research Center, Kolar between August 2017 and May 2018. Serum levels of placental protein 13 (PP13), asymmetric dimethylarginine (ADMA), nitric oxide and caspase-3 which represents placentation and endothelial function were measured and compared between trimesters.

Results and Discussion: There were 86 pregnant women enrolled at first trimester, the baseline values of women who developed preeclampsia were PP13 (477.54 ± 62.72 pg/ml), ADMA (21.06 ± 19.42 ng/ml), nitric oxide (3.71 ± 2.76 nmol/μL) and caspase-3 (6.15 ± 0.55 ng/ml). In the second trimester on follow up, the mean serum concentration of PP13 was 530.64 ± 69.10 pg/ml in preeclampsia versus 518.26 ± 49.33 pg/ml in normal pregnancy. The mean serum concentration of ADMA was 73.43 ± 30.95 ng/ml and nitric oxide 4.10 ± 2.70 nmol/μL in preeclampsia versus 21.06 ± 19.42 ng/ml and 4.85 ± 2.19 nmol/μL in normal pregnancy. The mean serum concentration of caspase-3 in preeclampsia was 22.65 ± 0.91 ng/ml versus normal pregnancy 18.45 ± 2.62 ng/ml were recorded. In preeclampsia, PP13 was positively correlated with nitric oxide and negatively correlated with ADMA.

Conclusion: Decreased PP13, nitric oxide and elevated ADMA in first trimester and increased PP13, ADMA and decreased nitric oxide at second trimester reflects altered placentation and endothelial function.

Keywords: Asymmetric dimethylarginine, Placental protein 13, Preeclampsia, Caspase-3D.

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Introduction

Preeclampsia contributes to the global incidence of 2-8% of pregnancies with maternal/neonatal morbidity and mortality in developing countries with poor antenatal care. The criteria for diagnosis of preeclampsia are blood pressure ≥ 140/90 mmHg with renal insufficiency, impaired liver function, haematological and neurological complications [1]. The adverse outcomes of preeclampsia are fetal growth restriction with oligohydramnios, preterm birth, low birth weight, severe birth asphyxia, still birth or intrapartum death. Many etiologies linked to this syndrome, *viz.* insufficient trophoblast invasion, uteroplacentalischemia, vascular disorders of the placenta, insulin resistance, systemic maternal inflammation, endothelial dysfunction and antiangiogenic state [2]. Even though, the exact reason for this pregnancy disorder is not known, preeclampsia research is striving to address this issues related to early diagnosis, underlying mechanism and management of the disorder. Therefore, the early assessment of preeclampsia by using biomarkers is effective and has drawn much attention.

Human placental protein 13 (PP13) is 32 kDa β-galactoside binding soluble-type galectin synthesized in the syncytiotrophoblast. The presence of carbohydrate recognition domain (CRD) in its structure enables binding to the glycans of endometrial membrane and annexin II of the extracellular matrix during implantation and embryogenesis. PP13 binds to β and γ actin proteins of cytoskeleton in the syncytiotrophoblast and facilitates its migration by increasing prostaglandin release, which is important for vascular remodelling during placental development. Besides, it is also known to provide immune tolerance at the maternal-fetal interface [3]. Nevertheless, theexact aetiology of preeclampsia is unclear, abnormal remodelling of uterine blood vessels and immunological tolerance between fetal and maternal tissues play possible role in the pathogenesis of this disorder [4]. Therefore, early assessment of PP13 concentrations seems advantageous.

Asymmetric dimethylarginine (ADMA), a methylated compound generated by post translational modification of

Relevance Of Placental Protein 13 In Combination With Asymmetric Dimethyl Arginine, Caspase 3 And Xanthine Oxidase Activity In Early Prediction Of Preeclampsia And An Evidence Of LGALS13 Gene Promoter Variant

Background: Preeclampsia is a pregnancy specific hypertensive disorder and its complications are linked to maternal morbidity and mortality.

Objectives: The aim of the study is to determine early placental and endothelial dysfunction markers in first and second trimesters of pregnancy in the prediction of preeclampsia.

Materials and Methods: Study design was longitudinal nested-case control, consisted of 268 pregnant women enrolled during ante-natal screening. Blood samples were analysed for total oxidant status (TOS) total antioxidant status (TAS), Placental protein13 (PP13), Caspase-3 and Asymmetric dimethyl arginine (ADMA) by ELISA, Xanthine oxidase (XO) activity and Nitric oxide (NO) by colorimetric assay. Oxidative Stress Index (OSI) was denoted as the ratio of TOS/TAS. On follow-up, 22 subjects developed preeclampsia and 12 had spontaneous abortions.PP13 gene, LGALS13 was sequenced to know any polymorphism. Results were analysed by Wilcoxon Signed Ranks test know the level of statistical significance, correlation analysis by Spearman's correlation and diagnostic performance of the measured parameters by receivers operating characteristic (ROC) curve.

Results: Preeclampsia onset women had low PP13 levels in first trimester and elevated OSI, XO activity, Caspase-3 and ADMA in second trimester ($p<0.001$). PP13 gene revealed 55% of preeclamptic cases with -98C/A SNP at promoter region. Elevated ADMA:NO ratio represents vascular endothelial dysfunction. ROC curve showed AUC for first trimester PP13 (0.932), second trimester PP13 (0.955) second trimester XO activity (0.967) and second trimester ADMA (1.000).

Conclusion: Our results suggest that oxidative stress, placental and endothelial dysfunction biomarkers would be helpful for early prediction of preeclampsia.

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