CIRCULATING ANGIOGENIC, OXIDATIVE STRESS MARKERS AND THEIR POSSIBLE ASSOCIATION WITH ENDOTHELIAL FUNCTION IN NORMAL PREGNANT AND PREECLAMPTIC WOMEN

Thesis submitted to

SRI DEVARAJ URS ACADEMY OF HIGHER EDUCATION
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For the requirements of the degree

DOCTOR OF PHILOSOPHY
IN
BIOCHEMISTRY

Under Faculty of Medicine

by

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April 2021

DECLARATION BY THE CANDIDATE

I, Rajeev Gandham hereby declare that this thesis entitled "**Circulating angiogenic, oxidative stress markers and their possible association with endothelial function in normal pregnant and preeclamptic women**" 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 of Biochemistry, Department of Allied Health Sciences, 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. Kiranmayee P** Assistant 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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The Central ethics Committee of Sri Devaraj Urs Academy of Higher Education and Research, Kolar has examined Ph.D synopsis titled "**Circulating angiogenic, oxidative stress markers and their possible association with endothelial function in normal pregnant and preeclamptic women**" and the detailed work plan of the project.

The central ethics committee has unanimously decided to approve the project and grant permission to investigator to carry out the research work. The interim and final report has to be submitted to the ethics committee after completion of the project for the issue of Central Ethics Committee certificate. Principal investigator should maintain the records of the Project and consent form for not less than 5 years from the date of completion or termination of the project.

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NOTE

The Department of Research & Innovation, Sri Devaraj Urs Medical College wish to inform Mr. Rajeev Gandham, Ph.D scholar (Reg No. 17Ph.D0301) of the Department of Biochemistry working on the Ph. D thesis titled "Circulating angiogenic, oxidative stress markers and their possible association with endothelial function in normal pregnant and Preeclamptic women" under the supervision of Dr. CD Dayanand and as per his representation was presented in the Institutional Research Committee on 23.05.2020 and has been approved.

In this regard Mr. Rajeev Gandham is hereby permitted to include healthy non-pregnant women to provide baseline values of Apelin-13 in his thesis. However, he is informed to communicate the same through his guide to Dean, Faculty of Medicine as it needs to be further approved in Central Ethics Committee. Mr. Rajeev Gandham can continue with his Ph. D thesis work in the interim period.

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LIST OF ABBREVATIONS

1.	ACE-II	Angiotensin Converting Enzyme - II
2.	ACOG	American College of Obstetrics and Gynecology
3.	ADMA	Asymmetric Dimethylarginine
4.	APLN	Apelin
5.	APLNR	Apelin Receptor
6.	APJ	Apelin - Apelin Receptor
7.	ATP	Adenosine Triphosphate
8.	AT1-AA	Angiotensin 1 receptor Agonistic Antibodies
9.	BH_4	Tetrahydrobiopterin
10.	CAT	Catalase
11.	CRP	C-Reactive Protein
12.	COX	Cyclooxygenase
13.	DNA	Deoxyribo Nucleic Acid
14.	DBP	Diastolic Blood Pressure
15.	ELB	Erythrocyte Lysis Buffer
16.	eNOS	endothelial Nitric Oxide Synthase
17.	FRAP	Ferric Reducing Ability of Plasma
18.	GA	Gestational Age
19.	GPCR	G-Protein Coupled Receptor

20.	GPx	Glutathione Peroxidase
21.	HELLP	Hemolysis, Elevated Liver enzymes and Low Platelet count
22.	HIF	Hypoxia Inducible Factor
23.	H/R	Hypoxia/Re-perfusion injury
24.	HTN	Hypertension
25.	IL-6	Interleukin-6
26.	IL-10	Interleukin-10
27.	IUGR	Intrauterine Growth Restriction
28.	LBW	Low Birth Weight
29.	MAP	Mean Arterial Pressure
30.	MAPK	Mitogen Activated Protein Kinase
31.	MDA	Malondialdehyde
32.	NHBPEP	National High Blood Pressure Education Program
33.	NO	Nitric Oxide
34.	NOX	NADPH Oxidase
35.	NOS	Nitric Oxide Synthase
36.	PAPP-A	Pregnancy Associated Plasma Protein- A
37.	PCR	Polymerase Chain Reaction
38.	PE	Preeclampsia
39.	PGI_2	Prostacyclin I_2
40.	PlGF	Placental Growth Factor

41.	RAAS	Renin-Angiotensin Aldosterone System
42.	RDS	Respiratory Distress Syndrome
43.	RFLP	Restriction Fragment Length Polymorphism
44.	ROS	Reactive Oxygen Species
45.	SBP	Systolic Blood Pressure
46.	sEng	soluble Endoglin
47.	sFlt-1	soluble Fms like tyrosine kinase-1
48.	SGA	Small for Gestational Age
49.	SOD	Superoxide Dismutase
50.	SNP	Single Nucleotide Polymorphism
51.	TAS	Total Antioxidant Status
52.	TBARS	Thiobarbituric Acid Reactive Substances
53.	TOS	Total Oxidant Status
54.	TXA_2	Thromboxane A_2
55.	TNF-α	Tumor Necrosis Factor-Alpha
56.	VEGF	Vascular Endothelial Growth Factor
57.	WHO	World Health Organization

CHAPTER-1

INTRODUCTION

1.0. BACKGROUND

Preeclampsia and eclampsia are two variants of hypertensive complications seen in pregnancy condition. Preeclampsia condition occur prior to eclampsia (Greek word "eclampsis" means sudden flashing). It is a potentially dangerous pregnancy disease, characterized by onset of *de novo* hypertension (≥140/90 mmHg) and proteinuria (≥0.3 g/day), this condition usually begins after twenty weeks of pregnancy and associated with a high risk of maternal and fetal complications [1]. The symptoms of preeclampsia are headache or visual disturbances, epigastric pain or right upper quadrant pain, edema, oliguria, thrombocytopenia, if untreated, it leads to seizures and such condition is known as eclampsia [2].

The maternal complications listed in preeclampsia are cardiovascular diseases, kidney disease, liver disease, ischemic heart disease (IHD), stroke, seizures and death. The newborns of preeclamptic mothers exist with Intra Uterine Growth Restriction (IUGR), preterm delivery, neonatal Respiratory Distress Syndrome (RDS), retinopathy of prematurity, sepsis and still birth. Hence, such children are at increased risk of impaired cognitive function, metabolic syndrome, coronary heart disease and chance of developing stroke subsequently in their life [3,4].

1.1. Risk factors of Preeclampsia

Preeclampsia is associated with several risk factors, these include primiparity, nulliparity, multiple pregnancies, prolonged pregnancy intervals, maternal age 35 years or older, prior preeclampsia, chronic hypertension, renal disease,

pre-gestational diabetes mellitus, obesity (pre-pregnancy BMI≥30 kg/m^2), insulin resistance, antiphospholipid syndrome and thrombophilia, assisted reproductive techniques, chronic inflammatory conditions such as systemic lupus erythematosus, chronic infections and also genetic susceptibility [1,2].

1.2. Epidemiology

The global incidence of preeclampsia is around 2-8% and nearly 10% of mortality occur due to perinatal and neonatal cases[1]. WHO report indicated that the leading cause of maternal mortality is preeclampsia and related hypertensive disorders of pregnancy that claim the lives of nearly 76,000 mothers and 500,000 babies worldwide per year. A woman in a developing country is seven times likely to develop preeclampsia than in developed country and thus preeclampsia in developing countries are about 10 percent [5].

In India, the incidence of preeclampsia is 8-10% among pregnant women, and Preeclampsia and eclampsia accounts for 24% of all maternal deaths [6]. The neonatal mortality rate in India is around 43/1000 live births [4]. Whereas, in Karnataka state, the incidence of hypertensive disorders of pregnancy found to be 7.9 percent [5].

1.3. Guidelines for the Diagnosis of Preeclampsia

1.3.1. World Health Organization (WHO)

As per the report of WHO, preeclampsia is diagnosed as onset of new episode of hypertension during pregnancy characterized by persistent hypertension with diastolic blood pressure of ≥ 90 mmHg and ≥0.3g/24 hrs substantial proteinuria [7].

1.3.2. National High Blood Pressure Education program (NHBPEP)

Blood Pressure of ≥140/90 mmHg noted for the first-time during pregnancy on two occasions at least 4 hrs apart, after twenty weeks of gestation with proteinuria of ≥0.3g/24 hrs or ≥ 1+ by dipstick method in a random urine sample with no evidence of urinary tract infection [8].

1.3.3. American College of Obstetricians and Gynecologists (ACOG)

In November 2013, ACOG defined preeclampsia as blood pressure of ≥ 140/90 mmHg on two occasions at least 4 hours apart after twenty weeks of gestation in a woman with a previously normal blood pressure and accompanied by significant proteinuria ≥300 mg per 24-hr urine collection or protein/creatinine ratio ≥ 0.3, dipstick reading of 1+. Or in the absence of proteinuria, new-onset hypertension with thrombocytopenia, renal insufficiency, impaired liver function, pulmonary edema, cerebral and visual symptoms were considered [9].

In January 2019, ACOG defined preeclampsia as blood pressure of ≥140/90 mmHg on two occasions at least 4 hrs apart after twenty weeks of gestation in a woman with a previously normal blood pressure. Severe preeclampsia, blood pressure of ≥160/110 mmHg (severe hypertension can be confirmed within a short interval (minutes) to facilitate timely antihypertensive therapy) and thrombocytopenia, renal insufficiency, impaired liver function, pulmonary edema, or visual impairment [1]. This revised ACOG guidelines describe about the diagnosis of preeclampsia that does not require the detection of high amount of protein in urine.

1.4. Etiology of Preeclampsia

The pathophysiology of preeclampsia is multi-factorial proposed to have maternal, placental and fetal factors. The widely considered causes of preeclampsia is abnormal trophoblastic invasion of uterine vessels or shallow placentation as shown in figure 1. In addition to this, immunological maladaptive tolerance between maternal, paternal and fetal complications, maternal maladaptation to cardiovascular or inflammatory changes in pregnancy, whereas genetic factors including inherited predisposing genes and epigenetic influences [2,10].

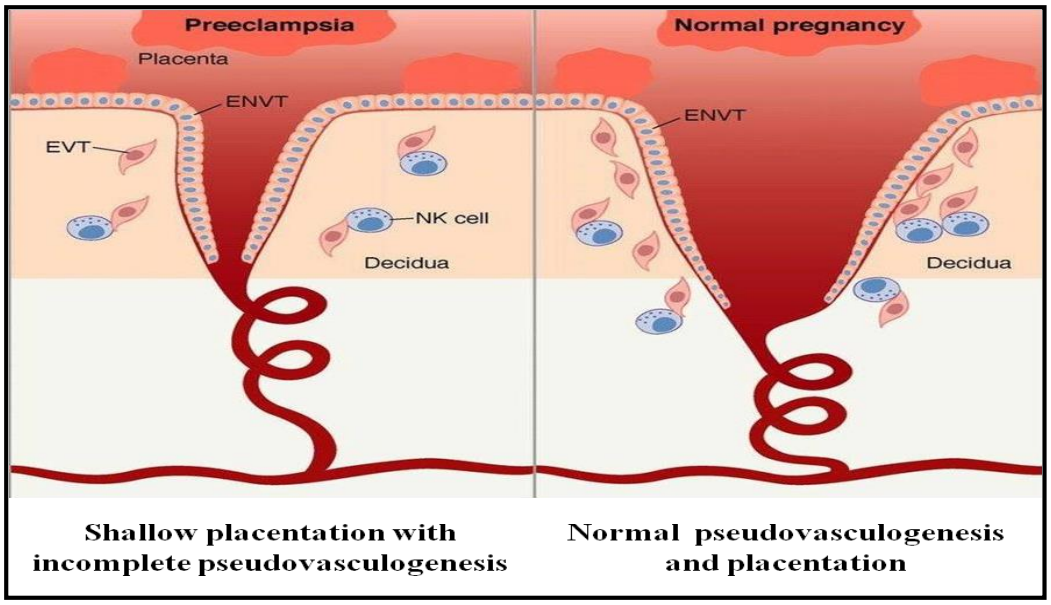


Figure 1: Placentation in preeclampsia and normal pregnancy
ENVT - endovascular trophoblast cells; EVT - Extravillous trophoblasts; NK- Natural killer cells
Source: Brandon Wang II. Preeclampsia and eclampsia

1.5. Pathophysiology of Preeclampsia

Placenta is connectivity for feto-maternal interaction through umbilical cord. The two components of placenta are fetal placenta (Chorion frondosum) and the maternal placenta (Decidua basalis). Placental bed contains the uterine spiral arteries that supply oxygenated blood to the growing placenta and fetus. Important physiological changes occur in the placental bed throughout the pregnancy and also plays an important role in the nutrient uptake, waste elimination, and gas exchange to fight against internal infection; and to produce hormones which are required for pregnancy. Therefore, abnormal placentation plays a central role in the pathophysiology of preeclampsia [11].

The pathophysiological mechanisms involved in preeclampsia are complex and has not been fully elucidated. The pathophysiology of this disorder is described in two stages [12]. Stage-1 is abnormal placentation, early in the first trimester and stage-2 is maternal preeclamptic syndrome as shown in the figure 2.

During the process of normal placentation, extravillous cytotrophoblast invades the lumen of the maternal uterine spiral arteries of the decidua and myometrium also known as endovascular invasion. These spiral arteries are the terminal branches of the uterine arteries and supply blood to the endometrium. The cytotrophoblast invasions replace the endothelial layer of maternal spiral arteries, transforming them into a low resistance, low pressure, and large capacitance vessels to increase utero-placental blood flow. This process of transformation is known as pseudo-vasculogenesis [13,14]. This cytotrophoblast invasion also accompanied by the replacement of arterial

smooth muscle and elastic tissue with fibrinoid layer in which trophoblastic cells are embedded. As a result of this, spiral arteries changes into flaccid tubes with a diameter at least four times greater than that of nonpregnant vessels, provides a low resistance circuit to the intervillous space. This process of vascular remodeling necessitates more or less 100-120 spiral arteries in the placental bed. Usually, this process begins at the end of first trimester (10 - 12 weeks) and ends by 18 – 20 weeks of pregnancy [15,16]. Trophoblast invasion in the spiral arteries deeper in the central region compared to the periphery of the placenta. Thus, makes a direct contact with maternal blood, this process involves many transcription factors, growth factors and cytokines. Such physiological changes are essential to provide adequate blood supply to the placenta and also for the fetal development [17].

In pathological conditions like preeclampsia, this transformation is incomplete and cytotrophoblast invasion of spiral arteries enter only to superficial decidua and does not reach the myometrial segments and retain their smooth muscle and elastic lamina. The control of this invasion of cytotrophoblasts depends on the interplay between maternal decidua and fetal trophoblast [18]. This shallow cytotrophoblast invasion of uterine spiral arterioles, leading to narrow bore size, high resistance vessels, reduced placental perfusion, relative placental ischemia and consequently placental insufficiency. These arteries further remain sensitive to humoral and neuronal vasoconstrictor influences. This affects the placental oxygen, nutritional status and fetal development [3,18].

These narrow spiral arteries are more prone to develop atherosis, which results in the formation of atheromatous plaques, and their lumens are occupied by lipid rich macrophages, inflammatory infiltrate and fibroid necrosis of vessels walls, which finally leads to further compromise in placental blood flow, with consequent uteroplacental ischemia and oxidative stress [12, 16]. This results in release of anti-angiogenic molecules such as sFlt-1 and sEndoglin, inflammatory cytokines, reactive oxygen species (ROS), Hypoxia Inducible Factor-1 alpha (HIF-1α), Angiotensin 1 receptor and Agonistic Autoantibodies (AT1-AA). sFlt-1, exerts anti-angiogenic effects by binding to and inhibiting the biological activity of VEGF and PlGF, thus preventing their availability to stimulate placental angiogenesis, neovascularization, maintains the homeostasis of endothelial cell and regulates the proliferation and induces the nitric oxide synthesis [19,20].

Placental apoptosis may be the final common pathway for this uteroplacental ischemia-reperfusion injury. Preeclamptic placentas show more apoptosis than healthy pregnant placentas. This placental apoptosis leads to the shedding of toxic placental debris into the maternal circulation that incites systemic inflammatory response and endothelial dysfunction. 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 [18,21].

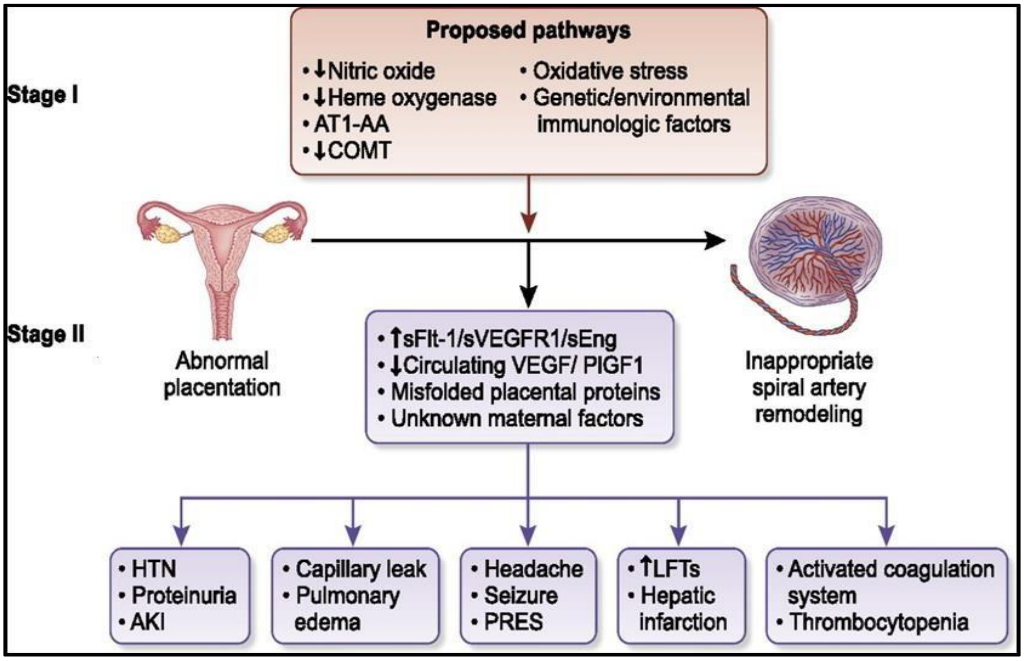


Figure 2: Pathogenesis of preeclampsia: two-stage model
AT1-AA - Autoantibodies to angiotensin receptor 1; COMT - Catechol-O-methyltransferase; HTN - Hypertension; LFT - Liver function test; PlGF1- Placental growth factor 1; PRES - Posterior reversible encephalopathy syndrome; sEng - soluble endoglin; sFlt-1- soluble fms–like tyrosine kinase 1; sVEGFR1 - soluble vascular endothelial growth factor receptor 1; VEGF- Vascular endothelial growth factor
Source: Jim B, Brahham K, Maynard SE, Hladunewich MA. Pregnancy and kidney disease. Nephrol Self Assess Program. 2016;11 (6): 1102-1113.

1.5.1. Genetic factors

Preeclampsia appears to be a multi-factorial disease and has genetic predisposition. This was first reported in the early 1960s. Both maternal and fetal genes are shown to be involved in the onset of preeclampsia. The overall preeclampsia heritability is estimated at ~55% (estimated at 35% and 20%, respectively), with both maternal and fetal genes contributes to the risk [2]. In relation to pregnancy complications, a few such genes polymorphisms are studied such as methylene tetrahydrofolate reductase (MTHFR) (C677T) gene, factor V (Leiden), angiotensinogen (AGT) (M235T), human leukocyte antigens (HLA various), endothelial nitric oxide synthase (NOS3) (Glu298Asp), F2 (G20210A), ACE (I/D[at] Intron 16) (Table 1) etc [2,22].

Table 1: Genes with possible association with Preeclampsia

Gene polymorphism	Function affected
MTHFR (C677T)	Methylene tetrahydrofolate reductase
F5 (Leiden)	Factor V_{Leiden}
AGT (M235T)	Angiotensinogen
HLA (Various)	Human leukocyte antigens
NOS3 (Glu298Asp)	Endothelial nitric oxide synthase
F2 (G20210A)	Prothrombin (factor II)
ACE (I/D^{at} Intron 16)	Angiotensinogen-converting enzyme

1.6. Oxidative stress and preeclampsia

Oxidative stress may be characterized by a state of imbalance between the production of reactive oxygen species (ROS) and endogenous antioxidant defense systems, leads to a disruption of redox signaling and molecular damage. The most common being superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\cdot}HO$) [17]. In the normal pregnancy, ROS are involved in the invasion of trophoblast, proliferation, energy production, organogenesis, regulation of cell growth, phagocytosis, synthesis of important biological substances and in intracellular signaling in the placenta. Therefore, homeostasis between oxidants and antioxidants plays an important role in preeclampsia [23].

The majority of reactive oxygen species are considered to be the by-products of cellular signaling and metabolism, located primarily in the mitochondria, or as specific products of enzymatic complexes such as NADPH oxidases, cytochrome P450, xanthine oxidases [24].

In normal pregnancy, the generation of ROS is known to be increased and is necessary for proper placentation [25]. Preeclampsia is associated with abnormal placentation, which in turn leads to reduced placental perfusion and ischemia, which triggers a condition of placental oxidative stress, leading to intravascular inflammatory response and endothelial dysfunction. Increased oxidative stress in preeclampsia starts as early as at the initiation of intervillous blood flow after 8 to 10 weeks of gestation [26].

Placental oxidative stress is considered to be a key intermediary step in the pathogenesis of preeclampsia, but the cause for the stress remains unknown. Hypoxia-reoxygenation injury, as a result of intermittent placental perfusion secondary to deficient trophoblast invasion of the endometrial arteries is a possible mechanism. Placental oxidative stress can be the consequences of fluctuations in oxygen concentrations after hypoxia and reoxygenation through the actions of ROS [17].

After placental reperfusion injury, re-established blood flow releases cytokines and other inflammatory factors such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and interleukin-10 (IL-10), C-reactive protein (CRP) and elevated levels of ROS like superoxide, in response to these events. Increased ROS may eventually trigger a redox signaling process to induce cell apoptosis. Increased superoxide generation by oxidative stress reacts with nitric oxide to produce Peroxynitrite ($ONOO^-$) and reduces the bioavailability of nitric oxide. Peroxynitrite is a strong pro-oxidant agent [25].

In the biological system, Peroxynitrite reacts slowly, selectively and it mostly reacts with tyrosine residues on proteins to produce 3-nitrotyrosine. This protein nitration causes post-translational modifications with pathological outcomes and Peroxynitrite can also cause damage to DNA and structural changes in lipids. It has been reported that, 3-nitrotyrosine residues have been observed in normal and pregnancies complicated with preeclampsia, especially in endothelium, surrounding smooth muscle and villous stroma. p38 MAPK has been significantly nitrated in preeclamptic placentas compared to normotensive controls [25]. p38 MAPK activation plays a role in release of pro-inflammatory cytokines and the induction of enzymes such as COX-2 which controls connective tissue remodeling in pathological conditions, inducible Nitric Oxide Synthase (iNOS) expression, induction of vascular cell adhesion molecules-1 (VCAM-1) and other adherent proteins along with other inflammatory molecules. Nitration of this p38 MAPK in preeclamptic pregnancies causes 65% drop-in catalytic activity. In addition to this, eNOS uncoupling also been shown as a source of superoxide generation and is linked with decreased nitric oxide production, when tetrahydrobiopterin (BH_4) is low or when post-translational modifications regulate eNOS function [25].

After reperfusion injury, re-oxygenation induces tissue and mitochondrial damage, indicating mitochondrial function is impaired in hypoxic placentas. This dysfunctional mitochondrion disrupts the fundamental processes which are essential for embryo development, and in turn, it has direct effect on fetal and placental growth and function [25]. This elevated systemic oxidative stress is responsible for the release of many factors from placenta into maternal circulation in preeclampsia (figure 3).

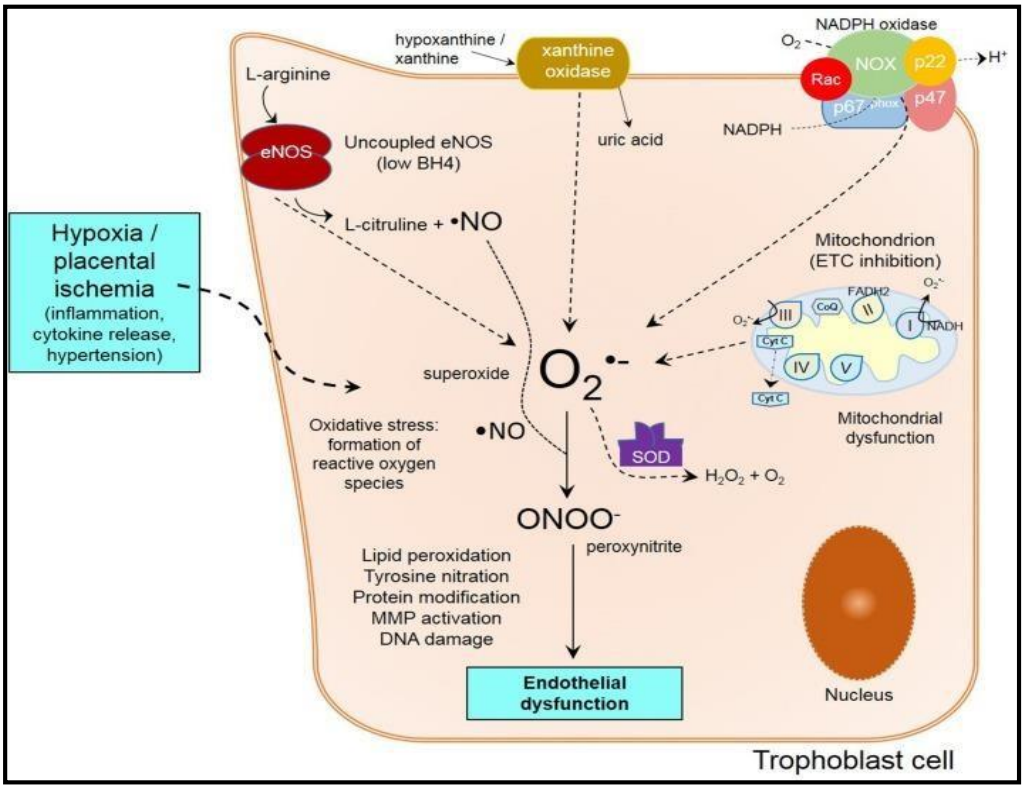


Figure 3: Mechanisms of free radical generation, oxidative stress and endothelial dysfunction in preeclampsia
eNOS - endothelial Nitric Oxide Synthase; NO – Nitric Oxide; SOD – Superoxide dismutase
Source: Sanchez-Aranguen LC, Prada CE, Riano Medina CE, Lopez M. Endothelial dysfunction and preeclampsia: role of oxidative stress. Frontiers in physiology. 2014;5:1-11.

The placental antioxidant imbalances have been strongly associated with the pathogenesis of preeclampsia. Oxidative stress markers like malondialdehyde (MDA) for lipid peroxidation, 8-hydroxy2-deoxy-guanosine (8-OHdG) for DNA damage and protein carbonyl for protein are found to be elevated in preeclampsia[26]. The enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-s-transferase (GST), thioredoxin (Trx), and thioredoxin reductase and non- enzymatic antioxidants vitamin C, vitamin E, β-carotene, glutathione (GSH), are found to be significantly decreased in preeclampsia [27].

In the recent years, cumulative evidences shown that biochemical imbalance in oxidative stress, lipid peroxidation and reduced antioxidant status as one of the factors involved in the preeclampsia pathophysiology [23]. Lipid peroxides, as products of an altered oxidative stress, are involved in endothelial cell injury, vasoconstriction and imbalance between thromboxane and prostacyclin. Endothelial contact with lipid peroxides would allow peroxidative damage of endothelial cell membrane lipids. This could ultimately reduce the ability of the endothelium to act as a permeability barrier to plasma components. Exposure of the vascular endothelium to lipid peroxides would begin to shutoff production of prostacyclin, increasing the propensity for vasoconstriction and platelet aggregation [26,27].

1.7. Apelin 13 - A circulating biomolecule

The term Apelin denotes **AP**J **e**ndogenous **li**ga**n**d. APJ receptor remained as "orphan receptor" until 1998, when Tatemoto K *et al*. identified this ligand and termed it as Apelin, for APJ endogenous ligand [28]. The Apelin receptor (also known as APJ or APLNR) is a class-A G-protein-coupled receptor discovered in 1993.

The gene encoding APJ is intron less and is termed as *APLNR* in humans. *APLNR* encodes a 380 amino acid protein and is located on the long arm of chromosome 11q12. The Apelin receptor is highly conserved across a range of species including human, rat, mouse and cow. This protein was named as APJ, contained the seven hydrophobic transmembrane segments characteristic of G-Protein Coupled Receptor genes. This Apelin receptor, most closely resembles the angiotensin II type-1 (AT1) receptor and overall, these two proteins share a

total of 115 amino acids (30%) and 86 amino acids (54%) in the transmembrane region. However, APJ does not bind angiotensin II. The APJ receptor containing seven hydrophobic transmembrane domains, with consensus sites for phosphorylation by protein kinase A, palmitoylation and glycosylation. The N-terminal glycosylation of GPCRs has been implicated in receptor expression, stability, correct folding of the nascent protein and ligand binding [29].

In humans, the *APLN* gene is located on chromosome X at Xq25-26.1 position; a 6 kb open reading frame is an Apelin gene (*APLN*) with one intron. Apelin is synthesized as 77 amino acid preproprotein (preproapelin), an immature single peptide, with hydrophobic rich N-terminal region. The amino acid sequence of Apelin is similar to that of angiotensin II. Amino proteases, are involved in the processing of Apelin peptides [29]. Bovine, human, rat and mouse preproapelin precursors have 76-95% homology and appear to exist endogenously as a dimeric protein, due to disulphide bridges. Dimerization of pre(pro) Apelin occurs by disulphide bridge formation. It has been reported that the dimerization is a prerequisite for proper processing in bioactive peptides, such as stomatostatin-II, suggesting that dimerization is necessary for proper processing of Apelin [30].

In the endoplasmic reticulum, 77 amino acid residue prepropeptide is cleaved to a 55 amino acid proapelin by cleaving N-terminal signal peptide, presumed to be an inactive precursor possessing the receptor binding site. The proapelin peptide further on processing produce smaller bioactive peptides such as Apelin 36 (Apelin 42-77), Apelin 17 (Apelin 61-77) and Apelin 13

(Apelin 65-77) as shown in figure 4. Additionally, pyroglutamate Apelin 13 [(Pyr^{1}) Apelin 13] is a post-translationally modified form of Apelin and it contains pyroglutamate group at N-terminus of the peptide [31, 32]. These peptides have distinct activities and shorter peptides are more potent activators for APJ. In adipocytes, proprotein convertase subtilisin (PCSK3) or furin directly cleaves proapelin to Apelin 13, with no production of longer isoforms [33]. Apelin peptides, which lack cysteine residues, are probably present in monomeric form. Apelin protein biological activity depends on carboxy terminal of the peptide comprising 12 amino acids [32]. Carboxy-terminal of preproapelin is rich in basic amino acids, which are potential cleavage sites during post-translational processing [28]. Among all Apelin peptides, Apelin 13 and (Pyr^{1}) Apelin 13 are the shortest, most prevalent and biologically active forms [32].

The biological inactivation of Apelin 13 and 36 occur by the action of angiotensin converting enzyme-II with high catalytic efficiency by cleaving bond between Proline-Phenylalanine and removes phenylalanine from C- terminus [32]. It has been reported that (Pyr^{1}) Apelin 13 is more resistant to enzymatic cleavage by angiotensin converting enzyme-II. In the circulation, Apelin is cleared very rapidly within a short plasma half- life [34,35]. The Neprilysin a metalloprotease involved in Apelin degradation at truncated region of Arg2-Leu5 region of Apelin and forms functional loss in activating APJ receptor.

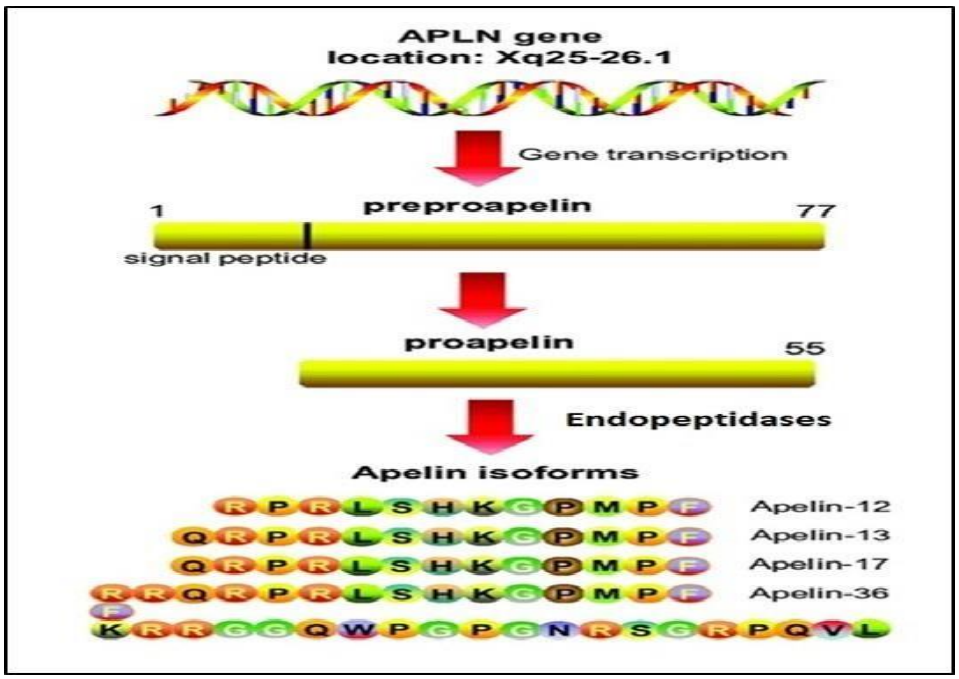


Figure 4: Synthesis of Apelin peptide isoforms and amino acid sequences
Source: Pedro Melgar-Lesmes, Meritxell Perramon and Wladimiro Jiménez. Roles of the Hepatic Endocannabinoid and Apelin Systems in the Pathogenesis of Liver Fibrosis. Cells. 2019; 8: 1311:1-24.

