PLASMINOLYTIC COMPONENTS AND THEIR RECEPTORS IN PATHOGENESIS OF PREECLAMPSIA

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I dedicate this work to my family and my teachers

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List of Abbreviations

APGAR- Appearance Pulse Grimace (reflex) Activity Respiration
ALX- Lipoxin receptor
ALXR- lipoxin A4 receptor
ANX1- Annexin A1
ANXA2- Annexin A2
BCA- Bicinchonic acid assay
BSA- Bovine Serum Albumin
cDNA- Complementary deoxyribonucleic acid
CRP- C-reactive protein
DAB- Diaminobenzidine chromogen
DC- Decidual cells
DEPC- Diethylpyrocarbonate
ECL - Enhanced chemiluminescence.
ECM- extracellular matrix
ECM- extracellular matrix
EGF- Epidermal growth factor
EGFR- Epidermal growth factor receptor
ELAM- Endothelial leukocyte adhesion molecule-1
ERBB- Erythroblastosis oncogene B
ET-1- Endothelin-1
EVT- extravillous cytotrophoblasts
EVTB- Extravilloustrophoblast
FDP- Fibrin degradation products

FPR2- formyl peptide receptor like-2
Gal-3- Galectin-3
GAPDH- Glyceraldehyde 3-phosphate dehydrogenase
G6PDH- Glucose-6-phosphate dehydrogenase
HELLP- Hemolysis elevated liver enzymes, and low platelets
HIF-Hypoxia- inducible factor
IHC- Immunohistochemistry
IUGR-intrauterine growth restriction
IRS- Immunoreactive score
IL-6- Interleukin 6
JAK-STAT- Janus kinase–signal transducer and activator of transcription
MAPKAPK- Mitogen-activated protein kinase-activated protein kinase 2
MMPs- Matrix metalloproteinases
mRNA- Messenger RNA
NADPH- Nicotinamide adenine dinucleotide phosphate
NCBI- National Center for Biotechnology Information
NK- Natural Killer
PAI-1- Plasminogen activator inhibitor-1 receptor.
PBMC- Peripheral blood mononuclear cells
PE- Preeclampsia
PlGF- Placental growth factor
PI3K- Phosphoinositide 3-kinase
PKCβ- Protein kinase C beta
RAS-RAF-MEK-ERK- Rat sarcoma–rapidly accelerated fibrosarcoma-

mitogen-activated protein kinase kinase- extracellular signal-regulated kinases
RNA- Ribonucleic acid
ROS- Reactive oxygen species
RT- Reverse transcription
SDS-PAGE- Sodium dodecyl sulphate–polyacrylamide gel electrophoresis
sENG- Soluble endoglin
sFLT1- fms-like tyrosine kinase-1
STB- Placental syncytiotrophoblas
TNF-α- Tumor necrosis factor-alpha
tPA- Tissue plasminogen activator
u-PA- Urokinase-type plasminogen activator
UPBF- Uteroplacental blood flow
VEGF- Vascular endothelial growth factor
VTB - Villous trophoblast

Abstract

Plasminolytic components and their receptors in pathogenesis of preeclampsia

Background

Preeclampsia (PE) is a multisystemic pregnancy disorder affecting 2-8% of pregnancies and remains a major cause of maternal and fetal morbidity and mortality. Despite decades of research the underlying cause of preeclampsia is still not clear. The pathophysiology of preeclampsia is complex wherein the placenta plays a central role. The primary pathology appears to be at the maternal fetal interface and is characterized by poor trophoblastic invasion of the uterus. Preeclampsia is associated with failure of endovascular invasion and spiral artery remodelling and plays the central role in pathogenesis of disease. VEGF, a well-known angiogenic factor produced by placental cells, plays a central role in placental pathogenesis of PE. Annexin A2 (ANXA2) is a profibrinolytic receptor required for plasminolysis, which is an important step in the formation of new blood vessel along with VEGF.ANXA2 increases tissue plasminogen activator (tPA) mediated plasmin generation and plasminogen activator inhibitor (PAI-1) inhibit the tPA.Preeclampsia is also associated with maternal, placental aggravated inflammatory response and generalized endothelial damage

AnnexinA1 (ANXA1) is glucocorticoid regulated protein regulates a wide range of cellular and molecular steps of the inflammatory response and is implicated in resolution of inflammation.Galectin-3(Gal-3), β-galcotoside-binding lectin participates in many functions, both intra- and extracellular. Recently it has been shown that galectin-3 modulates the inflammation. Role of ANXA1 and Gal-3 is poorly studied in context with human reproductive disease like PE.

Therefore, the present study examined the expression of above proteins which are involved in plasminolysis, angiogenesis and modulation of inflammation and their association in the placental bed of pregnancy with and without PE

Objectives

a. To evaluate the alterations in gross placental morphology of PE compared to placenta of normal women.
b. To demonstrate expression pattern ofANXA2, ANXA1, VEGF, tPA, PAI-1, EGFR and Gal-3 in placental tissue from women with and without Preeclampsia.
c. To evaluate the correlation between expression of these downstream plasminolytic proteins at the membrane of the placental cellular component to get insight into their possible relationship to placental angiogenesis and inflammation to verify whether it has some role in the development of Preeclampsia.

Material and methods

The study group comprised of placental tissues procured from gestations with PE (n = 40) and without (n = 30) PE. The expression of ANXA2, ANXA1, VEGF, tPA, PAI-1, EGFR and Gal-3 in the placental villous tissue was evaluated quantitatively by means of IHC, Western blotting and RT-PCR.

Results

Expression analysis illustrated that significant decrease in the expression of growth proteins VEGF, EGFR and profibrinolytic receptor ANXA2 in PE group and increase expression of tPA and PAI-1 compared with the normotensive control group. Expression of inflammation modulatory proteins ANXA1 and Gal-3 in PE group was more compared with the normotensive control group ($P < 0.05$)

Conclusion

Decreased expression of ANXA2 and VEGF with increased expression of PAI-1 is mainly responsible for altered angiogenic and fibrinolytic activity inPE. The increased expression of AnxA1 and Gal-3 in placental bed may be associated with a systemic inflammatory response in PE, suggesting role of above proteins in PE pathogenesis.

Keywords

Preeclampsia, VEGF, Annexin, Galectin-3, Tissue plasminogen activator, Plasminogen activator inhibitor, Epidermal growth factor receptor

Chapter 1

Introduction

Plasminolytic components and their receptors in pathogenesis of preeclampsia

1. Introduction

Preeclampsia (PE) is a multisystemic pregnancy disorder affecting 2-8% of pregnancies and remains a major cause of maternal and fetal morbidity and mortality.[1] Despite decades of research the underlying cause of PE is still not clear.[2] It is suggested that preeclampsia also increases the risk for cardiovascular diseases, stroke and neurological disorders in children in later life.[3]

The pathogenesis of PE is thought to involve three components: abnormal placentation, decreased blood flow to uterine artery leading to placental ischemia and dysfunction of endothelial cells (defective angiogenesis) leading to complications at the placental vascular level.[4] The primary pathology appears to be at the maternal fetal interface and is characterized by poor trophoblastic invasion of the uterus. The endovascular invasion of the spiral arteries is incomplete. Specifically, the failure of the cytotrophoblasts to penetrate deep appears to explain the relative reduction in uteroplacental blood flow, which is widely believed to lead placental hypoxia. Additional pathologic findings include placental infarcts.[5] During normal pregnancy; the placenta undergoes dramatic vascularization to enable circulation between fetus and mother. Placental vascularization involves vasculogenesis, angiogenesis and pseudovasculogenesis or maternal spiral artery remodeling.[6]

Extracellular proteolysis is an indispensable requirement for the formation of new blood vessels during neovascularisation.[7] The extracellular proteolytic system comprises serine proteases and their activators, containing tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA).[8] Plasmin, a central component of this system, is a broad-spectrum trypsin-like serine protease that

degrades several components of the extracellular matrix (ECM) -like laminin and fibrin, activates inactive pro- matrixmetalloproteinases (MMPs) and also increases the bioavailability of isoforms 165 and 189 of the angiogenic growth factor vascular endothelial growth factor (VEGF).[9]

ANXA2, a member of a family of Ca+2 –regulated phospholipid-binding proteins, is a cell surface co-receptor for tPA and plasminogen.[10] Catalytic efficiency of plasmin formation is increased by 60 fold when ANXA2 protein by binds to both plasminogen and tPA. This results in highly effective plasmin-facilitated proteolytic events which stimulates neovascularization by increasing the efficiency of endothelial cell invasion and degradation of ECM.[11]

Previous study has shown the involvement of ANXA2 in VEGF-induced neovascular responses and the role of ANXA2 as a cell-surface catalytic centre for the accelerated conversion of plasminogen into plasmin is directly connected in these processes.[12] VEGF is known to regulate many steps in the angiogenic process and is primarily involved in inducing extracellular proteolysis by up regulating the expression and activityof plasminogen activators.[13] VEGF and ANXA2 transcription are upregulated by hypoxia inducible factor (HIF). VEGF directly affects the expression of ANXA2, or a combination of VEGF and VEGFR2 induced the expression of ANXA2 on the surface of the cell membrane. VEGF and VEGFR2 also may be promoting ANXA2 expression by Protein kinase C (PKC) pathway. ANXA2 further influenced neovascularization.[12]

60% of the PA-inhibitory activity in the plasmais due to PAI-1 and is the main inhibitor of fibrinolysis compared with PAI-2 and PAI-3 during pregnancy.[14]

PE is also associated with maternal and placental aggravated inflammatory response and generalized endothelial damage.[15] AnnexinA1 (ANXA1) was predominantly delineated as a glucocorticoid-regulated protein having anti-phospholipase activity, but the protein also exhibits many other anti-inflammatory and pro-resolving properties, which primarily include profound inhibitory action on leucocyte transmigration and activation, leading to resolution of inflammation.[16] Gal-3, a member of galectin family, is a multifunctional protein which is involved in various biological processes including cell proliferation, apoptosis, and angiogenesis. Recently it has been shown that galectin-3 modulates the inflammation.[17] Though it might contribute to resolution of inflammation by clearing apoptotic neutrophils.[18] Considerable body of evidence illustrates that ANXA1 and Gal-3 participates in anti-inflammatory and pro-resolving function.

It is also observed that ANXA2–Gal-3 interaction at the membrane lattice is very critical in EGFR downstream signalling regulation, which has a major role in survival, growth.[19] Epidermal growth factor (EGF) is a 53-amino acid protein and is a ligand for the epidermal EGFR. Ligand binding activates a plethora of downstream signaling cascades involved in cellular proliferation, migration, and survival. [20] The EGF signalling system regulates trophoblast differentiation, and its alteration could contribute to perinatal disease. In one study it is reported that this pathway is altered in PE, a disorder associated with trophoblast apoptosis and failure to invade and remodel the uterine spiral arteries.[21]

VEGF, ANXA2, tPA, PAI-1, Annexin A1, Gal-3 and EGFR (epidermal growth factor receptor) are multifactorial proteins which play a role in cell proliferation, cell differentiation, apoptosis, inflammation, fibrinolysis and angiogenesis. Most of

these processes do occur during the normal development of the placenta. It is proved in various studies that there is molecular interaction between ANXA2 and Gal-3 with EGFR, ANXA1 with Gal-3, and tPA with ANXA2 and PAI-1 which are required for normal angiogenesis, fibrinolytic system and inflammation. But the biological significance of these interactions has not been well determined in normal pregnancy and also in relation to placental tissue of Preeclampsia.

Field literature considers PE is an angiogenic and exacerbated inflammatory disorder. We have focused our attention on angiogenesis and inflammatory response. Since in PE is a disorder of exacerbated inflammation and defective angiogenesis and fibrinolytic system, we hypothesized that there will be altered expression of VEGF, ANXA2, tPA, PAI-1, ANXA1, Gal-3, and EGFR in placental tissue of PE.

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Chapter 2

Aims and Objectives

Plasminolytic components and their receptors in pathogenesis of preeclampsia

2. Aims and Objectives

2.1: The following specific aims were proposed to accomplish our goal

a) To evaluate the alterations in placental morphology of Preeclampsia compared to placenta of normal women.

b) To demonstrate expression pattern of plasminolytic component proteins VEGF, ANXA2, tPA, PAI-1, ANXA1, Gal-3, and EGFR in placental tissue from women with and without Preeclampsia.

c) To evaluate the correlation between expression of these downstream plasminolytic proteins at the membrane of the placental cellular component to get insight into their possible relationship to placental angiogenesis and inflammation to verify whether it has some role in the development of PE.

Therefore, the purpose of present study is to add information pertaining to the expression of above proteins from placental tissue of women with and without Preeclampsia and thereby attempt to delineate the molecular mechanism of plasminolysis and evaluate the expression of these related proteins in the pathophysiology of preeclampsia in order to find out new targets for research into Preeclampsia prevention

2.2: Research hypothesis

Incidence of the PE globally is about 2 to 8%. High risk cases of PE may threaten the survival of the mother and newborn. Its etiology is poorly understood. As per Field literature, PE is an angiogenic and inflammatory disorder. For the placental development, there is a need of coordinated vascularization.

ANXA2 is a pro-fibrinolyticreceptor required for plasminolysis, which is important step in the formation of new blood vessel, it also increases the bioavailability of VEGF which is the most potent endothelial growth factor induces angiogenesis and endothelial cell proliferation and has a basic role in angiogenesis. ANXA2 is associated with other proteins like EGFR and Gal-3 at the membrane lattice are also involved in angiogenic pathway. ANX1 is glucocorticoid regulated protein regulates a wide range of cellular and molecular steps of the inflammatory response and is implicated in resolution of inflammation. Gal-3, β-galcotoside-binding lectinparticipates in many functions, both intra- and extracellularly. Recently it has been shown that Gal-3 modulates the inflammation along with its angiogenic role. Role of ANXA1 and Gal-3 is poorly studied in context with human reproductive disease like Preeclampsia. At present it is not clear whether and what extent ANXA2 and associated proteins are altered in PE.

Therefore, the purpose of the study is to lend the support to the existing information pertaining to the expression status of above proteins in placenta of PE compared to normotensive placenta and provide the new information (the importance of these proteins in placental development), So the purpose of the present study is to evaluate expression of these proteins in placenta tissue of normal and preeclamptic women, in order to find out new target for research into PE prevention.

Chapter 3

Review of Literature

Plasminolytic components and their receptors in pathogenesis of preeclampsia

3. Review of Literature

3.1: Hypertension in pregnancy and its classification

Hypertension is the most common medical condition encountered during pregnancy, complicating up to 10% of pregnancies.[1] Hypertensive disorders during pregnancy are classified into four groups, as suggested by the National High BP Education Program functioning Group on Hypertension in Pregnancy:[2]

Chronic hypertension

Chronic hypertension is diagnosed by BP of at least 140/90 mm Hg on two incidents recorded at least four hours apart at 20 weeks' gestation or previously. Chronic hypertension is associated with PE, intrauterine growth restriction (IUGR) and placental abruption. However, treating mild to moderately elevated BP does not provide benefit the foetus or prevent PE.[3]

Gestational Hypertension

Females who develop hypertension after 20 weeks of gestation and who do not have increased levels of protein in the urine or other criteria for PE are diagnosed with gestational hypertension. This is a temporary diagnosis that includes women who eventually develop PE, those with concealed chronic hypertension (diagnosed by continuously elevated BP beyond 12 weeks postpartum), and women with transient hypertension of pregnancy. Approximately 50% of females diagnosed with gestational hypertension between 24 and 35 weeks' gestation ultimately develop PE.[4]

Preeclampsia superimposed on chronic hypertension

This condition occurs in women who have been diagnosed with chronic high BP before pregnancy, but then develop worsening high BP and protein in the urine or other health complications during pregnancy.[1]

3.2: Preeclampsia

PE, a systemic vascular disorder of pregnancy characterized by hypertension in association with proteinuria, affects 5% to 10% of all pregnancies. This condition can affect virtually every organ system, causing preeclampsia-related adverse complications such as seizures (eclampsia), HELLP (hemolysis, elevated liver enzymes, and low platelets) syndrome, abruptio placentae, and fetal growth restriction.[5]PE/eclampsia is described as a pregnancy-specific systemic disorder of unknown aetiology and is a potentially serious disease with symptoms related to a generalized vascular endothelial activation. The placenta seems to be a most important component in the pathophysiology of the disease. PE is a multisystemic disease characterized by the development of high BP after 20 weeks of gestation, with the presence of proteinuria or, in its absence, of signs or symptoms indicative of target organ injury.[6, 7] PE can be defined as a new onset of hypertension (>140/90 mmHg) after gestational week 20 together with significant proteinuria (300 mg/24 h).[8, 9]

3.2.1: Classification of preeclampsia

Mild and severe PE

PE was diagnosed according to the criteria from International Society of Hypertension in Pregnancy.[10]

Mild PE is characterized by hypertension with a systolic blood pressure ≥ 140 mmHg, diastolic blood pressure ≥ 90 mmHg, and proteinuria with a urine dipstick of ≥ 1+ or ≥ 0.3 g per day after 20 weeks of gestation in a patient who was previously normotensive. Severe PE is characterized as the presence of one or more of thefollowing: newonset cerebral or visual disturbance, severe persistent right upper quadrant or epigastric pain unresponsive to medication and not accounted for by an alternative diagnosis or serum transaminase concentration ≥ twofold normal, or both,

systolic blood pressure ≥ 160 mmHg or diastolic blood pressure ≥ 110 mmHg on two occasions at least 4 h apart while the patient is on bed rest unless the patient is on anti-hypertensive therapy, 1.1 mg/dL or doubling of serum creatinine concentration in the absence of other renal disease, pulmonary edema.[11]

3.2.2: Subclassification of Preeclampsia

Early onset and late onset preeclampsia

PE is a heterogeneous disorder. Early-onset PE, defined as onset of clinical symptoms before 35 weeks of gestation, is associated with more pronounced symptoms and a poorer outcome than late-onset PE. A growing body of evidence suggests that early-onset PE diverges from late-onset PE to such an extent that in fact they may be two different diseases or subgroups sharing a similar clinical outcome.[12,13] Signs of abnormal placentation are more frequently observed in early- than in late-onset PE. Additionally early-onset PE is associated with IUGR and abnormal changes in the flow of blood of the umbilical arteries, whereas late-onset PE is associated with a normally developeing fetus and usually no changes in blood flow in umbilical artery.[14]

3.3: Placenta

3.3.1: Placental development

The placenta is part of the pregnancy from the second that the embryo consists of a few cells up until it is discharged after birth of a child. As the formationa of placentaalready starts at implantation, at which point the embryo invades the wall of the endometrium, disorders during the time of implantation may cause abnormalities in the placenta in location and anatomy.[15] Fertilization is a sequence of synchronized

events involving 1) sperm preparation, 2) sperm to egg binding, and 3) fusion and activation of the fertilized egg.[16] After ovulation, the oocyte is surrounded by the zonapellucida and the corona radiata. The sperm penetrates both layers causing a calcium wave throughout the cytoplasm of the oocyte.[17] Due to the calcium wave, a rapid activation of glucose-6-phosphatedehydrogenase (G6PDH) occurs and large quantities of the reduced co-enzymenicotinamide adenine dinucleotide phosphate(NADPH) are immediately produced. This is used as a substrate for a peroxidase enzyme, which instantly catalyzes the hardening of the zonapellucida, preventing polyspermy and thus fatal paternal triploidy.[18] Followingfertilization embryo is formed, which is the beginning of the fetus and its placenta. While it undergoesdivisions of cells, the embryo is inactively transported near the uterus. Around day five, the embryonic cells are unbound from the zonapellucida and the blastocyst is formed, which is ready for implantation.[19] At implantation, 7-12 days after the time of ovulation, the blastocyst comprises cavity of blastocyst, the inner cell mass or the embryoblast and the trophoblast at the periphery. The latter grow into the placenta.

Implantation is a extremelyplanned process which involves multifaceted interactions between the activated blastocyst and the receptive uterus.[20] Implantation can be defined as “the process by which the embryo attaches to the endometrial surface of the uterus and invades the epithelium and then the maternal circulation to form the placenta”.[18] The inadequate period of time during which the uterine receptivity for implantation is at peak is alled the “window of implantation”.[21] Within this window, suitable modifications of the blastocyst and endometrium create a atmosphere of uterus that is favorable for the embryo development and is immune-tolerant for the semi-allograft.[21]

For implantation, multifaceted communications between endometrium and embryo are indispensable. Synchronous endometrium development and embryo that is competent to implant is obligatory. The implantation procedure consists of “apposition”, “adhesion” and “invasion”.[22]

Dysfunction in apposition, adhesion and invasion during implantation might result in abnormal placenta developement, which can affect the placental architecture as well as the shape of the placental. Both can have long-lasting clinical concerns with impaired placental function. This is connected with maternal and fetal complications such as PE and IUGR, which are revealed in the placenta both macroscopically and microscopically.[23] Women with PE have defective transformation of the spiral arteries and can have a placental shape that is more oval than round, with a decreased surface area, whereas in IUGR the umbilical cord is inserted into the placental border or the fetal membranes rather than into the main placental mass.[24, 25]

3.3.2: Placental structure

The fetal side of the placenta is composed of the chorionic plate and the basal plate on the maternal side. The intervillous spaces eperates fetal side and maternal side.[26] The chorionic plate consists of dense mass of connective tissue and contains the amnion, main stem villi and the chorionic arteries and veins, which are ramifications of the umbilical arteries and umbilical vein. The chorionic arteries and veins branch into the arterioles and venules of the main stem villi. The main stem villi project into the intervillous space and are connected to the maternal basal plate by anchoring villi.[27] (Figure 1)

The basal plate on the maternal side of the placenta is composed of a mixture of trophoblastic cells and decidual cells and contains the decidua basalis. In the third trimester of pregnancy, Nitabuch's layer develops. This is the precise area from where the placenta separates itself from the uterus at birth. From the basal plate, placental septa bulge into the intervillous space, creating a system of furrows which delimit 10-40 raised areas, also known as cotyledons or maternal lobes.[28,29] The basal plate is pierced by endometrial vessels. The exchange of substances between fetal and maternal circulatory systems happens between the main stem villi and the maternal endometrial arteries and venules in the intervillous space (Figure 1).[26]

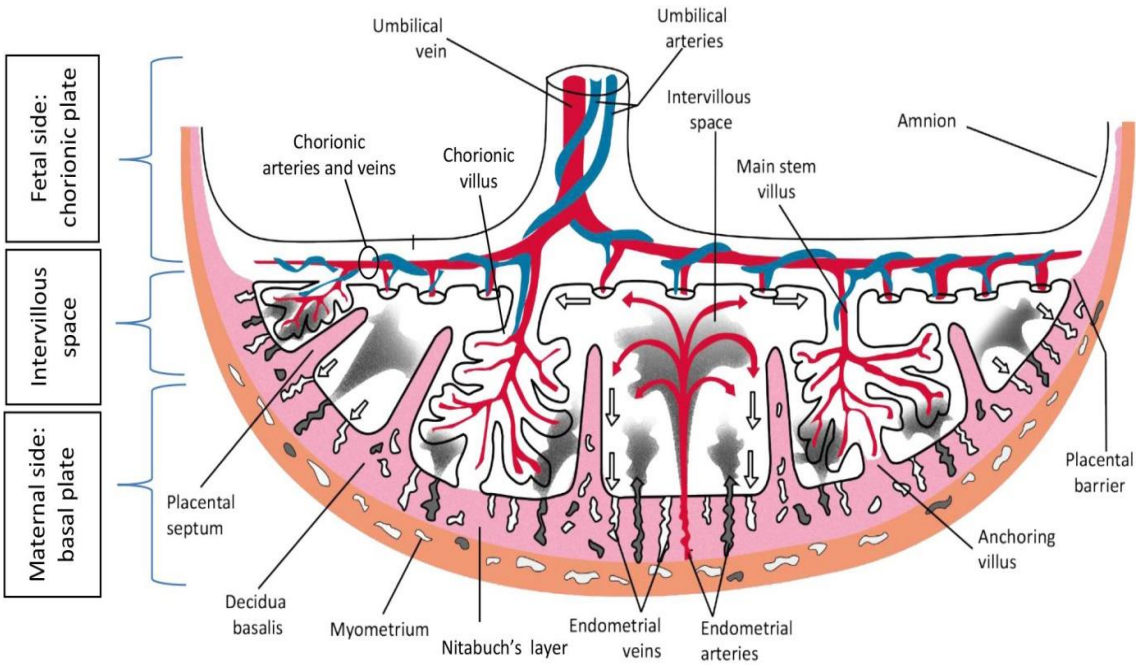

Figure 1: Represatational diagram of the fetal and maternal side of the placenta in the second half of pregnancy. Fetal side shows Chorionic plate that contains the amnion and main stem villi (chorionic villi). Maternal side shows Basal plate that comprises placental septa and decidua basalis.[30]

3.4: Pathophysiology of Preeclampsia

The accurate pathophysiology of PE remains unidentified. However, there are numerous theories that have been put forth that may clarify most of the abnormalities seen in this disease process.[31]

3.4.1: Abnormal placentation

PE is primarily a disease of the placenta as it may be encountered in molar pregnancies.[32] One of the most believed theories in PE revolves around abnormal development of placenta. In normal pregnancies, trophoblast initiates invasion into the myometrial blood vessels by maternal spiral arteries remodelling, transforming them from small, muscular, higher resistance arterioles into large diameter arteries with high capacitance and free flow of blood.[33] Remodeling usually begins in the late first trimester and is completed by 18-20 weeks of gestation. Failure of this process of complete remodeling leads to persistence of high resistance spiral arteries that impede placental perfusion thus leading to a state of "relative hypoxemia" which ends into dysfunction of maternal endothelial cell. Maternal systemic endothelial cell dysfunction manifests in signs and symptoms that are reflective of maternal vasoconstriction and multi-organ damage. Decreased perfusion of the placenta is both a cause and result of abnormal Placentation,[34,35] that becomes more marked with growing needs of the feto-placental unit as pregnancy progresses. Late pathologic variations that are seen in the placental tissue relate with ischemia including atherosis, fibrinoid necrosis, thrombosis, sclerosis of the arterioles, and infarction.[36]

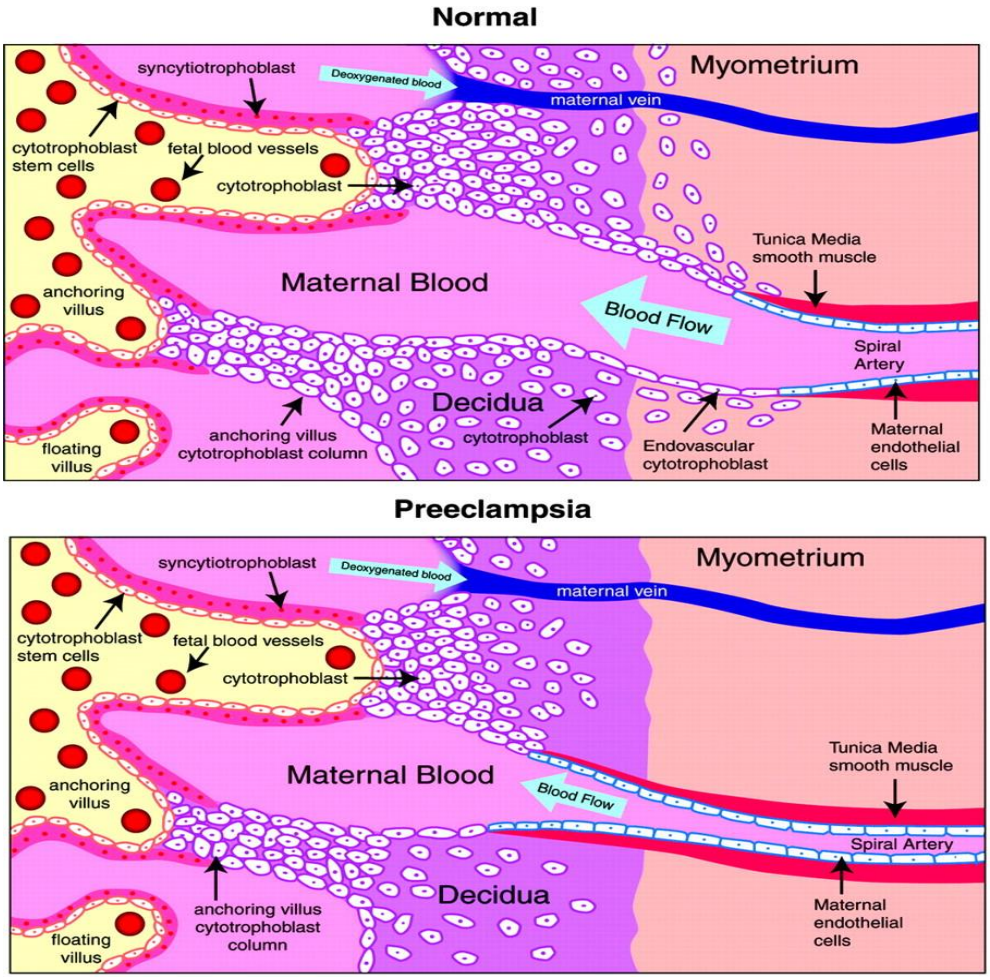

Figure 2: Abnormal developmentof placenta in PE. In the normal development of placenta, invasive cytotrophoblast of fetal origin invade the maternal spiral arteries, converting them from small diameter resistance vessels to high diameter capacitance vessels, capable of providing adequate placental perfusion to withstand the growing fetus. During the procedure of vascular invasion, the cytotrophoblasts separate from an epithelial to an endothelial phenotype, a process stated to as "pseudovasculogenesis" or "vascular mimicry" (above panel). In PE, failure of cytotrophoblasts to assume an invasive endothelial phenotype. Instead, invasion of the spiral arteries is shallow, and they remain small diameter resistance vessels (lower panel).[37]

3.4.2: Oxidative stress in Preeclampsia

Pregnancy increases oxidative stress, a condition that can be aggravated with PE, because free radicals are damaging to the integrity of the endothelium, causing dysfunction of maternal vessels. According to this knowledge, PE is a condition where there is a loss of balance between the endogenous antioxidant system and free radicals, mostly ROS. These species are caused by: mitochondrial aerobic metabolism, activation of NADPH oxidase, xanthine oxidase (XO), cytochrome P450 and lipid peroxidation process.[38]

During normal gestation, ROS generation are known to be increased and necessary for proper physiology.[39] However, a whole different story occurs when the balance between our antioxidant host defenses and the pro-oxidant species is damaged, like in PE. The process in where the relative pro-oxidant species called ROS are much higher than the antioxidant army defenses, is called oxidative stress.[40, 41]

In PE, placental reperfusion injury converges into a damaging inflammatory response that is responsible for inflammation and oxidative damage set up by oxidative stress. Immediately after placental reperfusion injury, reestablished blood flow releases cytokinesand other inflammatory cytokines like tumor necrosis factor-alpha (TNF-α), interleukin (IL)-6, and IL-10, C-reactive protein (CRP), and damaging levels of ROS like superoxide, in response to these events. Increased ROS canfinally trigger a redox signaling process to induce cell apoptosis. Scientific evidences have suggested that reduced perfusion due to aberrant placentation and shallow trophoblastic invasion, triggers a condition of placental oxidative stress leading to intravascular inflammatory response then the endothelial dysfunction.[42]

3.4.3: Endothelial dysfunction in preeclampsia

The maternal vascular endothelium appears to be an important target of factors triggered during PE.[43, 44] Both endothelium-derived relaxing and contractile factors play an important role in the regulation of arterial compliance, vascular resistance and BP. When abnormalities in the production or action of these factors occur, the vasculature is susceptible to vasoconstriction, adherence of leukocytes, oxidative stress and vascular inflammation.[45] When immune cells get adhered to the activated vascular endothelium a series of cellular communications occur inducing junction widening between cells and permitting immune cell penetration into the vascular wall thereby occupying local tissues.[46] As a result the endothelium becomes morepermeable allowing for leakage of fluid, known clinically as edema. Hence, endothelial dysfunction markers may serve as predictors of the syndrome in women that develop PE since many are often raised weeks previous to observation of clinical manifestations.[47] Such markers contain Endothelin-1, soluble vascular adhesion molecule and interleukin-8, ELAM-, or endothelial leukocyte adhesion molecule-1.