1.7.1. Regulation of Apelin gene

The Apelin gene expression is regulated by various transcription factors. A single-nucleotide polymorphism (SNP) study reported specificity protein 1 (Sp1) role in the regulation of Apelin gene expression. The expression of Apelin gene is enhanced by tumor necrosis factor-α (TNF-α), via phosphoinositide-3 kinase (PI3K), C-Jun-N-terminal kinase (JNK) and MEK1/2 (mitogen activated protein kinase 1 or 2) in adipocytes. Apelin core promoter sequences in rat and humans contain putative binding sites for upstream stimulatory factor (USF) 1/2, and overexpression of USF up- regulates Apelin transcription [36].

Hormone Response Elements (HREs) are present in promoter and intron sequence of Apelin gene in various species that causes up-regulation of Apelin gene expression. In hypoxic conditions, Apelin expression is up-regulated in cardiac myocytes wherein Hypoxia Inducible Factor-1α (HIF-1α) binds to HRE (-813/-826) located within the first intron of human Apelin gene and increases Apelin expression in vascular cells. In white adipocytes, peroxisome-proliferator-activated receptor γ co-activator-1α also up-regulates the Apelin gene expression [36].

1.7.2. Secretion and distribution of Apelin peptides

The Apelin expression widely takes place in placental syncytiotrophoblasts, cytotrophoblasts, and endothelial cells of fetal capillaries, lung, and less expressed in the heart, liver, adipose tissues, and brain. Immunoreactive Apelin is present in vascular endothelial cells of large conduit vessels, such as coronary artery and saphenous vein, blood vessels of kidney, adrenal gland, and vascular and endocardial endothelial cells of atria and ventricles [36].

1.7.3. Brief mechanism of Apelin and Apelin receptor interaction

Apelin is believed to be the only endogenous ligand for APJ. All Apelin peptides bind to APJ receptor [33]. Binding of Apelin peptides leads to conformational changes in the receptor, which causes activation of its associated G-protein that induces Guanosine Diphosphate (GDP) dissociation and Guanosine Triphosphate (GTP) binding. N-terminal domain of APJ plays a critical role in ligand binding. APJ is suggested to couple with G1α subunits family or also known as Gi/o protein, this finding was supported by the inability of (Pyr^1) Apelin 13 and Apelin 36 to generate calcium mobilization or

to release Arachidonic acid metabolites into the cells which leads to the positive or negative regulation of various intracellular effectors. By using transfected cells, similar results were obtained with neurons differentiated from NT2 (NTERA-2) cells and to a lesser extent with astrocytes. Interestingly, the rate of receptor recycling to the surface is faster for Apelin 13 than for Apelin 36 [37].

1.7.4. Molecular and Cellular signaling pathways of Apelin/APJ

Apelin peptides plays a key role in angiogenesis, vasodilation, cardiovascular system, fluid homeostasis, neuro-endocrine response to stress, metabolic actions and constriction. APJ activation leads to the activation of phospholipase Cβ, the phosphatidylinositol-3-kinase (PI3K)/Akt (protein kinase B) pathways and the Na^+/H^+ exchanger type 1. On the other hand, inhibits adenylyl cyclase and subsequent cyclic adenosine monophosphate production. Phospholipase Cβ triggers protein kinase C (PKC) and downstream Ras/Raf/MEK/ERK, which together with Akt via target of rapamycin (mTOR) are involved in the activation of P70S6K and endothelial nitric oxide synthase, causing the release of nitric oxide required for vasodilation and cell proliferation [35]. However, APJ activation associated with inflammation as shown in figure 5.

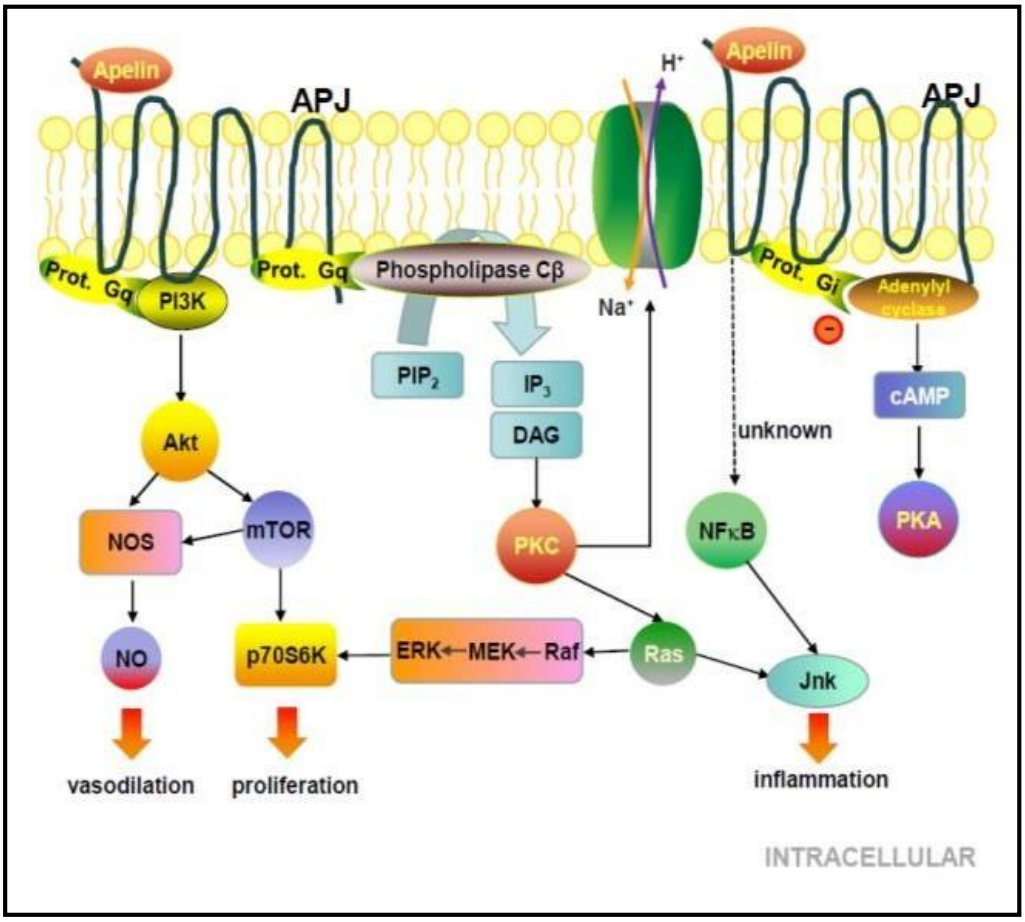


Figure 5: Schematic representation of intracellular signal transduction pathway and cellular signaling pathways of Apelin/APJ system

cAMP-Cyclic adenosine monophosphate; PKA-Protein Kinase A; PI3K-Phosphatidylinositol 3-Kinase; Akt-Protein Kinase B; NOS-Nitric Oxide Synthase; mTOR-Mechanistic Target of Rapamycin; PKC-Protein Kinase C; ERK-Extracellular Regulated Kinase; MEK-Mitogen-activated Protein/Extracellular Signal-regulated Kinase, NFkB – Nuclear factor kappaB, PKA-protein kinase A.

Source: Pedro Melgar-Lesmes, Meritxell Perramon and Wladimiro Jiménez. Roles of the Hepatic Endocannabinoid and Apelin Systems in the Pathogenesis of Liver Fibrosis. Cells. 2019; 8: 1311:1-24.

The Apelin peptides regulation in placenta is not fully elucidated. The expression of Apelin/APJ system in syncytiotrophoblasts, cytotrophoblasts, and endothelial cells of fetal capillaries of human chorionic villi, suggesting a paracrine role of Apelin in the uteroplacental unit. The angiogenic properties of Apelin as well as Apelin/APJ activation of cell migration and proliferation may be beneficial during placentation and embryogenesis [32].

During normal pregnancy, placental Apelin/APJ is more abundant during early gestation, suggesting its functional role in implantation or placentation. Plasma Apelin level is detectable in maternal circulation during pregnancy, but its level sharply drops in late pregnancy, suggesting that the circulating levels are strictly regulated during pregnancy. Altered expression impairs angiogenesis leading to onset of preeclampsia [38].

Cobellis *et al*. reported Apelin expression in human placenta was much higher in cytotrophoblast cells compared to syncytiotrophoblast. Cytotrophoblast are mononucleated cells that represent the proliferative population inside placental villi; suggesting direct role of Apelin/APJ on human placental cells proliferation [39]. In addition to this, immuno histochemistry on human placenta showed a strong signal for Apelin in the cytoplasm of the endothelial lining of the blood capillaries and in maternal blood, while cells of the placental artery exhibited a moderate intensity of the Apelin signal. A moderate to weak signal intensity for APJ was localized in the cytoplasm of epithelial cells of vesicles and mesenchymal cells and a strong signal for APJ was observed in cells of syncytiotrophoblast, suggesting Apelin role in placental cell proliferation [40].

Apelin plays an important role in placenta cell cycle progression. Apelin stimulates the transition to G2/M phase, which is required for cell division, activating cyclins D and E. Therefore, the modulation of cell cycle and expression of cyclins by Apelin is strictly connected with its biological activity. Apelin can enhance the cell cycle by promoting the switch of cells from S phase to G2/M phase. It is well known that increase in expression of cell cycle machinery is a key event in regulating cell proliferation [40].

Apelin plays a significant role in angiogenesis and vasodilation [34,41]. Increased expression of Apelin/APJ during embryogenesis provided initial support for the importance of Apelinergic signaling during angiogenesis. Apelin induces the phosphorylation of extracellular regulated kinase (ERK1/2) proteins, Akt, Stat3, and AMPK-α, indicating Apelin behaves as a mitogenic peptide for primary cultured umbilical endothelial cells. These findings suggest that Apelin-induced stimulation of trophoblast cell proliferation and regulates early placental development. As a result, the condition of the placenta directly indicates the well-being of the developing fetus [40]. Apelin may be implicated in placental vascular tone and influences subsequent maternal-fetal exchange of oxygen and nutrients. It has been reported that intravenous injection of Apelin 13 to rat pregnant mothers increases the transplacental transport of glucose from mother to fetus, suggesting that placenta derived Apelin participates in fetal glycaemia [42].

It has been reported that Apelin favors the glucose uptake in muscle through nitric oxide induced placental vasodilation [42]. In addition to this, hypoxia-induced Apelin gene expression also increases proliferation and differentiation of stem cells and progenitor cells, suggesting the possible therapeutic potential of Apelin in ischemic reperfusion injury. Apelin induces the proliferation of an immortalized endothelial cell line [41]. It has been reported that Apelin also involved in the regulation of caliber size of blood vessels by inducing their enlargement. During tumor neo-angiogenesis, overexpression of Apelin not only promotes an extension of the vascular network but also increases the number of large vessels. Since, Angiopoietin 1 (Ang 1) /Tie 2 regulates the enlargement of the vessel caliber, a link between

this pathway and Apelin signaling was expected. Ang 1 was found to upregulate the Apelin gene expression in endothelial cells and Apelin expression was increased in Ang 1 over expressing transgenic mice [43,44]. Enlargement of the vessel is one of the mechanisms by which hemodynamic changes can respond to an increase in oxygen demand. VEGF is the initiator of angiogenic process, which induces the sprouting of endothelial cells from mature vessels and the concomitant expression of Apelin receptor. Ang 1 through its Tie 2 receptor triggers the Apelin expression, which is secreted outside the endothelial cell. The synergic activation of VEGF and Apelin would lead to proliferation of endothelial cell and formation of cell-to-cell contacts. In this scenario, the precise role of Apelin signaling would concern the mobilization of endothelial cells in this assembly process and the construction of enlarged vessels [44].

Apelin causes endothelium dependent vasodilation by stimulating the phosphorylation of endothelial Nitric Oxide Synthase (eNOS) at Serine 1177 position and releases NO [45]. Apelin induced endothelium dependent vasodilation is reduced by co-administration of L-Nitro-Arginine- Methyl-Ester (L-NAME), suggesting an endothelium-derived NO dependent mechanism. L-NAME is an inhibitor of eNOS [32]. In isolated rat aorta, Apelin caused concentration and time-dependent increases in eNOS activity and NO synthesis [45]. Activation of mechanosensor pathways by flow-mediated shear stress increases APJ receptor expression, Apelin and NO synthesis in endothelial cells.

Apelin levels were found to be altered in acute coronary syndrome, essential hypertension, due to some common pathophysiological mechanisms in both conditions. Therefore, Apelin levels may reflect cardiac function in preeclamptic women [46,47].

Based upon the existing information on role of Apelin in placentation, angiogenesis and vasodilation**,** a few studies have reported circulating Apelin levels in preeclampsia. However, study findings were found to be controversial and needs to addressed. The studies reported decreased level of Apelin 13 [32,48-50], or increased [51] and found that in majorityof the reports emphasized the low levels of Apelin 13 in preeclampsia condition.

1.8. Apelin gene description

In humans, the *APLN* gene is located on chromosome X at Xq25-26.1 position, comprises 3 exons and 1 intron within its open reading frame of ~ 6 kb as depicted in figure 6. The single nucleotide polymorphism of Apelin gene in promoter region is related to plasma Apelin levels [52]. Therefore, Apelin gene polymorphisms may be related to lower circulating levels of Apelin and this low level may be associated with the abnormal placentation and reduced vasodilation in preeclampsia.

However, A few studies reported on the Apelin gene polymorphisms are linked with hypertension [53,54]. The Apelin - 1860T>C (rs56204867) polymorphism established in hypertensive conditions like heart disease [52,55]. Li WW *et al*. first reported at the promoter region of *APLN* gene -1860T>C (rs56204867) polymorphism in the Han Chinese hypertensive population [56].

It has been shown that Apelin -1860T>C (rs56204867) polymorphism was related to the plasma Apelin levels [52].

A few research reports emphasized on the relationship of low levels of Apelin and cardiovascular complications such as coronary artery disease, coronary artery ectasia, heart patients with systolic left ventricular dysfunction [52]. The evidences also exist about onset of cardiovascular complications by preeclamptic mother after delivery [57]. Hence, the present study is focused to know Apelin 13 concentrations and Apelin gene polymorphism in maternal blood. In addition to this, other markers with endothelial function are interest in the study.

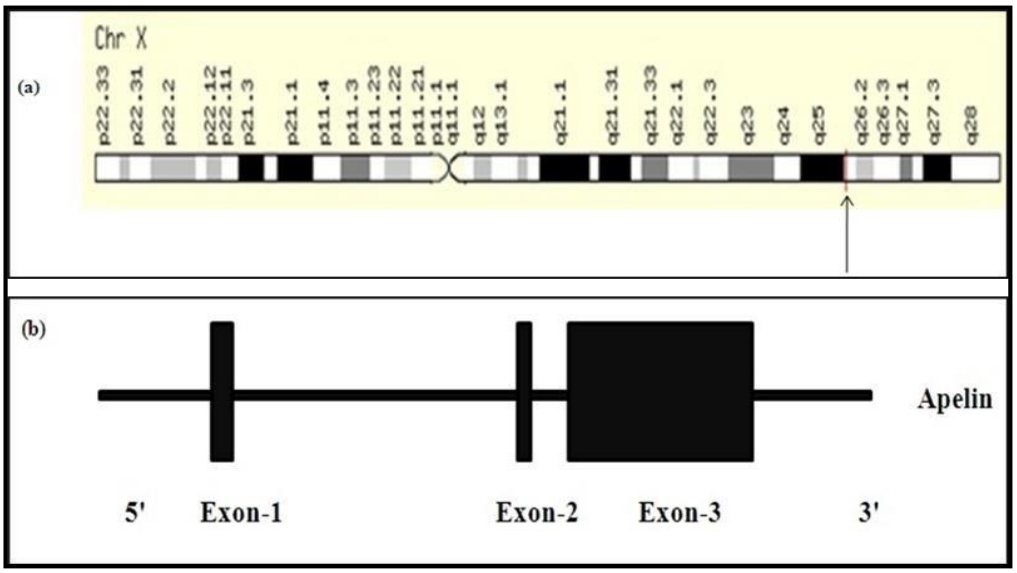


Figure 6: (a) Structure of chromosome X with location of *APLN* gene (arrow); (b) Structure of *APLN* gene showing exons and introns

Source: Jin W, Su X, Xu M, Liu Y, Shi J, Lu L *et al*. Interactive association of five candidate polymorphisms in apelin/APJ pathway with coronary artery disease among Chinese hypertensive patients. Plos One. 2012;7(12):e51123.

1.9. Endothelium

The vascular endothelium is the inner most structure and it lines the entire vascular system such as arteries, capillaries, and veins. It is a monolayered, squamous cells, called endothelial cells, and is originated from mesoderm. Endothelial cells are directly exposed to blood constituents are called vascular endothelial cells, whereas those in direct contact with lymph are known as lymphatic endothelial cells. Endothelium, forming an interface between circulating blood or lymph in the lumen and the rest of the vessel wall. In adults, approximately ten trillion (10^{13}) cells form an almost one-kilogram organ [59].

1.9.1. Endothelial cell structure

The shape of endothelial cells varies along with the vascular tree. In general, the endothelial cells are thin, slightly elongated and approximately 30-50 μm in length, 10-30 μm wide and a thickness of 0.1-10 μm as shown in figure 7. The orientation of the endothelial cells is along the axis of the vessel in the blood vessel wall in order to minimize the shear stress exerted by the flowing blood [59,60].

The cytoskeleton consists of microtubules and a network of actin and intermediate filaments. This provides a strong, dynamic intracellular scaffold that organizes integral membrane proteins with the cells interior, and responds to environmental cues to orchestrate appropriate cell shape. The actin cytoskeleton is comprised of three distinct, but interrelated structures, including actin cross-linking of spectrum within the membrane skeleton, the cortical actin rim, and actomyosin-based stress fibers. In quiescent endothelium, actin forms a cortical rim that interacts with both cell-cell and cell-matrix adhesion complexes, and tethers these structures to intracellular organelles. Actin tethering to adhesion complexes is absolutely essential to maintaining a functional endothelial cell barrier [60]. Imbalance between the adhesive force and contractile force results in barrier dysfunction. Pathophysiological conditions, such as inflammation, trigger a series of signaling events in endothelial cells that propagate to the cytoskeleton and promote cell contraction. Many inflammatory mediators also induce phosphorylation and disorganization of junction molecules [61].

Endothelial cell is lined with a very fine and fragile layer called the glycocalyx, which consists of glycoproteins, glycosaminoglycans and proteoglycans. Glycosaminoglycans, including heparin sulfate, a cofactor for antithrombin III that amplifies its anti-thrombotic properties, and dermatan sulfate, which interacts with heparin cofactor II. Destruction of the glycocalyx leads to increased capillary permeability. Endothelial cells are joined to each other and form junctional complexes consisting of tight junctions and adherence junctions, which are the sites of diffusional transport of solutes [62].

1.9.2. Functions of Endothelium

Endothelium performs wide range of homeostatic functions with its ability to act on both sensory and effector capacities. Endothelium has many functions in vascular biology including endothelium transport function, regulation of blood clotting, regulation of vascular tone, inflammation, immunity and angiogenesis. Endothelial cells play an important role in vasoconstriction by releasing endothelin and thromboxane A2. The vasoconstriction is followed by platelet adhesion to vascular wall. Endothelial cells synthesize Von Willebrand factor (VWF), fibronectin and thrombospondin. VWF forms bridges between the sub-endothelial structures and a specific receptor in platelet membrane. It also, serves as a 'glue' linking platelets to the sub-endothelial matrix and to other platelets. Endothelial fibronectin cross-links fibrin monomers, and endothelial thrombospondin reduces local fibrinolysis and promotes platelet aggregation. Endothelial cells synthesize factor V. They have, also, specific binding sites for factors IX/IXa and factor Xa. These binding sites localize the coagulation process to endothelial surface and suppress their circulation [62,63].

Endothelial cells produce thrombomodulin (TM), a thrombin-binding protein, on their surfaces. TM combines with thrombin to form thrombomodulin-thrombin complex. This complex convert's protein C to activated protein C. Protein C when activated, it inhibits both of active factor's V & VIII (in presence of protein S) and tissue plasminogen activator (t-PA) inhibitors. In presence of urokinase plasminogen activator (u-PA), convert plasminogen to plasmin. Plasmin breakdown fibrin clot into fibrin degeneration products which are excreted in urine. The absence of protein C leads to uncontrolled intravascular clotting and death in infancy [62]. In addition to this, thrombomodulin has its own intrinsic anticoagulant activity, since several works have demonstrated its ability to bind and directly inhibit activated factor X.

The factors like prostacyclin, nitric oxide and their synthesis in endothelial cells plays a role in the vasodilation, inhibits adhesion of platelets and neutrophils. The formed nitric oxide in the endothelial cells plays an important role in regulation of blood pressure. Vasodilatation caused by nitric oxide begins with stimulation of the soluble guanylate cyclase and subsequent formation of cyclic-GMP. Cyclic-GMP activates protein kinase C, which causes reuptake of Ca^{2+} and the opening of calcium-activated potassium channels. The fall in concentration of Ca^{2+} ensures that the myosin light-chain kinase can no longer phosphorylate the myosin molecule, thereby stopping the cross-bridge cycle and leading to relaxation of the smooth muscle cell. Nitric oxide has important role in immune system, nervous system, inflammation and blood flow. It contributes to vessel hemostasis by inhibiting vasoconstriction, platelet aggregation, and leukocyte adhesion to the

endothelium. Endothelium- Derived Hyperpolarizing Factor (EDHF) is a vasodilator, which hyperpolarizes the underlying smooth muscle. EDHF is released when endothelial cells are activated by agonists such as bradykinin and acetylcholine [62].

1.9.3. Endothelium and normal pregnancy

Endothelium plays an important role in vascular changes during pregnancy. The endothelium appears to be up-regulated in pregnancy, producing vasodilation either as a result of an increased production of vasodilators or decreased release of vasoconstrictors (figure 7). Normal pregnancy course includes several changes in anatomical and functional changes of hemodynamic and cardiovascular system in order to meet the oxygen and nutrient requirements of the growing fetus. There is increased blood volume, increase in heart rate and stroke volume and thus increase in cardiac output 30-50%, decrease in peripheral vascular resistance and blood pressure [63]. As a result of maternal vasodilatation peripheral blood flow increases in the uteroplacental circulation [25].

Placental vascular endothelium is also involved in the synthesis of angiogenic factors such as vascular endothelial growth factor (VEGF), placental growth factor (PlGF), vasodilators such as nitric oxide and prostaglandins, placental hormones like estradiol and progesterone and disposal of waste to keep the fetus safe [63]. The levels of growth factors and cytokines also increased at the site of implantation and placentation to assist in angiogenesis. Further changes in the uterine vasculature includes outward vascular smooth muscle cell hypertrophy; increase in vessel diameter, which is likely accompanied by

endothelial hyperplasia to cover the increased surface area of the outwardly growing vessel and vessel lengthening. The net result of this is reduction in vascular resistance in the tissue. This local drop in vascular resistance preferentially provides blood to the uterus, placenta, ensuring adequate gas, nutrient exchange and removal of waste materials. Also, thisdrop in vascular resistance favors the remodeling of the spiral arterioles [25].

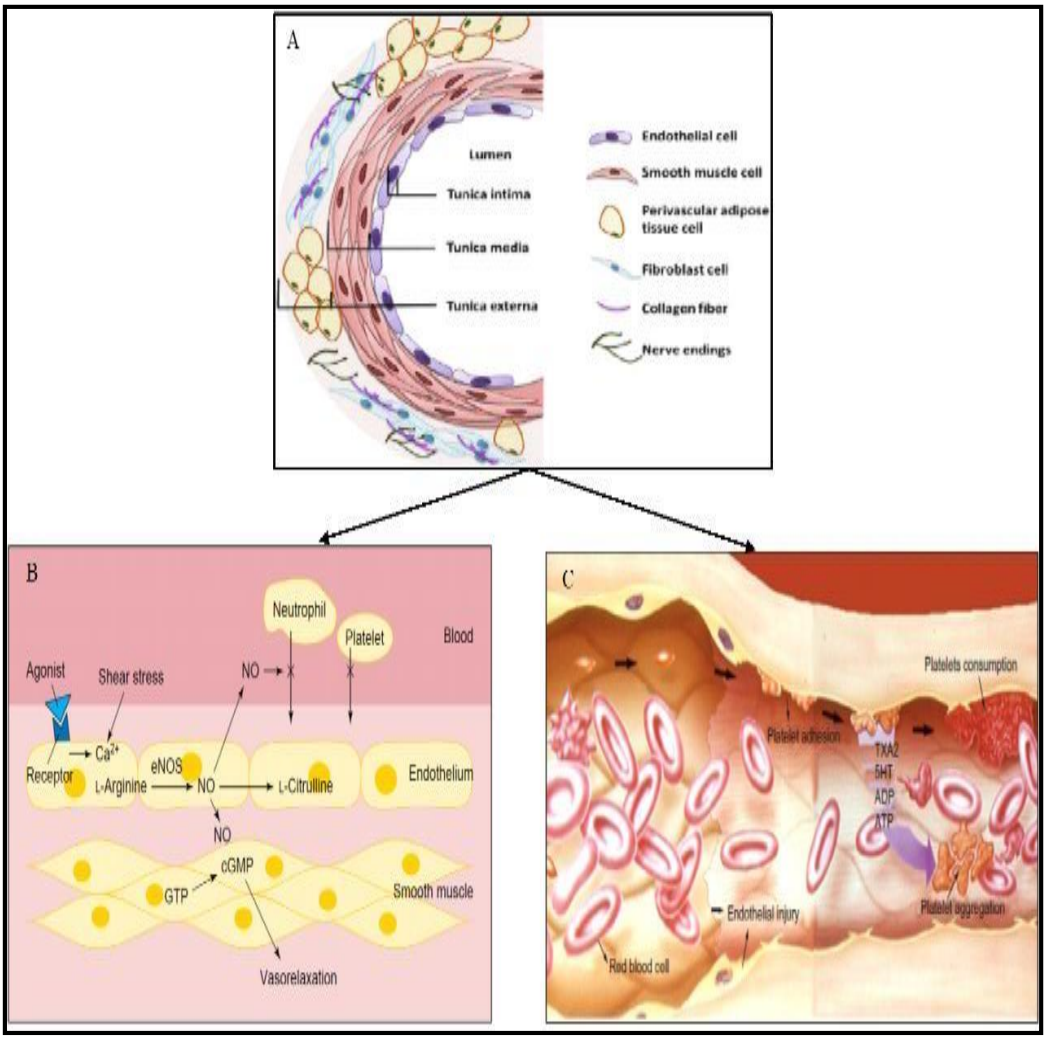


Figure 7: **(**a) Structure of arterial wall
(b) Endothelium in normal pregnancy
(c) Endothelium in Preeclampsia

GTP – Guanosine triphosphate; NO – Nitric Oxide; TXA2 – Thromboxane A2

Source: Fiona Lyall and Ian A. Greer. The vascular endothelium in normal pregnancy and preeclampsia. Reviews of reproduction. 1996;1:107-116.

1.9.4. Endothelium in preeclampsia

Functional alteration of endothelium is known in pregnancy complications [65]. This endothelial dysfunction is characterized by reduced vasodilation, a pro-inflammatory state, and prothrombic properties (figure 7). Preeclamptic women show systemic endothelial dysfunction and hypertension, glomerular endotheliosis causing kidney injury and proteinuria, cerebral endotheliosis leading to cerebral edema and seizures [66]. This suggest that endothelial dysfunction contributing to all major symptoms of preeclampsia such as hypertension, proteinuria, edema, improper platelet aggregation [67]. Studies have reported that increased levels of endothelial cell dysfunction markers such as soluble vascular cell adhesion molecule-1 (sVCAM-1), E-selectin and endocan [68,69].

During normal pregnancy, maternal spiral artery remodeling is necessary to access maternal blood supply. Abnormal placentation caused by shallow trophoblastic invasion associated with maternal vascular and endothelial dysfunction. The reduced placental perfusion in preeclampsia generates ROS and activation of endothelial cells and thereby causes endothelial dysfunction. Due to defective invasion of trophoblast, intermittency of blood flow occurs, results in ischemia/reperfusion injury, creating hypoxia, which favors oxidative stress, consequent oxidative damage and inflammation [67]. In addition, syncytiotrophoblasts derived extracellular vesicles (SDEVs) could stimulate neutrophils to produce superoxide free radicals that further injure endothelial cells in not only placental vasculature but also vessels in other organs. The key mechanisms involved in the endothelial dysfunction are release of soluble fms-like tyrosine kinase -1 (sFlt-1), an anti- angiogenic

factor, and endogenous inhibitor of vascular endothelial growth factor (VEGF). The concentrations of sFlt-1 were increased in preeclampsia, which leads to endothelial cell injury and dysfunction [70].

In preeclampsia, inflammatory response after ischemia and reperfusion injury converges into a damaging inflammatory response and is responsible for inflammation and oxidative stress. This leads to the release of inflammatory markers such as TNF-α, IL-6, CRP and elevated levels of ROS. This elevated ROS may trigger the redox signaling mechanism to induce cell apoptosis. Reduced placental perfusion and shallow invasion of trophoblast, triggers placental oxidative stress, leading to intravascular inflammation and endothelial dysfunction [25].

To enhance vascular relaxation, the endothelial cells release vasodilators such as nitric oxide, prostacyclin, endothelium-derived hyperpolarizing factor and also factors that cause vasoconstriction such as endothelin-1, thromboxane [71]. The imbalance of vasodilators and vasoconstrictors are associated with endothelial dysfunction (figure 8).

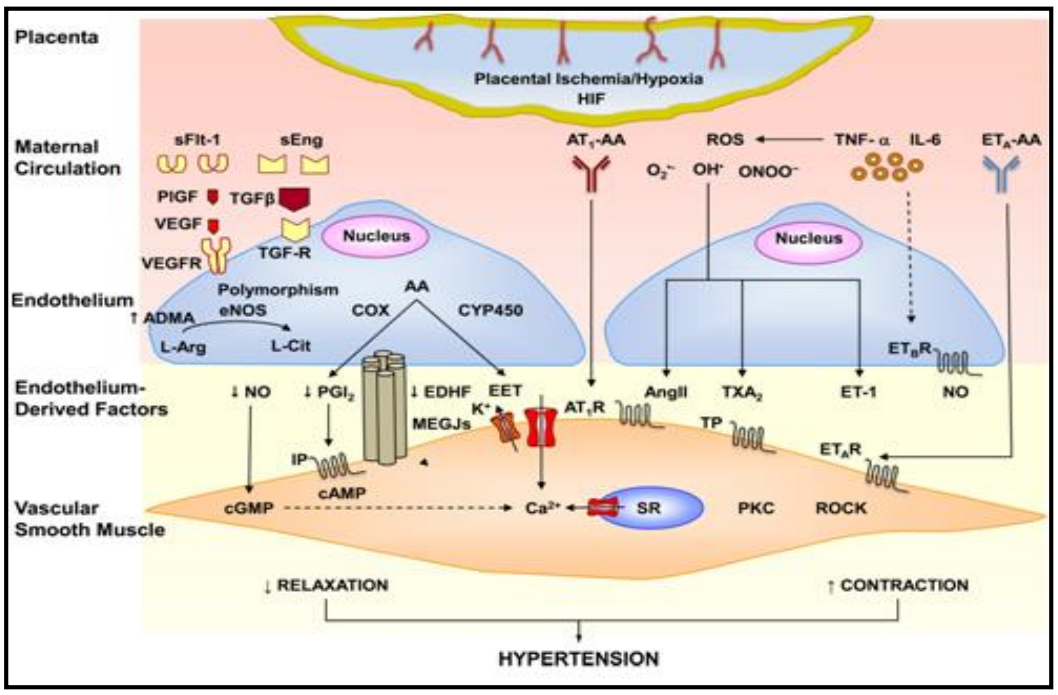


Figure 8: Circulating bioactive factors cause endothelial dysfunction and hypertension in preeclampsia

ADMA - Asymetric Dimethylarginine; Ang II - Angiotensin II; cAMP - cyclic Adenosine monophosphate; COX - Cyclooxygenase; ET-1 - Endothelin 1; PlGF - Placental growth factor; PKC - Protein kinase C; TGF -β - Transforming growth factor – β; VEGFR - Vascular endothelial growth factor receptor

Source: Jose S. Possomate-Vieira and Raouf A. Khalil. Mechanisms of endothelial dysfunction in hypertensive pregnancy and preeclampsia. Adv Pharmacol. 2016;77:361-431.