An important biomarker of endothelial activation is an endothelial-derived factor that may play a role in PE is the vasoconstrictor, endothelin-1 (ET-1). Though some studies have reported insignificant changes in circulating levels of ET-1 during moderate forms of PE, a possible role for ET-1 inPE remains worthy of consideration. [48,49]

Vascular manifestations of endothelial dysfunction may also affect the maternal liver and brain in severe forms of the disease.[50]

3.4.4: Immunologic factors

PE tends to affect the first pregnancy (naïve to the paternal &fetal antigens) and repeat exposure to paternal & fetal antigens inclines to lower the risk of the disease. In addition to that, other situations that limit exposure to paternal antigens such as a new partner in a following pregnancy and long inter-pregnancy breaks use barrier method of contraception, or conception through artificial insemination led to a higher risk of PE. It is also identified that women who conceive through egg donation have more than twofold the risk of PE than other forms of aided reproduction.[51] Pregnancies conceived through aided reproductive procedures had a four-fold increase in PE compared to naturally conceived pregnancies.[52] Abnormalities seen in PE are similar to those seen in graft versus host disease. The interaction between the extra villous trophoblast antigens and the natural killer cells dictates placental implantation. In PE abnormal placental implantation is believed to be due to the increased NK cell activity due to the conflict between the maternal and paternal genes. This may partially be facilitated through the dendritic cell activity as increased dendritic cell infiltration is seen in placental bed biopsies in PE.[53]

3.4.5: Altered angiogenic balance.

There is collecting evidence supporting the release of several placental anti-angiogenic factors including soluble fms-like tyrosine kinase-1 (sFLT1) and its synergist, soluble endoglin (sENG). These anti-angiogenic factors are induced or aggravated by placental ischemia, with sFLT1 being a circulating antagonist to both VEGF and PlGF. sFLT1, a spliced form of FLT1 antagonizes the function of VEGF and has been shown to decrease cytotrophoblast invasiveness in vitro.[54] It is likely

that dysregulation of these factors has multiple etiologies with more production of antiangiogenic factors being the final common pathway.[50]

The PE placenta releases excess amounts of antiangiogenic soluble Feline McDonough Sarcoma (fms) - like tyrosine kinase-1 (sFlt-1) into maternal circulation, which binds free circulating proangiogenic VEGF and PlGF, leading to an antiangiogenic state. [55,56]

VEGF and PlGF promote vascular development by activating proliferation of endothelial cell and migration, and maintenance of vascular integrity. Binding by sFlt1 prevents VEGF and PlGF signalling via the VEGF-1 receptor on endothelial cells, thus disrupting endothelial cell activation, leading to abnormal function and hence vascular dysfunction.[57,58]

3.4.6: Inflammation and/or infection

Signs of inflammation are seen in normal pregnancies at term, but these changes are exaggerated in women with PE. It is believed that debris of the outer layer of trophoblast contribute to maternal inflammation and some features of the syndrome. Placental DNA released into the circulation of the mother and the fetus (maternal circulation) could have a role in the characteristic inflammation involved with PE.[53] In studies that looked at the connection between maternal infection and PE, it was found that the risk of PE was increased in women with urinary tract infection and periodontal disease.[59,60,61]

3.4.7: Genetic factors

Genetic factors are thought to have a role in getting the disease.[62, 63] Observations that suggest this are that women who are pregnant for the first time and have a family

history of PE have a higher risk of getting it than women who are pregnant for the first time and do not have a family history of PE.[64] The risk of PEis significantly increased in women who previously had PE. The partners of men who whose mothers had PEare more likely to get PE. A woman who gets pregnant by a man whose previous partner had PE is at a higher risk of the disease. Maximum of the data proposes that the mother's and father's genes have a role in the defective formation of the placenta and subsequent development of PE.[65]

To review, placental hypoxia and ischemia are the eventual pathways in the pathogenesis of PE by release of vasoactive factors into the maternal circulation and endothelial cell dysfunction leading to the signs and symptoms of PE.[66, 67]

3.5: Epidemiology and Risk factors in preeclampsia

The worldwide incidence of preeclampsia is 3–4% of all pregnancies.[68] Most cases of preeclampsia occur in healthy nulliparous women, in whom the incidence of preeclampsia may be as high as 7.5%.[69] Multiparous females pregnantwith a new partner have a similar PE risk as nulliparous women[70]; this has been attributed to factors associated with a change in paternity or increased interpregnancy interval.[71] In addition, women with preeclampsia in a prior pregnancy continue to have a high risk of preeclampsia in subsequent pregnancies. Though most cases of PE occur even when there is absence of a family history, the presence of PE in a first-degree relative increases a woman’s risk of getting worsening PE two- to fourfold.[72] A history of PE in the father’s mother also confers an increased risk.[73]

Numerous medical illnesses are related with increased PE risk, including chronic hypertension, diabetes mellitus, renal disease, obesity, and hypercoagulable states,

such as antiphospholipid syndrome and factor V Leiden. Advanced maternal age is also an independent risk factor for preeclampsia.[74] Conditions associated with increased placental mass, such as multifetal gestations and hydatidiform mole also predispose women to PE. There seems to be no clear association between consanguinity and the incidence or severity of PE;[75] however, there are reports of familial aggregation of PE and IUGR in a genetically isolated populations.[76] Interestingly, smoking during pregnancy appears to reduce the risk of PE.[77] Although none of these epidemiological risk factors are well understood, they have helped to provide insight into the pathogenesis of PE.

Risk Factors for Preeclampsia

There are numerous well-studied risk factors for PE and the extent of risk is dependent on the individual factor, severity and the number of risk factors (Table 1). The maximum risk being maternal antiphospholipid antibody syndrome: a nine-fold increased risk for developing PE followed by history of PE in a previous pregnancy that confers a seven-fold increased risk. Additionally, the more severity of PE in previous pregnancy is associated with a higher risk for PE in subsequent gestation.[78] Others factors include diabetes, hypertension, multiple gestation, African American background, assisted reproduction and obesity.[79]

3.6: Maternal outcome in PE

Multiple clinical studies of women with preeclampsia show an increased risk of developing cardiovascular diseases later in life.[80] An often–quoted meta–analysis of prospective and retrospective cohort studies of 3,488,160 females indicated that the relative risk for hypertension was 3.70 (95% CI, 2.70 to 5.05) after 14.1 years weighted mean follow-up and that the relative risks for ischemic heart disease and

stroke were 2.16 (95% CI, 1.86 to 2.52) after 11.7 years and 1.81 (95% CI, 1.45 to 2.27) after 10.4 years, respectively.[81] Additional adverse outcomes, such as the increased risk of renal disease,[82] metabolic disorders and death,[83,84] have also been reported. Early-onset PE conferred a higher risk of end organ damage in terms of cardiovascular, respiratory, central nervous, renal, and hepatic systems compared with late onset.[85] These clinical studies, however, do not delineate whether preeclampsia is a cause or a marker for long–term vascular disease.

Table 1: Risk factors for preeclampsia.[78, 79]

1.	First pregnancy
2.	Prior preeclampsia
3.	Chronic hypertension
4.	Obesity
5.	Chronic renal disease
6.	History of thrombophilia
7.	Multiple gestation
8.	Assisted reproductive techniques
9.	Family history of preeclampsia
10.	Type I or Type II diabetes mellitus
11.	Obesity
12.	Age > 40
13.	Prolonged pregnancy interval

14.	African american race
15.	Molar pregnancy

3.7: Fetal outcome in PE

Placental perfusion is decreased in PE, and the primary consequences are IUGR of the fetus and oligohydramnios.[86] Perinatal death is primarily related to premature delivery, placental abruption, and intrauterine asphyxia. According to Liu, et al. the purported fetal death rate in a population-based cohort study is 10.8 per 1000 births.[87] with both fetal morbidity and mortality closely related to gestational age at the time of eclampsia.Outcome of fetus in pregnancies worsened by PE are largely influenced by gestational age at time of delivery. Neonatal complications such as necrotizing enterocolitis, respiratory distress syndrome, andintraventricularhemorrhage among women with PE are comparable to gestational age matched non-hypertensive controls.[88]

3.8: Management principles in preeclampsia

Given that the underlying disease process of PE lies in the placental tissue, the remedy is delivery of the placenta. For women diagnosed with PE without severe features, delivery is generally suggested at 37 weeks gestation,[89] and in the presence of severe features, delivery is recommended at 34 weeks or earlier for maternal or fetal instability. Labor induction and vaginal delivery is chosenwhen possible. Antihypertensive treatment is reserved for greater than 160 mmHg systolic or 110 mmHg diastolic. Most frequently used antihypertensive medicines for acute management contain labetalol and hydralazine. For seizure (eclampsia) prophylaxis Magnesium sulfate is indicated particularly in a setting of PE with severe features.[90] Magnesium sulphate is considered superior to other anticonvulsant agents.[91] The exact

mechanism of action of magnesium sulphate remains unknown; it is thought that it acts as a 1) Vasodilator 2) Protectant against cerebral edema and 3) Central anticonvulsant.[92]

3.9: Fibrinolysis:

At present, there is no consensus opinion on the changes in fibrinolytic activity during pregnancy. Several studies report that fibrinolysis is increased during pregnancy,[93, 94] whereas others report that it is suppressed, with normal levels being restored in 1 hour of placental delivery.[95] Additionally, some authors are of the view that none of the observed alterations in the components of the fibrinolytic system have an consequence on overall fibrinolytic activity.[96] Earlier reports of increased concentrations of fibrin degradation products (FDP) in pregnancy had directedprevious authors to conclude that fibrinolysis activity was increased during pregnancy.[93, 94] However, it has been suggested that increased levels of FDP such as D dimmers originate from the increased fibrin generation and degradation within the utero-placental unit (plasma D dimmer levels are higher in women who have a Caesarean section during labour compared to those who have an elective procedure), despite a reduced fibrinolytic potential in the systemic circulation.[95] A further possible explanation for increased levels of circulating FDP is impaired clearance.[96] Increased levels of plasminogen inhibitors PAI-1 and PAI-2 act to decrease levels of tissue plasminogen activator (tPA).[95, 97]

3.9.1: Fibrinolysis in Preeclampsia

Fibrinolysis is dependent upon the balance of basal levels of tPA, PAI-1 and tPA released from the endothelium as a reaction to vascular injury or thrombus formation.

[98] By the third trimester, there is a 4-5 fold increase in PAI-1 in the normal pregnant population.[99] PAI-1 inhibits the release of endothelial tPA, thus the increased levels of PAI-1 in normal pregnancy further reduce tPA levels.[99] PAI-2 is produced by the placenta, accordingly levels increase with gestation as the placental mass grows.[100] When affected by preeclampsia women exhibit an additional increase in PAI-1 compared to normotensive pregnant controls which precedes the onset of clinical symptoms.[101] Contrastingly, PAI-2 levels in PET are significantly lower than in normal pregnancy, most likely due to placental dysfunction.[102]

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3.9.2: Proteolysis involved in angiogenesis

Hypoxia-induced release of growth factors and cytokines and the generation of a matrix which permits angiogenesis assist the neighboring vasculature to reorganize and expand its capillary network. For vascular remodeling and tube formation in an extracellular matrix, regulated proteolytic activity is of key importance.[103,104] Capillary formation involves activation and focal detachment of the endothelial cells by (a) degrading the basement membrane of the existing vessel wall, (b) migration of the endothelial cells into the perivascular matrix, accompanied by (c) proliferation of endothelial cells, (d) formation of a lumen, and (e) stabilization of the microvascular structure by the deposition of a new basement membrane and the recruitment of pericytes or smooth muscle cells.[105,106] This vascular remodeling is critically dependent on the proteolytic activity of the endothelial cells, which can be attributed to the u-PA/plasmin system and members of the MMP family.[107]

3.10: Annexins

Annexins are generallyrecognized to be a large multigene family of Ca2+-dependent phospholipid-binding proteins.They were discovered in the late 1970s and before the name “annexin”, they were first introduced in diverse names which in Greek means “hold together”.[108] Over a hundred annexin proteins have been discovered in various species. Among these, 12 proteins are found in humans referred as A1–A13 (leaving A2 unassigned) each having a differently positioned calcium/membrane-binding site within the core domain and a different N-terminal domain.[109] Because of unique structure, Annexins allows them to locate on membranes reversibly. They contain a preserved calcium and membrane-binding unit, which constitutes the core domain. It contains of four annexin repeats of about 70–80 amino acids. The alfa-helical shape of Annexin forms a somewhat curved disc. The calcium and membrane-binding sites as well as binding sites for phospholipids, heparin, carried by the convex surface and F-actin.On the other hand the concave side is responsible for other interactions. Ahead of the core domain arises the N-terminal region which varies in length as well as in sequence. It mediates regulatory interactions with protein ligands and annexin-membrane association.[109] It has been recently demonstrated that a part of N-terminal area integrates into the folded core, permitting the N-terminal region to be exposed for additional interactions upon binding of calcium.[110] Annexins have diverged significantly, despite their gross structural similarity, in terms of their gene regulation, tissue-specific expression patterns, subcellular localization of various isoforms, and features peculiar to individual subfamilies. Annexins participates in numerous cellular functions, like membrane trafficking, exocytosis, endocytosis, membrane-cytoskeleton interactions, regulation of membrane protein activities, calcium channel activity and signal transduction, among others. Furthermore, though annexins are

predominantly cytosolic, they can as well be found as extracellular proteins exerting additional functions as anticoagulant and antiinflamatory proteins, or facilitating the interaction with other extracellular proteins.[109, 111]

Up to the present time, there is no confirmation to suggest that any single member of the annexin family is a disease-triggering gene, i.e., a gene that through loss, mutation, translocation or amplification leads to a known human disease. However, there is good evidence that in certain clinical conditions, changes in annexin expression levels or localisation may contribute to the pathological consequences and sequelae of disease.[112]

3.11: ANXA2, tPA, PAI-1 and VEGF and its relation to plasminolysis and angiogenesis

Failure of physiological spiral arteries transformation is seen in deep placentation disorders such as PE with or without IUGR. This spiral arteries transformation failure is characterized by the absence of spiral artery trophoblastic invasion and remodeling. [113] Angiogenesis is the process by which new blood vessels form from pre-existing vasculature.[114]

It occurs during embryologic development and in response to a wide variety of stimuli including inflammation, wound healing, hypoxia, tumor growth, and atherosclerosis. Angiogenesis encompasses a complex series of steps whereby activated endothelial cells dissociate from their underlying matrix, proliferate, and migrate toward a chemotactic stimulus.[115] Upon reassembly into tubular structures; endothelial cells lose their invasive phenotype, reassociate with matrix proteins, and develop cell–cell contacts in a tubular conformation. To penetrate extracellular matrices without sacrificing tissue integrity, migrating endothelial cells are thought to employ proteases

whose activities are restricted to the pericellular compartment.[116] Because fibrin forms a provisional matrix in many settings in which angiogenesis subsequently occurs [117], it has been assumed that the fibrinolytic system may play a pivotal role in the formation of new blood vessels. Recent studies suggest, however, that the role of the fibrinolytic system is quite complex and highly context specific.[118] The fibrinolytic activity is a unique sequential process that requires interaction between different components. Extracellular proteolysis is acrucial requirement for the development of new blood vessels during the process of neovascularisation.[119]

The extracellular proteolytic system comprises serine proteases and their activators, including tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA).[120]

Plasmin, a central component of this system, is a broad-spectrum trypsin-like serine protease that degrades several components of the ECM-like laminin and fibrin, activates inactive pro-matrixmetalloproteinases (MMPs) and also increases the bioavailability of isoforms 165 and 189 of the angiogenic growth factor vascular endothelial growth factor (VEGF).[121]

A fundamental tenet of cell surface fibrinolysis is the concept of fibrinolytic assembly, in which the tPA-dependent conversion of plasminogen to active plasmin is accurately coordinated through the formation of a multimolecular complex consisting of tPA, the ANXA2 heterotetramer, and plasminogen.[122] ANXA2 is a cell-surface protein, which, in complex with its binding partner p11 forms the receptor for both tPA , the inactive precursor of plasmin, and its activator, plasminogen. By assembling tPA, ANXA2, and plasminogen, this complex increases the catalytic efficacy of tPA, converting plasminogen to plasmin at least 60 times more competently than the same amount of

tPA alone.[123] PAI-1 is responsible for approximately 60% of the PA-inhibitory activity in the plasma and is the key inhibitor of fibrinolysis compared with PAI-2 and PAI-3 during pregnancy.[124]

ANXA2 plays a role in angiogenesis and neovascularization. In the first place, ANXA2 is a receptor for the angiogenic-related proteins such as angiostatin and tPA.[125.] Secondarily, ANXA2 is also involved in VEGF-facilitated neovascularization.

Zhao et al. stated that ANXA2 mRNA and ANXA2 protein were increased in a mice model of ischemic retinopathy through a VEGF/ VEGF-R2/PKCβ pathway.[126]

Xin et al Xin et investigated ANXA2 protein by using Immunohistochemisry, western blot analysis, and Real time PCR They found that expression of ANXA2 was significantly down regulated in placentas as well as maternal blood and found the anti annexin antibodies in the maternal blood in patients with PE. They speculated that the reduced expression of this protein and the presence of its antibodies provide clues to an impaired fibrinolytic function which may lead to increased placental thrombin formation in pregnancies complicated with PE.[127]

The above result is possibly at odds with those of, Sano et.al, who showed increased placental ANXA2 mRNA expression during the acute phase of PE. Immunohistochemical staining of placental ANXA2 was high regardless the severity of PE. Hence postulating that worsening of PE might alter ANXA2 expression at the transcription level.[128]

Marwa Abd El-Latif et.al, aimed to assess serum levels of ANXA2 in a cohort of PE patients and investigate their role as biomarkers for the development of the disease. They have found the significant positive correlation between ANXA2 levels and proteinuria in both mild and severe PE cases; indicate this may be used as a potential marker of the severity of the disease.[129]

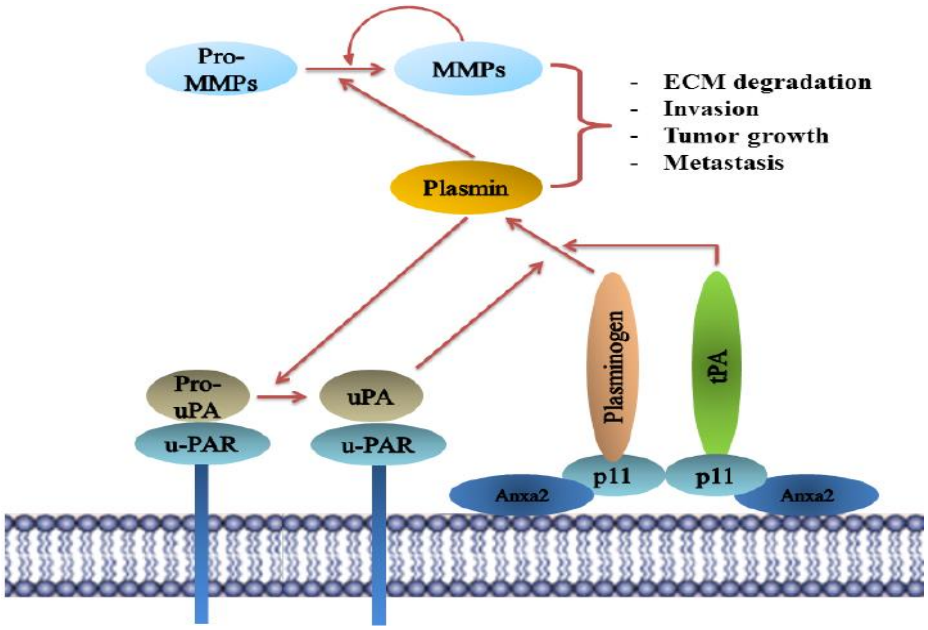

Figure 3: Experimental model of plasmin regulation by cell surface ANXA2 and p11. ANXA2 binds tPA and plasminogen at the carboxyl-terminal lysine residue of the p11 subunit. The ANXA2 subunit does not bind tPA or plasminogen but serves as cell surface receptor for p11. The co-localization of the tPAand plasminogen by ANXA2 results in enhanced conversion of plasminogen into plasmin. Plasmin converts pro-MMPs into active MMPs and further activates pro-uPA into active uPA.[130]

3.12: tPA and PAI-1 in normal pregnancy and Preeclampsia

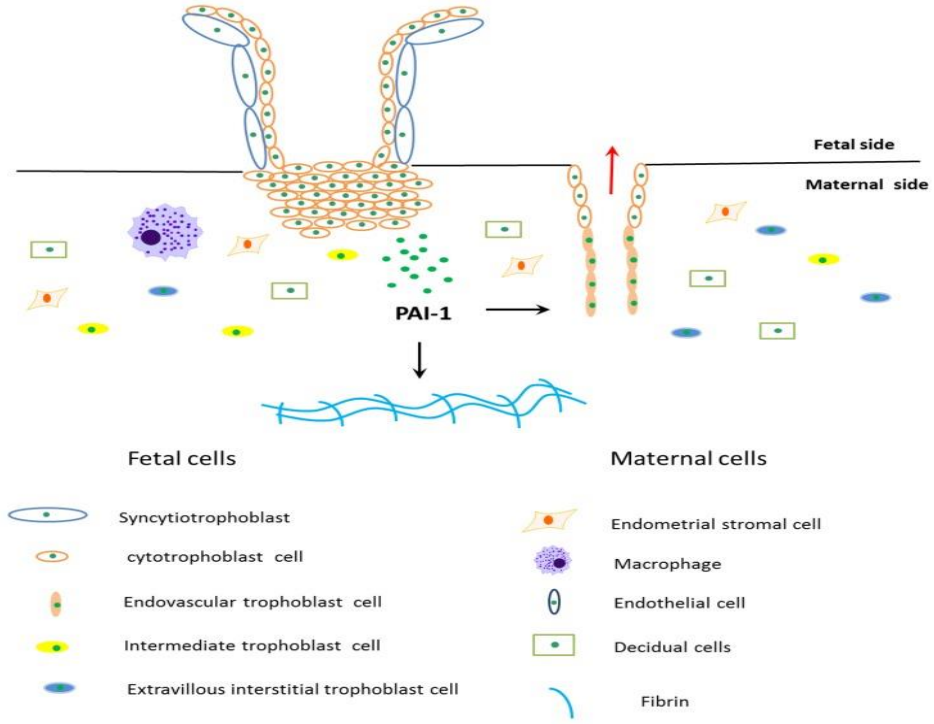

Figure 4: Role of PAI-1 in trophoblast invasion. The cytotrophoblasts invade into the maternal side and differentiate into extravillous interstitial trophoblasts, intermediate trophoblasts and endovascular trophoblasts. Among them, extravillous interstitial trophoblasts and endovascular trophoblasts express PAI-1. Moreover cells from the maternal side take part in trophoblast invasion, such as endometrial stromal cells, decidual cells, macrophages and endothelial cells. Extravilloustrophoblast invasion in early pregnancy is precisely controlled by many factors expressed by trophoblasts and maternal cells, where PAI-1 is the main anti-invasive factor. PAI-1 prevents trophoblast invasion by inhibiting extracellular matrix degradation, which leads to fibrin accumulation in the maternal side. PAI-1 may also play a role in remodeling maternal uterine spiral arteries.[131]

PAI-1 levels in the plasma steadily increase during the second trimester of pregnancy during the course of a healthy pregnancy, and reach a maximum at 32–40 weeks of pregnancy. Within 5–8 weeks after delivery, PAI-1 levels fall again to the levels as earlier the occurrence of pregnancy. PAI-1 is expressed in invading trophoblasts in the human placenta by immunostaining.[132] Invasion of the trophoblast at the maternal-fetal edge is a vital process during the time of implantation and placentation, and during this process extravillous cytotrophoblasts (EVT) obtain invasive properties, which are capable of invade and remodel maternal tissues (interstitial EVT) and uterine spiral artery (endovascular EVT).[133]

EVT can degrade extracellular matrix (ECM) to promote cell migration to the maternal side .This process is precisely controlled by many factors expressed by maternal cells and trophoblasts (Figure 2).[133] Trophoblasts and malignant tumors use the same biochemical mediators to help in invasion, including extracellular matrix degradation and immunosuppression of environmental conditions. PAI-1 can inhibit trophoblasts invasion while promoting tumor cell immigration. (Figure 4).[131]

Studies investigating PAI-1 levels in women with preeclampsia have conflicting results: some showing no difference.[134] whereas Oladosu et al, demonstrate a significantly higher maternal concentration of PAI-1 in women with preeclampsia compared to low risk pregnant women.[135]

YuditiyaPurwosunu et.al, measured the maternal plasma PAI-1 and tPA mRNAs concentration use of reverse transcription PCR assays. They had found that the levels of tPA, PAI-1mRNAs were significantly increased in patients with PE and are positively correlated with the severity of PE. The significantly increased PAI-1 and

tPA mRNA concentrations in maternal plasma in PE suggests that increased transcription of mRNA might be related to PE.[136]

A study conducted by K B Bodova et.al, found the increased maternal PAI-1 levels in the plasma in patients with PE during the second trimester of pregnancy.[137]

3.13: Role of Annexin A1 (ANXA1) in inflammation and pathogenesis of preeclampsia

In spite ofmany years of intensive research, the pathogenesis of PE remains to be explained, though dysfunction of placenta is considered to play a central role in the development of the disease. It has been proposed that the ischemic placenta can release soluble factors into the maternal circulation that cause endothelial cell activation and/or dysfunction and a systemic inflammatory response.[138]

Although a generalized systemic inflammation is common to all pregnancies.[139] Redman et.al, suggested that PE is not fundamentally different from normal pregnancy, but it is at the extreme end of a constant spectrum of inflammatory responses that are a feature of pregnancy itself.[140]

Originally described ANXA1 as a glucocorticoid-regulated protein with anti-phospholipase activity, but the protein reveals several other anti-inflammatory and proresolving properties, which include inhibition of neutrophils adhesion/transmigration through the endothelium and stimulation of macrophages phagocytic clearance of apoptotic neutrophils.[141] ANXA1 is also regulated by pro-inflammatory proteins, such as lipopolysaccharide (LPS) and interleukin (IL)-6, suggesting that it may act as a brake for controlling the inflammatory response.[142]

ANXA1 is a protein that limits initial steps of inflammation and also acts on the resolution phase of the inflammatory response.[143] Proresolving and anti-inflammatory

actions of Actiona of ANXA1 are mediated by a G-protein-couple receptor named formyl peptide receptor like-2 (FPR2)/lipoxin A4 receptor (ALXR), hereafter referred as "ALX".[144]

Cooray et al. showed that ALX/ALX dimer signature is stimulated by ANXA1 trough p38/MAPKAPK/Hsp27/IL-10 pathway. These authors debated that ANXA1 up-regulation may be ineffective to resolve inflammation if ALX/ALX dimerization fails.[145]Additionally, lower ALX expression level might be associated with reduced anti-inflammatory and proresolutive responses to ANXA1. Decreased ALX expression has been observed in patients with asthma, a chronic inflammatory disease.[146]

Luiza Perucci, et.al, evaluated ANXA1 plasma levels and ANXA1 mRNA expression in peripheral blood mononuclear cells (PBMC) in preeclamptic, normotensive pregnant and non-pregnant women. They found the increased levels of ANXA1 in the plasma which coincided with the higher us-CRP level of the plasma of preeclampticwomen. They concluded that the systemic inflammatory phenotype in PEwere associate with ANXA1 levels were associated suggesting deregulation of ANXA1 in PE pathogenesis.[147]

Jing Fenget.al,carried out the study in a model of a PE rats and the expression of ANXA1 in the PE rat model was detected. They explored the roles and molecular mechanism of ANXA1 in the Placental trophoblasts of PE rats. Their data revealed that the knockdown of ANXA1 decreased the apoptosis and inflammatory response of PE trophoblasts.[148]

3.14: Role of Galectin 3 (Gal-3) in placenta and PE

Gal-3 is a multifunctional protein, belongs to the galectin family. Its exclusive chimeric structure allows it to interact with a plethora of ligands and modifyvarious functions, including cell growth, adhesion, migration, invasion, angiogenesis, immune

function, endocytosis and apoptosis and it has impostant role in the process of progression of tumor [149] Studies have indicated that Gal-3 is involved in implantation of embryo, embryogenesis and formation of placenta, and is closely related with the maintenance and success of pregnancy.[150, 151]

Apoptosis was also noticed in placental tissue through the early stages of pregnancy (<12 weeks) through the expression of pro-apoptotic genes.[152] The apoptosis is linked with development of placenta, including trophoblast invasion, transformation of spiral arteries and differentiation of trophoblast cells, in addition to during birth.[153] During normal pregnancy, the grade of apoptosis of placental tissue progressively increases till delivery.[154] However, an disparity of the 'inhibition-induction' equilibrium leads to a pathological pregnancy

Numerous studies suggest that high expression of Gal-3 exerts inhibitory effects on apoptotic responses of various cells type;[155, 156] of note, intracellular Gal-3 has anti-apoptotic properties, while extracellular Gal-3 may possibly induce apoptosis.[155,157]

Stimulation of angiogenesis and the anti-apoptotic effect, among many biological functions of Gal-3 may play a critical role in the development of PE. Gal-3 stimulates angiogenesis through the VEGF receptor hooked pathway, which binds VEGF receptor 2 (VEGFR2), stops its internalisation and increases its VEGF sensitivity.[158]

During PE with considerably reduced VEGF bioavailability, this process may be very vital. There is also proof that Gal-3 located in cytoplasm of cells acts as an antiapoptotic factor, and decreased expression of Gal-3 in trophoblasts in first trimester of pregnancy is linked to more apoptosis in developing placental villi, leading to missed abortion.[159]

Kolla et.al, performed proteomic analysis of blood samples obtained from patients with high risk of developing PE during the first trimester and identified 10 proteins up-regulated in women who developed PE later in pregnancy, in contrast to those with uncomplicated pregnancies. Gal-3 binding protein was one of these proteins.[160]

Ž. Bojić-Trbojeviće et.al, from their data showed the relevance of Gal-3 for invasive trophoblast cell function in vitro.[161] Sattar et.al, demonstrated elevated serum Gal-3 levels in patients with PE that correlated with resistance forinsulin and dyslipidaemia. [162] On the contrary, Nikolov et.al, revealed no significant differences between serum galectin-3 levels in PE patients and women with uncomplicated pregnancy.[163] Another study revealed significantly increased serum galectin-1 and Gal-3 levels in patients with preterm premature rupture of membranes (pPROM).[164] Pankiewicz K et.al, described Gal-3 in their hypothetical model described the compensatory role of Gal-3in PE. Gal-3 Stimulates formation of new blood vessel through VEGFR2 and therefore it may decrease the severity of dysfunction of ndothelial cells. Apart from angiogenic activity Gal-3 has anti-apoptotic activity and therefore it might inhibit apoptosis in syncytiotrophoblast (STB) cells and decrease STB stress. Insufficient Gal-3 production leads to development of severe complications, such as HELLP syndrome and/or FGR.[165]

3.15: EGFR in placenta and preeclampsia

Epidermal growth factor (EGF) is a 53-amino acid protein and is a ligand for the epidermal growth factor receptor (EGFR). Ligand binding activates a plethora of downstream signaling cascades involved in cellular proliferation, migration, and survival.[166]

Collecting evidence proposes that survival and invasive ability of human trophoblast are associated to intercellular signaling by peptides related to EGF. EGF can protect

against apoptosis induced during in vitro culture of human term cytotrophoblast cells, [167] indicative of the ability of EGF and related proteins to act as survival factors. Peptide members of the EGF signaling system induce downstream signaling by binding to receptor trysosine kinases of the human EGF receptor (EGFR)/ERBB family, which contains four members.[168] The EGFR is a transmembrane glycoprotein and the founding member of the ErbB (erythroblastosis oncogene B) tyrosine kinase receptors.[168] Activation of EGFR ignites a plethora of downstream signaling cascades, including the RAS-RAF-MEK-ERK (rat sarcoma–rapidly accelerated fibrosarcoma–MAPK/ERK kinase), PI3K (phosphoinositide 3-kinase), Akt/mTOR, and JAK-STAT (Janus kinase–signal transducer and activator of transcription) pathways. The EGFR signaling pathway is one of the most versatile signalling units in mammalian biology where almost all cell types possess ErbB family members.[169]

The placenta, however, has the highest expression of EGFR compared with all other human nonmalignant tissues.[170] The EGFR plays critical roles in placental development and survival.

EGFR nullizygous mice have placental defects that can be embryonically lethal.[171] Aberrant previous studies have shown that EGFR signaling or ligand expression has been associated with fetal growth restriction,[172] gestational trophoblastic diseases, [173] and preeclampsia.[174]

Milchev et al. reported on lower EGFR expression in VTB of PE placentas, while Dong et al. reported on lower EGFR expression in VTB of placentas with pregnancy induced hypertension.[175,176]

Findings reported by Ferrandina et.al, suggest that hypertensive disorders in pregnancy are associated with elevated placental EGFR concentrations detected by the radioreceptor technique.[177] Indira Kosovic et.al, studied the Immunohistochemical

expression of EGF and EGFR of villous trophoblast (VTB), decidual cells (DC), and extravilloustrophoblast (EVTB) in the placentas from pregnancies complicated with preeclampsia (PE) and to compare them with placentas from normal pregnancies

EGF and EGFR expression of villous trophoblast (VTB), decidual cells (DC), and extravilloustrophoblast (EVTB) in the placentas from pregnancies complicated with PE and to compare them with placentas from normal pregnancies. Their result study showed no significant difference in the EGF and EGFR expression in DC, EVTB and VTB in term placentas from pregnancies complicated with PE compared with control group.[178]

Incidence of the Preeclampsia all over the world is about 2 to 8% and in the worst cases PE may threaten the survival of the mother and newborn, its etiology is not completely understood. Field literature considers PE is an angiogenic and inflammatory disorder.Coordinated Vascularisation is essential for placental development. ANXA2 is a profibrinolytic receptor required for plasminolysis, which is important step in the formation of new blood vessel, it also increases the bioavailability of VEGF which is the most potent endothelial growth factor induces angiogenesis and endothelial cell proliferation and has a basic role in angiogenesis. ANXA2 is associated with other proteins like EGFR and Gal-3 at the membrane lattice are also involved in angiogenic pathway. ANX1 is glucocorticoid regulated protein regulates a wide range of cellular and molecular steps of the inflammatory response and is implicated in resolution of inflammation. Gal-3, β-galcotoside-binding lectinparticipates in many functions, both intra- and extracellularly. Recently it has been shown that galectin-3 modulates the inflammation along with its angiogenic role. Role of AnxA1 and Galectin-3 is poorly studied in context with

human reproductive disease like Preeclampsia. At present it is not clear whether and what extent ANXA2 and associated proteins are altered in PE.

Therefore, briefly the purpose of the study is to lend the support to the existing information pertaining to the expression status of above proteins in placenta of PE compared to normotensive placenta and thereby provide the new information (the importance of these proteins in placental development), So the purpose of the present study is to evaluate expression of these proteins in placenta tissue of normal and preeclamptic women, in order to find out new target for research into PE prevention.

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Chapter 4

Material and methods

Plasminolytic components and their receptors in pathogenesis of preeclampsia

4. Materials and methods:

4.1: TYPE OF STUDY: OBSERVATIONAL

4.2: STUDY DESIGN: CASE CONTROL STUDY

4.3: DURATION OF COLLECTION OF DATA:

Prospective study –From 1st June 2016 to 30th Nov 2019.

4.4: PLACE OF CONDUCT OF RESEARCH:

Central research laboratory, SDM College of Medical Sciences and Hospital, Dharwad.

4.5: SAMPLE SIZE:

Sample size:

With 95% confidence level, anticipated prevalence of preeclampsia as 6.5% and desired precision as ± 10%, the minimum sample size per group is 24. (=25)

Normotensive placenta = 30, Preeclamptic placenta = 40

Sample size is calculated by using following formula

$n= Z^2P (1-P)/d^2$

Where d = Precision

n= sample size

Z= z statistics for level of confidence

P= Expected prevalence

A minimum number of 70 cases coming under inclusion criteria during the study period were included in the study

4.6: STUDY POPULATION AND SELECTION CRITERIA:

Inclusion criteria: Placenta of normotensive and different severity of preeclampsia of a women having age group of 19 to 35 were included.

Exclusion criteria- Patients with chronic hypertension, gestational diabetes, renal disease, collagen vascular disease, epilepsy and other pregnancy complications like fetal anomalies or chromosomal abnormalities were excluded from the study.

PE will be diagnosed according to the criteria from International Society for the Study of Hypertension in Pregnancy.[17]

Mild and Moderate PE - characterized by hypertension with SBP>/140 mm Hg and DBP is>/90 mmHg and proteinuria with a urine dipstick of >/1+or >/0.3 g per 24 hours, after 20 weeks' gestation in a previously normotensive parturient.

Severe PE-is characterized by a SBP≥160mmHg and ≥110 mmHg, proteinuria≥ 3 g per 24 hours or evidence of central nervous system disturbances, epigastric pain, liver dysfunction, Thrombocytopenia, and fetal growth restriction.

4.7: PLAN OF STATISTICAL ANALYSIS :

By using program SPSS 20 (USA Chicago) statistical analysis was carried out. Results for data which are normally distributed were shown as ±SD. Statistical significance between the groups was analyzed by one way ANOVA followed by Tukey's post hoc multi comparisons.

To see the neonatal gender difference chi-square test was used. Statistical analysis of expression of proteins for IHC was carried out with Kruskal–Wallis rank-sum test for more than two groups. Mann-Whitney U test were used for comparison between two groups. To detect correlation between expressions of proteins Spearman correlation coefficients were used. 'p-value'<0.05 was considered to be statistically significant

Ethical clearance was obtained by the Institutional Ethical Committee of BLDE (Deemed to be University) (IEC No-183/2016-17, dated-13-10-2016) and SDMCMS & H (SDM University) (IEC No-0748; 2016, dated 20-6-2016).

The fresh placenta villous tissue will be collected directly after the delivery of normal and preeclamptic females. Tissue was taken from the maternal side of the placenta.

The consent was taken for the use of tissues for the purpose of research after explaining in detail in a language (English / Kannada) understood by them.

4.8: DATA COLLECTION PROCEDURE

The present work investigated the expression of functionally associated protein related to angiogenesis, plasminolysis and inflammation ANXA2, ANXA1, VEGF, tPA, PAI-1, EGFR and Gal-3 in placental tissue from women with and without severe Preeclampsia.

3-4 µm thick section were obtained from the paraffin embedded tissue blocks and stained with hematoxylin and eosin to study the histology of placental tissue.

The localization of these proteins were demonstrated and assessed by immunohistochemistry.The technique of Western blot and RT-PCR was performed because the technology of IHC does not impart itself to quantification.

4.8.1: Immunohistochemistry:

By using scalpel, 4–5 biopsies of villous parenchyma (1 cm3 each) from the central and marginal regions of part of the placental disc are collected. Tissue fragments from the placenta consisting of homogeneous villous tissues were cut longitudinally from the maternal side to the foetal side and infarct areas were excluded from the study. Expression of above mentioned proteins was analyzed in placental villous tissues. 3-4 µm thick sections were obtained from formalin

fixed and paraffin embedded placental tissues. The sections were treated according to standard IHC staining procedure for the detection of protein. The endogenous peroxidase activity was blocked by incubating the tissue with 0.3% hydrogen peroxide.Nonspecific binding sites were blocked by incubating the sections with normal horse serum (vector laboratories) and then incubated with primary antibodyagainst above mmentioned proteins. This was followed by sequentially incubating the sections with biotynylated secondary antibody (Vector Laboratories, Burlingame, CA) and avidin biotin horse raddish peroxidase complex (ABC; VECTOR).The antigen of interest was detected by use of a 3, 31-diamino benzidine (DAB) chromogenand by counterstaining with haematoxylin. The primary antibody was replaced by anti-rabbit immunoglobulin-G (IgG) as a whole molecule (Sigma-Aldrich, USA, Catalogue No. A0545). The dilutions for IgG molecule at 1:1000 was used as a negative control. The tissues were evaluated under light microscope with Lieca image Centre.The intensity and localization of the staining reaction in syncytiotrophoblasts membrane chorionic villous stromal cells, and villous vascular endothelial cells and was assessed by using semiquantitativeimmunoreactive score (IRS) and all the samples were blinded.

The IRS was derived by multiplication of staining intensity graded (as 0 negative, 1 weak, 2 moderate, and 3 strong staining) and percentage of positively stained cells (0 as no staining, 1-10% as 1,11-50% as 2, 51-70% as 3,71-100% as 4). Immunoreactivity for antibodies was scored using a semi-quantitative scale for intensity of staining: 0 negative, no staining; 1+ weak; 2+ moderately positive; 3+ strongly positive. The localization protein was counted in 10 random fields in placental villi.

Table 2: Immunohistochemistry was performed by following protocol

Deparaffinization and rehydration	Deparaffinise the tissue sections at 65C for 2 hours in incubator. This is followed by immersing the slides in xylene for 10min. Xylene are replaced four times to completede-waxing. This is followed byrehydration.
Rehydration	Rehydrate the sections by incubating the slides descending grades of alcohol (100%, 95%, 80%, 70% of ethanol) 5mineach. Wash the sections in double distilled water for 5 min, followed by antigen retrieval.
Antigen retrieval	Treat the sections with 0.01 M of citrate buffer (pre warmed for 5min) for 45min at 1000C for unmasking the antigen before blocking.
Blocking	The endogenous peroxidase activity is blocked by incubating the renal tissue with 0.3% hydrogen peroxide (1:100 dilutions in methanol). Nonspecific binding sites are blocked by incubating the sections with normal horse serum (vector laboratories). Followed by incubation with primary antibody.
Primary antibody	The sections are incubated overnight with primary antibody against specific protein. Further treated with secondary antibody.
Secondary antibody	Sequentially incubate the sections with biotynylated secondary antibody (Vector Laboratories, Burlingame, CA) and avidin biotin horse raddish peroxidase complex (ABC; VECTOR).
Staining and Detection	Antigen of interest is detected byuse of a3, 31-diamino -benzidine (DAB) chromogen and by counterstaining with haematoxylin.

4.8.2: Western blot analysis

Human placental bed samples were homogenized at 4°C in 500µL RIPA lysis buffer. The lysates were centrifuged at 14000 rpm at 4°C for 45 min to remove the cell debris.Bicinchonic acid assay (BCA assay)was used to determine the protein concentrations. Whole cell lysates (40µg) were subjected to SDS-PAGE using Tris–Hcl buffer and the proteins were transferred to nitrocellulose membranes (Hi-media) using a transfer apparatus at 65 V for 90 min. The specific primary antibodies were used against above proteins of interest. Appropriate secondary antibodies conjugated to horseradish peroxidase (Bio-Rad) were incubated with respective membranes for 2 h at room temperature. The membranes were developed using ECL plus (Bio-Rad), and the image was captured using enhanced Chemiluminescence system, G: BOX Chemi-doc XX6/XX9. GAPDH housekeeping gene was used as an internal control. The densities of protein bands were determined with Image J, version 1.35d.

Table 3: Western blotting procedure

Preparation of lysate from tissues	1. Dissect the tissue of interest with clean tools, on ice preferably, and as quickly as possible to prevent degradation by proteases. 2. Store samples at -80°C for later use or keep on ice for immediate homogenization. 3. For a ~5 mg piece of tissue, add ~300 µL of ice cold RIPA lysis buffer rapidly to the tube, homogenize with an electric homogenizer 4. Centrifuge for 30 min at 14,000 rpm at 4°C in a cold microcentrifuge.
Determination of protein concentration	5. Perform a bicinchoninic acid (BCA) assay. 6. Determine the concentration of each sample and freeze them at -80°C for later use.
Protein separation by gel electrophoresis	7. Load equal amounts of protein 40 µg into the wells of SDS-PAGE gel, along with molecular marker 8. Run the gel for 90 minutes at 65V
Transferring the protein from the gel too the membrane	9. Place polyacrylamide gel in direct contact with a piece of nitrocellulose membrane. Next, the gel-membrane pair is "sandwiched" between two electrodes, which are typically submerged in a transfer buffer. Transfer for 90 min at 100 V.
Treatment with Ponceau-S Stain,	10. Briefly rinse the blot in DW and stain with Panceau S solution to see the success of transfer 11. Rinse off the Panceau stain with 1X wash buffer

Blocking and primary Antibody incubation	12. Block the membrane with 0.5 % of BSA in 1X wash buffer to prevent the non-specific binding of antibodie. 13. Incubate overnight with primary antibody which is diluted in 0.5% blocking buffer. 14. After incubation rinse the membrane 6-7 times in 1% wash buffer
Treatment with secondary antibody	15. Add secondary antibody which is diluted in 1% BSA in 1X wash buffer and incubate for 1 hour 30 minutes. 16. After incubation rinse the membrane 6-7 times in 1% wash buffer.
Imaging and data analysis	17. Prepare ECL mix (each of 700µl solution A and B provided by the manufacturer). Incubate the membrane for 1–2 minutes. 18. Capture the signal using the enhanced Chemiluminescence system, G: BOX Chemi-doc XX6/XX9. 19. Determine the intensity of bands of target protein by Image J software.

Table 4: Antibody used for IHC and Western blotting and their catalogue number.

Antibody	Catalogue no
ANXA2	Santa Cruz Biotechnology Inc; Santa Cruz, CA Catalogue No. SC-9061
ANXA1	Santa Cruz Biotechnology Inc; Santa Cruz, CA catalogue No. SC-12740
VEGF	Santa Cruz Biotechnology Inc; Santa Cruz, CA Catalogue No. SC-7269).
tPA	Santa Cruz Biotechnology Inc; Santa Cruz, CA Catalogue No SC-7269
PAI-1	Santacruz Biotechnology Inc; Santa Cruz, CA Catalogue NoSC -5297
GAL-3	Santa Cruz Biotechnology Inc; Santa Cruz, CA catalogue No. SC-23938).
EGFR	BD Biosciences CA catalogue No CA-9061
GAPDH	Santa Cruz Biotechnology Inc; Santa Cruz, CA catalogue SC-166574.

4.8.3: RNA preparation, real-time PCR

Total RNA was extracted using Trizol reagent (Thermofisher scientific invitrogen). CDNA was synthesized from 2µg of total RNA by using Takara cDNA synthesis kit. Template cDNA was subjected to PCR amplification using gene-specific sense and antisense primers.and reference gene β actin were generated (Juniper life sciences).RT-PCR conditions were at 95°C for 5 min, followed by 40 cycles of 95°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec in a Quant Studio 5 thermal cycle (Applied Bio systems). The expression of gene was standardized against the house keeping gene β-actin. The relative gene expression was calculated by 2ΔΔCT method for comparing the relative expression results between normotensive control and patients with PE.

RNA preparation, real-time PCR

RNA isolation:

Total RNA was extracted using Trizol reagent (Thermofisher scientific Invitrogen). Total mRNA was isolated from tissue samples following protocol (W.M. Keck Foundation Biotechnology Microarray Resource Laboratory at Yale University). Before isolating total RNA from tissue, tissue was washed with PBS, and then 1ml TRIZOL lysisreagent was added to the tissue and was homogenized using glass Teflon homogenizer in the TRIZOLreagent. Necessary precaution was taken to avoid DNA contamination. The homogenised mixture kept at RT for 5 minutes to permit whole dissociation on nucleo-protein complexes. Tissue debris was removed by centrifugation and supernatant preserved into eppendrof tube.

200 µl of chloroform per 1000 µl of TRIZOL was used to precipitate RNA from thesample (0.1 ml for 500 µl TRIZOL Reagent). Tubes were wrapped with Parafilmsecurely to avoid loss of lysate spillage. Samples were Vortexed strongly for 30seconds and waited for 2 to 3 minutes. Samples were centrifuged at <12,000xgfor 15 minutes at 4 ^{0} C. After centrifugation, aqueous phase was separatedcompletely with precaution and transferred into new tube.Precipitation of RNA was done by adding 500µl isopropyl alcohol. Samples were incubated at 15 to 30^{0}C for 10 minutes and later centrifuged at < 12,000xg for 10minutes at 4^{0}C. The RNA was precipitated and pellet preserved and decantedsupernatant completely. To remove impurities from RNA, pellet was once rinsedwith 75% ethanol. Mixed the samples by vortexing and centrifuged at <7,500 x gfor 5 minutes at 4^{0}C. Pellet of RNA after washing, dried the pellet for 5-10 minutes. RNA was dissolved in DEPC treated water by mixing several times using a pipette tip. Reconstituted 1 µl RNA was diluted with 39 µl of DEPC treated water (1:40 dilution) and then the conc. and purity of RNA was checked by Epoch analyzer.

cDNA synthesis:

cDNA was synthesized from 2µg of total RNA by using Takara cDNA synthesis kit.Single stranded RNA was converted into cDNA by Reverse transcription (RT). Tocarry out reverse transcription 20µl total reaction volume was selected, whichconsists; 1µg of total RNA + 2µl of 5× 1st strand buffer + 1µl DNase-I and 20minutes incubation at RT for complete digestion of genomic DNA. Then, 3µl ofMaster Mix1, and 300 ng/µl random hexamer primers, was added to the mix. Andkept in a thermal cycler, the program for this reaction was 5 minutes at 65°C

+ 10minutes at 25°C + initial incubation at 42°C for 60 minutes. Later, reaction was deactivated for 10 minutes at temperature 95°C.

Quantitative PCR & primer design:

PCR primers for qRT-PCR experiments were provided by Juniper Life Ciencesaspre designed primers or designed with the primer design tool from NCBI primerdesigning tool.

Real time PCR analysis:

RT-PCR reaction constituents: 10µl SYBR Green PCR mixture + 100 nanoMolarforward and reverse primers + 2µl cDNA template in a reaction volume of 12 µl.RT-PCR was performed by using DyNAmo Color Flash SYBR Green q-PCR kit(Thermoscientific). Template cDNA was subjected to PCR amplification using gene-specific sense and antisense primers. and reference gene β actin were generated (Juniper life sciences) RT-PCR conditions were at 95°C for 5 min, followed by 40 cycles of 95°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec in a Quant Studio 5 thermal cycle (Applied Bio systems). The expression of gene was standardized against the house keeping gene β-actin. The relative gene expression was calculated by 2ΔΔCT method for comparing the relative expression results between normotensive control and patients with PE.

Table 5: PCR protocol

Reagents	Volume
2X Master Mix	10µl
Prime mix	1.0 µl forward + 1.0 µl reverse
Template DNA	(5µl)10 µg cDNA (RT-PCR Product)
D/W (Nuclease Free)	3 µl
Total Volume	20µl

Table 6: Sequence of primers used for reverse transcriptase -polymerase chain reaction (RT-PCR)

Gene	Sequence
ANXA2	5'-CTGGCAAAGGGTAGAAGAGCA-3' 5'-CGTCATAGAGATCCCGAGCAT-3'
ANXA1	5'-ATCAGCGGTGAGCCCCTATC-3' 5'-TTCATCCAGGGGCTTTCCTG-3'),
VEGF	5'-GAGATGAGCT TCCTACAGCAC-3' 5'-TCACCGCCTCGGCTTGTCACAT-3'
tPA	5'-AGGAGCCAGATCTTACCAAGTGA-3' 5'-CGCAGCCATGACTGATGTTG-3'
PAI-1	5'-GGCCATTACTACGACATCCTG-3' 5'-GGTCATGTTGCCTTTCCAGT-3'
GAL-3	5′- CAATACAAAGCTGGATAATAACTGG-3' 5′-GATTGTACTGCAACAAGTGAG-3
EGFR	5'-GAGAGGAGAACTGCCAGAA-3' 5'-GTAGCATTTATGGAGAGTG-3'
β actin	5'-GGGAAATCGTGCGTGACATTAAG-3' 5'-TGTGTTGGCGTACAGGTCTTTG-3'

Chapter 5

Results

Plasminolytic components and their receptors in pathogenesis of preeclampsia

5. Results and observations:

Table 7: Demographic characters in preeclamsia (PE) and normotensives

Variables	Normal (N=30)	Mild PE (N=20)	Severe PE (N=20)	F value	*P*	*P1*	*P2*	*P3*
Age (yrs.)	25.53±3.9	25.75±2.9	25.55±3.3	0.025	0.975	0.836	0.987	0.843
Height (m)	1.53±0.049	1.51±0.03	1.52±0.02	1.36	0.262	0.153	0.337	0.495
Weight (kg)	60.63±4.0	60.05±4.3	63.05±5.7	2.375	0.101	0.630	0.08	0.07
BMI (kg)	25.83±2.0	26.05±1.6	25.45±2.1	2.88	0.06	0.602	0.020	0.05
GA(weeks)	37.67±0.9	36.85±1.4	34.60±1.5	35.09	0.000*	0.02	0.000	0.000
SBP (mmHg)	106.07±9.2	145.10±6.4	163.50±3.3	417.6	0.000*	0.000	0.000	0.000
DBP (mmHg)	70.10±6.8	91.00±5.7	102.30±4.3	191.8	0.000*	0.000	0.000	0.000
Nborn wt	2.9±0.29	2.72±0.22	2.2±0.40	33.22	0.000*	0.001	0.000	0.000
Placental wt	503±18.4	478±77.6	495±47.7	1.517	0.227	0.095	0.435	0.395
PLC (/µl)	2.77±0.4	2.55±0.5	2.35±0.4	4.775	0.012*	0.07	0.007	0.460
PT(s)	12.0±0.0	12.7±0.8	13.1±0.7	21.615	0.000*	0.000	0.000	0.102
APGAR score	7.8±0.68	7.1±0.6	6.8±0.61	18.01	0.000*	0.000	0.0001	0.139
APGAR score	8.8±0.68	8.3±0.65	7.9±0.307	16.67	0.000*	0.005	0.000	0.018

Note: BMI: Body mass index; GA: Gestational age; PT: Prothrombin time; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; PLC: Platelet count. Statistical significance between the groups was analysed by one way ANOVA followed by Tukey's post hoc multi comparisons (total comparison between groups) Statistical analysis is carried out by Student's unpaired t test between two groups (*p<0.05). (P1- Normotensives with mild PE, P2 –Normal to Severe PE, P3- Mild to severe PE) Above data is expressed as Mean ± SD

5.1: The Demographic characteristics of the normotensive women and preeclamptic patients. There were no statistical differences between the PE and normotensive control groups with respect to their age, BMI, neonatal gender. Almost all the deliveries in the control group were at full term. Compared to normal control group the mean gestational age is shorter in PE group. In patients with PE, when compared with the normotensive control group, Birth weight of the baby is reduced and the systolic and diastolic blood pressures were significantly higher.PT was increased and APGAR score was reduced in PE group ($P < 0.05$).

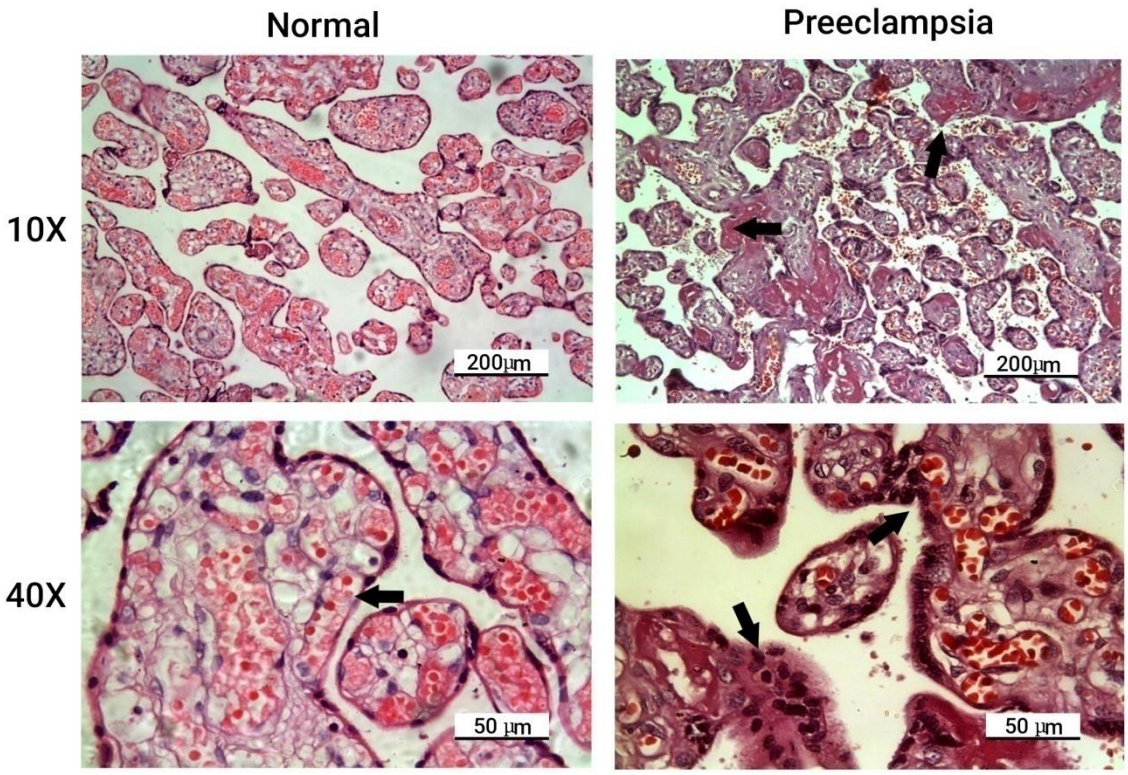

Figure 5: Representative photomicrography showing Hematoxylin and eosin staining showing the morphology of normal and preeclamptic placenta, Magnification 10x, bar =200 µm and 40x, bar= 50 µm. Black arrow shows the normal thickness of syncytiotrophoblast membrane (STM) which is increased in preeclamptic placental villi.Note increased increased formation of syncytial knots in PE placenta

5.2: Histology of Normal and Preeclamptic placenta

Microscopic examination showed the increase in the number, volume and density of villi in PE compared to controls. But villous surface area and diameter of villi reduced in PE compared to controls. Intervillous space volume is reduced in PE. The endothelial cells of the tunica intima were activated with a cuboidal morphology in the PE group, whereas the endothelial cells of the tunica intima in the control group appeared normal with a flattened morphology. This activation of endothelial cells means that the cells were damaged. Other villous abnormality observed in PE placenta was increased stromal fibrosis, fibrinoid necrosis, thickening of cytotrophoblast basement membrane and increased syncytial knot formation (Figure 5). All the changes observed in PE placenta may be a response of the placenta due to disturbance in the blood flow.