It has been reported that decreased nitric oxide production or its bioavailability in relation to preeclamptic pregnancies. The decreased bioavailability of nitric oxide often associated with endothelial dysfunction. This may be due to its low production or increased degradation, and its changes in metabolism [71]. Also, syncytiotrophoblasts derived extracellular vesicles (SDEVs) may reduce the production of nitric oxide from endothelial cells by blocking the endothelial nitric oxide synthase (eNOS) and contributes to the vascular endothelial dysfunction, hypertension and proteinuria in preeclampsia patients [70]. In addition to this, endothelial nitric oxide synthase gene polymorphism may also

affect the nitric oxide levels [71]. Shear stress-induced nitric oxide dependent vasorelaxation is also reduced in human myometrial arteries from preeclampsia patients [67].

Prostacyclin (PGI2) is a vasodilator produced from the endothelial Arachidonic acid metabolism by cyclooxygenase-1 (COX-1) and COX-2. PGI2 promotes vascular relaxation and inhibits platelet aggregation, promotes angiogenesis and also reduces hypoxic injury [71]. It has been reported that maternal plasma levels of PGI2 were decreased in preeclampsia. The decreased levels were due to impaired Ca^{2+} signaling in endothelial cells and increased oxidative stress in preeclampsia inhibits prostacyclin synthase, and this may contribute to endothelial dysfunction [67].

Endothelin -1 (ET-1), derived from endothelium could play a role in endothelial dysfunction in preeclampsia. Factors such as cytokines, placental hypoxia, and AT1-AA stimulate the synthesis of ET-1 from endothelial cells [62]. It has been reported that endothelin-1 concentrations were elevated in preeclamptic women. ET-1 induces trophoblastic cell apoptosis and increases oxidant and anti-angiogenic factors. It activates ET-1 receptor type A and B (ETAR and ETBR). Activation of endothelin receptor type A (ETAR) in vascular smooth muscle cells stimulates release of Ca^{2+} ions and its entry through Ca^{2+} channels, inhibits K^{+} channels though protein kinase C (PKC), which leads to increased Ca^{2+} concentration and vascular smooth muscle cell contraction. Activation of endothelin receptor type B (ETBR) stimulates secretion of vasodilators, which promotes vasodilation. ETBR may be reduced in endothelial cells and its down-regulation may impair the invasion of

trophoblasts in preeclampsia and production of vasodilators, especially nitric oxide. This could play a role in endothelial dysfunction in preeclampsia. Thromboxane A2, produced by the cyclooxygenase-1 (COX-1), in platelets. It stimulates platelet aggregation, proliferation of vascular smooth muscle cells and mitogenesis. TXA2 causes vasoconstriction by activating prostanoid receptors, Ca^{2+}, PKC, mitogen-activated protein kinase (MAPK) and rho-kinase (ROCK) in vascular smooth muscle cells [71].

Preeclampsia is associated with exaggerated inflammation and induces the secretion of proinflammatory markers such as interleukin (IL)-1, IL-6, IL-8, IL-10 and tumor necrosis factor-α, down-regulation of eNOS, mitochondrial biogenesis, which leads to mitochondrial dysfunction and exaggerated systemic inflammatory response [67]. These inflammatory markers can inhibit Ca^{2+} signaling mechanism, that are essential for the release of vasodilators, which finally induce endothelial cell injury, leads to tissue edema, hypertension and vascular leakage [70].

Hypercoagulable state is often seen in pregnant women, which is more severe in preeclamptic women. Tissue factor (TF) and anionic phosphatidylserine (PS) on the syncytiotrophoblast-derived extracellular vesicles (SDEV) could result in a systemic hypercoagulable state, which results in microvascular thrombosis, terminal organ ischemia and disseminated intravascular coagulation. Hypercoagulable state in preeclampsia could result in deposition of coagulation product fibrin in vascular endothelium, further damages endothelial function and increases the rigidity of vessel wall, which is a contributing factor for the development of hypertension. This TF and PS

driven hypercoagulable state was further enhanced by the over expression of serine protease plasminogen activator inhibitors-1 (PAI-I) on SDEVs. PAI-I inhibits tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) that activate plasminogen to trigger fibrinolysis and to re-establish blood flow of occluded vessels. PAI-I expressed on SDEVs could block or reduce fibrinolysis to prevent or delay tissue perfusion, thus propagating the preeclampsia induced hypercoagulable and prothrombotic state [64,70].

Functional alteration of endothelium is known in pregnancy complications. This endothelial dysfunction is characterized by reduced vasodilation, a pro-inflammatory state and prothrombotic properties. Preeclamptic women show systemic endothelial dysfunction and hypertension, glomerular endotheliosis causing kidney injury and proteinuria, cerebral endotheliosis leading to cerebral edema and seizures. This suggests that endothelial dysfunction contributing to all major symptoms of preeclampsia such as hypertension, proteinuria, edema and improper platelet aggregation.

Therefore, a major role of endothelial cells is to fight against vascular disease is by the action of endothelial nitric oxide synthase (eNOS), an enzyme that involved in the synthesis of a vasoprotective molecule regarded as nitric oxide.

1.10. Endothelial Nitric Oxide Synthase (eNOS)

Endothelial NOS (eNOS) (EC 1.14.13.39), also known as nitric oxide synthase 3 (NOS3) or constitutive NOS (cNOS), encoded by the NOS3 gene located in the 7q35-36, contains 25 introns and 26 exons, spanning 21 -22 kb. The gene, that

encode a 134-kD protein containing 1,205 amino acids and encodes an mRNA of 4052 nucleotides [72].

1.10.1. Structure of eNOS

eNOS is a bi-domain enzyme with two identical monomers of 134 kD constituted by a C-terminal reductase domain, which has binding sites for nicotinamide adenine dinucleotide phosphate (NADPH), flavinmononucleotide (FMN), and flavin adenine dinucleotide (FAD), and an N- terminal oxidase domain, which has binding sites for heme group, zinc, the cofactor tetrahydrobiopterin (BH_4), and the substrate L-arginine (figure 9). The reductase domain is linked to the oxidase domain by a calmodulin-binding sequence, which play an important role in the structure and function of the enzyme [73]. Dimerization is essential for eNOSenzymatic activity. The eNOS is activated by various physical and chemical stimuli such as fluid shear stress and increased intracellular Ca^{2+}, interaction with substrate and co-factors, protein phosphorylation [73-75].

The exact cellular location of the eNOS is not clear. However, it has been assigned to the plasma membrane, Golgi apparatus and plasmalemmal caveolae, most eNOS is located in the caveolae, where it is bound to caveolin, a resident coat protein. The caveolin binding leads to inhibition of eNOS activity by interfering with calmodulin (CaM) binding and transfer of electron to heam subunit. Increased intracellular Ca^{2+} concentration causes formation of Ca^{2+}/CaM complexes, resulting in the binding of the enzyme and consequently the dissociation of caveolin [73].

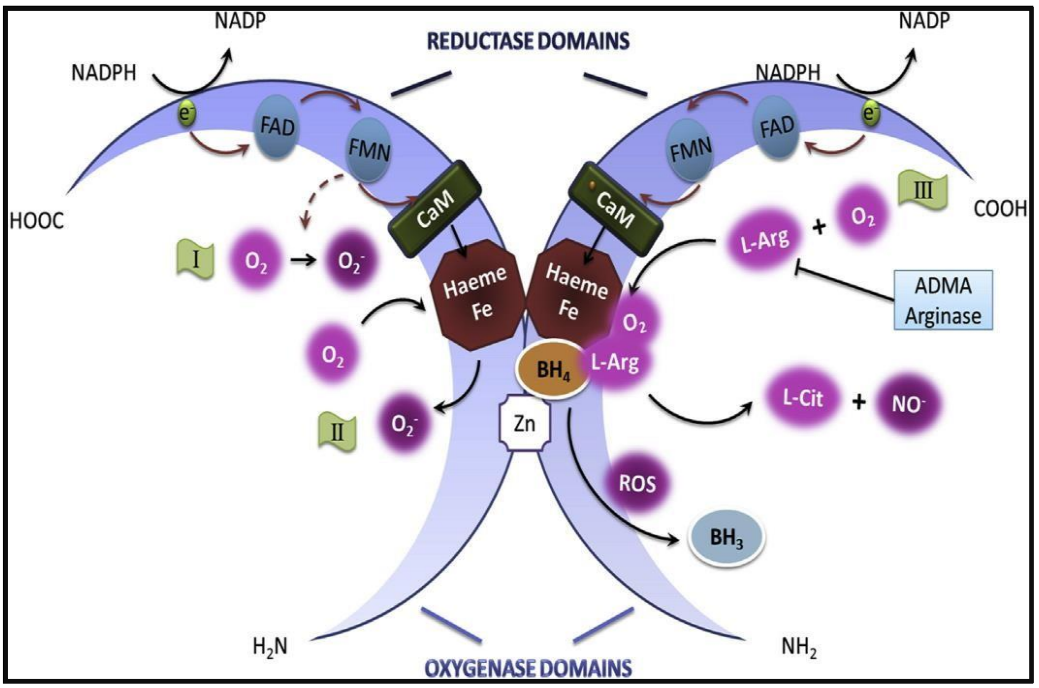


Figure 9: Homodimers of Nitric Oxide Synthase (NOS)
BH_4 – Tetrhydrobiopterin; Cam – Calmodulin; Zn – Zinc
Source: Yingzi Zhao, Paul M.Vanhoutte, Susan W.S. Leung. Vascular nitric oxide: Beyond eNOS. Journal of Pharmacological Sciences. 2015; 129(2):83-94.

In addition to this, eNOS can also be activated by stimuli that do not produce sustained increases in intracellular calcium ions, but still induce a long-lasting release of nitric oxide [76]. These stimuli are hypoxia, hormones such as estrogen, cyclic strain, G-protein, acetylcholine, bradykinin, LDLC, and mechanical forces. These molecules induce the transcription of eNOS or lead to eNOS activation by increasing the intracellular Ca^{2+} levels, caused by either influx of extracellular Ca^{2+} or Ca^{2+} release from intracellular stores, which binds to calmodulin and finally leads to the eNOS activation [73,75,77].

The electrons move from C-terminal reductase of one NOS monomer to N-terminal oxygenase domain of other NOS monomer [76]. In the setting of increased intracellular Ca^{2+}, the formation of a Ca^{2+}-calmodulin complex

disrupts NOS3 suppression from the NOS3- caveolin interaction, increasing the rate of electron transfer from NADPH via reductase domain flavins to the oxygenase domain. Within this N-terminal oxygenase domain, molecular oxygen is bound to heme and reduced and then incorporated into L-arginine to synthesize Nitric Oxide (NO) and L-citrulline [72]. For the efficient production of nitric oxide, eNOS must effectively coordinate the binding of multiple substrates and co-factors, especially BH_4 (figure 10).

Under oxidative conditions, ROS oxidizes BH_4, leading to a shift from dimeric form to monomeric form of the enzyme. Therefore, in the absence of this BH_4, eNOS thus becoming uncoupled [72]. In this conformation, instead of synthesizing NO, eNOS can result in generation of superoxide, which reacts with nitric oxide to form Peroxynitrite, a highly reactive free radical, contributes to endothelial dysfunction [73]. The bioavailability of nitric oxide is also decreased due to increased expression of arginase and ADMA. ADMA is a competitive inhibitor of NOS thereby decreases the synthesis of nitric oxide. ADMA represent a novel risk factors for the development of endothelial dysfunction [78,79].

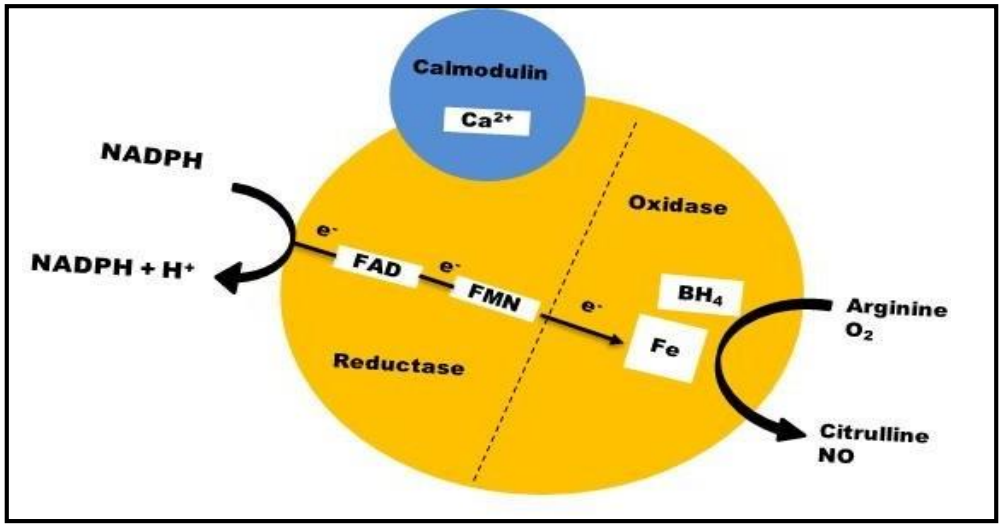


Figure 10: Overall reaction catalysed and cofactors of NOS
Source: Alderton WK, Cooper CE, Knowles RG. Nitric oxide synthases:structure, function and inhibition. Biochem J. 2001;357:593-615.

1.10.2. Regulation of eNOS activity

The regulation of eNOS activity is complex and it can be categorized into two: genetic and protein level. At genetic level, the expression and stability of eNOS genes are linked with eNOS activity. The eNOS promoter region contains binding sites for transcription factors such as activator protein-1 and 2 (AP-1 & 2), endothelin family, nuclear factor - kB (NK-kB) and neurofibromin-1 (NF-1). These transcription factors are involved in the regulation of eNOS expression. Along with this transcription factors, hypoxia, lipopolysaccharide, and cytokines can also induce the expression of eNOS. They bind with 3' untranslated region of eNOS mRNA, which is rich in cytosine, causes lower eNOS mRNA stability and shorter half-life [77].

At the protein level, the regulation is mainly through eNOS translocation, complex formation and phosphorylation of amino acid residues. The eNOS association with bradykinin B2 receptor and caveolin-1 (Cav-1) of the

endothelial plasma membrane leads inhibition of eNOS activity [77]. This inhibition is a result of functional interference with CaM binding and electron transfer (figure 11).

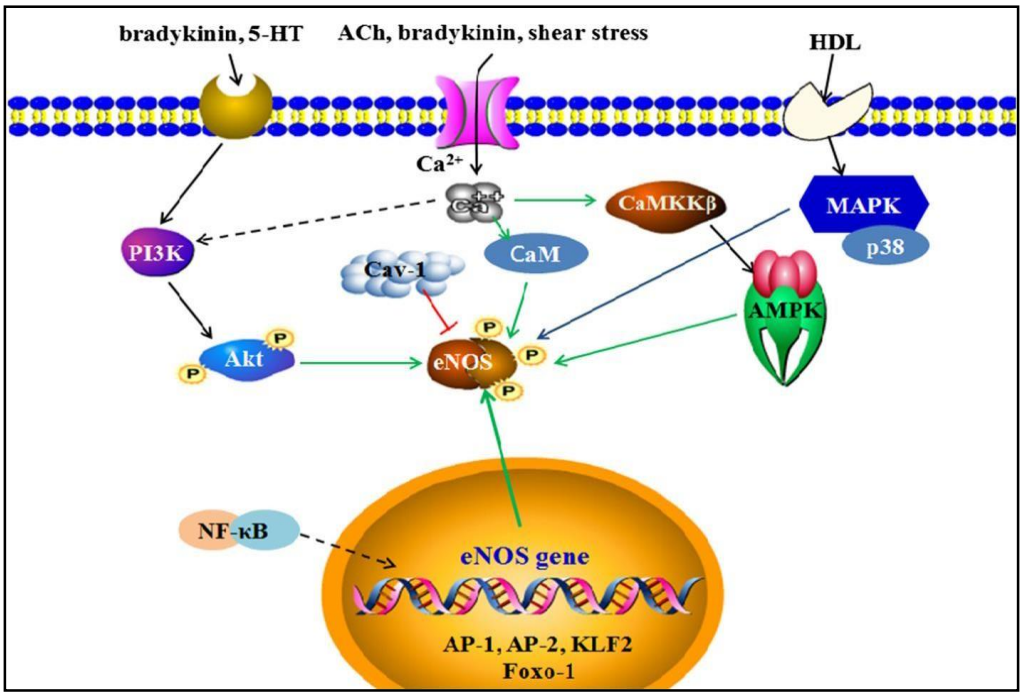


Figure 11: The regulatory mechanisms of eNOS activity
NF- κB: Nuclear factor-κB, AP-1: Activator protein-1, AP-2: Activator protein-2, KLF2: Krűppel-like Factor 2, Foxo -1: Endothelin family and Forkhead box O1, 5-HT: 5-hydroxytryptamine, Ach: Acetylcholine, HDLC: High density lipoprotein cholesterol, PI3K/Akt: Phosphatidylinisitol- 3kinase/protein kinase B, AMPK: AMP-activated protein kinase, MAPK: Mitogen-activated protein kinases pathway, CaM: Calmodulin Cav-1: Caveolin-1
Source: Jinqiang Zhu, Wanshan Song, Lin Li, Xiang Fan. Endothelial nitric oxide synthase: a potential therapeutic target for cerebrovascular diseases. Mol Brain.2016;9(30):1-8.

1.10.3. Phosphorylation sites of endothelial Nitric Oxide Synthase (eNOS)

The phosphorylation of eNOS at multiple positions, along with increased Ca^{2+} levels largely determine endothelial nitric oxide output. The eNOS activity can be regulated by protein phosphorylation. Studies reported that eNOS can be phosphorylated particularly at serine, threonine and tyrosine residues [76,77]. In humans, there are seven primary eNOS phosphorylation sites have been identified namely tyrosine81, serine114, threonine495, serine615, serine633,

tyrosine657 and serine1177. The phosphorylation of human eNOS serine1179 on C-terminal reductase domain is the first phosphorylation site identified as a positive regulator of eNOS activity (figure 12) [80]. Serine1177 phosphorylation stimulates the flux of electrons within the reductase domain, increases the Ca^{2+} sensitivity of the enzyme and leads to eNOS activation. Estrogen and vascular endothelial growth factor (VEGF) phosphorylate eNOS mainly via the serine/threonine kinase Akt, insulin probably activates both Akt (Protein Kinase B) and the AMP-activated protein kinase (AMPK), the bradykinin-induced phosphorylation of serine1177 is mediated by Ca^{2+}/calmodulin dependent protein kinase II (CaMKII), and shear stress elicits phosphorylation mainly by activating protein kinase A (PKA) [76,81].

Thus, although all the kinases mentioned can regulate eNOS serine1177 *in-vitro*, Akt is the only kinase proven to regulate eNOS function *in-vivo*. The phosphorylation of threonine495 occurs under non-stimulated conditions (mostly by protein kinase C) and this phosphorylation likely to interfere with the binding of calmodulin to the calmodulin-binding domain. In fact, dephosphorylation of threonine495 is associated with stimuli that elevate intracellular Ca^{2+} concentrations and increase eNOS activity. Substantially more calmodulin binds to eNOS when threonine495 is dephosphorylated. However, dephosphorylation of threonine495 has also been shown to favor eNOS uncoupling. Other phosphorylation sites of human eNOS include serine114, serine633, tyrosine81, and tyrosine657 residues. Phosphorylation of these residues is an intensively studied area and may have important consequences for enzyme activity [76].

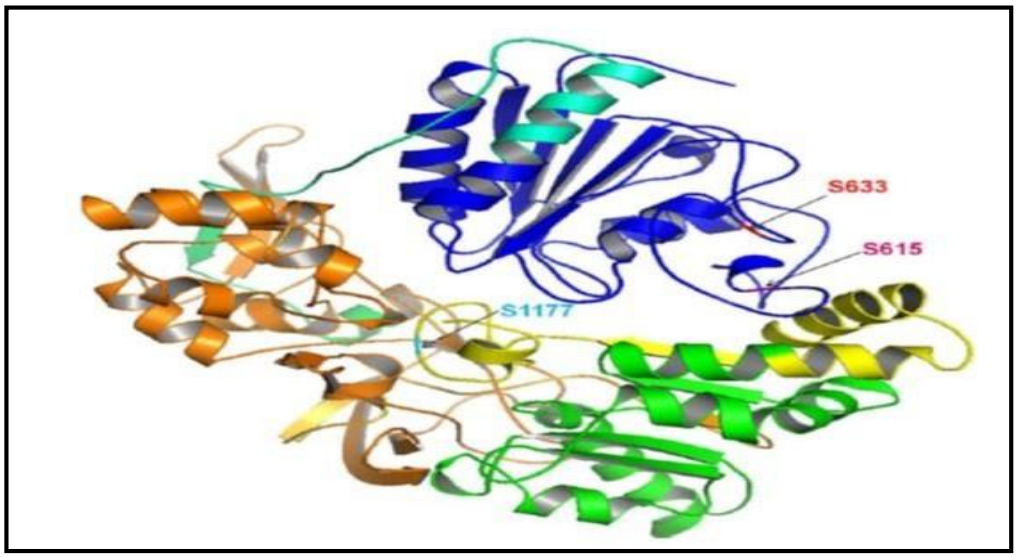


Figure 12: 3D structure of human eNOS reductase domain showing the serine phosphorylation sites generated using Modeller. Different binding domain regions have been identified and are color-coded: blue: flavodoxin, orange: FAD, green: NADPH
Source: NT Devika, P Amresh, MI. Imtiyaz Hassan, BM. Jaffar Ali. Molecular modeling and simulation of the human eNOS reductase domain, an enzyme involved in the release of vascular nitric oxide. J Mol Model.2014 20:2470.

Concerned with preeclampsia, expression of NOS is observed in syncytiotrophoblast, villous endothelium, macrophages and eNOS being the predominant isoform. eNOS and NO has been implicated in the preeclampsia pathophysiology. Preeclampsia is linked with impaired uteroplacental adaptations and abnormalities in the eNOS/NO pathway [78].

During pregnancy, adequate uteroplacental blood flow is required, which is dependent on vasodilation. Therefore, the production of vasodilator molecules from the endothelium is crucial to maintain a healthy pregnancy. However, in preeclampsia, endothelial function is compromised and contributes to the complications of preeclampsia such as hypertension,edema and proteinuria [67].
Studies on maternal serum eNOS concentrations in preeclampsia are limited and available studies have reported conflict of results about eNOS either as decreased, increased or unchanged [82-85].

1.11. Nitric oxide (NO)

Nitric oxide is produced from substrate L-arginine catalyzed by nitric oxide synthase into L-citrulline in presence of oxygen molecule, tetrahydrobiopterin (BH_4) and reducing equivalents NADH, FMN and FAD. Nitric oxide activates soluble guanylate cyclase in vascular smooth muscle cells, resulting in increased levels of cyclic guanosine monophosphate (cGMP) which mediates the dilation of vascular smooth muscles. This NO-cGMPpathway is present in the human uterus might be responsible for maintaining relaxation.

Increased ADMA and arginase expression decreases NO levels. It has been reported that ADMA is a mediator of endothelial dysfunction, because ADMA may contribute to eNOS blockade and NO deficiency [86]. In addition to this, eNOS gene polymorphisms also associated with altered levels of eNOS in preeclampsia [72].

Nitric oxide plays a key role in cytotrophoblast endovascular invasion and placentation through its unique angiogenic, vasculogenic properties and helps in placental blood flow. Evidence suggests that, nitric oxide may play an important role in implantation, placental perfusion and decidualization. The dilatation of uteroplacental arteries observed when invading trophoblast cells co-expressing eNOS and iNOS in the extravillous trophoblast, suggesting that NO mediates spiral arterial changes during pregnancy. Moreover, NO inhibits uterine contractility keeping the uterus relaxed throughout gestation [87].

Nitric oxide is a key transmitter for the endothelium dependent regulation of vascular tone that is controlled by humoral, metabolic and mechanical factors.

In response to elevated blood flow, nitric oxide inhibits adhesion and activation of platelet aggregation, abolishes the toxic activity of superoxide ions, acts as an anticoagulant and anti-atherogenic [86]. It is also considered to have major effects on the gestational endothelial functions, promoting embryo survival, tissue remodeling, immune suppression and vasoregulation of placental nutrient transport. The human feto-placental vasculature lacks autonomic innervations and therefore, NO confers autocrine and /or paracrine effects. In particular, NO is the main vasodilator that is involved in feto-placental vascular reactivity regulation, placental bed vascular resistance, trophoblast invasion and apoptosis, and platelet adhesion and aggregation in the intervillous space. Nitric oxide is also involved in vasculogenesis. VEGF is the key molecule in this process and its expression is mediated by NO. Low levels of NO in the feto-placental unit may leads to vasoconstriction of placental bed, abnormal placental perfusion, and its maternal consequences such as hypertension, systemic vascular resistance [86]. Studies pertaining to nitric oxide levels in preeclampsia have reported conflicting results either as increased or decreased NO levels [88-90].

1.12. Lacunae of knowledge

Preeclampsia is a pregnancy disease with multisystem involvement, characterized by hypertension, proteinuria, edema, which typically develop after twenty weeks of gestation. The etiology of preeclampsia is still unclear. Placenta plays a major role in the development of preeclampsia. Possible

pathogenic mechanisms implicated in preeclampsia include placental implantation with abnormal trophoblastic invasion of uterine vessels, oxidative and endoplasmic reticulum stress, endothelial dysfunction, intravascular inflammation, platelet and thrombin activation, coagulation abnormalities, genetic predisposition and the presence of an antiangiogenic state, among which an imbalance of angiogenesis has been emerged as one of the most important factors. Therefore, early detection of preeclampsia and understanding the pathophysiological mechanisms and management of the disease are very important.

Oxidative stress indicates the elevated MDA levels and low anti-oxidant status. Studies reported oxidants and anti-oxidants separately or in combination. This study focuses on oxidative stress markers, MDA and anti-oxidant status by FRAP levels in normotensive pregnants and preeclampsia.

Apelin levels and its expression in human placenta was studied in preeclampsia internationally. According to the research findings, expression of Apelin was reduced in preeclampsia, but maternal serum levels were yielded conflicting findings. However, existing reports not measure the biologically most active form of Apelin. i.e., Apelin 13. Therefore, measuring maternal serum Apelin 13 concentrations in normotensive pregnants and in preeclampsia in our population and also to find its association with endothelial function is an important aspect.

However, to the best of our knowledge, study on maternal serum Apelin 13 levels in preeclampsia under prevailing oxidative stress and the Apelin gene polymorphism in Indian population is not reported. Therefore, this research gap has become the need of this study.

Endothelial nitric oxide synthase (eNOS) regulates the nitric oxide synthesis in the endothelium. Studies have reported conflicting findings on maternal serum eNOS and nitric oxide concentrations under oxidative stress conditions in preeclampsia. Apelin has a direct activating effect on the L-arginine/eNOS/NO pathway and it also inhibits oxidative stress in preeclampsia. These results demonstrated the beneficial effects of Apelin in preeclampsia.

As per the available literature, study focusing on data relating to interaction of Apelin, eNOS and NO in normal pregnancy and its complications is the uniqueness and has become newer aspect of the study.

Therefore, the present study titled circulating angiogenic, oxidative stress markers and their possible association with endothelial function in normal pregnant and preeclamptic women with genetic screening of Apelin gene polymorphism is the basis of the research.

CHAPTER-2

REVIEW OF LITERATURE

2.0. REVIEW OF LITERATURE

The following are the recent literature reviewed in relation to oxidative stress, angiogenic, endothelial function and Apelin gene polymorphism.

2.1. OXIDATIVE STRESS

Sahay and his co-workers in 2015 conducted a cross-sectional study in Indian pregnant women to determine the placental levels of oxidative stress markers in terms of malondialdehyde (MDA), catalase and glutathione peroxidase (GPx), at 4 different places on placenta such as central maternal side and peripheral maternal region as well as central and peripheral fetal side of normotensive and pre-term and term preeclampsia groups. The study results showed that MDA levels were higher in all regions of the placenta in study groups than the normotensive groups. The study concluded that increased oxidative index in the two preeclampsia groups characterized by high level of lipid peroxidation and lower antioxidant defense. Study had few limitations that the parameters accountable to contribute antioxidant potential needs to be measured than alone catalase and glutathione peroxidase and its isoforms and also the similar observation needs to be tested for confirmation in larger samples. The unique observation in this study with respect to oxidative stress markers confined more to central portion of the placenta than the other regions [91].

Efher Oztas and co-workers in 2016 conducted a prospective case-control study in Turkey population to evaluate maternal serum total antioxidant status (TAS), total oxidant status (TOS) presented as oxidative stress index (OSI)

along with paraoxonase (PON) and arylesterase in severe preeclampsia and also to investigate their implication with respect to onset of perinatal morbidity or not. The study results concluded that increased maternal serum TAS, TOS and arylesterase associated with severe preeclampsia and correlated with adverse perinatal outcomes. Study suggested that in preeclampsia along with elevated oxidative stress that linked to perinatal outcomes, total antioxidants status also elevated as defensive mechanism to the prevailing oxidative stress in pregnancy complications in view of protecting the developing fetus against oxidative injury. However, study has main limitation with small sample size and also statistical non-significance between study groups with respect to study parameters [92].

Razia Sultana and co-workers in 2016 conducted a case-control study on Indian population to evaluate oxidative stress in normotensive pregnants and also in pregnancy induced hypertension. Oxidative stress measured using parameters plasma malondialdehyde (MDA) and serum superoxide dismutase (SOD) that represents the intensity of lipid peroxidation. The study observed that significant rise of the study parameters which have an impact on endothelial function. Major limitation of study was having less sample size and study results were almost similar to other studies [93].

Visala Sree Jammalamadaga and co-worker in 2016 conducted a cross-sectional analytical study between control, preeclampsia and eclampsia groups to evaluate various factors that triggering endothelial dysfunction in pregnancy induced hypertension. Study focused on measurement of oxidative stress in

terms of malondialdehyde (MDA), ferric reducing ability of plasma (FRAP), along with the factors such as tumor necrosis factor -α (TNF-α), soluble Fms-like tyrosine kinase -1 (sFlt-1) as anti-angiogenic factors and Vascular endothelial growth factor (VEGF), Placental growth factor (PlGF), Nitric oxide (NO) as pro-angiogenic factors. Study observations clearly emphasized a relationship between oxidative stress, pro-angiogenic factors and anti-angiogenic factors spectrum between normotensives, preeclamptics and eclamptic groups. Finally, study concluded the imbalance between these factors have enormous impact on endothelium has a prominent feature sets in preeclampsia. This study attempted to explain various biomarkers that serves as early indicators in assessment of pregnancy complications and its progression and also management. However, study missed to present the expression of endothelial nitric oxide synthase in association with VEGF known to participate in regulation of hypertension. Study has a limitation by having small sample size [94].

Mirjana Bogavac and co-workers in 2017 conducted a prospective case-control study in Serbia population to determine the oxidative stress markers in 1st trimester of pregnancy in preeclampsia and normotensive healthy pregnant group. The study results showed that the increased levels of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) in preeclampsia. However, total antioxidant status was reduced in preeclampsia and negatively correlated with GSH-Px and SOD. This study concluded that increased oxidative stress in the 1st trimester of pregnancy associated with initiation and development of pathophysiology of preeclampsia and start much earlier than the clinical

syndrome exhibit. The study limitation was unequal sample size between the groups [95].

Subandrate and co-workers in 2017 conducted an observational cross-sectional study in Indonesia population to evaluate plasma malondialdehyde (MDA) and glutathione (GSH) levels between healthy pregnant and preeclampsia may be used as a marker for appropriate evaluation of preeclampsia risk. The study results showed plasma MDA levels were significantly increased and glutathione levels were low in preeclamptic women compared to healthy pregnant women. The study concluded that increased MDA and reduced GSH may reflect vascular complications of preeclampsia. The limitation of the study was small sample size [96].

Asiltas B and co-workers in 2018 conducted a case-control study in Turkey population to determine the predictive value of pregnancy-associated plasma protein-A (PAPP-A), placental protein-13 (PP-13), human chorionic Gonadotrophin (β-hCG) and malondialdehyde (MDA) levels in 1st trimester of cases likely to develop preeclampsia. The study results showed that levels of PP-13 were significantly reduced and MDA concentrations were significantly elevated in preeclampsia. Area under the curve (AUC) is greater for MDA and PP-13 compared to PAPP-A and β-hCG. The combination of MDA + PP-13 model and MDA+PP-13+ PAPP-A+ β-hCG showed best predictor of preeclampsia compared to individual parameters. The study concluded that oxidative stress markers within combination of placental markers might serve

as ideal predictive indicators in assessment of risk associated in early onset and late-onset preeclampsia [97].