5.3: VEGF

Type of tissue		Overall Score	VEGF		
			STM	CVSC	VVEC
Normal (N=30)		0	0	0	0
		+1	0	0	0
		+2	8	17	28
		+3	22	13	2
Preeclampsia	Mild PE (n=20)	0	0	0	0
		+1	1	10	18
		+2	17	9	2
		+3	2	1	0
	Severe PE (n=20)	0	0	1	3
		+1	7	16	16
		+2	12	3	1
		+3	1	0	0
P			0.000	0.000	0.000
P1(N vs.M-PE)			0.000	0.000	0.000
P2(N vs.S-PE)			0.000	0.000	0.000
P3(M vs.S-PE)			0.628	0.013	0.107

Table 8: Statistical analysis of expression status of VEGF in normal and PE placental tissue. STM: Syncytiotrophoblast membrane; CVSC: Chorionic villous stromal cells; VVEC: Villous vascular endothelial cells. Immunohistochemical staining score: 0, no staining; 1+, 1–10%; 2+, 11–50%; 3+, 51–70%; 4+, 71–100%. P-Value is the level of significance calculated by Kruskal–Wallis rank-sum test. Mann-Whitney U test were used for comparison between two groups

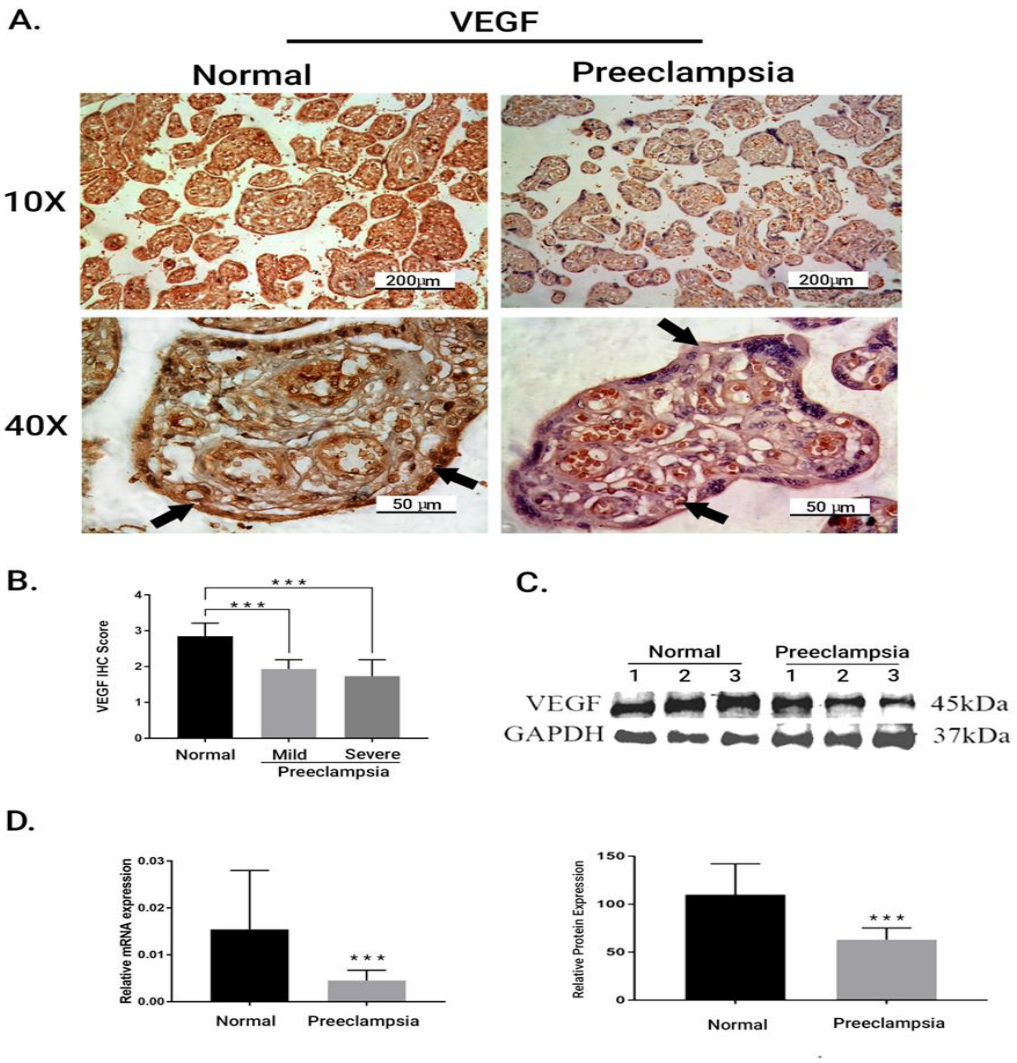

Figure 6: Expression of VEGF in normal and preeclamptic placenta. A. The black arrow heads indicate the intense immunostaining of VEGF165 in the membrane of syncytiotrophoblast in normal placenta, compared to weak immunostaining in the preeclamptic placenta. B. The VEGF staining intensity scores indicate the significant loss of its expression in the mild and severe preeclampsia, compared to normotensives. C &D. Western blot analysis and RT-PCR showed that VEGF in PE placentas were significantly reduced on protein and mRNA levels

5.3.1: Expression of VEGF in Placental villous tissue

Immunostaining of placental villous tissue confirmed the decreased expression of VEGF in the PE group compared to the normotensive control group. In chorionic villous tissue, VEGF staining was noticed in different cells, including membrane of syncyotiotrophoblastic cells, chorionic villous stromal cells and villous vascular endothelial cells. Expression of VEGF in placental villous tissues was semi-quantified. A statistically significant decrease in the VEGF expression was observed in placental villous tissue in both mild and severe PE groups, compared to normal pregnancies (P=0.000). The expression of VEGF in severe preeclampsia is slightly decreased compared to mild PE yet statistically not significant (P=0.628). In normal term placental villi, expression was strong and found chiefly in the membrane of syncytiotrophoblasts. In PE placentas, moderate staining was obtained with VEGF primary antibodies

5.3.2: Expression of VEGF protein in placentas by western blot and Real time PCR.

VEGF expression levels of were confirmed by densitometry. Expression of VEGF was significantly reduced in preeclamptic placentas by 1.7-fold in mild PE and 2.8-fold in severe PE compared to normal placenta (P = 0.0009, 0.007 respectively).

Placental levels of VEGF mRNA as determined by RT-PCR were reduced in women with PE (mild and Severe) compared with normotensive controls. Relative RNA expression of VEGF was reduced by 3.4-fold, P=0.0001. Levels of mRNA (expressed in arbitrary units relative to expression of Beta-actin mRNA). Unpaired t test is used to evaluate the statistical difference.

5.4: ANXA2

Type of tissue		Overall Score	ANXA2		
			STM	CVSC	VVEC
Normal N =30		0	0	0	1
		1	6	13	8
		2	19	17	21
		3	5	0	0
Preeclampsia N =40	Mild PE (n=20)	0	2	0	3
		1	11	14	17
		2	5	16	0
		3	2	0	0
	Severe PE (n= 20)	0	1	0	2
		1	11	15	17
		2	6	5	1
		3	2	0	0
P			0.005	0.048	0.000
P1(N vs.M-PE)			0.04	0.067	0.000
P2(N vs.S-PE)			0.010	0.029	0.000
P3(M vs.S-PE)			0.66	0.727	0.419

Table 9: Statistical analysis of expression status of ANXA2 in normal and PE placental tissue. STM: Syncytiotrophoblast membrane; CVSC: Chorionic villous stromal cells; VVEC: Villous vascular endothelial cells.Immunohistochemical staining score: 0, no staining; 1+, 1–10%; 2+, 11–50%; 3+, 51–70%; 4+, 71–100%. P-Value is the level of significance calculated by Kruskal–Wallis rank-sum test. Mann-Whitney U test were used for comparison between two groups

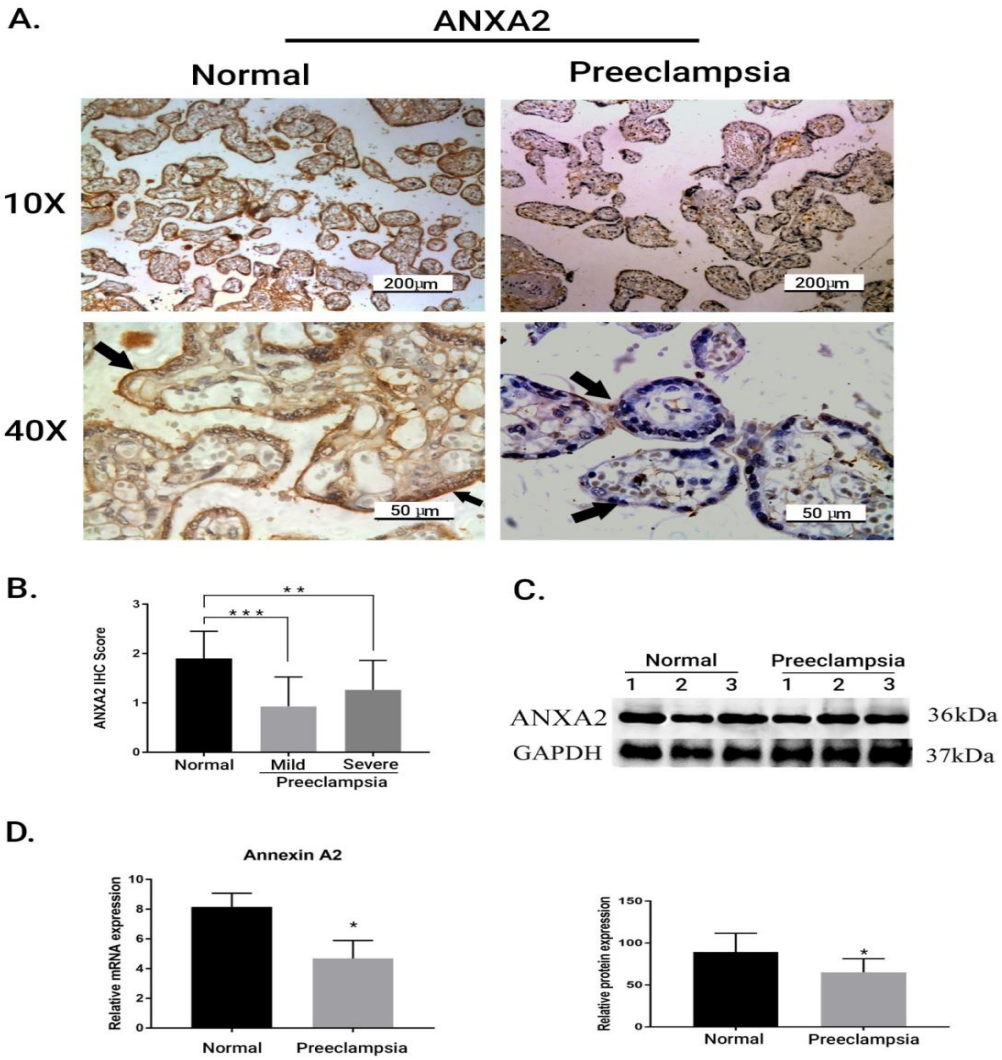

Figure 7 : Expression of ANXA2 in normal and preeclamptic placenta. A. The black arrow heads indicate the intense immunostaining of ANXA2 in the membrane of syncytiotrophoblast in normal placenta, compared to weak immunostaining in the preeclamptic placenta.B.The ANXA2 staining intensity scores indicate the significant loss of its expression in the mild and severe preeclampsia, compared to normotensives.C &D. Western blot analysis and RT-PCR showed that ANXA2 in PE placentas were significantly reduced on protein and mRNA levels.

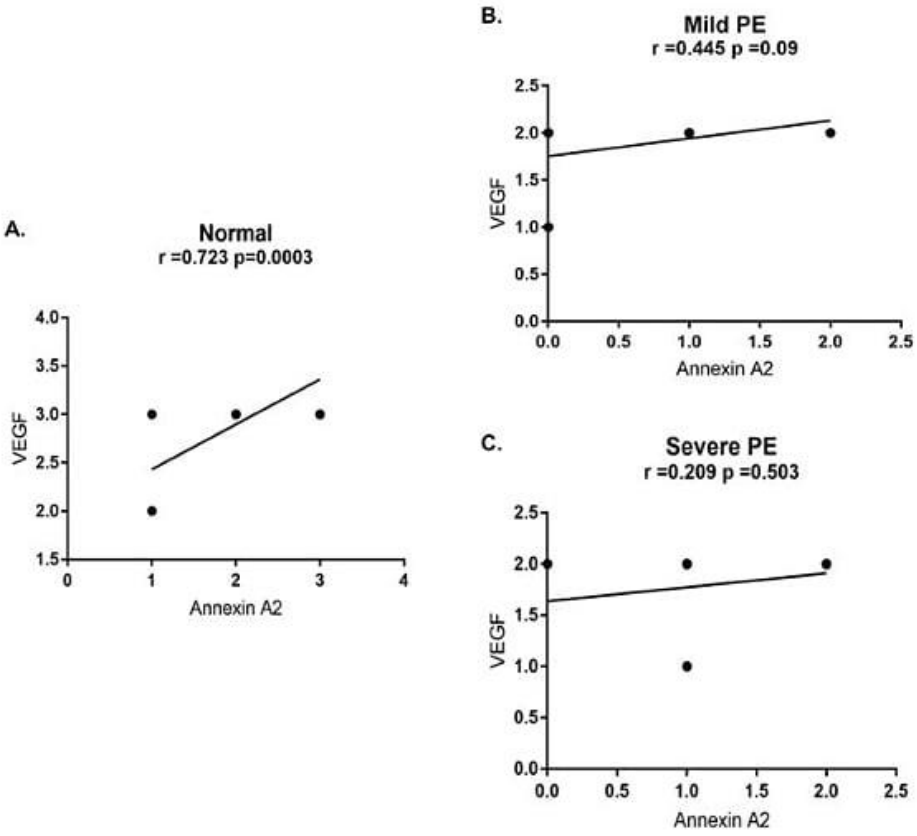

Figure 8: Analysis of correlation (spearman's) of expression of ANXA2 with VEGF in the syncytiotrobhoblast membrane in placental villous tissue showed a statistically significant positive correlation in the expression of VEGF and AnxA2 in in normal term placentas (A), But there was no significant correlation between proteins in PE group with either in mild (B) or in severe cases (C).

5.4.1: Decreased expression of ANXA2 in the placental villous tissue contributes to preeclamptic conditions.

Immunostaining of placental villous tissue confirmed the reduced expression of ANXA2 in the PE group (Mild and Severe) compared to the normotensive control group. Different cellular components were positive for ANXA2, including membrane of syncytiotrophoblastic cells, chorionic villous stromal cells and endothelial cells in both groups of placental villous tissue. Expression of ANXA2 in villous tissues was semi-quantified. A statistically significant decrease in the expression was observed in membrane of syncytiotrobhoblast, chorionic villous stromal cells and villous vascular endothelial cells in PE group (Mild and Severe P=0.04, 0.01) in syncytiotrophoblast compared with placentas of normal pregnancies. The expression of ANXA2 the placentas of severe PE is slightly more than mild PE however not statistically significant (P=0.663). But irrespective of severity ANXA2 is decreased in PE group. Staining wasmoderate in normal term pregnancy placentas, and observed mainly in the membrane of syncytiotrophoblasts. In PE placentas, weak staining was observed with ANXA2 primary antibodies.

5.4.2: Expression of ANXA2 protein in placenta by western blotting and Real time PCR

Expression levels of ANXA2 were confirmed by densitometry. ANXA2 expression was decreased by 1.3 and 1.4-fold in PE placentas (mild and severe) compared to normal placenta (P= 0.0191, P =0.0270). Placental levels of ANXA2 mRNA as determined by RT-PCR were reduced in women with PE (mild and Severe) compared with normotensive controls. Relative RNA expression of ANXA2 in PE (Mild and Severe) is decreased by 1.7-fold compared to normal placenta. Levels of mRNAs are

expressed as arbitrary units (P=0.0299). To evaluate the statistical significance unpaired t - test is used.

5.4.3: Association of expression of VEGF with AnxA2.

Results showed a statistically significant positive correlation in the expression of VEGF and ANXA2 in syncytiotrobhoblast membrane in normal term placentas (r = +0.723) (P<0.0003). But there was no significant correlation in the expression of ANXA2 and VEGF in PE group with either in mild and severe cases. This suggests that association which was maintained in normal placenta was lost in preeclampsia.

5.5: AnnexinA1 and Galectin- 3

Type of tissue		Overall Score	ANXA1		
			STM	CVSC	VVEC
Normal N =30		0	0	1	1
		1	8	24	24
		2	17	5	4
		3	5	0	0
Preeclampsia N =40	Mild PE N = 20	0	0	0	0
		1	0	11	14
		2	11	9	6
		3	9	0	0
	Severe PE N = 20	0	0	1	1
		1	2	11	13
		2	10	8	6
		3	8	0	0
P			0.011	0.075	0.278
P1(N vs.M-PE)			0.004	0.025	0.117
P2(N vs.S-PE)			0.042	0.116	0.241
P3(M vs.S-PE)			0.020	0.630	0.817

Table 10: Statistical analysis of expression status of ANXA1 in normal and PE placental tissue.STM: Syncytiotrophoblast membrane; CVSC: Chorionic villous stromal cells; VVEC: Villous vascular endothelial cells. Immunohistochemical staining score: 0, no staining; 1+, 1–10%; 2+, 11–50%; 3+, 51–70%; 4+, 71–100%. P-Value is the level of significance calculated by Kruskal–Wallis rank-sum test. Mann-Whitney U test were used for comparison between two groups

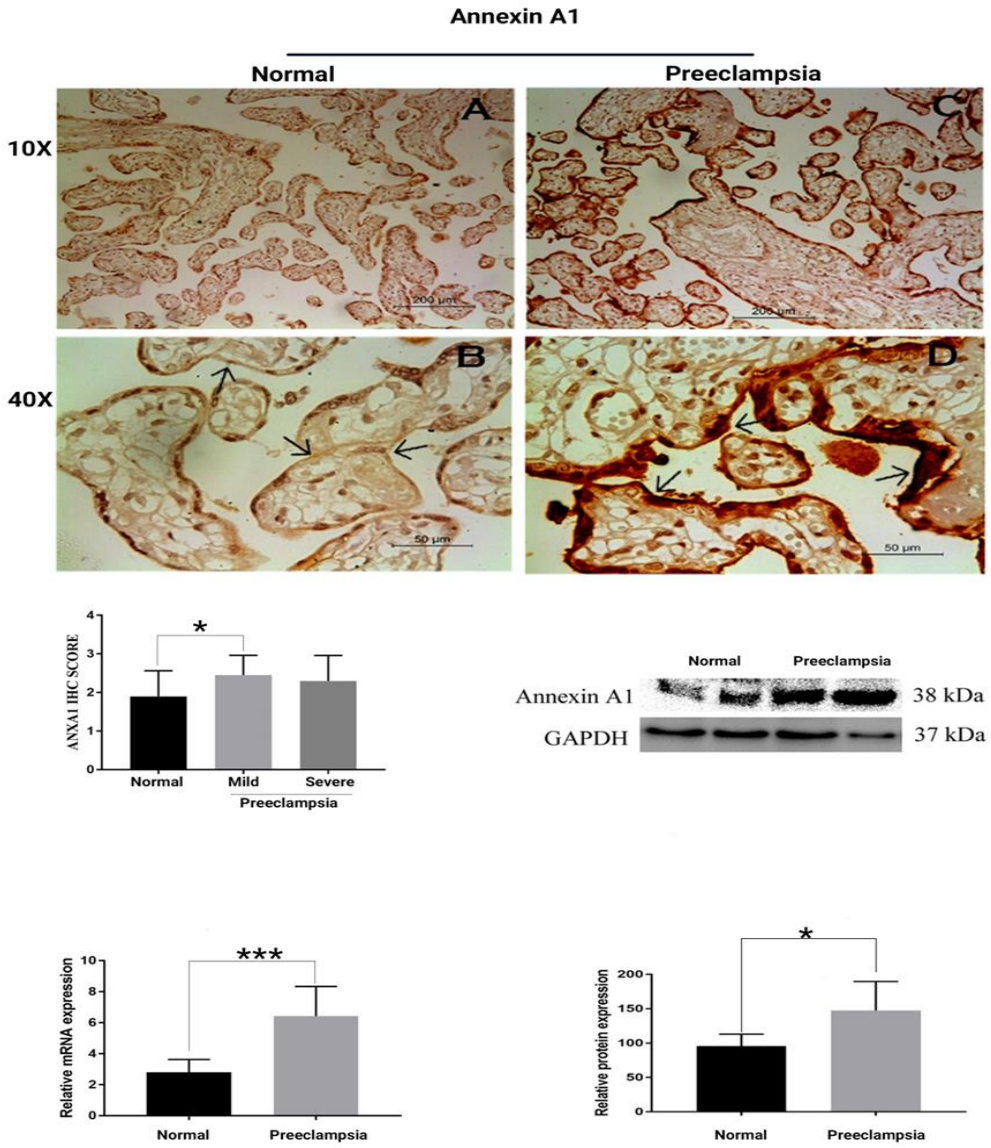

Figure 9: Expression of ANXA1 in normal and preeclamptic placenta. A. The black arrow heads indicate the intense immunostaining of ANXA1 in the membrane of syncytiotrophoblast in Preeclamptic placenta, compared to weak immunostaining in the normal placenta. B. TheANXA1 staining intensity scores indicate the significant increase in expressionPE compared to normotensives. C&D. Western blot analysis and RT-PCR showed that ANXA1 in PE placentas were significantlymore on protein and mRNA levels

Type of tissue		Overall Score	Galectin-3		
			STM	CVSC	VVEC
Normal N =30		0	0	10	1
		1	13	12	24
		2	17	8	4
		3	3	0	0
Preeclampsia N =40	Mild PE N = 20	0	0	4	0
		1	5	13	14
		2	9	3	6
		3	6	0	0
	Severe PE N = 20	0	0	1	3
		1	3	11	11
		2	11	8	6
		3	6	0	0
P			0.040	0.526	0.0253
P1			0.073	0.897	0.136
P1(N vs.M-PE)			0.017	0.317	0.207
P2(N vs.S-PE)			0.680	0.383	0.841
P3(M vs.S-PE)			0.798	0.503	0.805

Table 11 : Statistical analysis of expression status of GAL-3 in normal and PE placental tissue. STM: Syncytiotrophoblast membrane; CVSC: Chorionic villous stromal cells; VVEC: Villous vascular endothelial cells.Immunohistochemical staining score: 0, no staining; 1+, 1–10%; 2+, 11–50%; 3+, 51–70%; 4+, 71–100%. P-Value is the level of significance calculated by Kruskal–Wallis rank-sum test. Mann-Whitney U test were used for comparison between two groups

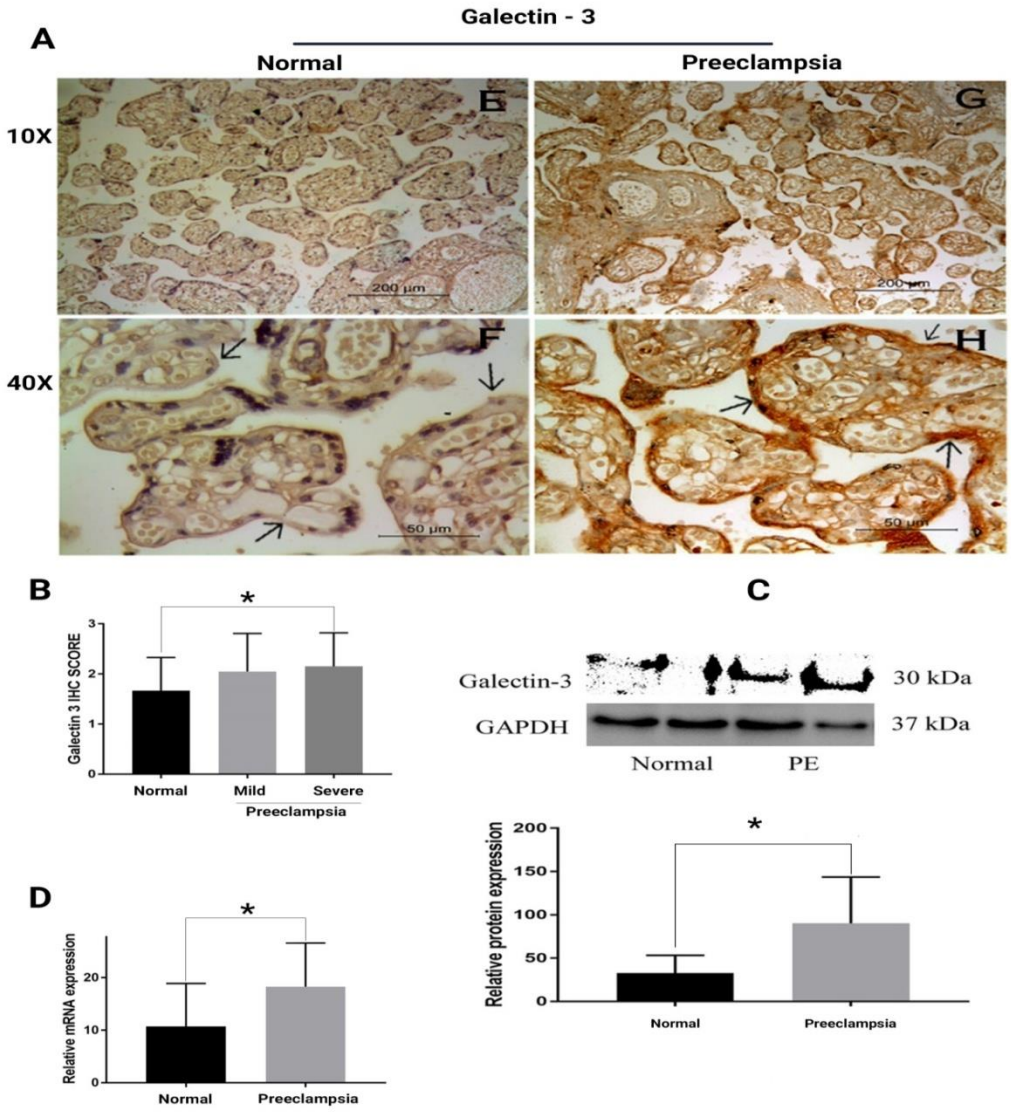

Figure 10 -Expression of Gal-3 in normal and preeclamptic placenta. A. The black arrow heads indicate the intense immunostaining of Gal-3 in the membrane of syncytiotrophoblast in Preeclamptic placenta, compared to weak immunostaining in the normal placenta. B. The Gal-3 staining intensity scores indicate the significant increase in expression preeclampsia, compared to normotensives.C &D.Western blot analysis and RT-PCR showed that GAL-3 in PE placentas were significantly more on protein and mRNAlevels

5.5.1: Expression of AnnexinA1 and Galectin 3 by Immunohistochemistry.

Immunostaining of placental bed sections confirmed the increased expression of ANXA1 and Gal-3 in PE group compared to the normotensive control group. Several different cell types in both placental villi of the PE and normotensive control groups were positive for ANXA1 and Gal-3 including of syncyotiotrophoblastic cells, chorionic villous stromal cells, and villous vascular endothelial cells Expression in placental villous tissues was semi-quantified. In preeclamptic placentas, staining was strong and located predominantly in the syncytiotrophoblasts and mild staining was observed in villous stromal cells. In normal placenta moderate staining was obtained with ANXA1 and Gal-3 primary antibodies.

5.5.2: Expression of Annexin A1 and Galectin 3 by western blot and Real time PCR in placentas

In western blot ANXA1and Gal-3 expression is increased in PE placenta by 3.2 and 3.14-fold respectively compared to normal placenta. Relative mRNA expression of ANXA1 and Gal-3 was increased by 2.2 9-fold and 1.6-fold and in placenta in PE placenta compared to controls ($P < 0.005$).

5.5.3: Correlation in the expression of Annexin A1 and Galectin 3

We also studied the statistical spearman correlation of expression level of ANXA1 and Gal-3 as both proteins are involved in modulation of inflammation. A statistically significant correlation in the expression of ANXA1 and Gal-3 was observed in syncytiotrophoblast membrane ($P< 0.05$).

5.6: tPA and PAI-1

Type of tissue		Overall Score	tPA	
			STM	CVSC
Normal N =30		0	11	11
		1	19	19
		2	0	0
		3	0	0
Preeclampsia N =40	Mild PE N = 20	0	2	2
		1	17	18
		2	1	0
		3	0	0
	Severe PE N = 20	0	3	3
		1	16	16
		2	1	1
		3	0	0
P			0.036	0.048
P1(N vs.M-PE)			0.023	0.037
P2(N vs.S-PE)			0.064	0.064
P3(M vs.S-PE)			0.697	0.965

Table 12: Statistical analysis of expression status of tPA in normal and PE placental tissue. STM: Syncytiotrophoblast membrane; CVSC: Chorionic villous stromal cells. Immunohistochemical staining score: 0, no staining; 1+, 1–10%; 2+, 11–50%; 3+, 51–70%; 4+, 71–100%. P-Value is the level of significance calculated by Kruskal–Wallis rank-sum test. Mann-Whitney U test were used for comparison between two groups

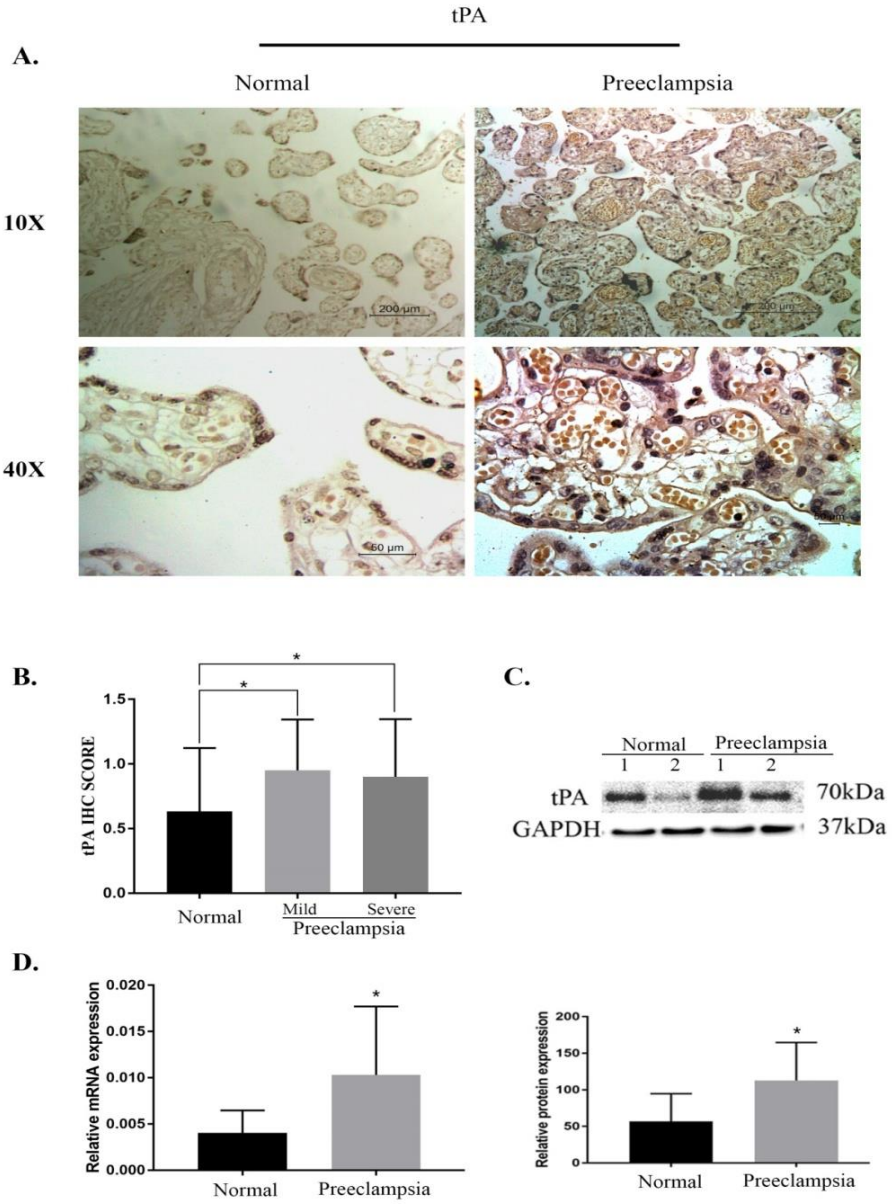

Figure 11: Expression of tPA in normal and preeclamptic placenta. A. Note weak immunostaining of tPA in the membrane of syncytiotrophoblast in normal placenta, compared to moderate immunostaining in the preeclamptic placenta. B. The tPA staining intensity scores indicate more expression of tPA in preeclamptic placenta compared to normal. C &D. Western blot analysis and RT-PCR showed that tPAin PE placentas were significantly more on protein and mRNA levels

Type of tissue		Overall Score	PAI-1	
			STM	CVSC
Normal N =30		0	9	11
		1	19	19
		2	2	0
		3	0	0
Preeclampsia N =40	Mild PE N = 20	0	1	2
		1	16	18
		2	3	0
		3	0	0
	Severe PE N = 20	0	3	3
		1	12	16
		2	5	1
		3	0	0
P			0.056	0.048
P1(N vs.M-PE)			0.031	0.011
P2(N vs.S-PE)			0.065	0.285
P3(M vs.S-PE)			0.946	0.211

Table 13: Statistical analysis of expression status of PAI-1 in normal and PE placental tissue. STM: Syncytiotrophoblast membrane; CVSC: Chorionic villous stromal cells. Immunohistochemical staining score: 0, no staining; 1+, 1–10%; 2+, 11–50%; 3+, 51–70%; 4+, 71–100%. P-Value is the level of significance calculated by Kruskal–Wallis rank-sum test. Mann-Whitney U test were used for comparison between two groups

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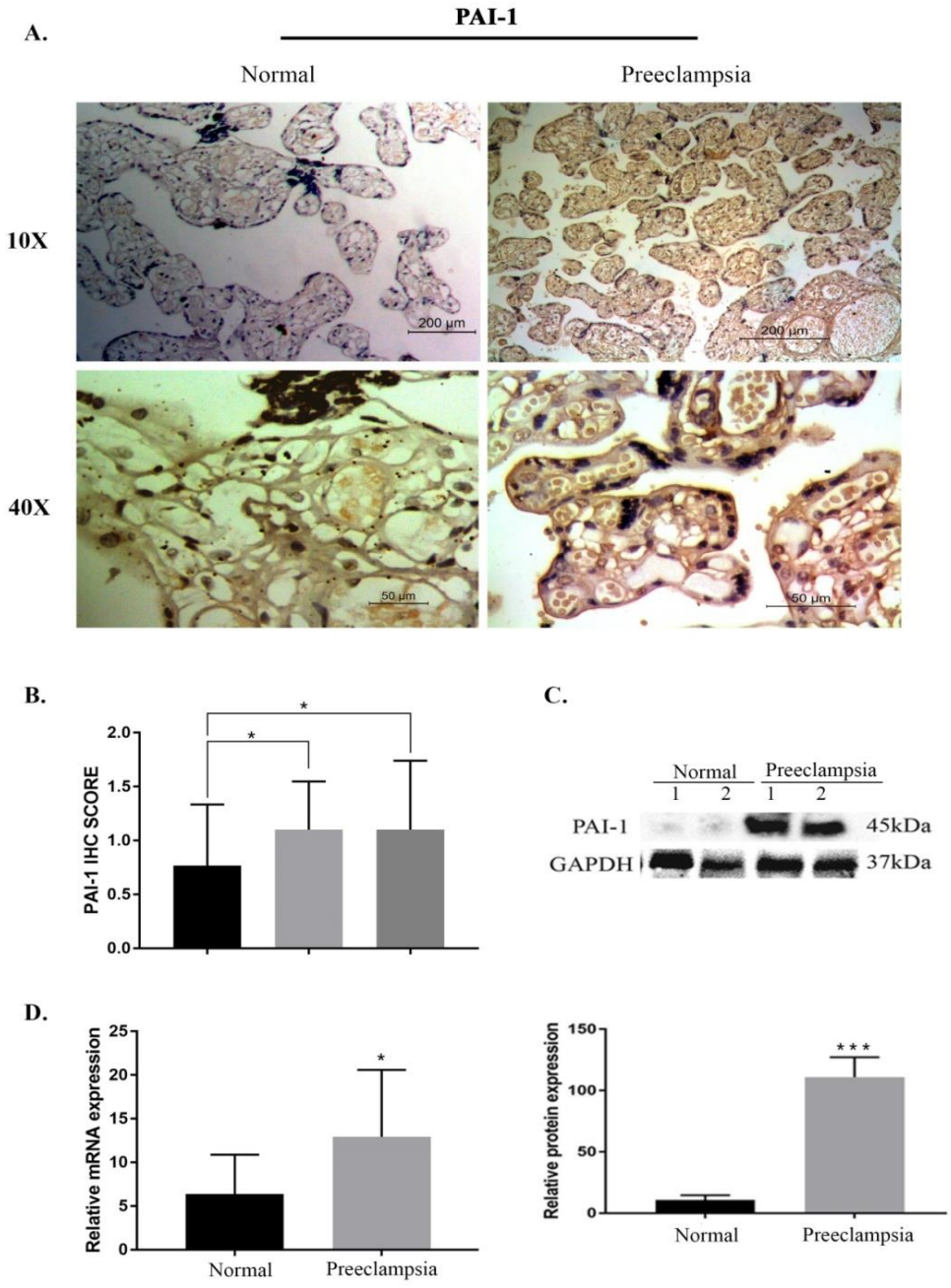

Figure 12 : Expression of PAI-1 in normal and preeclamptic placenta. A. Note weak immunostaining of PAI-1 in the membrane of syncytiotrophoblast in normal placenta, compared to intense immunostaining in the preeclamptic placenta. B. The PAI-1 staining intensity scores indicate more expression of PAI-1 in preeclamptic placenta compared to normal. C &D. Western blot analysis and RT-PCR showed that PAI-1 in PE placentas were significantly more on protein and mRNA levels

5.6.1: Expression of tPA and PAI-1 by Immunohistochemistry.

Most of the cases were negative or weak expressed for tPA in normal placenta, moderate expression was detected predominantly in syncytiotrophoblast membrane and villous stromal cells in preeclamptic placenta. PAI-1 staining was observed at the membrane of syncytiotrophoblast, villous stromal cells, and villous vascular endothelial cells. Qualitative analysis of PAI- 1 revealed weaker staining in the placentas of the control group than that in the PE (mild and severe PE) group ($P< 0.005$).

5.6.2: Expression oftPA and PAI-1 by western blot and Real time PCR in placentas

Expression levels of proteins were confirmed by densitometry. tPA and PAI- 1 increased by 1.7-fold and 9.7-fold so the increment of PAI-1 is much greater than increment of tPA in PE**.** The expression profiles of tPA and PAI-1 were examined by RT-PCR analysis. mRNA expression of tPA and PAI-1 was increased in pre-eclamptic placentas compared to normal placenta by 2.5 and by 2.03 fold ($P< 0.05$)

5.6.3: Correlation of tPA andPAI-1 with ANXA2

ANXA2 expression was positively correlated with tPA ($r=+0.895$, $p=0.002$) and negetively correlated with PAI-1($r=-0.905$, $p=0.020$) in controls. ANXA2 expression was negetively correlated with tPA (($r=-0.801$, $p=0.016$) and PAI-1 ($R=-0.831$, $P=0.010$) in PE group.

5.7: EGFR

Type of tissue		Overall Score	EGFR		
			STM	CVSC	VVEC
Normal N =30		0	1	4	6
		1	8	23	24
		2	17	3	0
		3	4	0	0
Preeclampsia N =40	Mild PE N = 20	0	2	10	9
		1	11	9	11
		2	4	1	0
		3	3	0	0
	Severe PE N = 20	0	3	0	8
		1	10	7	12
		2	7	13	0
		3	0	0	0
P			0.019	0.017	0.136
P1(N vs.M-PE)			0.056	0.009	0.061
P2(N vs.S-PE)			0.007	0.033	0.127
P3(M vs.S-PE)			0.614	0.465	0.752

Table 14: Statistical analysis of expression status of EGFR in normal and PE placental tissue. STM: Syncytiotrophoblast membrane; CVSC: Chorionic villous stromal cells; VVEC: Villous vascular endothelial cells. Immunohistochemical staining score: 0, no staining; 1+, 1–10%; 2+, 11–50%; 3+, 51–70%; 4+, 71–100%. P-Value is the level of significance calculated by Kruskal–Wallis rank-sum test. Mann-Whitney U test were used for comparison between two groups

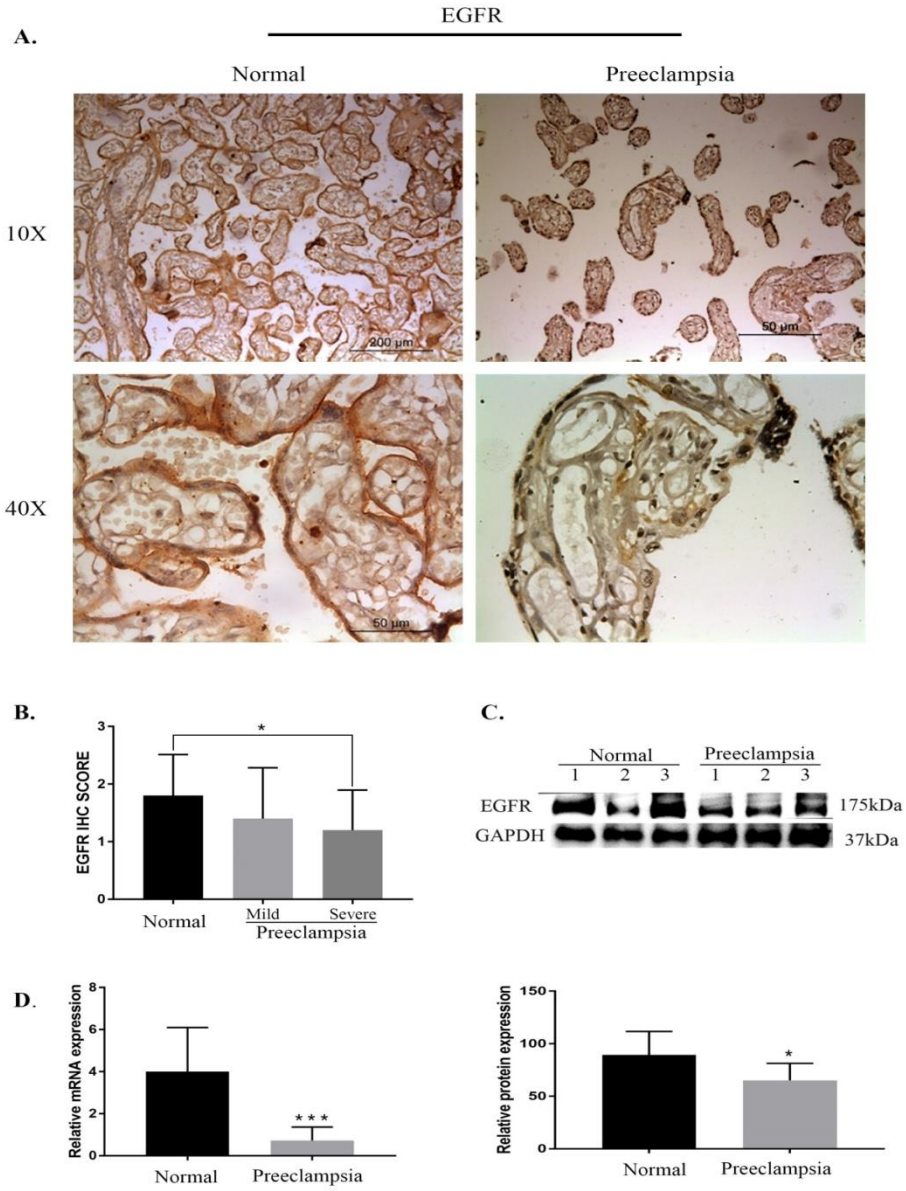

Figure 13: The expression of EGFR in normal and preeclamptic A. Immunohistochemical expression of EGFR antibodyshows strongimmunostaining in the membrane of syncytiotrophoblast in normal placenta compared to mild expression in preeclamptic placenta. B) The EGFR staining intensity scores indicate reduced expression of in preeclamptic placenta compared to normal. C&D: Western blot analysis and RT-PCR showed that EGFRin PE placentas were significantly lower on protein and mRNA levels.

5.7.1:EGFR expression by Immunohistochemistry

We identified a moderate staining intensity of EGFR in syncitiotrohoblast membrane and weak staining intensity in chorionic villous stromal cells, villous vascular endothelial cells, and vascular smooth muscle cells in normal as well as in PE placenta.In normal placenta staining was strong compared to PE placenta ($P<0.05$). The expression of EGFR in severe preeclampsia is slightly decreased compared to mild PE yet statistically not significant ($P=0.614$)

5.7.2 EGFR expression by western blotting and Real time PCR

In western blot expression of EGFR is decreased in PE placenta by 1.67-fold compared to normal placenta. Relative mRNA expression of was decreased by 4.8-fold in normal placenta compared to Preeclampsia ($P < 0.005$).

Chapter 6

Discussion

Plasminolytic components and their receptors in pathogenesis of preeclampsia

6: Discussion

Expression of ANXA2 and VEGF

This study showed decreased expressions of VEGF and ANXA2 in the third trimester placental bed from pregnancies with PE compared to normotensive control group. Although the mechanisms responsible for the pathogenesis of preeclampsia are poorly understood, there is an agreement that it is associated with reduced invasion and failed remodelling of maternal endometrial spiral arteries in the placenta. [1] There is growing evidence that deficient trophoblastic invasion due to altered fibrinolysis in the placental bed spiral arteries is crucial to the pathogenesis of PE.[2, 3] The primary established pathology in PE resides in the reduced trophoblastic implantation and placental perfusion.[4] In the present study, we observed the altered morphology of the villous vascular endothelial cells of the fetal capillary of preeclamptic placenta. Shape of endothelial cells was altered from normal flattened to cuboidal morphology showing that the endothelial cells of the placental villi were more damaged in the PE group compared to the normotensive group. VEGF has been shown to be involved in the regulation of trophoblast cell survival, migration, endovascular differentiation and proliferation. [5] Placenta endures dramatic vascularization in the course of normal pregnancy to allow the circulation between foetus and mother. The main pathogenic mechanism underlying PE is placental ischemia which results in hypoxia, which is a potent stimulator for VEGF production.[6] Several studies have reported levels of VEGF in the serum were increased in PE patients compared to normotensive patients because of placental hypoxia causing from placental ischemia.[7, 8] Though, other studies have presented decreased VEGF production in PE placenta compared to normotensive placenta.[9, 10] It may be a reason that as a compensatory mechanism to

hypoxia the production of VEGF may be initially increased. Nevertheless, it may cause damage to endothelial cells and gradually reduces the production of VEGF, if hypoxia becomes more advanced, finally resulting in development of PE.

For regulating early placental angiogenesis and remodelling of maternal artery VEGF family of angiogenic growth factors and the receptors are important molecules.[11,12] This result suggests the possibility that decreased VEGF expression in the placentas may be a cause of failure of remodelling of spiral artery, later resulting in PE. From a single VEGF-A, spliced variants of 121, 145, 165, 189 and 206 amino acids are generated.[13, 14] Study of placental villi of human first-trimester showed that VEGF isoforms 121, 165, and 189 are expressed in placenta, with a noted prominence of the VEGF-165 isoform.[15] Current evidence indicated, in patients with proliferative diabetic retinopathy the correlation between increased levels of VEGF, tissue plasminogen activator (tPA) and plasminogen activator inhibitor (PAI-1) indicating direct relation between VEGF and fibrinolysis.[16] There is also evidence that ANXA2 might induce neovascularisation through VEGF- VEGF R2 pathway in ischemia induced retina neovascularization.[17] For the formation of new blood vessels during neovascularisation, extracellular proteolysis is crucial requirement. Plasmin which degrades several components of the ECM like laminin and fibrin is a central component of neovascularisation. Plasmin further increases the bioavailability of angiogenic VEGF.[18] Interaction between different components are required for unique sequential process of fibrinolytic activity. ANXA2, is one of the key mediators converts plasminogen to plasmin. Profibrinolytic molecule ANXA2, serves as a cell surface coreceptor for plasmin generation and localizing fibrinolytic activity on the surface of the cell when it binds with plasminogen and its activator, tPA.[19, 20, 21] The efficiency in plasmin generation is increased by 60 fold when Purified ANXA2 binds

to plasminogen and tPA.[19] In both human diseases and animal models, it has been shown that deficient ANXA2 dependent fibrinolytic pathway has been connected to increased intravascular thrombosis.[20, 21] Probably ANXA2 deficiency may be one of the causes for increased clot formation in placenta. In acute promyelocyticleukemia cells abnormally high levels of ANXA2 leads to increased plasmin generation leading to haemorrhagic conditions.[22] Growing evidences suggests that ANXA2 expression and its binding with plasminogen and tPA play a crucial role in maintaining fibrinolytic balance on the surfaces of blood vessel.[23] In our study the expression of VEGF is reduced in the placental villi is reduced, probably this has triggered PE by reduced vascularisation in preeclamptic placenta. We confirmed the expression status of these proteins by IHC, Western blot and RT-PCR. Our immunohistochemical analysis showed that both ANXA2 and VEGF were mainly expressed on the membrane of syncytiotrophoblasts and weak expression was found in endothelial cells of foetal capillary in the placental villi. Location of VEGF and ANXA2 in placenta of preeclampsia was the same when compared to control group. But the expression of both VEGF and ANXA2 was lower in placentas in patients with PE irrespective of severity compared with normal pregnancies which suggested a decrease in production of these proteins. The decreased expression of ANXA2 protein in placental tissues may possibly weaken the local fibrinolytic activity by decreasing the plasmin generation. The PE placenta often shows infarctions and fibrin deposition which reveals haemostatic system failure. Defects of fibrinolytic system are well known risk factors for increased thrombosis and alterations of fibrinolysis have been shown to be present in PE, indicating a role for fibrinolytic abnormality in the development of the disease.[24,25] Recent evidence concluded that, in placentas as well as in maternal blood of patients with PE, expression of ANXA2 was significantly down regulated. The

decreased expression of ANXA2 provides evidence to an impaired fibrinolytic activity, which may lead to increased thrombin formation in placenta of PE.[26] The present study is consistent with these findings. We also observed that prothrombin time was prolonged in patients with PE suggesting altered fibrinolytic activity. In the process of angiogenesis, ANXA2 is known to perform as angiogenic regulator. VEGF up regulates ANXA2 production has been demonstrated in previous studies. In the current study, we observed the significant correlation in the expression of VEGF and ANXA2 in syncytiotrophoblast membrane in control group. This directs that, physiologically expression of these two factors may be dependent and regulated in the placental bed in response to the same stimulus like hypoxia, but this correlation is disturbed in preeclampsia. In the present study, placental bed biopsies showed significant difference among PE and normotensives for VEGF and ANXA2 expression. These results show the probable significance of expression of ANXA2 in placentas as a more effective biomarker in the prediction of the development PE. Although further studies with a greater number of patients, should be carried out to verify this possibility.

The present study showed decreased expression of VEGF and ANXA2 in the PE placental villous tissue in comparison with the normotensive control. The reduced expression of above angiogenic proteins in the placental tissue may be linked with the development of PE.

Expression of ANXA2, tPA and PAI-1

The present study clearly demonstrates that expression of ANXA2 is decreased while the expression of tPAand PAI-1 is increased in placenta of PE.

The placenta plays a crucial role in the pathogenesis of PE, particularly in the severe onset forms of the syndrome. The initial insult occurs early at the placentation site with shallow endovascular trophoblastic invasion and defective remodeling of the maternal spiral arteries, which leads to placental insufficiency caused by dysfunctional perfusion.[27] Placental dysfunction has been considered to play a vital role in the pathogenesis as well as in the prognosis of the PE disease.[28] The imbalance of haemostasis observed in normal pregnancy seems to be increased in PE. [29] Damage of endothelial cell or activation is believed to play an important role in PE and may underlie the haemostatic changes observed in this syndrome.[30] While normal endothelial cells participate in the regulation of haemostasis, perturbed vascular cells may express prothrombotic changes promoting pathologic events.[31] Microscopic and immunohistochemical studies have showed diffuse fibrin deposits in the placentas of patients with PE compared with those of normal pregnancies.[32] Fibrinolytic mechanisms is unique process that is rigidly controlled by a series of cofactors, inhibitors, and receptors of the plasminolytic components.[33] During normal pregnancy, overall fibrinolytic mechanism is depressed. Fibrinolysis activators t-PA and u-PA gradually increase, balanced by increased levels of PAI-1 which is a key regulator of fibrinolysis in vivo.[34] One of the key mediators involved in the conversion of plasminogen to plasmin is ANXA2. This profibrinolytic molecule serves as a surface receptor protein that binds both plasminogen and its activator, tPA, functioning as a co-factor for plasmin generation and localizing fibrinolytic activity to

the cell surface.[35] Plasmin is a highly reactive enzyme that is physiologically involved in the process of fibrinolysis and plays an important role in neoangiogenesis.

IHC analysis showed that ANXA2 was mainly expressed on the syncytiotrophoblast cell membrane, stromal cells and villous vascular endothelial cells in the placental villi of normal pregnancies. We observed that expression of ANXA2 protein was much lower in placentas in patients with PE compared with normal pregnancies. Subsequently the expression of ANXA2 mRNA was much lower as well. tPA and PAI- 1 was mainly expressed syncytiotrophoblast membrane and their detected expression was much lower in normal placenta. By IHC and Western blotting we found the expression of tPA and PAI-1 both are increased but the increment of PAI-1 is much larger than tPA. Accordingly their mRNA expression was increased in PE placenta. Despite the decreased fibrinolytic activity in pregnancy, a number of studies have shown that the expression of tPA which is the target molecule of ANXA2 was actually increased in patients with PE,[36] indicating predominant role of PAI-1 for depressing the fibrinolytic mechanism in PE.

Deficient ANXA2 dependent fibrinolytic pathway has been correlated to increased intravascular thrombosis in both human disease and animal models. Several animal studies support the hypothesis that ANXA2 regulates hemostasis in vivo. ANXA2 knockout mice, while displaying uncompromised development, fertility, and lifespan, accumulate fibrin in both intra- and extravascular locations within the lungs, spleen, small intestine, liver, and kidney.[37] ANXA2 alone or in combination with tPA enhances vascular patency and reduces infarct size in several rodent models of stroke.[38] The evidence suggesting that PAI-1 may play a role in cellular migration and invasion through the ECM pertains to both normal processes like placentation, as well as certain disease processes such as tumor invasion and metastasis. High levels of

PAI-1 indicate poor prognosis from a variety of malignancies including breast. Elevated levels of PAI-1 would result in fibrin deposition and occlusive lesions leading to thrombosis of the intervillous or spiral arteries and hence placental ischemia.[39] This is supported by the finding in the present study in which of expression endothelially derived tPA and PAI-1 were significantly higher in placenta of PE than the control placenta and the increment of PAI-1 is greater than the increment of tPA in PE, suggesting its decreased fibrinolytic mechanism in PE.

Expression of Annexin A1 and Galectin -3

This study showed increased expressions of ANXA1 and Gal-3 in the third trimester placental bed from pregnancies with PE compared with the normotensive control group. The result of western blotting and Real time PCR in the study revealed an increased expression of AnxA1 and Gal-3 in the preeclamptic placental samples compared to normotensive placenta. The increased expression of protein in different placental compartment was confirmed by IHC analysis. IHC findings revealed protein is expressed strongly in syncytiotrophoblast layer of the preeclamptic placental villous in comparison to controls. Expression of ANXA1 has been thoroughly studied in models of sterile inflammation, recognising its central role as key modulator of both of the innate and adaptive immune systems.[40] However, in the context of Preeclampsia only one study have been reported on the altered expression of ANXA1 in the plasma.[41] Very little is known about the expression status of ANXA1 in PE. Our study shows the differential expression of ANXA1 in the placenta of normotensive and preeclamptic women. This result was consistent with the study by Luiza O. Perucci et al who measured the protein in the plasma.[41] This result implies that the increase ANXA1 expression in PE placental bed could be an important factor in the aetiology of PE. In normal pregnancy it is established that there is a homeostatic

balance between inflammatory and regulatory response[42], which suggests that regulatory molecular mechanisms are sufficient to reduce the mild inflammatory response. PE is associated with chronic activation of immune system which leads to an increased production of inflammatory cytokines by pro-inflammatory T cells, and a decrease in regulatory and anti-inflammatory cytokines, which further promotes an inflammatory state during PE.[43,44] In preeclamptic pregnancy, the imbalance between pro-inflammatory and regulatory cytokines is correlated with placental ischemia. This imbalance exacerbates as the pregnancy progresses.[45] ANXA1 has been shown to be capable of regulating a large number of biological events such as chronic inflammation, growth of the tissue, and programmed cell death. It has been shown that the decreased expression of ANXA1 is associated with the development of more severe inflammation in inflammatory diseases.[46] Additionally, ANXA1 helps in monocyte augmentation and elimination of apoptotic leukocytes by macrophages, resulting in reduced production of pro-inflammatory cytokines and increased release of immunosuppressive and pro-resolving molecules.[47]

Our data may imply that ANXA1 is increased in patients with preeclampsia in an attempt to attenuate the exacerbated inflammatory response in these patients. Chronic inflammation in PE suggests that the resolution of inflammation pathway is dysfunctional. Consequently increased ANXA1 expression seems to be inadequate to resolve inflammation. In other chronic inflammatory disease such as inflammatory bowel disease and Alzheimer's disease systemic levels of proresolving mediators are increased.[48] In acute inflammatory response during the initiation phase, mediators derived from arachidonic acid become up-regulated and contribute to changes in vascular permeability and Polymorphonuclear leukocytes recruitment. However, the generation of these pro-inflammatory mediators is in due course terminated by

successive dynamic changes in prostaglandins E2 and D2.[49] This can be seen as switch where elevated levels of pro resolving mediators, including pro-resolving lipoxins A4 decreases inflammatory molecules such as prostaglandins, leukotrienes, and cytokines.[50] Pro-resolving and anti-inflammatory actions of ANXA1 are mediated by a G-protein-coupled receptor named formyl peptide receptor like-2 (FPR2)/lipoxin A4 receptor (ALXR).[51] Decreased ALX expressions has been observed in patients with asthma, a chronic inflammatory disease.[52] These mechanisms might explain the noticeable ineffectiveness of ANXA1 up-regulation in some human inflammatory diseases. More studies are required to solve the mystery whether these dysfunctional mechanisms in ANXA1 resolution pathway are present in PE.