Hanan MA and co-workers in 2018 conducted a cross-sectional study in Egypt population to evaluate placental visfatin gene expression and oxidative stress in pregnancy induced hypertension conditions such as gestational hypertension, mild and severe preeclampsia, preeclampsia superimposed on chronic hypertension and in healthy pregnant women. The study results showed that reduced placental visfatin gene expression in hypertensive disorders of pregnancy than healthy pregnant women. Serum catalase activity, total antioxidant capacity, reduced glutathione levels were decreased and serum malondialdehyde (MDA) levels were increased in all hypertensive disorders of pregnancy than healthy controls. Serum MDA and catalase were negatively correlated in mild preeclampsia group. Catalase and total antioxidant capacity were positively correlated in severe preeclampsia cases. Visfatin gene and oxidative parameters not showed any correlation. The study concluded that decreased visfatin gene expression and increased oxidative stress in preeclampsia than control may lead to free radical mediated endothelial dysfunction. Study also recommended supplementation of antioxidants to combat oxidative stress injury [98].

Ranjeeta Gadde and co-workers in 2018 conducted a longitudinal study in Indian population on 1st trimester of pregnant women with a gestational age of 11- 13 weeks to assess the levels of placental protein 13 (PP-13), Asymmetric dimethylarginine (ADMA), Caspase 3, Total oxidant status (TOS), Total

antioxidant capacity (TAC), nitric oxide (NO), xanthine oxidase (XO), uric acid (UA) and calcium. Study results showed that significant positive correlation observed between ADMA and Caspase 3, PP-13 and NO, TOS & TAC, UA & ADMA, UA & TAC. A significant negative correlation was observed between PP13 & ADMA, NO & TOS, UA & XO, UA & NO, UA & Caspase 3 and MAP & calcium. Study concluded that assessment of these markers in the 1st trimester of pregnancy and their correlations might predict the integrity of trophoblastic cell and endothelial function during placentation under prevailing oxidative stress conditions, which are also useful in identifying women who can subsequently develop preeclampsia [99].

Clara Barneo-Caragol and co-workers in 2019 conducted a study in Spain population and evaluated increased serum strontium level and altered oxidative stress during early onset of preeclampsia. Study revealed the diagnostic importance of increased strontium, sFlt-1/PlGF ratio, and decreased estimated glomerular filtration rate (eGFR) and antioxidant activity at early setup of symptoms in preeclampsia. However, study was not clearly focused on significance of serum strontium physiological role[100].

Moushira Zakia and his co-workers in 2019 conducted a case-control study in Egypt population to determine the levels of malondialdehyde (MDA), neutrophil elastase (NE) and vascular endothelial growth factor (VEGF) in preeclamptic women and healthy pregnant women. The study results showed that significantly elevated serum MDA, NE and VEGF in obese preeclampsia subjects than healthy pregnant women. The study concluded that obesity,

elevated MDA, NE and VEGF are risk factors for preeclampsia in the early 3rd trimester. The study had limitation of non-measurement of antioxidant status and consideration of small sample size [101].

Bhat PV and co-workers in 2019 conducted a case-control study in southwest Indian population to assess the effect of metabolic syndrome and oxidative stress factors in preeclampsia group. The study results showed that significantly elevated fasting glucose, insulin, insulin resistance, total cholesterol, triglycerides, low density lipoprotein cholesterol, and total antioxidant status in preeclamptic women. There is a reduced cardiac output and aortic wall distensibility in preeclampsia. The study concluded that dyslipidemia, increased lipid peroxidation in preeclampsia leads to increased oxidative stress and vascular endothelial dysfunction [102].

Iman M. Ahmed and co-workers in 2019 conducted a prospective, longitudinal study in USA population to assess the oxidative stress role prior to diagnosis of preeclampsia (i.e., 12-20 weeks). Study results showed that catalase activity, total glutathione (GSH) levels were reduced in preeclamptic group, and superoxide levels were elevated in mild preeclampsia. Study concluded that catalase, only antioxidant shown to be inversely associated with severity of preeclampsia, and may serve as predictor of preeclampsia [103].

Hao Feng and co-workers in 2020 conducted a study in China to investigate the effect of Keap1/Nrf2 pathway on the biological functions of trophoblast cells in oxidative stress model at the cellular level, and analyzed the expression

levels and clinical significance of Kelch-like ECH-associated protein 1 (Keap1)/ Nuclear factor erythroid 2-related factor 2 (Nrf2) related antioxidant factors in placental tissues of preeclampsia patients. Study results showed that under hypoxia conditions, the activities of catalase (CAT), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) in human chorionic trophoblast cell line (HTR8/SVneo) were reduced than those before treatment. The activities of CAT, GSH-Px, SOD in HTR8/SVneo cells in siRNA+H/R group reduced significantly, suggesting the defensive effect of Keap1/Nrf2 pathway in oxidative stress. Placental samples from preeclamptic women also showed that reduced levels of SOD, GSH-Px, and CAT than normal placental samples. Keap1 mRNA expression is slightly lower, whereas Nrf2 mRNA and heme oxygenase -1 (HO-1) mRNA expression was high in preeclampsia placenta compared to healthy pregnant women. Study concluded that reduced antioxidant enzymes under hypoxia conditions in HTR8/SVneo cells. The anti-oxidation related genes expression in-vitro cells Keap Nrf2 and HO-1 in HTR8/SVneo were varied. In support of this, the activities of the antioxidant enzymes CAT, GSH-Px and SOD were lower in preeclamptic placenta [104].

2.2. APELIN 13

Research studies on molecule Apelins drawn less attention globally and the information pertaining to Apelin levels and its functional role with involvement in preeclampsia is limited. Even though, a few studies focused on Apelin research are described here.

Bortoff KD and co-workers conducted a case-control study in 2012 in USA population to evaluate maternal serum total Apelin concentration in preeclampsia. The study reported low level of total Apelin concentration in preeclampsia cases at delivery compared with healthy pregnant women. This study indicated the probability of decreased Apelin peptides in the circulation and its association with pathophysiology of preeclampsia. However, study had the limitation of lack of information on type of biologically active Apelin and use of small sample size [105].

Yavuz Simsek and co-workers in 2012 conducted a case-control study in Turkey population to assess the relationship between the hypotensive peptides like serum Apelin, sulusin-α and salusin-β levels in healthy pregnants and in preeclamptics. The study results showed that serum sulusin-α and salusin-β levels were not significantly different between two groups. Apelin levels were significantly elevated in preeclampsia cases. No significant difference was observed between mild and severe preeclampsia in terms of serum Apelin. The study concluded that the differences in Apelin levels indicates the role in pathogenesis of preeclampsia. The limitation of the study was measurement of biologically active Apelin peptide, and less sample size [106].

Inuzuka H and co-workers in 2013 conducted a case-control study in Japan population to observe the placental expression of Apelin & its receptor and Apelin protein level in severe preeclampsia and healthy pregnant women. The study results revealed the reduced expression of Apelin mRNA in preeclamptic placentas compared with controls. In preeclamptic placenta,

Apelin protein levels are also lower than the controls, but the levels were higher in maternal circulation in preeclampsia subjects. The study concluded that dysfunctional Apelinergic system may contribute to the pathophysiology of preeclampsia by reducing the angiogenesis process in placental implantation. The limitation of the study was measurement of biologically active peptide in the circulation and small sample size [38].

Kucur M and co-workers in 2014 conducted a case-control study in Turkey population to measure the maternal serum levels of macrophage derived YKL-40, Apelin levels in early and late-onset of preeclampsia and with healthy pregnants group. The study results observed that, mean maternal serum YKL-40 concentrations were significantly decreased in early and late onset preeclampsia whereas mean maternal serum Apelin concentrations were higher in early and late onset preeclampsia compared with healthy pregnant. Significant negative correlation observed between YKL-40 and Apelin. The study concluded that Apelin and YKL-40 levels and their involvement in vascular pathogenesis of preeclampsia. The limitation of the study was requirement of larger number of participants to arrive at the conclusions [51].

Research group from North Carolina, Liliya MY and co-workers in 2015 conducted a case-control study, quantified by Radioimmunoassay (RIA) to compare the Immunoreactive Apelin content in preeclamptic chorionic villi tissue and from normal pregnants. The study results showed that total Apelin content was lower in preeclamptic chorionic villi compared to normal

chorionic villi. The expression pattern to Apelin peptides was characterized by HPLC-RIA revealed that (Pyr^{1}) Apelin 13 and Apelin 13 are the predominant isoforms in the chorionic villi. A low dose-Ang II reduced the Apelin release in normal villous explants that was blocked by the Ang II receptor (AT1) antagonist losartan. The study concluded that lower Apelin content in villi tissue of preeclampsia than the normal chorionic villi linked to negative regulation by Ang II. However, study proposes expression patterns of Apelin peptides as limitation [32].

Mieghem TV and co-workers in 2016 conducted an observational study in Canada population to assess maternal serum Apelin levels and hemodynamics such as cardiac output and total peripheral resistance between 20 and 34 weeks of gestational age at high risk of placental dysfunction. The study results showed that 30% reduction in serum Apelin levels in pregnancies complicated by Intra Uterine Growth Restriction (IUGR) than in uncomplicated pregnancies or in women with preeclampsia. Expression of placental Apelin gene was similar in IUGR, preeclampsia, preterm and term normal placentas. Apelin staining was observed in syncytiotrophoblast, stroma of the placental villi and Apelin staining was strongly decreased in both compartments of IUGR pregnancies than healthy pregnants. Preeclamptic placentas showed an intermediate staining. The study concluded that both placental and serum Apelin levels are lower in IUGR than normal pregnancy and both are highly similar between preeclampsia and controls. The limitation of the study demands larger population [107].

Wang C and co-workers in 2017 conducted an animal model experimental study in China to investigate the effects of Apelin in a rat model of preeclampsia induced by reduced uterine perfusion pressure (RUPP). Ratswith RUPP exhibited hypertension and poor pregnancy outcomes. The study results showed that Apelin 13 administration in preeclamptic rats significantly inhibited the elevation of SBP, DBP and MBP. Apelin administration reversed the decreased total fetal weight and total placental weight, while further increasing the embryo survival rate. Apelin administration to preeclamptic rats led to an upregulation of the protein and mRNA levels of eNOS. The study concluded that Apelin has a direct activating effect on the L- arginine/eNOS/NO pathway [108].

Colcimen N and co-workers in 2017 conducted a study in Turkey population to investigate role of vascular endothelial growth factor (VEGF), Annexin A5 and Apelin from placental tissue collected from normotensive pregnant, mild and severe preeclamptic women. The placental examination of VEGF, Annexin 5 and Apelin were done by streptavidin-biotin-peroxidase complex, immuno-histochemical methods. Immunoreactivity scores (IRS) were obtained. VEGF and Apelin IRS were increased and Annexin A5 IRS was reduced significantly in preeclamptic women than normotensive control group. The study concluded that increase in VEGF, Apelin, and reduced Annexin A5 correlated with intensity of the disease, which supports the roles of hemodynamic alterations in fetoplacental circulation and structural modifications in placental bed occurring in preeclampsia [109].

Hong Xie and co-workers in 2017 conducted a comprehensive meta-analysis in China, reviewed the literature on circulating Apelin levels in hypertension. The study reported that circulating lower levels of Apelin were significantly associated with the risk of hypertension. The study also addressed that validation of this findings in large, well designed studies are required, which might be useful for early identification of high-risk patients [110].

Beril Gurlek and co-workers in 2019 conducted a prospective case-control study in Turkey population to measure the maternal serum Apelin 13 and Apelin 36 levels in normotensive pregnants and preeclamptic women. The study results showed that maternal serum Apelin 13 and Apelin 36 concentrations were significantly lower in preeclamptic women compared with normotensive pregnant women. The Apelin levels were negatively correlated with blood pressure. Apelin 13 and Apelin 36 levels were not different between patients with and without adverse fetal outcomes in both the groups. The ROC analysis showed that, area under the curve for Apelin 13 was 0.673 and Apelin 36 was 0.634. The study concluded that reduced Apelin peptides may play a role in pathophysiology of preeclampsia. The limitations were placental expression patterns of Apelin peptides and sample size [48].

Deniz R and co-workers in 2019 conducted a study in Turkey subjects to determine the maternal blood levels of elabela, Apelin and nitric oxide in preeclampsia and in their newborns venous-arterial cord blood in comparison with healthy controls. The study results showed that Apelin, elabela, and nitric

oxide concentrations were significantly decreased in both mild preeclamptic and severe preeclamptic women compared with healthy pregnant women. This decrease was more prominent in the severe preeclamptic women. The levels of Apelin, elabela, and nitric oxide in newborns venous-arterial cord blood was parallel with maternal findings. The study concluded that elabela, Apelin and nitric oxide plays a significant role in pathophysiology of preeclampsia. The study limitations were specific active Apelin peptide and their measurements[49].

Yamaleyeva LM and co-workers in 2019 conducted an animal model study in North Carolina, USA to establish systemic outcomes of (Pyr^1) Apelin 13 administration in rats with preeclamptic features. Established the systemic outcomes of (Pyr^1) Apelin 13 administration in rats with preeclamptic features (TGA-PE, female transgenic for human angiotensinogen mated to male transgenic for human renin). (Pyr^1) Apelin 13 (2 mg/kg/day) or saline was infused in TGA-PE rats via osmotic minipumps starting at day 13 of gestation (GD). At GD20, TGA-PE rats had increased blood pressure, proteinuria, lower maternal and pup weights, lower pup number, kidney injury, and enlarged heart compared to a control group (pregnant Sprague-Dawley rats administered vehicle). (Pyr^1) Apelin 13 did not affect maternal or fetal weights in TGA-PE. The study results showed that the administration of (Pyr^1) Apelin 13 reduced blood pressure, and normalized heart rate variability and baroreflex sensitivity in TGA-PE rats compared to controls. (Pyr^1) Apelin 13 increased ejection fraction in TGA-PE rats. (Pyr^1) Apelin 13 normalized proteinuria in association with lower renal cortical collagen deposition,

improved renal pathology and lower immunostaining of oxidative stress markers (4-HNE and NOX-4) in TGA-PE. The study concluded that improved hemodynamic responses and renal injury without fetal toxicity following Apelin administration suggesting a role for Apelin in the regulation of maternal outcomes in preeclampsia [111].

Aamal Sattar Tahaa and co-workers in 2020 conducted a case-control study in Iran population to assess the correlation between maternal serum Apelin and galectin-3 levels with insulin resistance (IR) in women with preeclampsia. The study results showed that preeclamptic women had significantly lower Apelin and higher galectin-3 levels than the healthy pregnants. Preeclamptic group exhibited dyslipidemia and higher β-cell functions than the control group. Galectin-3 levels were significantly positively correlated with insulin, glucose, dyslipidemia and IR. The study concluded that these abnormalities are associated with preeclampsia. The study limitation was small sample size [50].

Mlyczynska E and co-workers in 2020 conducted an *in-vitro* study in Poland population to assess the effect of Apelin on cell proliferation, cell cycle, protein expression of cyclins and phosphorylation level of extracellular regulated kinases (ERK)1/2, phosphatidylinositol-3-kinase/protein kinase B (Akt), signal transducer and activator of transcription 3 (Stat3) 5´-monophosphate-activated protein kinase (AMPKα) by using syncytioprophoblast (BeWo) and cytotrophoblast (JEG-3) cells and immuno-histochemistry (IHC) in human normal placenta slides. The study

results showed that Apelin was elevated in JEG-3 than BeWo cells and APJ was same in both the placental cell lines. IHC revealed that high cytoplasmic and/or membrane Apelin localization in JEG-3 than BeWo, which showed weaker Apelin signal in cytoplasm. Apelin increased the cell proliferation, percentage of cells in G2/M phase of cell cycle, cyclin proteins and expression of all kinases required for early placentation by virtue of promoting trophoblast cell proliferation by APJ and ERK 1/2, Stat3 and AMPKα signaling mechanisms. The study attempted the possibility of involvement of adipokines for placentation in proliferation mechanism. However, it needs further supporting evidences *in-vivo* animal models or placenta explants for understanding the role of Apelin on placental physiology, since, the role of Apelin on animal/human placenta is unclear [40].

Muzaffer Temur and co-workers in 2020 conducted a case-control study in Turkey subjects to investigate the maternal serum Apelin levels between mild preeclampsia, severe preeclampsia and normotensive pregnants and also to assess its correlation with blood pressure. Study results showed that significantly reduced maternal serum Apelin levels in preeclampsia than controls. This reduction was more prominent in severe cases than mild preeclampsia. Strong inverse correlation was observed in preeclampsia cases between Apelin and systolic blood pressure. Study concluded that reduced Apelin may affect the deterioration cardiovascular function in preeclampsia[112].

2.3. ENDOTHELIAL NITRIC OXIDE SYNTHASE (eNOS)

Feng Li and co-workers in 2012 conducted an animal model study in Japan to test whether the reduction in nitric oxide levels occurring in female mice lacking eNOS aggravates the preeclampsia - like phenotype induced by increased sFlt-1. Untreated eNOS-deficient female mice had higher BP than wild-type mice. Adenovirus-mediated overexpression of sFlt-1 increased systolic BP by approximately 27 mmHg and led to severe loss of fenestration of glomerular capillary endothelial cells in both eNOS-deficient and wild-type mice. However, only the eNOS-deficient sFlt-1 mice exhibited severe foot process effacement. Compared with wild-typesFlt-1 mice, eNOS-deficient sFlt-1 mice also showed markedly higher urinary albumin excretion (4.6 v/s 1.7 g/d), lower creatinine clearance and more severe endotheliosis. Expression of preproendothelin-1 (ET-1) and its ETA receptor in the kidney was higher in eNOS-deficient sFlt-1 mice than in wild-type sFlt-1 mice. Furthermore, the selective ETA receptor antagonist ambrisentan attenuated the increases in BP and urinary albumin excretion and ameliorated endotheliosis in both wild-type and eNOS-deficient sFlt-1 mice. Ambrisent, improved creatinine clearance and podocyte effacement in eNOS-deficient sFlt-1 mice. Concluded that, reduced maternal eNOS/nitric oxide exacerbates the sFlt1-related preeclampsia-like phenotype through activation of the endothelin system [113].

Marzena Laskowska and co-workers in 2013 conducted a case-control study in Poland population to determine the maternal serum concentrations of endothelial nitric oxide synthase (eNOS), Asymmetric dimethylarginine

(ADMA), and homocysteine in preeclamptic pregnancies. The study results showed increased homocysteine and ADMA levels in the preeclampsia. This increase was more predominant in early onset preeclampsia, but not statistically significant. eNOS levels were slightly lower in early-onset and late-onset preeclampsia compared to normotensive pregnant women, but these differences were not statistically significant. The study concluded that increased concentrations homocysteine and ADMA in early onsetpreeclampsia may suggest a relationship between these molecules and may determine the earlier clinical onset of the disease. The increased ADMA and the unchanged levels of eNOS in severe preeclampsia may indicate nitric oxide deficiency in preeclampsia. The study limitation was small sample size [82].

A Zawiejska and co-workers in 2014 conducted a prospective observational study in Poland population to assess the association between angiogenic, oxidative stress and metabolic status in women with hypertensive disorders of pregnancy. The study results showed that decreased levels of endothelial nitric oxide synthase (eNOS), placental growth factor (PlGF), angiotensin converting enzyme (ACE) and increased levels of vascular endothelial growth factor (VEGF) in preeclamptic women compared with mild preeclampsia and healthy pregnant women. Significant positive correlation was observed between eNOS and PlGF in the preeclampsia patients. PlGF levels were positively correlated with maternal pre-pregnancy BMI and negatively correlated with PlGF in preeclampsia cases. The study concluded that hypertensive disorders of pregnancy are associated with alterations in angiogenic factors and oxidative stress markers. The limitation of the study were non-measurement of oxidative stress markers, nitric oxide levels and small sample size [83].

Marzena Laskowska and co-workers in 2014 conducted a cross sectional study in Poland population to determine the levels of endothelial nitric oxide synthase (eNOS), NOSTRIN (eNOS-trafficking inducer), Asymmetric dimethylarginine (ADMA) in pregnancies with intrauterine growth restriction in the presence or absence of preeclampsia in comparison with healthy pregnant women. The study results showed that eNOS and NOSTRIN concentrations were not statistically significant between two groups. ADMA concentrations were significantly increased in preeclampsia and pregnancies complicated with isolated IUGR. The study concluded that decreased levels of nitric oxide in preeclampsia and/or IUGR may not result from a decreased concentration or low activity of eNOS or altered intracellular transport, but from elevated ADMA levels [85].

Motta-Mejia C and co-workers in 2017 conducted a study in United Kingdom population to study the syncytiotrophoblasts extracellular vesicles-bound eNOS (STBEV-eNOS) capable of generating nitric oxide (NO) into maternal circulation. Study reported that *ex vivo*-derived syncytiotrobhoblast extracellular microvesicles (STBMV) and syncytiotrobhoblast extracellular exosomes (STBEX) isolated from placental perfused lobes to have less eNOS activity in preeclampsia compared with controls. Similarly, *in vivo*-derived plasma STBMV analyzed by flow cytometry showed less STBMV bound eNOS expression in preeclampsia compared with normal pregnancy. This may contribute to the decreased levels of nitric oxide in preeclampsia, which may affect the vascular functions [114].

Du L and co-workers in 2017 conducted a case-control study in China population to determine the placental protein expression of endoplasmic reticulum (ER) stress – markers and endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS) preeclampsia and normotensive pregnant women. The study results showed that iNOS upregulation was observed in preeclampsia placenta compared to controls. Placental protein expression of endothelium reticulum stress markers such as glucose regulated protein 78 (GRP78), GRP94, protein kinase-like endoplasmic reticulum kinase (p-PERK), eukaryotic translation initiation factor 2a (elF2a), p-eIF2a, spliced form of X-box binding protein 1 (XBP1) and protein kinase-like ER kinase (PERK) levels were similar in both groups. Upregulation of CCAAT/enhancer-binding protein homologous protein (CHOP) and iNOS wasobserved, and was consistent of apoptosis increasing indicated by deoxynucleotidyl transferase-mediated nick-end labelling (TUNEL) staining and also upregulation of caspase 4 in preeclamptic placenta. The study concluded that increased endoplasmic reticulum stress and iNOS upregulation may be associated with placental apoptosis of preeclampsia and may contribute to preeclampsia pathophysiology [115].

Ghazala Shaheen and co-workers in 2019 conducted a case-control study in Pakistan population to determine the expression of endothelial nitric oxide synthase (eNOS) and oxidative stress markers and their role in preeclampsia. The study results showed that significantly elevated guaiacol peroxidase (POD), thiobarbituric acid reactive substances (TBARS) and reactive oxygen species (ROS) in preeclampsia while no changes were observed in superoxide dismutase (SOD) and catalase (CAT) in preeclampsia than control groups.

Decreased eNOS immunoreactivity and mRNA abundance was seen in preeclampsia compared with healthy controls. The study concluded that reduced expression of eNOS and oxidative stress may play a role in pathophysiology of preeclampsia [116].

Paul Guerby and co-workers in 2019 conducted a study in Japan population to assess eNOS glutathionylation may linked with eNOS dysfunction in preeclampsia. The study reported that increased eNOS glutathionylation in preeclamptic placentas and mostly reversed by dithiotreitol (DTT), thus indicative of S-glutathionylation. In addition to this, to assess whether eNOS glutathionylation may alter trophoblast migration, cultured HTR-8/SVneo human trophoblasts (HTR8) were exposed either to low pO_2 (O_2 1%) or to pO_2 changes (O_2 1–20%), in order to generate oxidative stress. Trophoblasts exposed to low pO_2, did not undergo oxidative stress nor eNOS S-glutathionylation, and were able to generate nitric oxide and migrate in a wound closure model. In contrast, trophoblasts submitted to low/high pO_2 changes, exhibited oxidative stress and a (DTT reversible) S- glutathionylation of eNOS, associated with reduced NO production and migration. The autonomous production of NO seemed necessary for the migratory potential of HTR8, as suggested by the inhibitory effect of eNOS silencing by small interfering RNAs, and the eNOS inhibitor L-NAME, in low pO_2 conditions. Finally, the addition of the NO donor, NOC-18 (5 μM), restored in part the migration of HTR8, thereby emphasizing the role of NO in trophoblast homeostasis. The study concluded that increased levels of eNOS S-glutathionylation in preeclampsia placentas and may be a possible trigger for eNOS dysfunction and reduced nitric oxide availability. The benefit exerted by

NOC-18 confirms the potential interest of nitric oxide donors for compensating lack of nitric oxide and preventing the pathophysiological process of preeclampsia [117].

Mazloomi Sahar and co-workers in 2020 conducted a case-control study in Iran population to assess the activity of endothelial nitric oxide synthase (eNOS) and thioredoxin reductase (TrxR) and the serum levels of serum calcium, zinc, selenium in healthy pregnant women and preeclampsia. The study results showed that reduced levels of eNOS, TrxR, calcium, zinc and selenium in preeclampsia compared to healthy pregnant women. The study concluded that the activities of eNOS and TrxR in preeclampsia may be one of the ways to prevent and reduce the risks of preeclampsia. The limitation of the study was small sample size and measurement of oxidative stress parameters[118].

2.4. NITRIC OXIDE

Marjan Noorbakhsh and co-workers in 2013 conducted a case-control study in Iran population to determine the serum concentrations of asymmetric dimethylarginine (ADMA), vascular endothelial growth factor (VEGF), and nitric oxide (NO) in preeclamptic women in comparison with healthy pregnant women. The study results showed that significantly elevated VEGF and nitrite concentrations in preeclampsia compared with controls. Maternal serum ADMA level increased in preeclampsia, but not reached the significance level. The study concluded that elevated levels of these molecules may be involved in the pathogenesis of preeclampsia. The study limitation was small sample size [119].

Fabiana C. Bernardi and co-workers in 2014 conducted a case-control study in Brasil population to determine the levels of nitric oxide (NO), superoxide dismutase (SOD) activity, arginase activity and endothelin-1 in plasma and placenta of preeclamptic women and healthy pregnant women. The study results showed that plasma levels of NO and SOD activity were reduced significantly and endothelin-1 concentration and activity of arginase were significantly elevated in preeclampsia compared to normotensive healthy pregnant women. In the placental samples, there parameters were not different. The study concluded that these parameters are altered only at the systemic level, but not in placenta of preeclamptic women. The limitation of the study were single time point of sample collection and small sample size [120].

Adu-Bonsaffoh K and co-workers in 2015 conducted a cross-sectional case-control study in Ghana population to determine maternal serum levels of nitric oxide in non-pregnant, normal pregnant, and preeclamptic women. The study results showed significantly increased nitric oxide levels in preeclamptic women compared with normal pregnant and non-pregnant. The elevated levels were more profound in early onset preeclampsia than the late onset preeclampsia. The study concluded that nitric oxide up-regulation in preeclampsia, this may be due to dysregulated compensatory reaction to restore endothelial damage and persistent hypertension in preeclampsia. The limitations of the study were measurement of eNOS and small sample size [121].

Zeng Y and co-workers in 2015 conducted a cross-sectional study in China population to determine the serum homocysteine (Hcy), endothelin -1 (ET-1) and nitric oxide (NO) levels in hypertensive disorders of pregnancy. The study results showed that mean levels of Hct and ET-1 were significantly high in preeclampsia than the healthy controls. The mean NO levels were significantly decreased in preeclampsia compared to control group. These alterations were more profound in severe preeclampsia than mild preeclampsia. The levels of Hct and ET-1 were correlated positively with disease severity and NO levels were negatively correlated with disease severity. The study concluded that elevated levels of Hct and ET-1 and reduced NO levels were associated with preeclampsia pathophysiology. The study limitation was eNOS estimation [122].

Salmaakter and co-workers in 2017 conducted a case-control study in Bangladesh population to assess the nitric oxide levels in preeclampsia and in healthy pregnancy between 29 to 40 weeks of gestation. The study results showed that significantly reduced nitrite levels in preeclampsia compared to healthy pregnants. Serum nitrate levels were not correlated with systolic blood pressure, diastolic blood pressure and proteinuria in preeclampsia. The study concluded that, in preeclampsia nitric oxide levels decreases as pregnancy progresses. Urine uric acid to creatinine ratio elevated with reduction in nitric oxide levels may be used as marker for preeclampsia. The study limitation was small sample size [123].

Hodzic J and co-workers in 2017 conducted a prospective cross-sectional study in Bosina and Herzegovina population to nitric oxide concentrations during normal pregnancy and in preeclampsia. The study results showed that

nitric oxide levels were higher in 2nd and 3rd trimester of healthy pregnant than non-pregnant women. The nitric oxide levels were significantly low in preeclampsia than healthy pregnant women in 3rd trimester, but the levels were not significant. The nitric oxide levels were positively correlated with blood pressure, creatinine clearance, uric acid and inversely correlated with platelet count in preeclampsia. The study concluded that the production of nitric oxide was increased with advancing gestational age in normal pregnancyand slightly reduced in preeclampsia, indicating nitric oxide may modulate cardiovascular changes during normal pregnancy and in preeclampsia. The study limitation was small sample size [124].

Ebenezer Owusu Darkwa and co-workers in 2018 conducted a comparative study in Ghana population to compare nitric oxide (NO) levels in preeclampsia and healthy pregnant women. The study results showed that NO levels were decreased in preeclampsia compared with healthy pregnant women, but the levels were not statistically significant. Nitric oxide levels were negatively correlated with mean arterial pressure. The study concluded that NO concentrations may not play a key role in the pathophysiology of preeclampsia. The study limitation was a smaller number of subjects [90].

Hobiel HA and co-workers in 2018 conducted a cross-sectional study in Sudan population to evaluate the role of oxidative stress and dyslipidemia as an indicator of pathogenesis of preeclampsia. The study results showed that decreased levels of high-density lipoprotein cholesterol and increased low-density lipoprotein cholesterol increases the risk of preeclampsia. Reduced levels of nitric oxide and total antioxidant status in preeclampsia. The study

concluded that dyslipidemia and oxidative stress may contribute to the pathophysiology of preeclampsia. The limitation of study was measurement of oxidative stress markers [125].

Mohit Upadhye and co-workers in 2019 conducted a prospective case-control study in Indian population to determine the concentrations of asymmetric dimethylarginine (ADMA) and nitric oxide (NO) in preeclamptic and normotensive healthy pregnant women. The study results showed that concentrations of ADMA were significantly increased and NO levels were significantly low in preeclampsia. Significant correlation was not observed between nitric oxide and ADMA. Nitric oxide levels were negatively correlated with systolic blood pressure and mean arterial pressure, whereas ADMA levels were not correlated with blood pressure. The study concluded that the interplay between ADMA and NO may play a role in the etiopathogenesis of preeclampsia. The limitation of the study was measurement of arginine and less sample size [126].

Chaudhuri S and co-workers in 2019 conducted an observational cross-sectional study in Indian population to determine the serum nitric oxide and hydrogen sulfide in preeclampsia compared with healthy controls. The study results showed that the serum nitric oxide and hydrogen sulfide levels were reduced significantly in preeclampsia compared to healthy pregnant women and both were positively correlated in preeclampsia cases. The study concluded that alteration in these molecules may be associated with pathophysiology of preeclampsia. The study limitation was a smaller number of samples [88].

Ranjeeta Gadde and co-workers in 2019 conducted a nested case-control study in Indian population to determine the serum biomarkers as early markers of onset of preeclampsia. Study results showed that reduced levels of placental protein (PP13), nitric oxide (NO) and increased asymmetric dimethylarginine (ADMA) in 1st trimester and increased PP13, ADMA and reduced nitric oxide in 2nd trimester reflects abnormal placentation and endothelial function. In 1st trimester, significant positive correlation was observed between PP13 and nitric oxide. However, in 2nd trimester it was non-significant. An insignificant negative correlation was also observed between PP13 and ADMA in 1st and 2nd trimester. Even similar trend was observed between nitric oxide and ADMA in 1st and 2nd trimester. Study concluded that assessment of these markers in 1st and 2nd trimester may be useful to identify the risk of early onset of preeclampsia. The study limitation was a smaller number of preeclampsia cases [127].

Rahmawati Ita and co-workers in 2020 conducted a case-control study in Indonesia population to find out preeclampsia risk by measuring blood carboxy hemoglobin and hemoglobin levels in preeclampsia. Study results showed that significantly elevated blood carboxy hemoglobin and reduced serum nitric oxide (NO) levels in preeclampsia cases those who are exposed to smoke from combustion of tile or brick than the normotensive pregnant group. Study concluded that reduced serum NO levels due to carbon monoxide exposure increases the risk of preeclampsia. This reduced NO levels were associated with vascular endothelial dysfunction in preeclampsia. However, study limitation was small sample size [128].

2.5. APELIN GENE

The research work related to Apelin biosynthesis, structure, functional role, mechanism of action, involvement in disease process and applied aspects is less studied and the exact role of Apelin, biologically active peptide with respect to preeclampsia is less studied. Besides, genetic evidences on targeted polymorphisms on the candidate gene information also limited. Hence, present study is attempting to review the existing literature pertaining to study topic.

J Jia and co-workers in 2015 conducted a case-control in China population to determine whether the blood pressure response to losartan in an older Chinese population with essential hypertension was associated with Apelin gene polymorphisms. The study results showed that the following 24 weeks of treatment with losartan (50 mg/day), reductions in systolic, diastolic and mean arterial blood pressure were observed. In women, systolic blood pressure was significantly reduced, among the additive (CT vs CC vs TT), dominant (TT vs CC/CT), and recessive models (CC vs CT/TT). In the additive model, after the treatment, the greatest reductions in systolic blood pressure were observed in TT group, greater reductions in systolic blood pressure in CT group compared to CC group. Diastolic blood pressure did not show any difference in women. Systolic and diastolic blood pressure did not show any difference in males. After adjustment for confounding factors, female patients after 24 weeks of treatment, TT genotype showed greater reductions in systolic blood pressure compared to the patients with the C allele. The study concluded that the Apelin -1860T>C genotype may play an important role in the response to losartan in hypertensive women. The study limitation was measurement of circulating Apelin peptide levels [55].

Akcılar R and co-workers in 2015 conducted a case-control study in Turkey population to determine the association between the Apelin -1860T>C (rs56204867) polymorphism and plasma Apelin levels in coronary artery disease (CAD) patients. The study results showed that the CC genotype frequency and C allele of -1860 T>C was significantly high in CAD subjects compared to controls. Subjects with TC and CC genotypes were associated with the risk of CAD than TT in CAD cases. Plasma Apelin levels significantly decreased in CAD patients compared to healthy subjects. Apelin level of CAD patients with CC genotype of -1860T>C was significantly decreased compared to TT genotypes. The study concluded that homozygous CC genotype of Apelin gene was associated with CAD risk [52].

Pakizeh E and co-workers in 2015 conducted an observational cross-sectional study in Turkey population to investigate the association of 2 single nucleotide polymorphisms in Apelin gene with susceptibility to coronary artery disease. The study results showed that TT and AA genotypes of rs3115758 and rs3115759 of the Apelin gene were significantly associated with the CAD risk. The study concluded that the Apelin gene polymorphisms were associated with CAD risk. The limitation of the study was measurement of Apelin levels in the circulation and small sample size [54].

Gupta MD and co-workers in 2016 conducted a cross-sectional study in Indian population to investigate the association of two Apelin gene polymorphisms rs3761581 and rs2235312 and concentrations of Apelin in essential hypertension (EH) and acute coronary syndrome (ACS) patients. The study results showed that plasma Apelin 13 concentrations were significantly low in

patients with EH and ACS regardless of gender. Female subjects with EH and ACS had reduced Apelin 13 levels than the males. The G allele of rs3761581 T/G was more apparent in patients with ACS than healthy controls. The study concluded that decreased levels of Apelin 13 may enhance vasoconstriction to increase blood pressure and heart workload in EH and ACS. The variant allele rs3761581T/G may act as a risk factor in ACS, irrespective of gender. The study limitation was less sample size [46].

As per the available literature on Apelin are more precisely confined to hypertension and its related disorders, whereas Apelin research in the domain of pregnancy hypertensive complications are limited.

CHAPTER-3

AIM AND OBJECTIVES

3.0. AIM AND OBJECTIVES

3.1. Aim

Measurement of oxidative stress markers, serum Apelin 13, endothelial Nitric Oxide Synthase (eNOS), Nitric oxide (NO) levels and Apelin gene polymorphism in normotensive and preeclamptic women.

3.2. Objectives

1. To estimate and compare oxidative stress in terms of Malondialdehyde (MDA) and Total Antioxidant Status (TAS) in normal pregnant and preeclamptic women.
2. To estimate and compare Apelin 13 levels in normal pregnant and preeclamptic women.
3. To estimate and compare endothelial nitric oxide synthase (eNOS) and nitric oxide (NO) levels in normal pregnant and preeclamptic women.
4. To correlate the Apelin 13 levels with eNOS and NO in normal pregnant and preeclamptic women.
5. To associate Apelin 13 levels with maternal and fetal outcome.
6. To find out the frequency of occurrence of polymorphism of Apelin gene in normal pregnant and preeclamptic women.

CHAPTER-4

RESEARCH METHODOLOGY

4.0. RESEARCH METHODOLOGY

4.1. MATERIALS

The present prospective observational study was conducted in Department of Biochemistry in association with Department of Obstetrics and Gynecology at Sri R L Jalappa Hospital and Research Centre of Sri Devaraj Urs Medical College, a constituent unit of Sri Devaraj Urs Academy of Higher Education and Research (SDUAHER), Tamaka, Kolar, Karnataka. The duration of the study was three years commenced from August 2017 onwards. The study has been received approval by the University Central Ethics Committee in a letter vide No. SDUAHER/KLR/CEC/34/2018-19 dated 14-05-2018 and for non-pregnant group for the purpose to obtain basal Apelin 13 level, in a letter vide No. SDUAHER/KLR/R&I/19/2020-21 dated 12-06-2020 of SDUAHER. The participants were enrolled into the study after obtaining written informed consent.

4.1.1. Sample Size Calculation

Sample size was calculated based on a study conducted by Bortoff KD *et al*. taking mean and SD of Apelin levels in cases (0.66 ± 0.29 ng/mL) and in controls (0.78 ± 0.31ng/ml), with a power of 80% with Type I error of 5%, and the following formula was used for calculating sample size [105].

$$n = \frac{2S_P^2 [Z_1\text{-}\alpha/2 + Z_1\text{-}\beta]^2}{\mu_d^2}$$

$$S_P^2 = \frac{S_1^2 + S_2^2}{2}$$

S_1^2= Standard Deviation in first group

S_2^2= Standard Deviation in second group

μd^2= Mean difference between the samples

α = Significance level ($p<0.05$)

$Z_{1-\beta}$ = Power (80%)

$Z_{\alpha/2}$= 95% Confidence Interval (CI)

Based on the above calculation, the sample size arrived for study group is 98 for preeclampsia, 98 for normotensive pregnants. However, to have the basal data of serum Apelin 13 level, 90 non pregnants were considered.

4.1.2. Sample Collection

Five mL of venous blood was collected from antecubital vein under aseptic conditions using vacutainer and transferred 3mL into red tube to obtain serum and 2 mL into EDTA tube from preeclamptic and normotensive pregnant women who visits to Department of Obstetrics and Gynecology for antenatal checkup. The EDTA sample subjected to obtain plasma and the particulate fraction processed for genetic analysis.

4.1.3. Inclusion Criteria

The inclusion criteria of the study participants for preeclampsia cases are primigravida pregnant women clinically diagnosed with preeclampsia after twenty weeks of gestation were recruited from the Department of Obstetrics and Gynecology after detailed physical examination. The criteria for diagnosis of preeclampsia was with blood pressure of ≥140/90 mmHg noted for the first-

time during pregnancy on 2 occasions at least 4 hrs apart, after twenty weeks of gestation with proteinuria of ≥300 mg/24 hrs or ≥ 1+ by dipstick method in a random urine sample [129].

The inclusion criteria of the study participants for controls are with age and gestation matched primigravida normotensive healthy pregnant women, without proteinuria, with singleton pregnancy, no fetal anomaly, nonsmokers were recruited as controls.

4.4.4. Exclusion Criteria

The exclusion criteria of the study participants are pregnant women with multigravida, history of renal disease, liver disease, thyroid disorder, chronic systemic hypertension, gestational diabetics, epilepsy, hypertensive encephalopathy, cardiovascular disease, pregnancy with fetal anomaly, twin pregnancies, malignancy conditions and patients with history of smoking were excluded.

4.2. METHODS

The plain blood sample and EDTA sample were collected from the study participants, plain blood samples were allowed to retract at room temperature for two hours and centrifuged at 3000 rpm for 10 minutes to obtain the clear serum and were stored at -80 °C until analysis. Whereas EDTA blood samples were processed to obtain clear plasma. However, routine parameters were investigated in dry chemistry analyzer using appropriate methods.

The routine parameters include random blood sugar, urea, creatinine, uric acid, Aspartate transaminase (AST), Alanine transaminase (ALT), Lactate dehydrogenase (LDH) and Magnesium (Mg^{2+}) were measured in serum by dry chemistry analyzer (Ortho Clinical Diagnostics, Vitros 5.1 FS) and the plasma samples used for determination of Ferric Reducing Ability of Plasma (FRAP). In addition to this, the complete blood count (CBC) values were collected.

Study parameters like Nitric Oxide by Griess reaction, Malondialdehyde by thiobarbituric acid reactive substances (TBARS) and FRAP were measured by spectrophotometric method. Whole blood sample was used for the analysis of Apelin gene polymorphism. Urine samples were screened for protein content in normal pregnants and preeclampsia subjects by dipstick method.

Anthropometric parameters like BMI and blood pressure were used by the case files. In addition to this, family history and lifestyle parameters were recorded. Patients were followed and observed for any maternal complications such as acute renal failure, Hemolysis, Elevated Liver enzymes, Low Platelet count (HELLP) syndrome, eclampsia and fetal complications. The documented fetal complications such as preterm birth (<37 weeks), birth weight, Intrauterine Growth Restriction (IUGR), Respiratory Distress Syndrome (RDS), NICU admission and Intrauterine Death (IUD) were recorded and analyzed with respect to study parameters. Study participants viz mothers and neonates were treated in RL Jalappa Hospital and Research Centre.

The appropriate methodologies and the equipment used in the study for measurement of study parameters were listed and tabulated as shown in table2.

Table 2: Methods and instruments used for the biochemical analysis

NO	Parameters	Method	Make/ Catalog No	Instrument
1.	Malondialdehyde (MDA)	Thiobarbituric Acid Reactive Substances (TBARS)	Xeno Bio Solutions, Bengaluru, India.	Spectrophotometer, (Perkin Elmer lamda 1.2)
2.	Ferric Reducing Ability of Plasma (FRAP)	Benzie IF	Xeno Bio Solutions, Bengaluru,India.	Spectrophotometer, (Perkin Elmer lamda 1.2)
3.	Nitrite	Cortas NK Griess reaction	Xeno Bio Solutions, Bengaluru, India.	Spectrophotometer, (Perkin Elmer lamda 1.2)
4.	Apelin 13	Sandwich ELISA	SiNCERE Biotech Co. Ltd., Beijing,China. Catalogue No: E13652182	Merilyzer EIA Quant
5.	Endothelial Nitric OxideSynthase (eNOS)	Sandwich ELISA	SiNCERE Biotech Co. Ltd., Beijing,China. Catalogue No: E13652182	Merilyzer EIA Quant

4.2.1. ESTIMATION OF SERUM MALONDIALDEHYDE (MDA)

Method: Serum Malondialdehyde (MDA) level determined according to the method of Sinnhuber [130].

Principle: Free malondialdehyde, as a measure of lipid peroxidation. One molecule of MDA and two molecules of Thiobarbituric acid (TBA) reacts to form a red colored MDA-TBA complex which is measured spectrophotometrically at 530 nm.

Reagents:

1. 25% Trichloro acetic acid (TCA)
2. Thiobarbituric acid (TBA) reagent (0.75%)
3. Tetra methoxy propane (TMP) (20 μmol/L)
4. Sulfuric acid (H_2SO_4) (0.66N)

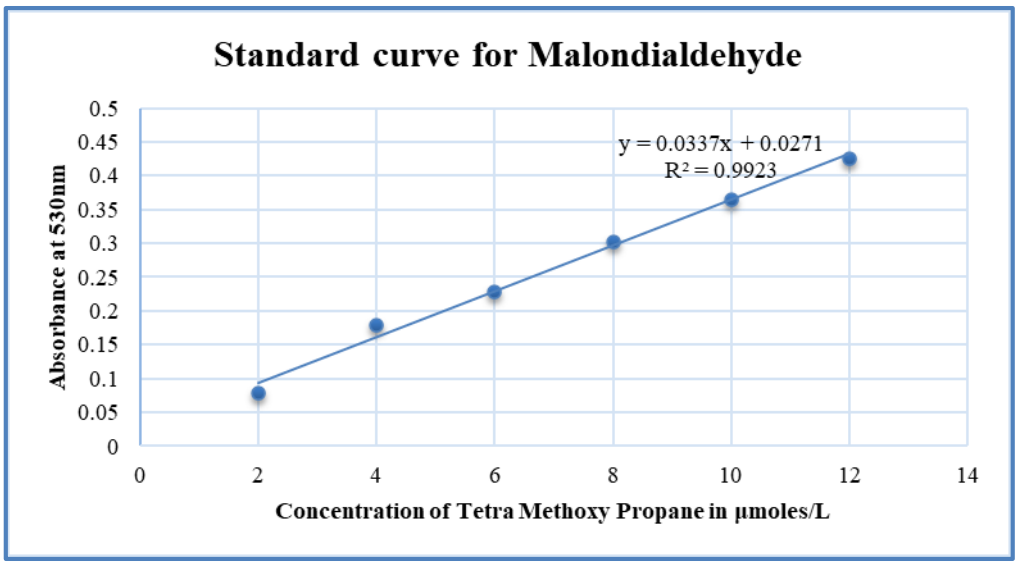
Procedure: 250 μL of serum sample was mixed well with 2.750 mL of 0.75% thiobarbituric acid. Kept in boiling water bath for 15 minutes. Then the tubes were removed and cooled. The absorbance of red colored MDA-TBA complex was read at 530 nm in spectrophotometer (Perkin Elmer lamda 1.2) against blank. The concentration of MDA calculated using standard Tetra methoxy propane (TMP).

Calculation:

$$\text{MDA in } \mu\text{moles/L} = \frac{\text{OD of Test} - \text{OD of Blank}}{\text{OD of Standard} - \text{OD of Blank}} \times \text{Concentration of Standard}$$

Preparation of standard curve for Malondialdehyde

Standard	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**
Concentration (µmoles/L)	2	4	6	8	10	12
Absorbance (530 nm)	0.079	0.199	0.229	0.303	0.375	0.425



4.2.2. ESTIMATION OF TOTAL ANTIOXIDANT STATUS BY FERRIC REDUCING ABILITY OF PLASMA (FRAP)

Method: The FRAP measured as per the method of Benzie IF [131].

Principle: At low pH, ferric tripyridyltriazine (FeIII - TPTZ) complex is reduced to the ferrous (FeII) form that produces an intense blue color which is measured spectrophotometrically at 593 nm.

Reagents:

1. Acetate buffer pH 3.6 (300 mmol/L)
2. Ferric Chloride ($FeCl_3.6H_2O$) (20 mmol/L)
3. Hydrochloric acid (40 mmol/L)
4. 2,4,6 – Tripyridyl-s-triazine (TPTZ) (10 mmol/L)
5. FRAP reagent: 25 mL of acetate buffer, 2.5 mL TPTZ and 2.5 mL $FeCl_3$

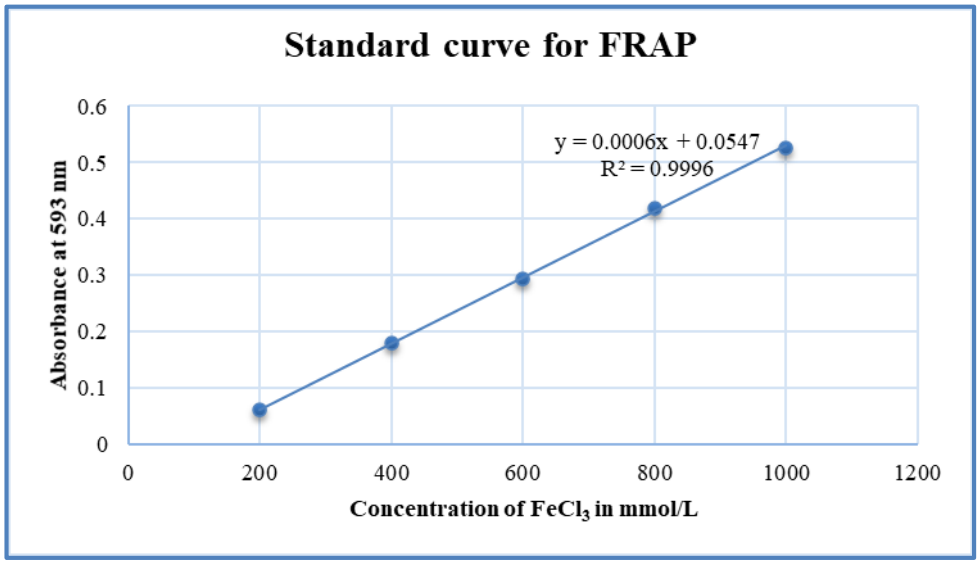
Procedure: To the 200 µL of plasma added 700 µL of distilled water and 2.1 mL of freshly prepared FRAP reagent. Incubated for 10 minutes at 37°C. The absorbance of the intense blue color produced was measured at 593 nm against blank in spectrophotometer (Perkin Elmer Lamda 1.2). The concentration of FRAP was calculated using standard Ferric Chloride.

Calculation:

$$\text{Total antioxidant capacity in mmol/L} = \frac{\text{OD of Test} - \text{OD of Blank}}{\text{OD of Standard} - \text{OD of Blank}} \times 1000$$

Preparation of standard curve for Ferric Reducing Ability of Plasma

Standard	S1	S2	S3	S4	S5
Concentration (mmol/L)	200	400	600	800	1000
Absorbance (593 nm)	0.091	0.180	0.294	0.419	0.526



5.2.3. ESTIMATION OF SERUM NITRITE

Method: Nitrite measured according to the method of Cortas NK Griess reaction [132].

Principle: Nitrate and nitrite concentration can be determined by using Griess reagent in which nitric oxide reacts with 3% sulphanilamine and 10% ethylenediamine dihydrochloride forming green color chromophore which can read at 540 nm in spectrophotometer (Perkin Elmer lamda 1.2)

Reagents:

1. 70% sulphosalicylic acid
2. 10% NaOH
3. Tris-HCl Buffer (pH: 9.0)
4. Sodium nitrite (NaNO2) (0.1mM)
5. Reagent A: 10% Ethylenediamine dihydrochloride
6. Reagent B: 3% Sulphanilamide in 1N HCl
7. Griess Reagent: Mix reagent A and B in equal volumes

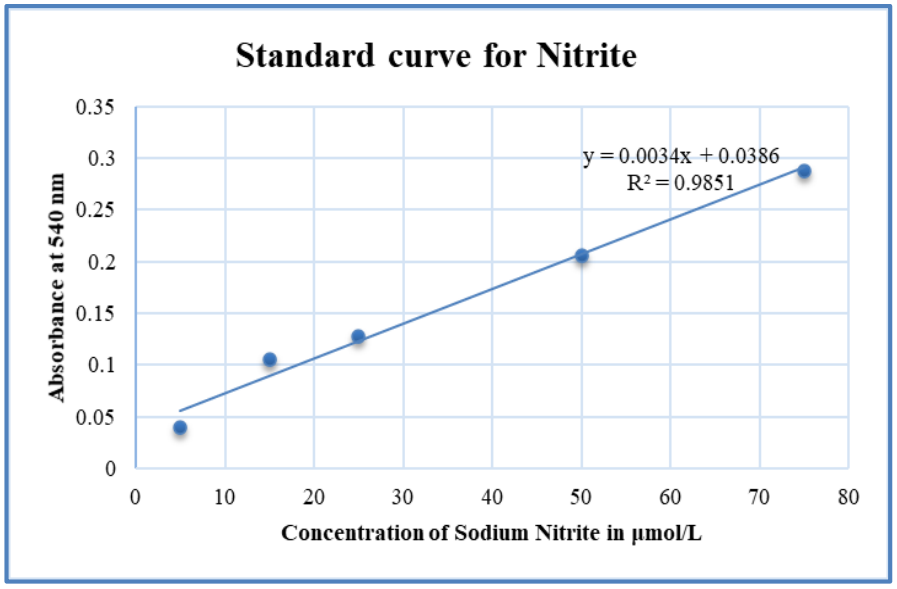
Procedure: To 500 µL of serum sample added 100 µL of 70% sulphosalicylic acid and mixed every 5 minutes for next 30 minutes. Centrifuged the tubes at 3000 rpm for 20 minutes. To 200 µL of supernatant and added 30 µL of 10% NaOH and 300 µL of Tris – HCl Buffer. To this added 530 µL of Griess reagent and kept in dark place for 10 minutes. The absorbance of the green color produced was read at 540 nm against blank in spectrophotometer (Perkin Elmer Lamda 1.2). The concentration of nitric oxide was calculated using standard 0.1 mM sodium nitrite.

Calculation:

$$\text{Nitrate in µmoles/L} = \frac{\text{OD of Test} - \text{OD of Blank}}{\text{OD of Standard} - \text{OD of Blank}} \times \text{Concentration of Standard}$$

Preparation of standard curve for Nitrite

Standard	**S1**	**S2**	**S3**	**S4**	**S5**
Concentration (µmoles/L)	5	15	25	50	75
Absorbance (540 nm)	0.039	0.105	0.127	0.206	0.288



5.2.4. ESTIMATION OF SERUM APELIN 13

Method: Human Apelin 13 concentration in serum was measured by enzyme-linked immunosorbent assay (ELISA) technique as per the procedure supplied by Sincere Biotech Co. Ltd, Beijing, China.

Principle: This assay was based on the principle of quantitative sandwich technique. Purified human Apelin 13 antibody was pre-coated onto the microtiter plate wells; make solid phase antibody, then added standards and samples to the wells. Apelin 13 present in the serum was bound to the human Apelin 13 antibody which with horse radish peroxidase (HRP) labeled, become antibody-antigen-enzyme-antibody complex, after removing any unbound substances by washing procedure, added 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate solution. TMB substrate solution becomes blue color at HRP enzyme-catalyzed. The reaction was terminated by adding stop solution (2 mol/L sulfuric acid) and then color changes to yellow, the absorbance of the color was measured at 450nm using microplate reader.

Reagents:

1. Standard
2. Wash solution
3. HRP-Conjugate reagent
4. Chromogen solution A
5. Chromogen solution B
6. Stop solution

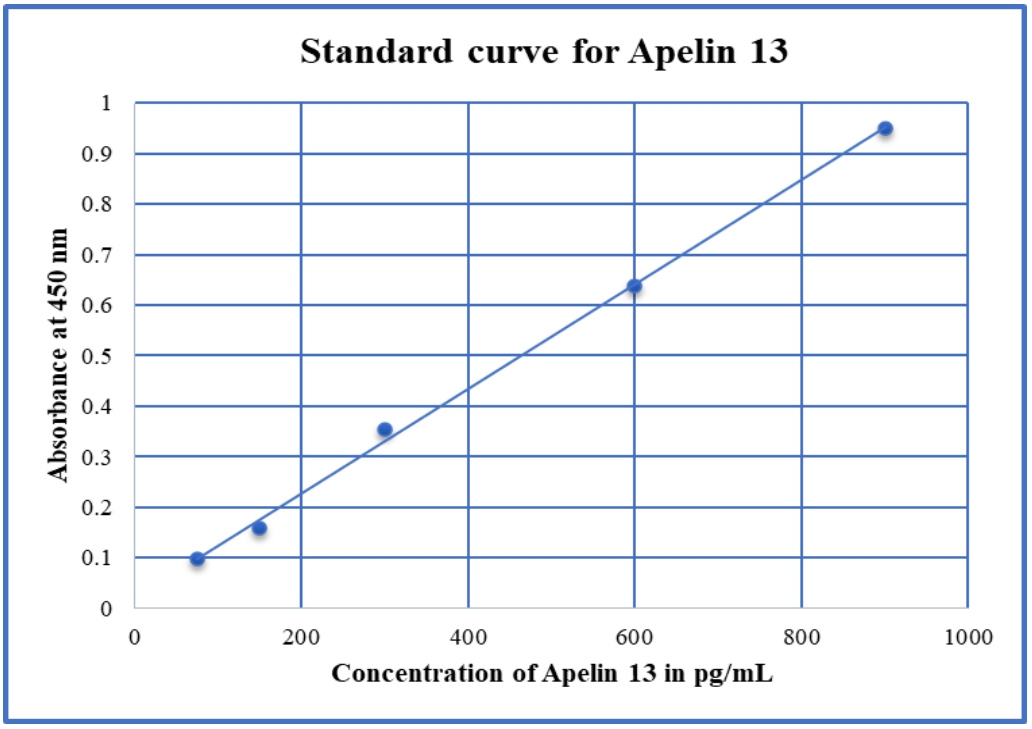
Procedure:

10 µL of serum sample, 40 µl sample diluent and 100 µL standard (S1-S5) and 50 µl of standard diluent were added to the wells and mixed. The plate was incubated for 30 minutes at 37ºC. The liquid was discarded, dry by swing, added washing buffer to each well, still for 30s then drain, repeated 5 times, dry by pat. HRP - conjugate reagent 50 µL was added to each well, except the blank well. The plate was incubated for 30 minutes at 37ºC. The liquid was discarded, repeated the washing procedure 5 times. Added TMB chromogen solution A and B 50 µL each well, mixed gently. The plate was light preserved for 15 minutes at 37ºC. The reaction was terminated by adding stop solution (2mol/L sulfuric acid) and then color changes to yellow, the absorbance of the color was measured within 15 minutes using microplate reader at 540 nm.

Calculation: The standard curve was plotted by taking the absorbance for each standard on the x-axis against the concentration on the y-axis. With the sample OD value in the equation ($y= 0.001 x + 0.0209$, $R^2= 0.9984$), the Apelin 13 concentration in the sample was calculated. The detection range was 13.5 - 1000 pg/mL. The result was represented as pg/mL.

Preparation of standard curve for Apelin 13

Standard	S1	S2	S3	S4	S5
Concentration (pg/mL)	75	150	300	600	900
Absorbance (450nm)	0.098	0.160	0.424	0.648	0.950



5.2.5. ESTIMATION OF SERUM ENDOTHELIAL NITRIC OXIDE SYNTHASE

Method: Human eNOS concentration in serum was measured by enzyme-linked immunosorbent assay (ELISA) technique as per the procedure supplied by Sincere Biotech Co. Ltd, Beijing, China.

Principle: This assay was based on the principle of quantitative sandwich technique. Purified human eNOS antibody was pre-coated onto the microtiter plate wells; make solid phase antibody, then added standards and samples to the wells. eNOS present in the serum was bound to the human eNOS antibody which with horse radish peroxidase (HRP) labeled, become antibody-antigen-enzyme-antibody complex, after removing any unbound substances by washing procedure, added 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate solution. TMB substrate solution becomes blue color at HRP enzyme-catalyzed. The reaction was terminated by adding stop solution (2 mol/L sulfuric acid) and then color changes to yellow, the absorbance of the color was measured at 450nm using microplate reader.

Reagents:

1. Standard
2. Wash solution
3. HRP-Conjugate reagent
4. Chromogen solution A
5. Chromogen solution B
6. Stop solution

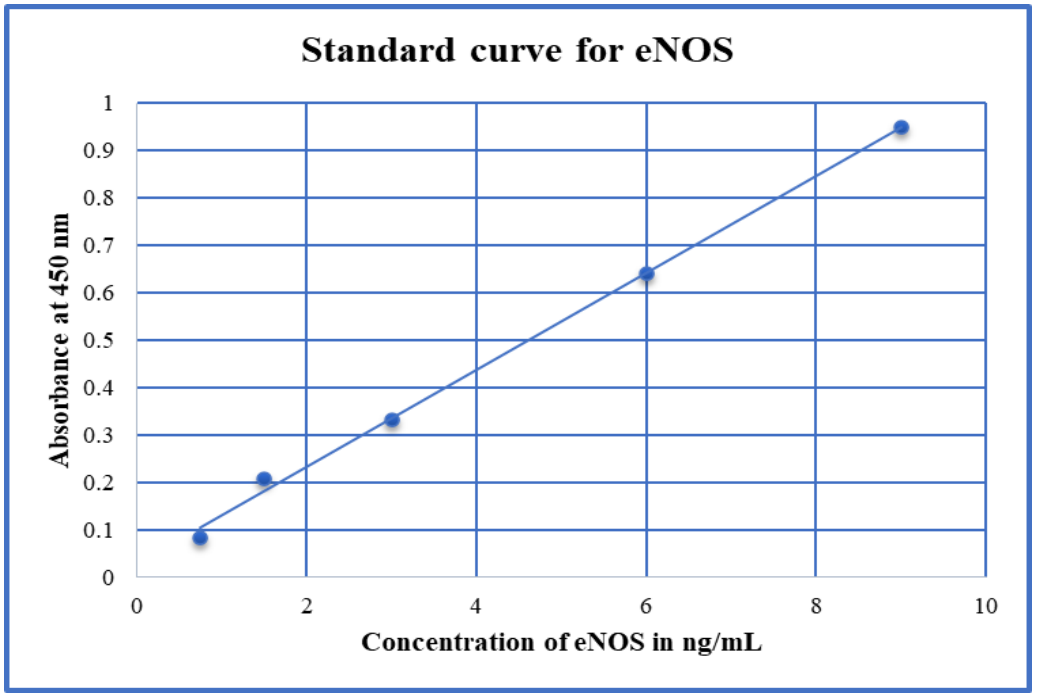
Procedure:

10 µL of serum sample, 40 µl sample diluent and 100 µL standard (S1-S5) and 50 µl of standard diluent were added to the wells and mixed. The plate was incubated for 30 minutes at 37ºC. The liquid was discarded, dry by swing, added washing buffer to each well, still for 30s then drain, repeated 5 times, dry by pat. HRP - conjugate reagent 50 µL was added to each well, except the blank well. The plate was incubated for 30 minutes at 37ºC. The liquid was discarded, repeated the washing procedure 5 times. Added TMB chromogen solution A and B 50 µL each well, mixed gently. The plate was light preserved for 15 minutes at 37ºC. The reaction was terminated by adding stop solution (2mol/L sulfuric acid) and then color changes to yellow, the absorbance of the color was measured within 15 minutes using microplate reader at 540 nm.

Calculation: The standard curve was plotted by taking the absorbance for each standard on the x-axis against the concentration on the y-axis. With the sample OD value in the equation ($y= 0.1025 x + 0.0271$, $R^2= 0.9976$), the eNOS concentration in the sample was calculated. The detection range was 0.152 -10 ng/mL. The result was represented as ng/mL.

Preparation of standard curve for eNOS

Standard	S1	S2	S3	S4	S5
Concentration (ng/mL)	0.75	1.5	3	6	9
Absorbance (450nm)	0.063	0.268	0.30	0.58	0.994



5.2.6. GENETIC ANALYSIS OF APELIN GENE

Human *APLN* gene is located on chromosome X at Xq25-26.1 was analyzed using maternal blood samples by adapting following molecular biology techniques.

1. Isolation of DNA from whole blood
2. Quantification and purity of DNA
3. Standardization of promoter region of apelin gene
4. Amplification of DNA by Polymerase Chain Reaction (PCR)
5. Electrophoresis
6. Restriction Fragment Length Polymorphism (RFLP)

1. Isolation of DNA from whole blood

Genomic DNA was isolated from the peripheral blood by salting out method[133].

Reagents required:

a) **Erythrocyte Lysis Buffer (1L, pH 7.4 stored at 4ºC):** 155 mM (8.29 g) Ammonium chloride (NH_4Cl), 0.1 mM (14.6 g) Ethylene diamine tetra acetic acid (EDTA) and 10 mM (1.0012 g) Potassium bicarbonate ($KHCO_3$)

b) **20% Sodium Dodecyl Sulphate (SDS):** 20 g of SDS is dissolved in 100 mL of milli-Q water, kept in water bath for 37ºC for 30 min to 2 hrs to dissolve completely and stored at room temperature to prevent precipitation.

c) **20 mg/mL Proteinase K:** 20 mg proteinase K dissolved in 1 mL of autoclaved water and transferred to Eppendorf micro tubes in aliquot manner and stored at -20ºC.

d) **5M Sodium chloride (NaCl):** 146.1 g of NaCl is dissolved in 500 mL of milli-Q water.

e) **80% Ethanol:** 80 mL of absolute ethanol diluted to 100 mL using milli-Q water.

f) **0.5 M EDTA:** Dissolved 186.2 g EDTA in 700 mL of double distilled water using stirrer. Adjusted pH 8.0 using NaOH and make the final volume to 1000 mL.

g) Tris EDTA buffer (100mL, pH 8.0) – 1M 121.14 Tris-Cl and 0.5M 186.2 EDTA:

Measure 1 mL of Tris-Cl and 2 mL of 0.5M EDTA in a reagent bottle. Make the final volume to 1000 mL by adding distilled water. Close the lid and invert bottle for few minutes and mix thoroughly.

Procedure: Genomic DNA was isolated from peripheral blood by salting out method. The 2 mL of blood sample was transferred to 15 mL falcon tubes at room temperature. The volume was made up to 12 mL with erythrocyte lysis buffer (ELB) and vortexed vigorously for 2-3 minutes to remove any debris or clumps formed in the tube. The tubes were centrifuged for10 minutes at 3000rpm at 4°C and supernatant was discarded. The obtained pellet was dissolved in ELB, centrifuged and supernatant was discarded. The pellet thus obtained was mixed using 0.27 mL of 20% sodium dodecyl sulphate (SDS)

and 30 µL of proteinase K (20mg/mL). The tubes were gently swirled, and incubated over night at 37 °C in water bath.

The pellet from the above step was mixed with 0.5 mL of 5M NaCl and equal volume of Isopropyl alcohol, mixed, and swirled gently until the DNA strands were visible, which were carefully transferred to an Eppendorf tubes containing 0.5 mL of 80% ethanol and centrifuged at 12,000 rpm for 7 minutes. This step repeated twice to get clear DNA pellet and subjected for drying at room temperature. To this, 0.5 mL of Tris EDTA buffer (TE) was added and allowed at 65°C in water bath for 30 minutes. Eppendorf tubes were parafilmed and placed on the rotator for solubilization of DNA in the Tris EDTA buffer. Finally, DNA obtained was preserved at -80°C until analysis.