Proteins from galectin family have emerged as master regulators of immune system homeostasis, playing central role in the amplification and/or resolution of inflammatory processes. Gal-3 functions as pro- or anti-inflammatory activities depending on various factors including its intracellular or extracellular localization and the target cell involved in these processes.[53] Even though it may contribute to resolution of inflammation by clearing apoptotic neutrophils,[54] this lectin exhibits mostly pro-inflammatory effects by increasing activation of macrophages, mast cells, and natural killer cells, as well as T and B lymphocytes.[55] Studies have shown that Gal-3 has a role in implantation of embryo, embryogenesis and placental formation, and is closely connected with the success and maintenance of pregnancy.[56] Proliferation and programmed cell death are crucial components of the trophoblast life cycle. There are aberrant cell turnover including an increased apoptosis in placental villous trophoblast of preeclamptic pregnancies.[57] Numerous studies suggest that high expression of Gal-3 exerts regulatory effects on apoptotic responses of various cell types.[58] Our results may suggest that the marked increase in expression of

Gal-3 in the syncytiotrophoblast cells in preeclamptic placenta could be important to turn on the intracellular machinery of these cells required for defence against a rapid process of apoptosis. In our earlier study, we have reported the interaction of Gal-3 and ANXA2 resulting in the cancer progression in Triple negative breast cancer cells.[59] ANXA2 is the pro-inflammatory molecule and ANXA1 being the anti-inflammatory, which are reciprocally regulated.[60] As far as PE is concerned, ANXA2, a (proinflammatoryfibrinolytic) protein level decreases and which should result in simultaneous increase in ANXA1 and which we are seeing in our current data. With respect to ANXA2 and ANXA1 reciprocal regulation, but increase in Gal-3 in preeclampsia reveals that it could be the apoptotic activity of the protein not the proliferative function which associates with ANXA2 is the causative factor in preeclampsia. Present study data illustrates that, there is a significant positive correlation in the expression of ANXA1 and Gal- 3.These facts presumably indicate that expression of these two factors may be dependent and regulated in the placental bed in response to the same pathogenic stimulator such as inflammation. ANXA1 and Gal-3 undergo changes in their content and localization when neutrophil adheres to the endothelium, and this could be indicative of a process of favouring and counter-balancing between two endogenous anti- and pro-inflammatory mediators.[61]

Expression of EGFR

EGFR is one of the most avidly studiedmolecules with important roles in both physiological and pathological states.[62, 63] After fertilization and repeated mitotic activity, the blastocyst is formed. The blastocyst which is outer layer is trophoblast (TB), a specialized epithelial cell layer with the capability to proliferate and differentiate near the placenta. In the post implantation period, TB is proliferating at an accelerated rate giving rise to two specific cell lines, mononucleated

cytotrophoblast (CTB) and multinucleated syncytiotrophoblast (STB). Later on, TB is divided into villous trophoblast (VTB) lining the placental villi and extravilloustrophoblast (EVTB) with the ability to invade maternal spiral arteries in the process of so-called endovascular remodeling, thus allowing for the newly formed placental tissue and embryo constant and fluent nutrition supply.[64] Numerous growth factors and their receptors, hormones, and cytokines regulate TB differentiation and the EVTB remodeling process. One of the crucial growth factors during placental development is the EGF and its receptor EGFR. EGF is a polypeptide composed of 53 amino acids with the ability to provoke the mitogenic effect on epidermal and mesothelial cells.[65] Binding of EGF and EGFR stimulates intracellular tyrosine kinase activity, which induces phosphorylation of the receptor resulting in cellular growth, proliferation, differentiation, migration, and sometimes apoptosis. [66] During the 4th and 5th weeks of gestation, EGF and EGFR are primarily expressed on the CTB while later on the expression is shifted to STB, demonstrating a dual role of the EGF–EGFR complex: TB proliferation and later on TB differentiation.[67, 68] The main idea behind the study was to study the expression pattern of EGFR in PE placenta indifferent placental compartment with respect to their severity and compare them with a control group. Our results show EGFR was expressed on the membrane of syncytiotrophoblast, Chorionic villous stromal cells and villous vascular endothelial cells of the placenta and the expression of EGFR is reduced in all the cellular compartments in PE placenta compared with normal with more reduction in severe PE compared to moderate PE.

So far, reports on the EGF and EGFR expression have been limited to the first trimester placental tissue and only a few of them refer to placentas from PE pregnancies. The results of those studies were dubious; some demonstrated higher

levels of studied factors in PE placentas, whereas others report on lower or unchanged levels. Proliferation is crucial in earlyplacental development both in TB and DC; furthermore, it is highly connected with the EGF–EGFR complex since they act synchronously.[69]

Untill now few studies have reported on this topic, findings reported by Ferrandina et al. suggest that hypertensive disorders in pregnancy are associated with elevated placental EGFR concentrations detected by the radioreceptor technique.[70]

Milchev et al. reported on lower EGFR expression in VTB of PE placentas, while Dong et al. reported on lower EGFR expression in VTB of placentas with pregnancy induced hypertension.[71, 72]

Our study points that the decreased expression of ANXA2 , and increased expression of tPA and PAI-1 , increased expression of ANXA1 and GAL-3 ,decreased expression of VEGF and EGFR shows altered plasminolytic,inflamematory, and angiogenic pathway. Altered expression of these proteins could be of clinical relevance in designing the new standard markers in PE. The expression of proteins in the syncytiotrophoblastmembrane, chorionic stromal cells and andfetal endothelial cells of the placental villi may presumably play essential role in cell signalling events in fibrin homeostasis, angiogenesis, and inflammatory activity.

The expression analysis of above proteins in normal and PE placenta could be of interest in PE research in delineating of the molecular networks that could bridge the gap between the involvements of these molecules in the development of PE

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Chapter 7

SUMMARY AND CONCLUSION

Plasminolytic components and their receptors in pathogenesis of preeclampsia

7. SUMMARY & CONCLUSION:

The principal conclusions from the present study were:

- Compared to normotensive subjects the mean gestational age and birth weight of the baby arereduced in PE group. In patients with PE, when compared with the normotensive control group the systolic and diastolic blood pressures were significantly higher.
- Microscopic examination revealed the increase in the number, density and volume of villi in PE compared to controls. But villous surface area and diameter of villi reduced in PE compared to controls. Intervillous space volume is reduced in PE. The endothelial cells of the tunica intima were activated with a swollen morphology in the PE group, whereas the endothelial cells of the tunica intima in the control group appeared normal with a flattened morphology. This activation of endothelial cells means that the cells were damaged. Other villous abnormality observed in PE placenta was increased stromal fibrosis, fibrinoid necrosis, thickening of syncytiotrophoblast basement membrane and increased syncytial knot formation .All the change observed in PE placenta may be response of the placenta due to inflammation and disturbance in the blood flow which leads to placental ischemia
- An increased prothrombin time patient of PE suggests altered fibrinolytic activity.
- The diminished expression of VEGF and ANXA2 in placenta may be associated with the defective angiogenesis and which may possibly play a vital role in development of PE by negatively influencing the plasmin generation.
- In the process of angiogenesis, ANXA2 is known to perform as angiogenic regulator. VEGF up regulates ANXA2 production has been demonstrated in

previous studies. The significant correlation in the expression of VEGF and ANXA2 in syncytiotrophoblast membrane in control group directs that, physiologically expression of these two factors may be dependent and regulated in the placental bed in response to the same stimulus like hypoxia, but this correlation is disturbed in preeclampsia.

- Decreased ANXA2 with increased expression of tPA, PAI-1 and altered association of ANXA2 with tPA in placental bed in PE is mainly responsible for altered fibrinolytic activity in PE and which may play a vital role in the pathogenesis of PE. Given their well-established role in regulating angiogenesis and fibrin homeostasis determining the fine details of cellular regulation of ANXA2, tPA and PAI-1 expression will likely contribute to a better understanding of normal placental biology and the pathogenesis of PE.
- Given the large body of evidence describing the anti-inflammatory and pro resolving actions of ANXA1, and knowing that PE is associated with an exacerbated inflammatory state, Our data may suggest that Anx1 is increased in PE women in an attempt to reduce the exacerbated inflammatory response in Preeclampsia
- The increased expression of ANXA1 and Gal-3 in placental bed may be associated with an altered systemic inflammatory response in PE, suggesting role of ANXA1 and Gal-3 in PE pathogenesis.
- ANXA2 and ANXA1 are reciprocally regulated in the placenta.
- With respect to ANXA2 and ANXA1 reciprocal regulation, but increase in Gal-3 in preeclampsia reveals that it could be the apoptotic activity of the protein not the proliferative function which associates with ANXA2 is the causative factor in preeclampsia.

- The expressions of EGFR in the placental cellular compartments of preeclampsia were reduced compared to normal. The altered morphology and morphometric changes observed in this study may be due to reduced growth factors like VEGF and EGFR.
- The purpose of our study points towards expression of above fibrinolytic and inflammatory proteins could be of clinical relevance in designing newer therapeutic molecules in treating PE.
- The correlation in the expression of above proteins gives an idea about the molecular mechanism in the preeclamptic placenta.

LIMITATIONS

- In the current study, we could analyze the expression status of proteins ANXA2,ANXA1,Gal-3, VEGF, EGFR, tPA, PAI-1 in the PE placenta compared to the normal but we have not assessed these proteins over the course of gestation to confirm the role of these proteins in the development of PE.

FUTURE DIRECTIONS:

- Additional studies with bigger sample size need to be conducted to improve the utility of profibrinolytic receptor ANXA2 as biomarker for pre-eclampsia.
- We consider that more research on ANXA2 must center not only on its contribution in regulating the mechanism of fibrinolysis but also on its role in regulating maturation and differentiation during the development of placenta.
- Further studies performed over the course of gestation are needed to confirm the role of these proteins in the development of preeclampsia and to determine whether assessment of these proteins may be a good predictive marker for the outcome of preeclampsia.

ANNEXURES

Plasminolytic components and their receptors in pathogenesis of preeclampsia

INFORMED CONSENT FORM TO PARTICIPATE IN THE RESEARCH STUDY

TITLE OF THE STUDY:"**PLASMINOLYTIC COMPONENTS AND THEIR RECEPTORS IN PATHOGENESIS OF PREECLAMPSIA"**

I completely, in my full senses, give my complete informed consent for microscopic study on placental tissue, for the purpose of research.

I hereby confirm that I have been informed (in the language understood by me) that a study is being conducted on "**Plasminolytic components and their receptors in pathogenesis of preeclampsia"**

The study has been explained to me in detail. I understand that the information regarding me collected during the course of this study will remain confidential. I understand that my participation in this study is voluntary and that I have the right to withdraw from the study at any time without giving any reason. I understand that the records maintained will be used only for research purpose. I hereby agree to participate in this study.

The refusal of my information will not affect my treatment in any way.

Date

Signature of Subject /Patient Signature of Witness Signature of Investigator

(Name) (Name) (Name)

Kannada consent form

PROFORMA FOR COLLECTION OF SAMPLE:

SOCIO DEMOGRAPHIC FACTORS:
Name IP/OPD no
Maternal age
Height: Weight:
BMI
Habits: Smoking/Alcohol/Tobacco
Obstetric factors:
Gestational age at present
Gestational age at disease onset
Previous history of preeclampsia
Parity
Obstetric history G P AL
SBP/DBP(mmHg)
RBS(mg/dl)
USG details
Mode of delivery Vaginal/Cesarian
Placental weight
Lab investigations:
Hematological investigation in relation to platelets, Prothrombin time
Proteinurea
Urea
Uric acid
Neonatal characteristics
Child gender Male/Female
Birth weight of the baby
Neonatal gender Male / Female
Apgar score at 5 min
Apgar score at 10 min

INFORMED CONSENT FORM TO PARTICIPATE IN THE RESEARCH STUDY

TITLE OF THE STUDY:"**PLASMINOLYTIC COMPONENTS AND THEIR RECEPTORS IN PATHOGENESIS OF PREECLAMPSIA"**

I completely, in my full senses, give my complete informed consent for microscopic study on placental tissue, for the purpose of research.

I hereby confirm that I have been informed (in the language understood by me) that a study is being conducted on "**Plasminolytic components and their receptors in pathogenesis of preeclampsia"**

The study has been explained to me in detail. I understand that the information regarding me collected during the course of this study will remain confidential. I understand that my participation in this study is voluntary and that I have the right to withdraw from the study at any time without giving any reason. I understand that the records maintained will be used only for research purpose. I hereby agree to participate in this study.

The refusal of my information will not affect my treatment in any way.

Date

Signature of Subject /Patient	Signature of Witness	Signature of Investigator
(Name)	(Name)	(Name)

ಸಂಶೋಧನಾ ಅಧ್ಯಯನದಲ್ಲಿ ಭಾಗವಹಿಸಲು ತಿಳುವಳಿಕೆಯುಳ್ಳ ಸಮ್ಮತಿ ನಮೂನೆ

ಅಧ್ಯಯನದ ಶೀರ್ಷಿಕೆ

"ಪ್ರೀಕ್ಲಾಂಪ್ಸಿಯಾದ ರೋಗಕಾರಕದಲ್ಲಿ ಪ್ಲಾಸ್ಮಿನೋಲಿಟಿಕ್ ಘಟಕಗಳು ಮತ್ತು ಅವುಗಳ ಗ್ರಾಹಕಗಳು"

ನಾನು ಸಂಪೂರ್ಣವಾಗಿ, ನನ್ನ ಪೂರ್ಣ ಅರ್ಥದಲ್ಲಿ, ಸಂಶೋಧನೆಯ ಉದ್ದೇಶಕ್ಕಾಗಿ ಜರಾಯು ಅಂಗಾಂಶದ ಮೇಲೆ ಸೂಕ್ಷ್ಮ ಅಧ್ಯಯನಕ್ಕಾಗಿ ನನ್ನ ಸಂಪೂರ್ಣ ತಿಳುವಳಿಕೆಯುಳ್ಳ ಒಪ್ಪಿಗೆಯನ್ನು ನೀಡುತ್ತೇನೆ.

"ಪ್ರೀಕ್ಲಾಂಪ್ಸಿಯಾದ ರೋಗೋತ್ಪತ್ತಿಯಲ್ಲಿ ಪ್ಲಾಸ್ಮಿನೋಲಿಟಿಕ್ ಘಟಕಗಳು ಮತ್ತು ಅವುಗಳ ಗ್ರಾಹಕಗಳು" ಕುರಿತು ಅಧ್ಯಯನವನ್ನು ನಡೆಸಲಾಗುತ್ತಿದೆ ಎಂದು (ನನಗೆ ಅರ್ಥವಾಗುವ ಭಾಷೆಯಲ್ಲಿ) ನನಗೆ ತಿಳಿಸಲಾಗಿದೆ ಎಂದು ನಾನು ಈ ಮೂಲಕ ದೃಢೀಕರಿಸುತ್ತೇನೆ

ಅಧ್ಯಯನವನ್ನು ನನಗೆ ವಿವರವಾಗಿ ವಿವರಿಸಲಾಗಿದೆ. ಈ ಅಧ್ಯಯನದ ಅವಧಿಯಲ್ಲಿ ನನಗೆ ಸಂಬಂಧಿಸಿದ ಮಾಹಿತಿಯು ಗೌಪ್ಯವಾಗಿ ಉಳಿಯುತ್ತದೆ ಎಂದು ನಾನು ಅರ್ಥಮಾಡಿಕೊಂಡಿದ್ದೇನೆ. ಈ ಅಧ್ಯಯನದಲ್ಲಿ ನನ್ನ ಭಾಗವಹಿಸುವಿಕೆಯು ಸ್ವಯಂಪ್ರೇರಿತವಾಗಿದೆ ಮತ್ತು ಯಾವುದೇ ಕಾರಣವನ್ನು ನೀಡದೆ ಯಾವುದೇ ಸಮಯದಲ್ಲಿ ಅಧ್ಯಯನದಿಂದ ಹಿಂದೆ ಸರಿಯುವ ಹಕ್ಕನ್ನು ನಾನು ಹೊಂದಿದ್ದೇನೆ ಎಂದು ನಾನು ಅರ್ಥಮಾಡಿಕೊಂಡಿದ್ದೇನೆ. ನಿರ್ವಹಿಸಿದ ದಾಖಲೆಗಳನ್ನು ಸಂಶೋಧನಾ ಉದ್ದೇಶಕ್ಕಾಗಿ ಮಾತ್ರ ಬಳಸಲಾಗುತ್ತದೆ ಎಂದು ನಾನು ಅರ್ಥಮಾಡಿಕೊಂಡಿದ್ದೇನೆ. ಈ ಅಧ್ಯಯನದಲ್ಲಿ ಭಾಗವಹಿಸಲು ನಾನು ಈ ಮೂಲಕ ಒಪ್ಪುತ್ತೇನೆ.

ನನ್ನ ಮಾಹಿತಿಯ ನಿರಾಕರಣೆಯು ನನ್ನ ಚಿಕಿತ್ಸೆಯ ಮೇಲೆ ಯಾವುದೇ ರೀತಿಯಲ್ಲಿ ಪರಿಣಾಮ ಬೀರುವುದಿಲ್ಲ.

ದಿನಾಂಕ

ವಿಷಯದ ಸಹಿ / ರೋಗಿಯ ಸಹಿ ತನಿಖಾಧಿಕಾರಿಯ ಸಾಕ್ಷಿ ಸಹಿ

(ಹೆಸರು) (ಹೆಸರು) (ಹೆಸರು)

PROFORMA FOR COLLECTION OF SAMPLE:

SOCIO DEMOGRAPHIC FACTORS:
Name IP/OPD no
Maternal age
Height: Weight:
BMI
Habits: Smoking/Alcohol/Tobacco
Obstetric factors:
Gestational age at present
Gestational age at disease onset
Previous history of preeclampsia
Parity
Obstetric history G P AL
SBP/DBP(mmHg)
RBS(mg/dl)
USG details
Mode of delivery Vaginal/Cesarian
Placental weight
Lab investigations:
Hematological investigation in relation to platelets, Prothrombin time
Proteinurea
Urea
Uric acid
Neonatal characteristics
Child gender Male/Female
Birth weight of the baby
Neonatal gender Male / Female
Apgar score at 5 min
Apgar score at 10 min

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2. **Title of the thesis: "Plasminolytic Components and Their Receptors in Pathogenesis of Preeclampsia."**
3. **Department: Physiology**
4. **Name of the Guide and Designation: Dr. Manjunatha Aithala, Professor**
5. **Name of the Co-Guide and Designation: Dr. Praveenkumar Shetty, Professor**

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(Declared vide notification No. F.9-37/2007-U.3 (A) Dated. 29-2-2008 of the MHRD, Government of India under Section 3 of the UGC Act,1956)

The Constituent College

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

IEC Ref No- 183/2016-17

13 October, 2016

INSTITUTIONAL ETHICAL CLEARANCE CERTIFICATE

The Ethical Committee of this University met on 30th June 2016 *at* 11 AM *to scrutinize the Synopsis / Research projects of Postgraduate student / Undergraduate student / Faculty members of this University / college from ethical clearance point of view. After scrutiny the following original / corrected & revised version synopsis of the Thesis / Research project has been accorded Ethical Clearance.*

Title: *"Plasminolytic components and their receptors in pathogenesis of preeclampsia".*

Name of Ph.D./ P. G. / U. G. Student / Faculty member: Ms.Komal Ruikar

Name of Guide: Dr. Manjunath Aithal, Prof.& HoD Dept. of Physiology.

Dr. Sharada Metgud
Chairperson, I.E.C
BLDE University,
VIJAYAPURA– 586 103

Dr.G.V.Kulkarni
Secretary, I.E.C
BLDE University,
VIJAYAPURA – 586 103.

Member Secretary,
Institutional Ethical Committee,
BLDE University, BIJAPUR.

Following documents were placed before Ethical Committee for Scrutinization:

- Copy of Synopsis / Research project
- Copy of informed consent form
- Any other relevant document's

ShriDharmasthalaManjunateshwara College of Medical Sciences and Hospital,
Manjushree Nagar, Sattur, Dharwad -580 009, Karnataka

Affiliated of Rajiv Gandhi University of Health Sciences, Bangalore & Recognized by Medical Council of India, New Delhi
GOI Notification No. U.12012/95/2001-ME (P-II) and MCI Notification No.MCI-34(41)/MED.2009/5527 dated 01.05.2009
Contact - Tel.No : +91836 2477574, 2477553, Tele fax: +91836 2461651 email: sdmcmshc@gmail.com Website: sdmmedicalcollege.org

Ref: SDMIEC: 0748: 2016

Date : 20-06-2016

To,
Dr. Komal Ruikar.,
Tutor,
Department of Physiology,
SDM College of Medical Sciences & Hospital,
Sattur, Dharwad.

Dear Dr. Komal Ruikar,

SUB: - Institutional Ethics Committee permission.

I am happy to inform you that **permission** is granted to you to carry out your study titled **"Plasminolytic components and their receptors in pathogenesis of preeclampsia".**

Thanking you,

Yours sincerely

Dr. M.A. Kamdod
Member Secretary - SDMIEC

Dr. H. Mallikarjun Swamy
Chairman – SDMIEC

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Organized By: Association of Physiologists & Pharmacologists of India (APPI), Belgaum Branch

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CERTIFICATE

This is to certify that Dr / Mr / Ms **Komal**

Bearing Reg. No. ..... Registerd with ..... Medical council from **SDMC , Dharwad** has

PRESENTED A POSTER TITLED **RELATIVE EXPRESSION OF CALCIUM DEPENDENT PHOSPHOLIPID BINDING PROTEIN ANNEXIN A2,ANNEXIN A1AND VEGF IN PLACENTA OF PREECLAMPSIA**

in 18th APPI National Seminar held on Saturday 18th August 2018 at Jawaharlal Nehru Medical College, Belagavi

Karnataka Medical Council has Accredited **"TWO (2)"** Credit Hours to this National Seminar, vide letter no. K.M.C./C.M.E./247/2018 dated 18-06-2018

Dr. Anil P. Hogade
President
APPI Belgaum Branch

Dr. Savita Hiremath
Organising Secretary
APPI Belgaum Branch

Dr. (Mrs) N.S. Mahantshetti
Principal
KAHER JN Medical College,
Belagavi

MANIPAL
ACADEMY of HIGHER EDUCATION
(Institution of Eminence Deemed to be University)

MANIPAL 25 1993-2018

64th Annual National Conference of
Association of Physiologists & Pharmacologists of India

APPICON 2018

'ADVANCING HORIZONS OF MEDICAL PHYSIOLOGY & PHARMACOLOGY IN RESEARCH AND TEACHING'

CERTIFICATE OF APPRECIATION

This is to certify that

Prof./Dr./Mr./Ms. KOMAL RUIKAR

has been awarded the **Best Scientific Free Paper/E-poster** titled

Relative expression of Ca^{2+}-dependent phospholipid binding protein Annexin A_2, Annexin A, & VEGF in placenta of Pre-eclampsia

during '**APPICON 2018**' organized by the Departments of Physiology & Pharmacology, Melaka Manipal Medical College and Kasturba Medical College-Manipal, Manipal Academy of Higher Education, Karnataka, India held on 29th, 30th November & 1st December 2018.

Dr Kirtana R Nayak
Organizing Secretary
Prof. & Head, Dept. of Physiology
Kasturba Medical College-Manipal
MAHE, Manipal

Dr Kiranmai S Rai
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Prof./Dr./Mr./Ms. ***Ms.Komal Gopal Ruikar*** *has presented poster presentation titled* ***Relative expression of inflammatory and anti-inflammatory proteins and VEGF in placenta of Preeclampsia.***

in the 6th Annual National Conference of Association of Physiologists of India organized by the Department of Physiology, JSS Medical College, Mysuru, Karnataka from 12th to 14th September 2019.

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Ms. *Komal Gopal Ruikar* has presented oral paper titled *"Plasminolytic components and their receptors in pathogenesis of Preeclampsia"*

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ORIGINAL RESEARCH ARTICLE

Placental Expression and Relative Role of Anti-inflammatory Annexin A1 and Animal Lectin Galectin-3 in the Pathogenesis of Preeclampsia

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Abstract Preeclampsia (PE) remains the major cause for maternal and foetal mortality and morbidity all over the world. Preeclampsia is associated with maternal, placental aggravated inflammatory response and generalized endothelial damage. AnnexinA1 (AnxA1) is glucocorticoid regulated protein regulates a wide range of cellular and molecular steps of the inflammatory response and is implicated in resolution of inflammation. Galectin-3 (Gal-3), β-galcotoside-binding lectin participates in many functions, both intra- and extracellularly. Recently it has been shown that galectin-3 modulates the inflammation. Role of AnxA1 and Galectin-3 is poorly studied in context with human reproductive disease like Preeclampsia. Therefore, the present study examined the expression of AnxA1 and Gal-3 which are involved in modulation of inflammation and their association in the placental bed of pregnancy with and without PE. The study group consisted of placental bed biopsy tissues obtained from pregnancies with PE (n = 30) and without (n = 30) PE. The expression of AnxA1 and Gal-3 in the placental bed tissues was evaluated quantitatively using Immunohisto-chemistry (IHC), western blot and mRNA expression analysis by quantitative RT-PCR. Our IHC, western blot and RT PCR analyses showed the increase in the expression of AnxA1 and Gal-3 in PE group compared with the normotensive control group ($P < 0.001$). The increased expression of AnxA1 and Gal-3 in placental bed may be associated with a systemic inflammatory response in PE, suggesting role of AnxA1 and Gal-3 in PE pathogenesis.

Keywords Preeclampsia · Annexin A1 · Galectin-3 · Placenta · Inflammation

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Introduction

Preeclampsia (PE) is a disorder of pregnancy characterized by onset of high blood pressure and proteinuria developing after the 20th week of gestation in a previously normotensive woman. It is a severe complication of human pregnancy with a worldwide incidence of 2–10%. It is one of the leading causes of maternal, as well as perinatal morbidity and mortality all over the world even in developed countries. In spite of in depth research, the pathophysiology of PE is not completely understood. An exaggerated maternal systemic inflammatory response to pregnancy with activation of both the innate and the adaptive arms of the immune system play a pivotal role in the development of the disease [1]. It has been proposed

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that the ischemic placenta can release soluble factors into the maternal circulation that cause endothelial cell activation and/or dysfunction and a systemic inflammatory response [2]. Redman and Sargent [3] earlier proposed that the features of the systemic inflammatory response seen in normotensive pregnant women are also seen in PE women, but in a greater severity. Annexin A1 (AnxA1), previously known as lipocortin-1 is a member of the calcium-dependent phospholipid-binding protein superfamily of Annexins, which regulate diverse cellular functions in various cellular types [4]. AnxA1 was predominantly delineated as a glucocorticoid-regulated protein having anti-phospholipase activity, but the protein also exhibits many other anti-inflammatory and pro-resolving properties, which primarily include profound inhibitory action on leucocyte transmigration and activation, leading to resolution of inflammation [5]. Gal-3 is involved in numerous biological processes associated with cell growth and differentiation [6]. This protein has also been implicated in numerous clinical states, such as inflammation [7]. Gal-3 has controversial pro- or anti-inflammatory activities depending on various factors including its intracellular or extracellular localization and the target cell implicated in these processes [8]. Although it may contribute to resolution of inflammation by clearing apoptotic neutrophils [9]. Gal-3 has also been identified in the human placenta and its abundance was found to be inversely correlated with trophoblast invasiveness during the course of gestation [10]. Considerable body of evidence illustrates that AnxA1 and Gal-3 participates in anti-inflammatory and proresolving function. PE is associated with an exacerbated inflammatory state, therefore it is rational to hypothesize that AnxA1 and Gal-3 may be altered in PE women. Therefore, the present study examined the expression of above proteins which are involved in modulation of inflammation and their association in the placental bed of pregnancy with and without PE.

Material and Methods

This study was approved by Institutional ethics committee at SDM College of Medical Sciences and Hospital Dharwad, Karnataka. PE was diagnosed based on increased blood pressure (140/90 mmHg) in a pregnant woman after 20 weeks of amenorrhea, accompanied by proteinuria (0.3 g/24 h or 1+ dipstick), as defined by the report of National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy [13]. Cases of chronic hypertension or superimposed PE were excluded from the study. Body mass index (BMI) was evaluated pre pregnancy and prior to caesarean delivery.

Placental Bed Biopsies

Fresh placental bed biopsy tissues were obtained from 60 term pregnancies at the time of caesarean delivery after the patient's informed consent between January 2017 and December 2018. The study groups consisted of pregnant women with PE (n = 30, PE group) and without PE (n = 30, normotensive control group). All pregnancies were also free of other complications, such as gestational diabetes, chronic hypertension, and autoimmune disease. For expression study, chorionic villous tissue from maternal side of the placenta is collected. The expression of AnxA1 and Gal-3 were analysed by using immunohistochemistry, western blot and real time PCR.

Immunohistochemistry

By using scalpel, 4–5 biopsies of villous parenchyma (1 cm^3 each) from the central and marginal regions of part of the placental disc are collected. Tissue fragments from the placenta consisting of homogeneous villous tissues were cut longitudinally from the maternal side to the foetal side and infarct areas were excluded from the study. Expression of Anx A1 and Gal-3 was analysed in 60 placental villous tissues. 3 µm thick sections were obtained from formalin fixed and paraffin embedded placental tissues. The sections were treated according to standard Immunohistochemical staining procedure for the detection of protein.

The endogenous peroxidase activity was blocked by incubating the tissue with 0.3% hydrogen peroxide. Non-specific binding sites were blocked by incubating the sections with normal horse serum (vector laboratories) and then incubated with primary antibody against AnxA1 (DIL 1:100, Santa Cruz Biotechnology Inc; Santa Cruz, CA catalogue No. SC-12740) and Gal-3 (DIL1:100 Santa Cruz Biotechnology Inc; Santa Cruz, CA catalogue No. SC-23938). This was followed by sequentially incubating the sections with biotynylated secondary antibody (Vector Laboratories, Burlingame, CA) and avidin biotin horse raddish peroxidase complex (ABC; VECTOR). The antigen of interest was detected by use of a 3, 31-diamino benzidine (DAB) chromogen and by counterstaining with haematoxylin.The primary antibody was replaced by anti-rabbit immunoglobulin G (IgG) whole molecule (Sigma-Aldrich, USA, Catalogue No. A0545), at 1:1000 dilution, as negative control, while triple negative breast cancer cell sections were used as positive controls. The tissues were evaluated under light microscope with Lieca image Centre. The intensity and localization of the staining reaction in syncytiotrophoblasts membrane chorionic villous stromal cells, and villous vascular endothelial cells and was assessed by using semiquantitative immunoreactive score

(IRS) and all the samples were blinded. The IRS was derived by multiplication of staining intensity graded (as 0 negative, 1 weak, 2 moderate, and 3 strong staining) and percentage of positively stained cells (0 = no staining, 1–10% as 1, 11–50% as 2, 51–70% as 3, 71–100% as 4). The localization of AnxA1 and Gal-3 protein was counted in 10 random fields in placental villi.

Western Blot Analysis

Human placental bed samples were homogenized at 4°C in 500 µL RIPA lysis buffer. The lysates were centrifuged at 14,000 rpm at 4°C for 45 min to remove the cell debris. Bicinchonic acid assay (BCA assay) was used to determine the protein concentrations. Whole cell lysates (40 µg) were subjected to SDS-PAGE using Tris–Hcl buffer and the proteins were transferred to nitrocellulose membranes (Himedia) using a transfer apparatus at 65 V for 90 min. The antibodies were used against AnxA1 (Mouse monoclonal, BD Biosciences, CA-12740), Gal-3 (Santa Cruz Biotechnology, SC-23938) and GAPDH (Santa Cruz Biotechnology, SC-166574). Appropriate secondary antibodies conjugated to horseradish peroxidase (BioRad) were incubated with respective membranes for 2 h at room temperature. The membranes were developed using ECL plus (BioRad) and the image was captured using enhanced Chemiluminescence system, G: BOX Chemi XX6/XX9. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene was used as an internal control for loading. The densities of protein bands were determined with Image J, version 1.35d.