2. Quantification and purity of DNA

The quality and quantity of the DNA samples were assessed by using UV spectrophotometer (Perkin Elmer Lambda 1.2) against TE buffer. 48 µL of TE buffer and 2 µL of DNA sample were mixed in the cuvette, the absorbance was measured at 260 and 280 nm. The ratio of absorbance at 260/280 represent the purity of DNA. DNA samples with 260/280 absorbance ratio between 1.7-2.0 were considered for amplification by PCR procedure.

3. Standardization of promoter region of Apelin gene

The referential APLN promoter gene sequence was retrieved Genbank (Accession No. NG_016718.1). To carry out PCR, sequence specific primer pairs were designed to amplify the promoter boundary regions of *APLN* gene

with the help of Primer Quest tool, IDT DNA software (Integrated DNA technologies, Inc, Coralville, IO, USA). PCR was carried out with these specific primers (Table 3). The primers were purchased from Sigma Aldrich Chemicals, USA [52].

Table 3: Designed primers on *ALPN* gene

Name of primer	5' to 3' sequence
Forward primer	GGGGAACAGTGAAGGGAGAATGGT
Reverse primer	AGAAGCGGGTCCTGAAGTTGT TTG

Reagents for PCR

a) 10x PCR buffer
b) dNTPs (10mM)
c) $MgCl_2$ (1.5mM)
d) Taq DNA polymerase (1 unit)
e) Primers (Sigma Aldrich Chemicals, USA)

Procedure: The reaction contained 100ng of genomic DNA, each primer (10 picomole) (1 µL), Milli-Q water 10.2 µL, $MgCl_2$ (1.5 mM) (1.5 µL), 10x PCR buffer (2.5µL), dNTPs (10 mM) (2.5 µL), and 1-unit Taq DNA polymerase (0.3 µL) (Bangalore Genei, India) (Table 4) and the conditions followed with an initial denaturation at 95° C for 5 minutes followed by 28 cycles of denaturation at 95°C for 30 seconds, annealing 56°C for 30 seconds, 72°C for 1 minute and final extension at 72°C for 5 minutes.

Table 4: Composition of PCR mix

Sl. No	Components	Final volume (µL)
1.	Forward primer (10 picomole)	1
2.	Reverse primer (10 picomole)	1
3.	Milli-Q Water	10.2
4.	$MgCl_2$ (1.5 mM)	1.5
5.	Taq buffer (10x)	2.5
6.	dNTPs (10 mM)	2.5
7.	Taq Polymerase	0.3
8.	DNA	6
Total reaction volume		**25**

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

The isolated DNA sample was subjected to PCR procedure by using primers (forward and reverse). The amplification was assessed by agarose gel electrophoresis and the bands were visualized in Bio Rad Gel Doc System (Figure 13).

5. Electrophoresis procedure

The PCR amplified products were subjected to agarose gel electrophoresis.

Reagents for agarose gel electrophoresis:

a) Agarose

b) **Loading dye:** 0.042% (W/V) Bromophenol blue powder, 2.5% Ficoll and 0.042% (W/V) Xylene Cyanol.

c) Ethidium bromide

d) **Tris Acetate EDTA (TAE) buffer:** 50x TAE stock solution: Dissolve 242 g of Tris base, 57.1 mL glacial acetic acid and 100 mL of 0.5 M EDTA (pH 8.0) in 600 mL distilled water, mix this by using magnetic stirrer bars.

Make the final volume to 1000 mL with double distilled water. Store at room temperature. 1x TAE buffer was prepared by diluting 20 mL of 50X stock solution into 980 mL of double distilled water.

Procedure: The promoter region of Apelin gene was standardized using gradient PCR reactions using primers. Annealing temperatures were calculated based upon the GC and AT content by the formula 4GC+2AT. The PCR amplified products were run on 2% agarose gel electrophoresis.

The PCR amplified products were subjected to 1% agarose gel electrophoresis. The gel was prepared by mixing 1g in 100 mL of 1x TE buffer in a microwavable flask until dissolved completely. Allowed to cool down to 50°C, mixed with 1.2 µL Ethidium bromide (EtBr). Poured the agarose into a gel tray with the well comb in place. The bubbles were removed from the surface of the agarose. Placed newly poured gel let sit at room temperature for 20-30 minutess, until get solidified. Gel was placed in the electrophoresis tank filled withsufficient 1x TAE to cover the gel to the depth of approximately 1mm and the comb was removed carefully. Added 1 µL of loading buffer to each PCR (5 µL) product placed the casted agarose gel into the pre-equilibrated electrophoretic unit. Carefully loaded 3µL DNA ladder into a well on the gel. The lid of the electrophoresis tank was closed and the gel was allowed to run using electric current of 130V until the dye line covers approximately 75-80% of the way down the gel[134]. Visualized the PCR separated products in gel documentation device under ultraviolet light (BIO-RAD, Gel Doc XR+).

6. Restriction Fragment Length Polymorphism (RFLP)

The PCR product (10 µL) was digested with XhoI (New England Biolabs, China) in a 20 µL volume mixture containing 1 µL restriction enzyme, 2µL CutSmart buffer and 7µL water (Table 5). The reaction mixture was incubated at 37°C overnight. After incubation, digested products were mixed with 2 µL loading buffer. The electrophoresis was performed with Ethidium bromide-stained 2% agarose gel. The size of the restriction fragments for RFLP was determined by using 1000 bp DNA ladder. The differences in polymorphic allele were determined by using UV light (BIO-RAD, Gel Doc XR+) (Figure 14).

Table 5: Composition of RFLP mix

Sl. No	Components	Final volume (µL)
1	Restriction enzyme (XhoI)	1
2	CutSmart Buffer	2
3	PCR product	10
4	Milli Q water	7
Total reaction volume		**20**

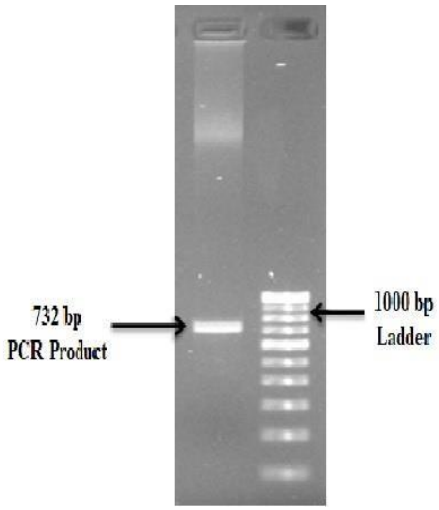


Figure 13. Agarose gel electrophoresis of PCR products

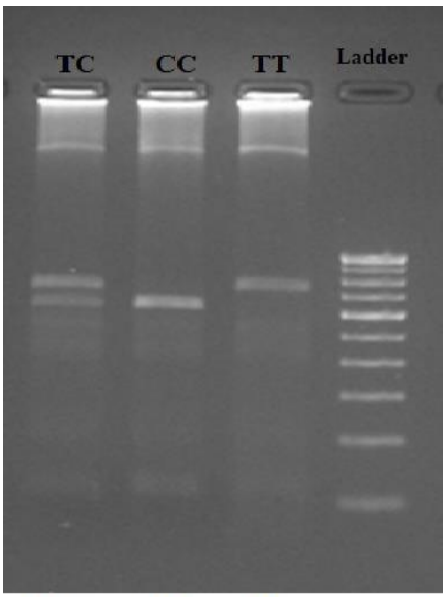


Figure 14. Agarose gel electrophoresis of RFLP products

4.3. Statistical analysis

The normality of data was checked using Shapiro-Wilk's test. The research data was not normally distributed. The results obtained in the study were represented as mean ± SD. Mann- Whitney U test was used for continuous non-normally distributed variables. Categorical variables were expressed as percentages. Spearman's correlation and linear regression was used to find the association of variables. Fisher exact test was used for comparison of categorical variables and to test the genotype frequencies, Hardy- Weinberg equilibrium was used. Chi- square ($\chi 2$) test was used for the comparison of the allele and genotype frequencies between preeclamptic cases and controls. The level of significance was $P<0.05$. Analysis was performed using licensed version of SPSS software, version 22.0

CHAPTER-5

RESULTS AND DISCUSSION

5. RESULTS AND DISCUSSION

5.1 Results

This prospective observational study was conducted in the Department of Biochemistry in association with Department of Obstetrics and Gynecology, Sri RL Jalappa Hospital and Research Centre, a teaching hospital of Sri Devaraj Urs Medical College, a constituent of Sri Devaraj Urs Academy of Higher Education and Research, Kolar, Karnataka, India. The current study includes 196 pregnant women, comprising of 98 preeclamptic women as cases and 98 normotensive pregnant women with age and gestation matched as controls. The study subjects were followed until delivery. To get the basal level of Apelin 13, 90 non-pregnant women were included. The study subjects were recruited from the Department of Obstetrics and Gynecology.

Table 6: Demographic, hematological, inflammatory and biochemical characteristics of normotensive pregnants and preeclampsia

Parameters	Normotensive pregnants (n=98) Mean±SD	Preeclampsia (n=98) Mean±SD	*P* values
Age (years)	22.93±3.05	22.62±3.30	0.317
Gestational age (weeks)	38.07±1.39	37.73±2.32	0.504
Systolic blood pressure (mmHg)	115.89±7.64	159.04±14.46	<0.001*
Diastolic blood pressure (mmHg)	73.95±6.52	104.91±10.74	<0.001*
Mean arterial Pressure (mmHg)	87.85±6.14	123.10±11.25	<0.001*
Presence of proteinuria (*n*, %)	Nil	98 (100%)	-
Pulse rate (bpm)	85.05±7.38	87.85±5.84	<0.001*
Hematological parameters			
Hemoglobin (g%)	11.35±1.77	11.07±1.98	0.477
White Blood Cells (cells/cu mm)	14.14±4.87	12.95±3.59	0.114
Platelets, x (10^9/L)	245.12±61.99	235.16±76.49	0.498
Inflammatory markers			
Neutrophil to Lymphocyte Ratio	5.21±2.46	5.93±2.11	<0.001*
Platelet to Lymphocyte Ratio	16.63±7.10	18.02±7.92	0.165
Biochemical parameters			
Random blood sugar (mg/dL)	82.66±15.66	83.58±21.19	0.655
Serum urea (mg/dL)	14.53±4.66	15.36±6.44	0.497
Serum Creatinine (mg/dL)	0.49±0.12	0.53±0.16	0.055
Serum uric acid (mg/dL)	4.81±1.45	6.25±1.51	<0.001*
Serum aspartate transaminases (U/L)	21.27±8.20	24.92±12.84	0.019*
Serum alanine transaminases (U/L)	14.54±7.51	19.41±9.94	<0.001*
Serum lactate dehydrogenase (U/L)	163.59±60.05	294.63±142.88	<0.001*
Serum magnesium (mg/dL)	2.19±0.62	2.00±0.39	0.099

*P<0.05 considered as statistically significant

Demographic, hematological, inflammatory and biochemical characteristics of normotensive pregnant and preeclampsia were illustrated in table 6. Systolic, diastolic, mean arterial pressure, pulse rate, Neutrophil to lymphocyte ratio, serum uric acid, Aspartate transaminase, Alanine transaminase, Lactate dehydrogenase were significantly increased in preeclampsia compared to normotensive pregnants. Serum magnesium levels were not significant between preeclampsia and normotensive pregnants.

Table 7: Serum Malondialdehyde (MDA) and Ferric Reducing Ability of Plasma (FRAP) levels between normotensive pregnants and preeclampsia

Parameters	Normotensive pregnants (n=98) Mean±SD	Preeclampsia (n=98) Mean±SD	*P* value
Serum MDA (µmoles/L)	10.24±5.70	18.12±6.98	<0.001*
FRAP (mmol/L)	1978.08±142.95	1077.71±417.85	<0.001*

*P<0.05 considered as statistically significant

In the present study, serum Malondialdehyde levels were increased significantly and ferric reducing ability of plasma levels were significantly reduced in preeclampsia compared to normotensive pregnants as depicted in table 7.

Table 8: Serum Apelin 13, endothelial Nitric Oxide Synthase (eNOS) and Nitric Oxide (NO) concentrations between normotensive pregnants and preeclampsia

Parameters	Normotensive pregnants (n=98) Mean±SD	Preeclampsia (n=98) Mean±SD	*P* value
Serum Apelin 13 (pg/mL)	482.50±240.98	236.73±125.88	<0.001*
Serum eNOS (ng/mL)	6.74±1.89	5.70±2.36	0.002*
Serum NO (µmoles/L)	13.29±3.66	6.23±1.58	<0.001*

*P<0.05 considered as statistically significant

In the present study, serum Apelin 13, serum endothelial nitric oxide synthase and nitric oxide concentrations were significantly reduced in preeclampsia compared to normotensive pregnants as depicted in table 8. The basal level of Apelin 13 in non-pregnant women were 107.51 ±33.85 pg/mL.

Table 9: Demographic, hematological, inflammatory and biochemical characteristics of mild preeclampsia and severe preeclampsia

Parameters	Mild Preeclampsia (n=36) Mean ± SD	Severe Preeclampsia (n=62) Mean ± SD	*P* value
Age (years)	22.86±3.48	22.48±3.21	0.686
BMI (kg/m^2)	27.00±4.21	27.46±3.45	0.266
Gestational age (weeks)	37.58±1.98	37.82±2.50	0.293
Systolic blood pressure (mmHg)	144.61±6.48	167.41±10.70	<0.001*
Diastolic blood pressure(mmHg)	92.55±4.98	112.09±5.16	<0.001*
Mean arterial Pressure (mmHg)	110.05±4.42	130.67±5.61	<0.001*
Pulse rate (bpm)	87.05±4.47	88.32±6.50	0.453
Hematological characteristics			
Hemoglobin (g%)	10.71±1.91	11.28±2.01	0.139
White Blood Cells (cells/ cumm)	12.67±3.19	13.12±3.81	0.825
Platelets, x (10^9/L)	230.72±67.49	237.74±81.68	0.601
Inflammatory markers			
Neutrophil to Lymphocyte Ratio	4.79±1.95	6.59±1.92	<0.001*
Platelet to Lymphocyte Ratio	15.44±6.75	19.53±8.20	0.023*
Biochemical parameters			
Random blood sugar (mg/dL)	84.16±21.65	83.24±21.09	0.903
Serum urea (mg/dL)	13.80±5.39	16.27±6.86	0.133
Serum Creatinine (mg/dL)	0.49±0.11	0.55±0.18	0.123
Serum uric acid (mg/dL)	5.54±1.26	6.66±1.49	<0.001*
Serum AST (U/L)	23.27±9.02	25.88±14.59	0.572
Serum ALT (U/L)	19.00±7.19	19.66±11.29	0.572
Serum LDH (U/L)	258.83±79.10	315.41±166.37	0.148
Serum magnesium (mg/dL)	1.90±0.34	2.06±0.41	0.030*

*P<0.05 considered as statistically significant

Demographic characteristics of mild preeclampsia and severe preeclampsia were illustrated in table 9. Systolic, diastolic, mean arterial pressure, Neutrophil to Lymphocyte Ratio, Platelet to Lymphocyte Ratio, serum uric acid and magnesium were significantly elevated in severe preeclampsia compared to mild preeclampsia.

Table 10: Serum malondialdehyde (MDA) and Ferric Reducing Ability of Plasma (FRAP) levels between mild preeclampsia and severe preeclampsia

Parameters	Mild Preeclampsia (n=36) Mean±SD	Severe Preeclampsia (n=62) Mean±SD	*P* value
Serum MDA(µmoles/L)	17.71±6.54	18.36±7.27	0.765
FRAP (mmol/L)	1148.85±431.43	1036.40±407.59	0.201

In the present study, serum malondialdehyde levels were increased and ferric reducing ability of plasma levels were reduced in severe preeclampsia compared to mild preeclampsia, the levels were not statistically significant as depicted in table 10.

Table 11: Serum Apelin 13, endothelial Nitric Oxide Synthase (eNOS) and Nitric Oxide (NO) concentrations between mild preeclampsia and severe preeclampsia

Parameters	Mild Preeclampsia (n=36) Mean±SD	Severe Preeclampsia (n=62) Mean±SD	*P* value
Serum Apelin 13 (pg/mL)	264.44±138.84	220.64±115.85	0.056
Serum eNOS (ng/mL)	7.09±1.92	4.87±2.27	<0.001*
Serum NO (µmoles/L)	6.51±1.34	6.06±1.69	0.314

*P<0.05 considered as statistically significant

In the present study, serum Apelin 13, eNOS and NO concentrations were reduced in severe preeclampsia, however, only eNOS levels were statistically significant as depicted in table 11.

Table 12: Correlation of Apelin 13 with eNOS, NO in normotensive pregnants and preeclampsia

Parameters	*r*-value	*P* value
Normotensive pregnants		
Serum eNOS	0.155	0.126
Serum NO	0.022	0.831
Preeclampsia		
Serum eNOS	0.214*	0.035
Serum NO	0.085	0.406

*. Correlation is significant at the 0.05 level (2-tailed).

In the present study, Apelin 13 levels were positively correlated with eNOS and NO in both groups. However, eNOS showed significant positive correlation as illustrated in table 12 and figure 15.

Table 13: Linear regression analysis of Apelin 13 and endothelial Nitric Oxide Synthase

Coefficients[a]

Model		Unstandardized coefficients		Standardized coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	4.669	0.507	0.217	9.267	<0.001
	Apelin 13	0.004	0.002		2.175	0.032

R Square value = 0.047

In the present study, linear regression analysis showed significant relationship of Apelin 13 with eNOS as shown in table 13.

Table 14: Linear regression analysis of Apelin 13 and Nitric Oxide

Coefficients[a]

Model		Unstandardized coefficients		Standardized coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant) Apelin 13	5.798	0356	0.140	16.306	<0.001
		0.002	0.001		1.390	0.168

R Square value = 0.020

In the present study, linear regression analysis showed significant relationship of Apelin 13 with Nitric Oxide as shown in table 14.

Table 15: Correlation of Apelin 13 with blood pressure

Parameters	*r*-value	*P* value
Systolic blood pressure	-0.241*	0.017
Diastolic blood pressure	-0.208*	0.040
Mean arterial pressure	-0.235*	0.020

*. Correlation is significant at the 0.05 level (2-tailed).

In this study, serum Apelin 13 concentrations were significantly negatively correlated with systolic blood pressure, diastolic blood pressure, mean arterial pressure as depicted in table 15 and figure 16.

Table 16: Correlation of eNOS with blood pressure

Parameters	*r*-value	*P* value
Systolic blood pressure	-0.380**	<0.001
Diastolic blood pressure	-0.372**	<0.001
Mean arterial pressure	-0.393**	<0.001

**. Correlation is significant at the 0.01 level (2-tailed).

In this study, serum eNOS concentrations were significantly negatively correlated with systolic, diastolic and mean arterial pressure as depicted in table 16 and figure 17.

Table 17: Correlation of Nitric Oxide with blood pressure

Parameters	*r*-value	*P* value
Systolic blood pressure	-.082	0.420
Diastolic blood pressure	-.127	0.214
Mean arterial pressure	-.093	0.360

In this study, serum nitric oxide concentrations were negatively correlated with systolic, diastolic and mean arterial pressure as depicted in table 17 and figure 18.

Table 18: Correlation of Apelin 13 with other study parameters

Parameters	*r*-value	*P* value
Serum urea	0.149	0.143
Serum Creatinine	0.027	0.793
Serum uric acid	-0.082	0.422
Serum LDH	0.069	0.502
Serum MDA	-0.031	0.762
FRAP	0.076	0.455
NLR	-0.249*	0.013
PLR	-.299*	0.003
Birth weight	0.069	0.497

*. Correlation is significant at the 0.05 level (2-tailed).

In this study, Neutrophil to lymphocyte ratio (NLR), platelet to lymphocyte ratio (PLR) were negatively correlated with Apelin 13 as depicted in table 18.

Table 19: Maternal/fetal adverse outcomes of normotensive pregnants and preeclampsia

Maternal /fetal adverse outcomes (*n*, %)	Normotensive pregnants (n=98)	Preeclampsia (n=98)	Chi-Square Test	*P* value
Acute renal failure	Nil	1 (1.0%)	-	1.000[b]
HELLP Syndrome	Nil	5 (5.1%)	-	0. 059[b]
Eclampsia	Nil	2 (2.0%)	-	0.497[b]
Fetal outcome				
Male	48 (48.9%)	51 (52.0%)	0.18	0.668[a]
Female	50 (51.0%)	47 (47.9%)		
Preterm birth (<37weeks)	2 (2.04%)	29 (29.5%)	27.93	<0.001[a*]
Birth weight (kg)	2.82±0.50	2.41±0.58	-	<0.001†*
IUGR	4 (4.08%)	20 (20.4%)	12.15	<0.001[a*]
Respiratory distress syndrome (RDS)	8 (8.16%)	23 (23.46%)	170.4	<0.001[a*]
NICU Admission	10 (10.2%)	38 (38.7%)	8.930	<0.001[a*]
Intrauterine death (IUD)	Nil	7 (7.14%)	-	0.014[b]

* Significant, † Mean±SD, HELLP Syndrome: Hemolysis, Elevated liver enzymes, Low Platelet count

a Chi-Square P value; b Fisher's Exact P value

In the present study, adverse maternal outcomes were significantly higher in preeclamptic group, such as HELLP syndrome 5 (5.10%), acute renal failure 1 (1%) and eclampsia 2 (2%). Adverse fetal outcomes were more in preeclamptic cases including significantly decreased birth weight (2.41±0.58), preterm birth (<37 weeks) in 29 (29.5%), IUGR in 20 (20.4%), RDS in 23 (23.4%), NICU admission in 38 (38.7%) and IUD in 7 (7.14%) babies (Table 19).

Table 20: Association of Apelin 13 with maternal and fetal outcome

Maternal/Fetal adverse outcomes	Chi-Square Test	P value
Acute renal failure	-	1.000[b]
HELLP Syndrome	-	0.660 [b]
Eclampsia	-	0.549 [b]
Fetal outcome		
Preterm birth (<37 weeks)	0.128	0.720 [a]
IUGR	1.443	0.230 [a]
Respiratory Distress Syndrome (RDS)	2.789	0.248 [a]
NICU Admission	0.279	0.597 [a]
Intrauterine Death (IUD)	-	0.685[b]

a Chi-Square P value; b Fisher's Exact P value

In the present study, Apelin 13 levels were not showed any association with maternal/fetal outcomes as represented in the table 20.

Table 21: Comparison of Apelin-1860T>C gene polymorphism in normotensive pregnants and preeclampsia

Apelin T1860C genotype frequency	Normotensive pregnants (n=90) (%)	Preeclampsia (n=91) (%)	Odds Ratio (95% CI)	*P* value
TT	84 (93.35%)	68 (74.72%)	-	0.0028
TC	5 (6.02%)	18 (19.78)		
CC	1 (1.20%)	5 (5.49)		
χ^2 = 11.69; df = 2				
Allele				
T	178 (96.21%)	154 (86.61%)	4.623 (1.965-10.88)	-
C	7 (3.78 %)	28 (15.38%)		0.00013
χ2 = 14.27; df = 1				

Table 22: Allele frequency/genotype frequencies and test of Hardy – Weinberg equilibrium

	Normotensive pregnants		Preeclampsia	
f(T)	0.9621		0.8461	
f(C)	0.0378		0.1538	
	O	**E**	**O**	**E**
TT	84	83.13	68	65.15
TC	5	6.72	18	23.69
CC	11	0.136	5	2.15
	χ^2 = 5.93, P=0.96		χ^2 = 32.23, P = 0.9202	

f = observed frequency of each allele (T or C); O = observed genotype numbers; E = expected genotype numbers under a Hardy- Weinberg equilibrium (HW) equilibrium assumption; χ 2 = Chi Square values

Table 21 and 22 depict the distribution of genotype/allele frequencies between normotensive pregnants and preeclamptic cases. In this study context, 68 (74.72%) of preeclamptic cases and 84 (93.35%) of normotensive pregnants were homozygote (TT), whereas 18 (19.78%) of preeclamptic cases and 5 (6.02%) of controls were heterozygote (TC), and also 05 (5.49%) of cases and 1 (1.0%) of controls were homozygous (CC) at this position were observed.

The distribution of Apelin-1860T>C genotype frequency was higher in preeclampsia in comparison with the normotensive controls. In the allelic distribution, T allele was observed in 154 (86.61%) of preeclamptic cases and 178 (96.21%) of the normotensive controls. C allele was seen in 28 (15.38%) of preeclamptic cases and 7 (3.78%) of the normotensive controls. Hence, C allele distribution was found to be significant (p=0.00013). The frequency of promoter APLN gene - 1860T>C polymorphism allele/ genotypes is higher in cases than controls and appears as risk allele.

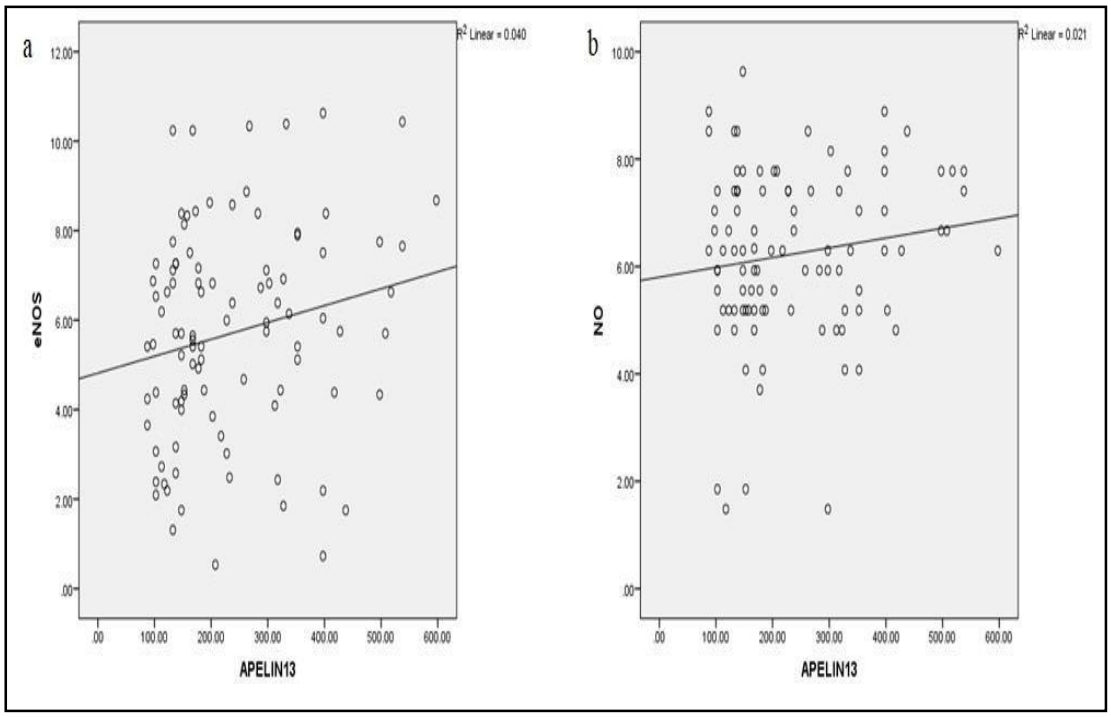


Figure 15: Shows the correlation of Apelin 13 with (a) eNOS and (b) NO in Preeclampsia

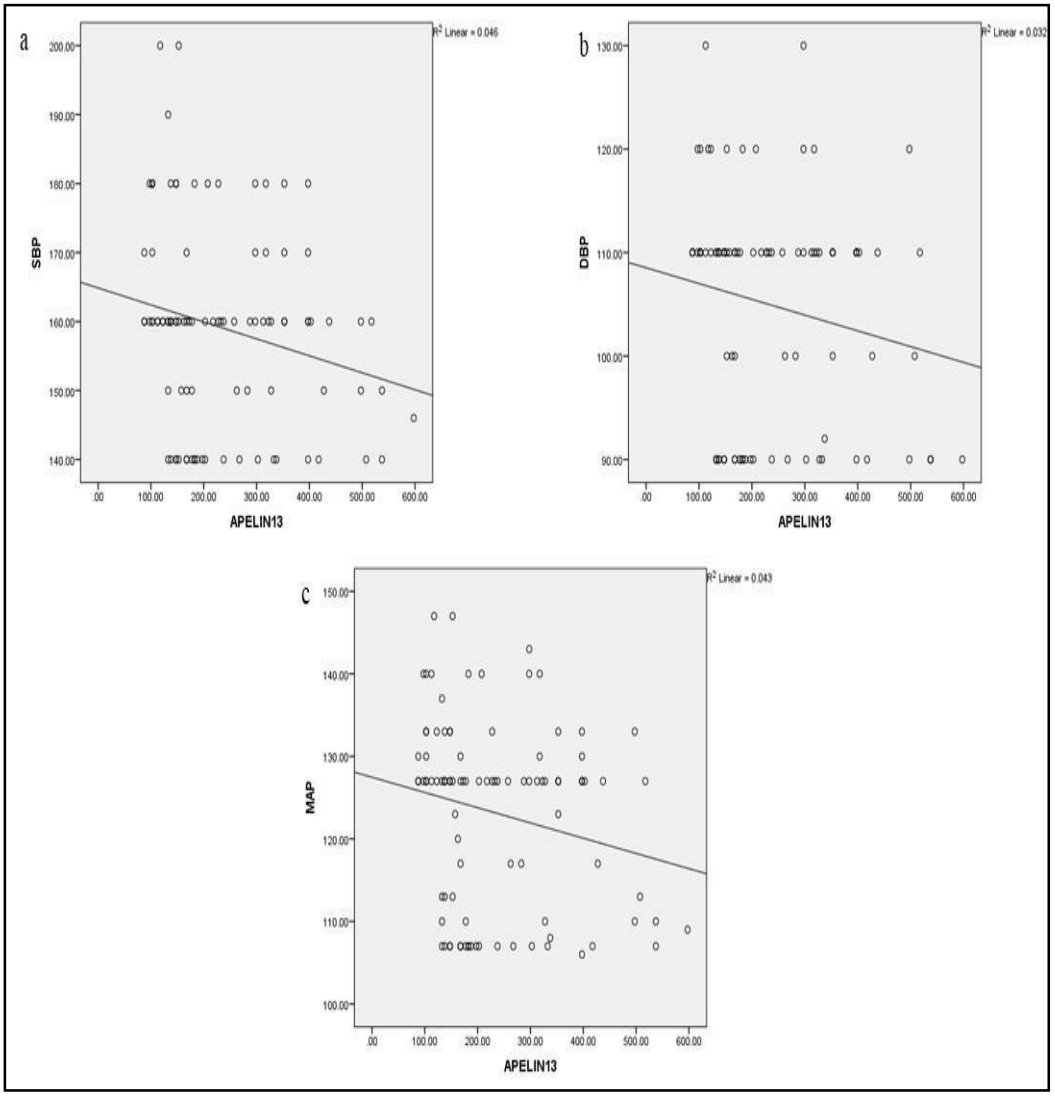


Figure 16: Shows the correlation of Apelin 13 with blood pressure
(a) Systolic blood pressure (b) Diastolic blood pressure (c) Mean arterial pressure

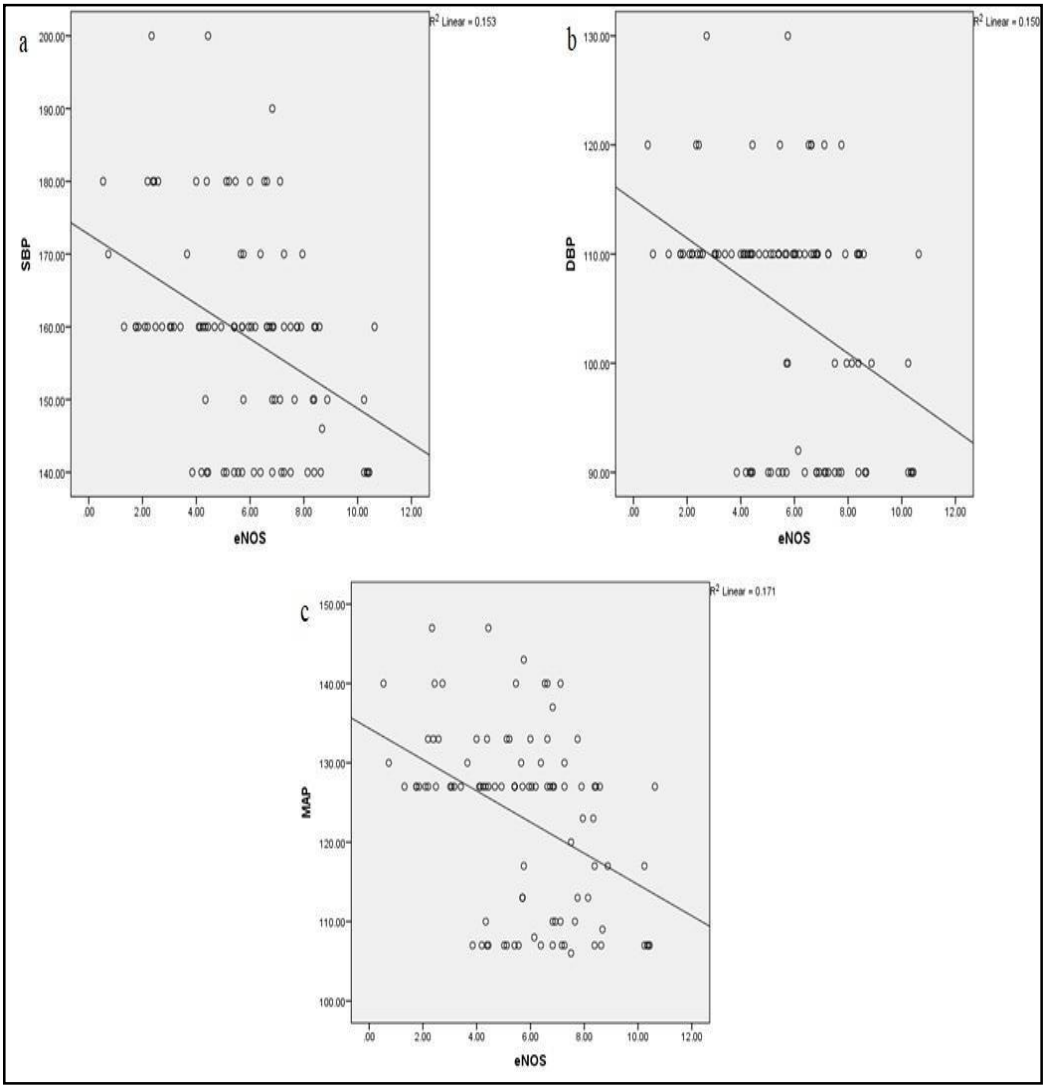


Figure 17: Shows the correlation of endothelial Nitric Oxide Synthase (eNOS) with blood pressure (a) Systolic blood pressure (b) Diastolic blood pressure (c) Mean arterial pressure