RNA Preparation, RT-PCR and Real-Time PCR

Total RNA was extracted using Trizol reagent (Thermofisher scientific invitrogen). Complementary DNA (cDNA) was synthesized from 2 µg of total RNA by using Takara cDNA synthesis kit using a random hexamer at 42°C for 1 h. Template cDNA was subjected to PCR amplification using gene-specific sense and antisense primers Annexin A1 (forward primer: 5′-ATCAGCGGTGA GCCCCTATC-3′ reverse primer 5′-TTCATCCAGGG GCTTTCCTG-3′), Galectin-3 (forward primer 5′-5CAA TACAAAGCTGGATAATAACTGG-3' reverse primer 5′-GATTGTACTGCAACAAGTGAG-3) and reference gene β actin (forward primer 5′-GGGAAATCGTGCGT GACATTAAG-3′, reverse primer 5′-TGTGTTGGCGTA CAGGTCTTTG-3′) were generated (Juniper life sciences) RT-PCR conditions were at 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s in a thermal cycle (Quant Studio 5 by Applied Bio systems). The quantitative amount of each gene was standardized against the house-keeping gene β-actin. The RNA levels were expressed as a ratio, using the 'delta-delta' method for comparing the relative expression results between normotensive control and patients with PE.

Statistical Analysis

All statistical analysis was carried out by using Graph Pad Prism version 7.04. Results for normally distributed data were shown as ± SD. Statistical analysis of expression of AnxA1 and Gal-3 for IHC was carried out with the Mann–Whitney U-test. Spearman correlation coefficients were used to detect correlation between AnxA1 and Gal-3 expression. "*P* value" less than 0.05 were considered to be statistically significant. Results for normally distributed data were analysed using student t test.

Results

The Demographic characteristics of the normotensive women and preeclamptic patients are shown in Table 1. There were no statistical differences between the PE and normotensive control groups with respect to their age, BMI, neonatal gender. Almost all the deliveries in the control group were at full term. Compared to normal control group the mean gestational age is shorter in PE group. In patients with PE, when compared with the normotensive control group, birth weight of the baby is reduced and the systolic and diastolic blood pressures were significantly higher ($P < 0.05$).

Histopathological Changes in Placenta of Preeclampsia

Microscopic examination revealed the increase in the number, density and volume of villi in PE compared to controls. But villous surface area and diameter of villi reduced in PE compared to controls. Intervillous space volume is reduced in PE. The endothelial cells of the tunica intima were activated with a swollen morphology in the PE group, whereas the endothelial cells of the tunica intima in the control group appeared normal with a flattened morphology. This activation of endothelial cells means that the cells were damaged. Other villous abnormality observed in PE placenta was increased stromal fibrosis, fibrinoid necrosis, thickening of syncytiotrophoblast basement membrane and increased syncytial knot formation (Fig. 1). All the change observed in PE placenta may be response of the placenta due to inflammation and disturbance in the blood flow.

Table 1 Demographic characters in preeclampsia (PE) and normotensives

Clinical data	Normotensives (n = 30)	Preeclampsia (n = 30)	P value
Age (years)	25.9 ± 3.9	24.93 ± 2.7	0.275
Gestational age	38.73 ± 0.94	35.07 ± 1.87	0.000*
BMI (kg/m2)	25.94 ± 2.01	25.83 ± 2.1	0.851
Gravid	2.0 ± 0.78	1 ± 0.0	0.000*
Parity	1.27 ± 0.45	1 ± 0.0	0.002*
Birth weight	2.94 ± 0.29	2.47 ± 0.35	0.000*
SBP (mm Hg)	105.9 ± 8.66	153.9 ± 10.71	0.000*
DBP (mm Hg)	71.67 ± 6.08	96.13 ± 7.4	0.000*
Platelet s (10^3/μL)	2.67 ± 0.21	2.66 ± 0.41	0.887

Statistical analysis is carried out by Student's t test (*P < 0.05). Above data is expressed as Mean ± SD

BMI Body mass index, *SBP* systolic blood pressure, *DBP* diastolic blood pressure

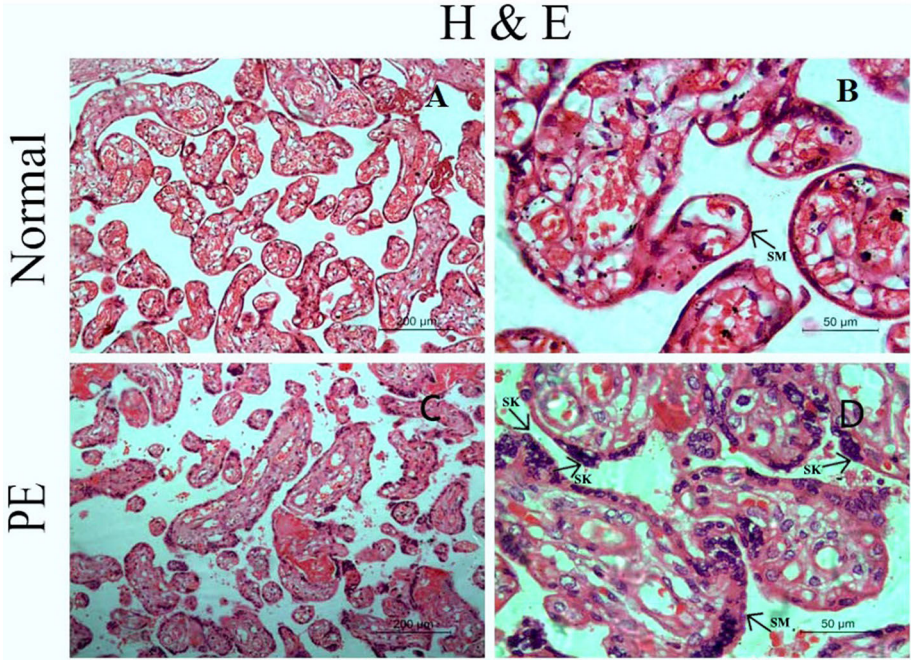

Fig. 1 **a**, **b**: H and E staining showing the morphology of normal placenta, **b**: black arrow shows the normal thickness of syncytiotrophoblast membrane (SM), **c**, **d**: H and E staining showing the morphology of PE placenta, **d**: increased thickness of syncytiotrophoblast membrane (SM) and increased syncytial knot (SK) formation. Magnification 10× = 200 μm and 40× = 50 μm

Expression of Annexin A1 and Galectin 3 in Placental Bed

Immunostaining of placental bed sections confirmed the increased expression of AnxA1 and Gal-3 in PE group compared to the normotensive control group. Several different cell types in both placental bed biopsies of the PE and normotensive control groups were positive for AnxA1 and Gal-3 including of syncyotiotrophoblastic cells, chorionic villous stromal cells, and villous vascular endothelial cells. Expression in placental villous tissues was semi-quantified (Table 2). In preeclamptic placentas, immunostaining was strong and located predominantly in the syncytiotrophoblasts and mild staining was observed in villous stromal cells and villous vascular endothelial cells. In normal placenta moderate staining was obtained with AnxA1 and Gal-3 primary antibodies (Fig. 2).

The technique of Western blot and RT-PCR was performed because the technology of immunohistochemistry does not impart itself to quantification. In western blot AnxA1 expression is increased in PE placenta by 3.2-fold ($P = 0.011$) while Gal-3 is increased by 3.14 fold ($P = 0.031$) compared to normal placenta.

Relative mRNA expression of AnxA1 and Gal-3 was increased in placenta in PE placenta compared to controls ($P = 0.0001$ and 0.035 respectively). Levels of mRNAs are expressed as arbitrary units. Unpaired t test is used to evaluate the potential difference (Figs. 3, 4). We also studied the statistical spearman correlation of expression level of AnxA1 and Gal-3 as both proteins are involved in modulation of inflammation. A statistically significant

Table 2 Localisation and Immunostaining intensity of AnxA1 and Gal-3 expression in placental villous tissues

	AnxA1				Gal-3		
	SCORE	STM	CVSC	VVEC	STM	CVSC	VVEC
Normal (n = 30)	0	0	4 (13)	4 (13)	0	11 (36)	9 (30)
	1+	7 (23)	20 (66)	21(70)	17 (56)	14 (46)	21 (70)
	2+	21 (70)	6 (20)	5 (16)	12 (40)	5 (16)	0
	3+	2 (6)	0	0	1 (3)	0	0
PE (n = 30)	0	0	1 (3)	1 (3)	0	7 (23)	7 (23)
	1+	0	7 (23)	17 (56)	7 (23)	15 (50)	20 (66)
	2+	15 (50)	22 (73)	12 (40)	14 (46)	8 (26)	3 (10)
	3+	15 (50)	0	0	9 (30)	0	0
P value		0.000*	0.000*	0.000*	0.000*	0.140	0.000*

Statistical analysis of expression of AnxA1 and Gal-3 for IHC was carried out with the Mann–Whitney U-test (*$P < 0.05$)

Immunohistochemical staining for AnxA1, 0 = no staining, 1–10% as 1+ , 11–50% as 2+ , 51–70% as 3+ , 71–100% as 4 + . (Numbers in bracket are in the percentage)

STM Syncytiotrophoblast membrane, *CVSC* chorionic villous stromal cells, *VSMC* vascular smooth muscle cell, *VVEC* villous vascular endothelial cells

correlation in the expression of AnxA1 and Gal-3 was observed in syncytiotrophoblast membrane ($P < 0.0048$).

Discussion

This study showed increased expressions of AnxA1 and Gal-3 in the third trimester placental bed from pregnancies with PE compared with the normotensive control group. The result of western blotting and Real time PCR in the study revealed an increased expression of AnxA1 and Gal-3 in the preeclamptic placental samples compared to normotensive placenta. The increased expression of protein in different placental compartment was confirmed by immunohistochemistry analysis. Immunohistochemistry findings revealed protein is expressed strongly in syncytiotrophoblast layer of the preeclamptic placental villous in comparison to controls. Expression of AnxA1 has been thoroughly studied in models of sterile inflammation, recognising its central role as key modulator of both of the innate and adaptive immune systems [11]. However, in the context of Preeclampsia only one study have been reported on the altered expression of AnxA1 in the plasma [12]. Very little is known about the expression status of Annexin A1 in PE. Our study shows the differential expression of AnxA1 in the placenta of normotensive and preeclamptic women. This result was consistent with the study by Perucci et al. [12] who measured the protein in the plasma [12]. This result implies that the increase AnxA1 expression in PE placental bed could be an important factor in the aetiology of PE. In normal pregnancy it is established that there is a homeostatic balance between inflammatory and regulatory response [13], which suggests that regulatory molecular mechanisms are sufficient to reduce the mild inflammatory response. Preeclampsia is associated with chronic activation of immune system which leads to an increased production of inflammatory cytokines by pro-inflammatory T cells, and a decrease in regulatory and anti-inflammatory cytokines, which further promotes an inflammatory state during PE [14, 15]. In preeclamptic pregnancy, the imbalance between pro-inflammatory and regulatory cytokines is correlated with placental ischemia. This imbalance exacerbates as the pregnancy progresses [16]. AnxA1 has been shown to be capable of regulating a large number of biological events such as chronic inflammation, growth of the tissue, and programmed cell death. It has been shown that the decreased expression of AnxA1 is associated with the development of more severe inflammation in inflammatory diseases [17]. Additionally, AnxA1 helps in monocyte augmentation and elimination of apoptotic leukocytes by macrophages, resulting in reduced production of pro-inflammatory cytokines and increased release of immunosuppressive and pro-resolving molecules [18].

Our data may imply that AnxA1 is increased in patients with preeclampsia in an attempt to attenuate the exacerbated inflammatory response in these patients. Chronic inflammation in PE suggests that the resolution of inflammation pathway is dysfunctional. Consequently increased AnxA1 expression seems to be inadequate to resolve inflammation. In other chronic inflammatory disease such as inflammatory bowel disease and Alzheimer's disease systemic levels of proresolving mediators are increased [19]. In acute inflammatory response during the initiation phase, mediators derived from arachidonic acid become up-regulated and contribute to changes in vascular

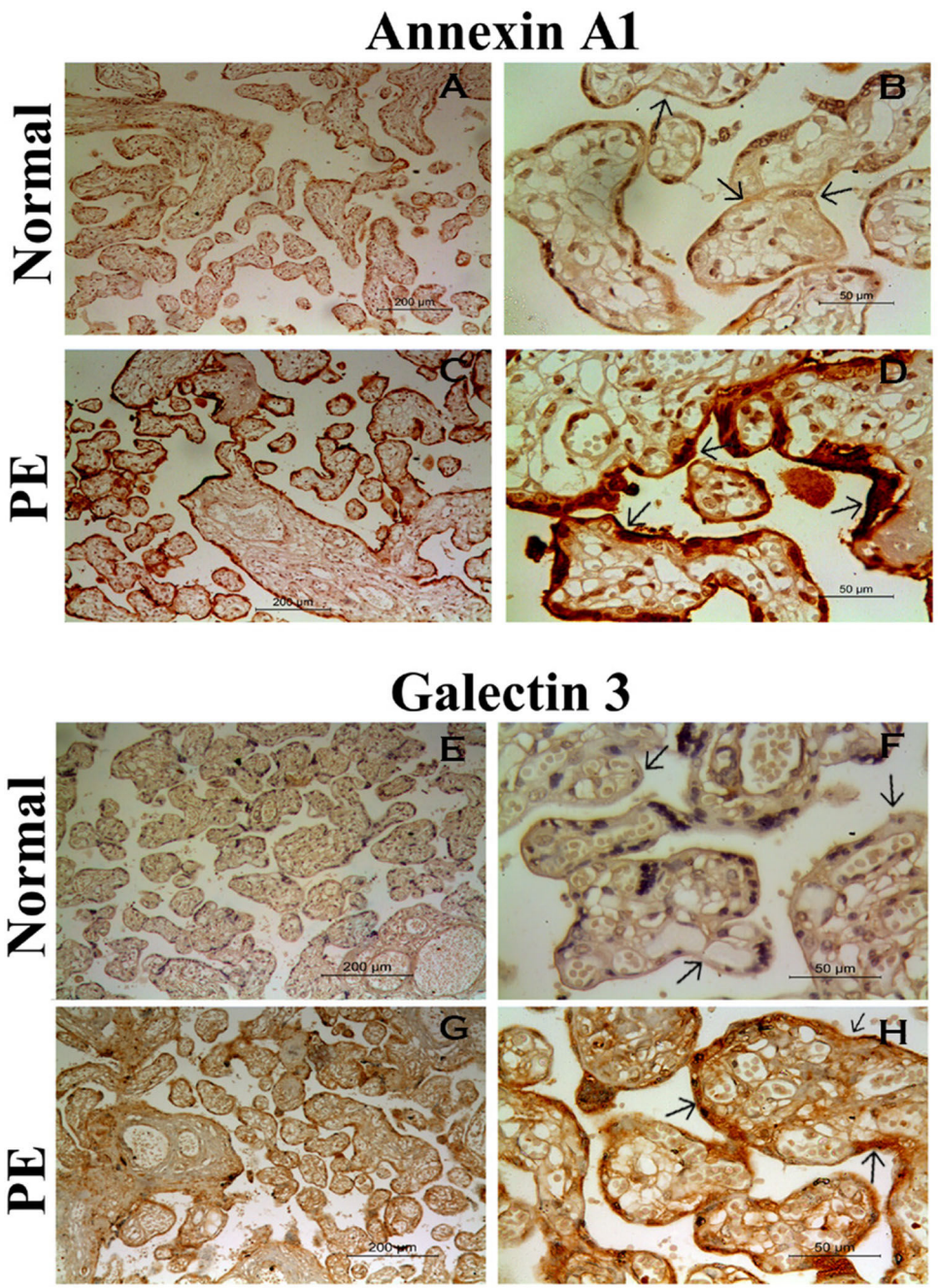

Fig. 2 Representative photomicrograph showing the expression of AnxA1 and Gal-3 in placental villi of normal and PE placenta under 10× and 40× respectively. **A**, **B**: note moderate AnxA1 immunostaining predominantly in the syncytiotrophoblastic membrane of normal placenta as indicated by black arrow, **C**, **D**: strong AnxA1 immunostaining in PE placenta, **E**, **F**: moderate membranous Gal-3 immunostaining in syncytiotrophoblast of normal placenta, **G**, **H**: intense Gal-3 staining in PE placenta. Bright-field microscopy images, representative of n = 30 per group. Mann–Whitney U-test was used to evaluate potential difference ($P < 0.05$)

permeability and Polymorphonuclear leukocytes recruitment. However, the generation of these pro-inflammatory mediators in due course terminated by successive dynamic changes in prostaglandins E2 and D2 [20]. This can be seen as switch where elevated levels of pro resolving mediators, including pro-resolving lipoxins A4 decreases inflammatory molecules such as prostaglandins, leukotrienes, and cytokines [21]. Pro-resolving and anti-inflammatory actions of AnxA1 are mediated by a G-protein-coupled receptor named formyl peptide receptor like-2 (FPR2)/lipoxin A4 receptor (ALXR) [22]. Decreased ALX expression has been observed in patients with asthma, a chronic inflammatory disease [23]. These mechanisms might explain the noticeable ineffectiveness of AnxA1 up-regulation in some human inflammatory diseases. More studies are required to solve the mystery whether these dysfunctional mechanisms in AnxA1 resolution pathway are present in PE. Proteins from galectin family have emerged as master regulators of immune system homeostasis, playing central role in the amplification and/or resolution of inflammatory processes. Gal-3 functions as pro- or anti-inflammatory activities depending on various

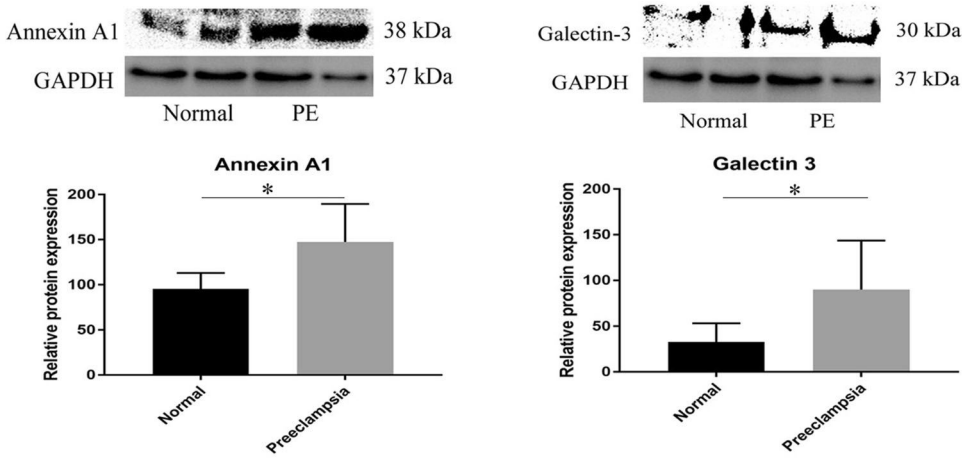

Fig. 3 Western blot analysis of AnxA1 and Gal-3 protein in Normal and Preeclamptic placenta. Expression levels were confirmed by densitometry. AnxA1 and Gal3 expression was significantly increased in PE placentas compared to normal placenta. Unpaired t test is used to evaluate the potential difference ($P < 0.05$)

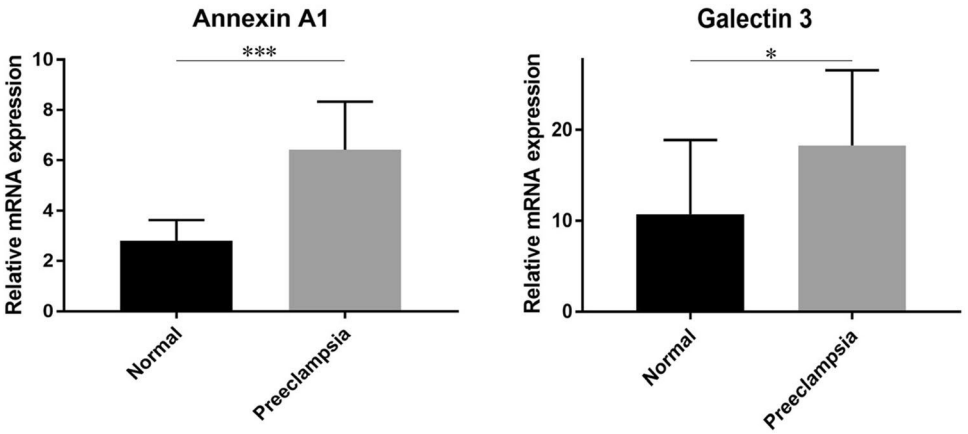

Fig. 4 Expression of Annexin A1 and Gal-3 mRNA in placental tissues as determined by RT-PCR. Levels of mRNAs are expressed as arbitrary units. Unpaired t test is used to evaluate the potential difference (*$P < 0.05$, ***$P < 0.001$)

factors including its intracellular or extracellular localization and the target cell involved in these processes [8]. Even though it may contribute to resolution of inflammation by clearing apoptotic neutrophils [9] this lectin exhibits mostly pro-inflammatory effects by increasing activation of macrophages, mast cells, and natural killer cells, as well as T and B lymphocytes [24]. Studies have shown that Gal-3 has a role in implantation of embryo, embryogenesis and placental formation, and is closely connected with the success and maintenance of pregnancy [25]. Proliferation and programmed cell death are crucial components of the trophoblast life cycle. There are aberrant cell turnover including an increased apoptosis in placental villous trophoblast of preeclamptic pregnancies [26]. Numerous studies suggest that high expression of Gal-3 exerts regulatory effects on apoptotic responses of various cell types [27]. Our results may suggest that the marked increase in expression of Gal-3 in the syncytiotrophoblast cells in preeclamptic placenta could be important to turn on the intracellular machinery of these cells required for defence against a rapid process of apoptosis. In our earlier study, we have reported the interaction of Gal-3 and Annexin A2 resulting in the cancer progression in Triple negative breast cancer cells [28]. Annexin A2 is the pro-inflammatory molecule and AnxA1 being the anti-inflammatory, which are reciprocally regulated [29]. As far as PE is concerned, Annexin A2, a (proinflammatory fibrinolytic) protein level decreases and which should result in simultaneous increase in AnxA1 and which we are seeing in our current data. With respect to Annexin A2 and AnxA1 reciprocal regulation, but increase in Gal-3 in preeclampsia reveals that it could be the apoptotic activity of the protein not the proliferative function which associates with Annexin A2 is the causative factor in preeclampsia. Present study data illustrates that, there is a significant positive correlation in the expression of AnxA1 and Gal-3.These facts presumably indicate that expression of these two factors may be dependent and regulated in the placental bed in response to the same pathogenic stimulator such as inflammation. ANXA1 and Gal-3 undergo changes in their content and localization when neutrophil adheres to the endothelium, and this could be indicative of a process of favouring and counter-balancing between two endogenous anti- and pro-inflammatory mediators [30].

Conclusion

The increased expression of AnxA1 and Gal-3 in placental bed may be associated with an altered systemic inflammatory response in PE, suggesting role of AnxA1 and Gal-3 in PE pathogenesis. Although more studies are needed to clarify the role of these inflammation modulatory proteins and other pro-resolving molecules in the context of the systemic inflammatory response in preeclampsia Additionally, further studies performed over the course of gestation are needed to confirm the role of these proteins in the development of preeclampsia and to determine whether assessment of these proteins may be a good predictive marker for the management of preeclampsia.

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Author contributions Conceived the idea and designed the experiments: PKS and KR; Helped in clinical sample collection and correlated the clinical relevance to the study: KR, PKS, MA and VK; Performed the experiments: KR, SE, AB, and RS; Analysed the data: USD, KR, PKS, PP, AB and VK; Manuscript preparation: KR, PKS and PP; Supervised the overall study: PKS and MA.

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Compliance with Ethical Standards

Conflict of Interest The author declare that they have no conflict of interest.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study was approved by the Ethical Committee of SDM College of Medical Sciences and Hospital, Dharwad, Karnataka, India (SDM IEC: 0748: 2016).

Informed Consent Informed consent was obtained from all individual participants included in the study.

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Decreased expression of annexin A2 and loss of its association with vascular endothelial growth factor leads to the deficient trophoblastic invasion in preeclampsia

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Abstract

Objectives: Preeclampsia (PE) remains the major cause for maternal and foetal mortality and morbidity. Invasion of endovascular trophoblast and remodelling of spiral artery are crucial actions of normal placental development. Non-fulfilment of these processes plays a leading role in the development of preeclampsia. Vascular endothelial growth factor (VEGF) is produced by extravillous trophoblastic tissue and decidual cell population is a well-known angiogenic growth which plays a fundamental role in placental pathogenesis of PE. Annexin A2 (ANXA2) is a profibrinolytic protein receptor required for plasminolysis, which is an important step in the formation of new blood vessel along with VEGF. Role of ANXA2 is poorly studied in context with human reproductive disease like preeclampsia. The purpose of the present study is to examine the expression and association of VEGF and ANXA2 in the term placentas of pregnancies with and without PE.

Methods: The study group comprised of placental tissues procured from gestations with PE (n=30) and without (n=20) PE. The expression of VEGF and ANXA2 in the placental villous tissue was evaluated quantitatively by means of IHC, western blotting and reverse transcriptase-polymerase chain reaction (RT-PCR).

Results: Our IHC, western blotting and RT-PCR analysis illustrated the significant decrease in the expression of VEGF and ANXA2 in PE group compared with the normotensive control group ($p<0.005$). We observed statistically significant positive correlation among the expression of ANXA2 and VEGF in placentas of normotensive control group ($p<0.0001$).

Conclusions: The diminished expression of VEGF and ANXA2 in placenta may be associated with the defective angiogenesis and which may possibly play a vital role in PE pathogenesis.

Keywords: Annexin A2; placenta vascular endothelial growth factor; preeclampsia.

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Introduction

Preeclampsia (PE) is a multisystem placental intermediated disorder affecting 5–8% of pregnancies entirely

over the world [1]. Though, the pathophysiology of PE has not yet been distinctly delineated. During the normal development of placenta, invasion of endovascular trophoblast and re-modelling of spiral artery are very essential events. Failure of these processes has been involved in the pathogenesis of PE [2, 3]. Highly regulated process of invasion of trophoblast has been shown to be controlled by various angiogenic growth factors including vascular endothelial growth factor (VEGF) [4, 5]. It is produced by extra villous trophoblast (EVT) and various decidual cell populations, which plays a critical role in placental angiogenesis [6–9]. Annexin A2 (ANXA2), a member of a family of Ca+2-dependent phospholipid-binding proteins, is a cell surface co-receptor for tissue plasminogen activator (tPA) and plasminogen [10]. Catalytic efficiency of plasmin formation is increased by 60 fold when ANXA2 protein by binds to both plasminogen and tPA. This results in highly effective plasmin-facilitated proteolytic events which stimulates neovascularisation by increasing the efficiency of endothelial cell degradation and invasion across the extra cellular matrix (ECM) [11]. Hypoxia inducible factor up regulates the transcription of VEGF and ANXA2. VEGF either directly or in a combination with VEGFR2 affects the expression of ANXA2. On the surface of the cell membrane VEGF induces the expression of ANXA2. By protein kinase C (PKC) pathway VEGF and VEGFR2 also may be stimulating ANXA2 expression. ANXA2 further influences neovascularisation [12]. There is an autocrine regulation of these factors where soluble ANXA2 acts as an upstream regulator of VEGF [13]. Coordinated vascularisation is essential for normal placental development. ANXA2 is a profibrinolytic receptor required for plasminolysis, which is important step in the formation of new blood vessel. VEGF is the most potent endothelial growth factor induces angiogenesis, endothelial cell proliferation and has a basic role in angiogenesis. Several studies have conveyed on the altered expression of VEGF in the preeclamptic placenta [14, 15]. There have been very few available reports on the ANXA2 expression in placenta and its relation with VEGF. Hence, the current study observed the expression of VEGF and ANXA2 which are involved in plasmin generation and their association in the placental bed of pregnancy with and without PE. As these factors are associated with invasion of extra villous trophoblast and re-modelling of spiral artery in the placental bed, we hypothesised that expressions of these proteins may change in PE placenta.

Materials and methods

This study was approved by institutional ethics committee at SDM College of Medical Sciences and Hospital Dharwad, Karnataka. PE was diagnosed based on increased blood pressure (140/90 mmHg) in a pregnant woman after 20 weeks of amenorrhea, accompanied by proteinuria (0.3 g/24 h or 1 + dipstick), as defined by the report of American College of Obstetricians and Gynecologists guidelines [16]. PE can have an early onset starting before 34 weeks of gestation and late onset after 34 weeks of gestational age and can be classified as mild or severe, depending on the severity of the symptoms present [17]. Mild PE is characterized by hypertension with systolic blood pressure ≥140 mmHg and diastolic blood pressure ≥90 mmHg accompanied with proteinurea ≥0.3 g per 24 h after 20 weeks of gestational age in a previously normotensive parturient. Severe PE is characterised by a systolic blood pressure ≥160 mmHg or and diastolic blood pressure ≥110 mmHg, and proteinuria >5 g per 24 h along with disturbances in central nervous system, epigastric pain, liver dysfunction and foetal growth restriction. The PE women group was divided, according to the aforementioned criteria, into two groups; mild PE group (15 patients) and severe PE (15 patients). Cases of chronic hypertension or superimposed PE were excluded from the study [Table 1].

Placental bed biopsies

Fresh placental bed biopsy tissues were obtained from 50 pregnancies at the time of caesarean delivery between January 2017 and December 2018. Written Informed consent was obtained from each patient. The study groups consisted of pregnant women with mild PE (n=15), severe PE (n=15) and normotensive group (n=20). All pregnancies were also free of other complications, such as gestational diabetes, chronic hypertension, autoimmune disease or intrauterine growth restriction. Women without PE were undergoing caesarean section because of breech presentation and cephalopelvic disproportion. The expression of VEGF and ANXA2 were analysed by using immunohistochemistry, western blot and real time polymerase chain reaction (PCR).

Immunohistochemistry

Biopsies (2–3) were collected from villous parenchyma (1 cm^3 each) at the central and marginal regions of part of the placenta using scalpel. Fragments of the placenta consisting of homogeneous villous tissues

Table 1: Sequence of primers used for reverse transcriptase-polymerase chain reaction (RT-PCR).