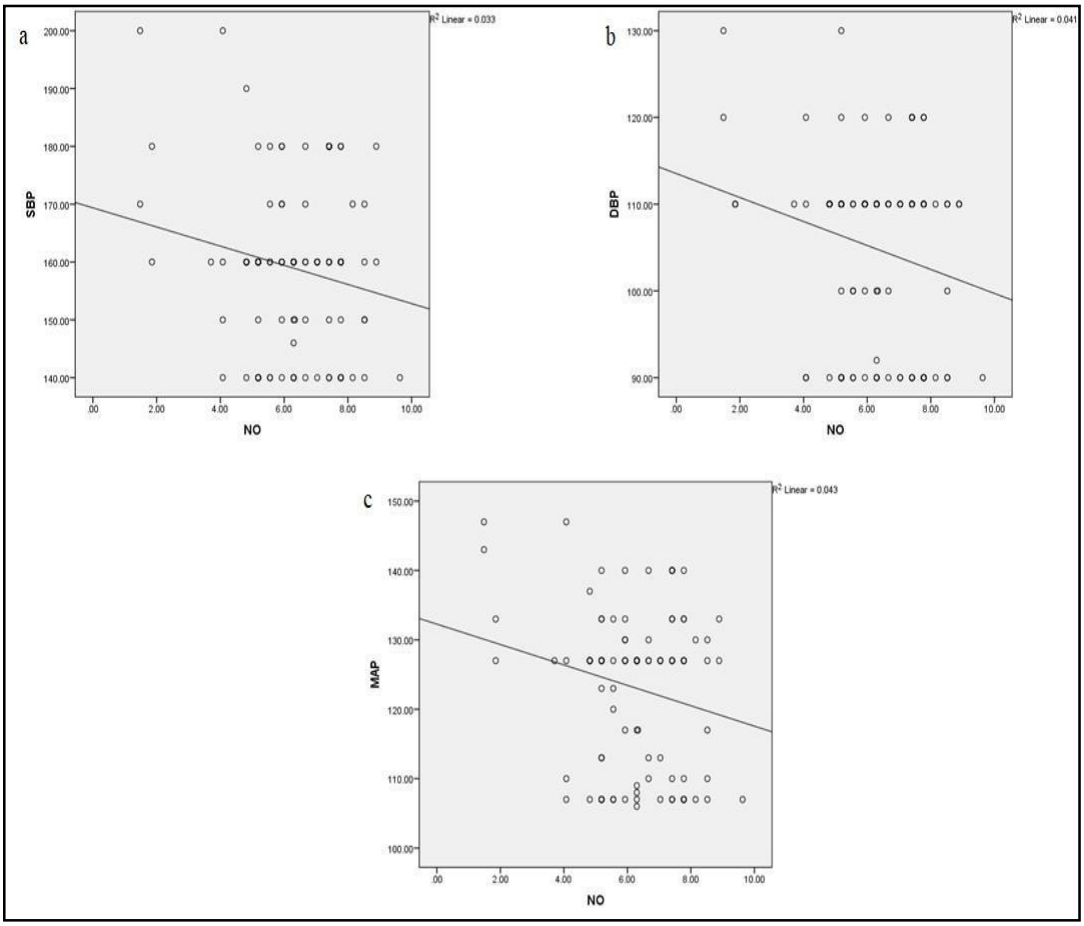


Figure 18: Shows the correlation of Nitric Oxide (NO) with blood pressure
(a) Systolic blood pressure (b) Diastolic blood pressure (c) Mean arterial pressure

5.2. DISCUSSION

The demographic details of the preeclamptic group and normotensive pregnant group showed that about 30.6% belong to the age group of 18-20 years and 28.5% respectively. Whereas in 21-25 years of age group about 53.0% were preeclamptic and 51.0% in normotensive pregnants. However, in the age group of 26 -30 years, 14.2% in preeclampsia and 19.3% in normotensives. Besides, 2.4% comprise more than 30 years of age in preeclampsia and 1.0% in normotensive group. Research reports indicated that the maternal age group of <25 years and >35 years contributing as a risk factor for preeclampsia [1,135]. In the present study, we observed the age group of <25 years of primigravida were associated with risk of preeclampsia.

In the present study, even though low level of hemoglobin and platelets were observed in preeclampsia found that statistically non-significant. The possible reason for low platelet count might be due to increased peripheral consumption, endothelial damage, reduced life span and stacking of platelets in the area with endothelial damage in preeclamptic women [136].

Neutrophil to lymphocyte ratio (NLR) and platelet to lymphocyte ratio (PLR) are proposed as inflammatory markers in preeclampsia. Similarly, in the current study elevated neutrophil to lymphocyte ratio and platelet to lymphocyte ratio were noticed. Amongst, NLR was found to be significant. However, NLR and PLR were significantly elevated in severe preeclampsia compared to mild preeclampsia. Activation of neutrophils, monocytes in

peripheral blood and in decidua under hypoxia or inflammatory conditions causes vascular damage. Increased activation of inflammatory cells and immunologic responses in preeclampsia may cause release of cytokines, autoantibodies and increased superoxide production may lead to endothelial dysfunction [137,138]. Preeclampsia is associated with dysregulation of T- helper 1 and T- helper 2 type inflammatory responses [137]. Preeclampsia is associated placental hypoxia, elevated oxidative stress and reduced anti-oxidants and exaggerated pro-inflammatory markers. Few studies have reported that, increased NLR and PLR may indicate subclinical inflammation in preeclampsia [139-141].

In contrast to above studies, Mehmet Toptas *et al*. reported that NLR and PLR were not significantly different between preeclampsia and controls. However, in comparison with NLR, PLR was found to be increased in severe preeclampsia [142].

Etiological factors that have been implicated in the pathogenesis of preeclampsia includes abnormal placentation, endothelial cell injury and endothelial dysfunction [143,144]. Endothelial dysfunction results in elevated systemic vascular resistance, and reduced placental perfusion, which aggravates the placental ischemia-reperfusion injury, that stimulates lipid peroxidation, and increased generation of ROS, or oxidative stress. Besides, compromised antioxidant status further exacerbates endothelial dysfunction[92].

And also, mitochondrial oxidative stress could be considered as another source of ROS generation, involved in placental dysfunction that leads to apoptosis which is the basis of pathogenesis of preeclampsia [25].

Placental dysfunction comprising due to defective trophoblast invasion, reduced blood flow and creating a hypoxic environment adds to oxidative stress, consequently oxidative damage results in inflammation. This irreversible damage of cellular components like enzyme activity, signal transduction and apoptosis [145].

Malondialdehyde (MDA) is a marker to represent intensity of lipid peroxidation. In line with other studies, the present study also evidenced prominent rise of MDA level in preeclampsia cases than the normotensives with statistical significance. Even though, moderate increase of MDA level in severe preeclampsia than mild preeclampsia but not showed statistical significance. Lipid peroxidation products are generally known to cause oxidative damage to endothelial cells *in-vivo* and leads to endothelial dysfunction. Hence, the results of the study justify the similar observation as described here in other research reports.

In a Nigerian cross-sectional study focused on preeclampsia, gestational hypertension and healthy pregnants. The study results showed elevated malondialdehyde and homocysteine level in preeclampsia. The levels of methylene tetrahydrofolate reductase, glutathione, superoxide dismutase and catalase were found to be low in preeclampsia than controls. Study highlights

the metabolic derangements linked to gestational hypertension in oxidative stress environment and thus high MDA level and low MTHFR serves as risk factors in pathogenesis of gestational hypertension complications [146].

Similarly, in another Nigerian cross-sectional study reported oxidative stress, inflammation and hematological profile between preeclampsia and control group. The study results showed that increased MDA ($P<0.05$), cardiac-specific Troponin I (cTnI), prothrombin time, activated partial thromboplastin time and reduced levels of enzyme antioxidants such as superoxide dismutase, catalase, and non-enzymatic antioxidant glutathione, in preeclampsia, suggests that preeclampsia is associated with oxidative stress, and metabolic derangements [147].

In addition, an Indian case-control study conducted between preeclampsia and healthy pregnant women reported increased levels of malondialdehyde along with glutathione peroxidase, superoxide dismutase, L-Ascorbic acid and low levels of lycopene in preeclampsia. This observation suggests that involvement of oxidative stress in pathophysiology of preeclampsia [148].

Besides, yet another US case-control study conducted in Bosnia and Herzegovina population between preeclampsia and healthy pregnant women to assess oxidative stress. Study results showed that significant increase of MDA level in preeclampsia than healthy pregnants. However, MDA level also positively correlated with blood pressure indicated oxidative stress is possible cause for preeclampsia [149].

Furthermore, many similar case-control studies evinced that oxidative stress as one of the underlying causes of preeclampsia and its complications [150-153].

The antioxidants neutralize the deleterious effect of free radicals contributes to oxidative stress. In the present study context, the total antioxidant status (accounted by ascorbic acid, α-tocopherol, uric acid, bilirubin and others) was found reduced and statistically significant ($p<0.05$) in preeclampsia compared to normotensive pregnants. This observation is also in agreement with several studies imparted high oxidative stress and low antioxidant status.

A cross-sectional study was conducted in Turkey population between preeclampsia, gestational diabetes mellitus and healthy pregnant women with 24 - 36 weeks of gestational age. The study results reported that total antioxidant status was significantly reduced in preeclampsia and gestational diabetes mellitus than normal pregnant women. Malondialdehyde (MDA) levels were elevated in preeclampsia and gestational diabetes mellitus than healthy pregnant women. Advanced oxidative protein products (AOPP) were elevated and unchanged myeloperoxidase and lipid hydroperoxide (LPH) were observed in gestational diabetes mellitus and preeclampsia, indicating that elevated oxidative stress and low antioxidant status in preeclampsia and gestational diabetes mellitus may contribute to the progression of both the disease conditions [154].

A cross-sectional study was conducted in Turkey population between mild preeclampsia and healthy pregnant women and their infants. They measured

mononuclear leukocyte DNA damage, and oxidative stress index (OSI) using maternal and cord blood. The results presented elevated DNA damage due to high OSI with concomitant reduction of total antioxidants. The extent of DNA damage and diastolic blood pressure were positively correlated in preeclamptic mothers. Results demonstrated that both feto-maternal oxidative stress and DNA damage are interlinked. Therefore, increased oxidative stress and DNA damage may be associated with fetal and maternal adverse complications [155].

A case-control study conducted in Indian population between neonates born to preeclamptic mothers and neonates born to normal pregnant women. The study results showed that increased oxidative stress and reduced total antioxidant level in neonates born to preeclampsia mothers than controls may be associated with adverse neonatal outcomes in preeclampsia [156].

A cross-sectional study conducted in Indian population between healthy pregnant women, preeclampsia and eclampsia patients to assess the ApoB/ApoA-1 ratio and nitric oxide in pregnancy induced hypertension. The study results showed significantly increased levels of malondialdehyde and lipid profile with apolipoproteins. Total antioxidant status represented as FRAP and nitric oxide levels were reduced in preeclampsia and eclampsia than the control groups. Results suggests that dyslipidemia along with oxidative stress may contribute to pathophysiology of preeclampsia [157].

In contrary to above studies, increased antioxidant status has also been reported in preeclampsia over well-known reduced antioxidant levels [92,158-160].

In support of the above overall observations, our study results confirm that elevated oxidative stress and low antioxidant status is a precise cause as observed in preeclampsia than healthy pregnant women. In addition to the parameters represents oxidative stress in terms of MDA and antioxidants status of plasma in terms of FRAP, few more markers like nitric oxide and endothelial nitric oxide synthase concentration represents as vasodilation of endothelium and also Apelin 13 level to indicate possible involvement with vasodilation through endothelial activation [32,161].

Apelin is an angiogenic peptide and binds through its cognate receptor APJ/AR causes endothelium dependent vasodilation by stimulating endothelial nitric oxide synthase (eNOS). eNOS is a dimeric protein consists of oxidase and reductase domain, Apelin peptide activates eNOS through phosphorylation at serine 1177 of reductase domain and releases nitric oxide [45,161].

We measured maternal serum Apelin 13 levels between the study groups and found results with statistical significance. It is evident that Apelin 13 concentration was low based on severity of preeclampsia, where Apelin 13 level was found high in normotensive than mild preeclampsia and severe preeclampsia.

Several mechanisms have proposed to be associated with pathophysiology of preeclampsia. Even then, there is a need to explore the pathogenic mechanisms involved the onset of preeclampsia. In view of Apelin 13 role in angiogenesis and vasodilation, understanding its molecular mechanisms could provide a

basis for therapeutic interventions that may decrease the incidence of preeclampsia and improve the maternal and fetal survival. Apelin peptides have showed their involvement in pathophysiology of preeclampsia [32].

To the best of our knowledge, this is the first study on Apelin 13 and its importance in tracing pathophysiology of preeclampsia in Indian population with respect to endothelial activation by considering Apelin gene analysis, relation between Apelin and endothelial nitric oxide under prevailing oxidative stress condition to explore the possible biological role with touch of therapeutical potency in pregnancy complication cases.

As an angiogenic factor, apelin stimulates blood vessel growth, differentiation and regulates caliber size of blood vessels by inducing their enlargement [41,162].

Preeclampsia is associated with imbalance in the angiogenic/anti-angiogenic factors[163]. Therefore, decreased Apelin levels might affect the migration of invasive trophoblasts along the spiral artery and impair their vascular invasion. This abnormal spiral artery remodeling, results in high resistance uteroplacental circulation, as observed in preeclampsia [38,164]. Apelin activates the cell transduction cascade extracellular signal-regulated kinase (ERKs), promotes Akt and p70S6 kinase phosphorylation, which leads to endothelial cell proliferation and formation of new blood vessels [165-167].

In the development of placenta, vascular endothelial growth factor (VEGF) has been studied extensively and shown to be involved in the angiogenic balance

in placentation [168,169]. It has beenreported that apelin/APJ system promotes the vascular endothelial growthfactor expression [170].

AMPK signaling is required for vascular endothelial growth factor (VEGF)-induced nitric oxide (NO) production, migration and differentiation under conditions of hypoxia. In addition, protein kinase Akt/protein kinase B (Akt) signaling participates in vascular homeostasis and angiogenesis [171,172]. Thus, it was hypothesized that AMPK and Akt signaling may participate in the regulation of angiogenesis stimulated by apelin 13 [165].

However, low concentrations of Apelin 13 in hypertensive disorders of pregnancy, especially in preeclampsia characterized by pathological angiogenesis associated with preeclampsia [48]. However, this low Apelin 13 levels could impair the endothelium dependent vasodilation induced by Apelin triggered NO release [38]. The low levels of Apelin 13 may be due to counter-regulation of Ang II, which may downregulate the Apelin expression or its release from the placenta and therefore interfere with its biological actions [32]. The reduced Apelin 13 levels may also be due to increased clearance by ACE-II [173]. Few studies reported that a counter- regulatory role for Apelin in relation to the rennin-angiotensin system (RAS) through the functional interactions between Apelin and angiotensin II (ANG II) [174,175].

This low level of Apelin 13 in preeclampsia may lead to reduced vascular endothelial growth factor expression. This might also play a central role in the abnormal angiogenesis in the onset of preeclampsia [170].

Hence, the results of the study justify the similar observation as described here in other research reports.

A cross-sectional study in China focused on Apelin expression in human placental samples from normotensive pregnants and hypertensive disorders of pregnancy. The study results showed that significantly reduced Apelin concentrations ($p<0.05$) and Apelin mRNA expression ($p<0.05$) in hypertensive disorders of pregnancy. The levels were also gradually decreased between mild and severe preeclampsia. The study findings indicate that abnormal placental expression of Apelin may be linked with pathogenesis of preeclampsia [176].

In addition, an USA case-control study between preeclampsia and normotensive healthy pregnant women focused on circulating levels of maternal plasma Apelin. Study results showed reduced Apelin concentrations in preeclampsia at delivery compared to normotensive controls. The study findings suggest that reduced Apelin levels may be associated with pathogenesis of preeclampsia and may have deleterious effects on fetal development [105].

Similarly, another case-control study in North Carolina, USA focused on placental expression of various Apelin peptides between healthy pregnant women and preeclampsia women at 37 to 38 weeks of gestation. The study results showed that reduced placental total Apelin content, Apelin 36, Apelin 13, and Apelin 12 in preeclampsia compared to normotensive controls. The

shorter forms of Apelin were more abundant. The study results suggest that Apelin may be a novel therapeutic agent to prevent development of preeclampsia [32].

Yet, another case-control study in Turkey between preeclampsia and healthy pregnant women focused to measure maternal serum Apelin 13 and Apelin 36 levels in both groups. Study results showed that maternal serum Apelin 13 and Apelin 36 concentrations were significantly lower in preeclamptic women compared with healthy normotensive pregnant women. Adverse maternal/fetal outcomes were more in preeclampsia than normotensive controls. However, Apelin 13 and Apelin 36 levels were not different between patients with adverse outcomes. The study highlights the lower levels of Apelin peptides may be linked with preeclampsia pathophysiology [48].

Similarly, another cross-sectional study in Turkey between normotensive pregnants, mild preeclampsia and severe preeclampsia cases focused to measure elabela, Apelin and nitric oxide levels in maternal blood, umbilical arteries and venules of newborns. The study results showed that Apelin, elabela, and nitric oxide concentrations were significantly decreased in both mild and severe preeclamptic women compared with healthy pregnant women. This decrease was more prominent in the severe preeclamptic women. Similar trend was observed in venous-arterial cord blood samples, suggesting endothelial dysfunction in preeclampsia pathophysiology [49].

Yet, another case-control study in Iran focused to assess the correlation between maternal serum Apelin and galectin-3 levels with insulin resistance (IR) in women with preeclampsia. The study results showed that preeclamptic women had significantly lower Apelin and higher galectin-3 levels than the healthy pregnants. Preeclamptic group exhibited dyslipidemia and higher β-cell functions than the normotensive control group. Galectin-3 levels were significantly positively correlated with insulin, glucose, dyslipidemia and IR. The study findings suggests that Apelin and galectin-3 involvement in preeclampsia, including future onset of cardiac diseases [50].

In contrast to the above, Yavuz Simsek *et al*. conducted a cross-sectional study in Turkey focused to measure serum levels of Apelin, salusin-α and salusin-β between mild and severe preeclampsia in comparison with healthy pregnant women with gestational age of 24 - 42 weeks. The study results showed that Apelin, salusin-α and salusin-β levels were significantly elevated in preeclampsia and no significant difference was observed between mild and severe preeclampsia, suggesting that this high level may be an adaptive response to hypertensive state to reduce the blood pressure [106].

Similarly, another case-control study in Turkey between healthy pregnants and preeclampsia focused to investigate the serum levels of Apelin and YKL-40 in both groups. The study results showed that mean maternal serum Apelin concentrations were higher in both early and late onset preeclampsia compared with healthy pregnant, suggesting that Apelin may be involved in the vascular pathogenesis of preeclampsia than YKL-40 [51].

Considering the effects of Apelin especially on angiogenesis and vasodilation, few animal model studies also reported the beneficial effects of Apelin 13 in reducing blood pressure and maternal and fetal outcome.

In an animal model experimental study in China investigated the effects of Apelin 13 in a rat model induced by reduced uterine perfusion pressure. Subcutaneous administration of Apelin 13 ($6x10^{-8}$ mol/kg, twice a day) in preeclamptic rats significantly inhibited the elevation of systolic, diastolic and mean arterial blood pressure. Apelin administration reversed the decreased total fetal weight and total placental weight. Apelin administration to preeclamptic rats led to an upregulation of the protein and mRNA levels of eNOS. Apelin has a direct activating effect on the L-arginine/eNOS/NO pathway suggested that apelin may be a novel drug for treating preeclampsia [108].

Yet, another animal model study in North Carolina, USA focused to establish systemic outcomes of (Pyr^1) Apelin 13 administration in rats with preeclamptic features. Established the systemic outcomes of (Pyr^1) Apelin 13 administration in rats with preeclamptic features (TGA-PE, female transgenic for human angiotensinogen mated to male transgenic for human renin). (Pyr^1) Apelin 13 (2 mg/kg/day) or saline was infused in TGA-PE rats via osmotic minipumps starting at day 13 of gestation (GD). At GD20, TGA-PE rats had increased blood pressure, proteinuria, lower maternal and pup weights, lower pup number, kidney injury, and enlarged heart compared to a control group (pregnant Sprague-Dawley rats administered vehicle). (Pyr^1) Apelin 13 didnot

affect maternal or fetal weights in TGA-PE. The study results showed that the administration of (Pyr^1) Apelin 13 reduced blood pressure, and normalized heart rate variability and baroreflex sensitivity in TGA-PE rats compared to controls. (Pyr^1) Apelin 13 increased ejection fraction in TGA-PE rats. (Pyr^1) Apelin 13 normalized proteinuria in association with lower renal cortical collagen deposition, improved renal pathology and lower immunostaining of oxidative stress markers 4-hydroxynonenal (4-HNE) and NADPH oxidase 4 (NOX-4) in TGA-PE. The study suggests that improved hemodynamic responses and renal injury without fetal toxicity following Apelin administration suggesting a role for Apelin in the regulation of maternal outcomes in preeclampsia [111].

Even though extrapolation of the similar strategy to the patient volunteers using recombinant Apelin is challenging in the management of disease needs to be established.

To assess the endothelial dysfunction, we measured the maternal serum eNOS and NO concentrations between study groups. In the present study, concentrations of eNOS and NO were significantly reduced in preeclampsia compared with normotensive pregnants. Furthermore, serum eNOS levels were reduced significantly in severe preeclampsia than mild preeclampsia. However, serum nitric oxide levels were also reduced in severe preeclampsia, but not reached statistical significance. The eNOS and NO concentrations were significantly negatively correlated with blood pressure. The decreased levels of eNOS may contribute to low levels of nitric oxide synthesis inpreeclampsia.

It has been reported that eNOS in the mother and in the fetus contribute uteroplacental vascular changes and increased uterine arterial blood flow, whereas preeclampsia is associated with abnormal placentation and abnormalities in the eNOS/NO pathway, which may result in vasoconstriction. Hence, the results of the study justify the similar observation as described here in other research reports [82].

A Korean case-control study between preeclampsia and healthy pregnant women focused to measure L-arginine, Asymmetric dimethylarginine (ADMA), Glu298Asp eNOS gene polymorphism and placental eNOS expression. The study results showed that reduced L-arginine and unchanged ADAM in preeclamptic cases and no association between Glu298Asp eNOS gene polymorphism and preeclampsia. Significant downregulation of placental eNOS expression in preeclamptic syncytiotrophoblasts compared with healthy pregnant women, suggesting that low L-arginine rather than elevated ADMA and reduced placental eNOS expression might constitute a characteristic finding in preeclamptic placenta [177].

Another prospective observational study in Poland between hypertensive disorders of pregnancy and healthy pregnant women in 3rd trimester of pregnancy, measured the concentrations of angiogenic factors and metabolic status. The study results showed that significantly decreased levels of maternal serum endothelial nitric oxide synthase, placental growth factor and increased levels of vascular endothelial growth factor in severe preeclampsia. Unchanged angiotensin-converting enzyme levels in preeclampsia. Endothelial

nitric oxide synthase and placental growth factor were positively correlated in preeclampsia cases. A weak correlation was observed between maternal body mass index and vascular endothelial growth factor. The study findings suggest that hypertensive disorders of pregnancy are associated with different pattern of alterations in concentrations of angiogenic factors [83].

Similarly, a cross-sectional study in India between healthy pregnant women and hypertensive disorders of pregnancy focused to study the expression of vascular endothelial growth factor and endothelial nitric oxide synthase in fetal end of umbilical cord. The study results showed that reduced expression of vascular endothelial growth factor and endothelial nitric oxide synthase in pregnancy hypertensive diseases than healthy pregnants. Severe preeclampsia showed high intensity of staining than mild preeclampsia and gestational hypertension. This highlights the reduced expression in pregnancy hypertension may cause hypoperfusion and subsequent hypoxia and their involvement in pathophysiology of preeclampsia [178].

In addition, a case-control study in Egypt between preeclampsia and healthy pregnants focused to examine the expression of endothelial nitric oxide synthase and hypoxia inducible factor 1 α. The study results showed that significant reduction in immunoreactivity of eNOS and increased hypoxia inducible factor 1 α expression, collagen fibers in preeclampsia than healthy pregnant women, suggesting compromised vasodilation and elevation of hypoxia inducible factor 1 α may be compensatory mechanism under hypoxic conditions [179].

In a United Kingdom cross-sectional study reported that *ex vivo*-derived syncytiotrobhoblast extracellular microvesicles (STBMV) and syncytiotrobhoblast extracellular exosomes (STBEX) isolated from placental perfused lobes to have less eNOS activity in preeclampsia compared with controls. Similarly, *in vivo*-derived plasma STBMV analyzed by flow cytometry showed less STBMV bound eNOS expression in preeclampsia compared with normal pregnancy. This may contribute to the decreased levels of nitric oxide in preeclampsia, which may affect the vascular functions [114].

In an Egypt cross-sectional study between non-pregnant, healthy pregnant and preeclampsia focused to determine the serum levels of tumor necrosis factor-α, C-reactive protein, and gene expression of vascular endothelial growth factor, endothelial nitric oxide synthase and p53. The study results showed that increased levels of tumor necrosis factor-α, C-reactive protein in preeclampsia and the placental expression of endothelial nitric oxide synthase, vascular endothelial growth factor and p53 were downregulated in preeclampsia, suggesting their possible involvement in compromised vasodilation and increased inflammatory response in preeclampsia [180].

Yet another case-control study in Pakistan between preeclampsia and healthy pregnant women at their 3rd trimester of pregnancy focused to measure the levels of oxidative stress markers such as malondialdehyde, superoxidase dismutase, catalase, guaiacol peroxidase and endothelial nitric oxide synthase expression. The study results showed that significantly reduced endothelial nitric oxide synthase expression and increased oxidative stress in preeclampsia

compared to healthy pregnant women, suggesting their role in promoting microvascular oxidative damage and favoring the abnormal placental perfusion, probably by contributing less placental blood flow and high vascular resistance to feto-maternal circulation [116].

In contrast to the above findings, a few studies have reported that increase endothelial nitric oxide synthase levels in preeclampsia than healthy pregnant women as described here [84,85,181,182].

Schiessl *et al*. reported significantly higher eNOS expression in preeclamptic placentas compared with healthy pregnant women [84]. Marzena Laskowska *et al*. reported that unchanged eNOS levels in preeclampsia than healthy pregnant women group [85]. Nasiell J *et al*. observed that the placental mRNA expression of eNOS was significantly increased in pregnancies complicated by preeclampsia, small for gestational age and both preeclampsia and small for gestational age compared with normal pregnancies [181]. K. Smith-Jackson *et al*. reported that increased placental expression of endothelial nitric oxide synthase in preeclampsia, suggesting this may be adaptive response in preeclampsia [182].

Nitric oxide is a vasodilator, produced by endothelial nitric oxide synthase in vascular endothelium. In the present study, maternal serum nitric oxide levels were reduced based on severity of preeclampsia. The decreased nitric oxide levels in preeclampsia may be due its low production or increased breakdown[67].

In the vascular endothelium, nitric oxide causes vasodilation. Therefore, its deficiency may be linked with pathogenesis of preeclampsia. Also, decreased concentrations of eNOS resulting in low nitric oxide production, which may contribute to vascular endothelial dysfunction, vasoconstriction and hypertension in preeclampsia [121].

In support of this study, a few studies reported that maternal serum nitric oxide levels were significantly decreased in preeclamptic women compared with healthy pregnant women [76,89,126,183,185,186].

In contrary to the above, a few studies reported that serum nitric oxide levels were significantly increased in preeclampsia [119,187-190].

Statistical analysis showed positive correlation of Apelin 13 with eNOS and NO. Linear regression analysis also showed significant relationship of Apelin 13 with eNOS and NO. This observation suggests that Apelin13/eNOS/NO pathway involvement in the endothelial dysfunction in preeclampsia. Our study results show that abnormal placentation and endothelial dysfunction in preeclampsia may be indicated by reduced Apelin 13, eNOS and NO under prevailing oxidative stress condition in preeclampsia.

Adverse maternal and fetal outcomes were significantly higher in preeclamptic women compared to normotensive pregnants. However, maternal serum Apelin 13 levels were not showed any association with adverse maternal/fetal outcomes.

Preeclampsia is complex genetic disorder. Many candidate genes were studied in relation to preeclampsia. Even though, there is a need to screen the genes and their products involved in the onset of preeclampsia and also to know their biological roles. Therefore, understanding the structure of gene and its expression is important to address etiopathogenesis linked to genetic causes. The involvement of many genes such as methylene tetrahydrofolate, factor V Leiden, angiotensinogen, eNOS and angiotensin converting enzyme etc. were studied and are known to be associated with preeclampsia [2].

However, Apelin (*APLN*) gene is one such gene and its biologically active product Apelin 13, almost untouched area in relation preeclampsia. Currently, there is a paucity of information on this gene polymorphism in preeclampsia. However, a few studies reported this gene polymorphism in hypertension and cardiovascular diseases [52,191-193].

The study results with respect to genetic analysis of Apelin gene exhibited APLN -1860T>C (rs56204867) polymorphism with concomitant circulating low level of Apelin 13 peptide precisely more in preeclamptic women than normal pregnant women. However, in support of this observation, blood pressure also altered proportionately. Li WW *et al*. first reported at the promoter region of *APLN* gene -1860T>C (rs56204867) polymorphism in the Han Chinese hypertensive population [56]. Apelin -1860T>C is documented in cardiovascular diseases with increased blood pressure and vascular complications [52].

However, similar observation needs to be screened with respect to preeclampsia. It has been shown that the Apelin -1860T>C gene polymorphism and plasma Apelin concentration are related. The possible Apelin genotype and allele frequencies of *APLN* gene was different between study groups ($\chi 2$ = 11.69; df = 2; p=0.0028 and $\chi 2$ = 14.27; df = 1; p= 0.00013, respectively). CC genotype and C allele of APLN -1860 T>C site was significantly higher in preeclampsia compared to normotensive pregnants. Thereby study results of -1860T>C at promoter region and low level of Apelin apparently projects the possible onset of cardiac problems in preeclamptic women after delivery.

There is a paucity of information on Apelin gene polymorphism in preeclampsia in Indian population. Therefore, there is a scope to study Apelin gene polymorphism in preeclampsia and in healthy pregnant women and also to explore functional role of Apelin 13 in early placentation, angiogenesis process has creates a scope for further research.

CHAPTER-7

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APPENDIX I

RESEARCH PUBLICATIONS FROM THE Ph.D. TOPIC

	Title	Journal	Indexation
1.	Apelin and its Receptor: An Overview	Journal of Clinical & Diagnostic Research 2019;13(6): BE01-BE06	Web of Science IF: 0.8
2.	Maternal serum biomarkers in early diagnosis of preeclampsia	Journal of Clinical and Biomedical Sciences. 2019; 9(1): 03-09	Index Copernicus
3.	Neutrophil and Platelet to Lymphocyte Ratio in Prevailing the Oxidative Stress and Its Relation with the Endothelial Dysfunction in Preeclampsia	Journal of Krishna Institute of Medical Sciences University. 2019;8:(4):89-97	Scopus Web of Science UGC Approved
4.	Impact of Oxidative Stress on Maternal Serum Apelin 13 and Endothelial Nitric Oxide Synthase in Preeclampsia	Biomedical & Pharmacology Journal. 2020;13(4): 2041-2048	Scopus UGC Approved
5.	Apelin 13 and Blood Pressure, Is there any Association in Pre-eclampsia? - A Case-control Study	Journal of Clinical & Diagnostic Research. 2021;15(1): BC01-BC04	Web of Science IF: 0.8
6.	Maternal serum Apelin 13 and APLN gene promoter variant -1860T > C in preeclampsia	The Journal of Maternal-Fetal & Neonatal Medicine. 2021; 1-9.	Scopus PubMed Web of Science IF: 1.7
7.	Evaluation of endothelial nitric oxide synthase and nitric oxide levels in preeclamptic and normotensive pregnant women	Journal of Krishna Institute of Medical Sciences University (Under Review)	Scopus Web of Science UGC Approved

IF: Impact Factor

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Review Article

Biochemistry Section

Apelin and its Receptor: An Overview

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ABSTRACT

Apelin protein is an endogenous ligand of Apelin Receptor (APJ). APJ is a member of G-protein coupled receptor family. Both apelin and its receptor express extensively in the human body. Apelin receptor activation occurs by its cognate peptide ligand, apelin and many physiological effects, including vasodilation, vasoconstriction, angiogenesis, fluid homeostasis, neuroendocrine response to stress and energy metabolism. Apelin derived from its precursor might yield a number of bioactive peptides. Apelin is synthesised as an immature single peptide (preproapelin) which consists of 77 amino acids. In the endoplasmic reticulum, preproapelin is cleaved by endopeptidases to a 55 amino acid proapelin and subsequently, to various biologically active apelin-36, apelin-17 and apelin-13 isoforms. Post-translation, the apelin containing the pyroglutamate group at N-terminus of the peptide is modified to pyroglutamate apelin 13. In adipocytes, proprotein convertase subtilisin/kexin 3 directly cleaves the proapelin to apelin 13 and does not produce any longer isoforms. In contrast, Angiotensin Converting Enzyme 2- (ACE-2) cleaves at proline-phenylalanine site at C-terminus and renders apelin 13 and apelin 36 inactive. To date, ACE-2 is the only known enzyme for apelin degradation. The C-terminal region is responsible for receptor binding and subsequent activation. Prior research suggests the role of apelin and its receptor in pathogenesis of various conditions including preeclampsia, hypertension, cardiovascular diseases, diabetes mellitus, obesity and cancer. Despite its established importance and link to therapeutic target, the precise role of this apelin/APJ remain obscure. In this attempt, we summarised the structure, chemistry, biosynthesis, expression and gene regulation, distribution, receptor binding mechanism, biological functions and therapeutic applications along with the associated recent advances.

Keywords: Angiogenesis, Angiotensin converting enzyme-2, Apelin receptor, G-protein coupled receptor, Vasodilator

HISTORY AND DISCOVERY

Apelin Receptor Structure and Properties

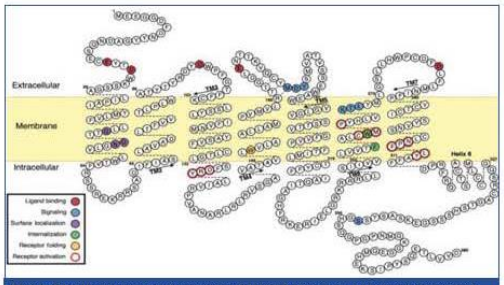
In 1993, O'Dowd BF et al., discovered a type of receptor protein and named it Apelin Receptor (APJ/AR) [1]. They characterised a 700 base pair sequence of the Polymerase Chain Reaction (PCR) fragment, which encoded a protein of 380 amino acids protein. The APJ gene (Gene symbol *APLNR*) is located on chromosome 11q12 and does not contain intron. APJ receptor resembles Angiotensin Type 1 receptor (AT1). APJ and AT1 have structural homology of 115 amino acids (30%) and 86 amino acids in the transmembrane regions (54%) [2].

APJ receptor contains seven hydrophobic transmembrane regions characteristic of G-Protein Coupled Receptor (GPCR) family [2]. GPCR is activated by neuropeptides, polypeptide hormones and non-peptides such as biogenic amines, nucleotides, lipids and ions [3]. Some novel GPCRs do not have their endogenous natural ligands, and are termed as "orphan receptors". The cAMP-dependent protein kinase phosphorylates the APJ receptor at its consensus sites for palmitoylation and glycosylation.

The glycosylation of N-terminal GPCR, has been implicated in the expression, correct folding of nascent protein, stability and ligand binding of receptor [4]. Studies on the APJ receptor structure showed that amino acids in the N-terminal (e.g., Asp23 and Glu20) and C-terminal portions of the APJ receptor are required for internalisation [5,6] [Table/Fig-1].

Regulation of APJ (*APLNR*) Gene

The regulation of APJ gene expression has not been extensively studied till date. At the transcriptional level, the highest, rat APJ gene promoter activity is found between -966 and -165 bp. Electrophoretic mobility shift, super-shift and competitive immunoassays have indicated that promoter is under complex regulation by specificity protein 1 (Sp1), oestrogen receptor, glucocorticoid receptor and CCAAT enhancer binding protein γ (C/EBP-γ or CEBPG) transcription



[Table/Fig-1]: Human apelin receptor (APJ/AR) structure, with seven delineated transmembrane (TM) helices [6].

factors, with Sp1 as a major regulator of rat APJ gene promoter activity [7]. Various studies on Single Nucleotide Polymorphisms (SNPs) of *APLNR* gene and reported an association between SNP (rs9943582 (G/A) in 5' flanking region of Sp1 binding site of *APLNR* gene with susceptibility to brain infarction [8,9]. Two *APLNR* gene polymorphisms, in Han Chinese population, rs7119375 (G/A) and rs10501367 (G/A), in Italian patients, rsG212A, suggests association of SNPs with hypertension [10]. In animal model, up-regulation of APJ gene, in response to acute and repeated stress is observed and these changes are likely to be dependent on glucocorticoids [11]. The endogenous ligand, apelin also regulates APJ expression within the Gastrointestinal (GI) tract and up-regulation of APJ expression was observed in animal adipose tissue by insulin [12].

APELIN STRUCTURE AND PROPERTIES

The term apelin denotes APJ endogenous ligand. APJ receptor remained as "orphan receptor" until 1998, when Tatemoto K et al., identified this ligand and termed it as apelin [2]. In humans, the *APLN* gene is located on chromosome X at Xq25-26.1 position; a 6 kb open reading frame is an apelin gene (*APLN*) with one intron.

Journal of Clinical and Biomedical Sciences

Review Article

Maternal serum biomarkers in early diagnosis of preeclampsia

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Abstract

Background: Preeclampsia (PE) is a pregnancy specific disorder, characterized by new onset of hypertension and proteinuria after 20 weeks of gestation. It is one of the leading causes of maternal and perinatal morbidity and mortality. The etiology of the disease process in not known. There is an urgent need for a 1st trimester marker for the prediction of preeclampsia. Recent studies have reported that this disease originate from abnormal placentation and maternal endothelial dysfunction. The intense research in this arena has unveiled some important serum biomarkers which play an important role in placentation. These markers include angiogenic and antiangiogenic molecules. However, these markers when they used alone are not effective for the prediction of preeclampsia, but in combination may help in predicting women who are likely to develop preeclampsia. This review summarizes the various maternal serum biomarkers available and utility in predicting preeclampsia.

Keywords: Preeclampsia, Biomarkers, Angiogenic markers, Antiangiogenic markers, Apelin

Introduction:

Preeclampsia [PE] is a pregnancy specific disorder characterized by new onset of hypertension and proteinuria after 20 weeks of gestation. [1] Globally, PE accounts for 3-5% of pregnancies and is the leading cause for maternal and perinatal morbidity and mortality. [2] In India, preeclampsia and eclampsia accounts for 24% of maternal deaths and neonatal mortality rate is approximately 43 per 1000 live births. [1] PE, a condition prior to eclampsia (Greek word "eklampsis" meaning sudden flashing), is a systemic syndrome, clearly shows the involvement of uteroplacental blood flow, vascular resistance, endothelial dysfunction, coagulation system. [1,3] The risk factors of PE include family history of hypertension, first pregnancy, chronic hypertension, diabetes mellitus, kidney disease, syndrome X, hypercoagulable state, maternal age, prolonged intervals between pregnancies, etc. [4] Symptoms of PE ranges from mild to severe. They include persistent headache, blurred vision, vomiting and abdominal pain. The complications include intrauterine growth restriction (IUGR), preterm delivery, maternal and fetal morbidity and mortality. [3]

Preeclampsia occurring at <34 weeks of gestation termed as 'early onset preeclampsia' and after 34 weeks of gestation termed as 'late onset preeclampsia'. However, in both conditions endothelial dysfunction is common and is responsible for hypertension and proteinuria. [1] Preeclampsia, occurs in two stages. Reduced placental perfusion, abnormal placentation, with improper trophoblast invasion and inadequate uterine spiral arteries remodeling occurs in stage one. Maternal inflammation, metabolic and thrombotic responses that converge to alter vascular function, results in multiorgan damage occurs in stage two. [5]

Pathophysiology of preeclampsia:

The exact mechanism of pathophysiology of preeclampsia is unknown. However, this disease involves multiple organ systems. [6] Placenta plays an important role in pathophysiology of preeclampsia. Many studies reported that, preeclampsia is mainly due to abnormal placentation rather than foetus, it occurs only in the presence of placenta and remits after delivery. [1] In early pregnancy, maternal spiral artery remodeling starts directly after implantation of blastocyst with invasion of extravillous trophoblast

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ORIGINAL ARTICLE

Neutrophil and Platelet to Lymphocyte Ratio in Prevailing the Oxidative Stress and Its Relation with the Endothelial Dysfunction in Preeclampsia

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Abstract:

Background: Preeclampsia (PE) is a pregnancy specific, hypertensive disorder. It affects 2-8% pregnancies. Oxidative stress and systemic inflammation are proposed to contribute significantly to the preeclampsia pathophysiology. The present study, aim is to determine and compare the markers of oxidative stress, endothelial dysfunction, systemic inflammatory markers Neutrophil/Lymphocyte Ratio (NLR) and Platelet/ Lymphocyte Ratio (PLR) in preeclampsia and gestational age matched healthy controls. *Material and Methods:* This study was conducted in the Department of Biochemistry and Department of Obstetrics and Gynecology, Sri Devaraj Urs Medical College, Kolar, Karnataka. The study included 98 preeclamptic women and 98 normotensive pregnant women. Five ml venous blood was collected from all the study subjects. Blood sample in EDTA vials was used for the complete blood count. NLR and PLR were calculated. Plasma was used for Ferric Reducing Ability of Plasma (FRAP) assay. Serum was used for the estimation of Malondialdehyde (MDA), nitric oxide, blood sugar, renal parameters and liver enzymes i.e., Aspartate Transaminase (AST), Alanine Transaminase (ALT), Lactate Dehydrogenase (LDH) and magnesium. Corresponding urine samples were collected for urinary protein analysis by dipstick method. Fetal outcome was recorded. *Results:* Gestational age was significantly low in preeclamptic women as compared to those of controls. Blood pressure (Systolic and diastolic), mean arterial pressure, body mass index, pulse rate, serum creatinine, uric acid, AST, ALT, LDH, MDA and NLR were increased significantly in preeclamptic women as compared to those of controls. In subgroup analysis, NLR was increased significantly in severe preeclamptics as compared to mild preeclamptics. Serum Nitric Oxide (NO) and FRAP levels were decreased significantly in preeclamptic women as compared to those of controls. Significantly decreased birth weight was observed in babies born to preeclamptic mothers compared with controls. *Conclusion:* The present study results conclude that increased oxidative stress in terms increased MDA, decreased NO and reduced antioxidant status (FRAP) in preeclamptic women, results in adverse perinatal outcome. In addition, maternal NLR could be considered as a marker for severity of preeclampsia.

Keywords: Ferric Reducing Ability of Plasma, Fetal Outcome, Malondialdehyde, Nitric oxide

Introduction:

Preeclampsia (PE) is a pregnancy specific hypertensive (Blood pressure ⩾ 140/90 mmHg on two occasions, at least six hours apart) disorder associated with proteinuria (0.3g/day or a dipstick of 1+) after 20 weeks of gestation [1]. It affects 2 to 8% of all pregnancies [2]. The incidence of PE in India is around 8-10% [3]. Preeclampsia and eclampsia contributes significantly to morbidity and mortality of mothers (24%) and fetal morbidity and mortality rate is around 43/1000 live births [4]. The symptoms include persistent headache, blurred

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Impact of Oxidative Stress on Maternal Serum Apelin 13 and Endothelial Nitric Oxide Synthase in Preeclampsia

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Preeclampsia (PE) is the most common hypertensive disease of pregnancy, leads to maternal, perinatal morbidity and mortality, which accounts for 2-8% of pregnancies. Preeclampsia is characterized by new onset of hypertension and proteinuria after 20 weeks of gestation.The exact cause of preeclampsia is not clear. Aim of this study is to investigate the association between maternal serum apelin13, endothelial nitric oxide synthase, markers of oxidative stress in healthypregnant womenand preeclamptic women. This prospective studycomprises 140 pregnant womenconsists of 70 preeclamptic women treated as cases and 70 normotensive healthy pregnant women as controls. Five mL blood sample was collected, centrifuged to obtain serum/plasma and was stored at -80°C for further testing. Plasma was used for Ferric reducing antioxidant power (FRAP) assay and complete blood countwas done. Routine parameters like random blood sugar, renal profile, liver enzymes, lactate dehydrogenase (LDH), malondialdehyde (MDA), nitric oxide (NO), apelin 13 and endothelial nitric oxide synthase (eNOS)were also analyzed. Corresponding urine sample was tested for protein. Study results showed lower gestational age (36.99 ± 3.48 weeks) and demographic details such as elevated blood pressure [systolic (156.80 ± 13.71 mmHg), diastolic (101.97 ± 10.70 mmHg),and mean arterial pressure (120.88 ± 11.02 mmHg)], BMI (27.42 ± 3.80 kg/m^2) and pulse rate (87.68 ± 5.74 bpm) were observed in cases than controls. The biological markers namely serum MDA (18.57 ± 7.52µmoles/L) levels were significantly increased and nitric oxide (6.47 ± 1.22µmoles/L), FRAP (1292.10 ± 525.38 mmol/L), apelin 13(312.42 ± 189.00pg/ml) andeNOS(5.07 ± 2.30 ng/ml)levels were significantly decreased in cases.Mean arterial pressure was negatively correlatedwithApelin 13 (r = -0.179), NO (r = -0.065), FRAP (r = -0.169), and birth weight (r = -0.281) and eNOS (r = 0.013), MDA (r = 0.022) were positively correlated with mean arterial pressure. The study concludesthat reduced levels of apelin 13, eNOS, FRAP,NO and high oxidative stress, contribute to pathogenesis of preeclampsia and adverse perinatal outcome. It also demandssufficient evidence for the functional role of apelin 13 as a target in hypertension regulation.

Keywords: Angiogenesis, Hypertension, Malondialdehyde, Nitric Oxide.

Preeclampsia (PE) is apregnancy specific complication and it causes maternal, perinatal morbidity and mortality, which accounts for 2-8% of pregnancies [1,2]. The incidence of preeclampsia in India varies from 5% to 10%[3].

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Original Article

Biochemistry Section

Apelin 13 and Blood Pressure, Is there any Association in Pre-eclampsia? - A Case-control Study

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ABSTRACT

Introduction: Pre-eclampsia is a pregnancy specific disorder, characterised by the onset of hypertension and proteinuria. Pre-eclampsia is the leading cause of maternal, perinatal morbidity and mortality. The exact cause of pre-eclampsia is not known clearly and needs to be explored.

Aim: To evaluate the maternal serum apelin 13 levels among pre-eclampsia and healthy pregnant women and also, to find the association between apelin 13 and blood pressure.

Materials and Methods: A case-control study was conducted between Department of Biochemistry and Department of Obstetrics and Gynaecology, RL Jalappa Hospital and Research Centre, Kolar, Karnataka, India. After approval from the Institutional Ethics Committee and written informed consent from study subjects, a total of 270 pregnant women were recruited for this study. Among them, 135 pre-eclamptic women were considered as cases and 135 normotensive healthy pregnant women served as controls. According to the pre-eclampsia severity, cases were grouped into mild (n=47) and severe pre-eclampsia (n=88). Blood samples were collected from all the study subjects and was analysed for apelin 13 by Enzyme Linked Immunosorbent Assay (ELISA) method. Maternal and foetal adverse outcomes were recorded. Results were expressed as mean±Standard Deviation (SD). Categorical variables were expressed in percentages. Spearman's correlation was applied and $p<0.05$ was considered significant.

Results: The mean gestational age was 36.66±3.69 weeks which was, significantly low in pre-eclamptic women compared with healthy pregnant women. BMI (26.94±3.81 kg/m^2), systolic (157.82±15.14 mmHg), diastolic (101.68±11.02 mmHg) and Mean Arterial Pressure (MAP) (120.20±11.12 mmHg), pulse rate (88.14±5.82 bpm), Aspartate Transaminase (AST) (25.25±12.49 IU/L) and Alanine Transaminase (ALT) (19.01±10.95 IU/L) were significantly increased in pre-eclamptic women when compared with control group. Mean maternal serum apelin 13 (341.44±218.63 pg/mL) concentrations were significantly lower in pre-eclampsia compared with healthy pregnant women. Maternal serum apelin 13 concentrations were negatively correlated with Systolic Blood Pressure (SBP) (r = -0.196), Diastolic Blood Pressure (DBP) (r = -0.172) and MAP (r =-0.204). Adverse maternal outcomes such as epigastric pain 75 (55.55%), oedema 62 (45.92%) and persistent headache 35 (25.92%) were higher in pre-eclamptic group. Additionally, adverse foetal outcomes were more in pre-eclamptic cases including significantly decreased birth weight (2.40±0.65), babies requiring Neonatal Intensive Care Unit (NICU) admission were 54 (40%), preterm birth (≤37 wks) in 50 (37.03%), Respiratory Distress Syndrome (RDS) 31 (22.96%), Small for Gestational Age (SGA) in 4 (2.96%) and Intra Uterine Death (IUD) in 11 (8.14%) babies.

Conclusion: It was concluded from the present study that there was low maternal serum apelin 13 concentrations in pre-eclampsia and had negative correlation with blood pressure, suggesting its potential role in the pathophysiology of pre-eclampsia.

Keywords: Angiogenesis, Endothelial dysfunction, Foetal outcome, Vasodilation

INTRODUCTION

Pre-eclampsia is a pregnancy disorder, characterised by new onset hypertension with or without proteinuria. It is characterised by blood pressure ≥140/90 mmHg and proteinuria (0.3g/day) after 20 weeks of gestation. In 2019, it has been reported that pre-eclampsia affects 2-8% of pregnancies worldwide [1]. Whereas in India, the incidence of pregnancy induced hypertension is 10.3% [2].

Pre-eclampsia is the leading cause of maternal, perinatal morbidity and mortality [3]. The major risk factors of pre-eclampsia include chronic hypertension, prior pre-eclampsia, cardiovascular disease, renal disease, diabetes mellitus, multiple gestations, advanced maternal age (>40 years) and obesity [4,5].

The precise mechanism of pre-eclampsia origin is not clear. The pathophysiology of pre-eclampsia is characterised by abnormal placentation, shallow trophoblast invasion, remodeling of spiral arteries and also maternal systemic inflammation, metabolic and thrombotic responses, links to altered vascular function, results in multi-organ damage [1,6,7]. Further, which is responsible for endothelial dysfunction and vascular inflammatory response, which causes disturbance in the haemodynamic changes necessary for maternal adaptation to pregnancy [8].

Apelin is a bioactive peptide, synthesised from preproapelin (77 amino acids) nascent single peptide, with hydrophobic rich N-terminal region. Further, preproapelin in the endoplasmic reticulum cleaves to generate 55 amino acid proapelin, containing receptor binding sites. Proapelin generates several biologically active short peptides. The short peptides include apelin 36, apelin 17, apelin 13 and Pyroglutamate apelin 13 (Pyr1-apelin 13) [9]. All these apelin peptides exhibit agonistic activity on the apelin receptor (APJ). However, apelin 13 being the most active peptide responsible for the biological activity of apelin [5]. The shorter peptides are more potent activators of APJ. The activation of apelin peptides by APJ promotes vasodilation, through Nitric Oxide (NO) pathway and the APJ is being targeted to treat heart failure and hypertension [10].

Studies have shown that apelin and its APJ receptor are expressed in endothelial cells, adipose tissue, heart and syncytiotrophoblast cells of placenta [9,11]. Apelin is an angiogenic factor in endothelial cells, stimulates vessel growth and endothelial cell proliferation [11,12]. Further, apelinergic system has been previously shown to be involved in the regulation of vascular bore size and integrity [13,14]. Even though, the role of apelin peptides in pre-eclampsia is not clear, but studies have reported conflicting results on apelin peptides [10,15,16].

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ORIGINAL ARTICLE

Maternal serum Apelin 13 and *APLN* gene promoter variant -1860T > C in preeclampsia

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ABSTRACT

Objective: To evaluate the apelin (*APLN*) −1860 T > C (rs56204867) polymorphism and maternal serum apelin 13 levels in preeclampsia and its association with blood pressure.

Methods: This case-control study was conducted in department of Biochemistry, Sri Devaraj Urs Medical College, Karnataka, India. A total of 181 subjects were enrolled in the study from department of Department of Obstetrics and Gynecology. The recruited women were grouped as: Group–I ($n = 91$) cases with preeclampsia and Group–II ($n = 90$) normotensive healthy pregnant women as controls. Under aseptic conditions, the collected 5 mL blood was distributed for serum separation (3 mL) and genetic analysis (2 mL). Serum was stored at −80 °C after centrifugation at 3000 rpm for 10 min. The collected five mL urine sample was used for urinary protein analysis by dipstick method. The *APLN* gene −1860 T > C polymorphism and Apelin 13 levels were analyzed by molecular methods and ELISA technique respectively. Birth weight and demographic details were recorded.

Results: In the present study, no significant difference was observed for mean gestational age and maternal age. Systolic (158.7 ± 14.0 mmHg) and diastolic (104.9 ± 10.7 mmHg) blood pressure, and mean arterial pressure (MAP) (123.0 ± 11.1 mmHg) (*p*-value .001) were significantly increased in preeclamptic women compared with healthy pregnant women. Birth weight (2.4 ± 0.5 kg) (*p*-value .001) was significantly decreased in babies born to preeclamptic mothers. Birth weights were also expressed in centiles, according to Fenton Chart. Number of small for gestational age (SGA) babies were more in preeclampsia ($n = 55$) than healthy pregnant women ($n = 28$). Mean maternal serum apelin 13 (239.4 ± 126.3 pg/mL) (*p*-value .001) concentrations were significantly lower in preeclampsia compared with healthy controls. Maternal serum apelin 13 concentration in preeclampsia was negatively correlated with systolic blood pressure ($r = -0.235$), diastolic blood pressure ($r = -0.172$) and mean arterial pressure ($r = -0.206$). However, maternal serum apelin 13 levels showed insignificant positive correlation with age, gestational age and birth weight. The genotype and allele frequencies of *APLN* gene were found significant between study groups as in preeclampsia ($\chi^2 = 11.69$; df = 2; $p = .0028$ and $\chi 2 = 14.27$; df = 1; $p = .00013$ respectively). CC genotype and C allele of *APLN* − 1860 T > C site was high in preeclampsia.

Conclusion: Study concludes that preeclamptic women have low level of serum apelin 13 and −1860 T > C polymorphism at *APLN* gene promoter site with increased allelic frequency of CC genotype and C allele compared to normotensive pregnant women. And this evidence may link to cardiac complications in preeclamptic women after delivery in later stage.

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KEYWORDS
Angiotensin; Apelin -1860T > C polymorphism; Apelin 13; Preeclampsia; Vasoconstriction

Introduction

Preeclampsia, a potentially dangerous pregnancy disease, characterized by new onset of hypertension and proteinuria after twenty weeks of gestation. Globally, the incidence of preeclampsia is around 5–8% and India alone accounts for about 10.3% of pregnancies [1,2]. Preeclampsia is the leading cause of maternal, perinatal morbidity and mortality. It also causes preterm birth and intrauterine growth restriction [3]. The symptoms include persistent headache, blurred vision, epigastric pain, vomiting and edema [4]. This multisystem disorder has the possible underlying pathophysiology such as abnormal placentation with improper trophoblast invasion, shallow remodeling of spiral arteries and also maternal systemic inflammation, oxidative stress and metabolic changes links to

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ORIGINAL ARTICLE

Evaluation of Endothelial Nitric Oxide Synthase and Nitric Oxide Levels in Preeclamptic and Normotensive Pregnant Women

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Abstract:

Background: Preeclampsia is a pregnancy specific disease, causes maternal, fetal morbidity and mortality. The exact cause of preeclampsia is not clearly known. However, endothelial dysfunction contributes significantly to the preeclampsia pathophysiology. The aim of the present study is to measure maternal serum endothelial Nitric Oxide Synthase (eNOS) and Nitric Oxide (NO) concentrations in preeclamptic and normotensive pregnant women and their correlation with blood pressure. *Materials and methods:* Prospective case-control study conducted in Department of Biochemistry in collaboration with Department of Obstetrics and Gynecology of RL Jalappa Hospital and Research Centre, Sri Devaraj Urs Medical College, Karnataka, India. Normotensive (n=120) and preeclamptic (n=120) women were included in this study. Based on preeclampsia severity, subjects were divided into mild (n=44) and severe preeclampsia (n=76). Blood samples collected from the study subjects allowed to obtain clear serum, stored at -80°C and processed. EDTA blood used to measure hemoglobin and platelet count. Routine parameters like Random Blood Sugar (RBS), urea, creatinine, uric acid, Aspartate Transaminase (AST), Alanine Transaminase (ALT), NO and eNOS concentrations were estimated. Corresponding urine samples tested for protein by dipstick. *Results:* Low gestational age (36.89 ± 3.27 weeks) and increased Body Mass Index (BMI) (27.28 ± 3.85 kg/m^2) were recorded. Elevated blood pressure [systolic (157.3 ± 15.06 mmHg), diastolic (101.31 ± 10.67 mmHg), mean arterial pressure (120.08 ± 11.18 mmHg)], pulse rate (88.00 ± 5.65 bpm), serum uric acid (5.83 ± 1.86 mg/dL), AST (25.04 ± 11.61 IU/L), ALT (18.92 ± 10.16 IU/L) levels were observed in preeclampsia than normotensive pregnant women. eNOS (4.89 ± 2.18 ng/mL) and NO (5.93 ± 2.25µmoles/L) levels were decreased significantly in preeclampsia than normotensive pregnant women. eNOS levels were significantly different between mild (5.80 ± 2.15ng/mL) and severe preeclampsia (4.33 ± 2.03 ng/mL). eNOS has negative correlation with blood pressure [systolic (r= -0.229), diastolic (r= -0.178) and mean arterial pressure (r= -0.197)]. NO levels were negatively correlated with systolic (r= - 0.250), diastolic (r= -0.208) and mean arterial pressure (r = -0.229). *Conclusion:* Study concludes that reduced eNOS and NO, negative correlation with blood pressure in preeclampsia indicated altered endothelial function.

Keywords: Endothelial Dysfunction, Normotensive Pregnant Women, Preeclampsia, Vasoconstriction

POSTER PRESENTATIONS FROM THE Ph.D TOPIC

	TITLE	Conference details	Type of presentation
1.	Maternal serum endothelial nitric oxide synthase, oxidative stress and inflammatory markers in preeclampsia	The Impact of Human Genomics on Current Medical Practice and Health Care, Special Interest Group-Human Genomics and Rare Disorders in association INDO-UK GENOMIC EDUCATION FORUM, JSS Academy of Higher Education & Research, Mysuru, held on 31st October 2019	Poster Presentation
2.	Relationship between maternal serum Apelin 13, oxidative stress and intrauterine growth retardation in Preeclampsia	17th Annual Meeting of Society for Free Radical Research (SFRR)-INDIA-2020, February 12-15, 2020, Baba Atomic Research Centre (BARC), Mumbai.	Poster Presentation

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Special Interest Group – Human Genomics and Rare Disorders

In association with

INDO-UK GENOMIC EDUCATION FORUM

Theme: "The impact of Human Genomics on Current Medical Practice and Health Care"

Certificate

of Participation

This is to Certify that **Mr. Rajeev Gandham**

of Sri Devaraj Urs Medical College, Kolar has presented titled Maternal serum endothelial Nitric Oxide Synthase, oxidative stress and inflammatory markers in preeclampsia held on 31st October 2019 at JSS Hospital, Mysuru.

Dr. Suma M N
Organizing Chairman

Dr. H. Basavanagowdappa
Principal
JSS Medical College

17TH ANNUAL MEETING OF THE SOCIETY FOR FREE RADICAL RESEARCH - INDIA

& CONFERENCE ON "ROLE AND MANAGEMENT OF OXIDATIVE STRESS IN HUMAN DISEASE" (SFRR-INDIA-2020)

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CIRCULATING ANGIOGENIC, OXIDATIVE STRESS MARKERS AND THEIR POSSIBLE ASSOCIATION WITH ENDOTHELIAL FUNCTION IN NORMAL PREGNANT AND PREECLAMPTIC WOMEN

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DOCTOR OF PHILOSOPHY
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by

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CHAPTER-6

SUMMARY AND CONCLUSION

6. SUMMARY AND CONCLUSION

6.1. Summary

All the efforts have been made during the analysis of the research data and our research findings were summarized as below.

Primigravida and maternal age ≤ 25 years are at increased risk of developing preeclampsia. Even though low level of hemoglobin and platelets were observed in preeclampsia found that statistically non-significant. The inflammatory markers Neutrophil to Lymphocyte ratio (NLR) and Platelet to Lymphocyte ratio (PLR) are elevated in preeclampsia, Amongst, NLR was found to be significant. However, NLR and PLR were significantly elevated in severe preeclampsia compared to mild preeclampsia.

Malondialdehyde (MDA) is a marker to represent intensity of lipid peroxidation. The present study evidenced prominent rise of MDA level in preeclampsia cases than the normotensives with statistical significance. The antioxidants neutralize the deleterious effect of free radicals contributes to oxidative stress. The total antioxidant status in terms of FRAP was found to be reduced and statistically significant ($p<0.05$) in preeclampsia compared to normotensive pregnants.

Maternal serum Apelin 13 concentrations were determined in both preeclamptic cases and normotensive pregnants. Baseline Apelin 13 levels were recorded in non-pregnant women. The results showed that significantly reduced Apelin 13 levels in preeclampsia compared to normotensive pregnants

and had negative correlation with blood pressure. Because of its direct activating effect on L-arginine/eNOS/NO pathway, Apelin may restore this pathway and inhibition of oxidative stress may be involved in the ameliorative effect of Apelin on preeclampsia.

Maternal serum eNOS and nitric oxide levels were significantly reduced in preeclampsia and had negative correlation with blood pressure. This suggests that reduced eNOS and NO may reflect endothelial dysfunction in preeclampsia and may serve as a marker for endothelial dysfunction. Maternal serum Apelin 13 levels were positively correlated with eNOS and NO.

Adverse maternal and fetal outcomes were higher in preeclamptic women compared to normotensive pregnants. However, maternal serum Apelin 13 levels were not showed any association with adverse maternal/fetal outcomes.

Preeclamptic women have low level of serum Apelin 13 and -1860T>C (rs56204867) polymorphism at promoter site with increased allelic frequency of CC genotype and C allele compared to normotensive pregnants linked with rise in blood pressure and future cardiovascular risk in preeclamptic women after delivery.

6.2. Conclusion

This thesis work presents measurement of Apelin 13, MDA, total antioxidant status, eNOS and NO involved in angiogenesis, oxidative stress and endothelial function in normotensive pregnants and preeclamptic women. These markers indicate the abnormal placentation and endothelial dysfunction under oxidative stress conditions. This study findings provide relation to low Apelin 13 levels and preeclampsia, indicating compromised angiogenesis. Reduced eNOS and nitric oxide levels represents endothelial dysfunction. Adverse maternal and fetal outcomes were significantly higher in preeclamptic women compared to normotensive pregnants. However, maternal serum Apelin 13 levels were not associated with adverse maternal/fetal outcomes. Genetic analysis of Apelin gene -1860T>C polymorphism at promoter region is high in preeclamptic women. Furthermore, study can be extended by using large sample size to study other polymorphism in Apelin gene, Apelin expression and its functional role associated with regulation of blood pressure.

Our research findings generated new knowledge about:

- Increased oxidative stress and reduced antioxidant status were observed in preeclampsia.
- Increased inflammatory markers were observed in preeclampsia.
- Maternal serum Apelin 13 levels were decreased in preeclampsia, suggesting abnormal angiogenesis and compromised vasodilatory function.
- Recorded the baseline value of Apelin 13 in non-pregnant women.
- Maternal serum eNOS and NO levels were reduced in preeclampsia, suggesting endothelial dysfunction in preeclampsia.

- Adverse maternal/fetal outcomes were higher in preeclamptic women and the maternal serum Apelin 13 levels were not associated with these adverse outcomes.
- Reported the Apelin (*APLN*) gene -1860 T>C (rs56204867) polymorphism in Indian preeclamptic women.
- CC genotype and C allele of *APLN* -1860 T>C site was significantly higherin preeclampsia and low level of Apelin apparently projects the possible onset of cardiac problems in preeclamptic women after delivery.