Gene	Sequence	Binding location
ANXA2	F – 5′-CTGGCAAAGGGTAGAAGAGCA-3′	Exon 8
	R – 5′-CGTCATAGAGATCCCGAGCAT-3′	Exon 9
VEGFA	F – 5′-GAGATGAGCT TCCTACAGCAC-3′	Exon 4
	R – 5′-TCACCGCCTCGGCTTGTCACAT-3′	Exon 7
β actin	F – 5′-GGGAAATCGTGCGTGACATTAAG-3′	Exon 4
	R – 5′-TGTGTTGGCGTACAGGTCTTTG-3′	Exon 5

were dissected longitudinally from the maternal side towards the foetal side and infarct areas were excluded from the study [18]. In 50 placental villous tissues expression of VEGF and ANXA2 were analysed. From formalin fixed and paraffin embedded placental tissues 3 µm thick sections were obtained. The IHC procedure was executed as described previously [13]. In primary antibody against ANXA2 (Dil. 1:100, Santa Cruz Biotechnology Inc; Santa Cruz, CA Catalogue No. SC-9061) the sections were incubated overnight and VEGF (Dil. 1:100, Santa Cruz Biotechnology Inc; Santa Cruz, CA Catalogue No. SC-7269). This was followed by consecutively incubating the sections with biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) and avidin biotin horse radish peroxidase complex (ABC; VECTOR). By using 3, 31-diamino benzidine (DAB) chromogen and by counterstaining with haematoxylin the antigen of interest was detected. The primary antibody was replaced by anti-rabbit immunoglobulin G (IgG) whole molecule (Sigma-Aldrich, USA, Catalogue No. A0545), at 1:1,000 dilution, as negative control, while triple negative breast cancer cell sections were used as positive controls [13]. Under light microscope with Lieca Image Centre the tissues were evaluated. The semi-quantitative immunoreactive score (IRS) was used to study the localisation and intensity of the staining reaction in chorionic villous stromal cells, vascular smooth muscle cells, villous vascular endothelial cells and syncytiotrophoblasts. All the samples were blinded. The IRS was calculated by multiplication of optical staining intensity (graded as 0-negative, 1-weak, 2-moderate and 3-strong staining) and of positively stained cells percentage (0-no staining, 1–10% as 1, 11–50% as 2, 51–70% as 3.71–100% as 4). By using a semi-quantitative scale for intensity of staining immunoreactivity for antibodies was scored: 0-negative or no staining; 1+ weak, 2+ moderately positive; 3+ strongly positive. The localisation of ANXA2 and VEGF was counted in different 10 random fields in placental villi.

Western blot analysis

For the protein expression analysis in placental tissue, placental samples of human were homogenised at 4 °C in 500 µL RIPA lysis buffer containing protease and phosphatise inhibitors. The lysates were centrifuged at 14,000 rpm at 4 °C for 15 min to remove the debris of cells. Bicinchoninic acid assay (BCA assay) was used to measure the protein concentrations. Tissue lysate (40 µg) were subjected to SDS-PAGE by using Tris–Hcl buffer. The proteins were transferred to nitrocellulose membranes (Himedia). The membrane was subsequently incubated with primary antibodies (Mouse monoclonal ANXA2 antibody, SC-9061 diluted 1:1,000, VEGF165 antibody, SC-7269 diluted 1:1,000, and GAPDH antibody, SC-166574 diluted to 1:3,000, all from Santa Cruz Biotechnology). Suitable secondary antibodies conjugated to horse radish peroxidase (Bio-Rad) were incubated with separate membranes for 2 h at room temperature. The membranes were developed using ECL plus (Bio-Rad), and the image was captured using enhanced Chemiluminescence system, G: BOX Chemi XX6/XX9. GAPDH immunoblot was considered as internal control for loading. The protein bands were normalized and quantified comparatively as the control band with Image J, version 1.35d (National Institutes of Health Image software).

RNA preparation, RT-PCR and real-time PCR

By using Trizol reagent the total RNA was extracted (Invitrogen, Carlsbad, CA, USA). From 2 µg of total RNA, complementary DNA (cDNA) was synthesized by using Takara cDNA synthesis kit using a random hexamer 42 °C for 1 h. By using gene-specific forward and reverse primers template cDNA was subjected to PCR amplification (Table 1). RT-PCR conditions were at 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s, annealing at 60 °C for 30s, and extension at 72 °C for 30 s in a thermal cycle (Quant Studio 5 by Applied Bio systems). The primers designed for ANXA2 and VEGF span exon–exon boundaries. Against the house-keeping gene β-actin the quantitative amount of each gene was standardised. The RNA levels were expressed as a ratio, using the delta–delta method for comparing the relative expression results between normotensive control and patients with PE [19].

Statistical analysis

By using program SPSS 20 (USA Chicago) statistical analysis was carried out. Results for data which are normally distributed were shown as ±SD. Statistical significance between the groups was analysed by one way ANOVA followed by Tukey's post hoc multi comparisons. To see the neonatal gender difference chi-square test was used. Statistical analysis of expression of VEGF and ANXA2 for IHC was carried out with Kruskal–Wallis rank-sum test. Mann-Whitney *U* test were used for comparison between two groups. To detect correlation between ANXA2 and VEGF expression Spearman correlation coefficients were used. 'p-value'<0.05 was considered to be statistically significant.

Results

There were no statistical significant differences with respect to their age, BMI (body mass index), neonatal gender in all three groups. All the patients were nulliparous in PE group. There was a statistically significant decrease in mean gestational age between severe PE in comparison with control group (0.000). In patients with PE group (Mild and severe), when compared with the normotensive control group, birth weight of the baby was decreased with more reduction in severe PE group (0.001). Prothombin time is increased in preeclampsia group (mild and severe) compared to control ($p<0.05$) with no statistical significant difference found among mild and severe PE group (0.992) as shown in Table 2.

Histopathological changes in placenta of preeclampsia

Microscopic examination showed the increase in the number, volume and density of villi in PE compared to controls. But villous surface area and diameter of villi reduced in PE compared to controls. Intervillous space volume is reduced in PE. The cells of endothelium of the tunica intima were activated with a swollen

Table 2: Clinico-demographic information of study subjects examined.

Variables	Normotensives, N, n=20	Preeclamptic, PE, n=30		p-Value	Level of significance		
		Mild, M, n=15	Severe, S, n=15		N vs. M-PE	N vs. S-PE	M vs. S-PE
Maternal age, years	25.5 ± 4.0	25.93 ± 2.4	23.9 ± 2.9	0.560	0.448	0.337	0.044*
BMI, kg/m_2	25.5 ± 2.14	26.3 ± 1.7	25.4 ± 1.8	0.391	0.485	0.980	0.424
Gestational age, weeks	37.6 ± 0.9	37.93 ± 1.5	34.67 ± 1.5	0.000*	0.324	0.000	0.000*
Birth weight	3.00 ± 0.31	2.7 ± 0.3	2.2 ± 0.38	0.001*	0.139	0.001*	0.004*
Gravidity	2.3 ± 0.6	1 ± 0.0	1 ± 0.0	0.000*			
Parity	1.3 ± 0.4	1 ± 0.0	1 ± 0.0	0.007*			
Prothrombin time, S	12.0 ± 0.3	13.6 ± 0.00	13.58 ± 0.47	0.000*	0.000*	0.000*	0.992

BMI, body mass index; PE, preeclampsia; M, mild; N, normal; S, severe. Data are expressed as mean ± S.D. p-Value is the level of significance obtained using one way ANOVA.

morphology PE. However the endothelial cells of the tunica intima in the control group appeared normal with a flattened morphology. These swollen endothelial cells suggest that the cells were damaged as shown in Figure 1.

Reduced expression of VEGF in the placental villous tissue triggers preeclampsia

Immunostaining of placental villous tissue confirmed the decreased expression of VEGF in the PE group compared to the normotensive control group. In chorionic villous tissue, VEGF staining was noticed in different cells, including membrane of syncyotiotrophoblastic cells, chorionic villous stromal cells and villous vascular endothelial cells. Expression of VEGF in placental villous tissues was semi-quantified (Table 3). A statistically significant decrease in the VEGF expression was observed in placental villous tissue in both mild and severe PE groups, compared to normal pregnancies (p=0.0001).The expression of VEGF in severe preeclampsia is slightly decreased compared to mild PE yet statistically not significant (p=0.335). In normal term placental villi, expression was strong and found chiefly in the membrane of syncytiotrophoblasts. In PE placentas, moderate staining was obtained with VEGF primary antibodies (Figure 3A, B).

Expression of VEGF protein in placentas by western blot and real time PCR

VEGF expression levels of were confirmed by densitometry. Expression of VEGF was significantly reduced in pre-eclamptic placentas by 1.7 fold in mild PE and 2.8 fold in severe PE compared to normal placenta (p=0.0009, 0.007 respectively). Placental levels of VEGF mRNA as determined by RT-PCR were reduced in women with PE (mild and

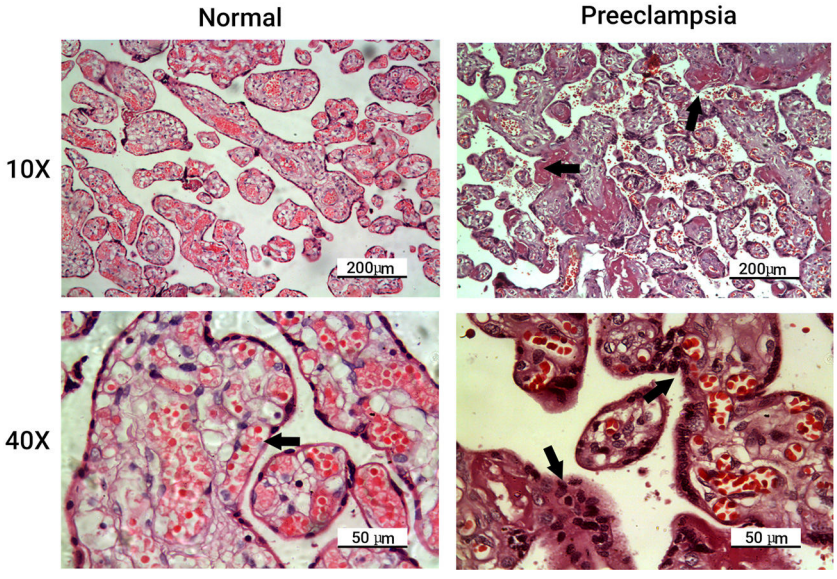

Figure 1: Hemotoxylin and eosin staining of normal and preeclamptic placenta. The thickness of syncytiotrophoblast membrane, the formation of syncytial knots and the stromal fibrosis are increased in the preeclamptic placenta, compared to normal placenta (black arrow heads).

Table 3: Localisation and immunostaining intensity of VEGF and ANXA2 expression in placental villous tissues.

Type of tissue		Overall score	VEGF			ANXA2		
			STM	CVSC	VVEC	STM	CVSC	VVEC
	Normal n=20	0	0	0	0	1 (5)	0	0
		1+	0	0	1 (5)	4 (20)	7 (35)	3 (15)
		2+	2 (10)	10 (50)	17 (85)	13 (65)	13 (65)	17 (85)
		3+	18 (90)	10 (50)	2 (10)	2 (10)	0	0
	Mild n=15	0	0	0	0	2 (13)	0	0
		1+	1 (6.6)	7 (46.6)	14	9 (60)	11 (73.3)	2 (13.3)
Preeclampsia n=30		2+	13 (86.6)	8 (53.3)	1	4 (26.6)	4 (26.6)	12 (80)
		3+	1 (6.6)	0	0	0	0	1 (6.6)
	Severe n=15	0	0	0	1	1 (6.6)	0	2 (13.3)
		1+	3 (20)	13 (86.6)	14	10 (66.6)	12 (80)	13 (86.6)
		2+	12 (80)	2 (13.3)	0	4 (26.6)	3 (20)	0
		3+	0	0	0	0	0	0
	p-Value	–	0.000	0.000	0.000	0.005	0.013	0.000

STM, syncytiotrophoblast membrane; CVSC, chorionic villous stromal cells; VVEC, villous vascular endothelial cells; PE, preeclampsia; M, mild; N, normal; S, severe. Immunohistochemical staining score: 0, no staining; 1+, 1–10%; 2+, 11–50%; 3+, 51–70%; 4+, 71–100%. Data are expressed as numbers (percentage). p-Value is the level of significance calculated by Kruskal–Wallis rank-sum test using one way ANOVA.

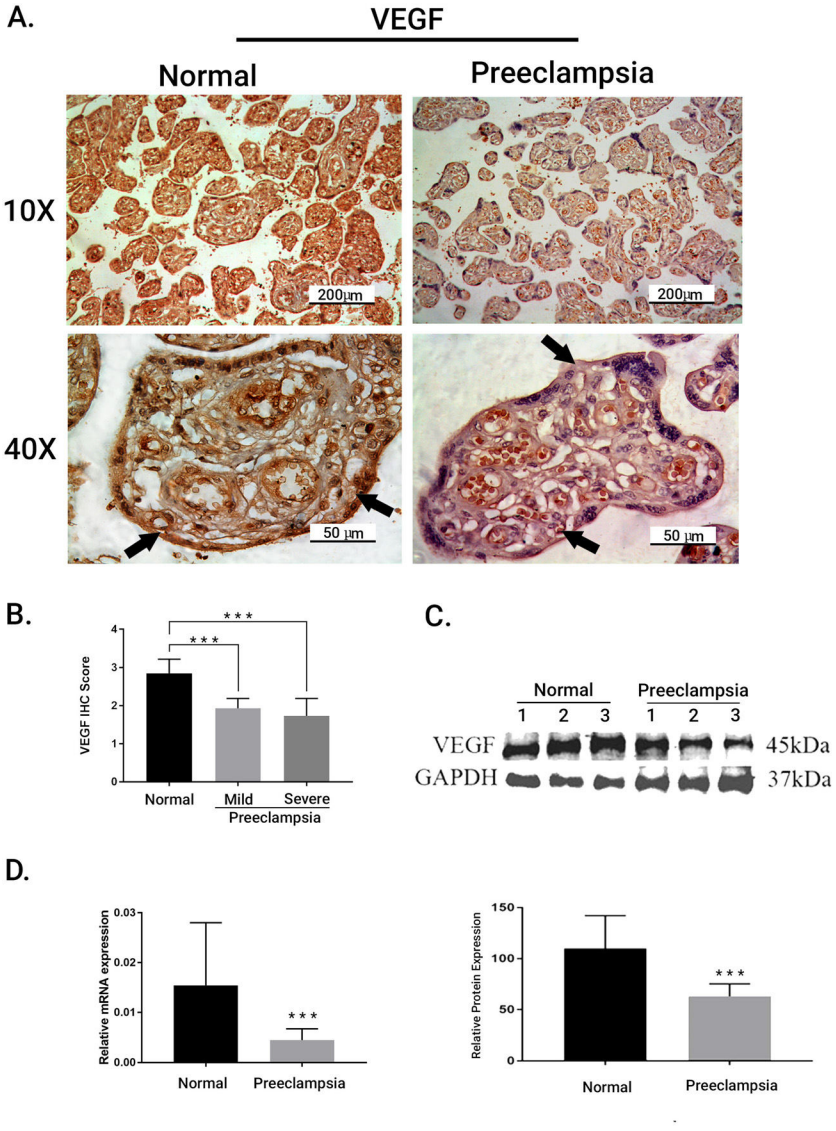

Figure 2: Expression of VEGF in normal and preeclamptic placenta. (A) The black arrow heads indicate the intense immunostaining of VEGF165 in the membrane of syncytiotrophoblast in normal placenta, compared to weak immunostaining in the preeclamptic placenta. (B) The VEGF staining intensity scores indicate the significant loss of its expression in the mild and severe preeclampsia, compared to normotensives. (C) Western blots analysis for VEGF protein expression in normal and preeclamptic placenta. (D) Relative mRNA expression of VEGF. ***p<0.0001.

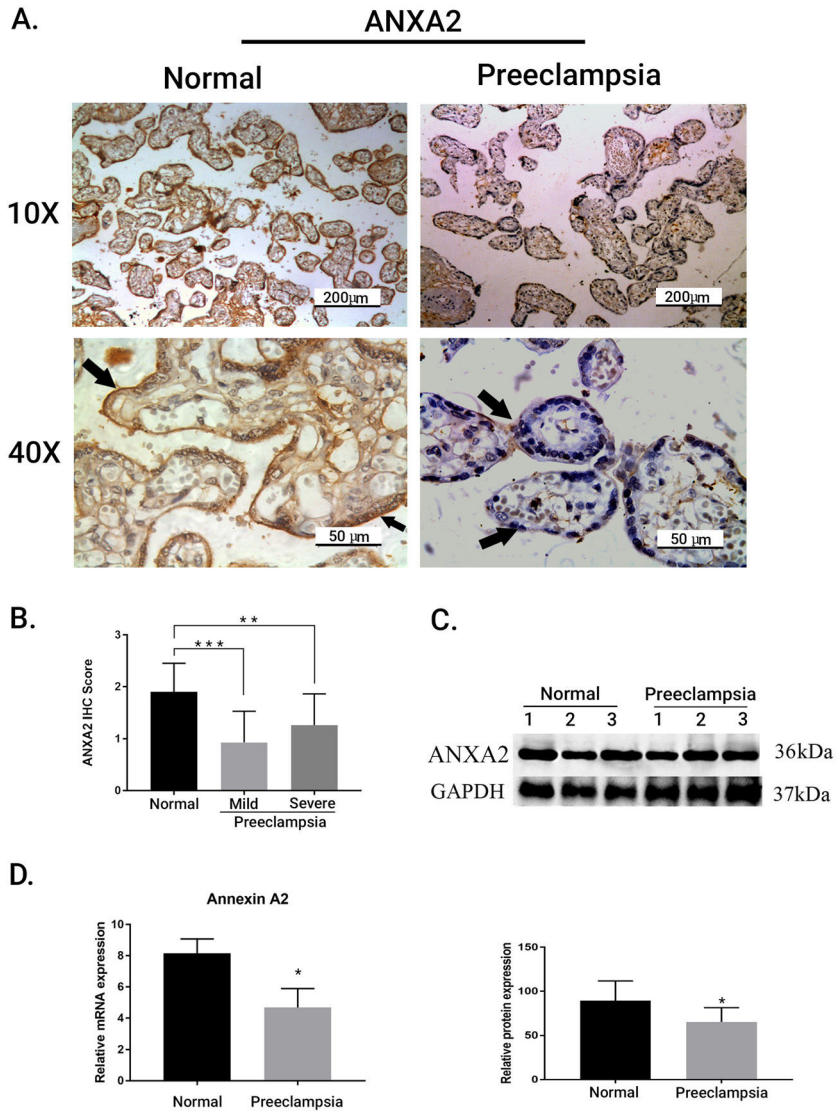

Figure 3: Expression of ANXA2 in normal and preeclamptic placenta. (A) The black arrow heads indicate the intense immunostaining of ANXA2 in the membrane of syncytiotrophoblast in normal placenta, compared to weak immunostaining in the preeclamptic placenta. (B) The ANXA2 staining intensity scores indicate the significant loss of its expression in the mild and severe preeclampsia, compared to normotensives. (C) Western blots analysis for ANXA2 protein expression in normal and preeclamptic placenta. (D) Relative mRNA expression of AnxA2. *p<0.05, **p<0.01, ***p<0.001.

Severe) (n=9) compared with normotensive controls (n=9). Relative RNA expression of VEGF was reduced by 3.4 fold, p=0.0001. Levels of mRNA (expressed in arbitrary units relative to expression of beta–actin mRNA). Unpaired t-test is used to evaluate the statistical difference (Figure 3C, D).

Decreased expression of ANXA2 in the placental villous tissue contributes to preeclamptic conditions

Immunostaining of placental villous tissue confirmed the reduced expression of ANXA2 in the PE group (mild and severe) compared to the normotensive control group. Different cellular components were positive for ANXA2, including membrane of syncytiotrophoblastic cells, chorionic villous stromal cells and endothelial cells in both groups of placental villous tissue. Expression of ANXA2 in villous tissues was semi-quantified as shown in Table 3. A statistically significant decrease in the expression was observed in membrane of syncytiotrobhoblast, chorionic villous stromal cells and villous vascular endothelial cells in PE group (mild and severe p=0.000, 0.003) compared with placentas of normal pregnancies. The expression of ANXA2the placentas of severe PE is slightly more than mild PE however not statistically significant (p=0.350). But irrespective of severity ANXA2 is decreased in PE group. Staining was moderate in normal term pregnancy placentas, and observed mainly in the membrane of syncytiotrophoblasts. In PE placentas, weak staining was observed with ANXA2 primary antibodies (Figure 2A, B).

Expression of ANXA2 protein in placenta by western blotting and real time PCR

Expression levels of ANXA2 were confirmed by densitometry. ANXA2 expression was decreased by 1.3 and 1.4 fold in PE placentas (mild and severe) compared to normal placenta (p=0.0191, p=0.0270) (Figure 2C). Placental levels of ANXA2 mRNA as determined by RT-PCR were reduced in women with PE (mild and severe) (n=9) compared with normotensive controls (n=9). Relative RNA expression of ANXA2 in PE (mild and severe) is decreased by 1.7 fold compared to normal placenta. Levels of mRNAs are expressed as arbitrary units (p=0.0299) (Figure 2D). To evaluate the statistical significance unpaired t-test is used.

Loss of association between ANXA2 and VEGF expression leads to preeclampsia

Results showed a statistically significant positive correlation in the expression of VEGF and ANXA2 in syncytiotrobhoblast membrane in normal term placentas (r=+0.723) (p<0.0003). But there was no significant correlation in the expression of ANXA2 and VEGF in PE group with either in mild and severe cases. This suggests that association which was maintained in normal placenta was lost in preeclampsia (Figure 4).

Discussion

This study showed decreased expressions of VEGF and ANXA2 in the third trimester placental bed from pregnancies with PE compared to normotensive control group. Although the mechanisms responsible for the etiopathogenesis of preeclampsia are poorly understood, there is an agreement that it is associated with reduced invasion and failed remodelling of maternal endometrial spiral arteries in the placenta [20]. There is growing evidence that deficient trophoblastic invasion due to altered fibrinolysis in the placental bed spiral arteries is crucial to the pathogenesis of PE [21, 22]. The primary established pathology in PE resides in the reduced trophoblastic implantation and placental perfusion [23]. In the present study, we observed the altered morphology of the villous vascular endothelial cells of the fetal capillary of preeclamptic placenta. Shape of endothelial cells was altered from normal flattened to cuboidal morphology showing that the endothelial cells of the placental villi were more damaged in the PE group compared to the normotensive group. VEGF has been shown to be involved in the regulation of trophoblast cell survival, migration, endovascular differentiation and proliferation [24]. Placenta endures dramatic vascularisation in the course of normal pregnancy to allow the circulation between foetus and mother. The main pathogenic mechanism underlying PE is placental ischemia which results

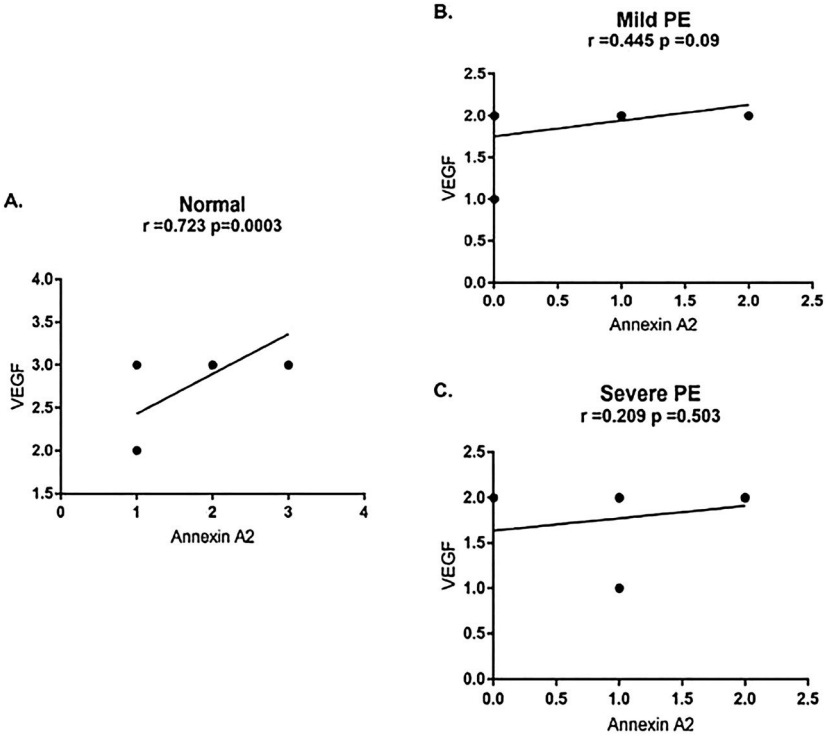

Figure 4: Analysis of correlation (Spearman's) of expression of ANXA2 with VEGF in the syncytiotrophoblast membrane in placental villous tissue showed a statistically significant positive correlation in the expression of VEGF and AnxA2 in in normal term placentas (A), but there was no correlation between proteins in PE group with either in mild (B) or in severe cases (C).

in hypoxia, which is a potent stimulator for VEGF production [25]. Several studies have reported levels of VEGF in the serum were increased in PE patients compared to normotensive patients because of placental hypoxia causing from placental ischemia [26, 27]. Though, other studies have presented decreased VEGF production in PE placenta compared to normotensive placenta [28, 29]. It may be a reason that as a compensatory mechanism to hypoxia the production of VEGF may be initially increased. Nevertheless, it may cause damage to endothelial cells and gradually reduces the production of VEGF, if hypoxia becomes more advanced, finally resulting in development of PE.

For regulating early placental angiogenesis and remodelling of maternal artery VEGF family of angiogenic growth factors and the receptors are important molecules [30, 31]. This result suggests the possibility that decreased VEGF expression in the placentas may be a cause of failure of re-modelling of spiral artery, later resulting in PE. From a single VEGF-A, spliced variants of 121, 145, 165, 189 and 206 amino acids are generated [32, 33]. Study of placental villi of human first-trimester showed that VEGF isoforms 121, 165 and 189 are expressed in placenta, with a noted prominence of the VEGF-165 isoform [34]. Current evidence indicated, in patients with proliferative diabetic retinopathy the correlation between increased levels of VEGF, tissue plasminogen activator (tPA) and plasminogen activator inhibitor (PAI-1) indicating direct relation between VEGF and fibrinolysis [35]. There is also evidence that ANXA2 might induce neovascularisation through VEGF-VEGF R2 pathway in ischemia induced retina neovascularisation [12]. For the formation of new blood vessels during neovascularisation, extracellular proteolysis is crucial requirement. Plasmin which degrades several components of the ECM such as laminin and fibrin is a central component of neovascularisation. Plasmin further increases the bioavailability of angiogenic VEGF [36]. Interaction between different components is required for unique sequential process of fibrinolytic activity. ANXA2 is one of the key mediators which converts plasminogen to plasmin. Profibrinolytic molecule ANXA2, serves as a cell surface coreceptor for plasmin generation and localizing fibrinolytic activity on the surface of the cell when it binds with plasminogen and its activator, tPA [37–39]. The efficiency in plasmin generation is increased by 60 fold when Purified ANXA2 binds to plasminogen and tPA, [37]. In both human diseases and animal models, it has been shown that deficient ANXA2 dependent fibrinolytic pathway has been connected to increased intravascular thrombosis [38, 39]. Probably ANXA2 deficiency may be one of the causes for increased clot formation in placenta. In acute promyelocytic leukaemia cells abnormally high levels of ANXA2 leads to increased plasmin generation leading to haemorrhagic conditions [40]. Growing evidences suggests that ANXA2 expression and its binding with plasminogen and tPA play a crucial role in maintaining fibrinolytic balance on the surfaces of blood vessel [41]. In our study the expression of VEGF is reduced in the placental villi is reduced, probably this has triggered PE by reduced vascularisation in preeclamptic placenta. We confirmed the expression status of these proteins by IHC, western blot and RT-PCR. Our immunohistochemical analysis showed that both ANXA2 and VEGF were mainly expressed on the membrane of syncytiotrophoblasts and weak expression was found in endothelial cells of foetal capillary in the placental villi. Location of VEGF and ANXA2 in placenta of preeclampsia was the same when compared to control group. But the expression of both VEGF and ANXA2 was lower in placentas in patients with PE irrespective of severity compared with normal pregnancies which suggested a decrease in production of these proteins. The decreased expression of ANXA2 protein in placental tissues may possibly weaken the local fibrinolytic activity by decreasing the plasmin generation. The PE placenta often shows infarctions and fibrin deposition which reveals haemostatic system failure. Defects of fibrinolytic system are well known risk factors for increased thrombosis and alterations of fibrinolysis have been shown to be present in PE, indicating a role for fibrinolytic abnormality in the development of the disease [42, 43]. Recent evidence concluded that, in placentas and in maternal blood of patients with PE, expression of ANXA2 was significantly down regulated. The decreased expression of ANXA2 provides evidence to an impaired fibrinolytic activity, which may lead to increased thrombin formation in placenta of PE [44]. The present study is consistent with these findings. We also observed that prothrombin time was prolonged in patients with PE suggesting altered fibrinolytic activity. In the process of angiogenesis, ANXA2 is known to perform as angiogenic regulator. VEGF up regulates ANXA2 production has been demonstrated in previous studies. In the current study, we observed the significant correlation in the expression of VEGF and ANXA2 in syncytiotrophoblast membrane in control group. This directs that, physiologically expression of these two factors may be dependent and regulated in the placental bed in response to the same stimulus such as hypoxia, but this correlation is disturbed in preeclampsia. In the present study, placental bed biopsies showed significant difference among PE and normotensives for VEGF and ANXA2 expression. These results show the probable significance of expression of ANXA2 in placentas as a more

effective biomarker in the prediction of the development PE. Although further studies with more number of patients, should be carried out to verify this possibility.

The present study showed decreased expression of VEGF and ANXA2 in the PE placental villous tissue in comparison with the normotensive control. The reduced expression of above angiogenic proteins in the placental tissue may be linked with the development of PE.

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Informed consent: Informed consent was obtained from all individuals included in this study.

Ethical approval: The study was conducted following informed consent and was approved by the Ethical Committees of SDM College of Medical Sciences & Hospital, Dharwad, Karnataka, India (SDM IEC: 0748: 2016).

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